AN INVESTIGATION OF CRITERIA FOR THE MORPHOLOGIC DIAGNOSIS OF INFECTION BY *Chlamydia trachomatis* IN THE UTERINE CERVIX

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Frontispiece: Ancient Chinese depiction of *Chlamydia trachomatis* conjunctivitis.

DECLARATION

I, Bryan Knight, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree is this or any other University.

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ABBREVIATIONS AND DEFINITIONS

α-DIG	α-Digoxigenin
AIDS	Acquired immuno-deficiency syndrome.
Alum	Aluminium ammonium sulphate [$Al(NH_2)\ SO_4$]
Alk Phos	Alkaline Phosphatase
APASD	Alcian blue periodic acid Schiff diastase stain, a combined stain for acid and neutral mucins, used in histology
B	Biotin
BCIP	ALK-PHOS-conjugated α-DIG/B (BCIP)
CAP	College of American Pathologists;
CDC	Centers for Disease Control and Prevention, a Federal Government Agency based in Atlanta Georgia, United States America. The Agency sets norms and standards of practice, particularly in relation to the control and management of Infectious Diseases;
CIN	Cervical intraepithelial neoplasia.
Cytospin	A specially adapted centrifuge system used commonly in cytology laboratories. A suspension of cells in a fluid specimen is placed in a special container that is in turn placed on the surface of glass slide. Centrifugation deposits the suspended cells onto the surface of the slide. The cells are dispersed in a circumscribed area making examination of large numbers of cells in a small area possible;
DFAT	Direct Fluorescent Antibody Test, a method devised to identify the presence of a specific antigen (for example *Chlamydial*) in a specimen by using an antibody with a fluorescent label attached;
DISH	DNA *in situ* hybridization, a technique for the *in situ* demonstration of the presence of a target DNA sequence;
DNA	Deoxyribonucleic Acid, a major constituent of the chromosome and the carrier of genetic information;
dATP	Deoxyribose Adenine tri-phosphate, the adenine building block for DNA, used in the PCR master mix;
dCTP	Deoxyribose Cytosine tri-phosphate, the cytosine building block for DNA, used in the PCR master mix;
dGTP	Deoxyribose Guanine tri-phosphate, the guanine building block for DNA, used in the PCR master mix;
dNTPs	Deoxyribose Nucleotide tri-phosphate, a generic term used to collectively describe the DNA building blocks used in the master mix;
dUTP	Deoxyribose Uracil tri-phosphate, the uracil building block for DNA, used in the Roche Amplicor® PCR master mix;
dTTP	Deoxyribose Thymine tri-phosphate, the thymine building block for DNA, used in the PCR master mix;

EA-65 Eosin Alcohol Stain, a polychromatic counter stain used in the Papanicolaou staining technique developed by Dr G Papanicolaou in 1942 and modified in 1954 and 1960. The stain contains Light Green, Fast Green, Bismarck Brown, phosphotungstic acid, lithium carbonate, Eosin, 95% ethanol, absolute methanol and glacial acetic acid;

EB Elemental Body, the infectious form of *Chlamydiae*, which is a non-replicative form and resistant to environmental conditions adverse to the growth and survival of *Chlamydiae*;

ELISA Enzyme-linked Immunosorbent Assay, a serologic method used for the detection of specific antigens. The antigen is bound by a specific antibody that is conjugated to an enzyme which catalyses a chromogenic reaction;

EM Electron microscopy was performed on a Hitachi H 600 Electron Microscope in the Electron Microscopy Unit, Department of Anatomical Pathology, University of Cape Town;

FDA The United States Government Food and Drug Administration, a statutory body that regulates and licenses for use, drugs, food additives, laboratory reagents, equipment and procedures. The FDA sets rigorous guidelines and requirements and approval by this body implies very thorough, exhaustive validation of the approved item or procedure;

H & E Haematoxylin and Eosin, the standard universal histology stain.

HIV Human Immuno-deficiency Virus, a retrovirus responsible for the acquired immuno-deficiency syndrome;

HPV Human Papilloma Virus, implicated in the aetiology of carcinoma of the human *cervix uteri*;

HgO Mercuric oxide

IATA The International Airline Transport Association, an international body regulating affairs of the international airline industry.

LCR Ligase Chain Reaction®, a DNA amplification method patented by Abbott Laboratories using a novel enzyme different to that used by the Roche PCR system;

LM Light microscopy;

LLETZ Large loop excision of the transformation zone. The LLETZ excision biopsy is a doughnut-shaped piece of tissue removed from the cervix using a hot wire loop attached to a special diathermy machine. The cutting effect of the loop removes a superficial piece of tissue including mucosa of the endocervix, the transformation zone between endo- and ecto- cervix, and part of the ectocervix. The diathermy effect causes tissue coagulation in the resection line preventing post-operative bleeding;

LPS Liposaccharide, a genus specific Chlamydial antigen present in the replicative (intra-cellular) form of all species of *Chlamydiae*;

Master Mix The cocktail of chemicals used in the polymerase chain reaction, consisting of primers, DNA building blocks, polymerase enzyme, and chemical buffers used to set reaction conditions (pH, molarity);

MOMP Major Outer Membrane Protein, a species-specific major structural protein present in the outer coat of Chlamydial elemental bodies

NBT Nitro-tetrazolium blue, a vital dye

OG-6 Orange G Stain, a polychromatic cytoplasmic counter stain used in the Papanicolaou staining technique developed by Dr G Papanicolaou in 1942 and modified in 1954 and 1960. The stain contains Orange G-6, 95% ethanol and phosphotungstic acid;

Organosilane Amino-propyl-triethoxy-silane, a compound used to coat glass slides used for microscopy in a process known as "subbing". The compound serves to increase adherence of tissue sections to the slide and to prevent the section from floating off the glass during the staining process. The compound is not susceptible to digestion by proteolytic enzymes;

Pap smear Papanicolaou smear, named after Dr. George Papanicolaou who developed the method of cytologic examination of exfoliated cells from the human *cervix uteri*;

PCR Polymerase Chain Reaction, the first method for DNA amplification, patented by Roche®;

Primer A synthetic, specially selected oligonucleotide sequence used to direct polymerization of a selected DNA sequence (target sequence) in the Polymerase Chain Reaction;

Polymerase An enzyme responsible for catalysing a polymerisation reaction;

SSC pH 7.0 Neutral Saline Citrate: A standard high concentration solution of sodium citrate in saline (3M solution of sodium chloride with 0.3M sodium citrate at pH 7.0) used to create high osmolality conditions in which non-homologous binding of nucleotide pairs in DNA become unstable. The concentration of SSC used in a particular reaction is expressed as a number of multiples of the standard solution, for example 0.1 x SSC or 20 x SSC.

STD Sexually transmitted disease, or venereal disease, an infection of the genital tract or lower urinary tract;

Taq A polymerase enzyme derived from a thermophilic bacterium, *Thermus aquaticus*. The enzyme retains its activity at the high temperatures necessary to denature DNA and maintain the split DNA in single strands (72°C)

The practice During the period that the study was conducted, the writer was a partner in a pathology practice in Cape Town South Africa, known as Drs Penman Kock and Knight.

Word count 77 177

1. STATEMENT OF THE HYPOTHESIS

1.1 THESIS:

θi:sis (Greek) : *A proposition to be maintained or proved, from the root of* ***tithenai,*** *'to place.'* [1]

A view that is widely held in the cytology literature is that the diagnosis of *Chlamydia trachomatis* infection using Papanicolaou smears of the uterine cervix is unreliable.[2] Further, the morphology of *Chlamydia trachomatis* infection is histology sections is not widely recognised. Traditionally, cytologists turn to histology to interpret and to corroborate their diagnoses.[3-5] The failure of histologists to recognise and report Chlamydial infections[6] strengthens resistance to cytologic reporting of the disease. Several workers in cytology have used other inappropriate tests[7] to corroborate diagnoses made by Papanicolaou smear.[8,9] These investigators failed to realize the limitations of their tests.

The thesis

Failure to appreciate the technical limitations and low sensitivity of confirmatory teats has resulted in the erroneous conclusion that cytologic diagnosis of Chlamydial infection is unreliable.

1.2 HYPOTHESIS:

HΛi'poθisis (*Greek*): A supposition made as a starting point for further investigation from known facts (theory).[1]

Recognition of *Chlamydia infection* in tissue sections will assist to dispel the concern over the validity of cytologic diagnoses. With use of more appropriate sensitive and specific tests, (*in-situ* DNA hybridization and the polymerase chain reaction) the morphologic criteria for the diagnosis of Chlamydial infection can be corroborated.

The hypothesis

Simple morphologic criteria can be applied in the examination of Papanicolaou smears and tissue sections of the uterine cervix to enable confident diagnosis of *Chlamydia trachomatis* infection.

2. ABSTRACT

Certain cytologic changes in Papanicolaou smears have been ascribed to *Chlamydia trachomatis* infection. These include intracellular vacuolar inclusions that occur predominantly in metaplastic squamous cells in the endocervical canal. If this is true, the Pap smear could play a role in the laboratory diagnosis of Chlamydial infection. However, many workers have described these changes as non-specific because cultures and immunologic tests for *Chlamydiae* failed to corroborate the morphologic diagnosis. The sensitivity of Chlamydial culture is low and many immunologic tests are not wholly satisfactory in detecting *Chlamydiae*. Newer DNA detection methods have surpassed these detection methods and are more appropriate gold standards for the diagnosis of Chlamydial infections. This study investigated the morphologic criteria (MC) for the diagnosis of *Chlamydia trachomatis* infections in both Pap smears and histology sections. A recurrent constellation of changes regarded as evidence of Chlamydial infection was identified. These included a characteristic inflammatory picture, peculiar epithelial changes and intra-cellular vacuoles containing three types of inclusion. Diagnostic criteria were refined to exclude possible non-specific changes due to inflammation, degeneration, and concomitant infections including HPV infection with and without CIN. A significant association of Chlamydial infection and CIN was demonstrated. The MC identified were interrogated by two modern DNA detection methods. DNA *in situ* hybridisation (DISH) and polymerase chain reaction (PCR) were used as a combined gold standard. DISH showed the presence of Chlamydial DNA in 92% of morphologically positive cases and absence of Chlamydial DNA in 93% of morphologically negative cases. The correlation between morphologic diagnosis and DISH result was significant in a high degree ($p < 0.001$). Using serial tissue sections from the same histology cases, PCR confirmed the presence of Chlamydial DNA in 88% of morphologically positive cases ($p < 0.005$) and the absence of Chlamydial DNA in 80% of morphologically negative cases ($p = 0.0255$). When challenged by a gold standard requiring concordant DISH and PCR results, the sensitivity of the MC was 95.5% and the specificity was 88.9%. It was concluded that a confident morphologic diagnosis of *Chlamydia trachomatis* infection can be made in Pap smears and in tissue sections of *cervix uteri*.

3. INTRODUCTION

3.1 THE POINT IN QUESTION

Several eminent American cytopathologists working in the Johns Hopkins Cytopathology Laboratory, including Prabodh K. Gupta and the late John K. Frost, causally ascribed cytologic changes seen in Papanicolaou smears of the female genital tract to infection by *Chlamydia trachomatis*.[1] The changes include intracellular vacuolar inclusions which occur predominantly in metaplastic squamous cells of the endocervical canal. If these are indeed caused by *Chlamydia trachomatis* infection, the Pap smear could play a role in the laboratory diagnosis of Chlamydial infection. However, many workers [3-14] considered the changes described by Gupta and Frost as non-specific, primarily because cultures and immunologic tests for *Chlamydiae* failed to corroborate the morphologic diagnosis.

The sensitivity of Chlamydial culture is low[15] and many immunologic tests, particularly immunoperoxidase tests, have not been assessed for sensitivity. Enzyme-linked immuno assays (ELISA)[2-4,16] and direct immunofluorescent tests (DFAT) [16-20] have been assessed for sensitivity and specificity are not wholly satisfactory. Newer DNA detection methods have surpassed culture and immunologic detection methods.[15,16,21-23] The view has been expressed that DNA testing offers a more appropriate "gold standard" for the diagnosis of Chlamydial infections.[15,16] The validity of many of the studies that refute the value of a morphologic diagnosis of *C trachomatis* may be called into question. Failure of these tests to corroborate the Papanicolaou smear diagnosis of Chlamydial infection can no longer be used to dismiss as non-specific, the changes described by Gupta and Frost. [24]

3.2 SETTING THE GOLD STANDARD

The selection of a particular test method to be used as *the* "gold standard" against which to compare other tests must be governed first by the specificity of the test. The term "gold standard" implies a pure and entirely reliable (specific) result. However, the selection of a "gold standard" should also take the sensitivity of the test into consideration.

Historically, the gold standard for laboratory confirmation of a Chlamydial infection has been culture of the organism,[15,16] principally because the specificity of this method is very high.[25] Culture of *Chlamydiae* entails inoculation of a specimen into cell culture of an immortalised cell-line, usually HeLa[26,27] or McCoy cells.[28-30] Demonstration of the successfully cultured organisms relies on observation by light microscopy of the organisms in the host cells. For many years, these organisms were stained non-specifically with iodine,[15] but use of fluorescent anti-Chlamydia antibodies has recently refined the demonstration of the cultured organisms.[30-32] More recently, immunologic tests have been developed which rely on direct detection of Chlamydial antigen in the test sample.[2-7,15,16,33-35] Although these methods are less time consuming and less expensive than the exacting techniques required for culture, neither ELISA[36-39] nor DFAT[18-20] are suitable as gold standard tests.

With the development of DNA molecular technology, testing for micro-organisms has been revolutionized.[15,22,23] DNA tests using the polymerase chain reaction (PCR) are significantly more sensitive methods[15,16,22,40-43] and DNA *in* situ

hybridization (DISH) is generally more specific than traditional culture methods.[15,16,23,36] Traditional gold standards in microbiology have been superseded by these new testing methods.

3.3 THE PRESENT INVESTIGATION

The purpose of this study was to investigate the morphologic criteria for the diagnosis of *Chlamydia trachomatis* infections in Pap smears. In the cytology laboratory, it is considered good laboratory practice that a cytology diagnosis should be corroborated by a tissue diagnosis using histology sections.[44-46] The present study therefore included an investigation of the morphologic features of Chlamydial infection in tissue sections. The morphologic criteria in both cytology and histology were to be interrogated by modern DNA testing.

4. REVIEW OF LITERATURE

4.1 INTRODUCTION

Laboratory detection of *Chlamydiae* is possible by a variety of methods. These include direct observation of the organism by microscopy, culture of the organism in immortalised cell lines (similar to viral cultures), various methods to detect Chlamydial antigen, and tests that detect Chlamydial DNA. All of these test modalities suffer limitations and interpretation of results requires an understanding of these limitations.

Historically, the gold standard for laboratory confirmation of a Chlamydial infection has been culture of the organism,[1] principally because the specificity of the method is high. Culture of *Chlamydiae* entails inoculation of a specimen into cell culture of an immortalised cell-line, usually HeLa or McCoy cells.[2] Demonstration of the successfully cultured organisms relies on observation by light microscopy of the organisms in the host cells. For many years, these organisms were stained non-specifically with iodine, but use of fluorescent anti-Chlamydia antibodies has recently refined the demonstration of the cultured organisms. More recently, immunologic tests have been developed which rely on direct detection of Chlamydial antigen in the test sample.[3-12] These methods are less time consuming and less expensive than the exacting techniques required for culture.

With the development of DNA molecular technology, testing for micro-organisms has been revolutionised. Tests using DNA technology are generally more specific than traditional culture methods. Further, DNA tests using the polymerase or ligase chain reactions are significantly more sensitive methods. Continuing improvements within the field of DNA technology have resulted in recently developed DNA tests that are more sensitive than ever before. Traditional gold standards in microbiology are increasingly being superseded by these new methods of testing.

Detection of unique sequences of Chlamydial nucleic acids by DNA probe became possible during the late 1980s.[13] DNA tests have been refined significantly with the development of DNA amplification technology. The improved sensitivity of DNA testing methods is so significant that culture methods can no longer be regarded as an acceptable "gold standard" for detection of *Chlamydia trachomatis*.[1,2,14-15]

This review of literature begins with a brief overview of the biology of *Chlamydiae*, upon which rational testing should be based. The various test modalities available for making a diagnosis of Chlamydial infection are examined. The tests that have been used to refute the value of the morphologic diagnosis of Chlamydial infection are evaluated. In addition, newer methods available for the diagnosis of Chlamydial infection are examined. The review highlights the many difficulties encountered in the laboratory diagnosis of Chlamydial infections. Shortcomings of many of the older tests, particularly culture and tests based on immunologic methods are identified. The literature reveals the superiority of DNA testing methods.

4.2 THE BIOLOGY OF *CHLAMYDIA TRACHOMATIS*

Rational testing for *Chlamydiae* should be based on an understanding of the unique biology of this organism. Further, an understanding of the biology of the organism may explain some of the problems encountered in the laboratory diagnosis of *Chlamydiae*.

4.2.1 THE ORGANISM

The *Chlamydiae* are bacterial organisms showing some characteristics of viruses. Like viruses, these bacteria are obligate intra-cellular organisms that appear to regulate to some extent the function of the host cell. During replication of the organism, Chlamydial antigens are expressed in the plasma membrane of the host cell. [2]

The life cycle of *Chlamydiae* is dimorphic and is unique among bacteria. [2] The organism is an obligate intracellular bacterium that only replicates within a membrane-bound vesicle ("inclusion") of eukaryotic host cells.[16] The organism takes two forms: the elementary bodies (EB) and reticulate bodies (RB).[2] The cell wall of the organism contains a glycoprotein known as the major outer membrane protein (MOMP) and other proteins rich in cysteine. The wall lacks peptidoglycan so the organism is resistant to β-lactam antibiotics. However, *Chlamydiae* are susceptible to both tetracyclines and to macrolides.[2, 16] Fifteen sero-types of the organism are recognised (A, B, Ba, C-K, L_1-L_3). [16] Serotypes A–K cause oculo-genital infections in man. They are fastidious organisms and require special handling for successful culture.[16] The serotypes L_1-L_3 cause lymphogranuloma venerium.

Elementary bodies[2]

EB are the infective form of the organism, are metabolically inactive, and are able to survive outside of host cells. They are small cocci that range in size from 200 to 300 nm in diameter. Numerous co-valent disulphide bonds (S-S) cross-link MOMP and the cysteine-rich proteins, rendering the cell wall rigid and resistant to the extra-cellular environment. [2] The wall consists of an external, tri-laminar rigid outer envelope with an inner trilaminar cytoplasmic membrane. The central core consists of DNA and small amounts of RNA. [16]

Reticulate bodies[2]

The RB are larger than the EB. They are coccoid, measuring between 600 and 1 000 nm in diameter, and resemble bacteria with a cell wall and featureless interior. RB are the replicative form of the organism, are extremely fragile and only survive within a vacuole the host cell. These bodies cannot initiate new infection of host cells, but may be carried into a new cell during host cell division. The RB accumulates glycogen, a phenomenon only found in the *trachomatis* species. The organism replicates by binary fission. [2]

4.2.2 THE LIFE CYCLE OF *CHLAMYDIAE*

Infection commences with the attachment of EB to the host cell membrane by numerous weak receptor-ligand interactions. A Chlamydial polycationic heparin-like molecule in wall of the EB attaches to glycosaminoglycans in the host cell membrane.[17] The attachment mechanism is flexible and enables infection of a variety of cell types. Trypsin treatment of host cells prevents attachment by digestion of the host cell receptor molecules. Entry of the EB into the host

cell occurs by parasite-initiated endocytosis and is mediated by host cell microfilament and is clathrin-dependent.[18] The process requires dephosphorylation of host cell ATP mediated by host cell tyrosine kinase.[16,19] The protein caveolin-1 appears to play a role in the endocytotic process by inactivating inducible nitric oxide synthase (iNOS) in the host membrane.[20] Inactivation of iNOS causes reduced production of nitric oxide in the host cell. This in turn inhibits the formation of free NO radicals, a key host cell defence mechanism against intracellular bacteria. Interferon-γ normally promotes iNOS activity thereby enhancing host cell defence mechanisms.[20]

After infection of a host cell, the morphology of the organism changes considerably. The RB cell wall retains the outer envelope and the cytoplasmic membrane. However, the outer envelope is much less rigid than that of EB because fewer S-S cross-links are present. Presumably, the three dimensional shape and structure of the MOMP and cysteine-rich proteins is changed by reduction of these covalent links (own observation). The organism becomes larger, chromatin becomes dispersed, transcription is initiated and transport of host derived amino acids follows with biosynthesis of proteins. Glycogen is also produced.

After phagocytosis of the organism, host cell lysosomes do not fuse with the endocytotic vesicle (endosome).[2] Instead numerous endosomes fuse together to form usually one large inclusion. Redistribution in the cell of the small endosomes for fusion is mediated by host cell clathrin and by annexins.[21] Occasionally, endosome fusion is not complete and two may be juxta-positioned, separated by a membranous septum.[16] The organisms remain within the endocytotic vesicle for the replicative phase of the cycle.

In cell culture systems, infection of a host cell leading to successful production of new organisms is dependent upon the "multiplicity of infection" (MOI).[16] If the MOI is in excess of 100 organisms per host cell, immediate cytotoxicity may occur. If the number of organisms infecting the cell is between 20 and 50, the load is too low, delayed cytotoxicity occurs and low numbers of EB are released. Where the number of organisms is less than 3 per host cell, asynchronous production of organisms occurs, only few cells are infected and these may be so sparse that they may not be detected when the culture is read.

Once in the endosome, the EB differentiate into an RB within 6 to 9 hours.[2] The S-S links in the envelope are reduced by proton-donators in the host cytoplasm rendering the envelope less dense. The MOMP acts as a porin permitting entry of host-derived energy rich nucleotides into the organism.[22,23] The metabolically active RB divides by binary fission every two hours for about 2 to 3 days.[16] Continued binary fission produces a microcolony of organisms that expands the endosome until it occupies most of the host cell cytoplasm and causes displacement of the nucleus to the periphery of the host cell.

The life cycle of different organisms in the endosome is asynchronous, with some RB maturing into EB after only one day of the cycle. Thus, both RB and EB and intermediate forms may be found in an endosome.[2] During the later part of the cycle, numerous forms intermediate between EB and RB are present. However, towards the end of the infectious

cycle, an unknown trigger induces maturation of most of the RB into EB. The maturation requires cysteine and up-regulation of synthesis of both MOMP and the cysteine-rich proteins. Maturation of one RB may give rise to more than one EB.[24] Not all organisms mature before lysis of the endosome occurs with release of EB into the extra-cellular space. In ideal circumstances, the increase in number of organisms in the course of one replicative cycle is in the region of 3 log. (1 000 fold).[16]

4.2.3 THE CHLAMYDIAL GENOME

The Chlamydial genome is one of the smallest prokaryotic genomes known. The circular DNA molecule consists of approximately 1 200 kilo-base pairs (kb) and codes for approximately 600 proteins. The entire sequence is not yet known. A "cryptic" plasmid genome of 7.5 kb is found in virtually all types of *Chlamydia trachomatis*.[25] Only very rarely (on two occasions) have organisms been isolated without this plasmid.[26] The plasmid genome has been fully sequenced[27] and is frequently the target for DNA-based laboratory testing for *Chlamydia trachomatis*.[2] The plasmid genome codes for proteins thought to be involved in plasmid replication and a 28 kilo-Dalton cell envelope antigen known and pgp3.[2] A Chlamydial bacteriophage Chp1 has been completely sequenced and offers opportunities for manipulation of the Chlamydial genome. A role for the Chp1 in Chlamydial metabolism is not known.

4.2.4 CHLAMYDIAL METABOLISM

The metabolism of *Chlamydiae* has been investigated mainly in experimental conditions using cell cultures.[16] Conditions ensuring successful passage of organisms in cultured cells have been standardised and differ markedly from *in vivo* growth conditions. These studies have shown that the RB of *Chlamydia trachomatis* is capable of synthesis of nucleic acids, proteins, glycogen and lipopolysaccharide (LPS).

Proliferation of *Chlamydiae* in the endosome is independent of many of the host cell functions,[28,29] but the Chlamydial organisms are dependent on the host cell as a source of energy. The organisms have some of the components required for Embden-Meyerhof glycolytic process, but they lack respiratory enzymes and flavoproteins.[30] The *Chlamydiae* are therefore regarded as "energy parasites" making use of host cell adenosine triphosphate (ATP).[31] By electron microscopy (EM), the endosome containing the organisms is frequently seen bound to host cell mitochondria, the source of host cell ATP.[32]

4.2.5 CHLAMYDIAL ANTIGENS[2]

The important Chlamydial antigens fall into two biochemical categories: the protein antigens and the glycolipid carbohydrate antigens. The protein antigens include MOMP, outer envelope proteins *envA* and *envB,* Chlamydial heat shock proteins hsps60 and hsps70 and MIP. The glycolipid carbohydrate antigen known to be of consequence is rough type LPS, although existence of a smooth type LPS is suspected. A third non-LPS glycolipid is as yet poorly defined, but appears to play a role in Chlamydial virulence.

Protein antigens [2]

(1) *Major outer membrane protein:* The gene (*omp1*) codes for MOMP that is the predominant antigenic determinant in EB. MOMP is a 40 kilo-Dalton protein that defines the 15 serotypes of *Chlamydia trachomatis* from one another. Sequencing of *omp1* shows five constant regions and four variable regions (VS I – IV) in the MOMP protein. Epitopes determined by VS I, II and IV are associated with B lymphocyte receptors. These epitopes are found on the surface of EB, in the exposed surface of the MOMP molecule and are accessible to antibody. *Chlamydiae* are divided into subspecies serogroup B (serovars B, Ba, D, E, F, G K, L1, L2) and serogroup C (serovars A, C, H, I, J, L3). These two groups are separated from one another by epitopes in V IV. The Chlamydial serovars are determined by specific epitopes expressed in VS I and VS II. Epitopes determined by V III are thought to be associated with T lymphocyte receptors.

MOMP antigen may be for development of a Chlamydial vaccine, but successful use will depend on the ability of the organism to undergo antigenic change. Antigenic drift has been documented and possible major antigen shifts have noted, indicating that development of a vaccine may be problematic.

(2) *Cysteine-rich outer envelope proteins:* There are two so-called "cysteine-rich" proteins. The larger outer envelope protein envB is coded for by the *omp2* gene and is a 60 kiloDalton protein. The smaller of the "cysteine-rich" proteins, the outer envelope protein envA, is a 12 – 15 kilo-Dalton lipoprotein coded for by the gene *omp3*. The RB synthesize these proteins as they mature into EB. It is thought that the lipid moiety of the envA lipoprotein is embedded in the Chlamydial cytoplasmic membrane with the protein moiety in the periplasmic space, available for S-S covalent bonding with MOMP. Both cysteine-rich proteins form S-S bonds with MOMP conferring osmotic stability on the EB. The structural alteration caused by the S-S bonding changes the thickness and permeability of the outer cell wall and reduces the size of the organism from between 600 and 1 000 nm to between 200 and 300 nm.

(3) *Heat shock proteins*: The three Chlamydial heat shock proteins are 12, 60 and 70 kilo-Daltons in size (hsps12, hsps60 and hsps70). The hsps60 and hsps70 proteins are expressed early in the life cycle of RB. They are located in the outer membrane complex and are thought to be involved in trans-membrane transport. Location of Chlamydial hsps in the cell membrane may be associated with host immune response (*vide infra*).

(4) *Chlamydial macrophage infectivity potentiator protein* (MIP): This 29 kilo-Dalton lipoprotein is embedded in the cell wall of the EB. The proteins has *cis / trans* isomerase activity and appears to be associated with protein folding. The protein plays a role in Chlamydial replication and possibly virulence by as yet uncertain mechanisms. Antibody to MIP weakly neutralizes *Chlamydiae*.

Glycolipid and carbohydrate antigens[2]

(1) *Chlamydial rough LPS*: LPS plays an important role in the pathogenesis of Chlamydial disease. This LPS is a Chlamydial genus-specific epitope consisting of a trisaccharide molecule encoded by the gene *gseA*. The trisaccharide consists of 3 molecules of 3-deoxy-D-manno-octulosonic acid (KDO) linked α KDO 2→8,

α KDO 2→4. The α KDO 2→8 linked molecule is uniquely Chlamydial and immuno-dominant. The trisaccharide epitope is exposed on the surface of both EB and RB.

(2) *Chlamydial smooth LPS:* Definite proof of the existence of a smooth LPS associated with virulence is lacking.

(3) *Chlamydial exoglycolipid*: This antigen has been recognised because an anti-idiotype antibody significantly reduces infectivity of Chlamydiae in mice. Little is presently known about this glycolipid.

4.2.6 HOST-CHLAMYDIA INTERACTIONS

Host-cell interactions

The interaction between the host cell and the micro-organism is complex and many of the factors that regulate parasitic replication, persistence of infection or elimination of infection are unknown.[16] Metabolic functions encoded by the Chlamydial genome are limited and replication of RB is only possible because some functions (particularly provision of energy requirements) are parasitised from the host cell.

The role of the endosome membrane is uncertain, but the organisms are frequently peripherally sited in the vesicle, near to the membrane.[16] Other intracellular parasites, for example *Rickettsiae*, proliferate free in host cell cytoplasm. The Chlamydial endosome probably provides a stable osmotic environment and confers resistance to β-lactam antibiotics. The membrane may also serve to regulate transport of nutrients and metabolites. Another possibly is that enzymatic activity essential to the organism may reside in the membrane. Although the membrane of the endosome originates from the host cell plasma membrane, expansion of the endosome membrane appears to be a function of the micro-organism. Endosome membrane synthesis continues even if protein synthesis in the host cell is prevented by cycloheximide,[28] or if the host culture cell is anucleate.[29]

In addition to Chlamydia-mediated alterations to the endosome membrane, Chlamydial MOMP and other antigens are expressed in the surface plasma membrane of the infected cells.[2] The mechanism and the purpose of this phenomenon is not known. Failure to appreciate this factor has resulted in misinterpretation of immuno-histochemical findings in several studies of the morphology of Chlamydial infection (*vide infra,* **Morphologic Study**).

Other host-Chlamydial interactions include a possible regulatory function by host prostaglandins on Chlamydial replication.[2]

Delayed infections and latency of disease

Julius Schachter, one of the foremost researchers in the Chlamydial disease, has carefully and extensively documented Chlamydial infections.[*inter alia* 33-35] Latency of infection is characteristic of Chlamydial infection.[34-36] Ocular and genital infections may be asymptomatic and persist for months or years.[33,34,36] It has been observed that successive infants born to the same mother have developed Chlamydial blepharopyorrhea while the mother remained asymptomatic. This and other cases indicate that the infection may persist for *at least* four years.[36]

There is evidence that interferon-γ and lymphokines limit Chlamydial development and are associated with delayed infections *in vitro*.[37-39] The mechanism of delayed infections occurring *in vitro* is partly a function of numbers of infectious organisms (MOI).[16] Similar mechanisms could explain latency of infection in the *in vivo* clinical setting. Chlamydial infection is thought to remain quiescent and sub-clinical unless associated with a super-infecting pathogen.[34] The disease is associated with other known cervical infective pathogens in at least 60 % of cases, frequently gonorrhoea.[34]

Delayed or latent infections may offer an explanation for many of the failures to corroborate the morphologic diagnosis of Chlamydial infection that are recorded in the literature. "Microbiological indices in chlamydial infection do not coincide perfectly with clinical signs. Clinically delayed infections would be characterised by positive assays for Chlamydial antigen or DNA but only intermittently positive isolation of infectious EBs by tissue culture. It follows that the isolation of *chlamydiae* in cell culture would no longer be a gold standard criterion for infection."[2]

Inflammatory response

The inflammatory infiltrate associated with Chlamydial infection is mixed with lymphocytes, plasma cells[33,40-42] and in the acute phase, neutrophils.[33] The infiltrate may show a follicular lymphoid infiltrate.[40,42] The Chlamydial hsps70 has an N-terminal ATPase domain and a C-terminal peptide-binding domain that is structurally homologous to the major histo-compatibilty class I proteins (MHC I). This may allow partial resistance to tumour necrosis factor (TNFα) and superoxide ions. Chlamydial hsps60 and MOMP cause proliferation of host $CD4^+$ T-lymphocytes resulting in γ-interferon production. Chlamydial hsps production is up regulated by γ-interferon, and because of the similarity of hsps70 to MHC I, this may confer a survival advantage over cytotoxic T-lymphocytes.

Chlamydial LPS engages the epithelial receptor CD14 resulting in production of pro-inflammatory cytokines by host epithelial cells. Cytokines up regulate lymphocyte receptors promoting lymphocyte adhesion and MHC II proteins. The cytokines include TNFα that induces cell necrosis and leads to inflammation with fibrosis and scarring. LPS is a non-specific activator of macrophages and persists in macrophages and in addition, stimulates B lymphocytes.

The absence in the literature of morphologic criteria to enable diagnosis of "cervicitis" as distinct from inflammatory cells "normally" present in the endocervical mucosa is discussed elsewhere (*vide infra.* **Morphologic Study**).

Immune response[41]

Some aspects of Chlamydial histology "may be more related to the inflammatory response than to direct host cell destruction as a result of chlamydial growth and spread."[16]

In contrast to the phagocytic process in epithelial cells, when macrophages phagocytose EB, lysosomes do fuse with the endosome. Subsequent B-lymphocyte interactions with these macrophages result in production of IgM, IgG and IgA antibodies directed against MOMP (not LPS). The efficacy of these antibodies in controlling or modifying the infection

is uncertain. However, IgA in the cervical mucus appears to reduce the number of EB shed. T-lymphocyte interaction results in clonal proliferation of cytotoxic lymphocytes (class II HLA restriction).[16] The role and efficacy of these cytotoxic cells remains unknown. Tissue necrosis due to production of TNFα by induced epithelial cells leads to necrosis, fibrosis and scarring.[2] There is also evidence of a delayed hypersensitivity reaction to recurrent Chlamydial infection and scarring and fibrosis appears to be partly mediated by this delayed hypersensitivity reaction.

Host tissue response

The clinical lesion most commonly associated with active Chlamydial cervicitis is an erosion."[34] Cervical erosions indicate reactive proliferation of immature metaplastic squamous epithelium, with some cell showing features intermediate between mucinous and squamous cells,[44] and frequently with mucinous cells overlying the surface.[34,45,46] While trauma, endogenous hormone fluctuations and the contraceptive pill are also associated with erosions, Chlamydial infection may be a frequent and important cause. The clinical observation that erosions are a feature of Chlamydial cervicitis is supported by the morphologic observation[44] that Chlamydial inclusions are found in metaplastic squamous epithelium. However, the Chlamydial inclusions found in many erosions have been "discounted as 'non-specific corpuscles' by some workers." [34]

4.3 FACTORS AFFECTING THE RELIABLE DIAGNOSIS OF CHLAMYDIAL INFECTION

Several factors conspire to reduce the sensitivity of all laboratory tests for *Chlamydia trachomatis.*[1] These factors include the site from which the specimen is collected, the type of device used for taking the sample, the type of specimen used, the adequacy of the specimen taken and the conditions of storage and transportation of the specimen. The impact of these factors varies with the type of test to be used. Of the factors affecting all laboratory tests, probably the most important is the adequacy of the specimen taken. The consequences to reliable testing for *Chlamydiae* of these potentially confounding factors are reviewed below.

Specimen adequacy

As many as 30% of specimens were inadequate to detect Chlamydia during infertility studies funded by the Centres for Disease Control and Prevention (CDC) in the United States.[1] This was found to be true despite special training of personnel gathering test samples. Several studies have shown that the sensitivity and the specificity of diagnostic testing for the organism are directly related to specimen adequacy. This was found for tests using direct immuno-fluorescent techniques,[47,48] enzyme-linked immunosorbent assays (ELISA)[49,50] or both methods in sequence.[51] Again, the sensitivity of DNA amplification methodologies has been directly related to the quality of the specimen.[1] If quality of the specimen affects the sensitivity of immunologic and nucleic acid technology detection methods, then the sensitivity of culture studies should similarly be affected. However, studies addressing this question have only arisen when culture of the organism (the traditional gold standard) has been compared with more recently developed DNA tests.

Collection of specimens for culture

Since the organism is an obligate intra-cellular pathogen, the specimen collected for the detection of *Chlamydia trachomatis* should include host cells. [1] Most commonly, infected cells are obtained from the endocervical canal in

women, or the anterior urethra in patients of both genders. Urethral specimens should not be collected after micturition as this reduces the number of columnar cells available for collection.[1] In women, multisampling and pooling of endocervical and urethral specimens improves sensitivity of culture by 23 %.[52]

The use of correct techniques to obtain infected cells is necessary.[1,53] The collection device should be capable of removing cells from the host, but at the same time should not be toxic to the organism.[54] Cytology brush samplers have been recommended because more cells are obtained,[55] but in the hands of well-trained specimen collectors, the brushes are not more advantageous to outcome than swabs. [56] Calcium alginate-tipped swabs, and Cotton, Dacron, or Rayon swabs may be used, but these should be tested prior to use for toxicity on cell culture line. [54,57] Swabs with wooden shafts are unsuitable.[25] The swab should be inserted 1 to 2 cm deep into the endocervical canal and rotated for at least 15 seconds, or 4 cm into the urethra and rotated two or three times.[1]

The sequence of sampling for different tests is debatable. The CDC recommends that a sample for the gram staining of *Neisseria gonorrhoeae* should precede all other procedures. Any purulent secretions should be wiped away prior to sampling for Chlamydial culture because bacterial contamination of the specimen and toxic effects of purulent secretions on Chlamydial organisms may influence the outcome of culture. Again, purulent secretions affect the appearance of the smears in direct immunofluorescent testing.[53,58] The CDC recommend making a Papanicolaou smear before scraping for cells to culture.[59] However, the device used to make the smear, especially a brush, may induce bleeding and the presence of blood in the specimen may inhibit polymerase chain reaction (PCR) tests for Chlamydial DNA.[60] On the other hand, a Papanicolaou smear made after scraping the cervix for cells to be cultured may not be representative and may be blood stained.

Transport of specimens for culture

Culture of the organism for diagnostic purposes requires that they remain viable after collection until inoculation into the cell culture line has been effected. The specimen must therefore be appropriately stored and transported.[53,54] The outcome of culture is optimised if the specimen is immediately cooled and transported at a temperature between 2 and 8 degrees centigrade. A specimen stored in these conditions should be cultured within 48 hours. The specimen may be stored for longer periods at –70° C, but freezing reduces the likely success of culture by at least 20%.[54,60] Freezing at –20° C has an even more deleterious effect.[61] These demanding requirements do not apply to those test modalities that rely on detection of Chlamydial antigen or DNA. Thus, inappropriate transport of specimens for culture may militate against a successful outcome. Poor conditions while transporting specimens may detract from the value of culture as a bench-mark against which other test modalities are to be measured.

Transport media recommended for the preservation of the organism include 2-sucrose phosphate [62] or sucrose-glutamate phosphate.[63] The addition of 2 % or 5 % foetal bovine serum may assist to preserve the viability of *Chlamydiae* in frozen specimens. Antibiotics to which *Chlamydiae* are resistant may be included in transport medium to prevent the growth of fungi or bacteria. Gentamycin or Vancomycin are effective against bacterial contamination and Amphotericin B or Nystatin prevent fungal over growth. Effective "universal" synthetic transport media (M4® transport media [MicroTest Inc.], and FlexTrans® media [Bartels Diagnostics]) are also commercially available.[63] Unlike sucrose-

glutamate phosphate, the "universal" media and 2 sucrose-phosphate are also suitable for non-culture tests, both ELISA and PCR.[64]

Collection of specimens for detection of Chlamydial antigen or DNA

Tests relying on the detection of Chlamydial antigen or DNA are usually commercially developed systems. Specific collection protocols using prescribed collection materials are recommended by the manufacturer. Most of the principles of specimen collection developed for culture of Chlamydia apply to these tests.[1] Specimens should be collected according to the manufacturer's instructions and the tests should not be performed on specimens for which the test has not been designed.[59] The inappropriate use of a test method on a particular type of specimen may lead to diagnostic confusion. For example, vaginal, rectal, nasopharyngeal and female urethral specimens are not regarded as appropriate for diagnosis of Chlamydia by antigen detection methods.[59]

Testing of urine is non-invasive and can be used for screening of asymptomatic populations. However, urine specimens have been shown to be of low sensitivity in detection of Chlamydia by culture or antigen detection methods.[65,66] Recently, a major advance has been the development of tests detecting Chlamydial DNA by amplification technology in urine specimens.[1] The amplification tests are able to detect very small amounts of Chlamydial DNA present in the urine because of urethral infection. Amplification of Chlamydial DNA in urine specimens has been shown to correlate[67] with cervical infection possibly because of contamination of the urine by cervical or vaginal secretions.

Although urine specimens are less likely to be inadequate than cervical or urethral samples, there are some restrictions on the collection of urine suitable for antigen detection or DNA amplification.[59] The specimens should preferably be "first catch", although mid-stream urine specimens have been successfully used for ligase chain reaction (LCR) testing of men attending a sexually transmitted diseases clinic.[68] In females, the perineum should *not* be cleaned prior to collection of the specimen. The urine should be collected not less than 1 hour nor more than 2 hours after previous micturition and should be of appropriate volume (10 to 20 ml). The time since previous micturition is of more importance in specimens from women than in men. Times in excess of 3 hours profoundly reduces sensitivity of antigen detection tests in women,[69] but not in men.[69,70]

Transport and storage of specimens for detection of Chlamydial antigen or DNA

The low pH and high urea content of urine rapidly denature Chlamydial DNA at ambient temperatures, especially above 25° C.[59] The urine should be voided into a clean container and immediately cooled to 2 to 8° C. Urine for PCR testing can be stored in these conditions for up to four days. Freezing and thawing of the urine prior to PCR testing may increase sensitivity by removal of transient inhibitory factors in the urine.[70]

Other factors affecting sensitivity of laboratory testing

The sensitivities of methods for the detection of *Chlamydia trachomatis* are enhanced by testing of multiple specimens.[52,71] Testing of either a single urine sample or an endocervical specimen by culture, direct fluorescent antigen

testing, PCR, and LCR did not identify all infected women, but combining of the results of both tests did.[71] Studies on whether endocervical specimens or urine specimens are more likely to detect *Chlamydia trachomatis* infections have been discordant.[71-73] Studies comparing the preferred site for obtaining a sample may underestimate the value of cervical specimens if they are not collected properly.[1] On the other hand, a more sensitive test methodology may allow specimens to be taken from sites traditionally regarded as unsuitable. Thus, urine tested by PCR or LCR is superior to urine tested by culture.[74,75] Again, vulval specimens *collected by the patient* and tested by PCR yield superior results to cervical specimens taken by specially trained personnel and tested by culture.[75]

Quality assurance for specimens

The CDC in the United States recommend that the quality of specimens collected for Chlamydia testing should be monitored.[1] Without assuring the quality of specimens over a period of time, up to 10% of samples may be found to be unsatisfactory.[49,56] Inadequate specimens have been defined by the CDC as those in which no columnar or metaplastic squamous cells are found by microscopic examination. Samples collected for DNA testing using commercially available methods may not be suitable for assessment by cell count because they frequently employ detergent-containing specimen collection media. The detergent is present to lyse cellular components and Chlamydial elemental particles. This facilitates the release of free Chlamydial DNA in the sample, upon which the test is performed.

4.4 METHODS FOR THE DETECTION OF *CHLAMYDIAE*

4.4.1 MICROSCOPIC DEMONSTRATION OF THE ORGANISM

4.4.1.1 CONJUNCTIVAL SMEARS

The presence of typical Chlamydial intracellular inclusions in direct smears of the conjunctival epithelium in neonates is a rapid and useful method to diagnose acute inclusion conjunctivitis.[1] Conjunctival smears are said to show numerous inclusions in a relatively clean debris-free preparation.[5] Cytologic demonstration in conjunctival smears is said to have a sensitivity of > 90 % in neonates, but in adults the sensitivity is much lower. These smears are stained using the Giemsa method and the use of control slides from Chlamydial cell cultures is recommended. Test smears should be of adequate cellularity (at least 10^3 cells per smear) if high sensitivity is to be achieved. The identification of the inclusions requires considerable experience and expertise and should be restricted to laboratories that perform the test regularly and in adequate numbers to maintain a high level of expertise.

4.4.1.2 FEMALE GENITAL TRACT SMEARS

The seminal cytological work by Gupta *et al*[76]

Gupta *et al* indicated in 1979 that the diagnosis of Chlamydial infection could be made using Pap smears. They described in detail the morphologic features to be found in Pap smears. Three stages were described, each apparently with different morphologic features:

(1) *The first stage of infection* was characterised by the presence of intra-cytoplasmic coccoid bodies:

(a) infected cells were enlarged, 15 to 20 mμ in diameter and infected by intra-cytoplasmic coccoid bodies;

many of the infected cells were metaplastic squamoid cells. Columnar cells were usually involved and in some cases, only columnar cells were affected;

(b) the cells occurred singly or small groups of two to six cells;

(c) infected metaplastic squamoid cells could be multinucleate and four to eight nuclei were common. The nuclei did not mould one another. Multinucleated infected columnar cells were uncommon;

(d) nuclei of infected cells were classified as "atypical" in the vast majority of cases. The nuclei were well preserved, enlarged and hyperchromatic with undulated nuclear envelopes. The chromatin was diffuse and resembled cells undergoing repair. Nucleoli were rare and inconspicuous in both metaplastic and columnar cells;

(e) particularly in the absence of inflammation, the nuclear changes resembled mild or moderate atypia (CIN I or CIN II), or folic acid deficiency;

(f) the cells showed distinct outlines and the cytoplasm was usually cyanophilic and rarely acidophilic. The cytoplasm was often "rarified" showing a "porous appearance" in the peri-nuclear region and occasional the entire cell was affected;

(g) finely granular, uniformly sized, coccoid bodies were present in the rarified areas of cytoplasm. The coccoid bodies were cyanophilic or acidophilic;

(2) *The second stage of infection* was characterised by the presence of free-lying Chlamydial organisms in the cytoplasm and by cytoplasmic Chlamydial "inclusion bodies":

(a) infected cells showed textured and finely vacuolated cytoplasm contained granular coccoid eosinophilic or basophilic *Chlamydiae* in a peri-nuclear distribution. These organisms occurred in both loose and compact forms;

(b) cells contained single or multiple inclusion bodies 1 to 2 mμ in diameter. The photomicrographs of these inclusions depicted small intra-cytoplasmic vacuoles containing central targetoid formations;

(3) *The third stage of infection* showed

(a) infected cells containing free-lying acidophilic and dense aggregates of *Chlamydia* particles with multiple larger inclusion bodies randomly or in peri-nuclear distribution;

(b) the intra-cytoplasmic inclusion bodies were uniform, frequently moulding or overlapping, with crisp borders;

(c) The inclusions contained a central basophilic or a diffuse, uniformly staining acidophilic condensation with a clear zone around them.

This description noted that the inflammatory infiltrate associated with Chlamydial infection was usually neutrophilic and that lymphocytes and plasma cells were uncommon. Gupta *et al*[1] indicated that follicular lymphoid cervicitis was not always associated with the presence of Chlamydial organisms (coccoid bodies).

Emphasis was placed on the recognition of intra-cytoplasmic Chlamydial coccoid bodies. Chlamydial "inclusion bodies" (vacuolar inclusions) were regarded as a degenerative phenomenon. In their discussion of Chlamydial changes, these authors indicated that caution was necessary in diagnosing Chlamydia where inflammation was present because inflammation is associated with degenerative cytoplasmic vacuolisation. Indeed, Chlamydial (vacuolar) inclusion bodies were regarded as degenerative in nature. However, the inclusion bodies were regarded as diagnostically useful because the morphology was constant and distinct. Inclusion bodies without the presence of intra-cytoplasmic coccoid bodies were not regarded as sufficient to diagnose the infection. The description emphasised the necessity to separate these degenerative but Chlamydia-specific forms from other degenerative changes.

The morphologic diagnoses based on their criteria were corroborated with three test modalities. Positive cultures were obtained in 25% of cases tested. Immuno-fluorescence to demonstrate Chlamydial antigen was performed on 20 cases but the number confirmed by this method was not indicated. EM was successfully performed on cells lifted from two smears.

In standard microbiology texts, the usual form taken by the intra-cellular forms of *Chlamydiae* Chlamydial are described as inclusion bodies.[5,6] Apparently for this reason, most subsequent investigators have concentrated their attention on the diagnostic importance of the (vacuolar) inclusion bodies.[2-4, 7-10, 12-15] Little, if any attention has been paid to the diagnostic value of finding Chlamydial coccoid bodies.

Subsequent to this seminal paper, numerous workers have investigated the morphologic diagnosis of Chlamydial infection in Pap smears. Attempts to confirm the morphologic diagnosis have made use of a variety of corroboratory methods including culture, serology, immuno-fluorescence, immuno-peroxidase staining and electron microscopy (EM). A number of such studies have not shown correlation with the Pap smear result. [*inter alia* 4-6, 9-15] Some researchers agree with Gupta *et al*[1] that the vacuolar inclusion bodies are not specific indicators of Chlamydial infection.[9,10,12-15] Other authors regard the vacuolar inclusions as non-specific, possibly degenerative or inflammatory changes.[5,7,11] Several papers have concluded that an insufficient proportion of Pap smears have been corroborated and actively discourage use of the Pap smear as a diagnostic test. [*inter alia* 4,7,8]

Immunoperoxidase confirmation in Pap smears

The publication in 1985 by Shiina[77] specifically addressed the problem first articulated by Gupta *et al*, [76] that of distinguishing Chlamydial vacuolar "inclusion bodies" from mucus inclusion vacuoles and inflammatory/degenerative vacuoles. Five types of vacuolar inclusion were identified in Pap smears and described in detail. Smears showing these inclusions were re-stained by the immuno-peroxidase (IP) staining method using a anti-Chlamydia species-specific, monoclonal mouse antibody. Subsequently, the same smears were exposed to amylase digestion to destroy Chlamydial glycogen before they were stained with PAS to demonstrate mucin. In this work, positive controls were HeLa 229 cells infected by Chlamydia and negative controls were un-infected HeLa cells.

In the study by Shiina,[77] Chlamydial antigen was demonstrated by IP within vacuolar inclusion in only 13 of 183 inclusions. Positive staining vacuoles inclusions were almost exclusively of the "nebular-type" and the antigen was found in 85.8 % of these inclusions. However, antigen was not confined to the vacuolar inclusion. Antigen was also found in the cytoplasm of intermediate squamous cells, metaplastic squamous cells, "tissue repair cells," parabasal cells and columnar cells. The distribution of antigen in the cells was categorized into several patterns, only one of which showed antigen with a surrounding membrane (the "nebular inclusions"). This cytoplasmic staining may have been due to the presence of coccoid bodies in the cell cytoplasm, but Shiina apparently did not recognise these nor did he discuss this possibility.

Shiina[77] found PAS-staining material in up to 82% of non–nebular inclusions and regarded this as evidence that the non-nebular inclusions contained mucin and were not Chlamydial. He concludes that only the nebular inclusions should be regarded as diagnostic of Chlamydial infection. Other types of vacuolar inclusions were regarded as non-diagnostic because these inclusions contained mucin and did not show the presence of Chlamydial antigen within the inclusion. However, this author notes that 12% of nebular inclusions also contained this mucin and that PAS-staining material also occurred in control HeLa cells containing nebular inclusions. The author apparently assumed that all Pas-staining material is mucin. In this publication it is not stated whether smears without nebular inclusions, but with other types of inclusion, showed antigen in the cytoplasm of cells.

Shiina[77] did not indicate which Chlamydial antigen was demonstrated using the commercially acquired antibody (Ortho Diagnostic Systems). However, in the discussion he notes that while IP was the best available method to show Chlamydia, selection of antisera, and some technical aspects of immuno-staining remained problematic. If the antibody used in his study was directed against Chlamydial MOMP, it could be expected that some Chlamydial inclusions would not stain positively (own observation). After infection of a host cell, Chlamydial MOMP is markedly reduced[78] but the cell wall protein is synthesized and expressed in the host cell membrane after approximately 18 hours in the life cycle.[2,78] Prior to that, the main antigenic determinant being synthesized is Chlamydial lipopolysaccharide (LPS).[2,78]

Although it is not stated in his publication, it seems likely that the antibody used in the Shiina study was indeed against MOMP. For technical reasons, antibodies directed against Chlamydial LPS antigen employ immunofluorescent chromogens.[79,80] Shiina used a peroxidase-antiperoxidase enzyme labeling system, not a fluorescent system. Unfortunately, unfixed fresh frozen cells would not be easily available in Pap smear form for immunofluorescent staining for LPS.

Meta-analysis by Bernal *et al*[81]

In 1989, Bernal *et al*[81] conducted a study using two endocervical samples taken from each patient at the same time. An endocervical smear was prepared for cytology and a swab taken for immunofluorescent detection of Chlamydia trachomatis by the Microtrak-Siva method. Various different cyto-morphologic criteria published, including those of Gupta *et al*[1] and Shiina,[77] were applied to the cytology smear. The findings using each method were recorded separately.

The cytology results were subsequently compared with the immunofluorescent test and the sensitivities and specificities of the different sets of criteria were calculated. Using the Microtrak-Siva method for immuno-fluorescence as a gold standard, the sensitivity of cytologic detection of the infection was not high (19% using the criteria of Gupta *et al*[76] and 38% using the criteria of Shiina[77]). However, evaluated against this Microtrak-Siva system, both sets of criteria were found to be highly specific compared to culture methods.[81] The specificity of the criteria enunciated by Gupta *et al* was 86% and those of Shiina, 91%.

In a meta-analysis, these authors evaluated nine publications in which the morphologic criteria published by Gupta *et al*[76] had been used for the diagnosis of Chlamydial infection. This revealed that using the criteria defined by Gupta *et al*, cytology has an average sensitivity of only 27% in the diagnosis of Chlamydial infection. They compared this outcome with culture, which had a sensitivity between 70 and 85%, [81] and concluded that cytology is not a good screening test for Chlamydial infection. Although the combined *specificity* of cytology derived from these publications was 79%, the conclusion drawn by Bernal *et al* was that Pap smear diagnosis of Chlamydial infection is not sufficiently accurate. They stated that "because of the implications of a misdiagnosis of a sexually transmitted disease, it is concluded that cervical cytology is not useful for ascertaining the presence of *Chlamydia trachomatis*." [81] In the investigator's opinion, a more valid conclusion would have been that although Pap smear diagnosis of Chlamydial infection is accurate, because of low sensitivity, it is not be recommended as a *screening* procedure.

It was a crucial aspect of this investigation to address the contention by numerous workers that vacuolar inclusions are a non-specific and degenerative phenomenon, or that inclusions contain mucin not Chlamydial organisms. The full cycle of cell infection and replication of Chlamydial organisms leads eventually to cell lysis and is reported to take approximately 40 hours.[2,41] It was reasoned that many cells would show changes in the earlier part of the life cycle that could not be dismissed as evidence of impending cell death. Signs of cell death could be expected late in the life cycle of the organism, after about 30 hours.[2,41] Cell death is part of the process and the signs of imminent disruption of the cell constitute part of the evidence of Chlamydial infection. Although a possible source of diagnostic confusion, these late signs of cell death should not be dismissed as unimportant. In addition, it remained necessary to show that the cytoplasmic vacuoles and nuclear alterations in metaplastic cells were not merely non-specific degenerative changes.

4.4.1.3 HISTOLOGIC DIAGNOSIS IN FEMALE GENITAL TRACT.

The possible diagnosis of Chlamydial infection in the uterine cervix by histology has addressed in several articles.[*inter alia* 7,40,82,83,84-86] In the book by Mårdh *et al*,[86] the authors note that although Chlamydial infection is an important cause of cervicitis, very little has been written about the histologic appearance of the disease. These authors review several publications describing the inflammatory changes associated with Chlamydial infection without mention of Chlamydial inclusions in epithelial cells. Despite commenting on the paucity of literature until 1989, they did not mention the seminal work by Swanson *et al*[44] (published fourteen years previously). Several other publications have followed suit, de-emphasising the importance of Chlamydial inclusions.[40,82,83,85,87] In 1984, Crum *et al*[7] concluded that lymphoid follicular inflammatory changes in cervical biopsies should alert the physician to possible Chlamydial infection. Again,

in 1999 Crum[88] and co-authors emphasised that follicular cervicitis was suspicious of Chlamydial infection, and warned that "raising suspicion of Chlamydia based on these changes alone (inclusion vacuoles) is not recommended."

The seminal histological work by Swanson *et al*[44]

In 1975, during a study of nine cases of cervicitis due to *Neisseria gonorrhoea,* Swanson *et al*[44] found Chlamydial inclusions in cervical biopsies from two patients. They described the morphologic features of the Chlamydia infection by light and electron microscopy. These workers acknowledged that scanty Chlamydial inclusions had been seen in biopsy material from the transition zone of the uterine cervix for the first time in 1938. Braley[89] found cervical Chlamydial inclusions in six women whose neonates had inclusion conjunctivitis. Swanson studied biopsies taken from these two patients for EM 7 and 11 months after the gonorrhoea had been treated. At that time of EM, one of the two patients had a cervical 'erosion,' high-titre serum anti-Chlamydial antibodies and a positive Chlamydia culture. The other had a normal cervix, high titre serum anti-Chlamydial antibodies and negative culture. Chlamydial inclusions were demonstrated in cervical biopsies from both patients.

Swanson *et al*[44] identified Chlamydial inclusions in epoxy embedded sections by light microscopy. These were most easily demonstrated in columnar mucinous epithelial cells, but were also found in metaplastic squamous cells. The vesicular inclusions showed numerous bodies measuring approximately 1mμ in diameter. Paraffin-embedded tissues showed the same intra-epithelial vesicles but the intravesicular inclusions (organisms) were not as easily seen. The micro-organisms were eosinophilic or haematoxylinophilic, stained weakly with silver using the methenamine silver technique, and were stained blue-violet with Giemsa stains.

Using EM, the vesicles showed micro-organisms with features typical of *Chlamydiae*. RB, EB and intermediate forms were identified and are well illustrated in their publication. These workers compared and corroborated their morphologic findings with descriptions by other workers. The same appearances had been described in six publications of the EM morphology of *Chlamydiae* in cultures, and two describing the EM features in conjunctival infections. They noted that RB were frequently found close to the vesicle membrane. The Chlamydiae were identified in cells showing mucinous differentiation with surface microvilli, in metaplastic cells showing tonofilaments and intermediate cells with blunted vili and tonofilaments. Some of the host cells showed degenerative changes with cytoplasmic lysosomes, condensation of cytoplasm, dilated mitochondria and marginated nuclear chromatin.

These workers suggested that recognition of the Chlamydial inclusions in exfoliative cytology smears and biopsy could prove useful in the diagnosis of Chlamydial infection.

The view suggesting inflammatory changes are the key

As noted previously, the emphasis of diagnosis has not always been placed on the identification of the presence of the vacuolar inclusions in metaplastic squamous cells. Three publications by Kiviat *et al*[83,85,87] used immunofluorescent staining directed against MOMP. While Chlamydial inclusions were found, these were infrequent and not found to be a

useful feature in most cases.[85,87] These workers identified muco-purulent cervicitis due to Chlamydia as indicated by positive culture and immuno-fluorescent detection of MOMP in extra-cellular EB in smears of cervical mucus.[83,85] They compared the morphology of Chlamydial infection with the changes of cervicitis due to infections by *Neisseria*, *Trichomonas* and *Herpesvirus*. An important defining feature of Chlamydial cervicitis was the presence of neutrophils infiltrating columnar epithelium and lymphoid follicles with germinal centres. Other features were dense sub-epithelial inflammation with predominantly plasma cells. They noted that Chlamydial infection occurs predominantly in mucous epithelial cells and found that inflammation was maximal around mucinous glands proximal to the squamo-columnar junction.[85] Of interest was the observation that some glands were spared and did not show inflammatory changes. This may indicate focal involvement of the endocervix (own observation). Ulceration was rare.

Hare *et al*[82] investigated the colposcopic appearances of Chlamydial infections and examined colposcopically directed biopsies form culture-proven cases of Chlamydial cervicitis. Follicular cervicitis, seen colposcopically as creamy white nodules on the cervix and in the biopsies, as lymphoid follicles, was found to correlate with Chlamydial infection. These workers noted that the follicles were situated beneath immature metaplastic squamous epithelium, in contrast to the finding by Kiviat *et al*[85] that inflammation was maximal near mucus glands proximal to the squamo-columnar junction. They found that patients with cervicitis due to *Mycoplasma hominis* and *Ureaplasma urealyticum* did not show similar changes. In publications by Paavonen *et al*[40,86] the emphasis was similarly placed on the presence of a chronic lymphoid inflammatory infiltrate, sometimes with a reactive follicular component.

Crum *et al*[7] screened 102 cervical biopsy specimens with inflammation for evidence of Chlamydial antigen (CAg) (*vide infra*). They found only 7 CAg positive cases, but noted a strong association with severe inflammation. Of the 102 cases, a sub-group of 27 cases showed severe inflammation and 6 (22%) were CAg positive. They concluded that severe inflammation should alert the physician to possible Chlamydial infection. Only 9 the 27 cases showing severe inflammation showed follicular cervicitis with reactive lymphoid follicles (germinal centres). Unlike Kiviat et al,[83,85,87] Paavonen et al[40,86] and Hare et al,[82] they did not link the follicular lymphocytic inflammation with the presence of CAg. Furthermore, they emphasised the importance of Chlamydial inclusions and regarded these as "the most conspicuous feature."

The view suggesting mainly mucinous cells are affected

In the studies of Kiviat *et al,*[85] and Hare *et al,*[82] it was stressed that the cervical mucinous epithelium was mainly affected, supporting an observation made by Swanson *et al.*[44] However, Swanson *et al*[44] Gupta *et al*[76] and Crum *et al*[7] also described the presence of Chlamydial inclusions in metaplastic squamous cells. Crum et al found that Chlamydial antigen was most prominent in mucinous columnar cells.

Immunohistochemistry for Chlamydial antigen

Crum *et al*[7] screened 102 cervical biopsies using the immunoperoxidase technique to detect Chlamydial antigen (CAg). The primary antibody used was a polyclonal rabbit antibody directed against a species-specific antigen reactive with Chlamydial serotypes A through K. The primary antibody being polyclonal, it is difficult to know which antigen(s) were

targeted. However, the basis for classifying serovars A to K is by typing the MOMP antigen.[2] It probable that the antibody was directed at MOMP.

Seven of the cases 102 (6.9%) showed CAg. The CAg detected was found *predominantly in mucinous endocervical epithelium* and only rarely in the metaplastic squamous epithelium. The staining in mucinous cells was both cytoplasmic and within the Chlamydial inclusions. Many of the inclusions, particularly in the metaplastic and reparative cells, but also in some mucinous columnar cells did not stain for CAg. Mucicarmine and periodic acid Schiff stains (PAS) showed mucin in many of these vacuoles leading the authors to the conclusion that many CAg-negative vacuoles were mucinous in nature. This paper did not indicate whether or not the PAS stain was performed with or without diastase pre-treatment. Since *Chlamydiae* accumulate glycogen, [2,16] some of the PAS–positive staining may not have been due to mucin.

The MOMP antigen is most prominent in EB and is less abundant in RB.[2,16] If the primary antibody used by Crum *et al* did not detect Chlamydial LPS, some inclusions containing mainly Chlamydial RB may not have stained positively. The expression of Chlamydial MOMP in the host cell membrane[2,16] may explain the staining of both cells and inclusions (own observations).

The relationship of *Chlamydiae* and atypia/intra-epithelial neoplasia

Numerous workers have reported a strong association of Chlamydial infection with cellular atypia of host cell nuclei, and with CIN.[*inter alia* 90-104] Crum *et al*[7] noted that 3 of their 7 cases showed atypia in cells showing reparative changes in metaplastic squamous epithelium adjacent to cells positive for CAg. One case showed concomitant HPV infection with CIN II. The potential of Chlamydia to cause cellular changes, and the extent and severity of cellular changes due to Chlamydial infection has not been investigated. Immature squamous metaplasia manifest as "cervical erosion" and reactive changes is attributed to the organism.[34,44] The association of Chlamydial infection with HPV infection and intra-epithelial neoplasia, although well known has not been explained. Epidemiologic evidence suggests that any relationship between these entities is the consequence of both conditions being sexually transmitted diseases. The spectrum of morphologic changes from reactive through "atypical" to intra-epithelial neoplasia has not been investigated to identify whether Chlamydia has a causal role. Using *in situ* PCR, Schlott *et al*[92] demonstrated the presence of *Chlamydia trachomatis* DNA in CIN and cervical carcinomas. Schlott and the researcher are collaborating presently, using micro-array gene expression studies to investigate the potential for oncogene production in Chlamydia trachomatis infection (unpublished data).

Electron microscopic features

Only a few attempts to corroborate morphologic diagnosis of cervical Chlamydia infection by EM have been reported.[44,76,105] On the one hand, Gupta *et al*[76] and Swanson *et* al[44] had examined cervical tissues by EM and concluded that the organisms identical with those seen in similar inclusions in cell cultures are indeed present in the vacuolar inclusions. The study by Henry *et al*[105] only demonstrated Chlamydial organisms by EM in nebular type inclusions in

metaplastic squamous cells lifted from Pap smears. The latter workers concluded that non-nebular vacuolar inclusions in these cells were not diagnostic.

Concluding remarks

The majority of workers have not been able to convincingly corroborate the reliability of a morphologic diagnosis of Chlamydial infection. The current attitude in medical practice to morphologic diagnosis of Chlamydial infections is summed up by Crum *et al*: [88] "Whether these inclusion vacuoles can be identified and distinguished from noninfectious vacuoles, by histology or cytology, is controversial. Raising the suspicion of Chlamydia based on these changes alone is not recommended."

4.4.2 CULTURE OF *CHLAMYDIAE*

Specificity and sensitivity

The specificity of culture for detecting *Chlamydia trachomatis* approaches 100%.[106] For this reason, culture has been until recently, considered to be the "gold standard" for the laboratory detection of the organism. However, culture suffers several disadvantages compared with newer DNA detection methods. Firstly, culture detects only viable elementary particles of the *Chlamydiae*. Secondly, a major disadvantage of the method is its low sensitivity. Depending upon the expertise of the laboratory, the sensitivity of culture has been found to vary between 70% and 85% compared to DNA amplification techniques.[64,107] A further shortcoming is the necessity for strict "cold chain" conditions in the transportation of the specimen to the laboratory. Other problems include the expense of running a culture facility, the need for special expertise and the time taken to culture the organism (*vide infra*). [1] Perhaps most importantly, successful culture only occurs "if sufficient numbers of viable Chlamydial elementary bodies are present" in the specimen. The MOI for successful culture lies between 50 and 100 organisms per host cell.[16]

Immortalised cell line

The culture of Chlamydia requires inoculation of immortalised cell lines, usually HeLa 229 cells [108,109] or McCoy cells.[110-112] Ideally, the cells should be grown on coverslips contained in shell vials. Use of multi-well culture dishes increases the possibility of cross contamination of specimens.[1] Cells are grown in Eagle's growth medium supplemented with aminoacids and vitamins, 5% to 10% foetal calf serum, extra glucose (0,056M) and L-glutamine (2mM). HEPES buffer may be added to the medium to maintain a neutral pH. The sensitivity of culture is increased if the culture medium contains between 0,5 and 1,5 µg/ml cyclo-heximide to selectively inhibit host cell protein synthesis. [110,112] Also added to the culture medium are the same antibiotics as used in transport medium, Gentamycin or Vancomycin to prevent bacterial growth and Amphotericin B or Nystatin to prevent fungal growth.[64]

Inoculation of the cell culture

Inoculation of the cell culture should follow disruption of the host cells and Chlamydial inclusions in the specimen. This process releases the Chlamydial elemental bodies, and assists to separate them from one another. Disruption of cells is best achieved by sonication of the specimen [113] but may also be achieved by agitation with glass beads. The sonicated

specimen in transport medium is poured over a cell monolayer. For significantly better results, the inoculated monolayer is then centrifuged.[113] Centrifugation causes alterations in the host cell membrane that enhance penetration of the cells by the Chlamydial elemental bodies. The number of inclusions found in infected cells can be enhanced 100 to 1 000 fold by centrifuging at 3 000 G for one hour at temperature of 35°C.[108] The monolayer of HeLa 229 cells or McCoy cells is more susceptible to infection it is pretreated by DHEA-dextran.[108,109] The residual specimen is aspirated from the monolayer to prevent cytotoxic effects and fresh culture medium added.

Demonstration of Chlamydial growth

Chlamydial inclusions are visible by light microscopy in the host cells 48 to 72 hours after inoculation of the monolayer. The inclusions may be specifically identified as Chlamydial by the use of fluorescent antibody staining.[112,114,115] The monolayer is stained with a fluorescent antibody that binds Chlamydial LPS common to all species of *Chlamydiae*. Alternatively, the inclusions may be stained with a fluorescent monoclonal antibody that binds MOMP specific to *Chlamydia trachomatis*. "The direct visualization of inclusions that possess a very distinctive morphology contributes to the near 100% specificity of culture tests."[1] Culture results should not be assessed by enzyme-linked assays, since this results in the loss of the "high specificity produced when inclusions are directly visualized."[116] Other less specific stains have been used previously and include Grams, Giemsa and iodine stains. Use of these less specific stains is discouraged as misinterpretation of the morphology of inclusions in the cell monolayer may affect specificity of the culture result. Recognition of the organisms "requires a skilled microscopist" and depends on "the colour reaction, morphology and location of the inclusion."[1]

The paradox concerning culture

It seems paradoxical that many cytopathologists, by definition skilled microscopists, conclude that it is not possible to reliably identify *Chlamydia trachomatis* in a Papanicolaou smear using morphologic features and the location of the inclusion within the cells. However, they will accept the identification of Chlamydial infection when *microbiologists* apply the same *morphologic* criteria to decide the outcome of a Chlamydia culture.

Improving the sensitivity of culture

The sensitivity of the culture method can be enhanced by the further passage of apparently uninfected cell monolayers. The monolayer is sonicated and then used to inoculate a new monolayer. This process results in detection of a further 3% to 10% of positives cases.[117] Extending the duration of the culture may also increase sensitivity.[118]

4.4.3 DETECTION OF CHLAMYDIAL ANTIGEN

Detection of Chlamydial antigen is achieved by either direct fluorescent antibody tests (DFAT) or enzyme-linked immunosorbent assays (ELISA). These methods may be designed for sophisticated laboratory conditions, or for "point-of-care" testing in the physicians office, outpatient clinic or hospital ward. Immuno-histochemical detection of Chlamydial antigen is a further option, allowing demonstration of the organism in tissue sections or cytology preparations.

4.4.3.1 DIRECT FLUORESCENT ANTIBODY TEST (DFAT)

Methodology

The DFAT is generally performed on a smear made from a scraping of epithelial cells from the infected epithelial surface, although urine samples containing epithelial cells have been used successfully.[119,120] The smear is air-dried or acetone fixed.[121] The presence in the specimen of adequate numbers of epithelial cells is essential.[1,52,56] The organisms (Chlamydial antigen) are detected by anti-chlamydia antibody conjugated to a fluorescent label. The smear is examined under ultra-violet light (UV) to detect the presence of the fluorescent antibody/antigen complex. The wave-length of the UV to be used is prescribed for each particular commercial test product. The fluorescent bodies detected should be the appropriate colour for the fluorophore used, and show appropriate morphology and location. Reticular bodies (RB) should be seen within cells and elemental bodies (EB) should be the appropriate size and be located between cells and in mucus streaks. The sensitivity of the test can be improved by centrifugation of the sample directly onto a glass slide.[122-124] Centrifugation also allows the DFAT to be conducted on urine samples.[119,120]

Specificity and sensitivity

When both are performed optimally, compared to culture, the DFAT has a sensitivity of between 80% and 90%, and a specificity of 98% to 99%.[121,125,126] DFAT combines the detection of Chlamydia-specific antigen with direct localisation of the organism in the specimen. The high specificity of the test depends on specific antibody-antigen staining of inclusions and elemental bodies that, at the same time show the characteristic morphology of *Chlamydiae.* A further advantage of the test is that the number of columnar epithelial cells present in the specimen can be determined and the representivity of the specimen assessed. The most recently available tests employ monoclonal antibodies to the Chlamydial MOMP or LPS and are more specific than older tests that used polyclonal antibodies. Tests using anti-MOMP antibodies are superior to those using anti-LPS antibodies because LPS is sparsely present in EB. Species-specific antibodies to the *Chlamydia trachomatis*-specific epitope of MOMP specifically identify the organism.[1]

DFAT has been shown to detect *Chlamydia trachomatis* infections in specimens scraped from the endocervix,[122,127,128] the conjunctiva,[129,130] the urethra,[127,131] the rectum[132] and the respiratory tract in infants,[133] and in urine.[119,120] The DFAT method requires expert examination of each specimen by fluorescence microscopy. The technique is subject to inaccuracies occasioned by various problems. These problems were highlighted in a College of American Pathologists (CAP) quality assurance study and include method of fixation of the specimen, the number of EB present, the serotype of the Chlamydia present, the fluorophore used and importantly, the expertise of the laboratory personnel.[128] A minimum number of ten EB is considered necessary for a reliable clinical diagnosis of infection. Reducing the cut-off to only two EB may be used to increase sensitivity,[134] but results in lower specificity.[135]

The DFAT is no longer widely used as a primary diagnostic test because the test is labour intensive and requires highly skilled personnel to perform the microscopy.[1] However, because of its high specificity, the DFAT tends still to be used to confirm positive results obtained using other non-culture techniques.[123,124] Further, the DFAT has proved useful in studies using PCR or LCR, including studies comparing PCR and LCR, to set threshold levels for diagnosis of

Chlamydial infection.[64,67,107,136,137] In these comparative situations, and when used as a corroboratory test, positive cases have been defined as those where only two (not 10) EB have been seen by DFAT.

4.4.3.2 ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

During the 1980s, ELISA tests for Chlamydia were developed and the technique is the most widely used in commercially available tests for *Chlamydiae*.[1] ELISA testing employs the use of an antibody bound to an enzyme. Detection of the target antigen results in the formation of an immune-complex linked to the enzyme. A second step in the test employs the enzyme to catalyse a chromogenic reaction, converting a colourless substrate to a coloured end product. A positive test is indicated by a change in colour that can be measured by a luminometer.

Methodology

The ELISA tests are immuno-chemical reactions in which enzyme-labeled antibodies detect Chlamydial LPS, which is a genus-specific antigen. LPS is more soluble than MOMP and considerable quantities of the LPS antigen are expressed in the surface membranes of infected cells.[138] For these reasons, LPS is more abundant in most specimens and is a useful marker for the presence of *Chlamydiae*. The enzyme is bound to the specific anti-LPS antibody. This conversion of a colourless substrate to a coloured end-product is catalysed by the enzyme in the test medium. The presence of coloured end-product is detected by a spectrophotometer. Some variations of the ELISA test use the enzyme to convert a colourless substrate to a fluorescent end-product which is detected by a fluorescence spectrometer.

Two varieties of ELISA are available: the direct and the indirect methods. The direct methods utilize anti-LPS labeled directly with the enzyme. The indirect tests employ a layer technique in which the LPS antigen/antibody (primary) complex is detected by a secondary antigen which is enzyme linked. Usually, the primary antigen is monoclonal murine IgG directed against LPS, and the secondary antibody is anti-murine, directed against murine IgG.

Specificity

Anti-chlamydial LPS antibodies are not always entirely specific and may cross-react with LPS from both gram-negative bacteria and yeasts.[116,117,139,140] To overcome this problem and to improve specificity, so called "blocking assays" have been used to verify *positive* ELISA tests. In this modification, an aliquot of the specimen that has been found to be ELISA-positive is pre-treated with monoclonal antibody specific for Chlamydial LPS. This step inhibits competitively or "mops up" or "blocks" Chlamydial LPS. Compared with the initial positive test, the blocked test yields a reduced spectrometric optic density because some of the end-product has been specifically blocked. To confirm the positive test a minimum inhibition is necessary. It is noted that in some specimens the amount of Chlamydial LPS in the specimen may be in significant excess of the blocking agent. The concentration of blocking agent may not be sufficient to suppress reactivity in such specimens. This may result in incorrect interpretation of the blocking test.[1,138] Positive ELISA tests have also been validated using DFAT in place of the blocking test.[123,141,142] It may be better to confirm positive ELISA tests with DFAT rather than with the blocking test (*vide infra*).

The blocking step has improved the specificity of ELISA tests for *Chlamydiae* and is recommended for ELISA used for screening tests. [143] However, even if the blocking step is used, some authors do not recommend ELISA for use on urine specimens from females because their studies appears to have shown a high rate of *false positive* results. [144] On the other hand, other workers have indicated that the sensitivity of culture for Chlamydia infection is improved using female urine contaminated with endocervical secretions.[51] For this reason, these so-called "false positive" results may indeed have been correct.

The specificity of most commercially available ELISA tests is in the region of 97% without the blocking step and better than 99% with the blocking step.[143]

Sensitivity

The sensitivity of ELISA tests varies with the particular brand of detection kit. The sensitivity of the "Chlamydiazyme Test®"[Abbott Diagnostics] is considerably improved by inclusion of the blocking step.[123,124,139,143-145] However, meta-analysis of several studies using different commercial tests showed that the best possible sensitivity when the blocking test is employed is only in the region of 73%.[1] Use of the blocking step is essential for urine specimens. Confirmation of the Chlamydiazyme Test® (detecting LPS) using the DFAT technique (detecting MOMP) has produced conflicting results. One study [124] gave good correlation, while another,[146] revealed that 24% of ELISA-positive but blocking-test-negative specimens were DFAT positive. The CDC recommends confirmation of positive ELISA tests in a low prevalence setting.[59] The relatively low sensitivity of this test and these conflicting findings suggest that use of the Chlamydiazyme® test is questionable.

The "Mikrotrack"® ELISA system, also marketed as "Syva," ® [Behring] has been evaluated in a series of publications.[123,145147-150] Meta-analysis of these papers indicated that the overall sensitivity is 83% for endocervical specimens, 97% for male urethral specimens and 82% for male urine specimens.[1] Once more, the specificity of Mikrotrak® ELISA has been improved by using confirmatory Mikrotrak® DFAT.[141] In direct comparative studies, the Mikrotrak® ELISA has been shown to be superior to Chlamydiazyme® ELISA.[142,143,148]

Other commercially available ELISA test systems detecting Chlamydial LPS have been marketed particularly for testing of urine specimens. These tests require centrifugation of the urine specimen and re-suspension of the sediment in buffer supplied with the test kit. ELISA testing of urine is reported to yield better results in symptomatic males than in asymptomatic patients.

The performance of these other test kits has been less extensively evaluated[1] than Chlamydiazyme® and Mikrotrak® tests. In one study,[151] the performance of the "IDEIA Test" ® [Dako Diagnostics] has been reported to be better than Chlamydiazyme®, but similar to Mikrotrak®, while another publication[145] reports that the three tests are on a par with each other. Sensitivity and specificity figures for the "Prima EIA Test" ® [Baxter Diagnostics] have been better than those for Chlamydiazyme® and Mikrotrak,®[152] but the three kits were not compared directly with each other.

Second generation ELISA tests using automated methods and more sophisticated technology have become available.[66] One of these, the "IMx Select" ® [Abbott] was reported to be more sensitive than culture. [66] However, in this particular study, culture was only 60% sensitive. It is clear that the performance varies considerably between the ELISA tests that are commercially available. The CDC notes that no single study has compared all available technologies.[1] In the same article, it is recommended that selection of an ELSIA test should be based on tests directly comparing the methodologies on the same specimens.

In summary, the CDC recommends confirmation of positive tests using any ELISA method in a low prevalence setting.[59] Further, it is recommended that the gold standard when evaluating ELISA tests should be expanded to include amplification of Chlamydial DNA.[1]

4.4.3.3 POINT-OF-CARE (RAPID) TESTING

Rapid ELISA tests for the detection of *Chlamydia trachomatis* have been developed for use in "point-of-care" or "near-to-patient" situations. These tests can be conducted by the physician or his assistant in his office and require neither sophisticated laboratory equipment nor special expertise.

Limitations

These tests use ELISA methods and detect Chlamydial LPS, which means that these tests are subject to similar limitations as laboratory ELISA methods, particularly cross reactions with non-Chlamydial LPS. The "near-to-patient" tests depend on visual interpretation of the ELISA reaction and are therefore qualitative (ie. positive or negative).

Specificity and Sensitivity

Several rapid tests are marketed commercially, but the CDC reports that none has been well evaluated.[1] However, several studies indicate that the rapid tests are significantly less sensitive and less specific than laboratory performed ELISA. [153-157] Since laboratory tests are more reliable, the use of rapid tests is not recommended unless immediate results are required for decisions regarding immediate patient management. [1] Quality assurance of such testing is essential, and a positive result should be subsequently confirmed by a laboratory method. The potential for false positives precludes use of such tests in low prevalence situations and in asymptomatic patients. [1]

The results of sensitivity testing of rapid tests vary widely and also differ with the specimen type. The sensitivity of these tests using endocervical swabs varies between 52% and 85% compared with culture. [153-155] Similarly, the sensitivities for tests done using male urethral swabs vary between 65% and 85%.[154,158] The specificity of these tests is reported as 95%. These sensitivity and specificity figures have been derived in studies conducted by experienced laboratory staff, not unskilled persons who would normally perform "near-to-patient" testing.[1]

4.4.3.4 IMMUNO-HISTOCHEMICAL DETECTION OF CHLAMYDIAL ANTIGEN

Introduction

Antibodies have been used to localize antigens in tissues since the 1940's and immuno-histochemistry (IH) has been particularly widespread since the advent of monoclonal antibodies in the 1970's.[159] IH is used to locate tissue antigens by interaction of the antigen with a specific antibody. The antigen/antibody complex formed in the tissues may be visualised by applying a label directly to the antibody or by means of a secondary immuno-detection and labelling method. The technique enables study of the function and chemical composition of cells by identifying the proteins and other complex molecules (eg. steroid and other hormones, enzymes, structural molecules, membrane receptors) within the cells. The tool has enhanced the understanding of the physiology of tissues and the process of many diseases. Antibodies have been used to identify antigens of normal cells, malignant cells and pathogens, and to identify alterations of gene expression regulating cell differentiation.[159]

IH detection of antigens is widely practiced in histopathology laboratories. The technique is readily available and is easy to apply. Further, many of the principles and systems used in IH are also employed in DISH methods. "We have an exciting future to look forward to when we can identify one gene, its mRNA and the encoded protein molecule within a cell which contains millions of copies of each. This will have a profound effect on the diagnosis of disease and its therapy in the practice of medicine."[160]

Both methods were seriously considered for use in this study. Therefore, some of the literature describing this technique and the methods available for both detecting and labelling of Chlamydial antigen and/or Chlamydial DNA has received detailed attention in this review.

Sensitivity and choice of method

The sensitivity of IH depends upon three main factors: the amount of and state of preservation of the antigen, the avidity, affinity and titre of the primary antibody, and the sensitivity of the immuno-detection system. A diversity of methods is available for the application of this technique. Extremely sensitive systems that will reveal small amounts of antigen are available, but frequently are not practical and are too costly in the setting of the routine pathology laboratory. All possible refinements of the technique may serve no purpose if the antigen is not preserved or available for binding to the labeled antibody.[159]

The technique requires well-preserved morphology and preservation of the target antigen so that the antibody can bind to it. However, preservation of tissue morphology by aldehyde, acetone or alcohol fixation may degrade or mask the antigenic binding sites. The formalin-fixed, wax-embedded tissue block used universally in pathology imposes limitations on the possible applications of immuno-histochemistry. Loss of the target antigen by diffusion or chemical alteration during the fixation and embedding process may prevent or severely limit the use of this technique. The range of antibodies available for detection of antigens in routinely prepared sections from formalin-fixed, paraffin-embedded tissues is wide and ever increasing.

IH with maximum sensitivity usually requires frozen sections of unfixed tissue. For routine application and to ensure reliable and reproducible results, the choice of the method to be used is determined by such practical considerations as the nature of the specimen available for examination, the cost of the reagents and the equipment used. Other important factors are the need to limit the number of steps required, the complexity of the solutions required and systems used for automation and batch staining. In the routine laboratory setting, these factors frequently prevent application of more sensitive and elegant IH methods. [159] Choice of a research method may be limited by the means and needs already established in the routine laboratory.[159]

Considerations for tissue sections

(1) *Preservation of the specimen*

Tissue for IH requires prompt fixation or freezing to retain antigenicity of target molecules. Small tissue samples may be kept at 4 °C in saline-soaked gauze for 24 hours without deleterious effect. Autopsy tissue frequently yields adequate results. The ideal means of tissue preservation varies according to the antigen being sought and the antibody available. The best IH results are obtained with cryostat sections of frozen tissue subsequently fixed in absolute acetone at 4 °C for optimal preservation of the broadest spectrum of antigens.[159] Once fixed and air-dried at room temperature, frozen sections may be wrapped in tin foil and stored at – 20 °C for years if necessary.[159] However, it is rare that tissue can be collected in ideal circumstances and in most cases, IH can be achieved with conventional fixation methods. Tissues fixed in formaldehyde and embedded in paraffin wax may be stored at room temperature for years. Tissues embedded in wax for 150 years have been successfully used for retrospective IH.[159]

Formaldehyde based fixatives are most widely used because preservation of morphology is good and the fixative is most stable in routine handling situations. During formalin fixation, hydroxymethyl groups form on the reactive species on the side chains of amino acids, or on the peptide bonds in the tissue proteins.[161] These hydroxymethyl groups react with un-reacted amino acid side chains to form methylene bridges between amino groups in the tissue proteins,[159,200] resulting in inter- and intra-molecular cross-links. These cross-links affect the three dimensional structures of proteins and can mask the sites for binding between antigen and antibody, or destroy the antigen completely. This problem becomes more severe if fixation is prolonged. Antigen retrieval *(vide infra)* is possible in cut sections if the cross-links between amino groups are digested by a proteolytic enzyme,[159,163] or by exposure to microwave radiation,[164] or by heating to at high temperatures in an autoclave or domestic pressure cooker. Alternatively, more sensitive IH methods can be used to detect the reduced amount of antigen present in fixed tissue.[159]

Bouin's fluid, periodate-lysine-paraformaldehyde (PLP) and PLP with dichromate are recommended as alternative fixatives for IH[165-168] but their use requires strict control of fixation times and this is frequently not practical. Further, since most tissue specimens in the pathology lab are routinely fixed with formalin, IH antibodies and methods adapted to this fixative allow retrospective staining of archived tissues.[159]

Fixation frequently continues after dissection of the specimen. Preparation of small to medium sized blocks of tissue for fixation and processing allows more uniform fixation. IH results are undoubtedly better on blocks prepared in this way. Further, reduced amounts of immuno-chemical reagents are required for small section. Blocks or sections that are too large may be trimmed prior to IH staining. [159]

Recently, use of microwave assisted fixation was reported.[169] Using this method, a wide range of antigens is preserved and fixation time is considerable shortened. Microwaves appear to act by heating protein molecules to melting point causing structural changes including the formation of disulphide bonds between amino-groups in the molecules.[170] Since this discovery, microwave techniques have been applied to all processes involving preparation of tissue for histology.[170,171] Rapid fixation using microwave irradiation for immunocytochemistry has been reported.[170,172] It was found that a greater range of antibodies detect target antigen after microwave fixation than after formalin fixation.[172]

Conformational changes in antigen structure can be caused by heat during the processing of tissues. Experimental studies have shown that epitopes of vimentin detected by monoclonal antibodies have a half life of 10 to 15 minutes at 60 °C.[173] Rapid fixation of tissues in hot formalin (usually 60 °C) should be avoided. Embedding of tissues in paraffin wax, which melts at temperatures between 50 and 60 °C, is particularly responsible for destruction of antigenic epitopes.[159] Careful control of the temperature of wax during the impregnation and embedding of tissue is necessary. Overheating of tissues during drying of sections can also be responsible for destruction of antigen. Drying of sections should preferably be done at 37 °C for 8 to 24 hours. Rapid drying of sections at 60 °C should be restricted to 15 minutes. Particularly sensitive antigens are preserved best by drying the section at room temperature for 24 hours.

(2) *Preparation of sections*

High quality thin 3 to 6 μm sections are essential for IH techniques. Thick, compressed, folded or scored sections may affect interpretation and are more likely to wash off the slide during processing. To prevent lifting of sections, slides with adhesive coatings are widely used. Gelatin-chrome alum-coated slides[174] are effective for most purposes, but proteolytic pre-treatment of sections may digest the gelatin. Amino-propyl-triethoxy-silane (Organosilane) is an effective coating [175,176] if a proteolytic step is required. Sections with small surface area adhere better to slides, staining is more uniform and use of reagents is more economical. The complete removal of wax in three washes of xylol is essential. Traces of wax cause incomplete penetration of antibody and result in patchy staining. Re-hydration is achieved through three washes of graded alcohol (100%, 100% 70% v/v) and thorough washing in tap water.

(3) *Antigen retrieval*

(a) Proteolytic enzyme digestion first described in 1976,[163] is the most frequently applied technique for antigen retrieval. The partial hydrolysis of proteins by protease digestion serves to un-mask antigens obscured during the fixation process. On the other hand, some antigens are destroyed by protease digestion. Further, the digestion process can seriously impair the tissue morphology. The concentration of enzyme used and the duration of digestion varies and is dependant on the conditions of fixation and the extent to which antigenicity has been altered by heat.[159] If a digestion

step is used, it must be carefully controlled. To achieve reproducibility of the method between batches, standardization of the digestion methods is necessary. For most histologic sections the following enzyme solutions are useful: a 0.1 % (w/v) Trypsin [ICN Biochemicals 150213] or a 0.0125 % (w/v) Protease [Sigma P-8038] or a 0.4% (w/v) Pepsin [Sigma P-7012]. Other enzymes used include Pronase, Proteinase K and Ficin. Solutions should be freshly prepared, the enzyme added to pre-warmed diluent, and slides heated to the working temperature before the enzyme is added. The use of a water bath to warm slides and solutions prevents undue evaporation and alteration of the concentration of solutions. [159]

(b) *High temperature antigen retrieval:* antigen retrieval for formalin fixed tissue can be achieved by application of high temperatures in combination with salt solutions of varying pH, molarity and type of buffer.[164,177,178] The heat may be applied by autoclaving,[179,180] microwave irradiation[164,172,177,181] or ultrasonic cavitation, [182] or domestic pressure cooker.[178,183] The mechanism of this effect is thought to be the removal of calcium ions bound to hydroxymethyl groups formed during formalin fixation.

(c) *Low temperature antigen retrieval by acid hydrolysis:* has recently been shown to be effective.[184] Use of low temperatures is made possible by using weak acid hydrolysis to remove calcium ions and break methylene bridges. The low temperature allows better preservation of tissue morphology and reduces lifting of sections from the glass slides.

Considerations for cytology specimens

IH is a useful technique in cytology specimens.[159] Smears may be derived from cells scraped or brushed from mucosal surfaces, cells exfoliated into body fluids, cells obtained by fine needle aspiration or cells from touch preparations of tissue.

(1) *Preservation of Specimens*

For many years, fixation of cytology smears was routinely by Papanicolaou's method using equal volumes of 95% alcohol and ether. This fixative has been abandoned because of the fire risk with ether. Routine fixation in most laboratories is with 95% ethanol or 100% methanol.[185] For gynaecologic Pap smear specimens, coating fixatives dispensed by aerosol spray[186] has found wide, almost universal acceptance.[187] These fixatives contain 95% ethanol, ether and carbowax (polyethylene glycol) or 100% ethanol and carbowax. The aerosolized ethanol solvent fixes the cells and after it evaporates, leaves a fine coating of carbowax over the smear that serves to protect the cells during transport of unstained slides to the laboratory. The coating is removed prior to staining by soaking slides in 95% ethanol. Aerosol cans are a convenient means of supplying fixative to sites where Pap smears are taken. The aerosol method allows for easy handling and rapid fixation is achieved, ensuring optimum preservation of cellular and nuclear detail.

Alcohols fix tissue by precipitation of proteins and prolonged exposure to ethanol or methanol may destroy antigenicity.[159] Thus, for IH work, the use of routinely fixed smears made from exfoliated cells of the *cervix uteri* is not always possible. The nature of the antigen being sought and the available antibody(s) to it governs the feasibility of

using IH on routine Pap smears. Recommendations for fixation of cytology smears for improved IH results include the use of mixtures of acetone:methanol (1:1 v/v) or acetone:methanol:formalin (19:19:1 v/v).[159]

(2) *Preparation of Smears*

IH on cytology smears made from fluids rich in mucins or protein, or with a large proportion of red blood cells present problems with background staining. This problem can cause significant problems with IH applied to Pap smears. Where possible, cells in protein rich specimens should be washed prior to staining and centrifuged onto the slide using a Cytospin centrifuge. Cytospin preparation also serves to concentrate cells from pauci-cellular fluids. Slides may be wrapped in aluminium foil and stored at –20 °C.[159]

Fluids for cytologic examination that are very rich in cells may be centrifuged and the aggregated cells suspended in agar prior to fixation in formalin, processing and embedding for routine histology as a cell block. Sections made from cell blocks are ideal for IH staining of cytology specimens.[159]

Newly developed commercially available systems for the collection of cell samples from the *cervix uteri* in a special fixative fluid have become available recently. The more widely available systems are "Thin-Prep®" and "Autocyte®" and both have been extensively validated and received FDA approval. These systems are not available for routine use in South Africa. For ease of examination, these methods produce a monolayer of well preserved cells placed in a circumscribed area on the glass slide. The techniques serve to wash away mucins and proteins, lyse red blood cells and concentrate cells. A very significant, quantum improvement (54%) in sensitivity of the Pap smear examination has been achieved with these techniques.[188-190] This technology allows the production of several smears from one fluid specimen and potentially offers the facility to do further testing on the same cell population.[191,192] This could offer huge advantages in the IH staining of cells exfoliated from the cervix.

Reagents for Immuno-histochemistry

(1) *Primary Antibody*

The primary antibody is used to localise the antigen being sought and can be directed against a wide range of molecules including cellular structures, cell products (hormones, enzymes, immunoglobulins), cellular receptors and infective organisms. These antibodies are usually monoclonal and are raised in the laboratory with a specific antigen being targeted. New antibodies become available continuously and prior to use, should be validated.[159] Use of a new antibody must be preceded by tests to establish efficacy and specificity. The method of fixation and processing of tissues or cytology smears must be shown to be compatible with the new antibody. Working dilutions, processing times, and the necessity for antigen retrieval must be established.

Appropriate storage of antibody is essential to maintain optimum activity and ideal conditions may vary between +4 °C and -20 °C.[159] Prolonged periods at higher temperatures, and repeated thawing and re-freezing of antibody results in rapid deterioration. Antibody should therefore be aliquoted into small working volumes prior to storage so that daily use does not lead to degradation of the entire batch of antibody.

(2) *Immuno-detection Systems*[159]

Numerous immuno-detection systems to attach a label the primary antibody have been evolved. The variations that have been developed aim at increasing the versatility and sensitivity of the tests.[159] Sensitivity may be improved by increasing the amount of label bound to the primary antigen. The choice of the immuno-detection system used to bind a label to the primary antibody may be critical in achieving successful detection of the target antigen. Several alternative systems have been developed for commercial use.[159]

(a) *Direct labelling*: is the simplest method of immuno-labelling. A label is conjugated directly onto the antibody. The target antigen is exposed to the conjugate and a signal is obtained if the antibody and target bind. This simple method is not sensitive and the antibody cannot be used in other more sensitive methods;

(b) *Indirect labelling*: makes use of an un-labelled primary antibody followed by a secondary antibody conjugated to label. The primary antibody is raised in one species of laboratory animal and the secondary antibody is raised in another species. The secondary antibody conjugates with the species-specific portion of the primary antibody. This allows the secondary antibody bearing the label to remain constant, provided the range of primary antibodies is raised in the same species of animal. Since several secondary antibodies conjugate with only one primary antibody, the number of labels attached to the single target antigen is increased. The signal is therefore brighter and the sensitivity enhanced;

(c) *Enhanced indirect labelling*: employs a three stage process where antibodies from three different species are used and results in further enhancement of the sensitivity;

(d) *Enzyme-anti-enzyme labelling*: uses the affinity of an enzyme-label and an antibody against that enzyme. Commonly used enzymes are peroxidase and alkaline phosphatase. The anti-enzyme antibody must be derived from the same species of laboratory animal as the primary antibody. The secondary antibody, raised in a different species, serves to link the anti-enzyme/enzyme complex (label) with the primary antibody and must be in excess. This enables the one of the Fab arms of the secondary antibody to bind with the Fc portion of the primary antibody and the other to bind with the Fc portion of the anti-enzyme antibody bound to the enzyme complex. If the target antigen is present in low amounts, the second and third layers of secondary antibody and enzyme/anti-enzyme complex may be applied repeatedly as many times as necessary to achieve required sensitivity levels;

(e) *Avidin-Biotin Complex (ABC) labelling*: utilizes the natural strong affinity of the glycoprotein avidin or the protein strepavidin to the small protein molecule biotin. Up to four molecules of biotin may bind to one molecule of avidin or strepavidin to form the ABC. The ABC is linked to the primary antibody by a secondary antibody. The secondary antibody must be raised against the species of animal in which the primary antibody was produced. A molecule of biotin is covalently bound to its Fc portion of the secondary antibody. The Fab arm of the secondary antibody binds to the Fc part of the primary antibody, and the avidin/strepavidin binds to the biotin on the Fc of the secondary antibody. Strepavidin is said to be better than avidin in this system because it has an isoelectric point close to neutral. This means that non-specific binding to charged groups is minimal. Further, strepavidin is not a glycoprotein and so does not bind with tissue lectins to cause non-specific signal.

(3) *Labelling System:* [159]

The labelling system is used to make visible the reaction between the target antigen (Ag) and the primary antibody (Ab). Labels that may be used include fluorescent dyes, enzymes, metallic precipitates, metalloprotein compounds and radioisotopes. The four more commonly used labels[159] are fluorescein isothionate (FITC), the enzymes horseradish peroxidase (HRP) and alkaline phosphatase (ALP) and gold precipitate:

(a) *FITC*: fluorescent dyes require florescence microscopy in which correlating the site of the antigen expression with the morphologic location is more complex because details of tissue morphology is lost under fluorescence conditions. The preparation is not stable and fluorescence fades. For these reasons FITC is not widely used in pathology; [159]

(b) *HRP*: is a plant-derived enzyme and is the most widely used enzyme-label. The enzyme label is invisible. However, it releases free O˙ radicles from H_2O_2 that oxidize a colourimetric agent to produce a visible deposit in the tissue at the sites where Ag-Ab-ABC-HRP binding has occurred. A number of colourimetric agents may be used, including 3,3-diaminobenzidine (DAB), 3-amino-9-ethylcarbazole, 4-chloro-1-napthol, p-phenylenediamine pyrocatechol (Hanker-Yates reagent), tetra-methyl-benzidine, homovanillic acid and napthol pyronine. DAB is most widely used because the brown precipitate is insoluble in organic solvents (alcohols and xylol) and preparations can be mounted permanently using synthetic mounting medium (DPX). To further increase sensitivity, post-incubation of the final reaction product in heavy metal salts can be enhance the intensity of the colour reaction or changed the colour of the DAB precipitate. Nickel chloride ($NiCl_2{\cdot}6H_2O$) is most commonly used.[159]

Endogenous (human) peroxidase is present in normal red blood cells, neutrophils and macrophages and may cause precipitation of DAB in non-target sites. The precipitate is usually intensely dark, is confined to these cells and can be ignored when the sections are interpreted. If the cells of interest contain large amounts of endogenous peroxidase, or inflammatory cells or numerous red blood cells due to haemorrhage are present in the section, the endogenous enzyme can be "blocked" prior to exposing the target antigen to the primary antibody. Flooding the section with 3% H_2O_2 in methanol will mop up endogenous peroxidases. Acid alcohol, sodium azide, sodium cyanide, potassium ferricyanide, nitroferricyanide and picric acid are enzyme poisons that destroy peroxidase, but must be carefully washed away before the primary antibody is used. Alternatively, Hanker-Yates reagent can be used because it only reacts with plant-derived enzymes. (Staining with this chromogen is capricious and fades over time.) DAB is suspected to be carcinogenic and requires careful handling and other colourimetric agents may be preferred for this reason. More than one chromogen can be used for simultaneous staining using two or more different primary antibodies to demonstrate multiple target antigens; [159]

(c) *ALP:* is less widely used than HRP. ALP used in IP is derived from calf intestine and is resistant to levamisole [Sigma I-9756], while human alkaline phosphatase is not. Addition of levamisole solution (between 1 and 5mM) to ALP is sufficient to block human endogenous peroxidase, except in tissues with high concentrations of the endogenous enzyme (gastro-intestinal tissues and placenta). The blocking agent can be added to the chromogenic

solution and a pre-incubation step is not required. Chromogens used with ALP are either napthol-AS-MX phosphate [Sigma N-4875] or napthol-AS-BI-phosphate [Sigma N-2250]. These chromogens can both be coloured red using Fast Red® [Sigma F-1500] or New Fuchsin [R. Lamb CI 42520] and can be turned blue using Fast Blue® [Sigma N-1300]. However, the substrate precipitate is soluble in organic solvents and sections must be mounted in aqueous media (glycerol-gelatin). New Fuchsin is stable enough to be mounted in DPX but may fade when stored; [159]

(d) *Gold*: immuno-gold labelling (IG) has been used for IP, mainly but not exclusively for EM. The metal label is used in EM because it is electron dense and shows as black granules in electron photomicrographs. The technique is not very sensitive but post-incubation with colloidal silver solution results in silver precipitation on the gold molecule and improves sensitivity (IGSS). The black precipitate is highly stable and potentially very sensitive,[193] especially if the deposits are examined under polarized light.[194] Disadvantages of this method include the cost of the gold and silver reagents and the tricky colloidal silver step that requires careful time and temperature control.

Controls for IH

Controls for IH are necessary to confirm that negative results are due to absence of the target antigen and that positive results are due to specific binding of antibody.[159] Controls for reagents, the tissue and the procedure are necessary.

(1) *Reagent controls*

Controls Commercial reagents should be tested against known positive tissue to check that staining is specific. Working dilutions of antibody and antigen retrieval procedures should be optimized using known positive controls; [159]

(2) *Procedure controls*

(a) A *known positive tissue section*, fixed and treated in the same way as the test section should be included with each staining batch. Staining in the known positive tissue should be appropriate: target cells should stain positively and non-target cells negatively. Background staining should be minimal or absent. Positive control sections should not stain too intensely so that assessment of the sensitivity of the IP result in the test section can be assessed. A negative result in the known positive control indicates a fault in one or more of the steps in the procedure. Careful selection of positive control blocks may allow testing of multiple antibodies against sections from the same block.[159] Sections of the intestinal appendix with reactive lymphoid hyperplasia for example, show multiples tissue elements, including epithelium, smooth muscle, vascular endothelium, nerve, fat, peritoneal mesothelium and lymphoid elements (histiocytes, B and T lymphocytes, small lymphocytes, centrocytes, immunocytes, lymphocytes from the mantle and marginal zones, plasma cells). It is common practice for laboratories to participate in external quality assurance schemes. Test tissues submitted regularly by an external agency are stained using IH procedures and the sections submitted for expert external scrutiny;

(b) A *primary antibody ("negative") control* using a non-immunogenic substitute for the primary antibody should be run on a serial test section. This control sections should stain negatively to confirm the specificity of positive staining in the section being tested. Four options[159] are available:

(i) use of a primary antibody reagent in which active antibody has been absorbed by adding specific purified antigen. This elaborate step is not necessary in the routine setting when known, previously tested reagents are being used;

(ii) use of an irrelevant antibody, achieved in the practical setting by using a panel of antibodies to identify a tissue by its characteristic positive and negative reactions. Epithelial cells for example, should stain positively for epithelial antigens and negatively for non-epithelial antigens;

(iii) use of non-immune serum from the same species used to raise the primary antibody;

(iv) omission of the primary antibody;

(c) *Other reagent controls* for secondary and tertiary linking and labelling reagents may become necessary if non-specific staining or un-explained negative staining occurs in test and/or control sections.

Laboratory procedures and technique [159]

IH can be successfully performed with relatively simple standard laboratory equipment. When performing IP procedures manually, best results are achieved with precise attention to accurate and careful application of the staining protocols. It is necessary to apply protocols with precise control of reaction times and regulation of temperature conditions. Standardization of reaction conditions allows comparison of the intensity of staining of target cell with different antibodies, or between cases. Preparation of reagents with special care taken to ensure correct osmolarity is essential for best results. Storage of reagents in optimum conditions to prevent degeneration or contamination is vital. Careful handling of slides is necessary and the numbers of tests being performed at a time should be limited to enable attention to precise technique.

Mounting several sections on one slide may allow improved technique by reducing the number of slides to be handled. Individual sections can be isolated from one another by application of line of hydrophobic wax around each section. The wax is applied easily using a special pen. The hydrophobic line serves to contain reagents in a circumscribed area over the tissue section. This method allows use of different reagents on different sections placed on the same slide. Smaller volumes of reagent are required by reducing the surface area to be covered by the reagent. The smaller reagent surface area minimises evaporation and serves to prevent drying out of the sections during incubation. Evaporation is also reduced if incubations are performed in covered containers.

Careful handling of slides reduces lifting of sections from the slide during staining. To wash slides, they should be soaked in buffer in Coplin jars or butter dishes. Slides should not be agitated to prevent lifting of sections. Immunologically inert coatings can be used to "sub" slides and reduce lifting of tissue. Buffer fluid should be carefully removed by tapping slides on edge over tissue paper. This prevents dilution of antibody serum or reagents by excess fluid. Once wet, slides should not be allowed to dry out. Once the target antigen has been stained, tissues should be counterstained to enable visualization of the morphology so that the precise location of Ab-Ag-label complexes can be identified. If the precipitated chromogen is insoluble, aqueous counterstaining can be performed. The nuclei and

cytoplasm of the cells is usually stained with haematoxylin. If the label precipitate is blue, methyl green or neutral red can be used to counterstain. More complex counterstains can be used if necessary.

Automation [159]

IP techniques are readily automated. Various commercial automated systems are available and automation frequently achieves excellent results by minimising deviations from standardised staining protocols.

Innovative techniques in IH [159]

When limited tissue is available, particularly in cytologic specimens, several innovations can be used to make the best use of tissue. Papanicolaou-stained smears or haematoxylin & eosin-stained (HE) sections can be re-stained using IH after being de-stained in weak citric acid which also serves to retrieve antigen by acid hydrolysis (own observation).

Smears and even sections may be split so that more than one antibody can be applied to one slide. The slide may be cut into several separate pieces for staining. Alternatively, sectors of the slide can be separated by lines of hydrophobic wax, or by carefully applied lines of molten paraffin wax. The sectors can then be stained using different antibodies.

It is also possible to re-stain tissue sections or smears with a second and even a third antibody. IH preparations may be double or triple stained using two or more different antibodies sequentially or simultaneously. Sequential staining makes use of two or three steps using one antigen at a time. The result of the first procedure is photographed before the section or smear is de-stained and then re-stained using a second (or third) antibody. Some robust antigens (for example cytokeratin or leukocyte common antigen) may retain their IP staining, but use of a second chromogenic label with a different colour is possible to differentiate the products of the different Ag-Ab reactions.[159]

Simultaneous staining is a most effective means of showing two or more antigens. If antibodies from the same species are used, the first Ag-Ab reaction must be labelled, before the second antibody is applied. A different chromogenic label is used for each subsequent Ab-Ag reaction. If binding sites on the first primary antibody are not saturated, it is possible that the secondary antibody labelled with the second chromogen could bind to the first target, causing false positive staining.

Preferably, primary antibodies raised in different species should be used. In these circumstances, the primary antibodies can be mixed allowing the two or more Ag-Ab reactions to occur simultaneously. The different chromogenic labels must be conjugated to appropriate species-specific secondary antibodies corresponding to the species of primary antibody used for each target antigen. Careful selection of enzyme substrates allows good contrast between the reaction products of the different primary antibodies. It may be necessary to dilute one or more of the primary antibodies so that a strong reaction does not mask a weaker reaction. The sequential method, using primary antibodies raised in different species yields best results.[159]

Combination of IH and DISH on the same section is possible but has limited application at present.[195,196]

Quality assurance in IH

IH is a mainstay in modern anatomical pathology. The science and technology used for IH work continues to improve and innovations that improve the quality and range of tests available may be expected to continue for some time. Development of new antibodies and new detection systems are the most likely means of improved quality in the future.[159] Quality of staining is indicated by intense staining with precise localisation of the Ab-Ag complex in both control and test sections. Steps to improve quality should include attention to optimal tissue fixation and antigen retrieval methods. Pre-digestion steps should use correct proteolytic enzymes for appropriate times with optimum temperature conditions. Particular care is necessary with high temperature antigen retrieval methods and if possible low temperature acid hydrolysis is preferable (own observations).

(1) *Incubation time:* Prolonged incubations times with both primary antisera and secondary antibodies can improve staining. Recommended incubation times for most commercial antisera should be regarded as *minimum* times rather than critical or optimum times.[159] Prolonged incubations, for several hours may be effective. Incubation overnight at 4°C to prevent evaporation of antisera may be necessary. Prolonged incubations should be done in a sealed moist incubation chamber to prevent drying of the sections;

(2) *Incubation temperature:* Incubation with antisera is frequently performed at room temperature (20°C) but incubation at 37°C may enhance staining considerably. This effect is probably due to increased movement of molecules improving the chances of Ag-Ab reactions. Care to prevent over heating or drying out of the sections is essential.[159] Microwave heating has been successfully used to reduce incubation times to 30 seconds for a range of antibodies;[197]

(3) *Antibody titration:* The titre of antisera greatly affects the staining in IH. Intensity of staining is improved with higher concentrations, but increased cost is a consideration and background staining can be a problem;[159]

(4) *Agitation during incubation:* Agitation during incubations is effective and may be due to increased movement of molecules. Agitation is difficult when the staining method is manual, may require greater volume of antisera and cause increased evaporation. Incubation of several sections in a Coplin jar or other container is more amenable to agitation. Some automated systems make successful use of agitation;[159]

(5) *Non-specific staining*: Non-specific binding of primary or secondary antibody to tissue due to charged groups on proteins or mucins can cause non-specific staining. The charged groups can be swamping with non-immune serum (bovine serum albumin or foetal calf serum) to prevent the non-specific staining. Non-immune serum can be added to the primary antibodies when they are diluted or can be applied in an extra step before incubation with the primary antibody. Another cause of non-specific staining is the presence of other antibodies in the primary or secondary

antibody sera. These can be absorbed out of the antisera by adding homogenized tissue extracts (brain or liver). Non-specific staining due to aldehyde groups present in the tissue section can be blocked by sodium borohydride; [159]

(6) *Removal of Pigments confused with reaction product:* Pigments in tissue are frequently brown and may be confused with the brown precipitate when DAB is used to stain the Ag-Ab reaction product.[159] Comparison of the test section with the negative control section will reveal the presence of pigment. The main culprits are bile salts, iron salts, lipofuscin, carbon, and melanin. Mercuric chloride or formalin pigment from fixation may also be a problem. Awareness of this problem may be sufficient to allow appropriate interpretation of the sections (own observation). The most satisfactory way to prevent confusion between pigment and chromogen precipitate is to use an alternative chromogen that is not brown, for example ALP.[159] However, it may be necessary to remove pigments from the sections. In such cases, care is necessary to ensure that removal does not interfere with tissue antigenicity and compromise the IH procedure. Frequently, steps to block endogenous peroxidase with H_2O_2 are sufficient to remove formalin pigment and mercuric chloride.[159] The standard method to remove formalin pigment using alcoholic picric acid can be used prior to incubation with the primary antibody. However, even when applied for only 10 minutes with thorough subsequent washing, this may be detrimental to the IH procedure. Again, if H_2O_2 pre-treatment does not effectively remove mercuric chloride, use of alcoholic iodine for 5 minutes followed by decolourisation with 5% sodium thiosulphate can be used. Removal of melanin pigment can be more problematic. Bleaching melanin in 0.25% potassium permanganate for 5 minutes and decolourisation with 2% oxalic acid can cause loss of antigenicity.[198] If use of this method is necessary, a known control should be treated similarly to ascertain the effect on antigenicity;

(7) *Use of detergents:* Access of antibodies to antigens has been improved by the use of detergents mixed with buffers and in the antibody diluent solutions.[199] Detergents also reduce antibody binding to serum proteins present in tissue sections; [159]

(8) *Section quality:* Thin sections (4mμ) taken from small, well-fixed tissue blocks consistently give better results. [159] Small sections allow use of less reagent while the risk of drying of the section or part of it during incubation is reduced. Smaller sections are more economic because less antiserum is required;

(9) *Repetition of labelling layers:* Improvement of the staining intensity can be achieved by repeating either of the antibody incubation steps. [159] This entails performing the usual incubation step, careful washing with buffer and then repeating the incubation with fresh antiserum. The primary or the secondary or both of the antibody incubation steps can be repeated. Repetition of the primary incubation with fresh primary antibody can enhance detection of the target antigen while repetition with the secondary antibody enhances labelling of the target Ag-Ab complex. A variation of this measure is to use two different labelling systems to detect the target Ag-Ab complex.

Trouble shooting

As the understanding of immunologic mechanisms has advanced, IH methods have markedly improved. Advice from manufacturers is freely and readily available. The difficulties most likely to be encountered are poor morphology, weak or no staining or nonspecific heavy background staining.

(1) *Background staining:* Background staining is difficult to prevent unless the step at which it arises can be identified. Poor tissue fixation or preparation should be excluded before embarking on tests to eliminate the cause. Serial sections can be taken through the steps of the procedure, with each step being excluded one at a time: [159]

Slide #	1	2	3	4	5	6	7	8
Blocking serum	+	0	+	+	+	+	0	+
Primary antibody	+	+	0	+	+	+	0	0
Secondary antibody	+	+	+	0	+	+	0	0
Tertiary reagent	+	+	+	+	0	+	0	+
Substrate	+	+	+	+	+	0	+	+

A useful list of potential problems and their possible causes is included in the description by Heryet and Gatter; [159]

(2) *"Negative" results:* Careful assessment of negative results is as necessary as ascertain the validity of a positive result. If control sections do not stain appropriately, it should be established that the fixation and processing of the tissue has been appropriate. Next, the proteolytic digestion should be investigated, ensuring that the enzyme applied was appropriate and active and the reaction conditions were correctly maintained. The third step is to check the primary and secondary antibodies. Storage conditions and the date of expiry of the reagents should be investigated. The antibody can be tested on new control frozen sections fixed in acetone, and then on new control tissue properly formalin-fixed to establish the reaction occurs when the target antigen is present. [159]

If control sections stain appropriately but test sections are negative, several possible causes may require investigation:

(a) Absence of the target antigen in the test section should be considered;

(b) The target antigen is present in levels too low for detection. Routine use of the most sensitive methodology available should be routine. However, incubations at 37°C (or even higher), prolonged incubation, or double incubations can be considered (*vide supra*). Innovative or alternative methods for antigen retrieval may prove useful; [159]

(c) Differences in fixation of control tissues and test tissues may explain the problem. Use of different types of tissue and different fixation regimes to prepare a range of control tissue blocks has been recommended. [159] This allows use of appropriate control tissue types when known alternative fixation protocols have been used (for example B5 fixation for lymph node specimens). Establishing the source of formalin used for a troublesome specimen may resolve a problem. Standard fresh, buffered formalin fixative should be used for all specimens (own observation).

A pertinent and useful observation is that most problems in IH are due to operator error. [159] Complete familiarization with the IH method is essential and dedicated, meticulous attention to details of the staining protocol is key to successful IH. Validation procedures and evaluation of the limitations of new commercial products is advised before embarking on testing with newly available antibodies. "If literature indicates that the antibody is only applicable to frozen sections, one should not be too surprised if it does not work satisfactorily on fixed, paraffin-processed sections." [159]

4.4.4 DETECTION OF CHLAMYDIAL DEOXYRIBONUCLEIC ACID (DNA)

Detection of Chlamydial deoxyribonucleic acid (DNA) may be achieved the specific annealing of a specially selected nucleic acid probe to a complimentary sequence of species specific Chlamydial DNA (target DNA). The hybridization of probe to target DNA is extremely specific if the conditions in which the reaction occurs are stringently set. The hybridization may be achieved both in solution and in histologic tissue sections (DNA *in situ* hybridization). Amplification of the amount of annealed product by use of a chain reaction procedure (PCR or LCR) can considerably enhance the sensitivity of the DNA detection. PCR is more commonly performed in solution using DNA extracted from a tissue specimen, but can also be applied to tissue *in situ*. In both techniques, detection of the annealed end product requires a visualization procedure.

Since DNA *in situ* hybridization and polymerase chain reaction techniques for tissue were selected as the tools to investigate the hypothesis in this study, literature describing these methods has been reviewed in depth.

4.4.4.1 DNA HYBRIDIZATION USING CERVICAL MUCUS & BRUSHINGS

Methodology

In the USA, the first commercially licensed DNA probe for *Chlamydia trachomatis* was marketed as the "PACE 2 test®" [Gen-Probe] and is the most commonly used probe in that country.[1] The DNA probe detects a species-specific sequence of Chlamydial 16S rRNA and employs a chemiluminescent detection system. The DNA-rRNA hybrid is absorbed on to a magnetic bead and the chemiluminescence's is measured by a luminometer. A "grey zone" reading on the luminometer is defined as a result falling between 0.6 and 1.0 times the cut off factor. Results falling within this zone are equivocal and a further confirmatory test is necessary. A confirmation assay has known as the Probe Competition Assay has been developed. This confirmatory test is based on probe competition for the same target sequence, similar in principle to the blocking antibody tests used in ELISA testing. A more ideal test would be a confirmation test using an alternative target sequence. [1]

Specificity and sensitivity

The specificity of the PACE 2 Test® is relatively high (98 or 99%).[13,200-202] Since actively dividing *Chlamydiae* contain up to 10^4 copies of 16S rRNA, the PACE 2 system should be more sensitive than antigen detection systems. Indeed, using purified chlamydial organisms, the PACE 2 test[19] is reported to be 1 log unit mores sensitive than ELISA methods and theoretically able to detect 10^3 Chlamydial elemental bodies.[1] Compared with DNA amplification methods, the PACE 2 DNA probe is less sensitive (between 77 and 93%).[13,203] Testing of urethral specimens from male patients was more sensitive if rigid swabs were used and half of the recommended transport medium was used.[204] Blood contamination can cause false positive results because red blood cells auto-fluoresce in the luminometer. Care in interpreting test results from bloodstained specimens is necessary. [1]

Advantages of PACE 2 test®

The PACE 2 test® has been designed to simultaneously detect DNA from *Neisseria gonorrhoea*. Using the same swab. In the clinical setting, this single test offers differentiation between the two most common pathogens causing sexually

transmitted disease (STD). In common with all non-culture methods of laboratory diagnosis, the PACE 2 test® does not depend on maintenance of the cold chain and specimens may be stored. The total processing time is short (2 to 3 hours), technical expertise is similar to that required for ELISA tests, and an automated system is available from the manufacturer.

Disadvantages of PACE 2 test®

PACE 2® is less sensitive than DNA amplification methods.[1] In this regard, the CDC recommends that positive PACE 2® tests in a low prevalence setting should be confirmed by another method.[59] The confirmation of results in the "grey zone" adds to the expense of this test.

Combined PACE 2 test®

The combined variant of the PACE 2 test® uses combined probes for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* for simultaneous testing on one specimen. If positive, the test indicates the presence of either or both of the two organisms and further testing is necessary. Evaluation of the test in a high prevalence population showed a sensitivity of 89% compared with culture, and a sensitivity of > 95%.[1]

4.4.4.2 DNA *IN SITU* HYBRIDIZATION [205]

Introduction

In situ hybridization, first described in 1969, enables the detection and precise location in fixed tissue of specific DNA or ribonucleic acid (RNA) sequences.[206] A specific labeled nucleic acid probe anneals with the target nucleic acid and is retained *in situ*. The position in the tissue of the labeled probe is subsequently visualised. This technique can locate DNA sequences on chromosomes, detect cellular RNA and demonstrate the presence of, precisely identify and morphologically locate micro-organisms or viruses present in the tissue.

Perhaps the greatest value of DNA *in situ* hybridization is the relative inertness of the DNA molecule. At temperatures below 64 °C, aldehyde fixatives do not affect cellular DNA.[207,208] RNA is more reactive, and is affected at temperatures as low as 45 °C. The relative inertness of DNA is due to the presence of hydrogen bonds between the purine and pyrimidine base pairs. These bonds are broken at high temperatures making the bases available to react with aldehydes.[209] The non-reactivity of DNA allows successful DNA *in situ* hybridization with tissues fixed with formaldehyde.[210]

Another very important value of *in situ* hybridization is its ability to locate the target at its morphologic location in the tissue. The demonstration of a specific target in its specific location renders the test highly specific, elegant and valuable. "The specificity and the sensitivity obtained is so high that reduced numbers of tests yield significant results without resorting to fastidious statistical analysis."[211]

The technique requires both well-preserved morphology and biochemical conditions that favour specific hybridization. However, optimal hybridization conditions are usually detrimental to preservation of tissue morphology. Successful *in*

situ hybridization technique therefore represents a compromise. On the one hand, it is necessary to preserve the tissue morphology and the integrity of target sequences whilst on the other, to provide conditions that allow maximal accessibility of the probe to target.[212] Further, it is necessary to maintain stringent biochemical conditions to ensure specificity of the probe-target complex.

Since this method was selected for the purposes of the study, detailed review of the method follows.

Methodology

There are certain conditions critical to successful *in situ* hybridization.[205] These include the following:

(a) the target DNA or RNA is retained *in situ* during and after fixation of the tissue;

(b) the target DNA or RNA is not degraded by nucleases prior to fixation of the tissue;

(c) access of the probe is not hindered by the complex matrix of the tissue;

(d) the stability of the hybrid within the tissue is retained.

Factors affecting these conditions are largely unknown, and the techniques used to achieve successful *in situ* hybridization are in part, empirical.[205] The methods used for *in situ* hybridization depend on the nature of the tissue, the nature of the target DNA or RNA and on which of the following matters are of priority:

(1) *Sensitivity:* the specificity depends on the accessibility of the target, the method by which the probe is labeled, the method used to detect the annealed end-product, the length of the probe, whether the target is RNA or single stranded DNA or double stranded DNA. Sensitivity may be limited by non-specific background staining. The abundance of target may allow reduced sensitivity or dictate a need for maximal sensitivity;[205]

(2) *Resolution:* the signal generated by the detection system may vary from a sub-cellular level to a particle size greater than a cell diameter. The method of probe labeling and detection are largely dictated by this consideration;[205]

(3) *Specificity:* the specificity of signal for target depends upon the stringency of the conditions during washing *(vide infra)*, and upon the presence or absence in the tissue of non-target sequences similar to target sequences. Non-specific signal can originate from a variety of sources. The setting of precise stringency conditions and use of controls to differentiate non-specific signal is essential;[220]

(4) *Convenience* : use of various labels and probes differs and selection of these is largely dictated by the sophistication and expertise of the laboratory;[205]

(5) *Safety:* a consideration regarding use of radio-active probes and of carcinogenic chemicals is safety, particularly in laboratories with large volumes of work;[205]

(6) *Simultaneous detection:* two or more targets can be detected in the tissue or cell, but depends on appropriate choice of reagents. Hybridization of target with probe labeled by different methods, or combination of the *in situ* technique with immuno-histochemistry is possible;[195,196]

(7) *Three dimensional arrangement:* the spatial pattern of target can be demonstrated, particularly in embryos or whole mounts of small organisms.[213] Serial sections may assist such studies.[205]

Selection and preparation of probe

Probes may be RNA or DNA, single or double stranded, and may be labeled by various methods. Further, several methods of probe synthesis may be used. The length of the probe to be used is an important consideration and is determined by the method of probe synthesis.

Four types of probe may be used for *in situ* hybridization techniques:

(1) *Double-stranded DNA probes:* theses are less sensitive than others but are sufficient for many purposes and are widely used. They may be prepared by nick-translation, random priming or PCR, using labeled nucleotides;[211,212]

(2) *Single-stranded DNA probes:* these are more sensitive than double-stranded probes, especially if long. They can be prepared by extension on single-stranded templates, but a purification step to remove the template is necessary.[205] PCR may also be used to prepare these probes[213] and this method has the advantage of being possible with small starting amounts. PCR is also flexible and allows the manufacture of a wide choice of probes by careful selection of appropriate primers;

(3) *Oligonucleotide probes:* usually between 20 and 30 base pairs in length, are designed and synthesized once the target sequence has been determined.[214] The label may be incorporated during synthesis or by adding a "tail" of labeled nucleotides.[214,215] These probes are less sensitive because their length is restricted by the synthetic procedure. The sensitivity of hybridization tests using oligonucleotides can be increased by the use of several probes that are complimentary to different regions in the target.[205]

(4) *Single-stranded RNA probes:* these are produced by using purified RNA polymerase to transcribe sequences downstream of a particular polymerase initiation site.[215,216] The sequence is usually cloned into a plasmid vector with two different RNA polymerase initiation sites flanking it, one on either side. Thus sense-strand or anti-sense probe-RNA can be synthesized. Background staining may occur in the fluorescent electrophoresis gel, due to transcription of plasmid sequences. To prevent plasmid sequences from being transcribed, a restriction enzyme is used to linearize the plasmid.

The type of probe to be used depends on the expertise of the laboratory, and the requirements of the procedure. Oligonucleotide probes are most easily synthesized as cloning techniques are not necessary.[205] If long probes are required, PCR-amplification of cloned or genomic DNA is necessary. Single stranded probes are more commonly used because of the ease of synthesis.

Length of Probe

The length of probe affects the sensitivity and the specificity of the procedure. Longer probes are more specific, but longer probes yield weaker signal, probably because they penetrate less effectively into cross-linked (fixed) tissue.[205] Penetration of tissues by longer probes is affected by the nature of the tissue, the method of fixation and whether pre-treatment of the tissue with proteases is used. The optimum length for good, specific sensitivity is between 50 and 150 base-pairs. As indicated, the length of the probe can be pre-determined in the synthetic process or regulated by subsequent partial cleavage. Probe length should be checked before use since short probes may result in low signal and /or high background (non-specific) staining.

Labeling of Probe

Probes may be labelled with radioactive labels, with hapten labels or with hapten-metal complexes. The choice of probe label dictates the sensitivity and the resolution of signal.

^{35}S-labelled probes offer the most sensitive detection system for *in situ* hybridization. Tests using radioactive-labelled DNA hybridization probes generally require ten thousand copies of the target DNA sequence for detection.[217] If single cell, or sub-cellular detailed resolution is necessary, or safety and convenience are considerations, use of hapten labels is preferable.

(1) *Radioactive labels:* these are detected by autoradiography. Several different isotopes have been used.[205] The isotope used affects the resolution of the signal, the speed of obtaining the autoradiograph, and the stability of the probe:

(a) *^{3}H-labelled probes* allow sub-cellular resolution of the signal, but require autoradiograph exposure periods of several weeks. ^{3}H has a half life of 12 years which means probes can be stored for use over several years;

(b) *^{35}S-labelled probes* yield resolution of whole cells and allow rapid results requiring shorter exposure periods. Oxidation of the ^{35}S is prevented by the addition of reducing agents to the probe. The half-life of ^{35}S is 87 days, so that probes must be used within one month of preparation. Probes labelled with ^{35}S are up to50-fold more sensitive than those labelled with ^{3}H;[216]

(c) *^{32}P-labelled probes* provide less resolution than ^{35}S. ^{32}P is a poor agent for autoradiography and has a half-life of only 14 days so that probes must be used within one week of preparation. Using special film (Kodak NTB-2® emulsion), ^{32}P can produce efficient autoradiographs. The β–particles emitted by ^{32}P produce a wide "spray" effect and this isotope is used where macroscopic resolution is required.

It is possible to quantitate autoradiographic results obtained with radioactive probes. The intensity of autoradiographic signal is compared with control autoradiographs made using known dilutions of target cells prepared from rat brain ("brain paste").[214]

(2) *Hapten-labelled probes:* these are detected by immuno-cytochemical methods and offer several advantages over radionucleotide-labelled probes. These probes are safe, stable, achieve rapid results and yield single-cell and sub-cellular resolution. Although conceptually possible, quantitation of immuno-cytochemical methods by preparation of control dilutions of brain paste has not been reported in the literature.

The most sensitive techniques employ a hapten against which a specific antibody or other binding protein (ligand) is available. Examples of these haptens include strepavidin-biotin and digoxigenin. Enzymes used include β-galactosidase, alkaline phosphatase and peroxidase. The hapten-binding antibody or protein ligand is conjugated to the enzyme before the procedure begins.

Following the hybridization reaction, un-bound probe is washed away. In exactly the same way as Ag-Ab complexes are detected in IH, hapten-binding antibody or protein ligand enzyme complex is added to the system.[159] It binds to the hapten-labelled probe-target complex to form a target-probe-hapten-enzyme sandwich. Subsequently, a soluble colourless chromogenic substrate is added to the mixture. In the presence of the enzyme it is converted into an insoluble, coloured end-product that is localized at the site of the target.[159,215] Multiple enzymes using different substrates which generate end-products of differing colour may be used to simultaneously demonstrate the positions of two or more target sequences in one tissue section.

Alternatively, the probe, or an anti-probe antibody is conjugated with fluorescein or another fluorescent tags to highlight the target-probe complex. Fluorescent methods are widely used for chromosome *in situ* hybridization.[211] These fluorescent methods are less sensitive than enzyme-linked methods. Fluorescent tags of different colours may be used to highlight two or more different target-probe complexes in the same section.

(3) *Hapten-metal complexes*: gold-labelled antibodies particularly, are helpful for *in situ* hybridization at the electron microscopic level.[212,218] The hapten-metal compound is used to position metallic deposits over the probe-target complex. The gold molecules are precipitated at the site of the target-probe complex, localise specific sub-cellular organelles and structures and appear in electron micrographs as electron-dense particles. Recently, use of the platinum molecule has increased the sensitivity of hapten-labelled *in situ* hybridization techniques.[219] Patented as the "universal linkage system" (ULS®), this method employs strong electro-valent binding sites on the platinum molecule to bind the nucleic acids guanine and adenine in the probe to haptens, enzymes or fluorescent tags (*vide infra* **4.4.4.3**).

Preparation of tissues[205]

Tissues suitable for *in situ* hybridization techniques include cells in cell culture, cell suspensions centrifuged down onto glass slides, cryostat frozen tissue sections, paraffin wax-embedded tissue sections and plastic-embedded tissue sections.

Frozen tissue sections offer the advantage of the absence of cross-linkage of tissue molecules found in fixed tissue. However, work with un-fixed tissue is limited by potential loss of morphologic detail caused by the formation of water crystals that may disrupt cellular morphology. Storage of frozen tissue is problematic and dehydration or lyophilization of the tissue may occur relatively quickly in stored frozen tissue. There is a further risk of degeneration of target molecules before freezing is effected. Frozen sections may be fixed after cutting, allowing very rapid fixation and preservation of morphology in material to be stored. Different fixation procedures for *in situ* hybridization and immuno-histochemistry can be applied to serial frozen sections. Freezing medium used during the cutting of frozen tissue must be washed away to prevent excessive background staining.[218]

Fixation for *in situ* hybridization techniques is best achieved by aldehyde fixation (paraformaldehyde, formaldehyde or gluteraldehyde). This process promotes cross linkage of tissue molecules and retains the structure of DNA and RNA molecules. Fixation preserves morphology of the tissue but restricts access of the probe to target molecules in the tissue. For successful *in situ* hybridization, compromise must be found between the fixation process necessary for preservation of morphology, and techniques used to facilitate access of probe. Fixation by alcohol or acetone causes precipitation of tissue proteins and is less restrictive to the access of probe,[218] but loss of small nucleic acid targets may occur, and preservation of morphology is compromised.

RNA is most sensitive to denaturation and best preservation of RNA is achieved by use of 1% paraformaldehyde, 4% formaldehyde or 1% gluteraldehyde. For RNA work, tissue should be cooled as soon as possible to temperatures between 0 and 4° C to inhibit endogenous ribonucleases.[216]

For *in situ* hybridization, the duration of fixation varies with the nature and size of the tissue specimen. Ideal fixation of cells and cell cultures occurs adequately in 20 minutes. Tissue specimens up to 1mm thick require 2 hours to fix while slides less than 1cm thick fix in 12 hours. Perfusion of tissue with fixative is recommended for thicker tissue specimens.[205] Selection of ideal fixation conditions is not possible when archival tissue is to be used. In this situation, manipulation of subsequent steps in the procedure is necessary to optimize hybridization results.[220]

To allow better penetration of probe after fixation, partial digestion of protein cross-links induced by the fixation process may be achieved by means of protease digestion.[215] Pre-digestion may cause degradation of tissue morphology and once more a balance between preservation and accessibility of probe must be achieved.

Embedding and sectioning procedures[205]

Frozen cryostat sections of fresh tissue offer several advantages including high penetration by probe, selection of different fixation procedures on serial sections[205] and preservation of RNA, particularly messenger RNA (mRNA). Paraffin-embedded tissue sections offer better preservation of morphology, thin and ultra-thin sections can be prepared, orientation of specimens is easier and ribbons of serial sections can be cut with relative ease.

The sections may be mounted is a small rectangular well formed by a thin Teflon® coat on special glass slides [C A Hendley PH106]. The well serves to contain and localise the reagents used during the hybridization procedure, thereby minimizing the volumes of reagent required. Alternatively, a similar effect can be achieved by constructing a small trough around the section using hydrophobic wax dispensed from a special pen [Pap Pen®, Japan]. If a wax trough is to be used, it must be constructed after the tissue sections have been de-waxed.

Standard paraffin wax used for histology is washed out of the tissue prior to hybridization. It allows cutting of sections as thin as 1 μm. Since there is likely to be less target DNA in thinner sections, it is usual to use sections as thick as 6 or 8 μm for hybridization techniques. Thicker sections allow less probe accessibility and are more likely to wash off the glass slide during the hybridization procedure. Resolution of tissue morphology and precise location of the target-probe complex is progressively compromised in thick (more than 5 μm) and very thick (more than 10 μm) sections.

In situ hybridization on ultra thin sections (100 to 200 nm) for electron microscopy requires embedding in plastic matrix. The plastic cannot be washed out of the tissue and so impedes access of the probe to target. The thinness of the section so reduces the amount of target that only high abundance DNA or RNA can be detected.[212]

Subbing of slides

"Subbing" (*sub Latin: under or close to, denoting support*)[221] is a technique of coating glass slides with an adhesive substance prior to mounting the tissue section on the slide. Sections, especially thick sections (more than 5 μm), are liable to wash off the glass slides during the harsh conditions applied in hybridization procedures, particularly the soapy high pH washing steps.[205] Various subbing agents are used to prevent sections from washing off and/or disintegration of the tissue section. Subbing agents commonly used include gelatine,[214] polylisine,[213] or 3-aminopropyltriethyloxysilane[218,222] ("organosilane"). Sections placed on subbed slides may also be incubated for 24 to 48 hours at 60 °C to maximize adhesion of the sections.[218,220]

Pre-treatment of specimens

Pre-treatment procedures are used to increase the efficiency of the hybridization process and to reduce non-specific background staining. Sections of wax-embedded tissue are de-waxed using an organic solvent, commonly xylol. Pre-heating the sections to 75° C for 15 minutes prior to submersion in xylol is said to improve results.[220] Xylol residues are removed by washing with 100 % ethyl alcohol, and hydrated using "graded" alcohol/water mixtures from 100% ethanol, through 90%, and 70 % ethanol before washing in distilled water. Pigment granules in tissue may obscure signal from the target-probe-indicator complex and are bleached[221] if possible. Various standard histologic techniques are available for removal of pigments (eg. melanin).[198]

Pre-digestion of the tissue section is performed to increase the accessibility of the target, especially if the probe is more than 100 base pairs in length. Techniques using oligonucleotide probes frequently do not require pre-digestion.[214] Proteinase K and Pronase are the most commonly used enzymes. The concentration of the proteinase enzyme and the

duration of digestion may be varied to optimize pre-digestion of the particular tissue for each particular *in situ* technique.[218] Post-digestion fixation to prevent total disintegration of the tissue during the hybridization process is regarded as essential by some authors.[205,218] A further step to improve permeability to probe is incubation in dilute HCl. This mild acid hydrolysis is thought to render highly cross-linked acidic nuclear proteins more soluble, enabling easier access of probe.[218] Incubation with detergent solution is also helpful in rendering cell membranes more permeable.[215] Incubation in acetic acid after the pre-digestion step removes endogenous alkaline phosphatase, essential if the target-probe signal is generated by an alkaline phosphatase-catalysed procedure.[205]

An optional step to reduce background signal is to wash with acetic anhydride.[205] This procedure may reduce background staining due to binding of probe to positively charged amino-groups. Pre-hybridization incubation of large sections in hybridization solution without probe may assist dispersion of the probe when it is subsequently applied. However, in small sections this step may only serve to dilute the small amount of probe used, causing weak signal. Indeed, some workers dehydrate the sections prior to adding the probe so that the sections soak up the probe efficiently.[205] A similar effect can be achieved in frozen sections or cell smears by pre-incubation in glycerol. Enhanced hybridization occurs, presumably because the glycerol excludes water from the cells allowing rapid access of the probe to the cells.[218]

An essential measure is to prevent degradation of target nucleic acids during the pre-hybridization steps. Aldehyde fixation of the tissue effectively inhibits nuclease activity. However, if RNA in unfixed tissue is the target, ribonuclease-free conditions must be used.[216] Water must be double de-ionized, charcoal filtered, and also filtered through a 0.45 µm millipore filter. Glassware must be baked to destroy RNase and disposable plastic microfuge tubes must be used. Pre-hybridization solutions must be amine-free and treated with diethylpyrocarbonate (DPEC) to destroy RNase. Subsequent to the pre-hybridization steps, the conditions prevailing during the *in situ* techniques are such that RNase is deactivated (proteinase K, acetic anhydride and high (0.5M) salt concentrations all inhibit RNase activity).

Pre-hybridization DNA denaturation

To allow the probe access to, and anneal with target DNA, double stranded target DNA (and double stranded probe) is heat denatured at 90 to 95 °C for 10 minutes to split the double strands into two single strands.[206,218] This incubation can be performed on a good quality hot plate that provides stable thermostatically controlled temperatures, or on a hot tray placed in a thermostatically controlled oven. The *temperature and duration* of this step is *critical* if morphology is to be preserved.[218]

Hybridization

Hybridization must be performed under optimal conditions to allow specific annealing of probe sequences to homologous complimentary target sequences in the tissue. Subsequent washing removes non-specifically bound probe that causes non-specific background staining.[218] Background staining is the limiting factor reducing sensitivity and

specificity of the procedure. The stringency of the conditions prevailing during the hybridization step and the subsequent washing are critical to successful in situ hybridization.

Optimizing conditions for *in situ* hybridization has been a largely empirical process.[205] However, tests to optimize the procedure can be performed and are based on factors known to affect the stability of hybrids and on factors affecting the kinetics of hybridization.[223] Hybridization times may vary from minutes to up to 40 hours.[132] Inclusion of Dextran sulphate polymer of molecular weight greater than 8000 increases the concentration of probe by molecular exclusion and is widely used.[220] Addition of unlabelled DNA (human DNA for viral probes, salmon sperm DNA for human genomic probes) may be necessary to bind un-hybridized probe and reduce non-specific staining.[135] Various other macromolecules may be used from time to time to reduce background staining (eg. Polyvinylpyrollidine).[205]

(1) *Stability of hybrids*: hybrid stability is affected by various factors.[205] RNA-RNA hybrids are most stable, RNA-DNA hybrids of intermediate stability and DNA-DNA hybrids least stable. The melting temperature (T_m) of hybrids depends on the length of the probe, longer probes forming more stable hybrids. The base-pair composition of the probe affects T_m, with higher melting temperatures occurring with higher concentrations of Guanine-Cytosine (GC) base pairs. Probe-target homology affects T_m, with the most stable hybrids occurring with exact homology. This effect is greater with short probes. The stability of hybrids is increased in high concentrations of monovalent cations (eg Na^+ sodium ions). Formamide de-stabilizes hybrids by reducing T_m, the melting point of DNA-DNA hybrids being affected more than for RNA-RNA hybrids. Inclusion of formamide in the post-hybridization wash renders non-homologous (non-specific) hybrids unstable at lower temperatures and thereby improves the stringency of the system. Hybrids are less stable in tissue sections, presumably because the probe is prevented from annealing along its full length by the three-dimensional shape of the cross-linked target. The T_m for RNA-RNA hybrids may be as much as 5° C lower in tissue sections than in solution. Several empirical formulas have been derived to assist predict the T_m of hybrids.[224,225] For example, for DNA probes longer than 22 base pairs, the T_m of a homologous hybrid in solution is predicted by:

$$T_m = 81.5 + 16.6 \log (\text{molarity monovalent cations}) + 0.41 (\%GC) - 500 (\text{number probe base pairs}) - 0.62 (\% \text{ formamide}).$$

For oligonucleotide DNA probes between 11 and 22 base pairs, the T_m of a homologous hybrid in solution is predicted by:

$$T_m = 4 (\text{number of GC residues}) + 2 (\text{number of AT residues})$$

(2) *The kinetics of hybridization*: hybridization kinetics is less well understood, especially since the main factor is the accessibility of the target. This depends on the extent of cross linkage in the tissue. Cross-links in the tissue depend on the nature of the tissue, the fixative used, the duration of the fixation, the degree of pre-digestion and the extent of post-digestion re-fixation. Other factors related to the accessibility of probe are the size of the probe and the

concentration of the probe.[205] If the stringency of conditions during hybridization are high, time taken for hybrid formation is longer, and the stringency of the subsequent wash may be reduced.[226]

Post-hybridization washing

The post-hybridization washing step serves to remove probe that has not annealed to the intended target. Generally neutral saline citrate at high concentration is used.[220] A further requirement at this stage is to impose stringency conditions that de-stabilize and break non-specific probe-nucleic acid sequences (non-homologous binding of probe). The higher the stringency of these conditions is, the more specific the result of the hybridization will be. It is critical that only the non-specifically bound probe is removed. The post-hybridization wash is usually performed at temperatures a few degrees below T_m of the target-probe complex so that only target remains annealed to probe.[205]

For RNA probes, excess free probe is digested by ribonuclease, leaving only double stranded hybrids in tact. The presence of inhibitors of ribonuclease, for example formamide or reducing substances must be removed.

Demonstration of hybrids[205]

The demonstration of hybrids is the last step of the *in situ* hybridization technique.

(1) *Radioactively labelled probes:* for convenience, radioactive labelling of hybrids is usually performed with ^{35}S. The labelled hybrid can be exposed to X-ray film over night for a low-resolution, rapid result when optimizing conditions or trouble-shooting.[205] Better resolution is obtained by dipping the slides in liquid nuclear track emulsion[226] and drying them slowly to avoid background staining. Since auto-radiography is more efficient at low temperature, the slide is exposed at 4°C and developed. Coarse grades of liquid emulsion are more sensitive but resolution is lower. Again, thicker emulsion is more sensitive but yields lower resolution. Emulsion is sensitive to mechanical stress and to background radiation, both of which can cause high background of silver grains. Over exposure leads to a reduced signal-to-noise ratio so exposure time must be optimized. Developing the film at higher temperature, or with more agitation, or for longer time develops larger silver grains, but reduces resolution. After developing the emulsion for the silver granules, cells nuclei are stained and the section is mounted under a coverslip. Stains include toluidine blue for conventional light microscopy and propidium iodide for fluorescence microscopy. Bright-field illumination is sufficient to visualize high-density silver grains. Dark-field illumination is more sensitive and visualizes smaller silver grains. Epipolarization allows resolution of the tissue in the dark-field, or dual-exposure photography can be used to superimpose the location of silver grains in the tissue.

(2) *Hapten-labelled probes:* hapten label systems using a stable enzyme, such as alkaline phosphatase or β-galactosidase, the reaction generating insoluble coloured precipitate may be carried out for several days to achieve high amplification of signal.[205] Non-specific background staining is however, a limiting factor. Although theoretically possible, simultaneous *in situ* hybridization for two targets using different hapten-labelling systems has not been described to date.[205]

Artifacts and controls in DNA *in situ* hybridization

The interpretation of results obtained is only as good as the controls used. No single control is best for *in situ* experiments and it is preferable to use as many as possible.[214] Both positive and negative controls are necessary.

Positive controls include hybridization controls and known positive cases. Hybridization controls ("housekeeping" controls) are essential because they indicate whether or not suitable conditions for hybridization have occurred during the procedure.[218] A total DNA probe that should hybridize every nucleus in the section may be used to indicate that the procedure has worked. A section from a known positive case will indicate whether the probe system for the particular target has worked. Further, the potential for signal to occur because of non-specific hybridization of probe to non-target ("cross-hybridization") makes use of this known positive control essential.[205, 218] Levels of background due to "cross hybridization" in test cases can be compared with sections from a particular, good quality known positive tissue block.

Negative controls may include a known good quality negative case. This is used as a further indicator of the process itself, and to compare the level of "cross-hybridization" background due to non-specific effects inherent to the particular method.[218] Negative controls can be used to test each step of the procedure.[227] A useful negative control step to test the specificity of the system is to digest the target with DNAse or RNAse.

A problem that is more difficult to solve is the distinction of low-level expression of target from non-specific background. Each different probe binds non-specifically to a different extent making assessment of low-level expression even more difficult.[205] This can be solved by inclusion of a control in which a ten-fold excess of unlabelled nucleic acid identical to the probe is present during the hybridization. The unlabelled nucleic acid effectively competes with the probe for specific binding sites, removing the low-level specific signal, but not altering the background signal.[205] By comparing this control with the test, the presence or absence of low-level signal becomes assessable. This technique does not find favour with some workers.[214]

One artifact specific for radioactive probes is background staining over the entire slide, not only that part of it covered by the tissue section. This phenomenon is due to mistreatment of the emulsion. Deposition of silver grains in the emulsion may be due to exposure during storage to radiation, to mechanical stress of the emulsion in storage, or to rapid drying of the emulsion.[205] Another specific artifact is background staining that occurs at the edges of the tissue due to mechanical stress to the emulsion during drying of the emulsion. By allowing the emulsion to dry slowly, this problem may be reduced. Spurious destruction of silver grains may occur if moisture is present, particularly during staining of the nuclei, or after cover slipping.[214]

Background staining over the entire tissue section is usually due to non-specific binding of the probe.[205] The size of the probe, the concentration of the probe used and/or the stringency of hybridization and washing may be altered to overcome this problem. Certain probe sequences give higher background signal and use of a probe from a different region of the target gene may be necessary. Some tissues seem to be more "sticky" than others: use of a different probe

sequence or adjusting the hybridization or washing conditions may resolve the problem. Background staining in techniques using RNA probes may occur if the post-hybridization ribonuclease treatment is not effective.

Background staining specific for hapten-labelled probes may be due to endogenous hapten or endogenous enzymes in the tissue section. A non-hybridized control section put through the signal generating steps should reveal artifactual signal.[214] Endogenous enzymes may be inactivated by pre-treatment with hydrogen peroxide or levamisole[214] or by pre-treatment at high temperature to inhibit the enzyme.[205] "Edge-artifact" at the periphery of tissue sections is a phenomenon of hapten-labelled and enzyme immuno-histochemical techniques and must be taken into account. The cause of the edge artifact is not known. However, *in situ* hybridization signal artifact occurs in tissue placed near to the edge of the protective coverslip during the hybridization procedure and is due to drying of probe.[205]

Low signal may reflect scant amounts of target in the tissue, but may also be caused by degradation of target. Contamination of pre-hybridization steps by nuclease enzymes must be avoided. Fixation and protease treatments must be optimized to preserve target. Low signal levels may also be improved by manipulating the size of the probe, the concentration of the probe used, and the stringency of the hybridization and washing steps.[205]

Sensitivity of DNA *in situ* hybridization

Although specificity of the method may approach 100 % when strict stringency is applied, the sensitivity of this method is variable and is in part dependant on the detection system used.[205] Where signal is not amplified, more copies of target DNA may be required for resolution of the signal. Tests using radioactive-labelled DNA hybridization probes generally require ten thousand copies of the target DNA sequence for detection. Many clinical specimens containing significant numbers of the target sequence (but less than 10 000 copies) would be falsely negative using radioactive labelled DNA probe methods. On the other hand, use of an enhanced labeling system that produces an easily resolved, bright signal improves sensitivity dramatically. Thus, hapten-labelled probes using immuno-detection systems that amplify the signal and are safer and easier to handle are more widely used. The recently developed platinum-linked hapten-labeled DNA probes (ULS®) are even more sensitive. These are said to detect as few as 10 copies per cell.[219,228] However, even using a platinum-labelled DNA probe method, specimens containing less than 10 copies of target DNA would be falsely negative.

4.4.4.3 ULTRA-SENSITIVE DNA *IN SITU* HYBRIDIZATION (KREATECH ULS®)

The Kreatech ULS® DNA *in situ* hybridization technique has significantly increased the sensitivity of DISH by a factor of approximately 3 log (1 000 times).[219,228] An affinity between purines and *cis*-Pt was discovered as a side effect of *cis*-Pt anti-tumour therapy.[229] The *cis*-Pt molecule forms a monofunctional covalent bond with purine molecules at the N7 position,[230,231] and also binds to pyrimidines with less avidly. The anti-cancer *cis*-Pt molecule is a Pt II complex stabilized with two covalent amine ligands bound by strong covalent bonds. The Pt II complex exhibits two free biding sites that can cross link DNA strands, preventing DNA replication. In the ULS method,[232] the Pt II complex stabilized by the chelating diamines is linked by the third covalent bond to the hapten marker biotin (ULS-BIO). The fourth un-

occupied binding site readily binds to the N7 on purines in the DNA probe. The platinum moiety serves as a linker molecule between the probe and the hapten.

The binding of ULS® to probe is a one step procedure, does not involve heat labile enzymes, and is easily achieved. The resulting ULS-BIO® molecule is stable and easily handled in the laboratory. Optimal binding of ULS-BIO® to probe is achieved if probe and ULS-BIO® are in a ratio 1:1. In these conditions, a labelling density of 1 in 10 bases is achieved. This high density labelling of the DNA probe has resulted in a very high sensitivity DISH procedure.[233] The ULS® DISH has been used in a variety of settings for the detection of specific human genes [234,235] and for *Chlamydia trachomatis* and HPV in the uterine cervix.[228, 236]

Remarks

The enhanced sensitivity of the commercially available Kreatech ULS® made this test a very attractive option for the present investigation. The high specificity of DISH, now combined with a high sensitivity, made an ideal laboratory test for investigating the morphology of Chlamydial infection in pap smears and tissue sections.

4.4.4.4 DNA Detection by the Polymerase Chain Reaction

Introduction

Detection of DNA by PCR was first described in 1985[237] and significantly improved by the same workers in 1988[238] and 1991.[239] PCR is an enzymatic technique for the *in vitro* amplification of specific DNA sequences. The method can amplify by a factor of a million or more, a single copy gene sequence occurring only once in a genome. The method can detect the single sequence from a background of many unrelated sequences using a vanishingly small specimen, such as DNA extracted from a tissue biopsy or even a single cell. The elegance of the technique was well demonstrated when specific gene sequences were amplified from the DNA in a single hair root.[240] The use of this technique and its numerous applications has expanded exponentially as a tool in clinical diagnosis and in research.[174,217]

The PCR technique vastly amplifies the number of DNA sequence of interest to easily detected amounts. The advantages of this application are the very high sensitivity and the relatively short duration of the assay.

(1) *Sensitivity*: The method allows demonstration of the presence of as few as one copy of target DNA in a specimen of tissue containing between one hundred thousand and one million cells.[217] This sensitivity surpasses all other molecular detection methods and microbiological culture or serology techniques. ULS® *in situ* hybridization is said to be sensitive enough to detect 10 copies of target DNA per cell, while PCR demonstrates one target sequence per hundred thousand cells.[217] PCR is between a million and 10 million times more sensitive than *in situ* hybridization.

(2) *Duration of the test:* The technique employs repeated cycles during which the temperature is changed, typically between 2 and 5 minutes in duration. For most purposes, between 25 and 50 cycles are sufficient so most assays are complete in a period of 2 to 4 hours.[217]

(3) *Limitations:* The amplification technology detects DNA targets and does not depend on the presence of viable intact organisms. Thus, PCR may detect DNA from a non-viable organism(s) in a specimen that could not be cultured. This fact can explain discrepancy between the results of tests using culture techniques and PCR techniques, and partially explains the enhanced sensitivity of PCR testing over culture. However, a positive PCR test does not necessarily indicate the presence of viable organisms in the sample tested. Tests after cure of infection by *C. trachomatis* must take this into account. A "window" period for culture-negative but PCR positive tests following successful doxycycline treatment of Chlamydial infection has been documented and appears to last for about 3 weeks.[241,242] Since the PCR procedure can detect minute amounts of DNA, it is vulnerable to contamination by spuriously present DNA. The laboratory is the most likely source of such spurious DNA and precautions to prevent contamination are necessary.

The following section of this review concentrates on the use of PCR applied to tissue specimens.

Principles of PCR[217,243]

The key to successful PCR was the discovery of a polymerase enzyme ("*Taq*" polymerase) [Roche] in a thermophilic bacterium (*Thermus aquaticus*). This polymerase retains its activity at the high temperatures necessary to denature DNA and to maintain the split DNA in single strands (72°C).[237] Other thermophilic enzymes have since been identified.[243]

The PCR method employs the thermophilic DNA polymerase enzyme to promote replication of a particular sequence of target nucleic acid in an *in vitro* system. The selected segment of target DNA is replicated under the direction of specially selected synthetic oligonucleotide primers ("amplimers"). Two primers are prepared, with sequences complimentary to the 3' end of each strand (the "sense" and the "anti-sense" strands) of target DNA. When the target DNA denatured by applying heat, and then cooled, the primers anneal to their complimentary sequence on the target DNA. The sequences of the primers are selected so that overlapping replication occurs. The primers anneal at selected positions on the sense and anti-sense target DNA strand so that a particular, relatively short segment of target DNA lies between them. This short intervening segment serves as a template for DNA replication. In the presence of excess primer, the complimentary target sequences readily anneal with the primers. Under the influence of the DNA polymerase enzyme, in the presence of nucleotide triphosphate molecules (dNTPs) and appropriate temperature conditions, the intervening segment of DNA is replicated. The reaction is promoted by sequentially altering the temperature of the system and is repeated, usually for 30 to 50 cycles.

The technique employs cycles of heat denaturation of DNA at 95°C, followed by annealing of the primers to the target nucleic acid sequence at temperatures between 40 and 60°C, and then enzymatic synthesis or "extension" of the target DNA sequence at 72°C.[217] Since the primers remain in excess, each replicated segment of target DNA binds with primer at the beginning of each new cycle. With each cycle, the original target sequence and the previously replicated sequences ("amplicons") are replicated again and again. The number of replicated segments is therefore doubled with each cycle, resulting in exponential replication of the original target sequence.[217] The chain reaction can potentially produce millions of copies of the selected target sequences. The detection of the amplified segment confirms the

presence of the specifically targeted DNA sequence. The high numbers of amplicons produced render the technique exquisitely sensitive.

(1) *Manual technique:* The thermal cycling can be performed using such simple equipment as three water baths at three different temperatures. Moving the reaction cups manually through the cycles is time consuming and tedious.

(2) *First generation thermal cycler:* The PCR reaction is most commonly achieved by use of a programmable computerised machine with a special heating and cooling plate with small wells that snuggly accommodate the Eppendorf® reaction cups [Perkin-Elmer Cetus Instruments®, Techne® and Koch-light®]. [217] The thermal cycle required for each particular reaction is selected and automatically performed by the machine. The number of cycles, the time and the temperature of each step in the cycle can be specifically programmed. The special heating and cooling block moves rapidly through the cycle and the snug fit of the Eppendorf cups ensures rapid temperature changes in the reaction mix. The use of such machines has brought the time necessary for most PCR reactions to 2 or 3 hours.[244]

After the amplification cycles, the PCR products are separated from the background on the basis of their molecular weight by electrophoresis.[174,244] The PCR products are passed through an agarose gel containing ethidium bromide, a fluorescent mutagen which binds to DNA. The products are separated into bands according to their molecular weight and can be viewed by exposure of the gel to ultra violet light. A mix of DNA fragments of known molecular weight is used to produce a ladder of bands of known molecular weight against which the size of the of the amplification product is compared. A fragment of known target DNA may also be used as a control to ascertain that the PCR product and the control migrate similarly in the agarose gel. The amplification of a product of a particular size is usually sufficient to identify the target sequence.[174,244] Subsequently, the PCR product can be extracted from the gel and its sequence determined directly, confirming the identity of the product and demonstrating its homology with the target sequence.[174,244]

(3) *Second generation technology*: The second generation technology improves the speed and ease of the PCR and provides real time computerized melting curve analysis of the T_m of the amplicons. The best established known second generation PCR machine known as the Light Cycler® [Roche] employs much small volumes of reaction mix contained in fine glass capillary tubes with high heat conductivity properties.[244] The tubes containing the reaction mix are heated and cooled in a chamber by circulating hot or cold air. The reagents used in this system are labelled with tags that fluoresce only once incorporated into double stranded DNA. The computerised system measures the reaction products during each thermal cycle and if different fluorescent tags are used, can monitor more than one reaction-product at a time. The computer monitors the melting point T_m of the reaction products and can plot the concentration of reaction products after each cycle. This new generation of PCR cycler offers the advantages of smaller volumes of reaction mix, considerably reduced reaction times and real time computerised monitoring and analysis of the reaction products.[244] The analysis of reaction products by T_m allows quantitation and precise identification of different products. This system is presently being marketed in an upgraded and improved model, (not considered third generation) and manufacture of fluorescent-labelled primers requires collaboration with the manufacturer. The equipment is expensive but its use

appears to be growing rapidly. Several other real-time PCR systems have become available subsequently, including one, the Smart Cycler® that can run different tests for different target sequences concurrently.

Factors and conditions affecting PCR

Important factors affecting PCR include the number of cycles, the time at temperature profiles, and the concentrations of the chemical participants in the reaction (the "master mix").[217] The mix includes primers, magnesium salts, deoxynucleoside triphosphates (dNTPs) and the polymerase enzyme. Reaction mixes are commercially available or may be specifically designed or adjusted for a particular purpose. Outside of the research setting, customised design of the mix is usually not necessary. Routine clinical testing is more convenient using standardised commercially available tests.

(1) *Selection of Primers*: Primers are generally 18 to 20 nucleotides long and manufactured using automated DNA synthesizers. Very small amounts of primers (0.2 to 1.0 μM) are sufficient for many hundreds of tests. Primers are also commercially available from many sources [DuPont, Perkin-Elmer Cetus, Roche]. The primer that is selected defines the sequence to be amplified and detected. Selection of the primer sequences is a critical factor for successful PCR. By altering the primer sequences, any number of pathogens or molecular genetic alterations can be detected using the same assay method.

The size of the amplicons is determined by the choice of primers and their precise location along the complimentary strands of DNA. In general, a convenient size for synthesis and detection of amplicons is between 100 and 300 nucleotides. [174,244] Long segment amplicons take more time to be synthesized and use more deoxynucleoside triphosphates in the mix. For long segment amplicons, the mix must be adjusted and that the part of each cycle in which synthesis occurs must be extended, prolonging the overall reaction time. The commonly used *Taq* DNA polymerase enzyme replicates between 35 and 100 nucleotides per second.[217] The longer the target, the less likely it is that intact target sequences will remain to be detected in a tissue specimen that has been preserved ("fixed"). Target sequences of 1,000 base pairs can be synthesized.[217]

Successful PCR depends on selection of a target sequence that is "genetically conserved" between individuals or microbiologic organisms (ie. a target sequence that does not vary between individuals). A genetically variable or "polymorphous" target sequence may result in false negative PCR results. Mismatches in target sequence at the 3′ portion of the primer are more critical, but can be tolerated at the nucleotide triphosphate (dNTP) concentrations normally used in the PCR mix.[245] Once a genetic polymorphism has been detected, usually by analysis of many DNA isolates, appropriate primers can be designed to avoid them. Genetic polymorphism may on the other hand be the target of PCR for the diagnosis of somatic germline mutations (DNA fingerprinting).

Design of primers to detect micro-organisms should ideally target a species-specific DNA sequence, thereby excluding closely related but different species. A strategy to overcome false negative results due to polymorphism is to employ

two sets of primers aimed at separate segments of DNA. The chance of polymorphism occurring in both target sequences in the same organisms is unlikely. Another use for two sets of primers is to target both species-specific and a type-specific DNA sequences.

The use of "*nested*" primers can improve specificity and sensitivity. A second PCR is performed on the amplicons of a first amplification, using primers specific for a second, shorter sequence occurring within the confines of the first sequence. However, manipulation of PCR products from the first PCR in order to perform the second amplification increases the chance of contamination so that a second PCR should be avoided if possible.

(2) *Number of Cycles*: The sensitivity of PCR is in general improved by increasing the number of cycles. However, the concentration of substrate in the mix and the prevalence of the target are factors that limit the end point of the reaction. Optimal sensitivity cannot be obtained by simply increasing the number of cycles. Most reactions are completed within 20 to 50 cycles.[217]

(3) *Fidelity of Taq Polymerase*: DNA polymerase derived from *Thermus aquaticus* lacks 3′ and 5′ proof reading activity. An error rate of approximately 1 in 10 000 bases occurs during synthesis of DNA using this enzyme. Other polymerase enzymes possess higher fidelity. However at this level, *Taq* replication is sufficiently accurate for practical purposes.[217] The performance of Taq polymerase from different manufacturers may vary.[147]

(4) *Concentration of target DNA:* the primer molecules become incorporated in to the amplicons and are consumed by each cycle, small quantities of target DNA are preferable. In general 250 ng of target DNA or less are best suited to the concentrations of primer and substrate in a standard PCR mix.[244]

(5) *Contamination by inhibitors of DNA polymerisation:* The most significant problem with the development of tests for routine use has been false negative results. These have usually been due to substances in the specimen that cause inhibition of the chain reaction.[50,246-248] The presence of inhibitors is not predictable and inhibition may be difficult to detect but can be suspected if amplification controls are used (eg. β-globin primers) to prove that the reaction is working. If the presence of inhibitors is suspected, PCR may be repeated on a diluted specimen. The inhibiting substances are thus diluted and their untoward effect obviated. Alternatively, a duplicate specimen may be spiked with known target and inhibition confirmed.[217]

(6) *Contamination by amplicons:* Contamination of specimens or the laboratory surfaces and equipment by amplicons that are present in huge numbers at the end of a chain reaction may cause false positive results. Contamination is relatively easily overcome by physical separation of areas for reagent preparation and DNA product amplification. In addition, contamination is avoided by use of latex gloves that are worn in each area, and removed prior to moving from one area to another. Further, careful pipetting techniques are necessary to prevent carry over from one

specimen to another. If false positives are identified, measures to prevent contamination include adoption of careful techniques and decontamination of all surfaces and equipment by wiping with dilute bleach.[217]

Commercial applications of PCR

The development of PCR techniques in the research laboratory has been followed by the development of tests for the clinical situation. In order to take advantage of the superior sensitivity of PCR, several commercial tests have been developed and approved by the FDA for routine clinical use in the United States. The development of these methods has however, not been smooth.[1] False positive results due to contamination with amplicons have proved to be less of a problem than was expected. On the other hand, substances commonly in human specimens inhibit the polymerase chain reaction and have been found to cause significant problems in the development of clinically reliable tests. These false negative results are unpredictable and without the use of amplification controls are more difficult to detect. The cost implications of performing the checks necessary to monitor false positive results are prohibitive in the routine clinical setting.

(1) *Amplicor® PCR* [Roche Diagnostics]: The first commercial FDA approved test for PCR on clinical specimens in the USA was a test for *Chlamydia trachomatis* (Amplicor®) [Roche Diagnostics].[249] Metanalysis[1] of several publications[119,147,201,203,241] shows this test to have a sensitivity of 90% and specificity of between 99 and 100%. The test, which is approved for female endocervical, male urethral and male urine specimens, has set a high standard for the future application of PCR in the routine clinical laboratory setting. The Amplicor® primers target a 207 base pair segment of the cryptic plasmid DNA present in seven copies per Chlamydial genome. Tests detecting Chlamydial genomic sequences from the MOMP gene have been compared with the test detecting cryptic plasmid.[250-252] *The sensitivity of the test detecting plasmid is higher*, probably because there are seven DNA target copies per organism. Although plasmid-free strains of *C trachomatis* have been identified,[241,253] these are rare and appear not to be clinically relevant.[1,241,249]

The Amplicor® test is innovatively designed for automation and colourimetric analysis of the test result.[241,249] The amplification product is biotinylated and is captured by an oligonucleotide probe that is complimentary to the amplified product. The capture probe is bound to the test container ("solid phase"). Avidin-horseradish peroxidase conjugate is added and binds to the product. After washing to remove unbound conjugate, a colourimetric substrate reaction catalysed by horseradish peroxidase occurs. The results are read by a standard colourimeter.

A further refinement of the Amplicor® test is the use of a novel system to prevent amplification of contaminant carryover product from previous amplifications.[241,249] The Amplicor® master mix substitutes dTTP with dUTP so that in the amplicons, uracil is substituted for thymine. Thus any DNA templates carried over from a previous amplification (contaminant) contain uracil, whereas naturally occurring templates (target) contain thymine. The Amplicor® master mix also contains Uracil-N-glycosylase. This enzyme cleaves uracil-containing DNA by opening the deoxyribose chain. The opened chains are broken during the initial heating step of the reaction so that previously produced templates are

destroyed and only naturally occurring templates (target DNA) can be polymerized. The initial heating step also destroys the Uracil-N-glycosylase. Thus, before the chain reaction commences, contaminants are eliminated by a protective self-destruct mechanism.

This protective system does not prevent contamination of specimen in the laboratory by naturally occurring Chlamydial DNA (eg. splash over from other sample wells, contamination of pipette tips or laboratory surfaces). Careful laboratory techniques are therefore essential. The potentially serious problem of contamination in the laboratory by vast numbers of amplicons from previous reactions is however, effectively eliminated. Contamination of specimens outside of the laboratory is minimised if appropriate sampling and specimen handling techniques are used (*vide supra*).

(2) *The Ligase Chain Reaction (LCR®)* [Abbot Laboratories]: is a novel system also licensed by the FDA for use in clinical testing.[254] Metanalysis[1] of early evaluations of the test,[64,120,137] shows a sensitivity of 94% and a specificity of 99 to 100 %. The system employs two pairs of synthetic oligonucleotide probes, one pair for each strand of cryptic plasmid DNA. The target sites of the paired probes have been selected so that there is a one or two nucleotide gap between the pair when the probes have annealed. The gap is filled by DNA polymerase and closed by ligase enzymes. This two-step procedure is said to improve the specificity of the test. After heat denaturation, the annealed probe pairs act as template during thermal cycling, resulting in logarithmic amplification of the target sequence. The test requires an automated instrument and is not easily available for non-automated, in-house laboratory use.

The LCR® product is measured using an immuno-colourimetric method with the immuno-capture antibody bound to magnetic beads. To prevent contamination of the instrument and laboratory environment with amplicons, at the end of the procedure, the automated colourimetric instrument pierces the caps of the tubes containing LCR product and injects an oxidizing agent and a chelated metal complex that denatures the amplicons.

4.4.4.5 PCR on Tissue Specimens

Although *in situ* PCR in tissue sections is possible (*vide infra*), analysis of nucleic acid from tissues is generally performed on DNA extracted from the sample. Most tissues can be successfully tested by extraction of DNA, but problems have been encountered with specimens taken from brain and spleen, or specimens that are extremely bloody or necrotic.[217] Typical normal human cells contain approximately 7pg of genomic DNA and a variable amount of mitochondrial DNA.[255] Genomic DNA can be separated from mitochondrial DNA if necessary by isolation of nuclei from cell by ultra-centrifugation methods. Generally, the presence of mitochondrial DNA in a sample is not an issue.

Specific RNA sequences in cells can be detected using reverse transcriptase PCR (RTPCR).[244] RNA is extremely labile in tissue and is denatured rapidly by endogenous RNases. Fresh tissue or tissue snap-frozen in liquid nitrogen is generally required for this process. Target RNA is initially transformed in to dDNA using reverse transcriptase. Subsequent detection of the dDNA is performed by PCR in the usual way. Approximately 15 pg of RNA are present in cells, 80 – 85 % being ribosomal RNA while only 1 to 5% of RNA in cells is mRNA.[255]

Preparation of tissue for PCR

(1) *Fixation of Tissue:* Amplification of DNA extracted from fresh tissue yields optimal results. Efficient PCR has been performed on frozen sections taken from fresh tissue specimens mounted in optimal cutting temperature compound (OCT).[217] PCR using DNA extracted from formaldehyde-fixed tissue is less efficient and caution is necessary when comparing results obtained using differently treated tissues.[217] However, PCR techniques may be readily applied to formaldehyde-fixed, paraffin embedded tissues used for routine histological purposes.[256,257] Provided there is careful extraction of DNA, tissue from paraffin embedded surgical specimens submitted for routine histopathological examination may be subjected to sophisticated DNA detection methods, including both PCR[217] and Southern blot analysis.[258,259] There are vast reserves of such material archived in anatomical pathology facilities all over the world.[259] Up to 20% of fixed tissue specimens may not be suitable for PCR because of inadequate or excessive fixation.[244] However, DNA from tissues fixed after a *post mortem* interval of seven days has been amplified and PCR has even been successful on archeological specimens.[257]

Optimum results on fixed tissue are achieved with fixation for 24 hours in neutral buffered 4% formaldehyde in physiological saline ("10% formol saline").[217, 244] Prolonged fixation should be avoided. Formaldehyde fixation results in the formation of Schiff bases in the DNA, but this step is reversed in aqueous conditions. However, with prolonged fixation, alterations in the DNA structure become irreversible.[217] Despite fixation and paraffin embedding, slow degeneration of DNA continues and PCR performed on very old histology tissue blocks (older than 10 years) may have reduced amplification. On the other hand, successful PCR has been performed using 40 year-old tissue blocks.[217]

Tissues fixed in ethanol or acetone yield adequate DNA extracts for PCR. Fixation by Zenker's Fluid, Carnoy's solution, Bouin's fixative and B-5 precludes successful PCR.[260] Acidic solutions used for the decalcification of bone degrade DNA template and usually preclude PCR on decalcified tissues.[217]

(2) *Processing of tissue:* During the routine preparation of tissue for embedding in paraffin, specimens are fixed, dehydrated and impregnated with paraffin wax in automated tissue processors. The tissue specimens are usually placed in special cassettes. These facilitate access to the tissue by these processing fluids. The tissue are sequentially impregnated through and through with alcohol, xylol and paraffin wax. It is possible that carry-over of DNA from one specimen to another (particularly DNA from micro-organisms) could occur during this process. Investigation of this problem has not received attention in the literature.

(3) *Cutting tissue sections:* Sections are cut between 5 mμ and 10 mμ thickness[217] (8 mμ thickness[244]) and placed in a sterile 1,5 ml micro-centrifuge tube or a 0,5 ml PCR capped reaction cup [Eppendorf®].[217,244] Once the dry section has been placed into the capped reaction cup it may be stored for months at room temperature. A specific portion of tissue block may be selected for testing by scoring the block prior to sectioning.

Contamination of the tissue section must be avoided. The routine use by the operator of latex gloves during sectioning however is not considered necessary but the use of paper towel to clean fingers between cases is recommended. [217] Potential contamination of sections by exfoliated squames from the operator's hands must be considered in certain dermatological conditions associated with exfoliation, hyperkeratosis or Human Papillomavirus infection. These conditions may cause significant contamination, depending on the intended DNA target. [217]

Cross-contamination between specimens is potentially a more significant problem. Sterile, pre-labelled capped reaction cups should be laid out prior to sectioning. Carry over of tissue fragments between different specimens must be avoided at all costs. Some workers have recommend that the forceps, the brush and the microtome blade should be wiped with acid or chlorine bleach to denature residual DNA, as well as with ethanol or xylol to remove residual fragments of wax-embedded tissue.[246] Other workers regard such measures as unnecessary, but wiping the instruments with paper towels has been recommended. [217,244] It has been demonstrated that normal trimming and facing of the wax block prior to cutting sections adequately cleans the blade between subsequent specimens. [217] In this study, negative control specimens were cut alternately with positive controls. Cross-contamination of the negative control by material from the positive controls did not occur, confirming the safety of routine cutting processes. [217]

An alternative method to obtain tissue for PCR from laboratory specimens is to use archived, glass-mounted stained or unstained sections or even cytology smears. The coverslip of such a section or smear is removed by soaking the slide in xylol. The entire tissue section or desired sample from the section or smear is then carefully scraped from the slide into a dry, capped Eppendorf® reaction cup. [217, 244]

(4) *Removal of wax*: The paraffin wax is removed from the tissue sections with solvent, usually xylol or octane. The sections must be dry as the presence of water may inhibit removal of wax. About 1 ml of solvent is added to un-mounted tissue sections in an 1,5 ml Eppendorf® cup and shaken vigorously using a vortex mixer. The paraffin wax is allowed to dissolve completely. Thorough removal of the paraffin is essential as traces of wax may inhibit DNA extraction.[244] Different recommendations to facilitate removal of wax have been made and the time and conditions for this step vary from 2 minutes at room temperature[217] to over night in a water bath at 60°C. [244] The tissue sample becomes translucent in xylol.

Subsequent to de-waxing, thorough removal of wax-solvent by washing with ethanol is essential as these solvents also inhibit PCR. [244] If the tissue sample is small or fragmented, the capped reaction cup containing the sample and xylol may be centrifuged to pellet the tissue prior to decanting the xylol. [217] Alternatively, xylol may simply be aspirated from the Eppendorf® cup.[244] One millilitre of absolute ethanol is used to wash the sample and is aspirated away. This step is repeated two[217] or three times [244] The tissue sample becomes white and is more easily visible. If the tissue is to be stored prior to undergoing PCR, it should be vacuum desiccated. [217]

Factors affecting PCR on tissue specimens

(1) *Amount of DNA extracted:* The presence of excessive amounts of extracted DNA in the specimen, or other tissue components may prevent successful PCR.[217,244] The mechanism of inhibition is not always known, but inhibition of enzyme reactions, particularly DNA restriction enzymes has been described.[156] For this reason, small test samples generally produce better results. For tissue sections larger than $1mm^2$, use of between one tenth and one fiftieth of the extract results in successful PCR.

If precise determination of sample size is necessary, the amount of DNA and of DNA degradation products in a tissue extract can be determined by electrophoresis.[217,255] A sample of the extract (5 to 10 µl) is run through 0.7% agarose gel and stained with ethidium bromide.[255] During electrophoresis, nucleic acids migrate through the gel at a rate inversely proportional to the $\log_{10}$ of their molecular weights. Since the molecular weight of nucleic acids is proportional to their length in base pairs, the size of the DNA fragments can be plotted against the distance they migrate. A standard curve can be constructed plotting the distances that marker fragments migrate against the $\log_{10}$ of their known molecular weight. Using the standard curve, the approximate molecular weight and concentration of the extracted DNA can be estimated.[255]

(2) *Target size:* The size of the target sequence is critical, and in samples from fixed tissue, smaller targets are more successfully detected. The target should be less than 200 base-pairs in length and best results are achieved with targets less than 100 base-pairs.[217]

Typical PCR protocols for tissue specimens

(1) *Master mix:* The mix is made initially and distributed into capped reaction cups before the addition of target DNA. This reduces the possibility of cross contamination in the reaction cups.[244] A typical PCR master mix is given in the following protocol:[217]

- 10 µl buffer : 100mM Tris-HCl, pH 8.3, 500mM KCl, 25mM $MgCl_2$
- 10 µl of dNTPs: 2mM each of dATP, dCTP, dTTP and dGTP
- 0.2 – 1 µM primer: 20-100 pmol of each primer per reaction
- 0.4 µl (2 units) *Taq* polymerase®
- Tissue DNA extract between one tenth and one fiftieth of its volume
- Make up to 100 µl with sterile distilled water
- Overlay with 2 drops mineral oil
- Place reaction cups in thermal cycler

To obtain optimum results, the concentration of magnesium, dNTPs and primers may require manipulation for particular target DNA tissue extracts using known positive and negative controls. The amount of *Taq* polymerase® may be increased to 4 units or more if inhibition of the PCR reaction by factors in the tissue extract is suspected. The entire mix

may be scaled down to 50 µl to save reagents. Precision micro-pipetting is not vital and up to 10% variation in volumes is an adequate level of accuracy.[217]

(2) *Thermal cycle:* A typical thermal cycle is given in the following protocol:[217]

- Initial denaturation 5 minutes @ 95°C
- 20 to 50 cycles of
- denature @ 95°C for 45 seconds
- anneal @ 56°C for 45 seconds
- extend @ 72°C for 60 seconds
- cool to 2°C

The initial heating is prolonged to ensure that complete denaturation of target is achieved. Insufficient heating during the denaturation steps is a frequent cause of failed PCR.[217] The specificity of the PCR reaction depends on the stringency of the hybridization of primer to target sequences. It is critical that there is exact homologous annealing of the primer to target. The annealing temperature should be as close as possible to the T_m, high enough that non-specific annealing of primer to target is unstable and does not occur. It is therefore vital that the annealing temperature is optimised. T_m is governed by the length of the primer and its G-C content. The approximate annealing temperature of the primer can be calculated using the formula:

T_m = 4 (number of GC residues) + 2 (number of AT residues).

Demonstration of the PCR product

The commonly used technique for the demonstration and analysis of PCR products is agarose gel electrophoresis. Electrophoresis is a simple and quick technique with several advantages. It indicates the presence or absence of reaction product, the approximate size of the product can be estimated, and the use of radioactive isotopes is avoided.[217] After separation of the target sequence form other DNA in the sample, the purified reaction product can be sequenced for absolute identification.[217]

Agarose gel electrophoresis: The first step is removal of the amplification product from the Eppendorf reaction cup If necessary, the mineral oil can be removed by adding chloroform, shaking the mixture and then aspirating the aqueous phase floating on the oil/chloroform mixture. Alternatively, product can be aspirated from beneath the mineral oil layer.[244] Under the influence of an electrical gradient of 10 to 15 volts/cm, the product migrates through 2% agarose gel at a rate inversely proportional to the $\log_{10}$ of the molecular weight of the DNA fragment.[255] The smaller the fragment, the faster the DNA migrates and the further the fragment travels from the starting point. The identity and the approximate molecular weight of the product is assessed by comparing the migration of the product with control DNA and a mix of standard sized fragments. A standard curve can be plotted using know concentrations of control DNA and a semi-quantitative assessment can be made of the amount of product produced. The addition of bromophenol blue ro the

reaction products and controls allows monitoring of the migration of the blue-stained DNA. Ethidium bromide is added to the gel before it is poured and allowed to solidify. The ethidium bromide binds to the migrating DNA fragments. Once the products have been separated by electrophoresis, the gel is exposed to UV light. The ethidium bromide-stained DNA is auto-fluorescent and the migration pattern of the bands of test and control DNA can be photographed.[255] Ethidium bromide is carcinogenic and careful handling to prevent exposure to the chemical is necessary. Agarose gel containing purified reaction product can be cut from the gel and the DNA content subjected to DNA sequencing for confident identification.[244,261]

Remarks

The techniques used in PCR have been standardised and the method is widely accepted as the most sensitive and reliable modality for the detection of Chlamydial DNA.[inter alia 1,17,217,240] Since PCR is infinitely the most sensitive detection method, yet at the same time a highly specific test, it has been recommended as the gold standard for confirmation of the presence of *Chlamydiae*.[1,2] PCR was an ideal means to corroborate the morphologic criteria for Chlamydial infection and also serve to confirm findings using the relatively untried DISH ULS® method.

4.5 DISCUSSION

4.5.1 SELECTION OF THE GOLD STANDARD

The selection of a "gold standard" implies a test method that yields entirely reliable (specific) results against which to compare other tests. The selected test must first be extremely specific, but also should show the highest possible sensitivity. The historical gold standard for confirmation of a Chlamydial infection has been culture of the organism[1,44] principally because the specificity of the method is near 100%.[1] However, compared with DNA amplification techniques, the sensitivity of culture has been found to vary between 70 and 85%.[40,82] The improved sensitivity of DNA testing methods is so significant that culture is no longer regarded as an acceptable "gold standard" for detection of *Chlamydia trachomatis*.[1,2] The concept of using an expanded gold standard employing two DNA detection methods has been put forward the CDC in the USA.[1]

The following considerations were made in the process of selecting the corroboratory tests used in the present study.

4.5.1.1 CULTURE OF *Chlamydiae*

The specificity of culture for detecting *Chlamydia trachomatis* approaches 100%. Chlamydial inclusions are visible by light microscopy in the host cells 48 to 72 hours after inoculation of the cell culture. The end point of culture is the morphologic identification of Chlamydial inclusions by the demonstration of inclusions in the cell culture. "The direct visualization of inclusions that possess a very distinctive morphology contributes to the near 100% specificity of culture tests."[1] Swanson *et al*[44] showed that the morphology by EM of vacuolar inclusions in control cultures and Pap smears were identical and yet the identification of the Chlamydial inclusions in a Pap smear is not generally accepted as diagnostic.[81]

Until recently, the specificity of culture of almost 100%, has resulted in cultures being used as the "gold standard" for the laboratory detection of *Chlamydiae*.[1] However, the method has a relatively low sensitivity compared to newer DNA detection methods. Depending upon the expertise of the laboratory, the sensitivity of culture has been found to vary between 70 and 85% compared to DNA amplification techniques.[40,82]

Many factors are likely to contribute to the low sensitivity of culture have been reviewed. A particularly important factor is specimen adequacy.[1] Since the organisms are obligate intra-cellular pathogens, specimens taken to detect *Chlamydia trachomatis* must include host cells. The use of correct techniques to obtain infected cells is necessary. [1] Up to 30% of specimens were found to be inadequate to detect Chlamydia despite special training of personnel taking the test samples. [1] Inadequate specimens are also a problem for direct immuno-fluorescent, [2,7] and ELISA techniques.[83,125] In addition, culture of *Chlamydiae* requires that the organism remains viable after collection until inoculation into the cell culture. The specimen must therefore be appropriately stored and transported.[43,87] Perhaps most importantly, successful culture only occurs "if sufficient numbers of viable Chlamydial elementary bodies are present" in the specimen. [1]

Without assuring the quality of specimens, up to 10% of samples may be found to be unsatisfactory.[9] The CDC recommends that the quality of specimens collected for Chlamydia testing should be monitored. [1] Inadequate specimens have been defined by the CDC as those in which no columnar or metaplastic squamous cells are found by microscopic examination. None of the studies using culture techniques to corroborate Pap smear diagnoses[6,8,15,42,44,106,122,262,263] indicated whether the quality of the specimens had been monitored by checking for the presence of columnar or metaplastic squamous cells. (All of theses studies were conducted before this recommendation was made.) It is likely that some specimens cultured in these studies were not adequate. A proportion of false negative cultures would reduce the apparent specificity of Pap smear diagnoses.

During the early stages of the study, culture for *Chlamydiae* was no longer available in Cape Town. However, Lancet Laboratories, a commercial laboratory in Johannesburg, was still performing cultures. This review indicated that limited use of Chlamydial cultures might be appropriate in the present study.

4.5.1.2 Electron microscopy

EM examination has been used successfully to reliably detect the presence of and to identify various micro-organisms[1, 264] including *Chlamydiae*.[21,44,105] One study successfully confirmed the presence of *Chlamydia trachomatis* organisms in McCoy cells by EM.[22] Another demonstrated the organisms by EM in tissue sections of the uterine cervix.[10] A study using EM techniques to corroborate the presence of *Chlamydia trachomatis* organisms in cells lifted from Pap smears showed organisms in only one of several smears.[105] Never the less, EM in that study had been able to identify *Chlamydiae* accurately. It was concluded that use of electron microscopy in the present study would be useful.

4.5.1.3 DETECTION OF CHLAMYDIAL ANTIGEN

Corroboration by DFAT

Several studies critical of the sensitivity and specificity of Pap smears for the diagnosis of Chlamydial infection have used direct immunofluorescent testing.[81,83,87,105] The DFAT is performed on a smear made from a scraping of epithelial cells from the infected epithelial surface. The smear is air-dried or acetone fixed.[105] The presence in the specimen of adequate numbers of epithelial cells is essential. [1,43]

The DFAT method combines detection of Chlamydia-specific antigen with direct localisation of the organism in the specimen, achieving a high specificity of 98 to 99%.[1,14,105] The value of the test is the specificity of antibody-antigen staining of inclusions and elemental bodies that, at the same time show the characteristic morphology of *Chlamydiae* in the correct location. However, compared to optimally performed culture, DFAT has a *sensitivity* of between 80 and 90%.[1,14,105] Since culture has a sensitivity of 70% to 85% compared to DNA amplification techniques,[13] the sensitivity of DFAT compared with DNA detection methods can be predicted to be between 56% (70% x 80%) and 76.5% (85% x 90%). The *low sensitivity* of DFAT calls into question the validity of the numerous studies[21,125,126] critical of the value of Pap smear diagnosis of Chlamydial infection.

The DFAT is no longer widely used as a primary diagnostic test because it has a low sensitivity and is labour intensive requiring highly skilled personnel to perform the microscopy.[1] The use of DFAT requires that a second specimen of fresh cervical mucus be obtained and this was not practical in the study setting. Further, experienced personnel in the Penman laboratory reported that reliable interpretation of the fluorescent Chlamydial particles was extremely difficult. Although DFAT was available in the practice, this review of the literature showed that when these methods had been used as corroboratory tests for Pap smear diagnosis of Chlamydia, results had been sub-optimal.[*inter alia* 6,87,125] It was decided not to repeat these studies.

Corroboration by ELISA

The ELISA tests detect Chlamydial LPS. The specificity of most commercially available ELISA tests is in the region of 97% without the blocking step and better than 99% with the blocking step.[1] The blocking step is necessary to prevent cross-reactions with LPS that occurs in both gram-negative bacteria and yeasts.[*inter alia* 116,139] However, meta-analysis of several studies using different commercial tests showed that the best possible *sensitivity* when the blocking test is employed is only in the region of 73%.[1]

On this basis, those studies that evaluated Pap smear diagnosis[105,265] using ELISA methods for the gold standard were regarded as being of questionable value. For example, the Chlamydiazyme Test® (detecting LPS) used by Henry *et al*[105] was shown by other workers to have 24% false negative results when the blocking test was used.[146] This method had been found to be sub-optimal by several workers.[*inter alia* 1,50,51,59] "Results should not be assessed by enzyme-linked assays, since this results in the loss of the high specificity produced when inclusions are directly visualized."[123] An ELISA method for *Chlamydiae* was available in the practice. However, because of poor sensitivity when used in

a high specificity mode (with the blocking test), ELISA testing was not contemplated for studies to corroborate the morphologic findings.

Immuno-histochemical demonstration of Chlamydial antigen

Numerous practical considerations are necessary to ensure reliable and reproducible IH results. These considerations include the nature of the specimen available for examination, the cost and availability of the reagents, and the equipment required. In the routine laboratory setting, these factors frequently prevent application of more sensitive and elegant IH methods. [159]

Assessment of the specificity of immuno-peroxidase techniques for the demonstration of Chlamydial antigen does not appear to have been published.[1] The specificity should be assessed using known positive and negative control sections on the same glass slides. The sensitivity of the procedure varies according to numerous factors including the amount of antigen present, the preservation of the structure of the antigenic molecule, the avidity, affinity and titre of the primary antibody, and the sensitivity of the immuno-detection system.[159] Well-preserved morphology and preservation of the antigen is essential and antigen retrieval may be required using proteolytic enzyme digestion, high or low temperature pretreatment techniques or acid hydrolysis. IH staining using fluorescent detection systems (IF) provides maximum sensitivity but must be performed on unfixed tissue. IF is not possible in alcohol or formalin-fixed specimens. All possible refinements of the technique may serve no purpose if the antigen is not preserved or available for binding to the labeled antibody. [159]

Routine fixation of Pap smears in most laboratories is with 95% ethanol or 100% methanol.[45] Alcohols fix tissue by precipitation of proteins and prolonged exposure to ethanol or methanol may destroy antigenicity.[167] Thus, for IH work, the use of routinely fixed Pap smears made from exfoliated cells of the *cervix uteri* is not always possible. [159]

The MOMP antigen is not present in high concentrations during all stages of the life cycle of *Chlamydiae*.[2,16,43] The intra-cellular vacuolar inclusions seen in smears and sections contain RB. Synthesis of MOMP antigen in the RB commences after 16 hours and is present in low concentrations during the early phase of the life cycle.[1,2,43] Chlamydial LPS antigen is more abundant in RB. [1,2,16,43]

The main factor determining the decision not to use IH in the present study was the problem with availability of suitable antibody. Use of anti-MOMP antibody in the present study was regarded as inappropriate because many inclusions may not be well stained during the first half of the infective cycle. Enquiries to several commercial concerns[79,80] revealed that antibodies commercially available for IH detection of Chlamydial antigen in alcohol fixed smears and in formalin-fixed paraffin sections were directed against MOMP. Antibodies for IH directed against LPS made use of fluorescent detection systems[79,80] and required fresh frozen tissue sections that were not available in the circumstances of the study.

4.5.1.4 DETECTION OF CHLAMYDIAL DNA

Corroboration by DISH

The cellular DNA molecule is relatively inert and remains largely unaltered in tissues preserved by aldehyde fixatives at temperatures below 64 °C.[207,208] This enables the detection and localisation in fixed tissue of specific DNA sequences by *in situ* hybridization.[209,210] The ability of DISH to locate the target DNA sequences at a precise morphologic location in the tissue is very important. The demonstration of a specific target in its specific location results in a highly specific, elegant and valuable test.[1] The specificity and the sensitivity obtained are high enough to allow reduced numbers of tests to yield significant results without resorting to fastidious statistical analysis.[211] These were regarded as extremely important points for consideration in the present study.

DISH requires well-preserved morphology on the one hand. On the other hand, DNA hybridization requires conditions that allow maximal accessibility of the probe to target and stringent biochemical conditions that favour specific hybridization.[212] Optimal hybridization conditions are usually detrimental to preservation of tissue morphology. Successful DISH technique therefore represents a compromise between preservation of tissue morphology and the integrity of target sequences.

The sensitivity of DISH depends on the accessibility of the target DNA, the method by which the probe is labeled, the method used to detect the annealed end product, the length of the probe, and whether the target is RNA or single-stranded DNA or double-stranded DNA. Non-specific background staining may limit sensitivity. Abundance of target may allow use of a method with relatively low sensitivity but dictate a need for maximal specificity.[212] The signal generated by the detection system may vary from a sub-cellular level to a particle size greater than a cell diameter. Resolution and detection of the signal is largely dictated by the method used to label the probe. The specificity of the signal for target depends upon the stringency of the conditions during the post-hybridization washing step and upon the presence or absence in the tissue of non-target sequences similar to target sequences. Non-specific signal can originate from a variety of sources. The setting of precise stringency conditions and use of controls to differentiate non-specific signal is essential.[212]

Hapten-labelled probes are detected by immuno-cytochemical methods. They are safe, stable, achieve rapid results and yield single-cell and sub-cellular resolution. The most sensitive techniques employ a hapten against which a specific antibody or other binding protein (ligand) is available. Examples of these haptens include strepavidin-biotin and digoxigenin. Hapten-metal complexes, particularly gold-labelled antibodies, have been used for in DISH at the electron microscopic level.[193,194] During the course of the study, an improvement of the DISH method became available.[219] The new method used the "universal linkage system" (ULS^{R}) to improve sensitivity of the detection system to signal DNA-probe complexes. Strong electro-valent binding sites on a *cis*-platinum molecule are used to bind the nucleic acids guanine and adenine in the probe to haptens, enzymes or fluorescent tags. This technique has dramatically increased the sensitivity of hapten-labelled DISH techniques and can detect as few as 10 copies of target DNA per cell.[219]

The most frequently used means of corroboratory testing in histologic work is immuno-histochemistry. Since the antibodies available for detection of Chlamydial antigens were inappropriate, a reliable alternative to IH was required to corroborate the presence of Chlamydial infection in Pap smears and tissue sections. Of the DNA detection methods available, DISH for Chlamydia-specific DNA was regarded as one of the best. The method has a high specificity and sensitivity and was ideal for use in the type of specimens available in the study. DISH technique has a high specificity combined with the ability to morphologically localize the target DNA. Unlike DISH, routine PCR does not localise the target DNA and *in situ* PCR is a complex and difficult procedure.[92] Because of the inherent advantages of the technique,[206,210,211] DISH was regarded as the most likely means to provide reliable proof that the vacuolar inclusions in Pap smears and tissue sections indeed contained Chlamydial RB. DISH was therefore selected to be the main corroboratory test for the presence of Chlamydial DNA in smears and sections. Further, studies using DNA methods for detection of *Chlamydiae* had not been reported in the literature. The opportunity existed to break new ground and to assist in resolution of the controversy regarding the morphologic diagnosis of Chlamydial infection.

At the commencement of the study in 1993, a Rembrandt® DISH kit suitable for the detection of *Chlamydia trachomatis* was available from Kreatech Laboratories in Amsterdam, The Netherlands. During 1992, the writer received initial training in the use of this kit at a seminar conducted by Kreatech in Cape Town at the Tygerberg Medical School Campus, University of Stellenbosch. Later in 1993, further training was provided when the investigator visited Kreatech Laboratory in Amsterdam. Subsequently in 1994, the improved Rembrandt® DISH kit using the "universal linkage system" (ULS®) became available.[219] In 1997, after the morphologic study had been completed, the investigator returned to Amsterdam to personally conduct the corroboratory tests using the ULS® kit in the Kreatech Laboratories.

Corroboration by PCR

The PCR technique vastly amplifies DNA sequences resulting in a highly *sensitive* assay. The method allows detection of one copy of target DNA in a specimen of tissue containing between one hundred thousand and one million cells.[205] This sensitivity surpasses microbiological culture, all serology techniques and other molecular detection methods. Compared to the ULS® *in situ* hybridization system that is said to detect 10 copies of target DNA per cell,[219] PCR is between a million and 10 million times more sensitive than *in situ* hybridization.

Since DNA amplification technology detects specific DNA sequences it does not depend on the presence of viable intact organisms.[16] DNA may be detected from non-viable organism in a specimen that could not be cultured. This partially explains the enhanced sensitivity of PCR testing over culture. Subsequent to the PCR, product can be extracted from the gel to be sequenced to confirm homology of the product with the target sequence.[217,244]

For these reasons, PCR was selected as a second DNA detection method so that an "expanded" gold standard could be applied to the morphologic criteria. Use of the PCR method in this study was restricted to tissue sections. The ability to cut serial sections from tissue blocks enabled testing of the same specimen by morphology, DISH and PCR. Although DISH could have been applied to Pap smears, there was limited cytologic material from each patient. However, if PCR

testing corroborated the DISH findings in tissue sections, there would be no reason to argue that the same morphologic criteria are not valid when applied to Pap smears.

4.5.2 THE EXPANDED GOLD STANDARD

Black[1] at the CDC in the USA has proposed the concept of using a combination of two tests for a gold standard in Chlamydial work. The specificity of DISH approaches 100%[1, 205,206,220] and PCR is the most sensitive test available, able to detect only one copy of Chlamydial DNA in vast numbers of cells.[205] The gold standard selected for the present study was therefore a combination of two complimentary tests: a highly specific test (DISH) and a highly sensitive test (PCR).

4.6 CONCLUSION

It is not surprising that previous workers have failed to adequately corroborate the use of Pap smears for the diagnosis of Chlamydial infection. In the past, the only tests available to corroborate Chlamydial infection[1,2] have been relatively insensitive non-DNA tests. Culture is difficult and has a low sensitivity.[2] The sensitivity of ELISA method has been described as not wholly satisfactory.[1,50,51,59] Several attempts that could not corroborate the presence of Chlamydial Ag using immuno-fluorescence[6,42,127] or immuno-histochemical methods[8,10,42,81] have used antisera directed against MOMP while antibodies against LPS would have been more appropriate because only scant amounts of MOMP are present in most Chlamydial vacuolar inclusions.[1,2,16,43] Only two studies reported successful demonstration of Chlamydial Ag.[14,76] In the years since these studies were performed, new testing methods, particularly testing for DNA had become available. The investigation would therefore address corroboration of morphologic criteria using two DNA tests.

5. MORPHOLOGIC STUDY

5.1 AIMS

The purpose of the morphologic study was to develop reliable cytologic and histologic criteria for the diagnosis of Chlamydial infection in the uterine cervix. Previously published cytologic[1-3] and histologic [4-6] descriptions were used as starting points to search for cases showing features of Chlamydial infection in Pap smears and in tissue sections. These cases would be carefully evaluated and diagnostic criteria for the morphologic diagnosis of Chlamydia infection formulated. These diagnostic criteria would subsequently be used to find cases that would undergo corroboratory testing using an expanded gold standard[7] to confirm that the morphologic identification of the organism by microscopy can be reliable and specific.

5.2 INTRODUCTION

5.2.1 MORPHOLOGIC DIAGNOSIS OF CHLAMYDIAL INFECTION

In 1975, Swanson *et al*[4] described the morphologic diagnosis by light and electron microscopy of Chlamydia infection in tissue sections of the uterine cervix. Chlamydial inclusions were demonstrated in two cervical biopsies and Chlamydia culture was positive in one of the two patients. The serum of both patients tested positively for antibodies to Chlamydia. These workers suggested that exfoliated cytology smears and biopsy could prove useful in the diagnosis of Chlamydial infection. Subsequently, in 1979, Gupta *et al*[1] described in detail the morphologic features of Chlamydial infection found in Pap smears. They emphasised recognition of intra-cytoplasmic Chlamydial coccoid bodies and regarded Chlamydial "inclusion bodies" (vacuolar inclusions) as a degenerative phenomenon that could easily be confused with inflammatory vacuoles or mucus inclusion vacuoles. Using their morphologic criteria, they were able to corroborate 25 % of cases tested by Chlamydia culture and an undisclosed proportion of cases showed Chlamydial antigen by immuno-fluorescence. EM was successfully performed on cells lifted from the smears in two cases.

Vacuolar inclusion bodies are the usual form taken by the intra-cellular forms of *Chlamydiae*.[8,9] Subsequent to the work by Swanson *et al* and Gupta *et al*, many workers have investigated the morphologic diagnosis of Chlamydia infection, particularly in Pap smears. Fewer researchers have addressed the diagnosis in tissue sections. Many have directed their attention (at least in part) to addressing the notion first expressed by Gupta *et al* that the diagnostic importance of these vacuolar inclusion bodies is questionable.[2,3,10-17] A variety of corroboratory methods including culture, serology, immuno-fluorescence, immuno-peroxidase staining and EM showed poor correlation with the Pap smear or biopsy result. Most of these investigations have concluded that vacuolar inclusion bodies are not reliable indicators of Chlamydial infection,[1,3,10-17] Many of these workers have concluded that the vacuolar inclusions are non-specific for Chlamydial infection.[10-15] Others have indicated that although the inclusions are Chlamydial, they are rarely seen and therefore are an insensitive morphologic marker of the infection.[16,17]

5.2.2 CYTOLOGIC DIAGNOSIS OF CHLAMYDIAL INFECTION

In 1985 Shiina[2] specifically addressed the problem of distinguishing Chlamydial vacuolar "inclusion bodies" from mucus inclusion vacuoles and inflammatory vacuoles. Shiina[2] identified five types of vacuolar inclusion in Pap smears. Smears showing these inclusions were re-stained by IP using a monoclonal, species-specific mouse anti-Chlamydia antibody. Amongst 183 cases suspected of showing Chlamydial infection, 13 showed expression of Chlamydial antigen by IP. The Chlamydial antigen was found in 85.8 % of "nebular type" vacuolar inclusions in these 13 cases. Antigen was not confined to the vacuolar inclusion and was also found in intermediate squamous cells, metaplastic squamous cells, "tissue repair cells," parabasal cells and columnar cells. Subsequently, using the same smears, glycogen was digested with amylase and the cells were stained with PAS to demonstrate mucin. Up to 82 % of non–nebular inclusions contained mucin and were regarded as non-Chlamydial. However, 12 % of nebular inclusions also contained mucin and control HeLa cells containing nebular inclusions also showed PAS-staining mucin.

Shiina[2] concluded that only the nebular inclusions should be regarded as diagnostic of Chlamydial infection. However, it seems likely that the antibody used in this study was against MOMP. In the review of literature, it was noted that Chlamydial MOMP is first expressed in the host cell membrane after approximately 18 hours in the life cycle and may only be found within the inclusion after 24 hours when assembly of daughter EB commences.[5,6] Therefore, it could be expected that some Chlamydial inclusions would not stain positively (personal observation).[15]

In 1989, Bernal *et al* [3] conducted a meta-analysis of 9 publications in which the criteria published by Gupta *et al*[1] had been used. The analysis revealed that using these criteria, cytology had an average sensitivity of only 27 % in the diagnosis of Chlamydial infection. The specificity of the criteria enunciated by Gupta *et al* was 86 % and those of Shiina, 91%. The combined *specificity* of cytology derived from these publications was 79%. The conclusion drawn was that Pap smear diagnosis of Chlamydial infection is not sufficiently accurate and is not useful for ascertaining the presence of *Chlamydia trachomatis* [3] "because of the implications of a misdiagnosis of a sexually transmitted disease."

It was a critical aspect of the current morphologic study to address the misdiagnosis of non-Chlamydial vacuoles. The purpose was to refine diagnostic criteria so that non-specific vacuolar inclusions could be separated from Chlamydial inclusions. In addition, it was necessary to show that the cytoplasmic vacuoles were associated with specific cellular and nuclear alterations in metaplastic cells that were not merely non-specific degenerative changes. It appears reasonable that in the earlier part of the life cycle, cells would show changes that could not be dismissed as evidence of impending cell death. On the other hand, cell death is part of the process of Chlamydial infection and the signs of imminent disruption of the cell constitute part of the evidence of infection. Signs of cell death during Chlamydial infection could be expected late in the life cycle of the organism, after about 30 hours.[5,6] Although a possible source of diagnostic confusion, these late signs of cell death could not be dismissed as unimportant. This view was supported in Gupta *et al*[1] when it was noted that the vacuolar changes were regarded as a degenerative phenomenon but specifically due to Chlamydial infection.

5.2.3 HISTOLOGLOGIC DIAGNOSIS OF CHLAMYDIAL INFECTION

Several published articles have addressed the possible diagnosis of Chlamydial infection in the uterine cervix by histology.[5,6,10,12,13,15,16] These descriptions have not placed emphasis on diagnosis of the infection by identification of the presence of the vacuolar inclusions seen in metaplastic squamous cells. In publications by Hare *et al* [5] and Paavonen *et al*,[6] the emphasis was rather on the follicular lymphoid inflammatory infiltrate. Further, in the histologic studies of Hare *et al*, [5] and Crum *et al* [10] it was stressed that the mucinous epithelium was mainly affected. The photographs shown in publications by Crum *et al* [10] and Swanson *et al*[4] show intra-cellular vacuolar inclusions in histology sections similar to those revealed in Pap smears. However, Crum *et al* found that the inclusions had not stained positively for *Chlamydia trachomatis* using IP.[10] These authors rejected the use of vacuolar inclusions in squamous cells as a morphologic manifestation of Chlamydial infection.

Only a few attempts to corroborate morphologic diagnosis of Chlamydia infection by EM have been reported.[1,4,17] On the one hand, Gupta *et al*[1] and Swanson *et* al[4] had examined cervical tissues by EM and concluded that the organisms were indeed present in the vacuolar inclusions. The study by Henry *et al*[17] only demonstrated Chlamydial organisms by EM in nebular type inclusions in metaplastic squamous cells lifted from Pap smears. The latter workers concluded that non-nebular vacuolar inclusions in these cells were not diagnostic.

The majority of workers have not been able to convincingly corroborate the reliability of a morphologic diagnosis of Chlamydial infection. The current attitude in medical practice to morphologic diagnosis of Chlamydial infections is summed up by Crum *et al*: [18] "Whether these inclusion vacuoles can be identified and distinguished from noninfectious vacuoles, by histology or cytology, is controversial. Raising the suspicion of Chlamydia based on these changes alone is not recommended."

The aim of the current morphologic study was to investigate this very question. The search for cases showing morphologic features of Chlamydial infection in Pap smears and tissue sections was based in part on the descriptions of several authors.[1-6] In addition, the search criteria included features predicated by extrapolation of cytologic features to histology sections. Refined criteria would subsequently be tested against a new expanded gold standard(s) using DNA testing as recommended by the CDC.[7]

5.3 MATERIALS & METHODS

5.3.1 PAP SMEARS

Commencing on 2 January 1993 and ending on 31 December 1996, all Pap smears submitted for examination in the practice of Drs Penman, Kock & Knight (the practice) were critically screened for features suggestive of Chlamydial infection. Physicians in private practice in the Cape Peninsula, Cape Flats, Gordon's Bay, Paarl, Somerset West, Stellenbosch, Strand and Worcester submitted specimens to the practice for routine examination (Appendix A). Consent from the patient to examine and do any appropriate further testing was obtained by the physician and included in the requisition form. Only the clinical, epidemiological and demographic information supplied on the request form by the

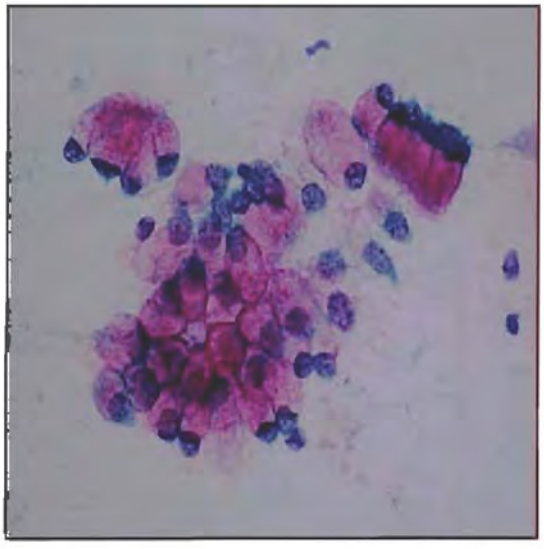


Figure 5.1. Mucinous endocervical cells stained with eosin (EA65) in Papanicolaou smears.

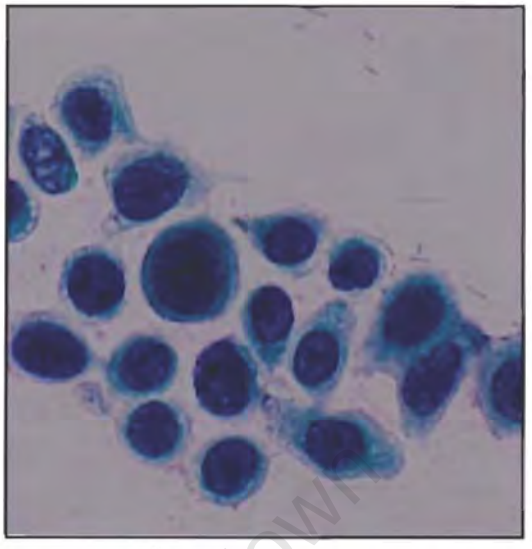


Figure 5.2. Single cells or small groups of two to six cells, 15 - 20mμ in diameter , showing distinct outlines.

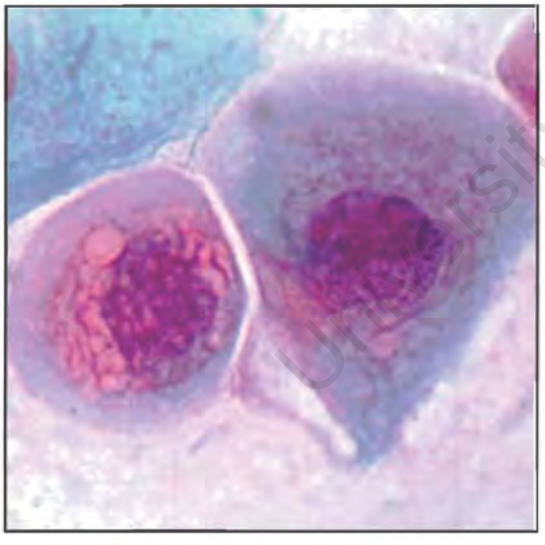


Figure 5.3. The first stage of infection characterised by cells with cyanophilic cytoplasm with a "porous appearance" in the peri-nuclear region, occasionally the entire cell affected.

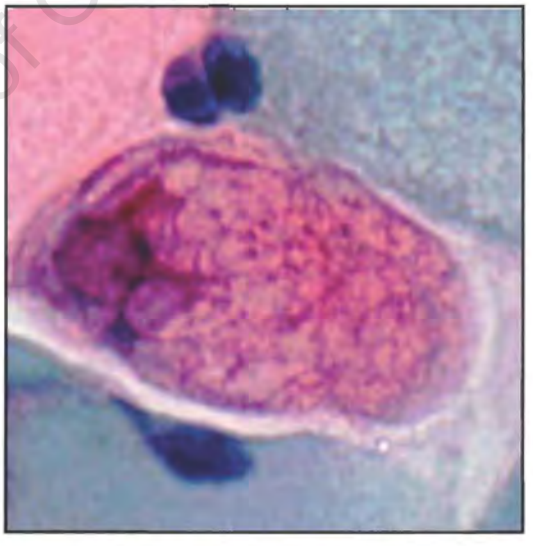


Figure 5.4. Cells with finely granular, uniformly sized, coccoid bodies present in the rarified areas of cytoplasm.

doctor or clinic submitting the smear was available. In most cases, the age and the race of the patient were known. In some cases the date of last menstrual period and an indication of hormonal therapy were given. Occasionally information about contraception was provided. This information and the reported findings were entered into the study database for cytology specimens. It was not possible to assess the socioeconomic status of these patients. However, all patients were paying a fee for the examination of the Pap smear, most of them by claiming from medical insurance/medical aid funds. This presumed a socioeconomic status of middle range income or above. The racial distribution of cases was known in most cases.

The first next Pap smear submitted to the lab after a smear showing evidence of Chlamydial infection was selected as a control Pap smear. Control smears by definition did not show changes of Chlamydial infection and could not be from the same patient nor be a repeat smear from a patient previously diagnosed with Chlamydial infection.

Pap smears were stained using Papanicolaou Stain (Appendix B) using commercially available Harris haematoxylin, Eosin Alcohol 50 containing Eosin Y (EA50) and Orange G6 (OG6) applying the protocol recommended by Koss[19] (Appendix B). The EA50 was used because intra-cellular mucin in columnar endocervical cells stain pink in routine smears, making identification of intracellular mucin possible without the use of special stains, (Figure 5.1). The smears were initially screened by one of two cyto-technologists. Areas on the slide that showed changes suggestive of Chlamydial infection or diagnostic changes of other cyto-pathology were identified on the surface of the cover slip by black marks (cyto-technologist) or green marks (author) made using fine tipped alcohol-soluble marking pens.

Diagnostic criteria for screening the smears were strictly applied (*vide infra*). All smears that according to these criteria showed evidence of inflammation with associated reactive cellular changes, and all smears showing features associated with HPV infection with or without cervical intra-epithelial neoplasia (CIN) were re-examined by the other technologist and then the writer. In addition, any smear submitted with a history of a previous diagnosis of a Chlamydial infection was reviewed by the writer, together with all of the previously submitted smears from that patient. All of the smears selected for control purposes were screened by both of the technologists and the writer. The findings reported included a statement of adequacy and of any evidence of infection by known pathogens and/or the presence of HPV infection. [20,21] The grade of the any intra-epithelial neoplasia ("dysplasia") found was reported using the long established CIN system.[22-24]

The nomenclature of the newer Bethesda system[25] was not in use by the laboratory between 1993 and 1996 and was not used during the study. Consultation with the National Cancer Association, gynaecology oncologists running the colposcopy clinics in Cape Town, and with general practitioners submitting the majority of Pap smears revealed a preference at that time for the CIN system. Their position was taken mainly because the Bethesda system had not been (and still has not been) recommended for reporting histology sections by the International Federation of Gynecologic Oncologists (FIGO). These practitioners indicated that use of the Bethesda nomenclature would cause confusion during

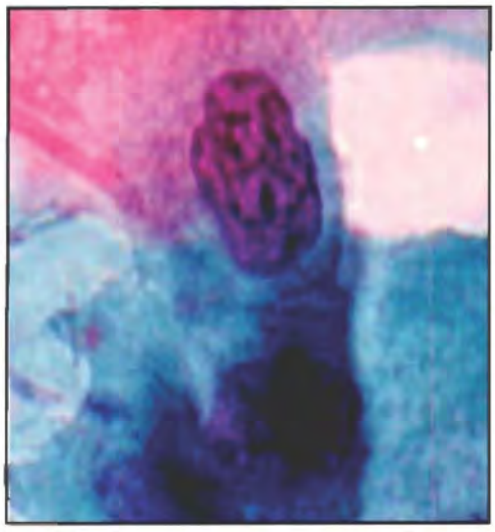


Figure 5.5. Nuclei of infected cells were "atypical." They were enlarged with mild hyperchromasia and undulated nuclear envelopes.

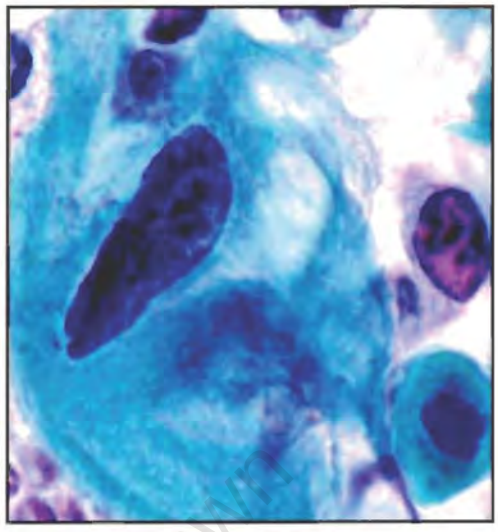


Figure 5.6. Nuclear changes resembled "mild or moderate atypia" (CIN I or CIN II), or sometimes changes ascribed to folic acid deficiency.

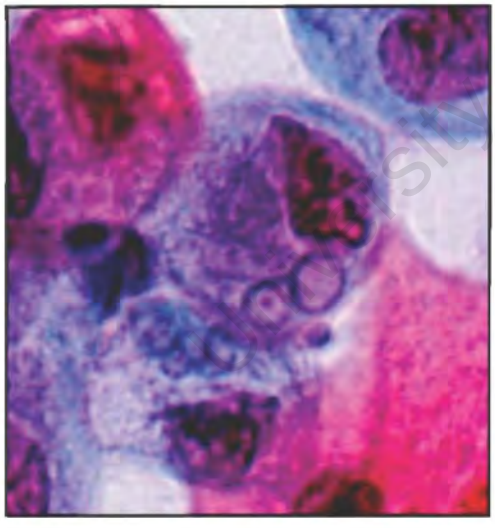


Figure 5.7. Cells containing single or multiple (vacuolar) "inclusion bodies" 1 to 2 mμ in diameter.

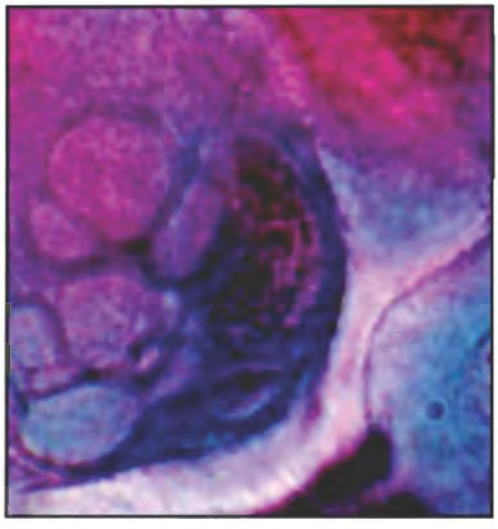


Figure 5.8. Inclusion bodies were uniform with crisp borders and frequently moulded or overlapped one another.

correlation of Pap smear reports with histology biopsy results. Introduction of the Bethesda system was negotiated subsequent to the end of the study during attempts to establish a national Pap smear screening programme.

The presence of other infecting organisms was reported according to established criteria.[26] The organisms recorded included *Actinomyces species*, *Candida species*, *Chlamydia trachomatis, Gardnerella vaginalis* with a shift in flora, mixed bacterial organisms with inflammation, *Neisseria gonorrhoeae, Trichomonas vaginalis* and Herpes Virus.

5.3.1.1 DIAGNOSTIC CRITERIA FOR DIAGNOSIS OF CHLAMYDIAL INFECTION IN PAP SMEARS [1-3]

Criteria for the morphologic diagnosis of Chlamydial infection were derived from two sources: Gupta *et al* [1] and Shiina.[2] The work by Bernal *et al* [3] evaluated the two sets of criteria put forward in these publications.

Criteria derived from Gupta *et al* [1]

A distillate of the description by Gupta *et al* [1] resulted in the following criteria for the diagnosis of Chlamydial infection in Pap smears:

(a) an inflammatory infiltrate, usually neutrophilic: lymphocytes and plasma cells uncommon and follicular lymphoid cervicitis not always associated with the presence of Chlamydial organisms;

(b) infected cells, occurring singly or small groups of two to six cells, enlarged, 15 to 20 mμ in diameter and showing distinct outlines, (Figure 5.2);

(c) infected cells mainly metaplastic squamoid cells but columnar cells also usually involved, in some cases, only columnar cells affected;

(d) *a first stage of infection* characterised by "rarified" cytoplasm, usually cyanophilic and rarely acidophilic, showing a "porous appearance" in the peri-nuclear region, occasionally the entire cell affected, (Figure 5.3);

(e) infected cells showing finely granular, uniformly sized, coccoid bodies present in the rarified areas of cytoplasm, (Figure 5.4);

(f) the coccoid bodies basophilic or acidophilic;

(g) infected metaplastic squamoid cells could be multinucleate, commonly with four to eight nuclei that did not mould one another, multinucleated columnar cells uncommon;

(h) nuclei of infected cells classified as "atypical" in the vast majority of cases, well preserved, enlarged and hyperchromatic with undulated nuclear envelopes, (Figure 5.5);

(i) chromatin diffuse and resembling cells undergoing repair, except that nucleoli rare and inconspicuous in both metaplastic and columnar cells;

(j) nuclear changes resembling "mild or moderate atypia" (CIN I or CIN II), or sometimes changes ascribed to folic acid deficiency, (Figure 5.6);

(k) *a second stage of infection*, characterised by the presence in the cytoplasm of both free-lying Chlamydial organisms and Chlamydial "inclusion bodies";

(l) cells containing single or multiple (vacuolar) "inclusion bodies" 1 to 2 mμ in diameter, (Figure 5.7);

(m) inclusion bodies uniform with crisp borders, frequently moulded or overlapped (Figure 5.8);

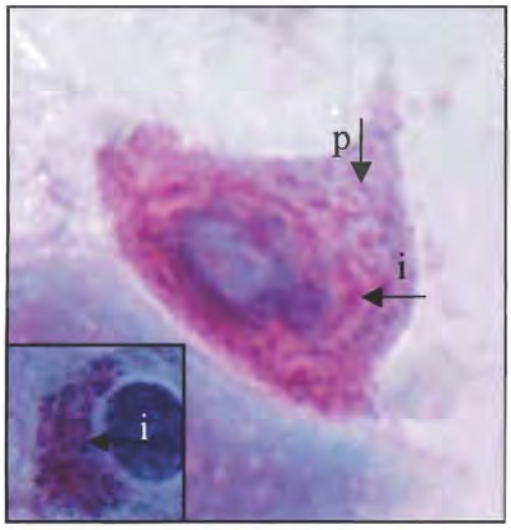


Figure 5.9. The *"third stage of infection"* was characterised by free-lying acidophilic dense aggregates of *Chlamydia* particles (← p) with multiple "inclusion bodies" (↓i).

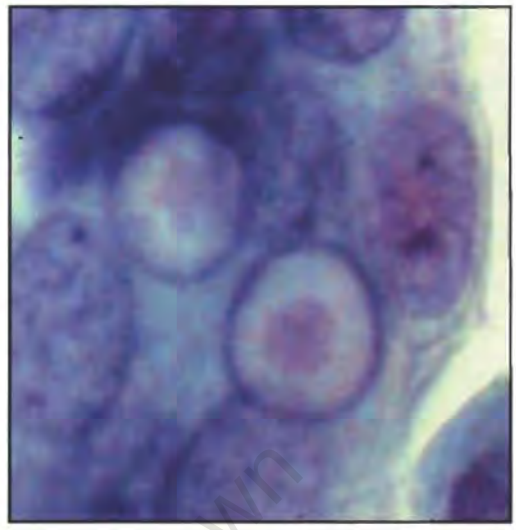


Figure 5.10. Inclusion bodies contained a central basophilic or acidophilic condensation with a clear zone around them.

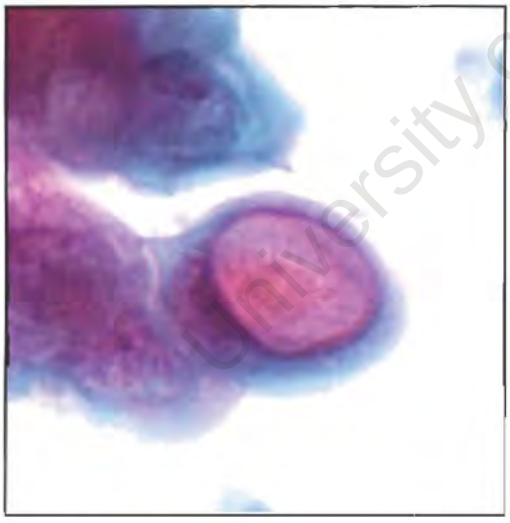


Figure 5.11. Eosinophilic inclusions ranged in size from less than 1 mμ to 15 mμ in diameter. The inclusion vacuoles showed a "thick margin" and contained numerous randomly distributed eosinophilic granular dot-like inclusions.

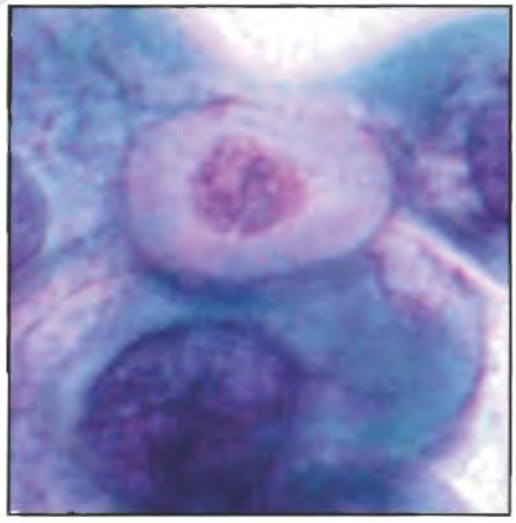


Figure 5.12. Haematoxylinophilic inclusions similar to eosinophilic inclusions, except that the dot-like inclusions are basophilic.

(n) *a third stage of infection* characterised by free-lying acidophilic and dense aggregates of *Chlamydia* particles with multiple "inclusion bodies" (Figure 5.9);

(o) inclusion bodies containing a central basophilic or a diffuse, uniformly staining acidophilic condensation with a clear zone around them, (Figure 5.10).

In their discussion, Gupta *et al*,[1] indicated that caution was necessary in making a diagnosis of Chlamydial infection where inflammation was present. The suggestion that diagnosis of infection can be made without inflammation seems paradoxical (personal observation). It was noted that inflammatory/degenerative cytoplasmic vacuoles could be confused with Chlamydial (vacuolar) "inclusion bodies." Indeed, Chlamydial inclusion bodies were regarded as degenerative in nature. However, because the morphology of inclusion bodies was distinct, they were considered to be diagnostically useful. They recommended that inclusion bodies without the presence of intra-cytoplasmic coccoid bodies should not be regarded as sufficient to diagnose the infection.

Criteria derived from Shiina [2]

Shiina[2] focused attention on the morphology in Papanicolaou stained smears of the vacuolar inclusions associated with *Chlamydia trachomatis* infection. These inclusions were classified into five groups according to morphology and staining characteristics. Illustrations of these, both diagrammatic and in photomicrographic, were available from the publication and proved useful in applying the criteria:

(a) inflammation with neutrophils only found in half cases confirmed to show Chlamydial antigen;

(b) eosinophilic inclusions ranging in size from less than 1 mμ to 15 mμ in diameter, comprising a vacuole with a "thick margin" containing numerous randomly distributed eosinophilic granular dor-like inclusions, (Figure 5.11);

(c) haematoxylinophilic inclusions similar to eosinophilic inclusions, except that the dot-like inclusions are basophilic (Figure 5.12);

(d) nebular inclusions similar "thick-walled" vacuoles, but much finer granular, haematoxylinophilic particles present within, (Figure 5.13);

(e) granular central target formations comprising a vacuole with a centrally placed dense granular inclusion, the tinctoral characteristics of these inclusions (eosinophilic or haematoxylinophilic) in Papanicolaou staining not mentioned, (Figure 5.11);

(f) homogeneous central target inclusions comprising a vacuole with a centrally placed dense homogeneous inclusion, the tinctoral characteristics of these inclusions (eosinophilic or haematoxylinophilic) in Papanicolaou staining not mentioned (Figure 5.14);

(g) mildly atypical nuclei with enlarged and hyperchromatic in half of smears positive for Chlamydial antigen;

(h) nuclei showing features of "repair" in a quarter of cases;

(i) associated mild dysplasia (CIN I) in one case.

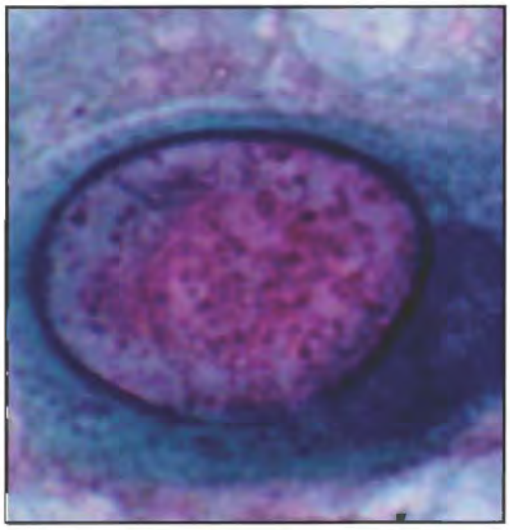


Figure 5.13. Nebular inclusions with similar "thick-walled" vacuoles, but much finer granular, haematoxylinophilic particles present within.

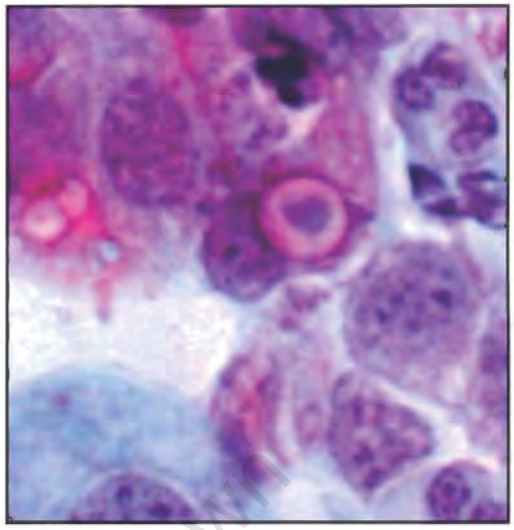


Figure 5.14. Homogeneous central target inclusions comprising a vacuole with a centrally placed dense homogeneous inclusion.

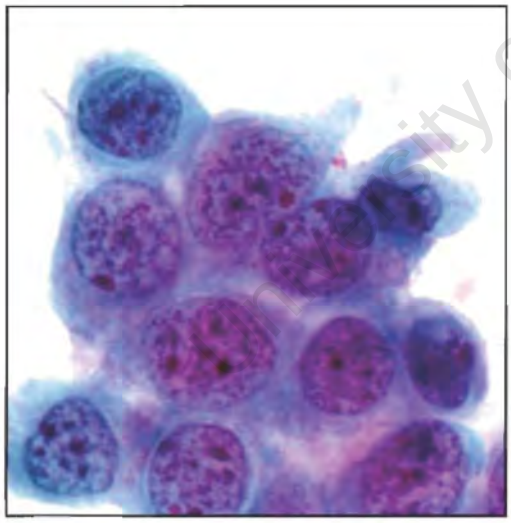


Figure 5.15. Cells showing repair with enlarged nuclei, up to 5 times the size of normal intermediate cell nuclei, showing dispersed cleared chromatin and conspicuous red nucleoli.

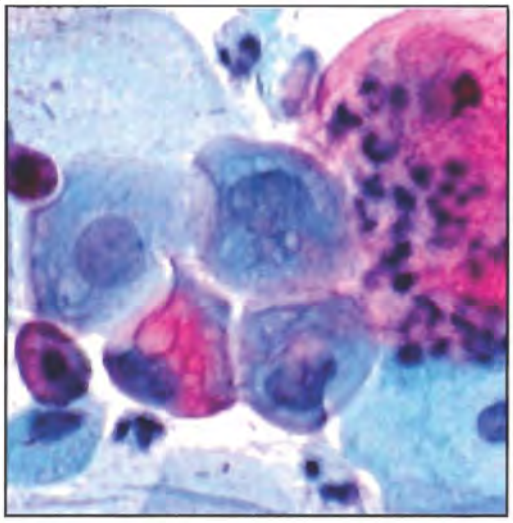


Figure 5.16. Degenerative changes showing extensive cytoplasmic vacuolisation with multiple, large vacuoles with smaller satellite vacuoles.

Shiina noted that in 85 % of cases, cells that showed nebular inclusions stained positively for Chlamydial antigen using IP. Shiina regarded nebular inclusions to be specifically indicative of Chlamydial infection. It is noted that all of the inclusion types described by Shiina appear to correspond to the third stage inclusions described by Gupta *et al*[1] (personal observation).

Evaluation of these criteria by Bernal *et al*[3]

In 1989, Bernal *et al*[3] evaluated the morphologic criteria for the diagnosis of Chlamydial infection described by Gupta *et al*[1] and by Shiina.[2] In this study, using the Microtrak-Siva immuno-fluorescence test as a gold standard, Bernal *et al*[3] showed the sensitivity of the cytologic criteria enunciated by Gupta *et al* was 19 % and specificity was 86 %. Similarly, the sensitivity of the criteria set by Shiina was 38 % and the specificity was 91%.

5.3.1.2 SEPARATION OF CHLAMYDIAL CHANGES FROM REACTIVE, DEGENERATIVE & INFLAMMATORY CHANGES

Reactive, degenerative and inflammatory changes indicative of an inflammatory process were to be expected in Chlamydial cervicitis as with any other cause of inflammation. It was therefore necessary to characterise inflammatory changes in order to identify features peculiar to Chlamydial infection if possible. The following criteria identifying inflammation were taken from standard cytology textbook literature: [27,28]

Reactive and reparative changes[27]

Reactive or reparative cells were recognised by features associated with active cellular metabolism, in particular the production of proteins. Specific features of protein metabolism included the presence of nucleoli directing cytoplasmic ribosomal RNA (rRNA) which causes cytoplasmic basophilia. The features used during the study were:

(1) enlarged nuclei, up to 5 times the size of normal intermediate cell nuclei, showing dispersed cleared chromatin, (Figure 5.15);
(2) the presence of well defined nucleoli; [27]
(3) basophilic or cyanophilic staining of cytoplasm due to the presence of ribosomes in the cell (rRNA); [27]
(4) increased numbers of mitoses.
(5) clusters of small young metaplastic cells, characteristically smaller than mature cells. [27]

Degenerative changes in cytoplasm due to cell injury[27]

Vacuoles occurring in a Pap smear showing inflammatory changes were described in Koss[27] as "inflammatory vacuoles." He regarded inflammatory vacuoles to be "a commonly encountered phenomenon" the "mechanism of which is not clear." Frost,[28] believed that inflammatory vacuoles were hydropic, indicating accumulation of water in the cytoplasm due to impaired cell function. Both speculated that these vacuoles were indicative of severe cellular injury, heralding cell death. Although, neither author offered a precise description of inflammatory vacuoles, Koss did describe degenerative vacuoles.[27] Further, it seems that these authors showed no distinction between "inflammatory" vacuoles and "degenerative" vacuoles, and used the terms interchangeably. In order to avoid confusion between observers in the

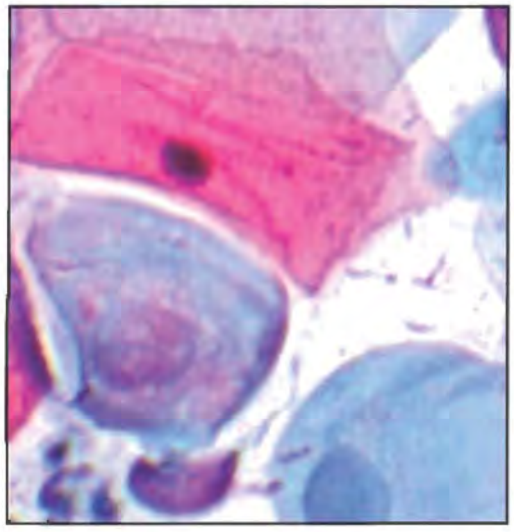


Figure 5.17. Fatty change due to accumulation of lipid droplets, recognised by the presence of "foamy" aggregates of numerous small, frequently fuzzy vacuoles, usually in epithelial cells.

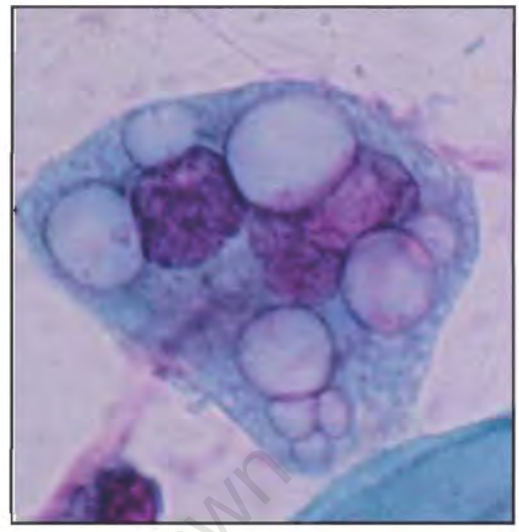


Figure 5.18. Phagocytic vacuoles containing *lipid* usually found in macrophages, and recognised by the presence of numerous fine vacuoles imparting a lacy or "foamy" appearance to the cells.

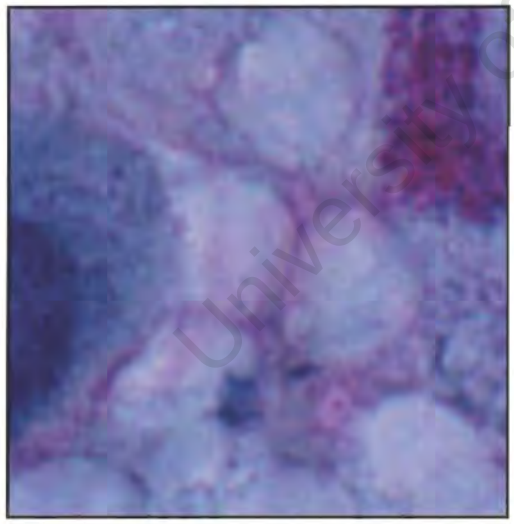


Figure 5.19. *Lipid* usually found in macrophages, but sometimes also extra-cellular, usually associated with use of vaginal lubricant or cream-based medication.

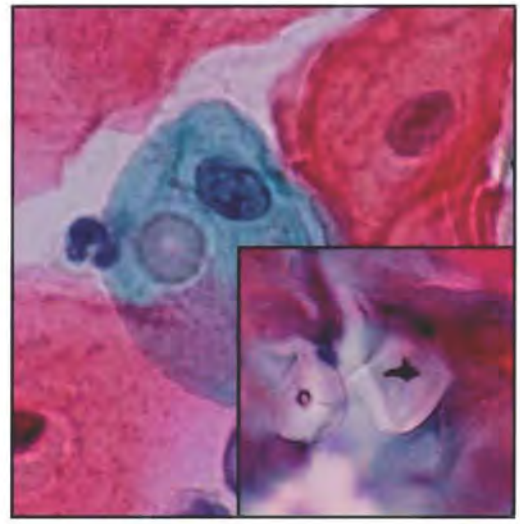


Figure 5.20. *Talc granules* occasionally seen within macrophages, usually found free-lying on top of metaplastic squamous cells and superficially resembling vacuoles. The talc granules characterised by a centrally situated refractile Maltese cross.

laboratory, clear distinction between the terms inflammatory and degenerative vacuoles was made. Inflammatory vacuoles were defined as vacuoles that contained neutrophils or neutrophil debris. Degenerative vacuoles were defined as occurring in a situation of cell injury, including inflammation, and associated with other signs of cellular degeneration .

Features regarded as indicating degenerative cellular changes were:

(1) extensive cytoplasmic vacuolisation with multiple, particularly large vacuoles with smaller satellite vacuoles, (Figure 5.16);[27]

(2) fatty change due to accumulation of lipid droplets, recognised by the presence of "foamy" aggregates of numerous small, frequently fuzzy vacuoles, usually in epithelial cells, (Figure 5.17);[27]

(3) phagocytic vacuoles containing *lipid* (often a vaginal lubricant or cream-based medication), usually found in macrophages, and recognised by the presence of numerous fine vacuoles imparting a lacy or "foamy" appearance to the cells, (Figure 5.18) sometimes also extra-cellular, (Figure 5.19); [27]

(4) phagocytic vacuoles containing recognisable *foreign material*, frequently talc granules seen within both macrophages and metaplastic squamous cells. The talc granules characterised by a centrally situated refractile Maltese cross. Usually associated with free-lying talc granules, some on top of cells, superficially resembling phagocytic vacuoles, (Figure 5.20);

(5) vacuoles containing *mucin*, showing a cyanophilic hyaline inclusion[26,29] that occupied most of the vacuole (ie. without a clear space around the inclusion)
and stained positively with mucicarmine or PAS diastase, (Figure 5.21);

(6) inflammatory vacuoles, recognised by the presence of neutrophils or leucocytoclastic debris within the vacuole, found in both macrophages and epithelial cells, (Figure 5.22).

Degenerative nuclear changes due to cell injury [27,28]

(1) nuclear enlargement seen with marked inflammation, (Figure 5.16);

(2) not associated with nuclear hyperchromasia;

(3) absence of irregular nuclear contours in intact nuclei;

(4) absence of nucleoli;

(5) nuclear enlargement frequently associated with cytoplasmic vacuolisation.

Changes attributed to cell death

(1) fragmentation of cells with fragmentation of the entire cell;

(2) progressive nuclear disintegration with characteristic stages *pyknosis, karyorrhexis and karyolysis* present in adjacent cells, (Figure 5.23);[27]

(3) *pyknosis* manifested by a darkly staining, condensed, homogenous slightly small nucleus;

(4) *karyorrhexis* with fragmentation of pyknotic nuclei;

(5) *karyolysis* with dissolution of the nucleus;

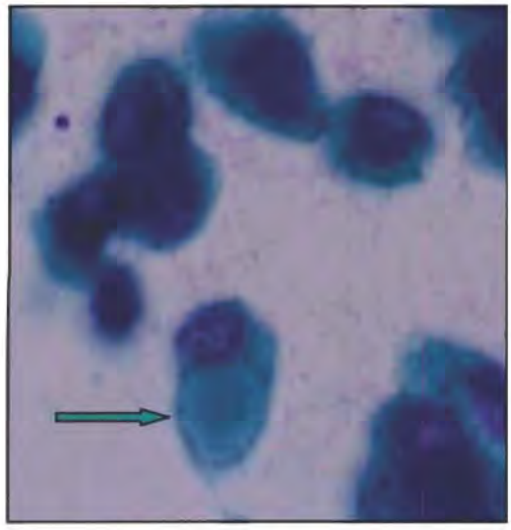


Figure 5.21. *Mucin* vacuole seen in a lobular carcinoma of breast, showing a cyanophilic hyaline inclusion occupying most of the vacuole (ie. without a clear space around the inclusion).

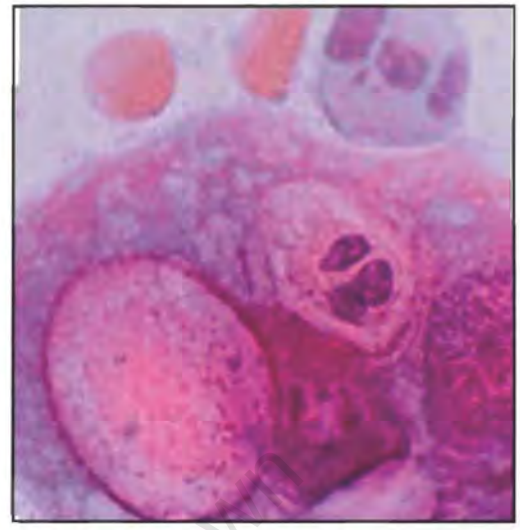


Figure 5.22. Inflammatory vacuoles, recognised by the presence of neutrophils or leucocytoclastic debris within the vacuole, found in both macrophages and epithelial cells

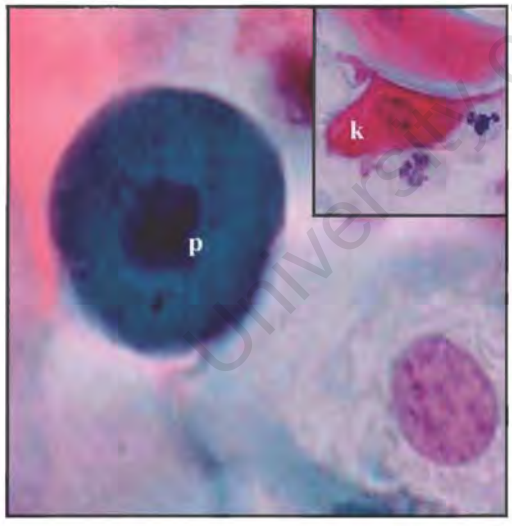


Figure 5.23. Cell death manifested by shrunken, prominent appearing cells with intensely stained nuclei and cytoplasm. Nuclear *pyknosis* (p) with absence of nucleoli, condensed, hyalinised, homogenous, intensely staining cell cytoplasm, usually intensely orangiophilic with the Papanicolaou stain, but occasionally intensely cyanophilic. Nuclear karyorrhexis (k) in a degenerate cells.

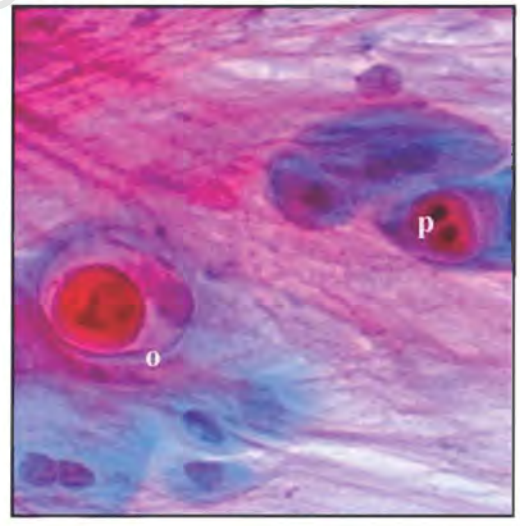


Figure 5.24. Apoptosis with shrinkage of the nucleus and development of intense pyknosis (p). Cells show cytoplasmic condensation with intense orangeophilia (o) and there is phagocytosis of membrane-bound cell fragments (apoptotic bodies) by the adjacent epithelial cells or macrophages.

(6) absence of nucleoli; [27]

(7) shrunken, prominent appearing cells with intensely stained nuclei and cytoplasm; [27]

(8) condensed, hyalinised, homogenous, intensely staining cell cytoplasm, eosinophilic with the HE stain, usually intensely orangiophilic with the Papanicolaou stain, but occasionally intensely cyanophilic, (Figure 5.23); [27]

(9) multiple cytoplasmic vacuoles with fuzzy outlines distinguished from well-defined Chlamydial vacuoles; [27]

Apoptosis

Characteristic progressive nuclear and cytoplasmic changes: [30,31]

(1) different stages present in cells in the same or adjacent microscopic fields; [27]

(2) commencing with condensation of chromatin at the periphery of the nucleus;

(3) dissolution of the nucleolus; [30,31]

(4) shrinkage of the nucleus with development of intense pyknosis; [30,31]

(5) cytoplasmic condensation with intense orangeophilia or eosinophilia; [27]

(6) finally, cell shrinkage and disintegration; [30,31]

(7) phagocytosis of membrane-bound cell fragments (apoptotic bodies) by the adjacent epithelial cells or macrophages (Figure 5.24); [30,31]

(8) apoptotic bodies appearing as small intracellular vacuolar inclusions containing relatively large, complexly staining cell fragments with pyknotic nuclear fragments and/or orangiophilic cytoplasmic remnants; [27]

(9) apoptotic bodies distinguished from Chlamydial inclusions by the narrow vacuolar space surrounding the inclusion;

(10) complex apoptotic bodies dissimilar from the characteristic Chlamydial intra-vacuolar inclusion bodies (compare Figures 5.22 & 5.24).

Acute Inflammatory changes [27]

An inflammatory exudate, together with evidence of both injury/degeneration of cells and regenerative/reparative changes, were the most important indicators of vaginitis or cervicitis. (Inflammatory cells also present in premenstrual and menstrual smears.) During the study, the following cellular changes were ascribed to inflammation:

Superficial and intermediate cells [27]

(1) necrosis of superficial and intermediate cells;

(2) enlarged, "blown-up" nuclei with loss of nuclear detail (Figure 5.25);

(3) nuclear pyknosis and karyorrhexis (Figure 5.25);

(4) narrow zone of peri-nuclear cytoplasmic clearing/pallor due to reduced intensity of staining;

(5) occasionally bi-nucleation of cells.

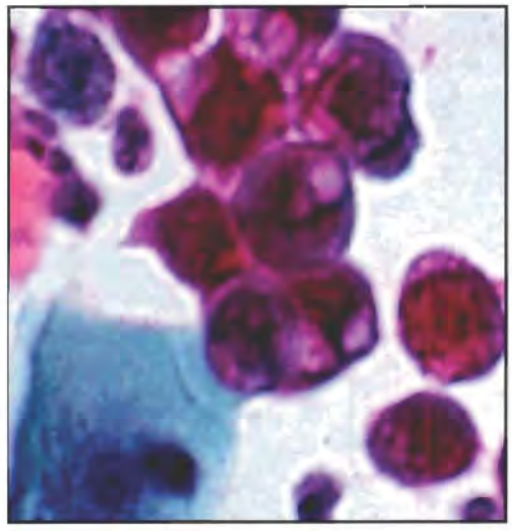


Figure 5.25 Necrosis of superficial and intermediate cells showing enlarged, "blown-up" nuclei with loss of nuclear detail, nuclear pyknosis and karyorrhexis and degenerative cytoplasmic vacuolation.

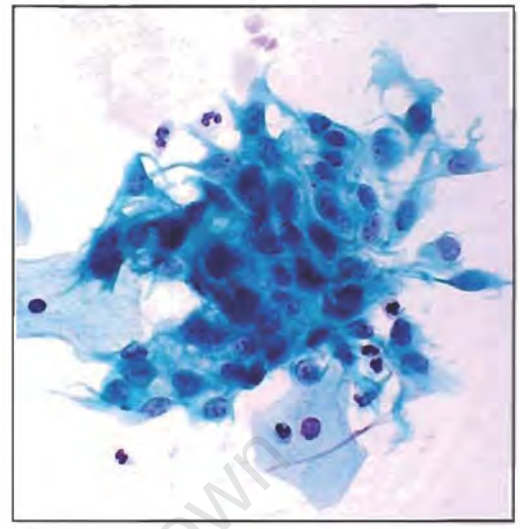


Figure 5.26. Aggregates or sheets of viable, basophilic para-basal cells showing "spongiotic change." The cell groups show wide inter-cellular spaces and long cytoplasmic extensions at the sites of desmosomes between the cells (indicating intra-epithelial oedema).

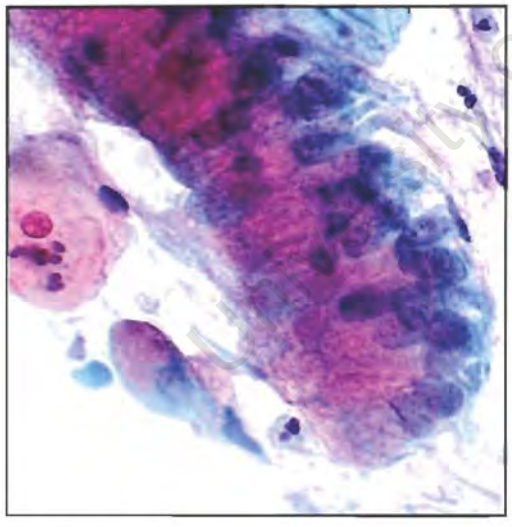


Figure 5.27. Inflammatory changes in a cohesive clusters of endocervical cells of variable size, with enlarged nuclei.

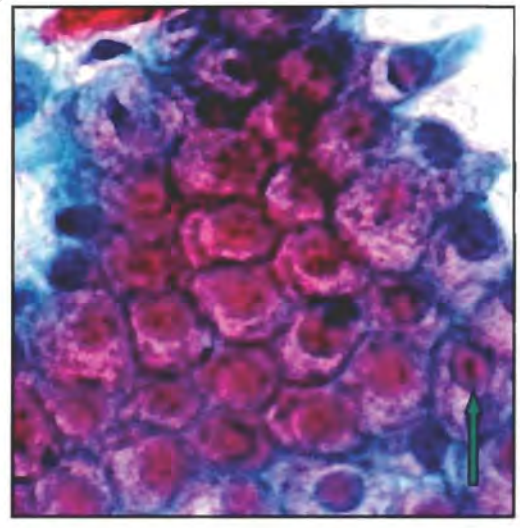


Figure 5.28. Mucinous endocervical cells showing inflammatory changes with enlarged nuclei and one or more large red nucleoli (↑). These changes may be confused with malignant changes.

Para-basal cells: [27]

(1) physiologic increase in numbers of para-basal cells replacing the losses of superficial and intermediate cells;

(2) rounded dead para-basal cells lying singly or in clusters, the cells showing contracted, usually intensely orangiophilic cytoplasm, sometimes containing fuzzy irregular cytoplasmic vacuoles, (Figure 5.25);

(3) aggregates or sheets of viable, basophilic para-basal cells containing cytoplasmic ribosomes. These aggregates showed wide inter-cellular spaces and long cytoplasmic extensions at the sites of desmosomes between the cells ("spongiotic change" due to intra-epithelial oedema), (Figure 5.26);

(4) enlarged nuclei with numerous chromocentres or even small single nucleoli indicating active cellular metabolism;

(5) occasional bi-nucleate or multinucleate para-basal cells;

Endocervical (mucinous) cells [27]

(1) cohesive clusters of cells showing variable size, sometimes striking enlargement of cells, (Figure 5.27);

(2) enlarged nuclei with numerous chromocentres and one or more large red nucleoli, sometimes confused with malignant changes, (Figure 5.28);

(3) mitoses;

(4) multinucleation of cells;

(5) bare nuclei, showing a similar appearances to nuclei within whole cells;

(6) occasional degenerate mucinous cells with pyknotic nuclei;

(7) fuzzy, small cytoplasmic vacuoles.

Chronic Inflammatory changes [27]

(1) parakeratosis, manifested by very small nucleated mature squamous cells with orangeophilic cytoplasm and small, eccentric pyknotic nuclei, (Figure 5.29);

(2) basal cell hyperplasia, (or "reserve cell" hyperplasia) with small clusters of small basaloid cells with scanty cytoplasm and hyperchromatic small nuclei, often with mature mucinous cells adherent to the periphery of these cell groups;

(3) sheets of metaplastic squamous cells with enlarged nuclei, prominent nucleoli and eosinophilic or cyanophilic cytoplasm. The metaplastic cells widely spaced with well-defined cell membranes and angular cell profiles resembling paving stones. Sometimes fine cytoplasmic "prickles" bridging the spaces between cells (desmosomes), (Figure 5.30);

(4) metaplastic cells frequently adherent to clusters of mucinous cells, with cells showing varying degrees of transition between squamous cells and mucinous cells. Some metaplastic cells with numerous small cytoplasmic vacuoles "nearly always positive with special stains for mucins," (Figure 5.31); [31]

(5) neutrophils, present in vacuoles in metaplastic cells and between metaplastic cells, (Figure 5.22 & 5.32);

(6) atypical metaplastic cells with bi-nucleation of cells, hyperchromasia of nuclei; [27]

(7) reparative metaplastic cells with enlarged, hypo-chromatic nuclei and multiple large and prominent nucleoli;

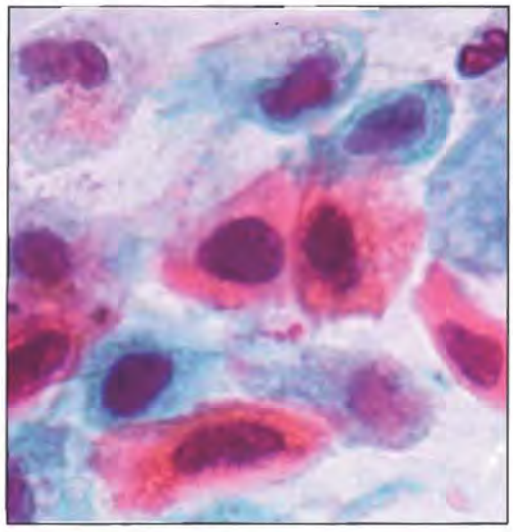


Figure 5.29. Chronic inflammatory changes manifested by parakeratosis, very small nucleated mature squamous cells with orangeophilic cytoplasm and small, eccentric pyknotic nuclei.

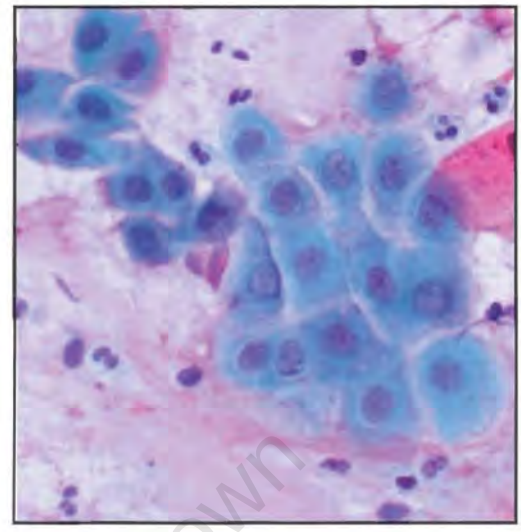


Figure 5.30. Chronic inflammation showing sheets of widely spaced metaplastic squamous cells with enlarged nuclei, prominent nucleoli. Well-defined cell membranes, angular cell profiles and fine cytoplasmic "prickles" bridging intercellular spaces (desmosomes).

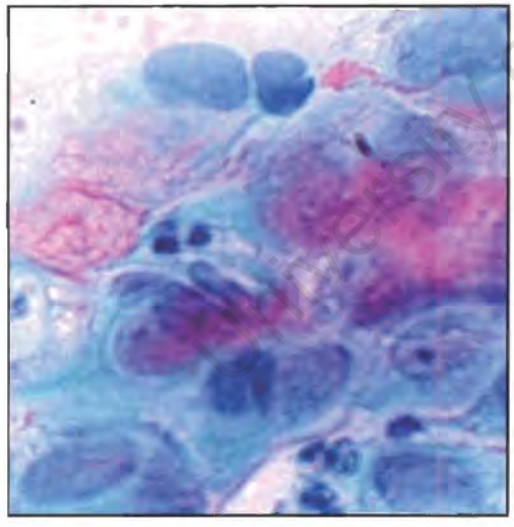


Figure 5.31. Chronic inflammation frequently associated with metaplastic cells adherent to clusters of mucinous cells (pink), with cells showing varying degrees of transition between squamous cells and mucinous cells.

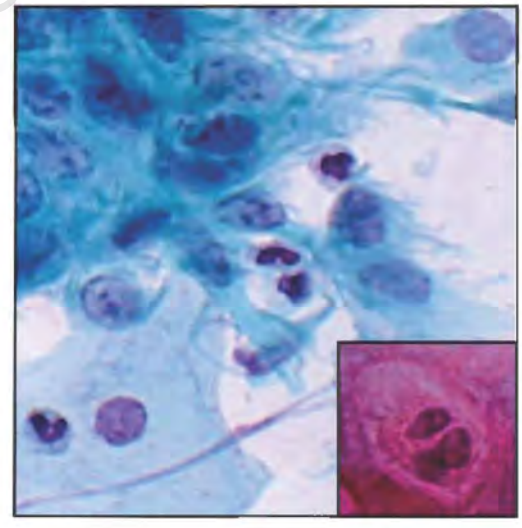


Figure 5.32. Chronic inflammation with neutrophils present between metaplastic cells and in vacuoles in metaplastic cells (insert from Figure 5.22).

(8) follicular cervicitis showing clusters of reactive lymphocytes including follicle center cells, macrophages containing phagocytic bodies, thought to be linked with Chlamydial infection;[10,12,27]

(9) plasma cell inflammatory infiltrates;

(10) infiltrates of large mono-nucleated or multi-nucleated macrophages, showing phagocytic features, (Figure 5.33);

(11) granulation tissue characterised by degenerate necrotic epithelial cells, inflammatory cells, macrophages and occasional elongated fibroblasts (Figure 5.34).

5.3.1.3 CRITERIA FOR THE RECOGNITION OF CHANGES DUE TO KNOWN PATHOGENS

Recognition of an infection by commonly encountered vaginal pathogens is frequent during the examination of Pap smears. Cases showing both Chlamydial infection and features of vaginitis or cervicitis due to other recognisable pathogens were recorded as showing dual pathologies (Appendix A). The organisms recorded were recognized according to the criteria of Koss[26] and included reactive changes associated with specific pathogens:

(1) *Actinomyces species,* recognised by the presence of aggregates of filamentous basophilic bacteria, sometimes showing terminal spores, and frequently associated with a history of use of an intra-uterine contraceptive device;

(2) *Candida species* infection with budding yeasts and pseudo-hyphae formed by germ tubes, a variable inflammatory exudate, and frequently associated with mild hypo-chromatic nuclear enlargement with condensation and increased orangeophilia of superficial squamous cells, (Figure 5.35);[26]

(3) *Gardnerella vaginalis* associated with proliferation of anaerobes ("shift in vaginal flora") with small coccobacilli adherent to squamous cells ("clue cells").[42] If severe, associated with an inflammatory exudate, cytolysis of superficial and intermediate squamous cells and reactive or reparative changes in mucinous and metaplastic squamous cells, (Figure 5.36);[26]

(4) *Neisseria gonorrhoeae* infection, suspected when small coffee-bean shaped diplococci adherent to the surface of cells and within the cytoplasm of neutrophils and squamous cells; [26]

(5) *Trichomonas vaginalis* infection recognised by the presence of grey flagellate organisms varying between 8 and 20 mμ in size, with cellular changes including eosinophilia of squamous cells, small perinuclear halos in squames, cytolysis, an inflammatory exudate, and atypia of cells, that especially in postmenopausal atrophy may cause confusion with dysplasia, (Figure 5.37);[26]

(6) *Herpes Simplex Virus* (HSV) with characteristic nuclear enlargement (up to 60 mμ in size), mosaicism due to moulding of multiple nuclei in squamous cells, vacuolation of the nuclei and accumulation of viral particles resulting in typical intra-nuclear eosinophilic viral inclusions (Cowdry's type A). Associated with degenerative cells showing bizarre hyperchromatic fused nuclei or pearls of cells showing cytokeratin, [26] easily confused with malignant squamous cells, (Figure 5.38);

(7) *Human Papilloma Virus* (HPV) *(vide infra).*

Figure 5.33. Chronic inflammation with large mono-nucleated or multi-nucleated macrophages, showing phagocytic features .

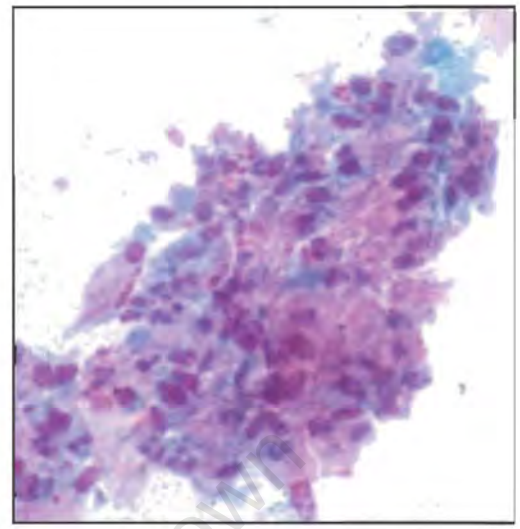


Figure 5.34 Granulation tissue characterised by degenerate necrotic epithelial cells, inflammatory cells, macrophages and occasional elongated fibroblasts.

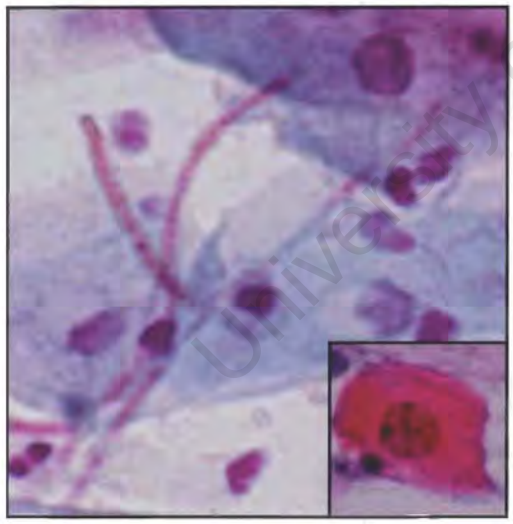


Figure 5.35. *Candida species* infection with pseudo-hyphae and a variable inflammatory exudate. Frequently superficial squamous cells show mild hypo-chromatic nuclear enlargement with condensed orangeophilic cytoplasm (insert).

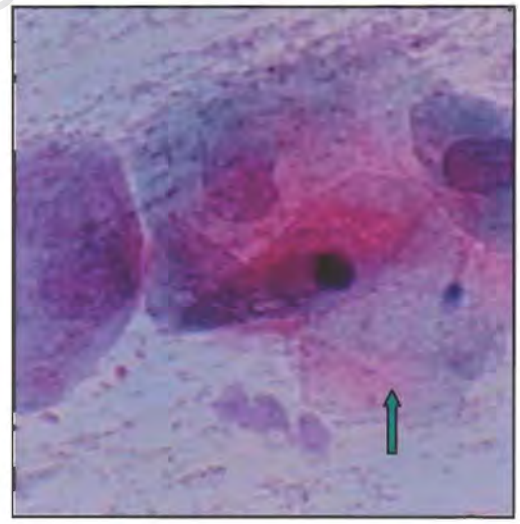


Figure 5.36. *Gardnerella vaginalis* associated with proliferation of anaerobes manifest by small coccobacilli, an inflammatory exudate, cytolysis of superficial and intermediate squamous cells (arrow) and reactive or reparative changes in mucinous and metaplastic squamous cells,

5.3.1.4 CRITERIA FOR THE RECOGNITION OF CHANGES ASSOCIATED WITH HPV INFECTION

Cases showing the features of both Chlamydial infection and HPV infection [20,21] with or without dysplasia (CIN) [22,25] were recorded as showing dual pathologies. Other cases selected for control purposes showed evidence of HPV infection without Chlamydial infection.

Changes defining HPV

Recognition of HPV infection was according to the criteria of Meisels *et al,* [20,21] of Richart[22] and Kurman & Solomon.[25] The features regarded as diagnostic of HPV infection included the following changes:

Cytoplasmic changes: [20,21, 25]

(1) cells showing distinct cytoplasmic borders and by definition showing nuclear atypia (*vide infra*);

(2) cells originating in both the superficial (eosinophilic cytoplasm) and the intermediate layers (cyanophilic cytoplasm) of the epithelium, either arranged in sheets or occurring as single cells;

(3) "koilocytic" changes in a proportion of cells, characterised by a peri-nuclear halo surrounded by a condensed rim of cytoplasm, and by definition, characteristic atypia of the nuclei, (Figure 5.39);

(4) a proportion of small parakeratotic cells showing intense orangeophilic staining of cytoplasm. The nuclei of these cells smaller than those of koilocytic cells, but relative to the size of the cell, large with intensely dark, smudged chromatin and irregular contours. (Figure 5.40);

(5) occasional very large cells similar in size to superficial cells with characteristic nuclear atypia, binucleation, multinucleation or giant nuclei (Figure 5.40).

Nuclear changes [20,21, 25]

(1) superficial and intermediate cells showing enlarged nuclei, by definition [23] three or more times the size of normal intermediate cell nuclei, resulting in an increased nuclear/cytoplasmic ratio, (Figure 5.41);

(2) nuclei showing variation in size and shape, occasionally bi-nucleate, or multi-nucleate or irregular giant nuclei cells present;

(3) the nuclear outlines distinct or smudged;

(4) cells with distinct nuclear membranes showing irregular "crumpled" nuclei with uniformly distributed, hyper-chromatic, slightly coarse chromatin without nucleoli;

(5) cells with ill-defined nuclear membranes showing dark, smudged chromatin without nucleoli, (Figure 5.41);

(6) nucleoli very rarely present, and if ever seen, very small and inconspicuous.

5.3.1.5 CRITERIA FOR THE RECOGNITION OF COINCIDENTAL CIN I, II & III IN CHLAMYDIAL INFECTION

Cases showing the features of both Chlamydial infection and CIN[22-25] were recorded as showing dual pathologies. It was important to recognise these cases so that nuclear atypia due to Chlamydia could be characterised and separated from CIN. Cases showing features of invasive carcinoma were excluded from the study.

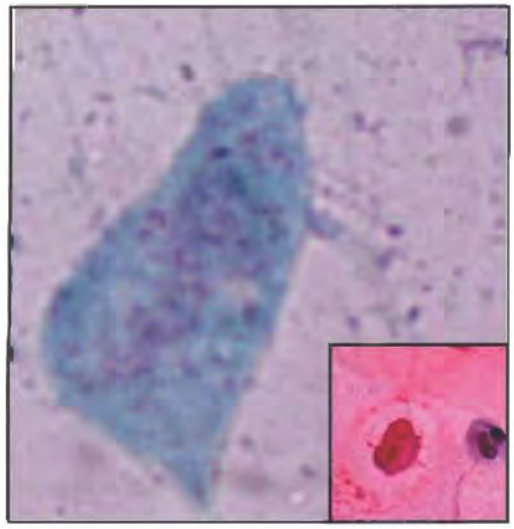


Figure 5.37. *Trichomonas vaginalis* infection recognised by the presence of grey flagellate organisms between 8 and 20 mμ in size. Cellular changes include small perinuclear halos in squames, an inflammatory exudate, and atypia of cells that may be confused with dysplasia.

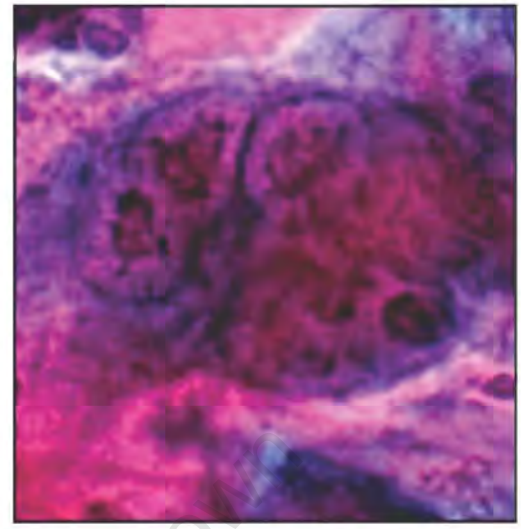


Figure 5.38. *Herpes Simplex Virus* with characteristic nuclear enlargement (up to 60 mμ in size) and mosaicism due to moulding of multiple nuclei in squamous cells. Typical intra-nuclear eosinophilic viral inclusions (Cowdry's type A).

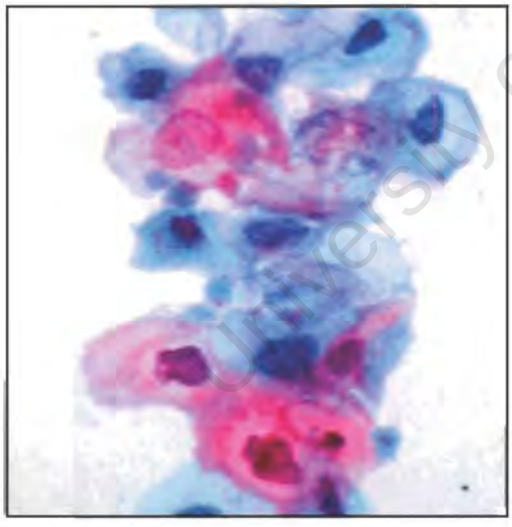


Figure 5.39. "Koilocytic" changes in superficial and intermediate squamous cells characterised by peri-nuclear halos surrounded by a condensed rim of cytoplasm, and by definition, characteristically enlarged nuclei with irregular "crumpled" nuclear membranes.

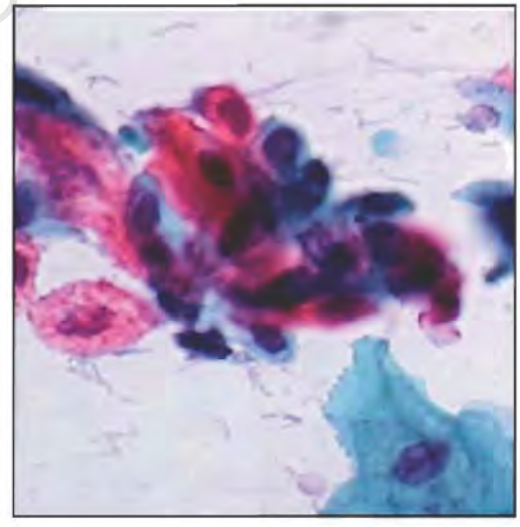


Figure 5.40. Small parakeratotic cells showing intense orangeophilic staining of cytoplasm. The nuclei of these cells smaller than those of koilocytic cells, but relative to the size of the cell, large with intensely dark, smudged chromatin and irregular contours.

Changes defining CIN I[22-25]

Cytoplasmic changes:

(1) affected cells frequently single cells, or arranged in loosely aggregated clusters or occasionally as cohesive groups, (Figure 5.42);

(2) characteristic condensation and intensely orangeophilic cytoplasm (Figure 5.42);

(3) cells show inconspicuous plasma membranes when in cohesive groups;

(4) cells variable size, but usually less than one third of the size of normal intermediate cells;

(5) cells irregularly shaped, usually with angular or elongated contours (Figure 5.42).

Nuclear changes:

(1) nuclei enlarged, by definition[25] more than three times the size of normal intermediate cell nuclei, (Figure 5.42);

(2) nuclei, by definition[25] less than half of the diameter of the widest cell diameter;
nuclei vary in size and shape;

(3) the nuclear contours distinct with irregular, thickened nuclear membranes;

(4) nuclei show uniformly distributed, hyper-chromatic, coarse chromatin;

(5) nucleoli not present;

(6) some cells showing ill-defined nuclear membranes with dark, smudged chromatin resembling the nuclei of HPV-infected cells.

Changes defining CIN II[22-25]

Cytoplasmic changes:

(1) cells smaller than in LSIL;

(2) cells occur singly, in sheets or syncytial aggregates, (Figure 5.43);

(3) some mature cells with marked keratinisation orangeophilic;

(4) mostly immature squamous cells with lacy or dense basophilic cytoplasm;

Nuclear changes:

(1) nuclei enlarged, more than three times the size of normal intermediate cell nuclei, (Figure 5.44) ;

(2) nuclear size characteristically variable;

(3) enlarged nuclei, by definition[25] more than half the diameter of the widest cell diameter (Figure 5.44) ;

(4) some nuclei less than three times the size of intermediate cells, but still more than half of the diameter of the widest cell diameter;

(5) nuclei show sharply irregular, angular, knobbly contours (Figure 5.43, 44);

(6) nuclei generally darkly hyperchromatic;

(7) chromatin evenly dispersed, sometimes smudged or granular;

(8) nucleoli not present.

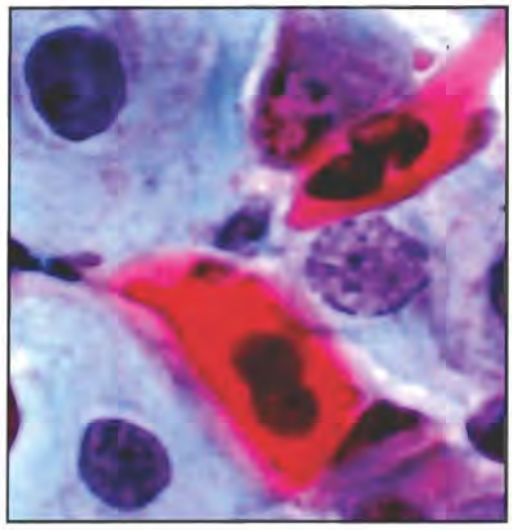


Figure 5.41. HPV change in superficial and intermediate cells showing enlarged nuclei, three or more times the size of normal intermediate cell nuclei, with variation in size and shape, occasionally bi-nucleate, or multi-nucleate. The nuclear outlines distinct or smudged.

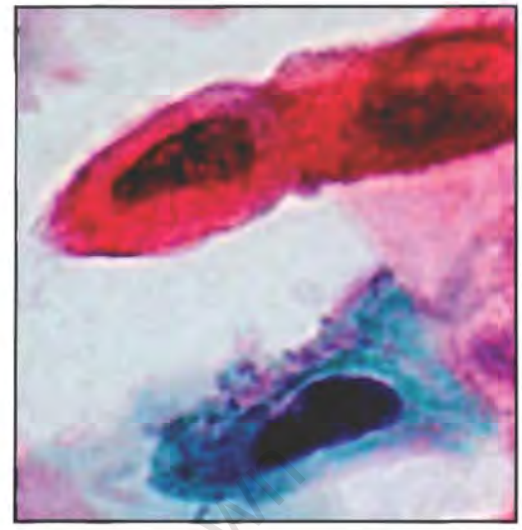


Figure 5.42 CIN I, frequently single cells, or arranged in loosely aggregated clusters, but occasionally as cohesive groups. Cells intensely orangeophilic, usually less than one third of the size of normal intermediate cells, irregularly shaped, usually with angular or elongated contours.

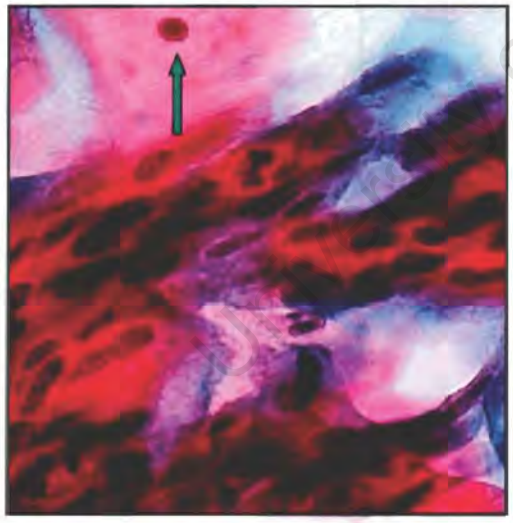


Figure 5.43. CIN II with cells in sheets or syncytial aggregates of mature cells with marked orangeophilic keratinisation. Nuclei darkly hyperchromatic, characteristically variably enlarged, by definition more than three times the size of intermediate cells (↑) nuclei sharply irregular, angular, knobbly contours.

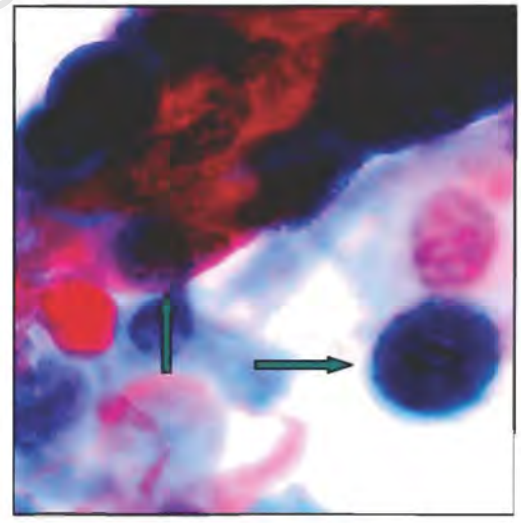


Figure 5.44. CIN II with immature squamous cells showing lacy or dense basophilic cytoplasm (→) with enlarged nuclei, by definition more than half the diameter of the widest cell diameter; and, chromatin evenly dispersed, sometimes smudged or granular (↑).

Changes defining CIN III[22-25]

Cytoplasmic changes:

(1) cells usually smaller than in CIN I and CIN II;

(2) cells usually in sheets or syncytial aggregates but may lie singly;

(3) cells usually showing immature squamous features with scant basophilic cytoplasm without keratinisation, (Figure 5.45);

(4) cells occasionally large with weakly orangeophilic cytoplasm;

Nuclear changes:

(1) nuclei usually enlarged, by definition more than three times the size of normal intermediate cell nuclei, (Figure 5.45);[25]

(2) enlarged nuclei, by definition[25] more than half of the diameter of the widest cell diameter;

(3) some cells show smaller nuclei (less than three times the size of intermediate cells), but the nuclei still more than half of the diameter of the widest cell diameter;

(4) nuclei ovoid or markedly elongated and usually show markedly irregular contours;

(5) the size of nuclei within the cell groups usually markedly variable;

(6) nuclei may show fairly uniform size and contour, (Figure 5.45);

(7) nuclei generally darkly hyperchromatic;

(8) the chromatin coarsely granular and evenly dispersed;

(9) nucleoli not present.

5.3.1.6 CRITERIA FOR THE RECOGNITION OF COINCIDENTAL ASCUS[25]

Inevitably, a proportion of cases showing atypia of metaplastic squamous cells in which it was not possible to make a firm diagnosis of HPV infection, or CIN or Chlamydial infection. These cases were assigned the diagnosis ASCUS[25] (atypical squamous cells of uncertain significance). Some cases showing Chlamydial infection showed metaplastic cells with atypia more severe than usual for Chlamydial infection alone, but not sufficient to confidently diagnose HPV infection or CIN. These cases were recorded as showing dual pathologies of Chlamydial infection and ASCUS. Criteria defining ASCUS had been defined in The Bethesda System:[25]

Changes defining ASCUS[25]

Cytoplasmic changes:

(1) absence of "koilocytic" changes in cells with mild atypia of nuclei not sufficient to meet the criteria of HPV infection or CIN (*vide* infra);

(2) "koilocytic" change without characteristic atypia of the nuclei (Figure 5.46);

(3) parakeratotic cells without characteristic atypia of the nuclei;

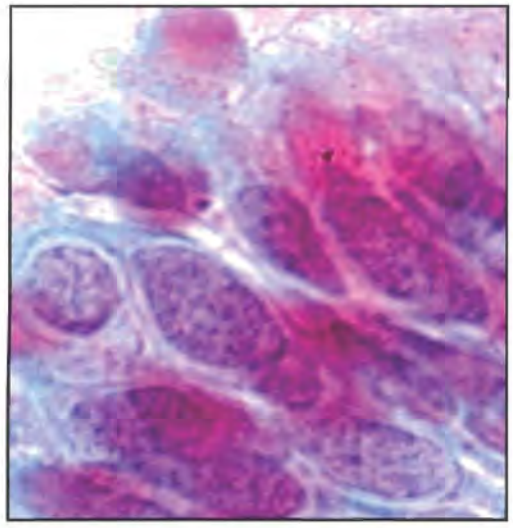


Figure 5.45. CIN III in syncytial aggregates of immature squamous cells with scant basophilic non-keratinised cytoplasm. Nuclei enlarged, elongated, fairly uniform in size with markedly irregular contours, coarsely granular, evenly dispersed hyperchromatic chromatin.

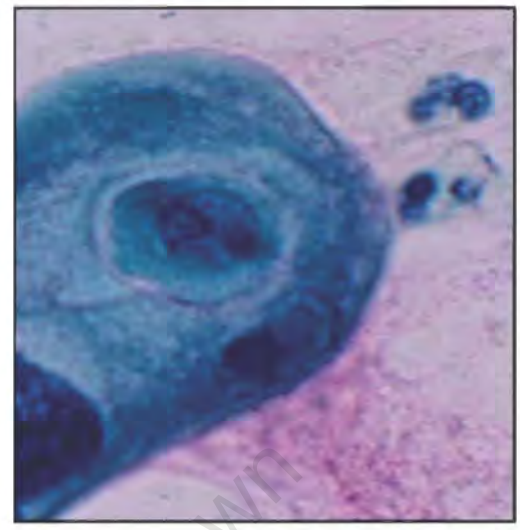


Figure 5.46. ASCUS resembling HPV change showing "koilocytoid" features without sufficient atypia of the nucleus which is neither hyperchromatic, nor smudged nor irregular in contour.

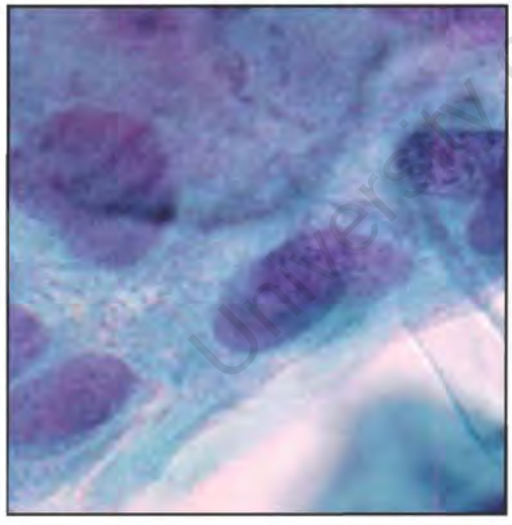


Figure 5.47. ASCUS showing immature squamous cells with abundant cytoplasm. The nuclei are enlarged (two and a half but not more than three times the size of normal intermediate squamous cell nuclei), with smooth nuclear outlines and evenly dispersed fine chromatin.

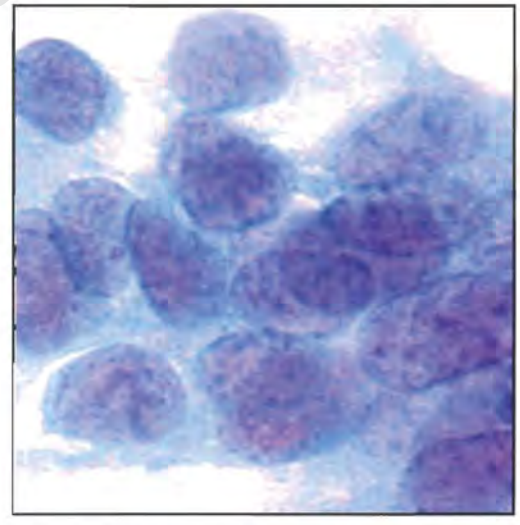


Figure 5.48. ASCUS showing immature squamous cells with scanty cytoplasm resembling CIN III. Nnuclei are enlarged with marked variation in size. Note the mild irregularity of nuclear outlines and mild disturbance of the chromatin which is not coarsely granular nor hyperchromatic (cf. Figure 5.45).

Nuclear changes:

(1) nuclear enlargement, by definition[25] two and a half but not more than three times the size of normal intermediate squamous cell nuclei, (Figure 5.47, 5.48);

(2) variation in the size and shape of nuclei (Figure 5.48);

(3) smooth nuclear outline with very limited irregularity(Figure 5.47);

(4) bi-nucleation of cells;

(5) mild hyperchromasia but evenly dispersed chromatin without granularity (Figure 5.48).

5.3.1.7 RECOGNITION OF CHANGES IN ENDOCERVICAL OR ENDOMETRIAL GLANDULAR CELLS

Benign mucinous endocervical cells with or without reactive changes are important indicators that the transitional zone has been adequately sampled.[19,25] A statement regarding the presence or absence of endocervical cells was regarded as an essential comment reflecting the adequacy of the smear.[25] Reactive or atypical endocervical cells are frequently seen in inflammatory smears[26] (Figure 5.49) and were found as part of the spectrum of changes associated with Chlamydial infection. Occasionally, mucinous cells could be confused with cells showing vacuolar changes and entered into the differential diagnosis of Chlamydial infection. Criteria for the recognition of mucinous endocervical cells were defined (*vide supra* **Acute inflammatory changes**).

Benign endometrial cells in Pap smears may show degenerative changes with vacuolation.[25] It was necessary to define criteria for the recognition of benign endometrial cells. Further, malignant glandular cells have been shown to contain mucinous inclusions within vacuoles.[29] Therefore it was important to carefully examine any cases found during the study to show evidence of both Chlamydial infection and endocervical adenocarcinoma *in situ* (AIS), endocervical adenocarcinoma or endometrial adenocarcinoma. Ciliated endocervical cells may occur normally, but ciliated glandular cells showing "tubal" metaplasia are notoriously confused with AIS[25] and required definition. In addition, changes suspicious for but indeterminate for AIS or adenocarcinoma were designated atypical glandular cells of uncertain significance (AGUS).

Features of benign endometrial cells in Pap smears[25]

Cytoplasmic features:

(1) small darkly staining epithelial cells in small three-dimensional clusters, less commonly single cells, when shed from the endometrial cavity;

(2) small darkly staining epithelial cells in sheets when sampled directly using brush samplers;

(3) ill-defined epithelial cell borders with scanty basophilic cytoplasm, sometimes vacuolated;

(4) round or oval stromal cells with scanty pale basophilic cytoplasm, singly or in sheets;

(5) stromal cells with decidual changes showing abundant foamy cytoplasm resembling macrophages.

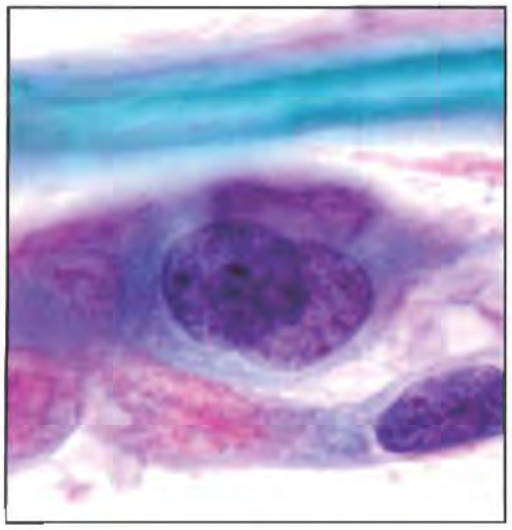


Figure 5.49. Reactive mucinous endocervical cells showing eosinophilic mucin when stained with Pap satin using EA50. Nuclei are round or slightly oval, enlarged, with a mildly disturbed chromatin pattern. Compare tinctoral differences between cells stained with EA50 (Fig 5.49) and EA65 (Fig 5.50).

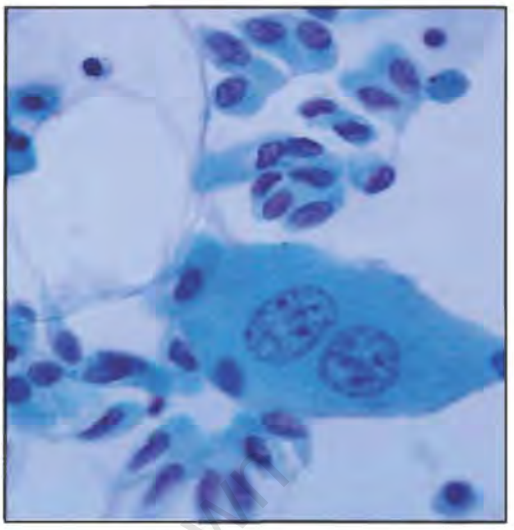


Figure 5.50. AIS showing spindle-shaped glandular cells in rosettes with loss of the normal honeycomb arrangement. Cells show scanty, grey-blue staining cytoplasm (Pap stain using EA65). Nuclei show enlarged, elongated, irregular contours and are coarsely hyperchromatic.

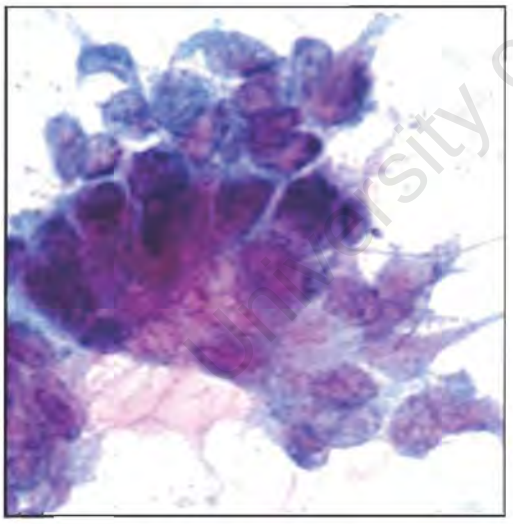


Figure 5.51. AIS showing hyper-cellular cluster with absence of the normal honeycomb pattern. The enlarged nuclei vary in size and shape, are crowded and overlapping, elongated and show granular dark chromatin with small or inconspicuous nucleoli. Stratification and palisading of nuclei, sometimes protruding at the periphery of cell group causes a "feathered" appearance.

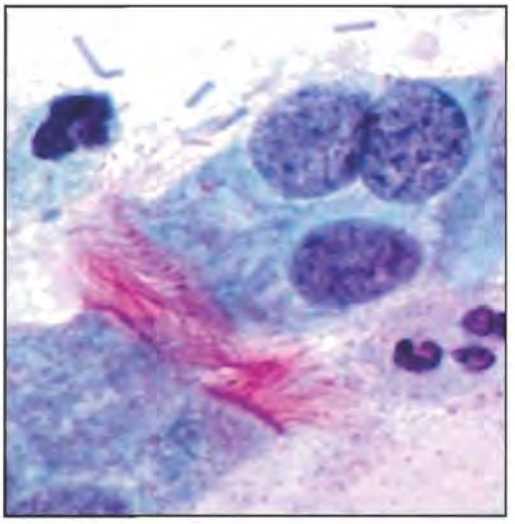


Figure 5.52. Ciliated metaplastic endocervical cells in strips, with cellular crowding and loss of honeycomb arrangement. Cells show cyanophilic cytoplasm with ill-defined cytoplasmic borders. Nuclei are relatively uniform in size and shape, enlarged, oval to round, normo-chromatic with small or inconspicuous nucleoli. The absence of stratification, palisading, rosettes and "feathered" appearance is noted.

Nuclear features:

(1) epithelial cells: small, rounded nuclei, similar in size to nuclei of normal intermediate cells, typically with small inconspicuous nucleoli;

(2) stromal cells: spindle-shaped, darkly basophilic nuclei, frequently with folds in the nuclear membrane.

Changes defining endocervical AIS [25]

Atypical endocervical cells showing features of AIS may be associated with synchronous HPV-related squamous abnormalities or CIN in 25 to 30% of cases. [26]

Cytoplasmic features:

(1) frequently, groups of glandular cells more abundant than is usual in a Pap smear;

(2) spindle shaped glandular cells in sheets, strips and rosettes, recognisably abnormal because of cellular crowding with loss of the normal honeycomb arrangement of cells, (Figure 5.50);

(3) scanty, characteristically grey-blue staining cytoplasm (Pap stain using EA65), or eosinophilic cytoplasm (Pap stain with EA50) with ill-defined cytoplasmic borders (Figures 5.48 and 5.49);

Nuclear features:

(1) enlarged, elongated hyperchromatic nuclei with high nuclear/cytoplasmic ratios;

(2) crowded overlapping nuclei, with absence of the normal honeycomb pattern;

(3) variation in size and shape of nuclei (Figure 5.51);

(4) stratification and palisading of nuclei, sometimes with nuclei protruding at the periphery of cell groups causing a "feathered" appearance (Figure 5.51) ;

(5) granular dark chromatin, small or inconspicuous nuclei;

(6) mitoses sometimes seen.

Features of benign ciliated cell "tubal" metaplasia in Pap smears[25]

Cytoplasmic features: (Figure 5.52)

(1) groups of pseudostratified glandular cells recognisably and characteristically ciliated;

(2) spindle shaped glandular cells in strips, abnormal because of cellular crowding with loss of the normal honeycomb arrangement of cells;

(3) cyanophilic cytoplasm with ill-defined cytoplasmic borders;

Nuclear features: (Figure 5.52)

(1) enlarged, oval to round normo-chromatic nuclei with high nuclear/cytoplasmic ratios;

(2) crowded overlapping nuclei, with absence of the normal honeycomb pattern;

(3) relatively uniform size and shape of nuclei;

(4) stratification and palisading of nuclei;

(5) absence of rosettes and absence of a "feathered" appearance;

(5) finely granular chromatin, small or inconspicuous nuclei;

(6) mitoses not commonly seen.

Changes defining endocervical adenocarcinoma

Changes associated with adenocarcinoma of the endocervix include those found in AIS. Endocervical adenocarcinoma is also frequently associated with HPV-related squamous abnormalities or CIN. Additional features include:

Cytoplasmic features:

(1) single cells, flat strips of cells and three-dimensional clusters of cells, (Figure 5.53);

(2) polygonal or columnar cells with eosinophilic or cyanophilic cytoplasm;

(3) sometimes a necrotic tumour diathesis;

(4) apoptotic bodies;

Nuclear features:

(1) enlarged oval or irregular nuclei with high nuclear/cytoplasmic ratios (Figure 5.53);

(2) crowded overlapping nuclei, with absence of the normal honeycomb pattern;

(3) variation in size and shape of nuclei showing irregular chromatin distribution with parachromatin clearing;

(4) prominent irregularly shaped macronucleoli.

Changes defining endometrial adenocarcinoma

Cells from endometrial adenocarcinoma are generally scanty in Pap smears. In addition, it may not be possible to separate well-differentiated endometrial adenocarcinoma from benign endometrial cells on cytologic appearances.

Cytoplasmic features:

(1) small dyscohesive clusters or single cells;

(2) small cells with scant, typically vacuolated cytoplasm;

(3) tumour diathesis with apoptotic bodies and characteristic foamy macrophages;

(4) neutrophils within epithelial clusters, appearing ingested.

Nuclear features:

(1) variably enlarged irregular nuclei with loss of nuclear polarity, depending on the degree of tumour differentiation;

(2) moderate hyperchromasia of nuclei, parachromatin clearing with variable nucleoli, depending on tumour differentiation.

Changes defining AGUS

Atypical glandular cells, either of endocervical or endometrial origin, were included in this group. AGUS was defined as cells showing nuclear changes exceeding in severity those of reactive or reparative changes, but not obviously malignant.

AGUS endocervical

Cytoplasmic features:

(1) small sheets and strips of endocervical cells;

(2) abundant grey/blue or eosinophilic cytoplasm (depending whether EA50 or EA65 used in staining) with distinct cell borders;

(3) polygonal or columnar cells.

Nuclear feature (Figure 5.54):

(1) enlarged nuclei, from three up to five times the area of normal endocervical cell nuclei;

(2) elongated nuclei, arranged in the long axis of columnar endocervical cells;

(3) mild variation in size and shape of nuclei;

(4) mild hyperchromasia of nuclei, often with nucleoli.

AGUS endometrial

Cytoplasmic features:

(1) small groups of small endometrial cells, five to ten cells per group;

(2) scanty blue cytoplasm with indistinct cell borders;

(3) occasional irregularly shaped vacuoles;

Nuclear features:

(1) nuclei slightly enlarged;

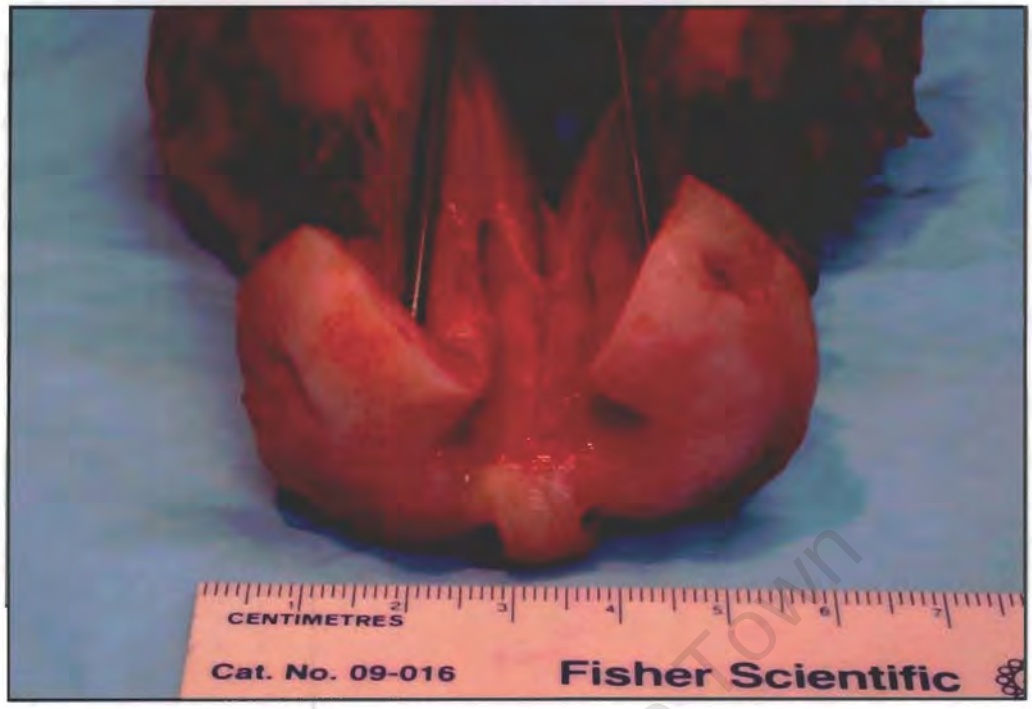
(2) slight hyperchromasia of nuclei, sometimes with small nucleoli present.

5.3.2 TISSUE SECTIONS

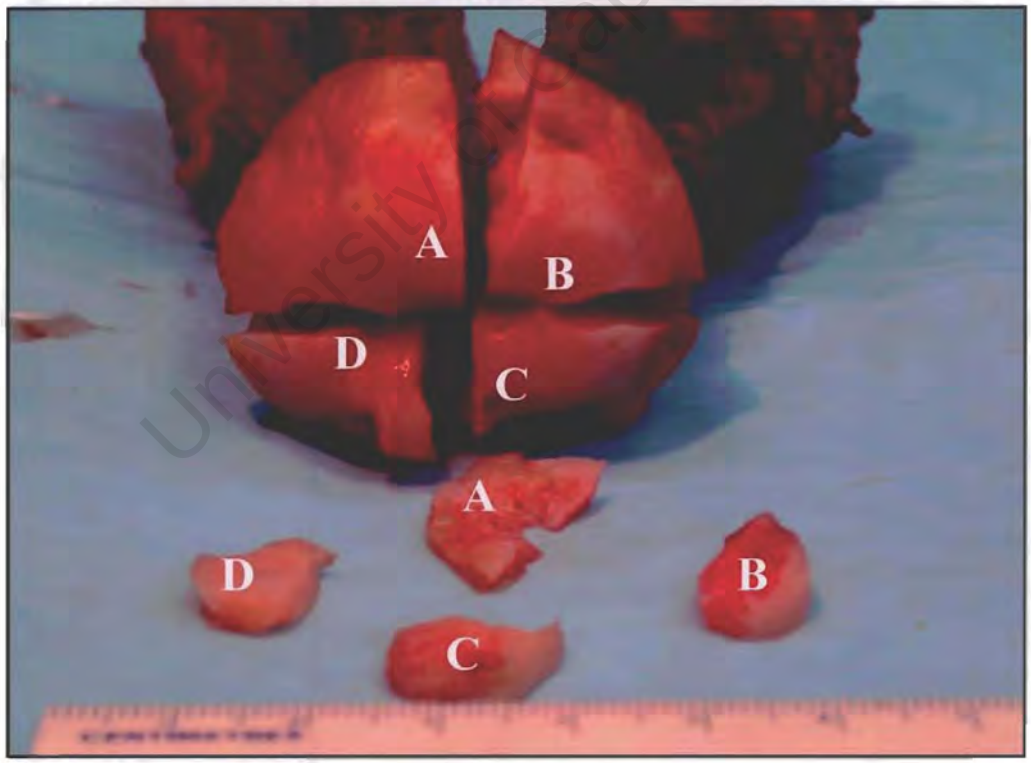
During the period between 2 January 1993 and 31 December 1996, all tissue specimens of the *cervix uteri* submitted for examination in the practice were critically examined for features suggestive of Chlamydial infection. Only specimens examined grossly and microscopically by the writer were included in the study. The clinical, epidemiological and demographic information supplied on the request form by the surgeon submitting the specimen was available. This information and the reported findings were entered into the study database for histology specimens. When available, pre-histology PS were reviewed after the surgical specimen had been evaluated. Pre-histology cytology were received in less than 10% of cases, usually submitted by general practitioners in the community. Gynaecologists sent specimens to a purely cytological laboratory so that correlation studies were subject to less bias. An analysis of the results of pre-histology PS was therefore not possible.

5.3.2.1 TISSUE SPECIMENS EXAMINED FOR CHLAMYDIAL INFECTION

Tissue specimens were submitted by surgeons in private practice to the practice for routine histopathology reporting. These practitioners made use of operating theatres in 25 privately owned hospitals situated in the Greater Cape Town Area and in the nearby towns of Atlantis, Gordon's Bay, Paarl, The Strand, Somerset West, Stellenbosch and Worcester (Appendix A). Pre-operative patient consent was obtained for the surgical and anaesthetic procedures and for the submission of the tissues to the pathology laboratory for pathologic examination. This consent was obtained from the patient by the surgeon.



Macro 5.1. . The cervix was opened at 12 o'clock but otherwise kept in one piece.



Macro 5.2. Radially orientated wedges were removed from 12, 3, 6 and 9 o'clock for microscopy, still keeping the remaining cervix in one piece. Sections were labelled so that location of significant changes could be anatomically identified. The section from the left anterior quadrant was marked A, the left posterior marked B, the right posterior block marked C and the left anterior block marked D. Pertinent inflammatory or dysplastic changes could be anatomically located in the cervix.

Tissues were fixed in 10% neutral buffered formol saline supplied by the practice to the operating theatres. Specimens were transported by the practice for processing in the central histology laboratory. During the period January 1st 1993 until December 31st 1996, the writer grossly and microscopically examined a total of 2682 surgical specimens of *cervix uteri*. Included in these specimens were 1037 hysterectomies and 1645 cervical biopsies, polypectomies, cold-knife cone biopsies and large loop resections of the cervical transformation zone (LLETZ). Routine procedures for processing tissues for LM were used. Histology sections were cut on a rotary microtome (Reichert) at 5µm in thickness. The sections were stained with haematoxylin and eosin (H & E) (Appendix C). One of the four blocks of cervix from hysterectomy, LLETZ or old knife cone specimens was routinely stained for acid and neutral mucins using the alcian blue, periodic acid Schiff diastase method (A-PAS-D) (Appendix D). At least one block of tissue from each biopsy specimen was similarly stained.

Hysterectomy specimens

The 1037 hysterectomy specimens were examined macroscopically and dissected by the writer. The presence or absence of inflammatory vascular proliferation in the cervical mucosa, evidenced by an easily visible mottled red appearance was noted. Blocks of tissue for histology from all hysterectomy specimens were taken from four quadrants of the cervix. The cervix was opened at 12 o'clock but otherwise kept in one piece (Macro 5.1). Radially orientated wedges were removed from 12, 3, 6 and 9 o'clock for microscopic examination, still keeping the remaining cervix in one piece (Macro 5.2). The wedges removed included a segment of ectocervix, the transition zone and a segment of endocervix. The wedges were labelled so that the location in the cervix of any significant changes could be anatomically identified. The section from the left anterior quadrant was marked A, the left posterior marked B, the right posterior block marked C and the left anterior block marked D. This procedure allowed pertinent inflammatory or dysplastic changes to be anatomically located in the cervix (Macro 5.2). If the findings on initial microscopic examination dictated, it was possible to take more tissue from an appropriate area of the cervix for further examination and subsequent corroboratory testing. Care was taken that the ectocervix, the transition zone and the endocervix were sampled in each block. In addition two sections of the endometrium and full thickness of the myometrium were taken. These were marked MA (anterior uterine wall) and MP (posterior uterine wall). The endometrial blocks were examined for evidence of endometrial Chlamydial infection. If the Fallopian tubes and ovaries were submitted, sections of each were taken and marked L (left) and R (right) respectively.

Biopsy specimens

A total of 1645 biopsy specimens examined in the study included cold knife cone biopsies, LLETZ, cervical polypectomies, wedge biopsies and colposcopically directed punch biopsies. In these cases, the entire specimen was processed for LM.

5.3.2.2 Diagnostic criteria for Chlamydial infection in tissue sections

Cervical *Chlamydia trachomatis* infections are amenable to diagnosis by histology.[18] During the study, histology specimens were examined for features described in several histological publications.[2,4-6,10,15,16] While some of these

writers did not describe Chlamydial inclusions at all,[5,6] and some workers questioned their reliability, [10,13,16] other authors recognised them as diagnostically useful.[2,4] It was decided that in the present study, the presence of Chlamydial inclusions would be accepted as being of diagnostic value.

In addition to the features described in these publications, a search was made for features more commonly described in Pap smears. Many of the changes ascribed to Chlamydial infection in Pap smears had not received emphasis in histologic descriptions.[6,12,13,16] In particular, a feature constantly seen in Pap smears but not reported in tissue sections was striking immature squamous metaplasia. Accordingly, sections showing marked squamous metaplasia were carefully scrutinised for Chlamydial inclusions.

The different fixation and processing techniques used in histology and cytology result in different morphologic appearances of cells. Consequently, the fine detail seen in cytology is not found in histology using paraffin-embedded sections.[4] Nevertheless, recognition of the same pathologic process as seen in the two modalities is a routine exercise to the cytopathologist. As far as it was possible, criteria defined above for cytologic examination of smears were applied to histologic sections.

5.3.2.3 Diagnostic criteria for the diagnosis of cervicitis in tissue sections

Inflammation of the uterine cervix is almost universal in women and in the majority of patients is regarded as inconsequential Crum *et al* 1999. [18] The incidence of a mild inflammatory infiltrate showing plasma cells and lymphocytes in biopsy specimens is so high that the infiltrate is regarded as "normal." In gynaecologic diagnosis of cervicitis, the emphasis is placed on detection of specific organisms known to be pathogenic.[18,32,34] Chlamydia is said not to elicit specific epithelial changes but is associated with a chronic follicular lymphocytic inflammatory infiltrate and reparative changes.[5,6,18,32] No complete and acceptable criteria for a histologic diagnosis of cervicitis were found in the literature, although some of the features described by Hare *et al*[5] and Paavonen *et al*[6] were useful. For the purposes of the study, general principles of pathology regarding what constitutes inflammation were applied.[33]

Criteria for a diagnosis of inflammation in the study included:

(1) oedema in sub-epithelial tissue or spongiosis in metaplastic epithelium;[33]

(2) an acute inflammatory infiltrate with margination of neutrophils, infiltration by neutrophils into the peri-capillary areas,[33] into squamous epithelium[32] (leucocytosis) or mucinous glands (crypt abscess);

(3) proliferation of capillary vessels in the sub-epithelial connective tissue;[33]

(4) ulceration of mucosa with necrosis of tissue, granulation tissue, inflammation and fibrosis;[33]

(5) a chronic inflammatory infiltrate with lymphocytes and/or plasma cells,[33] sometimes with reactive germinal centres,[5,6,15,16] and/or Russell bodies;

(6) degenerative cellular changes with necrosis of cells, [26-28,33] and apoptosis;[30,31]

(7) reactive epithelial changes manifest by squamous metaplasia;[4,26-28,33]

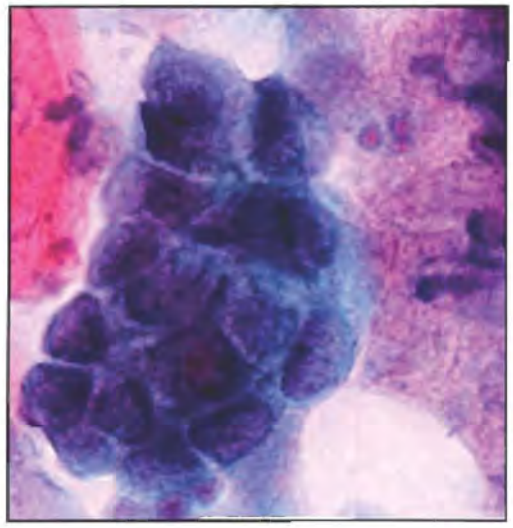


Figure 5.53. Endocervical adenocarcinoma showing a three-dimensional cluster of polygonal cells with high nuclear/cytoplasmic ratios. The oval, enlarged and irregular nuclei are crowded, overlapping. Note irregular chromatin distribution with parachromatin clearing.

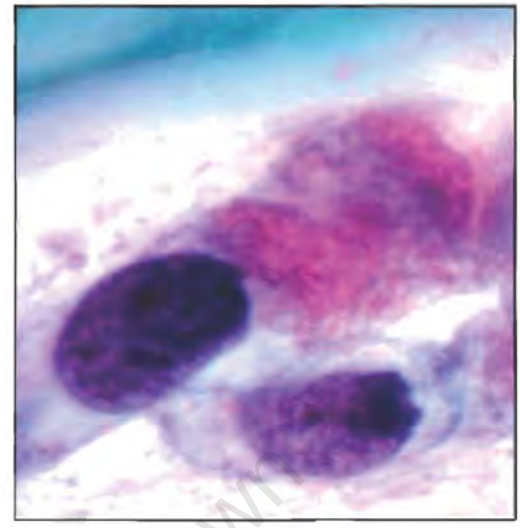


Figure 5.54. AGUS (endocervical) with columnar endocervical cells showing abundant eosinophilic cytoplasm (EA50 staining). The enlarged elongated nuclei are arranged in the long axis of the cells and show mild variation in size and shape, mild hyperchromasia often with nucleoli .

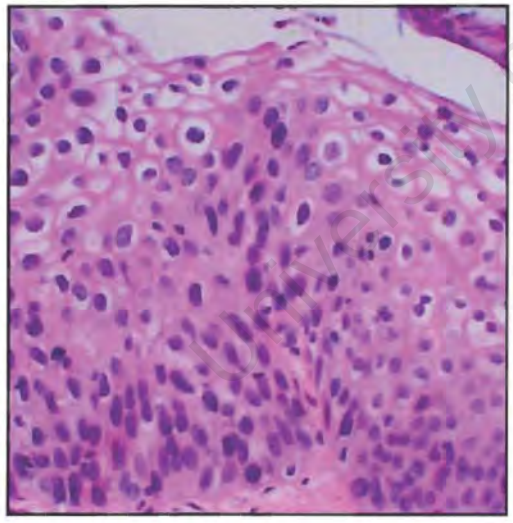


Figure 5.55. "Warty" changes with a thickened epithelial layer and subtle papillomatosis manifested by narrow reteform papillae. "Koilocytic" cytoplasmic changes in the superficial and the intermediate layers.

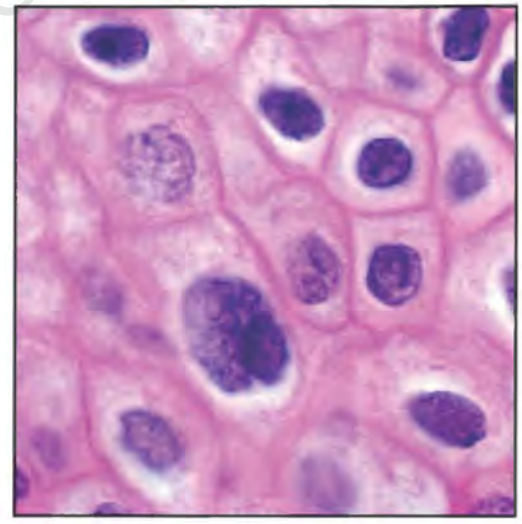


Figure 5.56. HPV changes characterized by cells showing distinct cytoplasmic borders, nuclear enlargement with intensely dark, smudged or granular chromatin and irregular "crumpled" contours, sometimes binucleation and giant nucleation.

(8) reactive epithelial changes with enlarged nuclei, the presence of nucleoli and immature cytoplasmic differentiation (loss of keratin or mucin production); [26-28]

(9) reactive connective tissue changes with oedema, proliferation of fibroblasts, and eventually fibroplasia with collagen deposition. [33]

5.3.2.4 DIAGNOSTIC CRITERIA FOR HPV INFECTION AND DYSPLASIA IN TISSUE SECTIONS

The criteria for the diagnosis of HPV infection and CIN in histologic sections were derived from the same sources as for cytology. [21-25] Recognition of HPV infection was according to the criteria of Meisels *et al*,[21] and of Richart.[22] Tissue specimens showing invasive carcinoma were excluded from the study.

Changes defining HPV[21,22] (Figure 5.55,56)

(1) "warty" changes found in the ectocervical area, usually immediately adjacent to the endocervical mucinous epithelium;

(2) frequently, mild thickening of the epithelial layer with subtle papillomatosis manifested by narrow reteform papillae with delicate submucous capillaries extending into the epithelium;

(3) the superficial and the intermediate layers of the ectocervical epithelium showing distinct cytoplasmic borders with "koilocytic" cytoplasmic changes;

(4) nuclear atypia manifested by large nuclei relative to the size of the cell, with intensely dark, smudged or granular chromatin and irregular "crumpled" contours, sometimes binucleation and giant nucleation.

Changes defining CIN I[22-25] (Figure 5.57)

(1) parakeratosis sometimes, with keratinised cells on the surface of the ectocervical epithelium;

(2) enlarged, elongated atypical nuclei in the lower third of the ectocervical epithelium, the long axis of these cells arranged perpendicular to the basement membrane;

(3) nuclei of the atypical cells mildly pleomorphic with coarse hyperchromatic chromatin and without nucleoli;

(4) cells with normal nuclei in the upper two thirds of the ectocervical epithelium, lying parallel to the basement membrane.

Changes defining CIN II[22-25] (Figure 5.58)

(1) enlarged atypical nuclei in the lower two thirds of the ectocervical epithelium, arranged perpendicular to the basement membrane;

(2) eosinophilic weakly keratinised cytoplasm usually present in the middle and upper thirds of the ectocervical epithelium;

(3) nuclei enlarged, characteristically variable in size with sharply irregular contours, chromatin coarsely granular and darkly hyperchromatic;

(4) chromatin evenly dispersed and nucleoli not present.

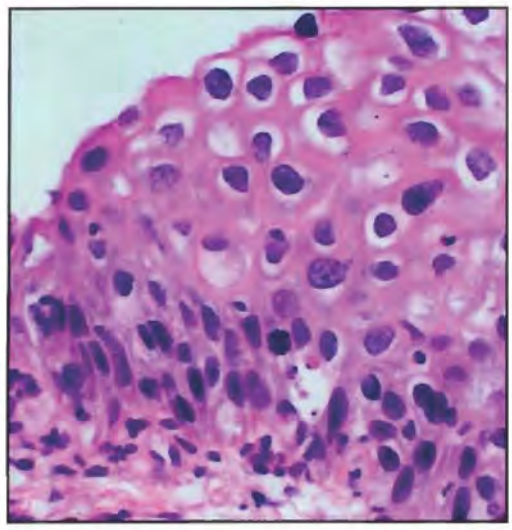


Figure 5.57. CIN I showing enlarged, elongated atypical nuclei in the lower third of the ectocervical epithelium, the long axis of these cells arranged perpendicular to the basement membrane. The atypical nuclei are mildly pleomorphic with coarse hyperchromatic chromatin without nucleoli. Note a mitotic figure.

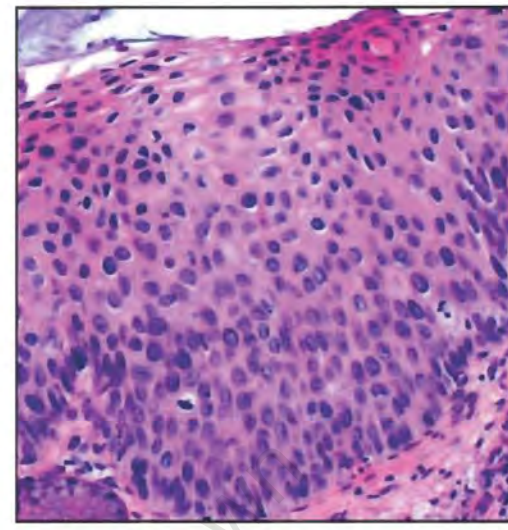


Figure 5.58. CIN II with atypical nuclei arranged perpendicular to the basement membrane in the lower two thirds of the epithelium. Nuclei are enlarged, variable in size with sharply irregular contours, chromatin is coarsely granular and darkly hyperchromatic.

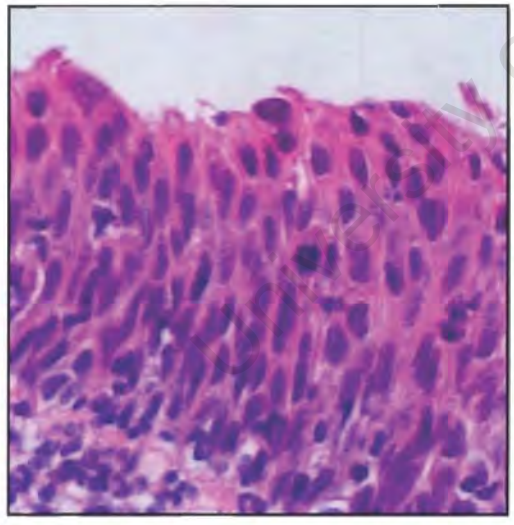


Figure 5.59. CIN III with atypical nuclei in the full thickness of the ectocervical epithelium. Cells show immature squamous features in a syncytial pattern. Nuclei enlarged, polygonal, variable in size, darkly hyperchromatic with irregular contours. Note a mitotic figure.

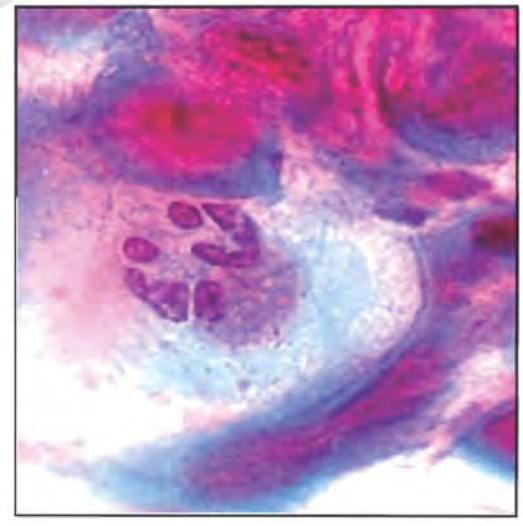


Figure 5.60 Neutrophil leucocytosis was frequently seen with neutrophils between acantholytic metaplastic squamous epithelium or within vacuoles in epithelial cells ("inflammatory vacuoles"). Care to separate such vacuoles from Chlamydial RB was necessary

Changes defining CIN III[22-25] (Figure 5.59)

(1) enlarged atypical nuclei present in the full thickness of the ectocervical or transitional zone epithelium;

(2) eosinophilic weakly keratinised cytoplasm sometimes present in the middle and upper thirds of the ectocervical epithelium;

(3) in weakly keratinised cells, nuclei enlarged, polygonal, variable in size, generally darkly hyperchromatic with sharply irregular contours;

(4) cells frequently showing immature squamous features with scant basophilic cytoplasm in a syncytial pattern in the full thickness of the epithelium;

(5) if immature cells, nuclei enlarged, enlongated/fusiform, fairly uniform in size, but showing irregular contours;

(6) nuclei of immature cells arranged vertically to the basement membrane,

(7) nuclei generally darkly hyperchromatic with coarse evenly dispersed chromatin without nucleoli.

5.4 RESULTS

5.4.1 PAP SMEARS

5.4.1.1 INCIDENCE AND DEMOGRAPHICS OF CHLAMYDIAL INFECTION

Incidence of Chlamydial infection found by Pap smear

During the study period a total of 48 958 Pap smears were submitted for examination in the practice. Amongst these, 1 141 cases (2.33%) were identified as showing features of infection by *Chlamydia trachomatis* (Table 5.1). The incidence in the population examined by Pap smear varied each year. In 1993 when the study commenced, the detected incidence was 0.6 %. During the third year of the study the detected incidence rose to 3.20 %. This suggested that the ability of the screeners and author to recognise the changes of Chlamydial infection improved over the course of the first two years. The incidence in the study population during the last two years of the study of Chlamydial infection was approximately 3.1%. The reported incidence of the infection in women in the USA is 3%.[36]

Table 5.1
Number of patients examined by year and number of cases found.

Year	No smears examined	Controls	No cases Chlamydia	Incidence of Chlamydia
1993	11 327	73	73	0.64 %
1994	12 493	283	283	2.26 %
1995	13 968	447	447	3.20 %
1996	11 077	338	338	3.05 %
Total (1993 - 1996)	**48 865**	**1 141**	**1 141**	**2.33 %**
Total (1995 - 1996)	**22 045**	**785**	**785**	**3.13 %**

Age distribution of patients with Chlamydial infection

The age distribution of patients showing evidence of Chlamydial infection (Table 5.2) was compared with the age distribution of the control patients (Table 5.3). There were significantly more patients with Chlamydial infection in the age group 41 – 50 years (24.8%) compared with the control group (18.6%) ($p < 0.005$). However, no other age group showed a significant difference in the number of patients with Chlamydial infection compared with the number of controls ($p > 0.20$ to 0.75).

Table 5.2
Age distribution of patients found to have changes of Chlamydial infection.

Year	Age of patient in years								No cases Chlamydia
	< 21	21- 30	31 - 40	41 - 50	51 - 60	61 - 70	> 70	Unknown	
1993	1	15	21	18	10	2	0	6	73
1994	8	49	89	67	24	9	2	35	283
1995	7	85	141	111	53	8	1	41	447
1996	5	98	82	87	25	10	1	30	338
Total	**21**	**247**	**333**	**283**	**112**	**29**	**4**	**112**	**1 141**

Incidence of Chlamydial infection in racial groups

The race of patients and controls was known in most cases (Table 5.4). There was no statistically significant difference detected when the proportion of patients with Chlamydia was compared with the proportion of controls (Asian $p = 0.0485$; Black $p = 0.4436$; Mixed $p = 0.3002$; White: $p = 0.3956$). The numbers of patients with Chlamydial infection in each racial group reflected the proportion of that racial group in the control population.

Table 5.3
Age distribution of control patients without evidence of Chlamydial infection.

Year	Age of control patients in years								No cases
	< 21	21- 30	31 - 40	41 - 50	51 - 60	61 - 70	> 70	Unknown	
1993	2	16	23	17	9	2	0	4	73
1994	7	74	92	54	19	8	1	28	283
1995	11	113	135	75	42	14	2	58	447
1996	8	90	94	66	28	16	2	34	338
Total	**28**	**293**	**344**	**212**	**98**	40	**5**	**126**	**1141**

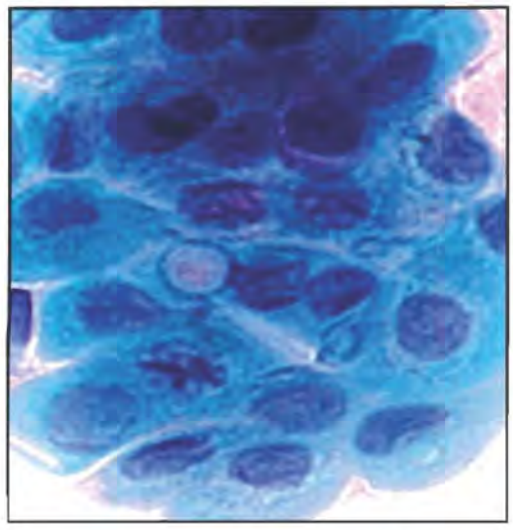


Figure 5.61. Metaplastic cells in Chlamydial infection occurred either in acantholytic, loosely aggregated clusters or occasionally as single cells. The cytoplasm stained pale cyan-blue and occasionally showed characteristic Chlamydial vacuolar inclusions.

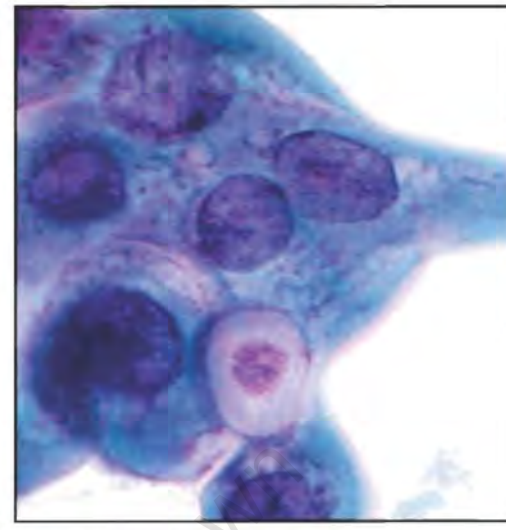


Figure 5.62. Nuclei of metaplastic cells were enlarged, sometimes more than three times the size of normal intermediate cell nuclei. Nuclear membranes were always well defined and fine, without coarse, dark thickening. Nuclear outlines are oval, smooth and regular, in many respects, qualifying for a diagnosis of ASCUS.

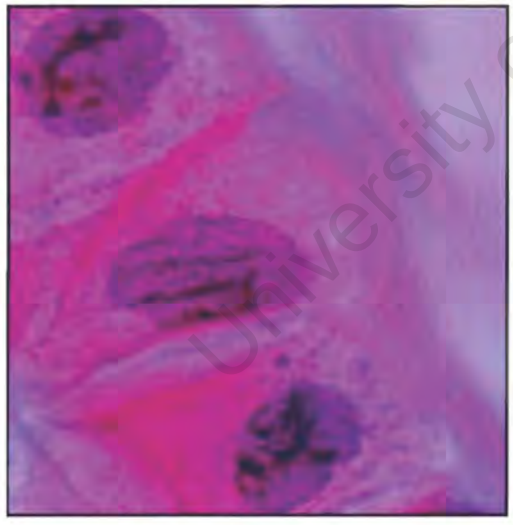


Figure 5.63. Metaplastic cells showed nuclear outlines with fine wrinkling, the wrinkles tending to be longitudinally arranged in oval nuclei. Chromatin was evenly dispersed and finely granular, with a mildly hyperchromatic appearance. Nucleoli were infrequently found

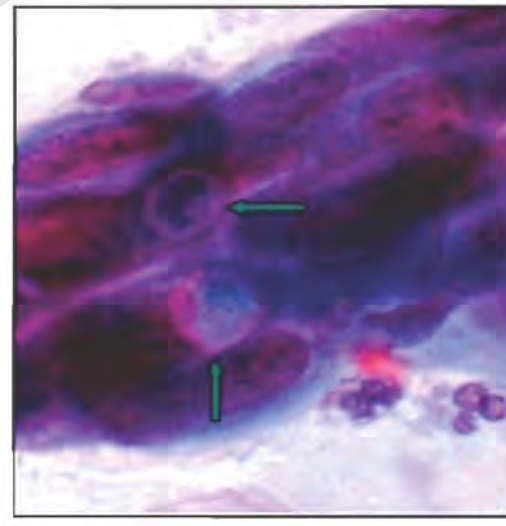


Figure 5.64 The chromatin was occasionally clumped and darkly basophilic, and rarely coarse and irregularly dispersed. Small inconspicuous nucleoli were infrequently found. Note the Chlamydial inclusion (↑) (not in sharp focus in this three dimensional cluster) and the inflammatory vacuole containing a neutrophil (←)

Table 5.4
Comparison by race of the incidence of Chlamydial infection compared with control patients.

Year	Patients with Chlamydia					Patients without Chlamydia				
	Asian	Black	Mixed	White	Total	Asian	Black	Mixed	White	Total
1993	0	1	19	53	73	2	0	25	46	73
1994	6	3	66	207	283	0	4	61	218	283
1995	1	6	116	324	447	0	1	135	301	447
1996	4	6	89	239	338	1	7	92	238	338
Total	**11**	**16**	**290**	**823**	**1141**	**3**	**22**	**313**	**803**	**1141**

5.4.1.2 MORPHOLOGIC FEATURES DIAGNOSTIC OF CHLAMYDIAL INFECTION

Some of the morphologic changes associated with Chlamydial infection reported below were new observations and served to refine and expand the diagnostic criteria for the morphologic diagnosis of Chlamydial infection.

Inflammatory infiltrate

An inflammatory infiltrate was almost always evident, but not invariably present. The infiltrate was of variable severity, either lymphocytic or mixed with lymphocytes, plasma cells, and/or neutrophils;

(1) neutrophil leucocytosis was frequently seen with neutrophils infiltrating between epithelial cells in sheets of acantholytic metaplastic squamous epithelium. Neutrophils were also found within vacuoles in metaplastic epithelial cells ("inflammatory vacuoles"). Care to separate such vacuoles from Chlamydial RB was necessary (Figure 5.60);

(2) most frequently, the infiltrate was a mixed exudate with numerous free-lying neutrophils, lymphocytes and plasma cells in the background;

(3) less frequently, a follicular lymphoid infiltrate was evident within exfoliated papillary fragments of endocervical mucinous mucosa.

Cellular changes

In Pap smears, cellular changes associated with the presence of Chlamydial vacuoles were seen in both mucinous and squamous cells. However, consistently specific and recognisable cellular changes were only found in the metaplastic squamous cells. In particular, the nuclear changes found in metaplastic cells with Chlamydial infection were distinct.

Characteristic changes in metaplastic endocervical epithelium

The metaplastic cells showed the following features, in many instances resembling / qualifying for a diagnosis of ASCUS:

(1) the cells occurred either in three-dimensional papillary clusters, in sheets, in syncytia, or in acantholytic, loosely aggregated clusters or occasionally as single cells (Figure 5.61; 5.64);

(2) the cytoplasm of the cells usually stained a pale cyan-blue and was not the darker blue of haematoxylinophilic or basophilic cells (Figure 5.61; 5.62);

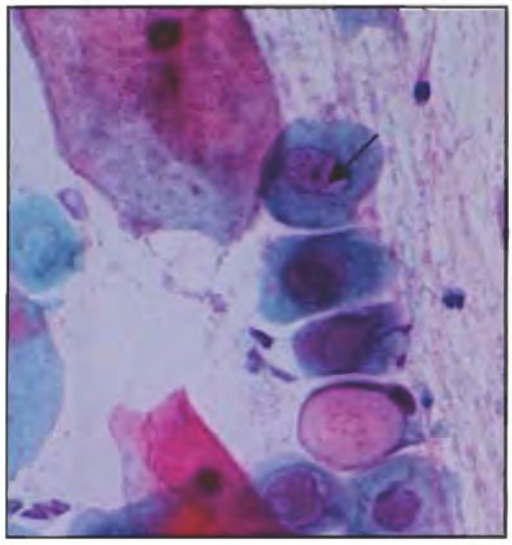


Figure 5.65. Metaplastic cells with Chlamydial inclusions infrequently showed small inconspicuous nucleoli.

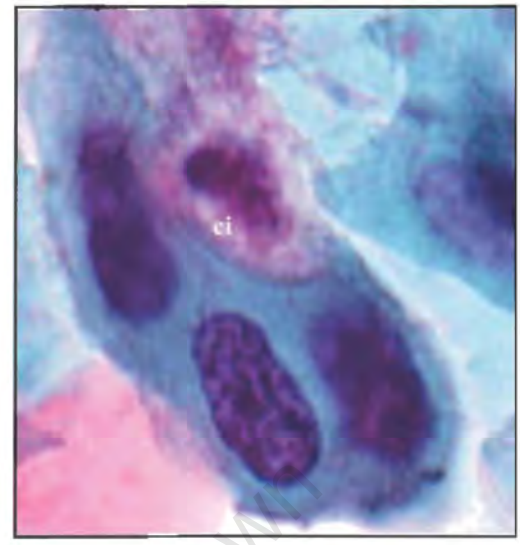


Figure 5.66. Chlamydial infection [vacuolar inclusion (ci) out of plane of focus] occasionally showed marked nuclear atypia with irregularly shaped, hyperchromatic nuclei and coarsely clumped chromatin. These cases frequently but not always showed evidence of HPV infection elsewhere on the Pap smear.

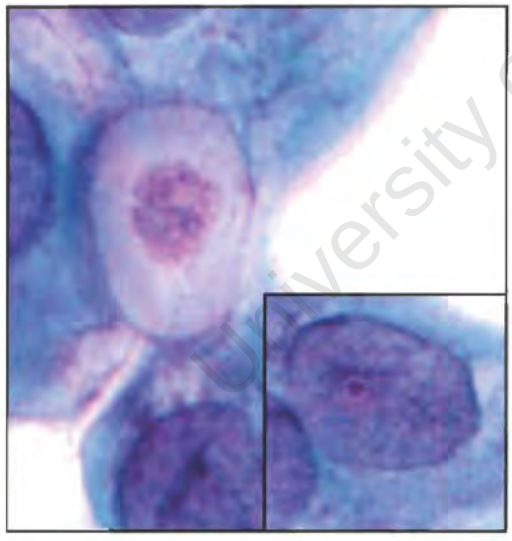


Figure 5.67. *Targetoid* inclusions showed one distinct central inclusion. These were either large eosinophilic or basophilic inclusion up to 5 μm in diameter or small dot-like coccoid basophilic inclusions < 1μm in diameter (insert).

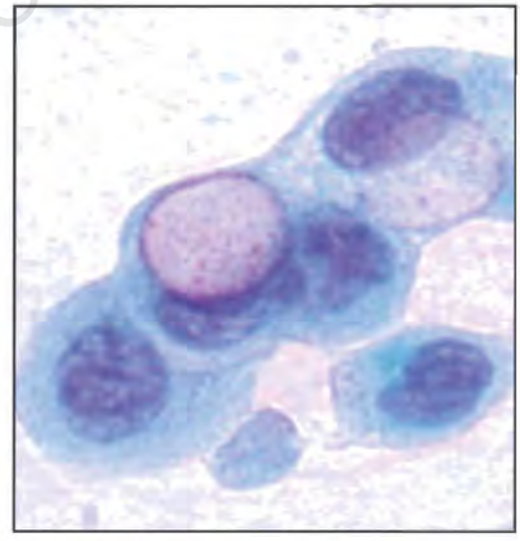


Figure 5.68. *Nebular* inclusions showed numerous small dot-like eosinophilic or basophilic coccoid bodies.

(3) these metaplastic cells occasionally showed characteristic Chlamydial vacuolar inclusions, described in more detail below (*vide infra*) (Figure 5.61; 5.62; 5.64);

(4) nuclei of the cells were enlarged, sometimes more than three times the size of normal intermediate cell nuclei (Figure 5.61 to 5.65);

(5) nuclear membranes were always well defined and fine, without coarse, dark thickening (Figure 5.61 to 5.64);

(6) nuclear outlines were usually oval, smooth and regular, but fine wrinkling of the nuclei was also regularly seen. The wrinkles tended to be longitudinally arranged in oval nuclei (Figure 5.61; 5.63);

(7) chromatin was evenly dispersed and finely granular, with a mildly hyperchromatic appearance (Figure 5.61 to 5.63). The chromatin was occasionally clumped and darkly basophilic, and rarely coarse and irregularly dispersed (Figure 5.64);

(8) small inconspicuous nucleoli were infrequently found (Figure 5.65).

(9) occasionally, a case with other features indicating Chlamydial infection did show marked nuclear atypia with irregularly shaped, hyperchromatic nuclei and coarsely clumped chromatin. These cases frequently but not always showed evidence of HPV infection elsewhere on the Pap smear (Figure 5.66).

Characteristic Chlamydial inclusions

The characteristic Chlamydial inclusions ranged from 1 μm to >15 μm in size. They were present in at least one viable metaplastic cell. The inclusions were usually found within a well-defined vacuole, called stage three "inclusion bodies" in Gupta *et al.*[1] These vacuoles assisted identification of the Chlamydial inclusion with certainty. Vacuoles acceptable as Chlamydial showed the following features:

(1) the vacuoles were demonstrably within the cytoplasm of the metaplastic cells and showed a distinct, well-defined, usually round enclosing membrane;

(2) only vacuoles that were in a viable cell showing no evidence of karyorrhexis or karyolysis were accepted;

(3) the vacuoles showed characteristic intra-vacuolar inclusions, details of which were often only visible with careful up-and-down focusing of the microscope through the thickness of the vacuole;

(4) three types of intra-vacuolar inclusion were identified:

(a) *targetoid* with one distinct central inclusion. These were either small dot-like coccoid basophilic inclusions < 1μm in diameter, or large eosinophilic or basophilic inclusion up to 5 μm in diameter (Figure 5.67);

(b) *nebular* with numerous small dot-like eosinophilic or basophilic coccoid bodies (Figure 5.68);

(c) *festooned* with large irregular basophilic or eosinophilic ribbons and stranded inclusions festooned in a well-defined enclosing membrane (Figure 5.69, 5.77);[37,38]

(5) care was taken to separate degenerative vacuoles and vacuoles containing neutrophils, apoptotic bodies, mucin, lipid or talc granules from true Chlamydial vacuolar inclusions (Figure 5.70).

Small, usually numerous coccoid bodies without distinct vacuoles were sometimes found within the cytoplasm of metaplastic squamous and/or mucinous cells (Figure 5.71 A & B). These inclusions showed a surrounding ill-defined

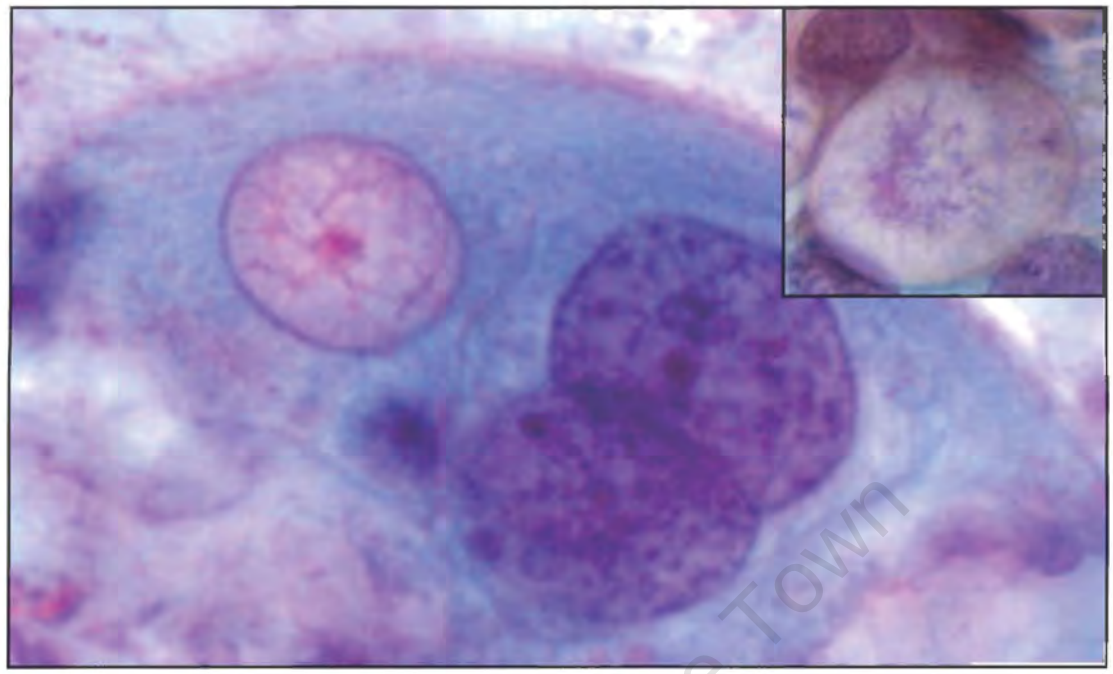


Figure 5.69. *Festooned* inclusions showed large irregular basophilic or eosinophilic ribbons and stranded inclusions festooned in a well-defined enclosing membrane

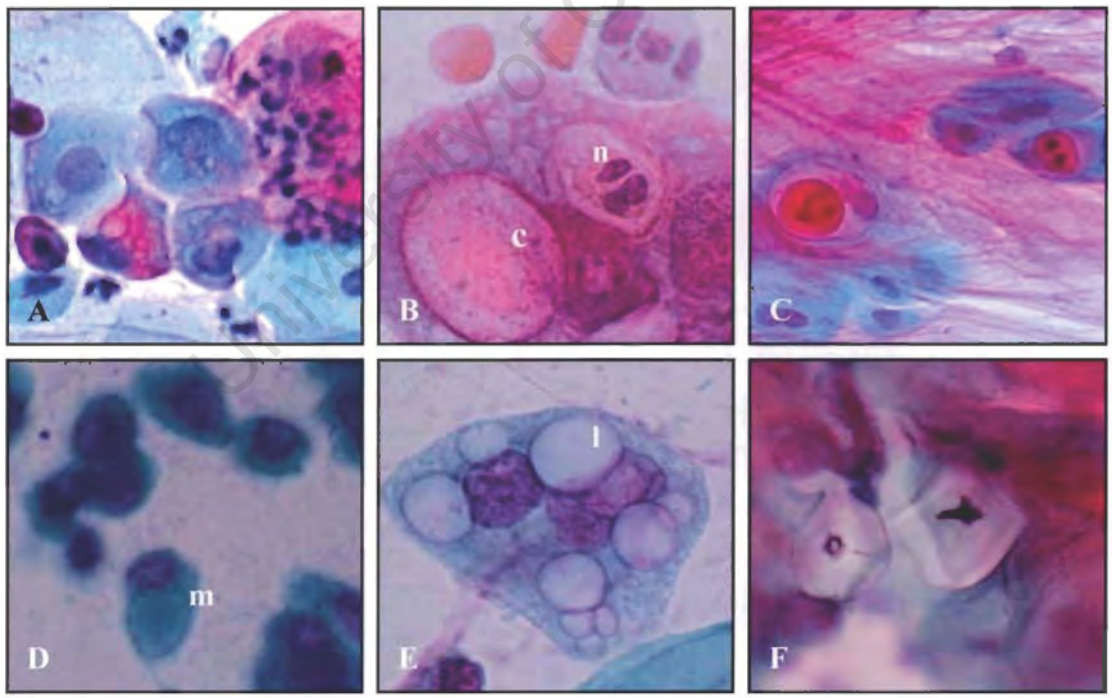


Figure 5.70. Care was taken to separate true Chlamydial vacuolar inclusions (B: c) from degenerative vacuoles (A), vacuoles containing neutrophils (B: n), apoptotic bodies (C), mucin (D: m), lipid (E: l) or talc granules (F)

area of pallor in the cytoplasm but a vacuole with a distinct membrane was not present. Cells containing numerous coccoid inclusions appeared pale with a lacy pattern in the cytoplasm. These coccoid bodies were called first stage inclusions in Gupta *et al.*[1] They were difficult to identify with certainty and were not regarded as a practical or useful feature of Chlamydial infection. When convincing examples of the first stage inclusions were present, they were almost always found in association with at least one of the types of vacuolar inclusions elsewhere on the same slide.

The inclusions designated as nebular in the present study were not separated into the fine granular and more coarsely granular inclusions described by Shiina.[2] Again, large targetoid inclusions were not divided into the granular and homogeneous types described in Shiina. The latter author did not describe nor investigate targetoid inclusions with small dot-like coccoid inclusions.

Changes in mucinous endocervical epithelium

Although reactive changes were also seen in mucinous endocervical cells, these were variable, appeared non-specific and were not considered useful in the recognition of *Chlamydia trachomatis* infection. Mucinous cells usually occurred in three-dimensional clusters and showed mild loss of polarity with crowding of nuclei. The nuclei were enlarged and showed mild hyperchromasia and small nucleoli. The nuclei were not elongated, palisaded or "feathered" and were not hyperchromatic. They resembled "reactive" mucinous cells seen in inflammatory smears. Immature mucinous cells were found within and at the periphery of groups of metaplastic squamous cells, as described by Koss.[27] These mucinous cells showed finely vacuolated grey/blue cytoplasm (EA 65) or eosinophilic cytoplasm (EA 50) and did not resemble cells containing well-defined Chlamydial inclusions. Occasionally vacuolar Chlamydial inclusions were found in mucinous cells (Figure 5.72).

Cardinal features necessary for the diagnosis of Chlamydial infection

The above results describe a constellation of changes that were regarded as important in arriving at a diagnosis of Chlamydial infection in any particular case. The features associated with Chlamydial infection varied from case to case, and all of the changes described above were not always present in any one case. However, the presence of *all* of the following features were regarded as *absolutely necessary* before a confident diagnosis of Chlamydial infection was made. These cardinal features are listed as follows (Figure 5.73; 5.74):

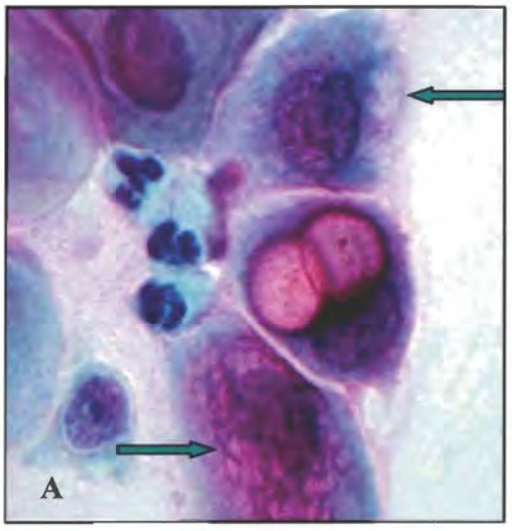
(1) a mixed inflammatory infiltrate with neutrophils, lymphocytes and plasma cells;

(2) sheets of immature metaplastic squamous epithelium, frequently acantholytic (Figure 5.74);

(3) neutrophil leucocytosis showing neutrophils in the interstices between acantholytic metaplastic cells (Figure 5.74) and sometimes within intraepithelial vacuoles (Figure 5.73);

(4) metaplastic cells showing enlarged, usually oval nuclei, with well defined, fine nuclear membranes, sometimes with longitudinally arranged wrinkles;

(5) metaplastic cells showed evenly dispersed and finely granular chromatin with a mildly hyperchromatic appearance and infrequently, small inconspicuous nucleoli (Figure 5.73);



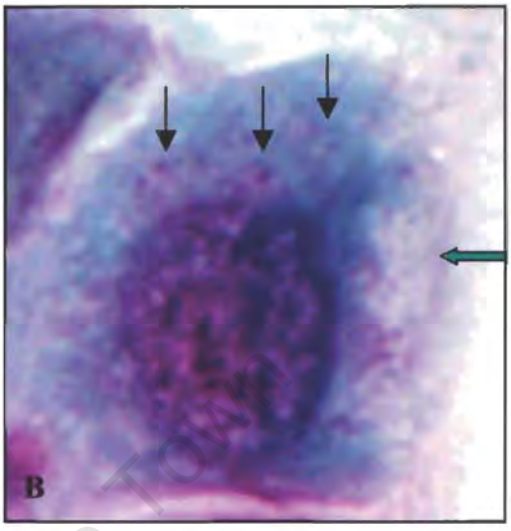


Figure 5.71 A. Cells with numerous small coccoid bodies without distinct vacuoles appeared pale with a lacy ill-defined areas of pallor in the cytoplasm (→). The coccoid bodies ("first stage inclusions in Gupta *et al*[1]) were difficult to identify with certainty but were sometimes found with vacuolar inclusions in nearby cells.
Figure 5.71 B. A closer view of coccoid bodies, difficult to identify with certainty (↓).

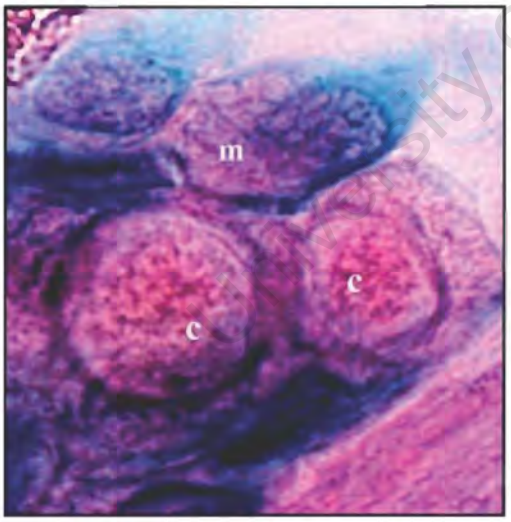


Figure 5.72. Mucinous cells showed finely vacuolated eosinophilic cytoplasm when stained with EA 50, (m) did not resemble cells containing well-defined Chlamydial vacuolar inclusions (c). Occasionally Chlamydial inclusions were found in mucinous cells.

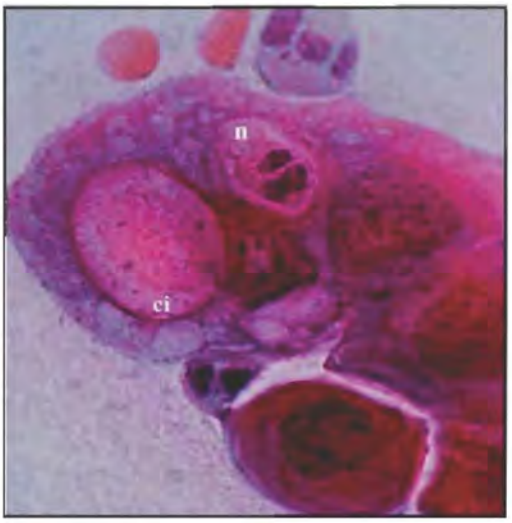


Figure 5.73 The constellation of changes included metaplastic cells with enlarged, oval nuclei, sometimes with wrinkled nuclear membranes, neutrophil leucocytosis sometimes within intraepithelial vacuoles (n), and characteristic Chlamydial inclusions (ci).

(6) characteristic Chlamydial inclusions, ranging from 1 μm to >15 μm in size with a round, distinct, well-defined enclosing membrane (Figure 5.73);

(7) only vacuoles demonstrably within the cytoplasm of viable metaplastic cells showing no evidence of karyorrhexis or karyolysis were accepted as Chlamydial;

(8) only vacuoles containing characteristic intra-vacuolar inclusions of three types were accepted as characteristic of Chlamydia:

(a) *targetoid* eosinophilic or basophilic

(b) *nebular* with numerous small round eosinophilic and/or basophilic coccoid bodies;

(c) *festooned* with large basophilic irregular inclusions;[36,37]

In many smears, dual pathologies were present. A confident diagnosis of Chlamydial infection was possible when sub-populations of cells were seen showing features of other pathologic processes. For example, the presence of some metaplastic squamous cells with coarse, granular hyperchromatic chromatin and irregular nuclear membranes characteristic of intra-epithelial neoplasia (CIN) did not prevent a diagnosis of Chlamydial infection. However, to diagnose Chlamydial infection with CIN, it was necessary to identify a sub-population of metaplastic squamous cells showing the blander nuclear changes of Chlamydial infection.

5.4.1.3 FEATURES SEPARATING CHLAMYDIAL CHANGES FROM HPV EFFECT & DYSPLASIA

In many cases, the morphologic changes due to HPV infection and intra-epithelial neoplasia (CIN) were present concomitantly with evidence of Chlamydial infection (Table 5.5). The atypical cells of HPV with or without CIN generally occurred in clusters separate from groups of cells showing Chlamydial changes. This allowed careful examination for the diagnostic features of both conditions. During the study, it became clear that Chlamydia-associated atypia was not always easy to interpret (Figure 5.75). On many occasions, Chlamydial atypia was labelled ASCUS. There was a tendency to "overcall" lower degrees of CIN when there was concomitant Chlamydial infection.

Using the published criteria for recognition of HPV infection[20,21] it was usually possible to separate changes due to Chlamydial infection from HPV effect. Again, the changes seen in CIN I, II or III[22-25] could usually be differentiated from the effects of Chlamydia (Figure 5.75). In a proportion of cases, cellular and nuclear features suggestive of Chlamydial infection were found, but the diagnostic vacuolar inclusions were not identified. In many of these cases, the cellular changes could be dismissed as "reactive" or "reparative" (Figure 5.75 B). In some cases the nuclear atypia was worrisome of possible intra-epithelial neoplasia and a diagnosis of ASCUS (Figure 5.75 F) was sometimes necessary. However, not all cases labelled ASCUS showed features suspicious of Chlamydial infection. Again, atypical metaplastic cells found with Chlamydia could often be confused with CIN III (Figure 5.75 F), especially if there was evidence of HPV infection with CIN I or II present elsewhere on the smear (Figure5.76).

The number of cases showing Chlamydia infection with HPV infection with or without CIN is tabulated (Table 5.5). Comparative figures in the control group without Chlamydial infection were also recorded (Table 5.6). The incidences

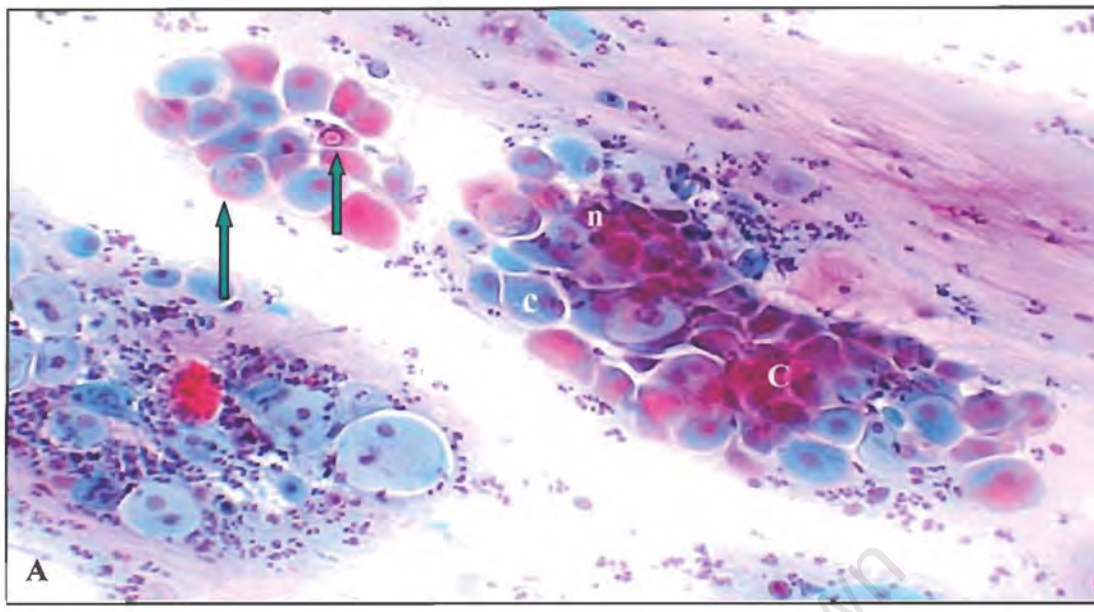


Figure 5.74. The constellation of changes was regarded as *absolutely necessary* for a confident diagnosis of Chlamydial infection. This included a mixed inflammatory infiltrate, acantholytic sheets of immature metaplastic squamous epithelium and characteristic Chlamydial inclusions (↑). Inclusions ranged from 1 μm to >15 μm in size, with a round, distinct, well-defined enclosing membrane and were present within the cytoplasm of viable metaplastic cells.

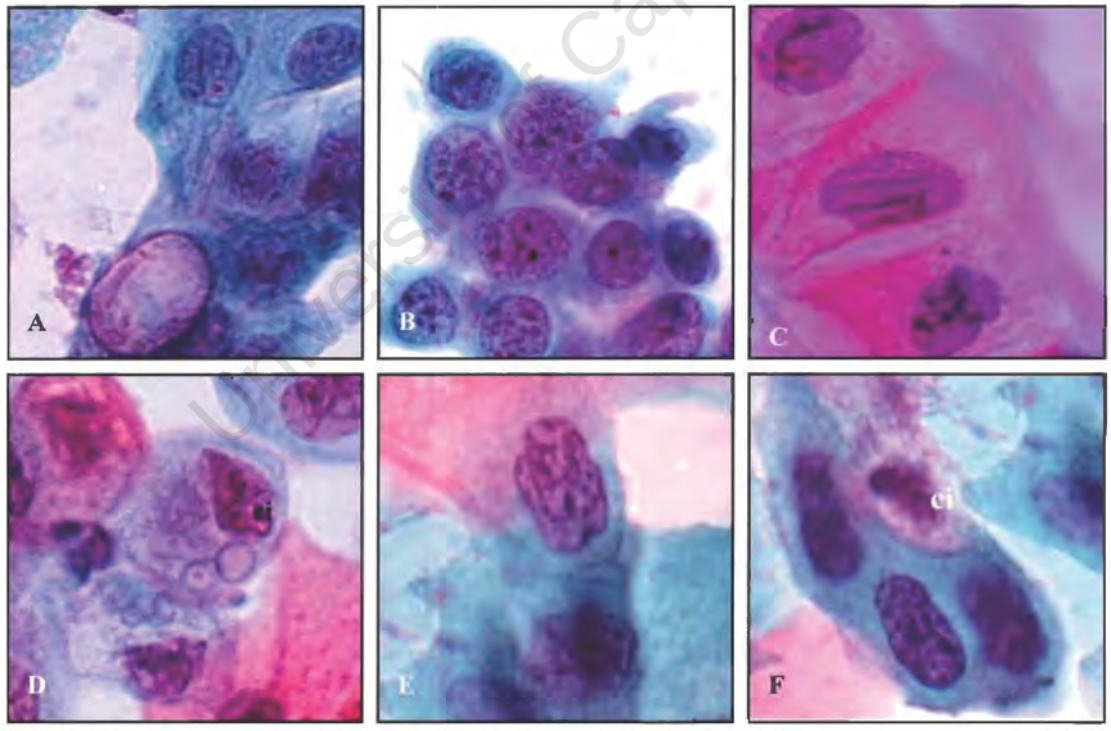


Figure 5.75. In many cases, Chlamydial changes (A) could be dismissed as "reactive" or "reparative" (B). Nuclear changes due to Chlamydiae included wrinkled nuclear membranes (C) and undulated nuclear membranes (D, E). However, in some cases (F), chromatin was hyperchromatic and coarse and the nuclear cytoplasmic ratio was increased (Chlamydial vacuole (ci) out of the plane of focus). In such cases a diagnosis of ASCUS was necessary, unless CIN was found elsewhere in the smear.

of CIN II & CIN III were similar in the Chlamydia-infected and control populations. However, in the Chlamydia infected population, the rates of ASCUS, HPV infection and CIN I were higher than those in the control group (Tables 5.5 & 5.6).

In the Chlamydia-infected population, there was a significantly higher incidence of HPV infection (106/1000) compared with the control population (58/1000) ($p = < 0.0005$, $X^2 = 16.83$). Similarly, comparison of ASCUS rates in the two populations showed a significantly higher rate in the group of patients infected with Chlamydia (53/1000) than in the control group (15/1000) ($P < 0.0005$, $X^2 = 16.628$). In the period 1994 – 1996, the rate of combined HPV/CIN disease in the Chlamydia infected group was 171/1000 while the rate in the control group 110/1000 ($p < 0.0005$; $X^2 = 15.938$). However, the difference in the incidence of CIN I in the group of patients with Chlamydia infection (56/1000) and the control patients (36/1000) was not found to be significant ($p < 0.0037$, $X^2 = 4.754$). Likewise, rates of CIN II and CIN III were similar in the two groups of patients. The rates of CIN III in the control population were comparable with rates in other studies on the South African population.[38,39]

Table 5.5
Incidence of HPV infection, CIN and ASCUS occurring in association with Chlamydial infection. The rate is reported as number of cases per 1000 smears.

Year	No cases Chlamydia	No of cases showing Chlamydia infection with associated HPV, CIN or ASCUS									
		HPV	Rate	CIN I	Rate	CIN II	Rate	CIN III	Rate	ASCUS	Rate
1993	73	14	192	10	137	1	14	0	-	11	151
1994	283	32	113	15	53	2	7	8	28	14	45
1995	447	41	92	14	31	1	2	11	25	17	38
1996	338	34	101	25	74	1	3	5	15	19	56
Sans'93	**1068**	107	**100**	54	**51**	4	**3.7**	24	**21**	51	**48**
Sans'93	**1068**	Combined HPV/CIN 107 + 54 + 4 + 24 = 189									**171**
Total	**1 141**	121	**106**	64	**56**	5	**4**	24	**21**	61	**53**

Table 5.6
Incidence of HPV infection, CIN and ASCUS occurring control patients without Chlamydial infection. The rate is reported as number of cases per 1000 smears.

Year	No cases	No of control cases with HPV, CIN or ASCUS									
		HPV	Rate	CIN I	Rate	CIN II	Rate	CIN III	Rate	ASCUS	Rate
1993	73	4	55	2	27	0	-	1	14	1	14
1994	283	12	42	6	21	1	4	3	11	5	17
1995	447	28	63	16	36	1	2	8	18	6	14
1996	338	22	65	11	33	1	3	9	27	5	15
Sans'93	**1068**	62	**58**	33	**31**	3	**3**	20	**19**	16	**15**
Sans'93	**1068**	Combined HPV/CIN 62 + 33 + 3 + 20 = 118									**110**
Total	**1 141**	66	**58**	35	**36**	3	**3**	21	**18**	17	**15**

The similar incidences of CIN I, CIN II and CIN III in the group showing Chlamydial infection and the control groups was a somewhat surprising finding. Several publications that have indicated there is an association between Chlamydial infection and CIN.[14,40,43-45] The present study corroborated this finding only for ASCUS, HPV infection alone and for all HPV/CIN disease combined.

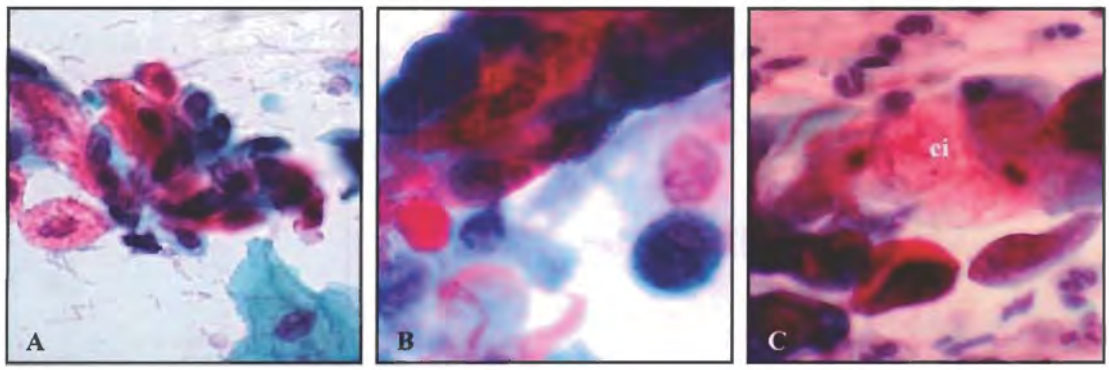


Figure 5.76 The changes seen in CIN I, II or III (A, B) could usually be differentiated from the effects of Chlamydia. However in a proportion of cases, Chlamydial vacuolar inclusions (ci) (out of focus, cell nuclei in focus) and inflammation were found, together with cellular changes probably indicative of intra-epithelial neoplasia (CIN).

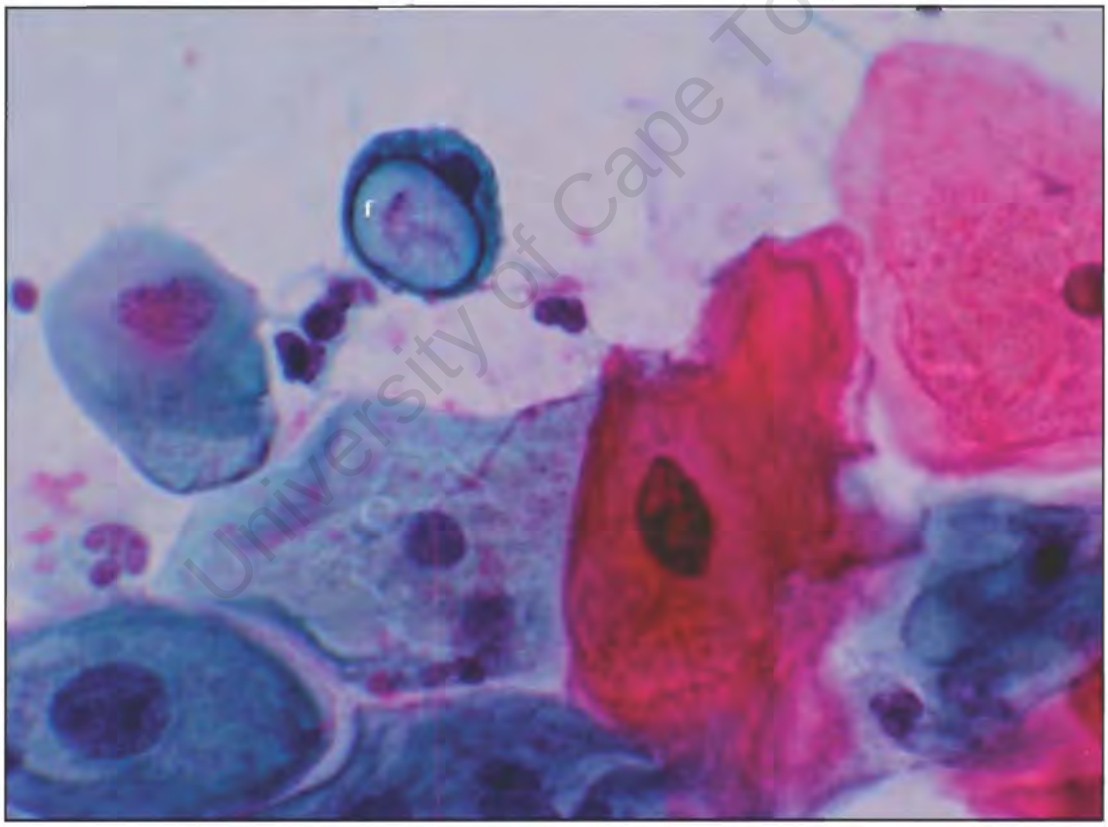


Figure 5.77. Sometimes, Chlamydial vacuolar inclusions could be recognised in degenerate cells, here a festooned inclusion (f). The host cell shows a small pyknotic nucleus displaced to the periphery of the cell by the large inclusion and shows characteristic intense cytoplasmic staining associated with cell degeneration. Attention to the morphology of the vacuole and its contents allowed distinction between Chlamydial vacuoles and degenerative vacuoles.

During the four years of the study, evidence of HPV infection without dysplasia was found in 121 patients with Chlamydia (10.6%). In the first year of the study, the proportion of cases reported as showing HPV infection (19.2%) was almost double that reported during the last year (10.1%). Although this difference was not statistically significant ($p = 0.0473$), there was a trend towards a lower reporting rate for HPV infection as the study progressed. The fall in the reported incidence of ASCUS in the Chlamydia-infected patients, from 15.1 % to 4.8 % over the last three years was significant ($p < 0.0005$). The reporting rate of CIN I fell from 13.7 % to 5.1 % over the last three years ($p = 0.0049$). Rates of CIN II and CIN III over the course of the study were statistically similar in the two groups of patients. These observations probably reflect a difference in the interpretation of the causes of mild to moderate atypia of nuclei early in the study compared with the last years of the study. The small number of cases found in 1993 was a limitation on the interpretation and statistical analysis of these figures. However, there appeared to be a "learning process" as the diagnostic criteria separating HPV effect from changes due to *Chlamydia trachomatis* were refined.

Cardinal features separating Chlamydial infection from HPV/CIN

As the study progressed, criteria were developed to separate HPV effects and CIN from Chlamydial changes (Table 5.7). The learning process leading to recognition of these cardinal difference between Chlamydial infection and HPV/CIN probably explains the lower rates of HPV infection, ACSUS and CIN I reported in 1995/6 compared with 1993. Although Chlamydial infection, HPV infection and CIN/SIL all showed enlarged nuclei, the severity of nuclear atypia in Chlamydial infection was milder than that seen in CIN (Figures 5.75, 5.76).

The differences between the features of HPV or CIN and Chlamydial infection are summarised as follows:

(1) the nuclear membranes in Chlamydial infection were only finely wrinkled or undulated and did not show sharply irregular, knobbly nuclear contours ;

(2) the nuclei within a group of cells showing Chlamydial changes did not vary in size as much as nuclei seen with HPV and CIN;

(3) the smudged chromatin seen with HPV infection was never found in cells showing Chlamydial infection;

(4) the chromatin of Chlamydia-infected cells was usually very mildly hyperchromatic, finely granular and evenly dispersed, never coarsely granular;

(5) darkly staining nuclei were almost never seen in viable cells showing Chlamydial vacuolar inclusions;

(6) degenerate cells occurring in the late stages of Chlamydial infection showed irregular, hyperchromatic pyknotic nuclei, often with karyorrhexis/lysis. These changes were regarded as degenerative and although part of the infective process, were not accepted as diagnostic of Chlamydial infection;

(7) an important clue to dual pathology was a Chlamydia-associated severe inflammatory infiltrate with epithelial leucocytosis. Although associated with invasive carcinoma, severe inflammation is not part of HPV infection or CIN;

(8) CIN III and Chlamydia infected cells were distinguished from one another by recognition of the presence of relatively abundant cytoplasm in Chlamydial infection (lower nuclear-cytoplasmic ratio).

Table 5.7
Comparison of the morphology of Chlamydial infection with HPV change, CIN and Repair. Singular and differentiating features highlighted red for Chlamydia, blue for HPV, green for CIN and pink for repair.

Feature	Chlamydia	HPV	CIN	Repair
Inflammatory changes				
Inflammation	+++	-	-	+++
Neutrophil leucocytosis into epithelial clusters	+++	-	-	-
Follicular lymphoid clusters	+	-	-	-
Cytoplasm				
Abundant cytoplasm	+++	++	-	+++
Prominent squamous metaplasia	+++	-	-	+++
Acantholysis	+++	-	-	+/
Vacuolar changes				
Cytoplasmic vacuoles with Chlamydial bodies	+++	-	-	-
Cytoplasmic vacuoles containing neutrophils	+++	-	-	-
Cytoplasmic vacuoles variable in size	+/-	-	-	++
Cytoplasmic vacuoles with fuzzy membranes	-	-	-	++
Cytoplasmic vacuoles in degenerate cells	+/-	-	-	++
Perinuclear halo/koilocyte condensed rim cytoplasm	-	+++	-	-
Nuclear features				
Enlarged nucleus	+++	+	+++	+++
Variation of nuclear size within cell group	+/-	+	+++	-
Fine nuclear membranes	++	-	-	++
Wrinkled nuclear membrane	++	-	-	-
Nucleolus small inconspicuous blue	++	-	-	-
Nucleolus prominent (sometimes red)	-	-	-	+++
Clearing of chromatin	-	-	-	+++
Karyorrhexis, karyolysis	++	-	-	+/-
Apoptosis	-	-	+++	-
Smudged chromatin	-	++	-	-
Hyperchromasia	+/-	+	+++	-
Coarse chromatin	-	+	+++	-
Crumpled nuclear contour	-	++	-	-
Irregular angular or nobly nuclear contours	+/-	++	+++	-
High N/C ratio	-	+	+++	-
Mitoses	-	-	+	-

5.4.1.4 FEATURES SEPARATING CHLAMYDIAL CHANGES FROM THOSE DUE TO OTHER INFECTIVE AGENTS

Cellular changes in Pap smears regarded as reactive and reparative were frequently associated with specific common pathogens found in Pap smears. When the well- described criteria for these pathogens[26] were strictly applied it was possible to identify Chlamydia infection separately from changes due infection by *Candida* species, *Neisseria gonorrhoea, Trichomonas vaginalis,* mixed bacterial infections, infection by anaerobic organisms associated with proliferation of *Gardnerella vaginalis* and *Herpes simplex virus* (HSV). Cases showing evidence of dual infection with Chlamydia and another infective agent were encountered (Table 5.8).

Table 5.8
Common pathogens found in Pap smears with and without evidence of Chlamydial infection.

Organism	*C trachomatis*		Controls		
	No	%	No	%	
Lactobacilli	209	18.3	701	61.0	p<0.0005
Actinomyces	19	1.6	13	1.1	
Candida spp	53	4.6	82	7.2	
G. vaginalis	71	6.2	103	9.0	
T. vaginalis	11	1.0	17	1.4	
N. gonorrhoea	0	-	2	0.2	
Bacterial vaginosis	47	4.0	87	7.6	
HSV	2	0.2	1	0.1	
No other organism	729	63.9	135	11.8	
Total	1141		1141		

No association between Chlamydial infection and occurrence of other pathogens was found. Patients showing Chlamydial infection were less likely to show normal vaginal flora (Lactobacilli) than the controls ($p < 0.0005$). The inflammatory infiltrates associated with these infections did not show epithelial neutrophil leucocytosis and follicular lymphoid clusters were infrequent.

5.4.1.5 Features separating Chlamydial changes from repair

Some cases showing both Chlamydial infection and repair were documented. Chlamydial infection and repair both showed prominent squamous metaplasia but could be differentiated from one another. Changes regarded as characteristic of "repair" in metaplastic cells were enlarged, hypo-chromatic, almost clear nuclei showing prominent, sometimes red nucleoli Figure 5.15, 5.75).[27] Acantholysis was not a prominent feature associated with repair. Cells showing clearing of nuclear chromatin and prominent large nucleoli characteristic of repair were almost never found to contain Chlamydial vacuoles (Figure 5.75) (Table 5.7). Infiltration by neutrophils into groups of metaplastic epithelium (neutrophil leucocytosis) was not usually found with repair. Although inflammatory cells were abundant in repair, they occurred in the background or were present overlying epithelial cell clusters.

5.4.1.6 Recognition of Non-Chlamydial vacuoles and pseudo-vacuoles

Degenerative vacuolar changes[26-28] associated with and due to the inflammatory process occurred in Pap smears from both the Chlamydia-infected and the control groups. The terms "degenerative vacuoles" and "inflammatory vacuoles" have been used inter-changeably by others.[26-28] As noted, to avoid confusion during the study, distinction between these entities was defined. Inflammatory vacuoles were defined as those containing neutrophils or neutrophil debris (Figures 5.22 & 5.73). Non-Chlamydial vacuoles occurring in an inflammatory background with evidence of cellular degeneration were regarded as degenerative vacuoles (Figure 5.25). Acceptance of this definition and nomenclature simplified the interpretation of vacuoles occurring in inflammatory smears and avoided inter-observer confusion in the laboratory.

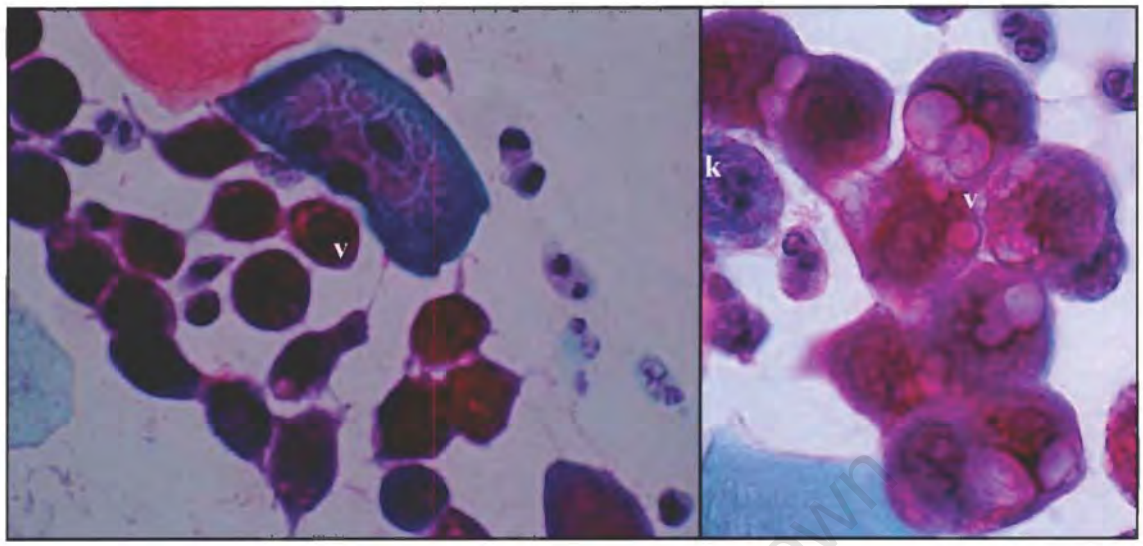


Figure 5.78. Degenerative vacuoles (v) were frequently accompanied by nuclear pyknosis, karyorrhexis (k), karyolysis or apoptosis. In addition, degenerative vacuoles were often multiple with large and small fuzzy vacuoles that appeared empty. Attention to the morphology of the host cell nucleus and the contents of the vacuole allowed distinction between Chlamydial vacuoles (see Figure 5.79) and degenerative vacuoles.

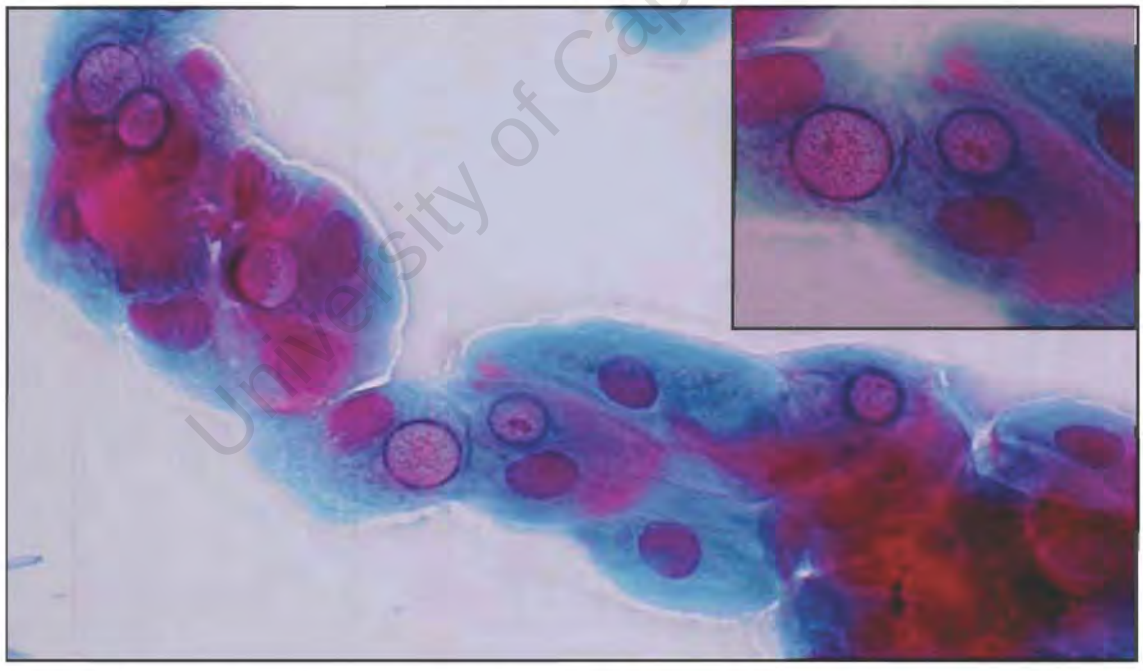


Figure 5.79. Viable metaplastic cells showing a variety of Chlamydial inclusions with crisp, well-defined surrounding plasma membranes. In contrast to degenerative vacuoles, Chlamydial vacuoles by definition always contained an inclusion, either a single targetoid or festooned inclusion, or numerous small nebular inclusions. Note the paucity of inflammatory cells in this case.

Separating Chlamydial vacuoles from inflammatory vacuoles

Neutrophil leucocytosis was almost always present in Pap smears showing convincing evidence of Chlamydial infection. Neutrophils could be seen in windows between immature spongiotic metaplastic cells, within intra-cellular vacuoles, or overlying the metaplastic cells (Figures 5.26; 5.32). Leucocytoclasis was usual in this setting and free-lying clusters of neutrophil nuclei or lobes of disintegrated nuclei were frequently found.

Although neutrophils were abundant in inflammatory smears showing other pathogens and when reparative changes were present, neutrophil leucocytosis was not. In non-Chlamydial inflammation, neutrophils were usually found in clusters or lying singly in the background and overlying epithelial cells (Figures 5.35 – 5.37). Epithelial leucocytosis with inflammatory vacuoles, where neutrophils had clearly infiltrated into and between the epithelial cells appeared to be specifically associated with Chlamydial infection.

Separating Chlamydial vacuoles from degenerative vacuoles

In the setting of Chlamydial cervicitis, it was unavoidable that degenerative vacuolar changes would be present (Figure 5.25). It was generally not difficult to separate Chlamydial vacuoles from degenerative vacuoles using the described morphologic criteria (Table 5.7 & 9). Sometimes, Chlamydial vacuolar inclusions could be recognised in degenerate cells (Figure 5.77). However, diagnosis of Chlamydial infection was never made with out finding typical inclusions in cells with viable nuclei. It was usually not difficult to find other inclusions in viable cells elsewhere in the same smear. This was regarded as crucial to avoid any possible confusion with degenerative vacuolar changes.

Degenerative vacuoles in the cytoplasm were frequently accompanied by nuclear pyknosis, karyorrhexis, karyolysis or apoptosis. In addition, attention to the morphology of the vacuole and its contents allowed distinction between Chlamydial vacuoles and degenerative vacuoles. Chlamydial vacuoles were usually round with a crisp well-defined surrounding plasma membrane. In contrast, degenerative vacuoles were frequently fuzzy (Figure 5.78). It was usual to find only one large Chlamydial vacuole within a cell. There were *usually* several degenerative vacuoles in a cell. In those cases where a cell did show several Chlamydial vacuoles, these tended to be smaller, of a similar size, each with a well-defined enclosing membrane (Figure 5.79). When more than one Chlamydial vacuole was present in a cell, the interface between the vacuoles was sometimes flattened. On the other hand, degenerative vacuoles were always of variable size and did not flatten one another. Degenerative vacuoles appeared empty while Chlamydial vacuoles by definition always contained an inclusion, either a single targetoid or festooned inclusion, or numerous small nebular inclusions (Table 5.9).

Separating Chlamydial vacuoles from apoptotic bodies

Apoptosis was not a frequently encountered phenomenon in association with Chlamydial infection. Apoptosis was usually found with CIN, particularly CIN III. Although in theory apoptotic inclusions in metaplastic dysplastic cells could have been confused with Chlamydial inclusions, recognition of the dysplastic process placed these peculiar inclusion vacuoles in context. Apoptotic inclusions were usually much smaller than Chlamydial inclusions and appeared denser. The complex nature of the apoptotic inclusions was characteristic. The remnants of condensed orangeophilic cytoplasm and densely

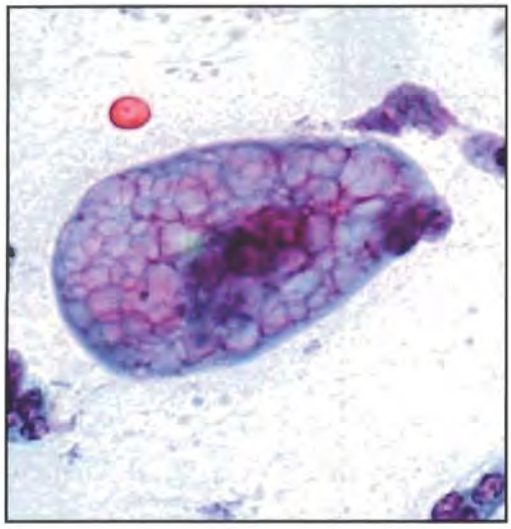


Figure 5.80. Numerous small ill-defined (fuzzy) lipid vacuoles almost invariably found in macrophages. Macrophage recognised by the presence of centrally placed, small round nucleus in a large cells with abundant pale cytoplasm.

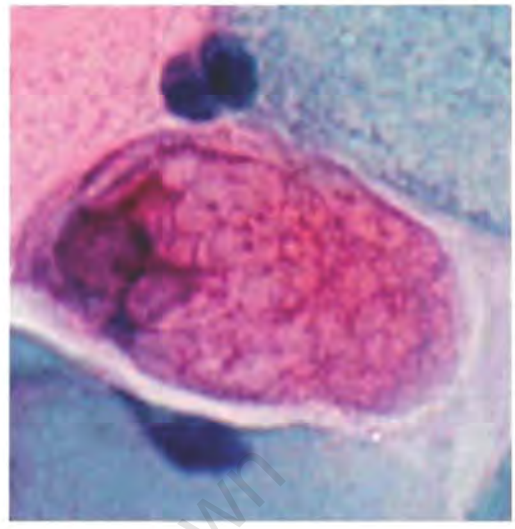


Figure 5.81. Epithelial cell with a peripherally placed nucleus shows finely granular, uniformly sized, coccoid bodies present in the 'rarified' areas of cytoplasm.

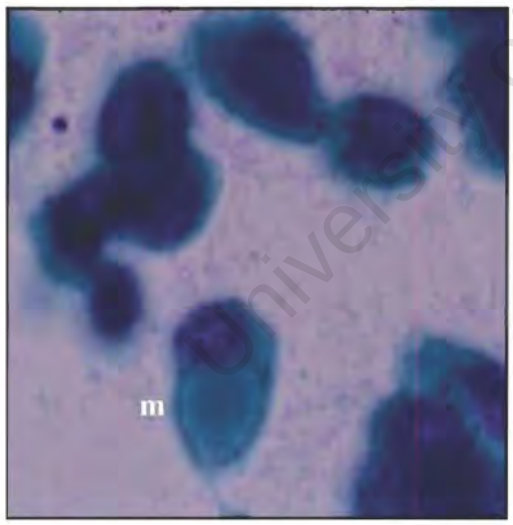


Figure 5.82. Mucinous inclusions in adeno-carcinoma cells [lobular carcinoma of breast] with malignant nuclear features (out of focus). Mucinous inclusions (m, in focus) showed a homogenous texture that was cyanophilic rather than basophilic with the Papanicolaou stain. Note the absence of a well defined vacuolar membrane and a clear peri-inclusional halo.

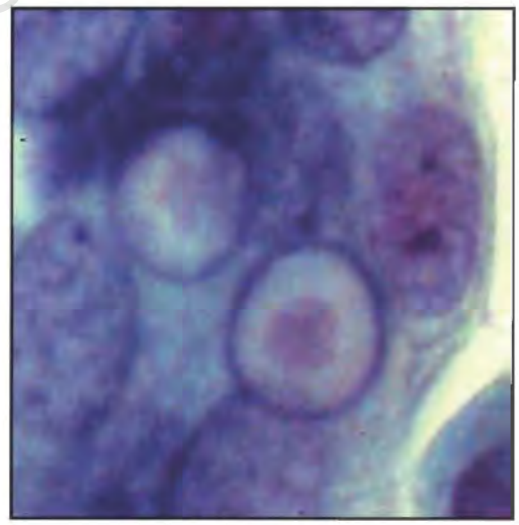


Figure 5.83. *Targetoid* basophilic inclusions in a well defined plasma membrane. Note the vacuolar membrane and the halo surrounding the inclusion body within the vacuole, which is not seen in Figure 5.82.

basophilic nuclear debris formed a distinct bi-coloured inclusion that virtually filled the intra-cellular vacuole (Figure 5.24; 5.70).

Table 5.9
Comparison of the morphology of Chlamydial, inflammatory , degenerative, apoptotic, mucinous and foreign material vacuoles.

Features of vacuole		Chlamydia	Inflamat	Degener	HPV & CIN III	Adeno ca	Foreign material	
							Lipid	Talc
Cell type	Mature squamous cell	+/–	+	+	+	–	–	–
	Metaplastic cells	+	+	+	–	–	+	+
	Mucinous cells	–	+/–	+	–	+	+/–	+
	Histiocytes	–	+	+	–	–	+	+
	Extra-cellular	–	–	–	–	–	–	+
Site	Peripheral cytoplasm	+	+	+	–	+	+	+
	Peri-nuclear	–	–	–	+	–	–	–
No.	Single	–	–	–	+	+	–	–
	Usually single	+	+	–	–	–	–	+
	Multiple	–		+	–	–	+	–
Size	Small only (1-3mμ)	–	–	–	–	–	+	–
	Intermediate (4 - 10mμ)	–	–	+	+	+	–	–
	Large (10 - 15mμ)	–	+	–	–	–	–	+
	Variable (1 - 20mμ)	+	–	–	–	–	–	–
Edge of vacuole	Well-defined	+	–	–	–	+	–	+
	Ill-defined/Fuzzy	–	–	–	+	–	+	–
	Either or both	–	+	+	–	–	–	–
Content of vacuole	Targetoid eosinophilic	+	–	–	–	–	–	–
	Targetoid basophilic	+	–	–	–	–	–	–
	Targetoid cyanophilic	–	–	–	–	+	–	–
	Nebular	+	–	–	–	–	–	–
	Festooned	+	–	–	–	–	–	–
	Neutrophils	+	+	–	–	–	–	–
	Complex (apoptotic)	–	–	+	+	+	–	–
	Refractile	–	–	–	–	–	–	+
	No content	–	–	+	–	–	+	–

Separating Chlamydial vacuoles from lipid vacuoles

The presence of lipid vacuoles seen in Pap smears did not appear to be a degenerative phenomenon due to fatty change in epithelial cells. Numerous small ill-defined (fuzzy) vacuoles regarded as lipid material was almost invariably found in macrophages. Macrophages were recognised by the presence of centrally placed, small round nuclei in large cells with abundant pale cytoplasm (Figures 5.33; 5.80). On occasion, free-lying globules of lipid material obscured the underlying cells. These changes were regarded as being due to the use of vaginal creams and there was little potential for confusion with Chlamydial infection.

Separating Chlamydial vacuoles from mucin-containing vacuoles

Intracellular vacuoles containing basophilic targetoid inclusions (Figures 5.10; 5.83) ascribed by Swanson[4] to Chlamydial infection bore striking resemblance to mucinous inclusions sometimes encountered in malignant effusions in

serous fluids.[29] This similarity was previously noted by *inter alia* Gupta *et al*,[1] and Shiina.[2] Mucinous inclusions in adenocarcinoma are found in cells with frankly malignant morphologic features, and usually show a homogenous texture that is cyanophilic rather than basophilic when stained with the Papanicolaou stain (Figure 5.82). Characteristically, a clear space was present within Chlamydial vacuoles, surrounding targetoid inclusions. Mucinous inclusions generally did not show a peri-inclusional halo (own observation). However, despite these subtle differences, using the Papanicolaou stain alone, there was no way to differentiate with absolute certainty between mucinous inclusions and targetoid Chlamydial inclusions. In cases of Chlamydial infection, it was usually not difficult to show the presence of other recogniseable forms of Chlamydial vacuole in the same smear.

During the study, a diagnosis of Chlamydial infection was only made if all of the cardinal features were present (*vide supra*). The diagnosis was never made on the presence of inclusions (targetoid basophilic or any other type) alone. Other morphologic cellular changes regarded as Chlamydial were always present. Further, almost always, other types of inclusion suggestive of *Chlamydiae*, (targetoid eosinophilic, nebular or festooned) were found in the same smear as basophilic targetoid inclusions. There were no cases of endometrial or endocervical adenocarcinoma included in the study group. Adenocarcinoma-associated "mucinous" inclusions could not have been a source of diagnostic confusion.

Separating Chlamydial vacuoles from talc pseudo-vacuoles

Talc granules were present in variable numbers in at least a third of Pap smears seen in the practice. The granules were of variable size, between 8 and 12 mμ in diameter with a well-defined refractile edge and frequently a refractile, central stellate fracture. The talc granules frequently overlay cells and on occasion, particularly at low power magnification, superficially resembled vacuolar inclusions in metaplastic cells. Close examination with fine focusing revealed the true nature of these granules (Figures 5.20; 5.70).

5.4.1.7 ASSOCIATION OF CHLAMYDIAL INFECTION WITH HORMONAL STATUS AND IUCD

The requisition form completed by the submitting physician seeking examination of a Pap smear included a request for information regarding use of contraceptives and the "hormonal status" of the patient. The information obtained (Table 5.10) indicated that significantly fewer patients with Chlamydial infection (11.6%) were using oral contraception (OC) compared with the control group (22.4%) ($p < 0.0005$). Fewer patients with Chlamydial infection were pregnant (1.2%) compared with the control group (5.2%) ($p < 0.0005$). On the other hand, there was no difference between the two groups in the number of patients who were *post-partum,* suggesting that during pregnancy, patients had been investigated and treated for sexually transmitted disease. More patients with Chlamydial infection were using intra-uterine contraceptive devices (IUCD), than patients without Chlamydial infection. However, the difference between the two groups was not significant. Slightly fewer women with Chlamydial infection were post-menopausal and using hormone replacement therapy (HRT) than in the control group (difference not significant).

Table 5.10
Association of contraceptive methods, hormone replacement and hormonal status in patients with and without evidence of Chlamydial infection.

Status	No	IUCD	OC	Pregnant	*Post- partum*	Post-menopausal	HRT
Chlamydia +	1141	79	164	14	31	89	67
Chlamydia –	1141	53	256	60	26	107	79
p value	–	–	< 0.0005	< 0.0005	–	–	–

5.4.1.8 RECOGNITION OF GLANDULAR CELL CHANGES

There were no cases of endocervical or endometrial adenocarcinoma found with evidence of co-existent Chlamydial infection. The number of cases of AGUS associated with features of Chlamydial infection with characteristic Chlamydial vacuoles varied, depending upon the threshold of the observer for atypical nuclear features in glandular cells. Concordant diagnosis of AGUS by the two screeners was poor. Strict application of published features defining AGUS[25] prevented over calling of AGUS. The diagnosis of AGUS was restricted to cases showing enlarged nuclei, 3 to 5 times the size of normal mucinous cell nuclei with nuclear hyperchromasia and a coarse chromatin pattern. In addition, diagnosis of AGUS required irregularities of nuclear size with variable nuclear contours within a group of mucinous cells. The groups of mucinous cells were required to show crowding and overlapping of nuclei with the presence of some oval or elongated nuclei. Using these criteria, only one patient received a diagnosis of AGUS (1996). This patient underwent colposcopy and endocervical curettage. Histology showed no significant glandular atypia.

5.4.2 TISSUE SECTIONS

5.4.2.1 INCIDENCE & DEMOGRAPHICS OF CHLAMYDIAL INFECTION

Incidence of Chlamydial infection found in tissue sections

During the study period 1993 to 1996, 2682 histology specimens of the uterine cervix were received in the practice (1037 hysterectomy specimens and 1645 cervical biopsies) (1). Evidence suggestive of infection by *Chlamydiae* was found in 257 (9.6 %) of these specimens (Table 5.12).

Table 5.11
Number and type of tissue specimens examined by year.

Year	Hysterectomy	LLETZ/Cone Bx	Colposcopic Bx	Polypectomy	Total
1993	225	59	291	8	583
1994	299	81	378	13	771
1995	229	58	291	16	594
1996	284	71	360	19	734
Total	**1037**	**269**	**1320**	**56**	**2682**

Table 5.12
Incidence of cases showing Chlamydial infection by specimen type

Specimen type	Number	Positive for *C. trachomatis*	Negative for *C. trachomatis*	Rate of Positives
Hysterectomy	1037	93	944	9.0 %
LLETZ/Cone bx	269	21	248	7.8 %
Colposcopic bx	1320	121	1199	9.2 %
Polypectomy	56	22	34	39.3 %
Total	**2682**	**257**	**2425**	**9.6 %**
Non-polypectomy	2626	235	2512	8.9 %

Over the four-year course of the study, the rate of reporting Chlamydial infection in tissue specimens increased marginally from 8.9 % in 1993 to 10.2 % in 1996 (Table 5.13).

Table 5.13
Number of specimens seen per year and the incidence of infection reported by year.

Year	Number	Positive for *C. trachomatis*	Negative for *C. trachomatis*	Rate of Positives
1993	583	52	531	8.9 %
1994	771	72	699	9.3 %
1995	594	58	536	9.8 %
1996	734	75	659	10.2 %
Total	2682	257	2425	9.6 %

The increased rate was not statistically significant. In comparison, the detection rate of Chlamydia in Pap smears showed a more marked increase in the detection rate, especially during the first two years of the study (the "learning curve"). The Pap smears were initially screened by one of the two cyto-technologists. All atypical smears were re-screened by a second technologist. During the study two technologists and the author examined all heavily inflamed smears with possible signs of Chlamydial infection. The learning curve noted in the cytology study may have been promoted by this screening policy. The number of cells on an average, well-made Pap smear is said range between 350 000 and 500 000 [Papnet computerized neural-net Pap smear screening system]. The screening of Pap smears has been compared to "searching for the proverbial "needle in a haystack" and is a more complex exercise than the examination of histologic sections. This also may account for the steadier rate of finding Chlamydial infection in tissue sections. The incidence of Chlamydial infection in the Pap smear screening process was approximately 3 % while about 10 % of tissue specimens showed the signs of the organism. On the other hand, the incidence of Chlamydial infection in Pap smears in the combined group showing HPV/CIN was approximately 17%. The reasons for taking a Pap smear are entirely different from the indications for hysterectomy, colposcopic biopsy, LLETZ/cone biopsy and endocervical polypectomy. The incidence of Chlamydial infection in these different specimens is therefore not un-expected. A group of 257 control

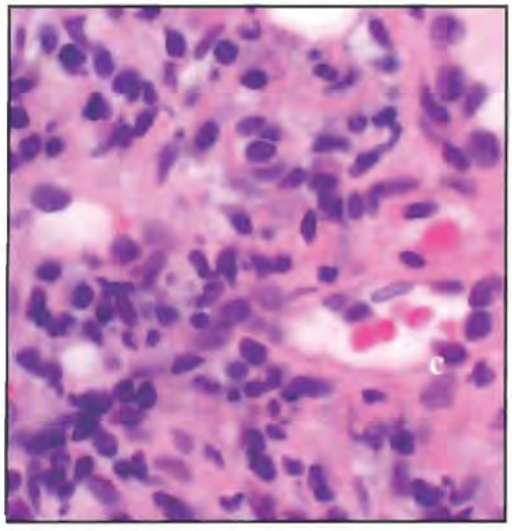


Figure 5.84. A mixed inflammatory infiltrate was usually, but not always present The inflammatory changes were associated with proliferation of small capillary vessels (c) in the sub-epithelial connective tissue.

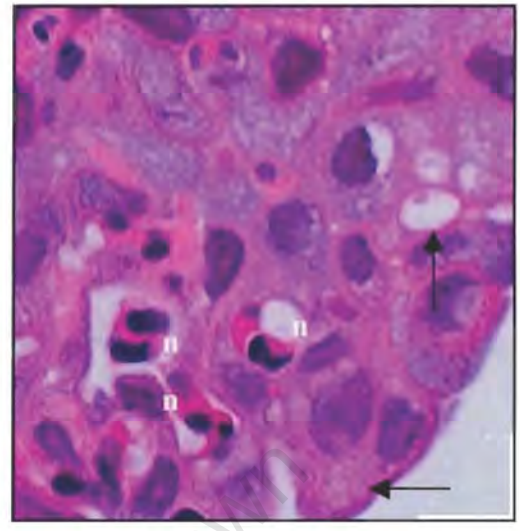


Figure 5.85. Inflammation usually included neutrophils (n), seen in sub-epithelial connective tissues and infiltrating the interstices between epithelial cells in spongiotic epithelium. Some were found within intraepithelial vacuoles. Note both small (←) and large (↑) targetoid Chlamydial inclusions.

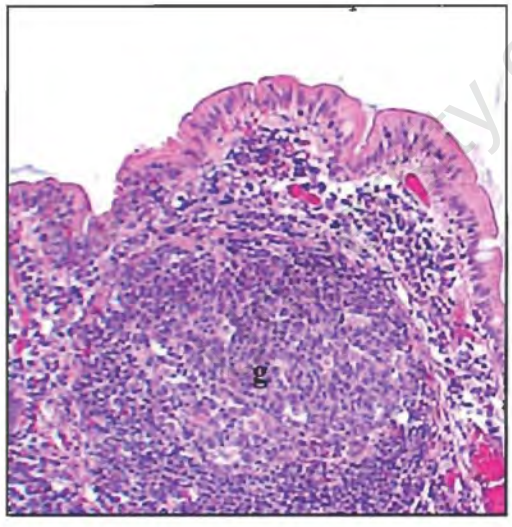


Figure 5.86. Follicular lymphoid aggregates that showed germinal centers (g) were present in some cases. Note the immature mucinous epithelial changes.

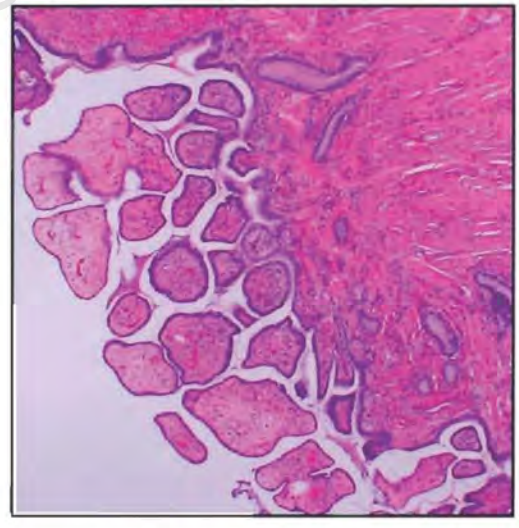


Figure 5.87 Sub-mucosal mature fibrosis was occasionally present indicating a very long-standing inflammatory process. In a large number of cases, changes of Chlamydial cervicitis occurred in a benign inflammatory fibro-epithelial polyp.

cases was selected from among the same specimens. The control for each positive case was selected by taking the first next specimen of similar type without evidence of Chlamydial endocervicitis.

Age distribution of patients with Chlamydial infection

The age distribution of patients in the group with features of Chlamydial infection was similar to that of the control group (Tables 5.14 & 5.15). The age distribution of patients with Chlamydial infection was wide, resembling the findings in Pap smears.

Table 5.14
Age distribution of patients found to have changes of Chlamydial infection.

Year	Age of patient in years								No cases Chlamydia
	< 21	21 - 30	31 - 40	41 - 50	51 - 60	61 - 70	> 70	Unknown	
1993	1	7	20	15	6	10	1	0	52
1994	0	12	34	16	4	2	0	4	72
1995	1	6	28	16	7	0	0	0	58
1996	2	9	36	21	5	1	0	1	75
Total	4	34	118	68	22	4	0	7	257

5.4.2.2 MORPHOLOGIC FEATURES DIAGNOSTIC OF CHLAMYDIAL INFECTION

The morphologic changes regarded as indicative of Chlamydial cervicitis comprised of a constellation of changes similar to those found in Pap smears.

Inflammatory infiltrate

An inflammatory infiltrate was usually, but not always present (Figures 5.84 – 5.86). The inflammatory changes were:

(1) of variable severity;

(2) were usually found in the region of the transition from ectocervix to endocervix;

(3) associated with proliferation of small capillary vessels in the sub-epithelial connective tissue (Figure 5.84);

(4) associated with a variable mixed inflammatory infiltrate usually including neutrophils, although the numbers varied. The neutrophils were found in both the sub-epithelial connective tissue and as intra-epithelial neutrophil leucocytosis. Neutrophils infiltrating the overlying metaplastic squamous epithelium were usually found in the interstices between the epithelial cells in spongiotic epithelium, but occasionally, neutrophils were found within intraepithelial vacuoles (Figure 5.85);

(5) the inflammatory infiltrate usually showed both lymphocytes and plasma cells and, in many cases, these cells predominated over the numbers of neutrophils. Follicular lymphoid aggregates that showed germinal centers were present in some cases (Figure 5.86);

(6) ulceration with granulation tissue was very rarely seen;

(7) sub-mucosal mature fibrosis was occasionally present suggesting a very long-standing inflammatory process (Figure 5.87);

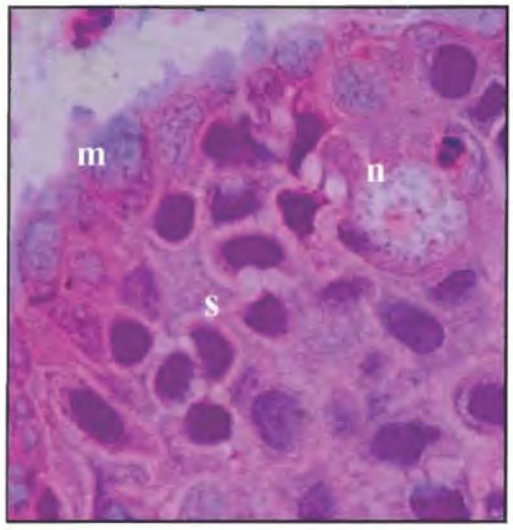


Figure 5.88. Immature squamous metaplasia was a cardinal change always present and invariably extensive. Note the mucinous cells (m) overlying the immature squamous cells (s). The metaplastic cells showed "reactive" nuclear changes characteristic of Chlamydial cervicitis. A *nebular* Chlamydial inclusion is present (n).

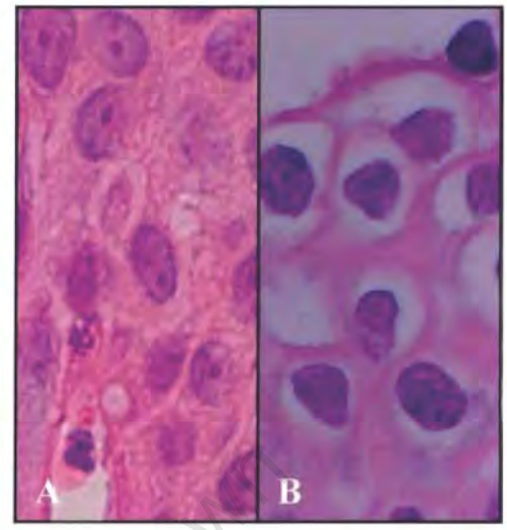


Figure 5.89. The enlarged nuclei in Chlamydial infection (A) were usually oval with fine nuclear membranes with smooth regular contours. Nucleoli were frequently found in Chlamydial infection. HPV infection (B) showed characteristic koilocytes with crumpled nuclear membranes and coarsely granular chromatin.

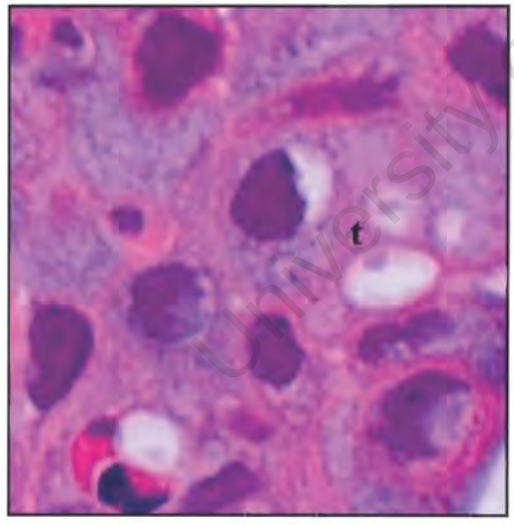


Figure 5.90. Some cases with features indicating Chlamydial infection showed marked nuclear atypia with irregularly shaped, hyperchromatic nuclei. Several *targetoid* inclusions are present (t).

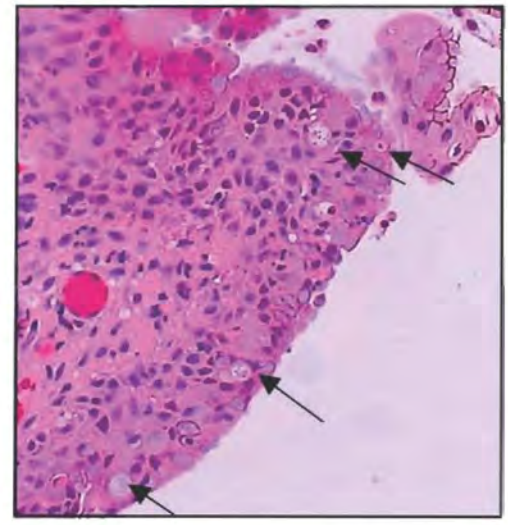


Figure 5.91. Vacuoles containing RB ranged from 1 μm to >15 μm in size.

(8) in a large number of cases, changes of Chlamydial cervicitis occurred in a benign inflammatory fibro-epithelial polyp (Figure 5.87), (Appendix C).

Table 5.15
Age distribution of control patients without evidence of Chlamydial infection.

Year	Age of patient in years								No cases
	< 21	21-30	31-40	41-50	51-60	61-70	> 70	Unknown	
1993	1	9	17	16	6	3	0	0	52
1994	2	11	31	21	4	1	1	1	72
1995	2	5	21	19	7	2	1	1	58
1996	2	12	31	21	5	3	0	1	75
Total	7	37	100	77	22	9	2	3	257

Characteristic cellular changes in immature metaplastic squamous epithelium

Immature squamous metaplasia was a cardinal change that was always present and was invariably extensive. The metaplastic cells showed recognisable "reactive" nuclear changes regarded as characteristic of Chlamydial cervicitis (Figure 5.88):

(1) the enlarged nuclei were usually oval in shape and showed fine nuclear membranes usually with smooth regular contours, whereas HPV infection characteristically shows crumpled nuclei with coarsely granular nuclear membranes (Figure 5.89);

(2) in well preserved specimens, cells showing Chlamydial infection showed fine wrinkling of the nuclei, with the wrinkles usually longitudinally arranged;

(3) generally, nuclei were not hyperchromatic and showed evenly dispersed, finely granular chromatin that was not clumped or coarse;

(4) small inconspicuous nucleoli were frequently found in Chlamydial infection and are not a feature of HPV infection (Figure 5.89);

(5) some cases with other features indicating Chlamydial infection did show marked nuclear atypia with irregularly shaped, hyperchromatic nuclei. These cases frequently but not always showed HPV infection elsewhere within the cervix (Figure 5.90).

Characteristic Chlamydial vacuolar inclusions

Chlamydial vacuolar inclusions were regarded as characteristic of *Chlamydia trachomatis* infection. Their presence was considered to be an essential feature (Figures 5.89 - 5.92) if the presence of a Chlamydial infection was to be reported. An intra-epithelial vacuole alone was insufficient: the presence in the vacuole of characteristic intra-vacuolar bodies Chlamydial bodies (RB) was required:

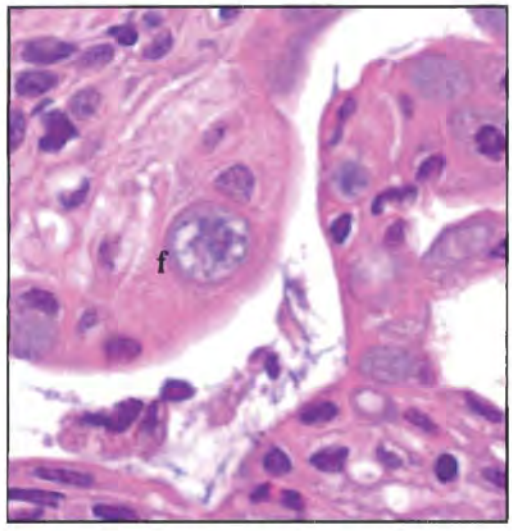


Figure 5.92. *Festooned* Chlamydial inclusion (f).

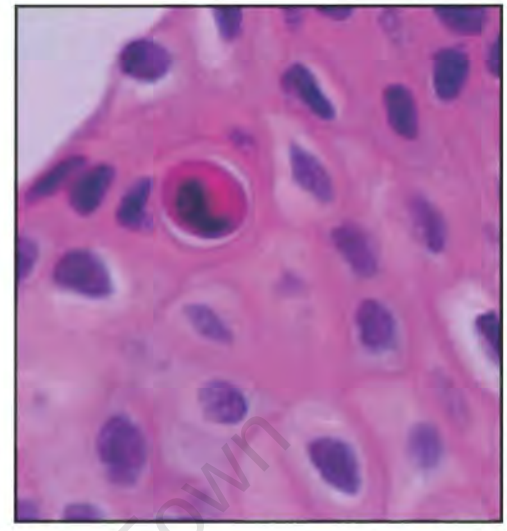


Figure 5.93. Vacuoles containing apoptotic bodies were carefully differentiated from true Chlamydial vacuolar RB.

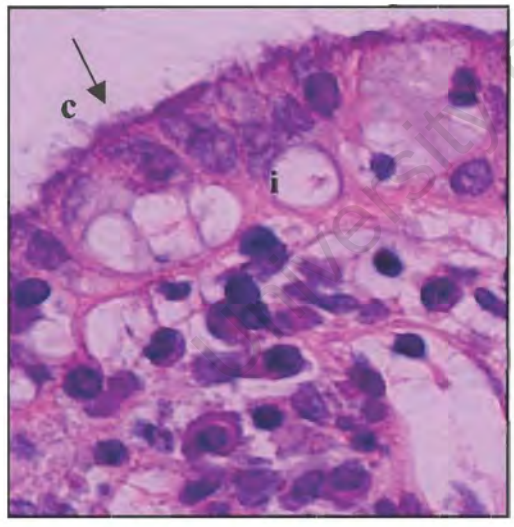


Figure 5.94. Mucinous endocervical epithelium showed variable reactive changes that were non-specific and not diagnostic of *Chlamydia trachomatis* infection. Occasionally, ciliated cell metaplasia was found (c). Mucinous cells showed enlarged, crowded nuclei, frequently with small nucleoli. Vacuolar inclusions (i) were not often found in mucinous cells.

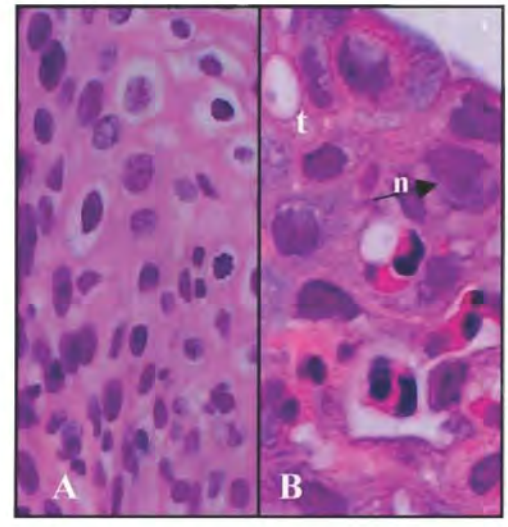


Figure 5.95. Separation of CIN I & II (A) from Chlamydial changes (B) was not usually difficult. An important clue to Chlamydial infection was the presence an inflammatory infiltrate and reactive nuclear changes with nucleoli (n) in immature metaplastic cells. A *targetoid* inclusion (t) is present.

(1) vacuoles containing RB ranged from 1 µm to >15 µm in size (Figure 5.91). They were demonstrably within the cytoplasm of the metaplastic cells and showed a round, distinct, well-defined enclosing membrane;

(2) most cells contained a single vacuole, but occasional more than one vacuole was found in a cell. In such circumstances, the adjacent vacuolar membranes were flattened (Figure 5.93);

(3) three types of intra-vacuolar inclusion were identified:

(i) *targetoid* eosinophilic or basophilic, (Figure 5. 90);

(ii) *nebular* with numerous small round coccoid bodies (Figure 5.89);

(iii) *festooned* with larger irregular festooned inclusions[36,37] (Figure 5. 92);

(5) vacuoles containing neutrophils (Figure 5.85) or apoptotic bodies were carefully differentiated from true Chlamydial vacuolar RB (Figure 5.93).

Changes in mucinous endocervical epithelium

Changes in mucinous endocervical epithelium were regarded as reactive. These changes were variable, appeared to be non-specific and were not found to be useful in the recognition of *Chlamydia trachomatis* infection. The mucinous endocervical epithelial cells showed mild enlargement and crowding of nuclei and frequently showed small nucleoli. Vacuolar inclusions were not often found in mucinous cells. Occasionally mucinous cells showed ciliated metaplasia (Figure 5.94).

5.4.2.3 Reactive changes in the endocervical transitional zone epithelium

Reactive and reparative changes were recognized in immature squamous metaplasia in the transformation zone of the endocervix.[25] These changes were sometimes associated with reserve-cell hyperplasia and micro-glandular hyperplasia. Such changes were frequently associated with a chronic inflammatory infiltrate.

As has been described previously,[25] mucinous cells were frequently found within immature squamous metaplastic epithelium lining the transitional zone of the endocervical canal. Usually these mucinous cells were arranged individually or in groups of two or three cells along the luminal surface of the metaplastic squamous cells (Figure 5.88), but they were also found deeper within the metaplastic epithelium (Figure 5.90). The cytoplasm of these mucinous cells appeared paler than that of the metaplastic squamous cells, and was usually finely vacuolated (Figure 5..88, 5.90). These mucinous endoeervical cells stained positively for neutral and weakly acidic mucin with the APAS-D stain. On superficial inspection, these cells could be confused for cells showing vacuolar inclusions. However, careful attention to morphologic detail at higher magnification allowed separation of mucinous cells from vacuolated metaplastic cells.

5.4.2.4 Changes due to other infectious organisms

Organisms responsible for cervical infectious causing morphologically distinct changes that may be found in histologic secrions include HPV, HSV and Chlamydia.[18] These organisms are obligate intra-epithelial infectors and exhibit a distinct cyto-pathologic effect. Very rarely, germ tubes of *Candida* species infiltrating cervical epithelium may be

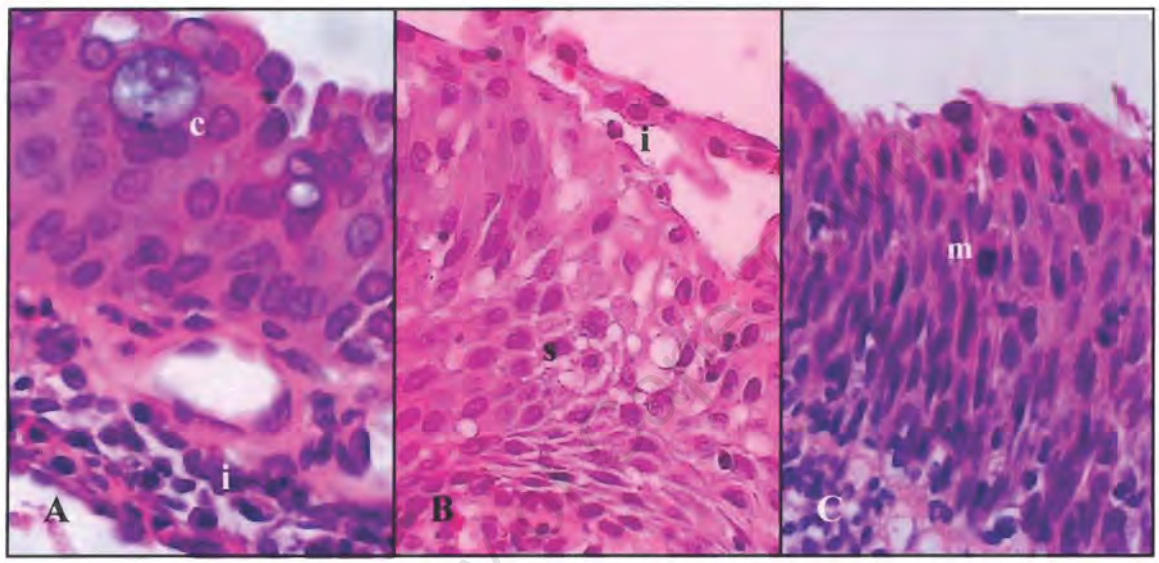


Figure 5.96 A & B. Chlamydial infection was associated with an inflammatory infiltrate (i) and spongiosis (s). Metaplastic squamous cells showed more cytoplasm than CIN III and nuclei tended to be round to oval while in CIN III (C) nuclei were more elongated. Nuclei did not show angular, irregular contours and did not vary markedly in size and shape. Immature metaplastic cells in Chlamydial infection showed nucleoli, a feature not present in CIN III. Chlamydial infection showed typical vacuolar inclusions (c).

Figure 5.96 C. The nuclear/cytoplasmic ratio of CIN III was much higher than for Chlamydial infection. Chromatin in Chlamydial infection was not as coarsely granular nor hyperchromatic as in CIN III. CIN III was associated with numerous mitoses (m), a feature not seen in Chlamydial infection.

demonstrated in tissue sections (personal observation). During the study, no cases of HSV or *Candida* were demonstrated histologically. HPV with Chlamydial infection was frequent (*vide infra* **5.4.2.5**).

Numerous tissue specimens showed inflammatory changes without histologically demonstrated cause. The causative organisms of many common cervical infections proliferate in exfoliated cells in the cervico-vaginal lumen.[18] Infections by *Trichomonas vaginalis*, organisms of *Candida species* and proliferation of *Gardnerella vaginalis* together with mixed anaerobes were potential causes of such "non-specific" inflammatory changes.[18] In addition, inflammatory changes may have been due to use of contraceptive creams, IUCDs, lubricants and cosmetic deodorants.

5.4.2.5 MORPHOLOGIC FEATURES SEPARATING CHLAMYDIAL CHANGES FROM HPV & DYSPLASIA

In a proportion of cases, evidence of HPV infection and "dysplastic changes" (CIN) was present together with features of Chlamydial infection. Using the published criteria for HPV infection[20-21] and for CIN,[22-25] it was possible to separate changes due to Chlamydia from those due to HPV/CIN (Figure 5.89). Further, the atypical cells of HPV and CIN generally occurred in anatomically different locations within the cervix. An important clue to Chlamydial infection was the presence of a severe inflammatory infiltrate.

Separation of Chlamydial changes from changes due to HPV infection, of CIN I, II (mild or moderate dysplasia) and the sub-type of CIN III previously known as severe dysplasia was not difficult. The diagnostic features of these lesions occur in mature squamous epithelium[21-25] while the changes of Chlamydial infection are present in immature metaplastic cells (Figure 5.95). However, the differential diagnosis of carcinoma *in situ* (CIN III) and Chlamydial infection was potentially more difficult. Differentiation between the two conditions was dependant on several features in the immature squamous (metaplastic) epithelium (Figure 5.96):

(1) while both lesions showed enlarged nuclei, Chlamydial infection showed relatively more cytoplasm than CIN III. The nuclear/cytoplasmic ratio of CIN III was much higher than for Chlamydial infection (Figure 5.96 C);

(2) nuclei in Chlamydial infection tended to be round to oval while in CIN III the nuclei were more elongated and sausage-shaped (Figure 5.96 C);

(3) the nuclear changes due to Chlamydia were almost always clearly not dysplastic. The nuclei did not show angular, severely irregular contours and did not vary markedly in size and shape (Figure 5.96 A,B);[23-25]

(4) the chromatin in Chlamydial infection was not as coarsely granular nor hyperchromatic as in CIN III;[23-25]

(5) immature metaplastic cells in Chlamydial infection showed nucleoli, a feature not present in CIN III;[23-25]

(6) Chlamydial infection showed typical vacuolar inclusions (Figure 5.96 A);

(7) CIN III was associated with apoptotic vacuoles and showed numerous mitoses, features not seen in Chlamydial infection (Figure 5.96 C).

The number of histology specimens showing features of Chlamydial infection with concurrent HPV infection with or without CIN was tabulated (Table 5.16). The rate of reporting concurrent Chlamydial infection and HPV infection with

or without CIN over the four years of the study varied between 46% and 81%. The incidence of HPV infection and CIN in controls was recorded (Table 5.17) and varied between 56% and 63%. Unlike the Pap smear study, the histology study did not show a trend toward reporting fewer cases of HPV or CIN as the study progressed. This suggests that it was less likely to confuse atypia due to Chlamydia with changes due to HPV infection or CIN. Interpretation of Pap smear morphology is more difficult and complex than it is for histology sections.

Table 5.16
Incidence of HPV infection and CIN occurring in association with Chlamydial infection.

Year	Number with *C. trachomatis*	HPV *s* CIN		CIN I		CIN II		CIN III		Total	Rate
		No.	Rate	No.	Rate	No.	Rate	No.	Rate		
1993	52	13	25.0	4	7.7	7	13.5	3	5.8	27	46.2
1994	72	24	33.3	9	12.5	12	16.7	2	2.8	47	65.2
1995	58	14	24.1	7	12.1	2	3.4	4	6.9	27	46.6
1996	75	31	41.3	17	22.7	5	6.7	8	10.7	61	81.4
Total	**257**	**82**	**31.9**	**37**	**14.4**	**26**	**10.1**	**17**	**6.6**	**162**	**63.0**

The overall incidence of HPV/CIN disease in specimens showing evidence of Chlamydial infection was 63.0% while in the control specimens the incidence was 59.5%. The slightly higher incidence in the specimens showing Chlamydial infection was not statistically significant.

Table 5.17
Incidence of HPV infection and CIN occurring in the selected controls (ie. without evidence of Chlamydial infection).

Year	Number	HPV *s* CIN		CIN I		CIN II		CIN III		Total	Rate
		No.	%	No.	%	No.	%	No.	%		%
1993	52	7	13.5	10	19.2	5	9.6	7	13.5	29	55.8
1994	72	10	13.8	14	19.5	8	11.1	11	15.3	43	59.7
1995	58	8	10.4	12	20.7	6	10.3	8	13.8	34	58.6
1996	75	11	14.6	13	17.4	10	13.4	13	17.4	47	62.7
Total	**257**	**36**	**14.0**	**49**	**19.1**	**29**	**11.3**	**39**	**15.2**	**153**	**59.5**

Table 5.18
Incidence in the different types of specimen of HPV infection and CIN occurring in association with Chlamydial infection.

Specimen type	No.	Chlamydia negative	Chlamydia positive						
			No.	HPV	CIN I	CIN II	CIN III	HPV/CIN	*Sans* HPV/CIN
Hysterectomy	1037	944	93	11	13	6	4	34	59
LLETZ/cone bx	269	248	21	1	3	8	7	19	2
Colposcopic bx	1320	1199	121	66	18	11	6	101	20
Polypectomy	56	34	22	4	3	1	0	8	14
Total	2682	2425	257	82	27	26	17	162	95

5.4.2.6 THE ASSOCIATION OF CHLAMYDIAL CHANGES WITH HPV & DYSPLASIA

There were 1 589 colposcopically directed biopsies or LLETZ/cone biopsies taken for the management of HPV/CIN. Only three of the 1 037 hysterectomy specimens were submitted for management of HPV/CIN disease (two cases with CIN I and one case with CIN II). Thus, useful comparison of the disease rates between hysterectomy specimens and LLETZ/colposcopic biopsy specimens was possible. The occurrence of CIN and HPV infection by specimen type were tabulated (Tables 5.18 & 5.19). The rates of Chlamydial infection with and without HPV infection and CIN in hysterectomy specimens were compared with specimens obtained at colposcopy (colposcopically directed biopsies and LLETZ/cone biopsies) (Table 5.20).

Table 5.19
Incidence in the different types of specimen of HPV infection and CIN occurring in specimens with out evidence of Chlamydial infection.

Specimen type	No.	Chlamy. positive	Chlamydia negative						
			No.	HPV	CIN I	CIN II	CIN III	HPV/CIN	*Sans* HPV/CIN
Hysterectomy	1037	93	944	29	19 (2)	11 (1)	0	59	885
LLETZ/cone bx	269	21	248	58	41	55	94	248	0
Colposcopic bx	1320	121	1199	209	150	207	339	905	294
Polypectomy	56	22	34	2	2	6	0	10	24
Total	2682	257	2425	299	212	279	433	1222	1203

CIN & HPV infection in patients with Chlamydial infection (Hysterectomies)

Amongst the 1 037 hysterectomy procedures performed during the study, two were done to treat CIN I and one to treat CIN II. None of these three cases showed evidence of Chlamydial infection (indicated in parentheses in Table 5.20). The three cases were not included in the statistical analysis. Evidence of Chlamydial infection was found in 93 hysterectomy specimens and four of these showed CIN III (4.4%). There were no cases of CIN III in the 941 (944 – 3) hysterectomy specimens without Chlamydial infection. The occurrence of the two cases showing both CIN III and Chlamydial infection was not a chance event ($p < 0.0005$, $X^2 = 31.155$). In respect of CIN II, there were six cases among 93 (6.7%) cases showing Chlamydial infection, while ten of 941 (1.1%) cases occurred without evidence of Chlamydial infection. The difference in these rates is significant ($p < 0.0005$, $X^2 = 40.237$). Similarly, thirteen cases of CIN I (13.9%) compared with seventeen cases in the non-Chlamydial group ($p < 0.0005$, $X^2 = 40.237$). Again, comparison of the eleven cases of HPV infection (11.8%) showing Chlamydial infection with the 29 cases without (3.1%) yielded a significant difference ($p < 0.0005$, $X^2 = 15.140$). Thus, in patients with Chlamydial infection, higher than expected incidences of HPV infection and all grades of CIN were demonstrated.

Table 5.20
The rates of Chlamydial infection with and without HPV infection and CIN in hysterectomy specimens were compared with specimens obtained at colposcopy (colposcopically directed biopsies and LLETZ/cone biopsies).

Specimen	No.	Chlamydia Positive						Chlamydia Negative						NIL
		#	*sans* HPV/CIN	*with* HPV	*with* CIN I	*with* CIN II	*with* CIN III	#	*sans* HPV/CIN	*with* HPV	*with* CIN I	*with* CIN II	*with* CIN III	
Hysterectomy	1037	93	59	11	13	6	4	944	885	29	17	10	0	885
Colposcopic	1589	142	22	67	21	19	13	1447	294	267	191	262	433	294
Total	2626	235	81	78	34	25	17	2391	1179	296	208	272	433	1179

Chlamydial infection in patients with HPV infection/CIN (colposcopic biopsies & LLETZ/cone biopsy specimens)
The number of colposcopically directed biopsies and LLETZ/cone biopsy specimens received during the study was 1 589. As expected, these specimens showed a high incidence of HPV infection (21.1%) and CIN disease (59.0%), since the purpose of these biopsy procedures was the management of these conditions. Among these specimens, 142 showed evidence of Chlamydial infection (8.9%). Of the 1 589 tissue specimens only 22 showed evidence of Chlamydial infection without evidence of HPV infection and/or CIN (1.4%). On the other hand 120 showed evidence HPV infection and/or CIN (7.6%) indicating a strong association between HPV/CIN disease and Chlamydial infection ($p < 0.0001$).

The rates of Chlamydial infection with CIN III, CIN II, CIN I and HPV infection alone were each compared with the rate of Chlamydial infection without evidence of HPV infection and/or CIN (1.4%). The association of CIN III and Chlamydial infection was not statistically significant. Amongst 446 patients with CIN III, there were 13 with evidence of Chlamydial infection (2.9%) ($p = 0.0508$). However, 6.7% of 281 patients with CIN II showed changes of Chlamydial infection, a significant association ($p < 0.0005$). Again in respect of CIN I, 9.9% of 212 patients showed a significant association with Chlamydial infection ($p < 0.0001$). The occurrence of HPV infection without CIN together with Chlamydial infection was 20.1% amongst 334 patients ($p < 0.0001$).

5.4.2.7 THE ASSOCIATION OF CHLAMYDIAL INFECTION WITH ENDOCERVICAL POLYPS

Of the 56 polypectomy specimens, 22 showed features of Chlamydial infection (39.3%) while 235 of the 2626 non-polypectomy specimens (8.9 %) showed these changes. Comparison of the rates of infection in these two groups shows a statistically significant difference ($p < 0.001$).

5.5 DISCUSSION

Changes ascribed to Chlamydial infection,[1-3] particularly the vacuolar inclusions, are regarded by many cytopathologists as non-specific, possibly degenerative or inflammatory changes. [*inter alia* 1,7,13,17] In a review of literature by Bernal *et al*,[3] meta-analysis of nine publications indicated that using criteria proposed by Gupta *et al*,[1] the sensitivity of Pap smear diagnosis of Chlamydial infection was 27% and the specificity was 79 %. Bernal *et al*[3] concluded that " . . . the examination of Papanicolaou-stained cervical smears can not be used at this time for diagnosing Chlamydial infections." Other authors regard the Pap smear diagnosis of Chlamydial infection as impossible. Geerling *et al*[43] state

that "Our results signify that the routine diagnosis of *C. trachomatis* is not only unreliable but that it is not possible. "

5.5.1 The Gold Standard

Most investigators who have concluded that Pap smear diagnosis of Chlamydial infection is unreliable have compared the morphology results with culture of the organism. Culture of the organism has been used as the gold standard for many years, but recently new techniques have shown that culture is less sensitive than previously realised.[7, 46-58] The possibility that cytology smears may have detected some cases with false-negative culture results was discussed by Dorman *et al.*[59] These workers recognised the high specificity of antigen detection using immuno-peroxidase staining (IP). A significant number (43%) of culture negative but morphologically positive Pap smears were shown by IP to be positive for Chlamydial antigen. Such a result could be interpreted in one of two ways. Either the culture results were false negatives, or the IP and Pap smear results were false positives. The high specificity of the IP where the antigen is located precisely in the appropriate location was emphasised by Black.[7] Dorman *et al*[59] accepted that the Pap smears had detected some false-negative cultures. Without stating it, these workers indicated that culture may not be the best possible gold standard. Silverman[60] also found that IP was a useful means to confirm the presence of Chlamydial infection in Pap smears.

The histologic study by Crum *et al*[10] using IP staining for Chlamydial MOMP found antigen in the cytoplasm and membranes of the metaplastic and mucinous cervical epithelium. Similar findings were made in Pap smears by Dorman *et al.*[59] Many of the vacuolar inclusions seen by Crum *et al* did not stain positively for MOMP. They regarded the inappropriate location of MOMP-staining as a serious problem militating against the identifying the inclusions as Chlamydial. However, as noted in the introductory review, MOMP is not expressed in the vacuolar inclusions during the vegetative phase of the life cycle of Chlamydia. The protein is synthesized by the host cell and is expressed in the host cell membrane.[8,9] These workers regarded their findings as anomalous, when in fact, the location of the antigen staining was entirely appropriate.

Most cytopathologists make use of histology as a gold standard for cytologic diagnoses on a routine basis. Therefore, the histologic study by Crum *et a,l*[10] and the electron microscopic report by Michael *et al*[17] probably had considerable impact on attitudes to Pap smear diagnosis of Chlamydial infection. The present morphologic study was undertaken to investigate the hypothesis that the Pap smear is a potentially excellent tool for the detection of Chlamydial infection and that it is being ignored because of inappropriate use of relatively insensitive technologies as the gold standard. Since histology is the first court of appeal in a questionable cytologic diagnosis, a complementary histologic investigation was deemed necessary. However, once the diagnostic morphologic criteria had been refined, the intention was to confirm them using an improved and expanded gold standard as recommended by the CDC. [7]

5.5.2 MORPHOLOGIC FINDINGS

Incidence

During the morphologic study, 48 958 smears were screened over a four year period and 1141 (2.33%) cases were regarded as showing Chlamydial-infection. Fewer cases were detected in the early part of the study (0.6% in 1993). As the study progressed, diagnostic criteria were refined, and "the eye" of the screeners was sensitised to the cardinal features found with the infection. This resulted in a higher rate of detection (3.2%) in 1996. The detection rate during the last two years was 3.1% and compared favourably with the rate reported (about 3%) in the general population in the US.[59] A recent study in Mexico showed the prevalence of Chlamydial infection in Pap smears was 3.2% of 125 patients attending an antenatal clinic.[61] Using ELISA and PCR testing, with confirmation by DFAT in uncertain cases, the same workers showed a prevalence of 2.4% of confirmed Chlamydial infection in these women. The incidence of Chlamydial infection in the South African population was not known. The histologic arm of the study examined 2682 histology specimens of the uterine cervix. Evidence suggestive of Chlamydial infection was found in 257 (10.43 %) of these specimens. This higher incidence in the histology specimens possibly reflects a selected population. Most of the hysterectomy specimens (1037) originated from patients with gynaecologic symptoms possibly related to Chlamydial infection (eg. menorrhagia or dysmenorrhoea). The pathology in specimens from colposcopy clinics (directed biopsies and LLETZ/cone biopsies) and in polypectomy specimens showed significant association with Chlamydial infection. These observations may explain the high rate of Chlamydial infection in the tissue specimens. It is possible that recognition and treatment of Chlamydial infections could reduce the rate of hysterectomies performed for symptoms that may be Chlamydia-associated.

Diagnostic features

A constellation of cardinal features useful to make a confident diagnosis of Chlamydial infection was defined during the study. As expected, the features characteristic of Chlamydial infection in pap smears and tissue sections were essential similar. The infection was associated with inflammatory changes, reactive epithelial changes and characterised by the presence of intra-epithelial vacuolar inclusions.

Inflammation

A characteristic feature was neutrophil leucocytosis with neutrophils infiltrating the interstices between acantholytic metaplastic cells and, sometimes within intraepithelial vacuoles. The association of Chlamydial infection with intra-vacuolar neutrophils was first noted in Pap smears by Henry *et al.*[17] In tissue sections, a similar infiltrate was seen and leucocytosis was also a feature. Capillary vascular proliferation was evident in the sub-epithelial connective tissues and sometimes margination of neutrophils in these vessels was noted. These features were regarded as indicators of active inflammation and could help differentiate true inflammation from so-called "normal" resident mucosa-associated inflammatory cell population in the genital tract. Occasionally follicular lymphoid aggregates with germinal centres were evident in smears and sections.

Several publications have characterised the inflammatory infiltrate found in Chlamydial infection. In particular, Kiviat *et al*[12,13,15] regarded the inflammatory changes of Chlamydial infection as characteristic. These workers reported that the presence in Pap smears of histiocytes and transformed lymphocytes was the most useful predictive feature of Chlamydial infection. Paavonen *et al*[6,12,16,40] also regarded inflammatory changes as the most useful feature in the diagnosis of Chlamydial infection. They regarded the presence of follicular lymphoid infiltrates in Pap smears and in histology sections as the most useful feature of Chlamydial infection. Paavonen has worked in both of these groups and the findings of the groups are complimentary. Transformed lymphocytes generally originate from a germinal centre in follicular lymphoid tissue.

In this morphologic study, lymphoid cells were almost always seen in the cases regarded as showing Chlamydia infection. There was no attempt made to study the phenotype of these cells using lymphoid markers. Application of immuno-cytochemistry to Pap smears could have been problematic. Firstly, alcohol fixation routinely used in Pap smear cytology requires refined laboratory procedures for successful immunoperoxidase staining. Secondly, almost always, only one Pap smear was available form each patient. A panel of lymphoid markers could not be performed easily on one smear. However, with patient and careful technique, smears can be divided into three, possibly four areas for different antibody markers. Protocols for such work could be implemented relatively easily in a good immuno-cytochemistry laboratory. On the other hand, studies of lymphoid phenotype could more easily be performed on serial sections in histology cases. Further, commercially available immunoperoxidase reagents and automated techniques are usually optimised for use in paraffin-embedded, formalin-fixed specimens. Such work may be valuable and could be performed on selected cases in the future.

Epithelial changes

The reactive epithelial changes were striking in both Pap smears and tissue sections and were usually the first clue that Chlamydial infection was present. This observation has not received emphasis in previous descriptions of the morphology of Chlamydial infection. The immature metaplastic squamous epithelium occurred as sheets or groups on Pap smears, and was found in the transition zone in tissue sections. The metaplastic cells showed reactive changes including nuclear atypia that could be severe. In cytology smears, it was possible, although sometimes difficult, to separate these nuclear changes from HPV effect or intra-epithelial neoplasia. Interpretation of these changes in histology sections was less difficult, although on occasion immature squamous metaplasia with evidence of Chlamydial infection could be confused with CIN III.

In many cases, both Chlamydial infection and HPV infection/CIN were present. It was usually possible to differentiate between the two conditions and recognise the cases in which both Chlamydia and HPV were present. In Pap smears, cells showing features of Chlamydia were usually separated in space on the smear from cells showing evidence of HPV/CIN. In histology sections, the two conditions were usually adjacent to one another, but in separate locations. On occasion in both smears and sections, it was not possible to be certain whether both or only one of the conditions existed.

The atypical immature metaplastic cells associated with Chlamydial infection may well contribute to an increased rate of ASCUS and intra-epithelial lesions in the cytology laboratory. Recognition of the morphology of Chlamydial infections in the cytology laboratory could offer an opportunity to significantly reduce in the incidence of ASCUS. This was demonstrated by the fall in the numbers reported cases of both ASCUS and CIN I as the study progressed. Further, even in tissue sections, an unwary pathologist may on occasion make a mistaken diagnosis of intra-epithelial neoplasia when the presence of immature metaplastic epithelium due to Chlamydial infection is confused with CIN III (own observation).

Clues to recognise the non-neoplastic nature of Chlamydial changes were identified. In both Pap smears and tissue sections, the metaplastic epithelium often showed inter-cellular oedema (spongiosis) and sometimes acantholysis with cells separated by oedema fluid between the cells. The severity of the spongiosis varied. Sometimes on Pap smears, the metaplastic cells were sometimes still attached to one another by "inter-cellular prickles" (desmosomes) or showed long cytoplasmic extensions that had been drawn out during the smearing process due to the desmosomes adherent to adjacent cells. On occasions the acantholytic cells were entirely separated, resembled cornflakes on a plate.

Criteria to separate atypia of nuclei due to Chlamydial infection from HPV atypia were formulated. In smears, the metaplastic cells seen in Chlamydial infection showed enlarged, usually oval nuclei, with well-defined, fine nuclear membranes, sometimes with longitudinally arranged wrinkles. The cells showed evenly dispersed and finely granular chromatin with a mildly hyperchromatic appearance and infrequently, small inconspicuous nucleoli. Although similar changes were seen in well-preserved tissue sections, frequently fixation was not adequate to discern such fine detail. Tissues were generally better preserved in small biopsy specimens, LLETZ specimens and curettings. Despite the relatively poorer preservation of nuclear detail in tissue sections, Chlamydial infection showed metaplastic cells with relatively abundant, frequently eosinophilic cytoplasm and normo-chromic nuclei. In comparison, CIN III showed darker more irregular nuclei with less cytoplasm. Careful attention to identifying these features assisted in reaching a correct diagnosis.

Chlamydial vacuolar inclusions

The characteristic Chlamydial inclusions ranged from 1 μm to >15 μm in size. The inclusions in both histology and cytology preparations showed a round, distinct, well-defined enclosing membrane and characteristic intra-vacuolar inclusions. The detail of these inclusions was almost always better seen in Pap smears. The entire inclusion was present in the intact cells and could be visualized by careful up-and-down fine focusing. In thin tissue sections (5mμ), the slice through the cells and the intra-epithelial vacuoles often only revealed scanty intra-vacuolar content. On occasion thicker sections (8mμ), showed more detail of the intra-vacuolar inclusions.

In both histology and cytology specimens, three types of intra-vacuolar inclusions were seen. This classification consolidated the five types described by Shiina[2] into two categories. This simplified division of inclusions collected "granular" and "homogenous central target formations" described by Shiina into *targetoid inclusions* (either *eosinophilic*

or *basophilic*). The vacuoles that contained numerous small round coccoid bodies were designated nebular and corresponded to the "eosinophilic" and "haematoxylinophilic" inclusions as well as the "nebular" inclusions described by Shiina.[2] The inclusions containing small coccoid bodies were not separated into fine granular and coarse granular inclusions as Shiina had done. Lastly, the *festooned* inclusions with large irregular basophilic threads had not been previously described. These were shown to be Chlamydial.[36,37]

Only vacuoles that were demonstrably within the cytoplasm of viable metaplastic cells that showed no evidence of karyorrhexis or karyolysis were accepted as Chlamydial. Before a diagnosis of Chlamydial infection could be rendered, the presence of at least one of these inclusions was regarded as essential. Usually several inclusions were demonstrated in a smear or section and very seldom was only one type of inclusion present. The reason for the variety of appearances remains conjectural.

5.5.3 THE CONTROVERSY REGARDING CHLAMYDIAL INCLUSIONS

Opinion regarding the nature of vacuolar inclusions seen in Pap smears showing Chlamydial infection varies. Some of the vacuolar inclusions are recognised as Chlamydial.[1-4,7-9,17,19,26,36,37,45,59-64] The variety of morphologies probably reflects different phases in the life cycle of the Chlamydial organism.[1-4,8,9,19,36] Others have expressed the opinion that many of the vacuoles contain mucinous inclusions,[1-3,10,17] or reflect degenerative or inflammatory changes,[1,17,26] or contain bacterial debris.[41,50] It is noted that in the textbook by Koss[26] (pp322, Figure 10-10A) the photomicrograph illustrating reactive mucinous cells in cervicitis shows a nebular type (Shiina)[2] Chlamydial vacuolar inclusion.

Mucinous inclusions

Gupta *et al*[1] warned that targetoid intra-vacuolar inclusions bore a close resemblance to mucinous inclusions described to sometimes occur in adenocarcinomas by Spriggs *et al*.[29] These workers offered the first cytologic description of mucinous vacuoles. The vacuoles were found in metastatic breast carcinoma cells present in peritoneal and pleural serous fluids.[29] The "vacuoles" contained mucinous material that stained with alcian blue and PAS with prior ptyalin digestion. With the Papanicolaou stain the inclusions were *eosinophilic*. Using EM, the "vacuoles" were found to be intra-cellular lumina lined by microvilli. Some of the intra-cellular lumina contained a central mass of electron dense material presumed to correspond to the mucin stained by PAS. Similar features had previously been described in an ultrastructural investigation of primary carcinoma of the breast,[65] and are also a useful diagnostic feature in malignant mesothelioma.[66] Intra-cellular lumina occur in various adenocarcinomas but have not been described in non-neoplastic proliferations.[67]

Gupta *et al*[1] investigated the possible "mucinous" nature of vacuoles seen Pap smears with Chlamydial infection without reaching a definitive conclusion. Variable numbers of the targetoid vacuoles were shown to be diastase-resistant PAS positive (PAS +) and mucicarmine positive. The results were considered equivocal because the results were variable. In Shiina,[2] a high proportion of the non-nebular vacuolar inclusions (between 67% and 82 %) were PAS + but <2% of nebular inclusions were PAS +. Shiina considered many non-nebular inclusions to be "mucinous inclusions". Both Gupta *et al* and Shiina indicated that diastase pre-treatment of the smears prevented detection of glycogen produced by

the organism. However, the PAS-diastase method does also stain complex carbohydrates including lipopolysaccharides (LPS) that are not susceptible to diastase digestion. Chlamydial inclusions containing reticular bodies contain LPS[8,9] and could be PAS + for this reason. More sophisticated histochemical characterisation of the content of targetoid inclusions in Chlamydial infection is required.

The investigation of Chlamydial vacuolar inclusions by Gupta *et al*[1] included IF staining. These authors noted that Chlamydial antigen was found by IF in dense intracytoplasmic inclusions bodies within large vacuoles (targetoid inclusions). Shiina[2] used a monoclonal mouse antibody to identify species-specific Chlamydial antigen (Ortho Diagnostic Systems). Although the target antigen was not stated in the paper, this antibody could only have been an anti-MOMP antibody. The species-specific sub-typing of the 15 sero-types *Chlamydiae* is determined by variations in the 40 KD MOMP protein.[68] A review in 1997 of product monographs from eight suppliers [Biodesign International (USA); Biogenesis (UK); BioGenix California; Capricorn Products Inc (USA); Chemicon International Inc. (USA); QED Bioscience Inc. (USA); Virostat (USA); Ortho Diagnostics (USA)] revealed that commercially available species-specific antibodies that were suitable for testing fixed tissue were all directed at MOMP. This being the case, the antibody used by Shiina would have limited his search to detection of elemental bodies (EB) and their immediate precursors.[8,9] The detection system used in his study excluded the early vegetative forms of Chlamydia (RB) likely to be present in many of the vacuoles seen in metaplastic cells. Shiina's conclusion that only so called nebular inclusions were diagnostic of Chlamydial infection was therefore not valid. Detection of LPS, the dominant antigenic determinant of early vegetative forms of Chlamydia,[8,9] requires detection systems that use immunofluorescence labeling and fresh-frozen tissue.

The EM appearance of large perinuclear inclusion bodies was briefly described by Gupta *et al*[1] as containing degenerative-type myelin bodies. Using EM, Henry *et al*[17] were able to find Chlamydial organisms (elementary and reticular bodies) in only one of six "Type III" (non-targetoid nebular-type) inclusions. This correlated with finding MOMP by the *Chlamydiazyme* (ELISA) test in one of the six patients with nebular inclusions. It is not clear whether the *Chlamydiazyme*-positive case was the same case showing elementary and reticular bodies. The targetoid inclusions investigated by Henry *et al* were shown by EM to contain amorphous material and showed no Chlamydial organisms, yet three of these five were *Chlamydiazyme*-positive. In their summary, Henry *et al* noted that EM "revealed Chlamydial organisms to be present in only nebular inclusions", thereby promoting acceptance of the nebular inclusion as the only diagnostically acceptable marker of Chlamydial infection. A more accurate conclusion may have been that since organisms were found in only one of 11 patients, results of the EM study were equivocal.

Swanson[4] examined the ultrastructure of Chlamydial inclusions from cell cultures and compared these controls with biopsy material from two cases of endocervical Chlamydial infection. He found the three typical forms of Chlamydial organism in electron micrographs from both the controls and the patients. The electron micrograph in figure 2 of this publication (Journal of Infectious Diseases, 1975, **13**: 682) shows an intraepithelial vacuole larger than the cell nucleus (perhaps 12 – 15 mμ in diameter). In the centre of the vacuole a large amorphous mass of intermediate electron density is present with numerous elementary, reticular and intermediate forms arranged toward the periphery of the vacuole. This vacuole with Chlamydial inclusions resembles the electron micrograph of a targetoid inclusion shown by Henry *et*

al[17] (Acta Cytologica, 1993, 37: 347). On examination of such a vacuole by light microscopy, it is likely that the dominant feature would be the large central targetoid amorphous mass. Swanson *et al* showed such an inclusion to contain *Chlamydiae* while Henry *et al* did not.

Intra-cellular mucinous vacuoles have been described in malignant glandular cells.[29] In the present study, there were no cases of cervical adenocarcinoma among the Pap smears and histology cases, nor among cases assigned to either of the control groups. It was therefore not possible for mucinous vacuoles due to endocervical adenocarcinoma to be confused with Chlamydial infections. Mucinous vacuoles occurring in adenocarcinomas, for example in the breast and stomach, (Figures 5.21; 5.70) were frequently encountered in both cytology and histology specimens submitted to the practice during the study period. In cytology smears, the Papanicolaou-stained adenocarcinoma cells showed mucinous vacuoles, in which the mucin inclusions were found to be cyanophilic rather than basophilic (Table 5.9). The cyan colour in Papanicolaou staining is distinctive and although the difference is subtle, the colour is different to haematoxylinophilic (basophilic) staining (Figure 5.21).

Papanicolaou developed his stain progressively over years of experimentation.[27] The empirical "design" of the stain and several published variations to achieve different effects has resulted in a wide range of tinctoral appearances of different cell types and other components part of the of the gynaecologic Pap smear. [27] Thus, keratinised cells are orangeophilic (Orange G) while intermediate and immature squamous cells are an aquamarine blue. Nuclei are dark blue/black, nucleoli red and chromocentres blue. Glycogen stains yellow (Orange G) and mucous streaks show a delicate malachite green appearance. Mucinous cells show grey slightly basophilic granular cytoplasm unless Eosin Y is used, when the mucinous content of cells is pink. (Figure 5.27). For this reason, the particular variation of the Papanicolaou stain used during the study made use of an EA solution containing Eosin Y. Without the necessity for special staining, this method allowed clear distinction of endocervical mucinous cells from non-mucinous metaplastic squamous cells that frequently occur together in a single cluster, [27] (Figure 5.31).

The mucinous vacuoles in malignant breast cancer cells stained using this staining method were cyanophilic not eosinophilic nor basophilic, presumably because the mucins in these two cell types are chemically different. However, without specific investigation of the chemistry of the mucin-types stained by Eosin Y, it seems possible that eosinophilic targetoid inclusions may have contained endocervical mucin. Further, it is not possible to totally exclude the possibility that mucinous vacuoles and Chlamydial basophilic targetoid vacuoles could be reliably differentiated from on another on the basis of colour alone.

The EM investigation by Henry *et al*[17] showed that thick-walled intracellular "vacuoles" with coarse granular inclusions showed microvilli without Chlamydial organisms. These intracellular lumina would appear to conform to the features of "mucinous" vacuoles described by Spriggs *et al*.[29] Thus, Henry *et al*[17] have demonstrated that some of the vacuoles examined were indeed mucinous, indicating that these vacuoles are not confined to neoplastic proliferations. However, the light microscopic appearance of the mucinous inclusions found by Henry *et al* was not targetoid. The proven existence of intra-cellular lumina/mucinous inclusions by EM reveals that these are the thick-walled coarsely granular type of inclusion reported by Shiina.

Although it was never properly proven, the suggestion first made by Gupta *et al*[1] and supported by Shiina,[2] that targetoid inclusions may be mucin vacuoles, the notion has found widely acceptance as a fact. The assumption that the targetoid vacuolar inclusions were mucinous inclusions was readily used to explain the failure to show Chlamydial antigen by IP or DFAT in a proportion of cases.[2,3,42] Other workers have speculated that the vacuoles contained cellular, neutrophil or bacterial debris.[17,42,43]

Reactive & degenerative Vacuoles

Gupta *et al* [1] also noted that Chlamydial vacuoles may resemble those found in Pap smears showing "reactive, reparative or degenerative changes." This idea was supported by other subsequent publications. [17,26,27,43] Accordingly, during the present study, the vacuolar changes ascribed to Chlamydia had to be characterised and separated from similar non-specific "reactive or degenerative" vacuoles. Criteria defining reactive, reparative or degenerative changes and cell death were taken from descriptions in standard cytology literature.[*inter alia* 22,27] Particularly useful features in separating reactive, reparative and degenerative changes were found in the nucleus of the cell (Table 5.7). Sometime very subtle differences in the appearances of the vacuoles were critical in ascribing a cause of vacuolar change. The cytologic features most useful in separating various vacuolar changes into different categories were defined (Table 5.9).

The Chlamydial vacuoles occurred almost exclusively in metaplastic cells so that confusion with mucinous vacuoles was not likely. Mixed groups of cells with both mucinous and metaplastic cells did occur but separation of mucinous cytoplasm from cells with well-defined mucinous vacuoles was easy. Misdiagnosis of inflammatory or degenerative vacuoles in metaplastic cells as Chlamydial vacuoles was a more likely possibility. Vacuolar changes in macrophages were less easily confused. Chlamydial vacuoles usually occurred singly in cells and showed well-defined enclosing membranes. They were very variable in size and contained characteristic inclusions. Frequently inflammatory vacuoles, defined in this study as containing neutrophils or neutrophil debris, were also seen in Chlamydial infection. This association had been previously described.[17] In contrast degenerative vacuoles were mostly multiple, variable in size, usually smaller, and did not contain inclusions. Apoptoses were sometimes seen in smears showing degenerative changes. To avoid mistaking degenerative vacuoles for Chlamydial infection, criteria for recognising cell death and apoptosis were strictly applied. The diagnosis of Chlamydia infection was only made if appropriate vacuoles were found in viable metaplastic cells.

The reason for the variable appearances of Chlamydial vacuoles remains conjectural. The different morphologies probably reflect different phases in the life cycle of the Chlamydial organism.[1,4,8,9,41,62] Gupta [1] and Henry et al[17] speculated that some of the vacuolar changes might represent degenerative phenomena occurring during the course of a Chlamydial infection. This view may explain why EM or immunologic marker studies were not able to demonstrate Chlamydia-specific components in some vacuoles. This view seems sensible, and in particular may explain the frequent occurrence of inflammatory vacuoles with neutrophils within them. It is possible that as part of the inflammatory response to the infection, neutrophils infiltrate Chlamydial vacuoles that have ruptured and released EB.

Another possible reason for some of the morphologic variants of vacuoles is artifactual changes occurring during the smearing and/or the fixation process. Cells and tissues fixed for light microscopy represent an artifactual snapshot of cellular appearance in a dynamic living process. Precipitation of proteins by alcohol during fixation of Pap smears probably alters to some extent the morphology of the intra-vacuolar components of Chlamydial inclusions. Similarly, cross-linking of cellular proteins by formaldehyde in the fixation of tissue for histology could induce certain cellular artefacts. The influence of osmotic forces on intra-cellular vacuoles during the fixation process may also be important in considering the variety of appearances seen. The morphologist examining any fixed tissue or cellular preparation recognises artifactual changes as the "normal" microscopic features. Although possible in some circumstances, the microscopist usually has no way to examine the cell in its native state. Investigation of the possible role of fixation artefact in causing or altering the appearance of the vacuolar changes seen in metaplastic cells showing Chlamydial infection may prove illuminating.

These speculations aside, the inclusions were recognised as part of a characteristic inflammatory process as seen in fixed tissues or cells. Since the various types of inclusions always occurred together and in the same setting, it seemed illogical to regard only one or other type as Chlamydial in origin. Rather the inclusions were regarded as variants occurring in the same disease process.

5.5.4 THE POSSIBLE RELATIONSHIP BETWEEN CHLAMYDIAL INFECTION AND CERVICAL CARCINOMA

There are numerous reports in the literature of an association between Chlamydial infection and "abnormal" Pap smears, intra-epithelial neoplasia and invasive squamous carcinoma.[*inter alia* 62-64,58,71] The rising incidence of cervical adenocarcinoma requires explanation.[72-74] Variable atypia of endocervical mucinous cells was seen in Pap smears and micro-glandular hyperplasia with atypia was found in tissue sections during the study. Fibro-epithelial endocervical polyps frequently showed Chlamydial infection. The presence of Chlamydial antigen in endocervical mucinous cells has been documented by IF and IP.[*inter alia* 1,10,15] However, any association between Chlamydial infection, glandular atypia and carcinoma is entirely speculative at the present time. If there is indeed an association between *Chlamydiae* and carcinogenesis, accurate diagnosis of the infection in both Pap smears and tissue sections could be of significant importance to patient care. Mecsi *et al*,[75] have indicated that epithelial atypia associated with Chlamydial infection is reversed by successful antibiotic therapy.

Differential diagnosis of Chlamydial epithelial changes and HPV/CIN

Assessment and separation of Chlamydial epithelial changes from HPV effects and/or intra-epithelial neoplasia became a very important part of the study. In the Chlamydia-infected population, there was a significantly higher ASCUS rate (53/1000) than in the control group (15/1000) ($P < 0.0005$). As the study progressed, the rate of ASCUS diagnoses fell from 151/1000 in the first year to 48/1000 over the last three years. This fall in the rate of reporting ASCUS in the Chlamydia-infected patients was statistically significant ($p < 0.0005$). It became clear as the study progressed that many cases resembling ASCUS could safely be ascribed to Chlamydial infection. The study showed that recognition of

Chlamydial atypia can reduce the ASCUS rate in a laboratory. Further, if Chlamydial atypia is recognised for what it is on the Pap smear, it can be reversed by antibiotic eradication of the organism.[64,75]
In the study by Mecsi *et al,*[75] 18 of 23 patients with culture-confirmed Chlamydial infection showed atypical smears that reverted to normal after Tetracycline treatment. These workers grouped inflammatory changes and CIN I changes and found 19.8% of Chlamydial infections showed such atypia. Their approach emphasises the view that separation of Chlamydial atypia from low-grade intraepithelial neoplasia may be difficult.

It appears that a proportion of unnecessary diagnoses of ASCUS and possibly CIN I could be avoided by recognition of Chlamydia-associated atypia. The management of HPV infection, ASCUS and CIN I is recall of the patient for repeat Pap smear. If the process progresses, or persists, colposcopy and other gynaecologic intervention may be considered. The economic and human resource costs associated with colposcopy clinics are considerable. Re-examination of the patient has significant impact in terms of the cost to the patient (or her medical insurance), contributes to escalating cost of medical services, and adds to work overload at colposcopy clinics. Antibiotic treatment for Chlamydia is relatively cheap, compared to the cost of overloading the gynaecology services

The anxiety caused to patients when they are given a diagnosis of ASCUS is not inconsequential. Likewise, an un-expected diagnosis of Chlamydial infection in an asymptomatic patient may cause a measure of anxiety regarding the cause and possible consequences of an STD. Criteria that successfully separate Chlamydial atypia from CIN I and reduce the rate of ASCUS, while not misdiagnosing an STD would therefore be of clinical value.

The diagnosis of Chlamydial infection may highlight a particular subset of patients at higher risk. On the other hand, misdiagnosis of HPV, ASCUS or CIN I because Chlamydial atypia is not recognised undue cost and anxiety is a regrettable consequence. Seen in this light, it seems to the author that if a Pap smear or tissue biopsy shows reasonable suspicion of Chlamydial infection it would be inappropriate if it were not reported so that appropriate further investigation could follow. Since the Pap smear is not a sensitive test for Chlamydial infections, it should not be regarded as a suitable test to screen for Chlamydia. On the other hand, if Chlamydia infection is found, should it not be reported ?

Chlamydia and cervical carcinogenesis

The present morphologic study in Pap smears revealed a significantly increased incidence of HPV infection (106/1000) in patients with evidence of Chlamydia infection compared with the control population (58/1000) ($p = > 0.0005$). The association of Chlamydia infection with HPV infection has also be reported by Alderling.[69] Since both conditions are acquired infections, this association may simply be related to exposure during sexual activity. Similarly, during the study the rate of CIN I Pap smears associated with Chlamydial infection (56/1000) was higher than in the control population (36/1000), although this was not a significant difference ($p < 0.0037$). As the study progressed and criteria were refined, the apparent incidence of associated HPV effect and CIN I fell. This indicated a probable improvement in

ability to separate Chlamydial atypia from HPV effect and CIN I. In Pap smears, the rates of CIN II and CIN III in the Chlamydial infected group and the control groups were not very different.

There are numerous reports in the literature of an association with "abnormal" Pap smears[63] and "dysplasia."[62,64,69-74] Although in the present study there was a notably higher rate of CIN I, and a slightly higher incidence of CIN III in the patients with Chlamydial infection, these differences were not found to be statistically significant. Similar findings were reported by Mecsi *et al.*[75] One possible explanation for the finding is that there is indeed no association between Chlamydial infection and CIN. This conclusion has been reached in several publications.[76-79] It is possible that other workers[40,63,69] have over-called a proportion of CIN cases, not recognising that the atypia due to Chlamydia may resemble intra-epithelial neoplasia.

The different opinions expressed in the cytology literature[63,69,70,76-79] may possibly be explained by findings in the present study. This cytologic morphology study showed that in respect of low-grade intra-epithelial neoplasia and HPV infection, a learning process occurred as diagnostic criteria were refined. It seems possible that in the past, other workers may have confused Chlamydial atypia with low-grade intra-epithelial neoplasia, or put another way, that the diagnostic criteria used for CIN I differed. The reproducibility of cytologic diagnosis of HPV infection and CIN I is known to be less than optimal with high inter-observer and intra-observer variability.[80] The Bethesda System[25] for the classification of cervical intraepithelial neoplasia has set down criteria to address this problem. The Bethesda *nomenclature* was not used in this work because it has not been accepted for histologic reporting and correlation of cytology reports and histology reports was necessary. However, the refined and precise Bethesda *criteria were* used in the present study. This may explain the failure to corroborate an association between Chlamydia and CIN I (low-grade intra-epithelial neoplasia). Certainly, use of the Bethesda criteria was regarded as critical in reducing the ASCUS rate and enabled clear separation of Chlamydial atypia from CIN I in most cases.

On the other hand, in respect of CIN III (high-grade intraepithelial lesions), use of the Bethesda criteria may not entirely explain the discrepancy between the present study and the findings of many other workers. Reproducibility of CIN III diagnoses between observers is known to be much better.[25,80] A possible explanation for the failure to demonstrate a significant association between Chlamydial infection and CIN III is that insufficient numbers (45) of patients with CIN III were studied. The control population included 21 patients with CIN III (rate 18/1000) and the Chlamydia-infected population revealed 24 patients (rate 21/1000). The difference between these rates was not significant ($p = 0.6770$). The rate of CIN III in the present study was higher than 4.6/1000 that was reported in a previous study by Learmonth *et al*[81] from Cape Town. The population screened and reported in the Learmonth *et al*[81] study was an established community that had been regularly screened and was likely to show lower rates of CIN III. On the other hand, the rate of CIN III in the present study was lower than 20.2/1000[38] and 28/1000[39] reported in two recent studies conducted in Johannesburg. The population screened in Johannesburg comprised of urbanised black women who had not been screened previously and were likely to have higher rates of CIN III. The rate in present study control group (18/1000) was intermediate between these two disparate reports.

The above points not withstanding, evidence reported in the literature for an association between HPV infection/CIN and Chlamydial infections appears to be sound and is well documented. For example, in a retrospective study conducted in Johannesburg, Harnekar *et al*[70] followed 54 patients with Chlamydial infection for at least two years. In comparison with 57 controls, these patients demonstrated a biopsy-proven accelerated progression to CIN III. It should be noted that apparent rapid progress of intraepithelial neoplasia as gauged by repeating smears may on occasion be due to improved representivity of the repeat smear. The question of whether or not Pap smears show an association between Chlamydial infection and cervical intra-epithelial neoplasia remains unanswered by this study. It appears therefore, that this question should be further investigated. The matter would probably be better investigated if the starting point of such a focused study were to be collection of numerous Pap smear cases of intra-epithelial neoplasia or carcinoma.
Contrary to the Pap smear study, the work using tissue sections from hysterectomy specimens, colposcopically directed biopsies and LLETZ/cone biopsies resulted in an unequivocal demonstration of an association between Chlamydial infection and HPV infection ($p < 0.0001$), CIN I ($p < 0.0001$) and CIN II ($p = 0.0005$). The higher incidence of CIN III with Chlamydial infection was not statistically significant in colposcopically directed biopsies and LLETZ/cone biopsies ($p = 0.0508$) but it was in the hysterectomy specimens ($p = 0.0005$).

The larger sections of tissue taken from hysterectomy specimens may explain the better detection of Chlamydial infection in hysterectomies. Evidence of Chlamydial infection was usually seen adjacent to the CIN, and was neither recognised nor recognisable within the focus of CIN. Colposcopically biopsies were much smaller specimens directed by skilled colposcopists at CIN lesions and were not intended to sample benign squamous metaplasia in which Chlamydia was found. Thus it was possible that Chlamydial infection was missed in some patients undergoing colposcopically directed biopsy. On the other hand, LLETZ/cone biopsies were larger specimens and possibly more likely to reveal an adjacent Chlamydial infection.

The morphologic features of HPV infection are seen adjacent to CIN III and cannot be recognised within the CIN III itself.[82] Early studies using immunoperoxidase detection of HPV antigen were not able to demonstrate HPV within the CIN. Only with subsequent testing using PCR was detection of integrated HPV DNA within CIN III possible.[23, 82-84] This could also be the case with Chlamydial infection.

Cervical carcinogenesis by oncogenic strains of HPV has been well described. [inter alia 23,82-86] Integration of HPV DNA sequences into the host cell genome results in disruption of mechanisms controlling the cell cycle. However, there is evidence that HPV is not the sole carcinogen.[87] Various candidate co-carcinogens have been put forward including *Treponema pallidum, Trichomonads,*[88] cigarette smoking,[82,89] *Herpes simplex virus,*[90] and spermatozoa.[90] Several publications[70,78-80,91-93] implicate Chlamydia in the genesis of cervical intraepithelial neoplasia and invasive squamous carcinoma of the cervix on an epidemiologic basis. It is possible that Chlamydia may be a co-factor with HPV in the development of cervical carcinoma. The presence of Chlamydial DNA in cells adjacent to CIN III and cervical carcinoma has been demonstrated by Schlott *et al*[68] using *in situ* PCR in tissue sections. However, as yet a possible causal relationship between Chlamydial infection and the HPV-CIN-carcinoma sequence has not been established.

Although carcinogenesis due to bacterial infection is not a widely recognised phenomenon, a strong association between *Helicobacter pylori* infection and gastric carcinoma and lymphoma has been established.[94-99] The mechanism of carcinogenesis appears to be indirect[98,99] and associated with chronic inflammation with prolonged immunologic stimuli, cytotoxin production, secretion of high levels of gastrin and gastric acid. There are some similarities and several obvious dissimilarities between the circumstances of *H. pylori-associated* carcinogenesis and Chlamydial infection. However, the *H. pylori* model reveals that the influence of an organism on the environment of cells vulnerable to other carcinogenic mechanisms may be profound.

Mechanisms of carcinogenesis have been ascribed to *Mycoplasma fermentans* and *M. penetrans*.[100] These organisms are close relatives of *Chlamydiae* and are similar obligate intracellular bacteria. These particular *Mycoplasmidae* are capable of transforming C3H mouse embryo cells by multistage progression. Increased expression of H-*ras* and c-*myc* mRNA, and prominent chromosomal alterations were associated with the malignant transformation of the C3H mouse embryo cells. The transformed cells were able to form tumours in animals. Similar studies using Chlamydia could prove illuminating in the search for co-factors in cervical carcinogenesis.

Further morphology-based study of Chlamydia infection occurring adjacent to CIN or cervical carcinoma might be augmented by *in situ* DNA hybridization or *in situ* PCR detection of Chlamydial DNA. Such a study could include a search for oncogene expression by use of PCR and or DNA hybridization techniques in micro-array. Integration of Chlamydial DNA in the host genome may be demonstrated, and could cause alteration of regulators of the cell cycle. Alternatively, epigeneic Chlamydia-mediated control mechanisms may influence the cell cycle. It has been established already that similar to viruses, Chlamydia subvert cellular metabolism, resulting in expression of Chlamydia-specific MOMP in the host cell membrane.[9]

5. 5.5 CORROBORATION OF MORPHOLOGY

A large number of publications have concluded that the morphologic diagnosis of Chlamydial infection on Pap smears is unreliable because of low sensitivity and specificity. *inter alia* 3,7,10-15,17,18,25,34,40-45 These publications indicate poor correlation between Pap smear diagnosis and other corroboratory tests. Frequently, culture of the organism has failed to confirm the diagnosis in a significant number of cases.[12,14,34, 41,43-45] Other workers have used direct immunofluorescent (DFAT)[11,12,14,42-45] or immuno-peroxidase staining for Chlamydial MOMP.[10] Poor correlation of Pap smear diagnosis and serology, usually using ELISA detection methods has also been reported. [11,17] On the other hand, a few publications have indicated that findings in Papanicolaou-stained have been corroborated by culture studies[45] and by immuno-peroxidase staining techniques.[35,44,61]

There is universal belief amongst practitioners of cytopathology in the process of confirmation of cytology by biopsy, and that histology is a reliable gold standard. Colposcopy was performed on patients showing CIN II and CIN III, and in those cases with associated Chlamydia infection, some biopsies did show the morphology of both processes. During the

study some colposcopists reported that they could recognise immature squamous metaplasia with inflammation and separate these changes from intra-epithelial neoplasia. However, it was not appropriate to subject patients to colposcopic biopsy to confirm Chlamydial infection alone. Histology could not be used as a gold standard in follow-up of individual patients showing Chlamydial infection on a Pap smear.

The parallel study on histology specimens of the cervix submitted to the laboratory did show the same morphologic changes seen in Pap smears. The presence in tissue specimens of a similar constellation of features was regarded as strong support for the cytologic criteria. However, it remained necessary to prove that this constellation of changes found in both cytology and histology specimens was due to *Chlamydia trachomatis* infection.

Clearly it was necessary to expose the diagnostic criteria for Pap smears developed in the morphologic study to corroboratory testing. After consideration of the in-depth review of literature pertaining to the laboratory diagnosis of Chlamydial infection (*vide supra*), two corroboratory methods were selected as the most appropriate corroboratory tests. The recommendation of the CDC in the US was that two methods of DNA detection be used as a "combined gold standard." The two methods chosen were DISH and PCR (see Chapter 6 "Corroboratory tests"). The DISH and PCR testing was performed at two *independent reference laboratories* that agreed to train the author in the use of these methods.

5.6 CONCLUSIONS

The conclusions in the morphologic study were:

(1) Pap smears and tissue sections showed a recurring constellation of changes regarded as evidence of Chlamydial infection. These included a characteristic inflammatory picture, peculiar epithelial changes and intra-cellular vacuoles containing three types of inclusion. Cytologic criteria were refined to exclude possible non-specific changes due to inflammation, degeneration, and concomitant infections including HPV infection with and without CIN;

(2) Of 48 958 Pap smears, 1 141 (2.33%) showed features of *Chlamydia trachomatis* infection;

(3) Among 2 682 specimens of uterine cervix, 257 (9.6 %) showed features of *Chlamydia* histology *trachomatis* infection;

(4) An association between Chlamydial infection and HPV infection with cervical intra-epithelial neoplasia was demonstrated, particularly in the histology specimens;

(5) Failure to recognise Chlamydia-associated changes may result in an increased incidence of reporting of ASCUS and low grade intraepithelial neoplasia with a consequent impact on the health care management of patients;

(6) If the association of Chlamydial infection with CIN reflects a causal relationship between *Chlamydiae* in cervical carcinogenesis, accurate diagnosis of the infection in both Pap smears and tissue sections could be of significant importance to patient care;

(7) Further research to investigate and elucidate a causal role of the organism in carcinogenesis should be undertaken in the future;

(8) It was necessary to obtain proof of the presence of Chlamydial organisms in smears and sections showing this constellation of changes. The use of two different DNA detection methods as a combined gold standard was most likely to gain acceptance of the diagnostic criteria by the scientific community.

6. CORROBORATORY TESTING

6.1 AIM

The aim was to corroborate the morphologic criteria for the diagnosis of Chlamydial infection developed during the first phase of the study (Chapter 5). The corroboratory tests were intended to show that these criteria could be confidently applied in every-day routine microscopy without the necessity to resort to elaborate and expensive testing. It was necessary to establish a suitable corroboratory DNA test in the laboratory, and to test cases selected using the established criteria. In accordance with the CDC recommendations, the corroboratory testing would use an "expanded gold standard." The same specimens would be subsequently tested in an independent laboratory using a second DNA method.

6.2 SELECTION OF THE GOLD STANDARD

An in-depth review of literature concerning the laboratory diagnosis of Chlamydial infection revealed that an "expanded gold standard" using two different DNA detection methods was the recommended[1] and preferred way to corroborate the morphologic criteria developed for Pap smears and tissue sections. The gold standard selected for the present study was therefore a combination of DISH and PCR.

6.3 MATERIALS & METHODS

Prior to the availability of DNA testing, it was necessary to use culture and EM techniques to confirm the morphologic diagnosis of Chlamydial infection in Pap smears and tissue sections. Use of these modalities was limited to a few specimens and served to confirm that further investigation and development of the DNA testing methods was justified. EM was used to confirm Chlamydial infection in positive control tissues that were subsequently used to establish and validate the DISH method.

6.3.1 CULTURE OF *Chlamydiae*

Several patients whose Pap smear results reported possible Chlamydial infection requested confirmatory testing. By arrangement with their physicians, cervical brushings from thirteen women were collected into M4® Chlamydial transport medium[2] and frozen at –70°C within two hours of collection. Within four days, the specimens were packed in polystyrene containers with dry ice and transported by air to Lancet Laboratories for culture in McCoy cells. Care was taken to maintain the cold chain during the transport of these specimens. The cultures for Chlamydia were performed at Lancet Laboratories using their protocol (Appendix E). The cell cultures were stained using an immuno-fluorescent technique with anti-LPS antibody to detect the presence of Chlamydial antigen.[3] It is noted that the Lancet protocol did not include evaluation of the quality of the specimens by checking for the presence of mucinous and metaplastic cells.

6.3.2 ELECTRON MICROSCOPY

Tissues were processed for EM using standard procedures in the Electron Microscopy Unit at the Department of Anatomical Pathology, University of Cape Town (Appendix L). Ultra-thin sections were examined using an Hitachi 5000 Electron Microscope.

6.3.2.1 PAP SMEARS

During routine microscopy, Pap smears showing large three-dimensional groups of metaplastic squamous cells containing vacuolar inclusions were identified. Many of these groups contained 50 or more cells (Figure 6.1). Cells for electron microscopy, particularly cells arranged in large three-dimensional clumps, were lifted from Pap smears. Five cytology smear showing such clusters of metaplastic cells were selected for EM investigation by this method. The groups of cells were located by making a circle around them on the back of the glass slides with a diamond pencil. The cover-slips were then soaked off the slides using xylol and the smears were air dried. A small bleb of DPX mounting medium was applied to each group of cells on the surface of the smear and allowed to dry. The dry DPX bleb was carefully scraped from the slide, lifting the clump of cells from the smear. The clumps of cells from each case were placed in small test tubes and the DPX mounting medium dissolved in xylol. These cell clusters were processed for EM. The remaining cells on the smears were left undisturbed by this procedure. The smears was re-mounted in DPX and retained in the study archive. Tissue fragments containing immature metaplastic squamous cells were successfully lifted from the five Pap smears and after processing and ultra-microtomy, examined using the Hitachi electron microscope.

6.3.2.2 TISSUE SECTIONS

HE sections were used to identify nine formalin-fixed hysterectomy specimens that showed good preservation and changes regarded as being Chlamydial in origin. These specimens were used for EM. Small blocks of tissue suitable for EM were obtained from the residual unprocessed hysterectomy specimen. The formalin-fixed tissue was taken from the transition zone in the same quadrants of cervix as those shown to be Chlamydia positive on light microscopy. This was possible because sections had been taken routinely from four quadrants of the cervix and labeled according to the protocol described (*vide supra* **Morphologic Study 5.3.2.1**).

Multiple small columnar blocks of tissue approximately 1mm by 1mm by 3 mm were cut under a dissecting microscope from the residual formalin-fixed tissue. The long axis of these small blocks was cut parallel to the mucosal plane so that the affected transitional epithelial membrane formed one of the long surfaces of the block. This served to assist orientation of the tissue during processing for electron microscopy. The tissues were post fixed in buffered 4% gluteraldehyde and processed for routine electron microscopy.

Thick sections (sections appeared silver in refracted light on the water bath) were sectioned using a glass knife on a Reichert Ultra-Microtome. These were stained with toluidine blue and examined by light microscopy to enable precise localisation of the metaplastic epithelial cell layer and identification of intra-epithelial inclusions. The blocks were then further trimmed and ultra-thin sections cut (sections appeared gold in refracted light) for transmission microscopy.

6.3.3 TO SET UP AND VALIDATE THE PROTOTYPE REMBRANDT DISH KIT

6.3.3.1 INTRODUCTION

Establishing the DISH method for detection of *Chlamydia trachomatis* was initially problematic. The method was set up using the recommended protocol for the Rembrandt® DISH kit from Kreatech Laboratories in Amsterdam (Appendix F).

Positive control slides supplied by Kreatech Laboratories were used to validate the Rembrandt kit method. These control slides were preparations of McCoy cells in which *Chlamydia trachomatis* had been cultured and identified by immunofluorescent antigen detection (Kreatech Catalogue CS375).[3] During the initial validation of the kit, the preservation of morphology of the positive McCoy cells preparations was noted to be sub-optimal. It became clear that various tests were necessary to refine the method to achieve optimum test conditions. There was considerable loss of specimen because cells washed off the surface of the glass slides. The loss of cellular material seemed to occur during the washing step after pre-digestion with protease.[4] Subsequently, when Pap smears and tissue sections were stained using the same method, loss of specimen occurred and preservation of morphology was poor. The loss of tissue was associated with the protease digestion step and poor morphology appeared to be due to heat-induced artifact.[5]

To resolve these problems, panels of tests were conceived. The tests were designed to confirm that loss of cells was due to the pre-digestion step and that poor morphology was in part due to the effects of heat during the denaturing of DNA. The tests also aimed to investigate whether less harsh pre-digestion and reduced hybridization temperature protocols would preserve morphology, yet be sufficient to allow access of probe to target DNA.[5] Therefore, the test panels included Pap smears, tissue sections and positive control slides (McCoy cell cultures). The aim was to find optimum conditions for the preservation of cell morphology in the smears and sections whilst exposing the tissue to conditions sufficiently harsh to allow access of the probe to target DNA. It became clear that purchase of specialized equipment to heat the glass slides to precisely 95°C was necessary if reliable DISH testing was to be performed in Cape Town. The refinements made to the protocol for cytology smears developed during this validation study, are appended (Appendix F).

When the new improved Rembrandt® DISH kit using the "universal linkage system" (ULS®) became available it was decided to use this method to improve the sensitivity of the DISH method. Kreatech Laboratories had no Pap smears or tissue sections available for control purposes to validate the application of ULS to their Chlamydia kit. However, the researcher identified suitable control tissue blocks and confirmed the presence of Chlamydial organisms in this tissue using EM. This tissue was sent to Kreatech to be validated for the DISH method. In return, for providing control tissue, Kreatech agreed that the researcher could perform the DISH testing of Pap smears and tissue sections in their Amsterdam laboratory. This obviated the necessity for purchase of expensive equipment. Again, arising from this exercise, Georg-August-University in Goettingen extended an invitation for the researcher to learn PCR techniques and to test all specimens in Germany. Serial sections from the original control blocks were taken to Georg-August University for independent testing using PCR. The researcher personally performed all of the tests done in Amsterdam and Goettingen.

6.3.3.2 PILOT STUDIES TO SET UP DISH TO DEMONSTRATE CHLAMYDIAL DNA

Funding for the pilot study was limited and initially, only two Rembrandt® DISH[6] kits could be purchased from Kreatech for the validation of the method and running hybridisation tests for the pilot study. Each kit had sufficient volumes of probe reagent to run 25 tests. Since the compositions of the other reagents used in the protocol were known and these reagents were available in the laboratory, tests without probe could be conducted in relatively un-restricted numbers.

However the volume of Chlamydial probe reagent for the validation and setting up of the method was restricted to a total of only 50 tests. The two kits were used in preliminary testing of Pap smears and tissue sections to validate, optimise and establish the DISH method.

Positive & negative controls

As a first step in establishing the DISH method, positive control slides using McCoy tissue culture cells infected by *Chlamydia trachomatis*[3,7] were used to validate the Rembrandt® DISH kits. Six slides with McCoy cells containing Chlamydial organisms and six slide with un-infected McCoy cells were stained according to the DISH protocol supplied by Kreatech (Appendix F). The Kreatech Chlamydia-specific probe CHLT-DIG-1 was compared with Kreatech non-Chlamydial probe PLD043. Initially, the protease pre-digestion was performed using 2.5 mg protease/ml diluent for 30 minutes. Subsequently, 0.1 mg protease/ml diluent was used for 10 minutes.

Refining the DISH protocol for Pap smears

The poor morphology of smears after they had been stained using the DISH protocol was thought to be due to two factors: excessive protease digestion, and heat-induced artifact. Tests were conducted on 36 normal Pap smears drawn from the archives of the practice. These smears were due to be discarded from the archives after the statutory retention period of three years. The ability to perform DISH on archival material is well documented [5] The two problems were addressed separately.

Protease pre-digestion tests

The usual means of preventing loss of cellular material during pre-digestion steps is the use of subbed slides.[5] Since the Pap smears for DNA testing were obtained from a routine diagnostic service, it was not possible to use smears made on subbed slides. A series of tests was devised to confirm that the pre-digestion step was a cause for loss of tissue. Thereafter, it was necessary to establish a minimum effective protease pre-digestion protocol. To achieve these objectives, several modifications of the DISH protocol were applied to the normal smears (See Table 6.1). In protocol P1, the entire protocol was applied. Protocol P2 did not include the pre-digestion step and protocols P3, P4, P5 and P6 used modified pre-digestion steps. In protocol P3, the concentration of Pepsin used was reduced from 2.5 mg/ml to 1.0 mg/ml of diluent. In protocol P5, the pepsin concentration was further reduced to 0.1 mg/ml of diluent. The P4 protocol used the lower concentration of pepsin used in P3, and reduced the digestion time to 10 minutes from 30 minutes. In protocol P5, the reduced concentration of pepsin in P4 was only applied for 10 minutes (Table 6.1). In total 24 smears and two controls were tested.

Hybridization temperatures

To investigate the influence of heat on the morphology of smears, protocols P7, P8 and P9 subjected the smears to temperatures of 90°C, 95°C and 98°C respectively on a heating block for exactly 180 seconds (Table 6.2). The P7,P8 and P9 control protocols served to indicate whether or not the changed hybridization temperatures affected the outcome of the DISH. The preservation of morphology was graded either as acceptable for microscopic interpretation (+) or

unacceptable for microscopy (–). Alternatively it was recorded that a substantial part of the smear had floated off the slide (#). The control slides were examined for successful hybridization (+) or negative for hybridization (–), for preservation of morphology as acceptable (+) or not acceptable (–) and for ability to localize precisely the hybridization product as suitable (+) or not suitable (–). In this panel of tests, six smears & three controls were tested.

Table 6.1
The panel of tests designed to optimise the pre-digestion step for Papanicolaou smears. Protocol P1 applied the entire protocol. Protocol P2 did not include the pre-digestion step. Protocols P3, P4, P5 and P6 used modified pre-digestion steps with lower concentrations of pepsin and reduced incubation times. P5 + Control and P6 + Control were run as controls using Chlamydia-infected McCoy cells

Protocol	Number smears	Pepsin mg/ml 0.01N HCl	Volume of reagent in µl	Time in minutes	Temperature °C
P1	4	2.5	350	30	37
P2	4	0.0	350	30	37
P3	4	1.0	350	30	37
P4	4	1.0	350	10	37
P5	4	0.1	350	30	37
P5 + Control	1	0.1	350	30	37
P6	4	0.1	350	10	37
P6 + Control	1	0.1	350	10	37

Table 6.2
The panel of tests designed to demonstrate the effect of heat during the step to denature DNA in Papanicolaou Smears. Protocol P7, P8 and P9 subjected the smears to temperatures of 90°C, 95°C and 98°C respectively on a heating block for exactly 180 seconds.

Protocol	Number	Pre-digestion	Time seconds	Temperature °C
P7	4	0.1 mg,10 min	180	90
P7 + Control	1	0.1 mg,10 min	180	90
P8	4	0.1 mg,10 min	180	95
P8 + Control	1	0.1 mg,10 min	180	95
P9	4	0.1 mg,10 min	180	98
P9 + Control	1	0.1 mg,10 min	180	98

Refining the DISH protocol for tissue sections

Application of the DISH method in tissue sections during the pilot study was difficult. First, there was considerable loss of specimen because sections floated off the surface of the glass slides during the washing step after pre-digestion with protease. Second, there was poor preservation of tissue morphology. The particular value of the DISH method is that it should allow the precise localisation of the hybridization product. It was therefore regarded as critical to achieve good preservation of morphology in the tissue sections so that the hybridization product could indeed be precisely located.

In order to refine the DISH protocol, panels of tests investigated the effect of changing the thickness of sections, the type of subbing agent used on slides (Table 6.3) and the maximum temperature applied for the step to denature DNA (Table 6.4). Four additional blocks of tissue were taken from four normal uterine cervices and serial sections from each were tested. For control purposes, use of Chlamydial cultures in McCoy cells, although not ideal, was the only option until tissue control blocks had been validated. The problems of the lifting sections and the poor morphologic detail were addressed as follows:

Subbing for the protease pre-digestion

Subbing of slides is a standard measure to prevent lifting of sections in DISH procedures.[5] The commercially available subbed slides (StarFrost®) used initially were not effective in this regard. Investigation revealed that StarFrost® produced two different coated slides using either polylysine or organosilane as subbing agents. Kreatech supplied organosilane-coated slides. Tests to compare the performance of these different coated slides were conducted (Table 6.3).

Thickness of tissue sections

Apart from the efficacy of the subbing agent, another factor that could have affected the adherence of sections to the slides was the thickness of the sections. Thinner sections exhibit better adherence, but since less tissue is present on the slide, the chances of detecting and localising Chlamydial DNA within intra-cellular vacuoles were less in the thinner sections. On the other hand, the resolution of fine cellular detail is not as good in thick sections. A compromise between thin sections (5 μm) and thick sections (10 μm) was necessary. The tests were designed to compare both the adherence and the morphologic outcome using sections cut at 5, 8 and 10 μm (Table 6.3). A total of 96 sections were tested in this panel of tests.

The influence of hybridization temperature

Experience with Pap smears suggested that the poor morphology was also in part due to heat-induced artifact. A panel of tests investigated the effect of heat on morphology of sections and the efficaey of the DISH protoeol if hybridization was performed at 90, 95 and 98 °C. Tissues from the four blocks of normal cervix were serially sectioned at thickness of 5μm, 8 μm and 10 μm and mounted on plain glass slides (G) or the three different types of commercially available subbed slides. The subbed slides were designated as brands SA, SB and K. Brand SA slides were StarFrost® with poly-lysine, brand SB slides were StarFrost® with organosilane and brand K slides were from Kreatech® with organosilane.

The sections of different thickness, on slides with or without subbing agent were subsequently taken through the steps of the *in situ* hybridization process. To determine which if any of the steps in the process proved critical to section quality, modified protocols were used. In protocol P1 the entire protocol was applied. Protocol P2 did not include the pre-digestion step (Table 6.3).

In protocols P10, P11 and P12 (Table 6.4), the sections were mounted either on un-subbed slides (G) or on K-brand organosilane-subbed slides and on a heating block for exactly 180 seconds were subjected to temperatures of 90°C, 95°C

and 98°C respectively. The preservation of morphology was graded as either acceptable for microscopic interpretation (+) or unacceptable for microscopy (–). If a substantial part of the smear or section had floated off the slide, this was recorded (#).

Table 6.3
The panel of tests designed to investigate the ability of subbing agents to resist the pre-digestion step on tissue sections cut at different thicknesses from four blocks of tissue.

Protocol	Subbing Agent												No
	G – no agent			SA – polylysine			SB – polylysine			K – organosilane			
	5µm	8µm	10µm	5µm	8µm	10µm	5µm	8µm	10µm	5µm	8µm	10µm	
P1	4	4	4	4	4	4	4	4	4	4	4	4	48
P2	4	4	4	4	4	4	4	4	4	4	4	4	48
TOTAL	8	8	8	8	8	8	8	8	8	8	8	8	96

Table 6.4
The panel of tests designed to demonstrate the effect of heat on section quality during the step to denature DNA using sections cut from four blocks of tissue.

Protocol	Subbing agent						Pre-digestion Protocol	Time secs	Temperature °C	No
	G – no agent			K – organosilane						
	5µm	8µm	10µm	5µm	8µm	10µm				
P10	4	4	4	4	4	4	2.5 mg,30 min	180	90	24
P10 + cont	0			1			2.5 mg,30 min	180	90	1
P11	4	4	4	4	4	4	2.5 mg,30 min	180	95	24
P11 + cont	0			1			2.5 mg,30 min	180	95	1
P12	4	4	4	4	4	4	2.5 mg,30 min	180	98	24
P12 + cont	0			1			2.5 mg,30 min	180	98	1
Total	12	12	12	12	15	12	-	-	-	75

6.3.4 DISH USING THE IMPROVED ULS® REMBRANDT KIT

The improved Ultradig Kreatech Rembrandt DISH® kit[8-10] was used for the detection of *Chlamydia trachomatis* in Pap smears and tissue sections. The Ultradig DISH® kit using DNA probe labelled with the new Universal Linkage System (ULS) was essentially the same kit as the prototype. Sensitivity of the method was enhanced by use of the ULS labeling system that allowed improved detection of hybridization product. The protocol for hybridization, the stringency conditions and the procedure to label the hybridization product were the same for the prototype and the ULS kits. The methodology developed during the experiments conducted to set up the DISH technique could therefore be applied to the new kit without modification. However, the use of Chlamydia-infected McCoy cells from tissue cultures for controls was regarded as inappropriate. Blocks of control tissue for control sections were required.

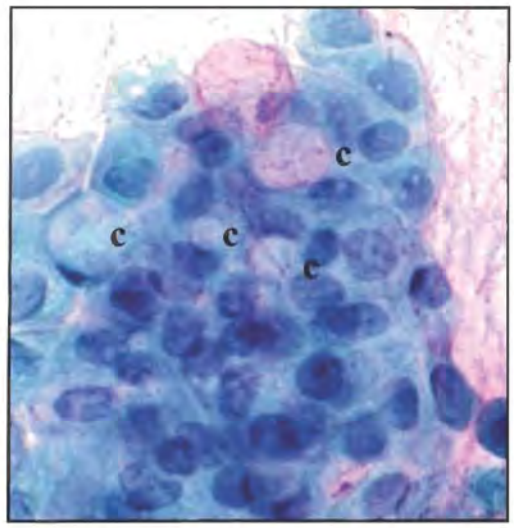


Figure 6.1. Cells arranged in large three-dimensional clumps were lifted from five Pap smears for electron microscopy. Many of these groups contained 50 or more cells. Note the Chlamydial vacuolar inclusions (c).

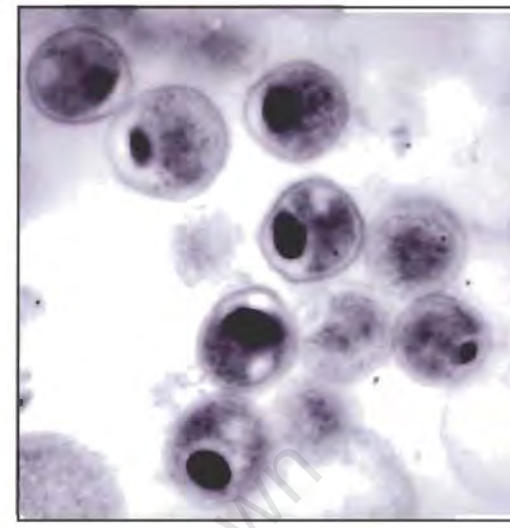


Figure 6.2. Controls for the PCR tests were McCoy cells shown to have Chlamydial inclusions by EM.

Figure 6.3. Kreatech probe CHLT-DIG-1® and Kreatech non-Chlamydial probe PLD043® were supplied in plastic dropper bottles that delivered a metered volume of 0.1 ml per drop.

Figure 6.4. Two sectors or "work areas" slightly larger than the size of a 18 x 18 mm cover slip were demarcated on each slide using a wax Pap Pen.®

6.3.5 CONTROLS FOR THE DISH METHOD

6.3.5.1 POSITIVE CONTROLS FOR PAP SMEARS - CHLAMYDIA-INFECTED MCCOY CELLS

Positive controls were necessary to demonstrate that the Chlamydial probe was specific and functioning to detect Chlamydial DNA. McCoy tissue culture cell preparations infected by *Chlamydia trachomatis*[3-7] (MC-1) were supplied with both the prototype and the ULS Rembrandt® DISH kits (Kreatech Catalogue CS375). These cells had been shown by Kreatech Labs to be infected by the demonstration of LPS Chlamydial antigen using immuno-fluorescence (Enzo BioProbe® Catalogue EN40530). McCoy cell culture preparations, being very similar to smears, were regarded as suitable for positive controls when testing Pap smears.

6.3.5.2 POSITIVE CONTROLS FOR TISSUE SECTIONS - ESTABLISHING A KNOWN POSITIVE CASE (7H0139)

The Kreatech Rembrandt Ultradig DISH® kit [8-10] method for the detection of *Chlamydia trachomatis* using a DNA probe labelled with the new Universal Linkage System (ULS) had not been validated in tissue sections prior to this work. No control tissue sections were available with the kit. As a first step to enable DISH testing of tissue sections, it was necessary to establish a known positive tissue control. Accordingly, tissue blocks to be used as a positive control had to be identified and validated by demonstrating the presence of Chlamydial DNA in the tissue.

After the collection phase of the morphology study had been completed, in January 1997, a potential tissue control block was identified (Practice Lab No 7H0139). Permission was obtained from the patient by the researcher to use this tissue for control purposes during the experimental phase of the main study. Seven blocks of tissue were taken from the endocervical transition zone in the hysterectomy specimen and examined for Chlamydial inclusions by EM at the University of Cape Town. Subsequently, blocks from case 7H0139 were submitted to Kreatech Laboratories. Kreatech personnel conducted independent tests using DISH to validate the control tissue. During this validation, the controls used were *Chlamydia trachomatis*-infected McCoy tissue culture cells (confirmed by immuno-fluorescence for MOMP). Serial sections of 7H0139 were submitted for independent reference PCR testing by staff in the Cytogenetics laboratory at the Georg-August-University in Goettingen Germany. At Georg-August-University, controls for the PCR tests were McCoy cells shown to have Chlamydial inclusions by EM (Figure 6.2). The PCR reaction product was sequenced using a DNA automatic sequencer (DNA Analyser Model ABI310, Applied Biosystems) to establish the specificity of the PCR.

6.3.5.3 CONTROL "HOUSEKEEPING" PROBE USING KREATECH HPV-DIG-1®

A control "housekeeping" probe was included with each batch of tests to demonstrate that the hybridization and stringency conditions during the procedure were appropriate. The housekeeping probe was required to make use of the same system as the test. Apart from the probe, these controls used the same reagents used with the Chlamydial probe.[11] Thus, if tests were negative, but the "housekeeping" controls were positive, this could not be attributed to failed hybridization conditions. Again, to demonstrate appropriate stringency conditions, the housekeeping probe was required to stain its intended target only. The "housekeeping" probe selected was HPV-DIG-1®. The probe was available from

The Kreatech Rembrandt Ultradig DISH® kit [8-10] had not been validated for diagnosis of Chlamydia prior to this work. Only one control specimen (7H0139) investigated by EM and PCR was used to validate the test. The use of the PCR test therefore also served to establish the validity of the Kreatech method.

For each of the tissue blocks selected for DNA testing, ten serial sections were cut at a thickness of approximately 8μm. To prevent cross contamination of Chlamydial DNA between cases, during sectioning of these specimens, the forceps, the brush and the microtome blade were cleaned with chlorine bleach between specimens.[16,17] The instruments were also wiped with ethanol and dried with paper towels to remove residual chlorine and fragments of wax-embedded tissue.[18,19] In addition, the blocks were carefully faced and initial sections discarded. This facing process alone has been shown to be sufficient to prevent cross contamination of specimens.[18]

During the cutting process, sections from each case were floated on newly dispensed distilled water in a new clean container, preventing cross contamination between cases. These sections were mounted on subbed slides coated with organosilane. The ten serial sections were used in a particular sequence for the testing. Section numbers one and ten were mounted singly on a slide each, and were stained with H & E. In positive specimens, the purpose of these two sections was to demonstrate the presence of *Chlamydia trachomatis* inclusions in the transitional metaplastic epithelium on both sides of the portion of tissue to be tested for Chlamydial DNA. In negative specimens, the purpose was to demonstrate the absence of Chlamydia-associated changes on both sides of the portion of tissue tested for Chlamydial DNA.

Section numbers two to nine were for DNA probing and PCR testing. Sections two and three, four and five, six and seven, eight and nine were placed into four pairs on four slides. Each pair of sections was mounted on one slide. The paired sections were positioned as widely apart as possible on the slide. This allowed use of different probes on each section without cross contamination of reagents during the DISH procedure. One pair of sections (serial sections 2 & 3) was used for the DNA *in situ* hybridization purposes. The next pair (serial sections 4 & 5) was available for repeat testing by DISH if sections washed off the slide during DNA probing, or other technical difficulties were encountered. The next pair (serial sections 5 & 7) was used for PCR. The last pair (serial sections 8 & 9) could be used for repeating the PCR if necessary. If not used, the spare serial sections (4 & 5 and 8 & 9) were kept in the study archive as spare sets for possible future use.

6.3.7 TO TEST THE MORPHOLOGIC CRITERIA IN PS & TISSUE SECTIONS WITH DISH

Smears and sections showing evidence of Chlamydial infection were selected from the study archives using the criteria developed in the Morphologic Study (*vide* chapter 5, **5.4.1.2, 5.4.2.2**). Control smears and sections were similarly selected using the criteria for HPV/CIN disease, inflammation and repair (*vide* **5.4.1.3-6, 5.4.2.3-6**). The DISH® kit made use of a DNA probe labelled with digoxigenin using the novel ULS® for the detection of the DNA of the cryptic plasmid of *Chlamydia trachomatis*.

6.3.7.1 DISH ON CYTOLOGY SMEARS

The test and control smears were divided into four batches and re-stained using the *in situ* hybridization technique on four consecutive days (Table 6.5). Twenty five Pap smears showing the features of *Chlamydia trachomatis* infection (PS+) were tested. Fourteen Chlamydia-negative cases (PS–), including four PS–HPV/CIN "housekeeping" controls were included in the test panel.

Three of the fourteen PS– showed no other organism, while the remaining eleven PS– showed evidence of other organisms. Four PS– showed changes of HPV infection, three with CIN (PS–HPV/CIN). These cases were used for "housekeeping purposes" and included one each of PS- HPV, PS–CIN I, PS–CIN II and PS–CIN III randomly assigned one per batch. Two of the PS– were smears showing organisms of *Candida* sp. (PS–C) and two showed organisms of *Gardnerella* sp. (PS–G). The three remaining PS- included one smear showing features of Herpesvirus (PS–H), one showing *Neisseria* sp. (PS–N), and one showing *Trichomonas* sp. (PS–T). These were randomly assigned amongst the four test batches. There were four MC+ positive controls, one of each for each batch. In total, 43 test and control smears were stained, involving 90 separate evaluations.

A unique Kreatech experiment number was used to identify the smears. The status of the smears as PS+, PS–, PS--HPV or PS- CIN was concealed during the staining stage and while undergoing subsequent examination and evaluation. The results were recorded by patient name and the Kreatech experiment number.

6.3.7.2 DISH ON TISSUE SECTIONS

Twenty five histology cases (TS) regarded as positive for Chlamydial infection (TS+) and 10 histology cases negative for Chlamydial infection (TS–) were selected for *in situ* hybridization. The testing was carried out on paired serial sections. Section numbers 2 & 3 of 10 serial sections were used.

The paired serial sections 4 & 5 were available for repeat testing if necessitated by technical problems (loss of section, excessive signal noise in the background or failure of hybridization). These sections were randomly assigned to four separate batches and stained using the DISH technique on four consecutive days. Chlamydia-positive (C+) control sections (7H0139) were included in each batch. "Housekeeping" controls included were HPV positive, Chlamydia negative cases (TS-HPV). The status of the cases as "positive" or "negative" was concealed during the subsequent examination. The sections were identified by patient name and a unique Kreatech experiment number only. The panel of tests conducted is shown below (Table 6.6). In all, 90 evaluations were performed.

6.3.7.3 DISH PROCEDURE

The protocol used for the DISH procedure is detailed in Appendix F. Supplied with the kit were Kreatech probe CHLT-DIG-1[®] and Kreatech non-Chlamydial probe PLD043[®]. Kreatech probe HPV-DIG-1[®] was used to demonstrate HPV DNA in PS-HPV and TS-HPV. Kreatech differentiation agent BC0013,[®] 12 % formamide and 0.01 x SSC (neutral saline citrate buffer pH 7.0), was used to set the stringency conditions in the post hybridization wash. All of these reagents and solutions were supplied in plastic dropper bottles (Figure 6.3) that delivered a metered volume of 0.1 ml per

drop. Other working solutions were prepared and stored in bulk and were pipetted using Eppendorf® micropipettes. Variations of technique were necessary for PS and TS.

Table 6.5

The tests performed on 25 Pap smears positive for Chlamydia on LM (PS+) using CHLT-DIG-1® to demonstrate Chlamydial plasmid DNA using the Kreatech Rembrandt® DISH kit. Pap smears negative for Chlamydia on LM (PS–) were probed with CHLT-DIG-1,® PLD043® and HPV-DIG-1.® Positive controls included were Chlamydia-infected McCoy cells (MC+) probed with CHLT-DIG-1,® PLD043® and HPV-DIG-1.® Housekeeping controls were Pap smears showing HPV effect on LM probed with HPV-DIG-1® and CHLT-DIG-1.®

Run	Sample	CHLT-DIG1 No. of slides	PLD043 No. of slides	HPV-DIG1 No. of slides	Differentiation BC0013 No. of tests	Detection α-DIG-AP No. of tests	Total No. of Tests
1	MC+	1	1	1	3	3	3
	PS-, PS-C	2	2	0	4	4	4
	PS+	6	4	2	12	12	12
	PS-HPV	1	0	1	2	2	2
2	MC+	1	1	1	3	3	3
	PS-G, PS-T	2	2	0	4	4	4
	PS+	6	4	2	12	12	12
	PS-CIN II	1	0	1	2	2	2
3	MC+	1	1	1	3	3	3
	PS-, PS-C,PS-H	3	3	0	6	6	6
	PS+	6	4	2	12	12	12
	PS-CIN I	1	0	1	2	2	2
4	MC+	1	1	1	3	3	3
	PS-,PS-G, PS-N	3	3	0	6	6	6
	PS+	7	4	3	14	14	14
	PS-CIN III	1	0	1	2	2	2
Total		**43**	**30**	**17**	**90**	**90**	**90**

Step 1a: Preparation of Pap smears

The slides were soaked in xylol to remove the cover slips. To duplicate treatment conditions, the McCoy cell cultures (MC+) were dehydrated through graded alcohols and soaked in xylol. The xylol residue was removed from all of the slides in two washes of absolute alcohol and slides were air-dried in an oven at 60 °C.

Table 6.6

The tests performed on 25 histology cases positive for Chlamydia on light microscopy (TS+) to demonstrate Chlamydial DNA using the Kreatech Rembrandt® DISH kit. Controls included a known positive case (C+) probed with CHLT-DIG-1, PLD043 and HPV-DIG-1, histology cases negative for Chlamydia on light microscopy (TS–) probed with CHLT-DIG-1, PLD043, and histology cases showing HPV effect without evidence of Chlamydial infection (TS-HPV) on light microscopy probed with HPV-DIG-1 and PLD043.

Run	Sample	CHLT-DIG1 No. slides	PLD043 No. slides	HPV-DIG1 No. slides	Differentiation BC0013 No. tests	Detection α-DIG-AP No. tests	Total No. tests
1	C+	1	1	1	3	3	3
	TS–	2	2	0	4	4	4
	TS+	6	6	0	12	12	12
	TS-HPV	1	0	1	2	2	2
2	C+	1	1	1	3	3	3
	TS–	2	2	0	4	4	4
	TS+	6	6	0	12	12	12
	TS–HPV	1	0	1	2	2	2
3	C+	1	1	1	3	3	3
	TS–	3	3	0	6	6	6
	TS+	6	6	0	12	12	12
	TS-HPV	1	0	1	2	2	2
4	C+	1	1	1	3	3	3
	TS-	3	3	0	6	6	6
	TS+	7	7	0	14	14	14
	TS-HPV	1	0	1	2	2	2
Total		**43**	**39**	**8**	**90**	90	**90**

Two sectors or "work areas" slightly larger than the size of a 18 x 18 mm cover slip were demarcated on each slide using a wax Pap Pen® (Figure 6.4). The work areas were placed as far apart as possible on the slide. The Pap Pen dispensed a hydrophobic wax line that served to contain small volumes of reagent solutions flooded onto the work area surface of the smear. This technique allowed economic use of small volumes of reagents. The method also allowed the two work areas on the same slide to be exposed to different probe reagents during the hybridization step of the procedure without contaminating one another. For the PS+ and PS , one area was a test area to be probed for Chlamydial DNA using CHLT-DIG-1[®]. The other sector was a negative control area probed with non-Chlamydial probe PLD043[®]. The Pap

smear HPV slides were stained in one sector with Kreatech HPV-specific probe HPV-DIG-1® and the other with PLD043®.

Step 1b: Preparation of Tissue Sections

Two serial sections were mounted side by side on one subbed (Kreatech organosilane) glass slide, one for testing with CHLT-DIG-1® and the other for probing with the non-Chlamydial probe PLD043®. These sections were placed as widely apart as possible so that separate treatments could be applied without contamination by the probes and other reagents occurring between the sections. The sections were incubated at 60°C for 12 hours (over night) to ensure best possible adherence to the glass slides. Sections were de-waxed in two changes of fresh xylol for ten minutes each, soaked in 100 % ethanol for five minutes and air-dried in an oven at 60 °C for 30 minutes. Using a Pap Pen®, hydrophobic wax lines were drawn around the section to delineate a square work area measuring approximately 18mm x 18mm, the size of a small cover-slip. If the section was larger than $18mm^2$, an appropriate part of the section was selected as the working area, ensuring that epithelium of the endocervix, transitional zone and ectocervix was included in the work area. The serial H & E sections were used to identify the portion of the section that had to be included in the work areas.

As for PS, the continuous linear deposit of hydrophobic material around the work area formed a trough that allowed use of small volumes of reagents to be used on the TS. The two working areas on the same slide could be exposed to different reagents during certain steps of the procedure without cross-contamination of one work area by reagent from another. The work area over the TS most distant from the frosted end of the slide was a test area to be probed with CHLT-DIG-1® for Chlamydial plasmid DNA, the other a negative control area probed with PLD043®.

Step 2: Pre-digestion of Pap smears and Tissue Sections

The smears and sections were hydrated in distilled water. On the PS, the test and control areas were flooded with a solution of 0.1 mg/ml pepsin in 0.01N hydrochloric acid and cells were pre-digested for 10 minutes at 37°C on a Fisher® slide warmer. The slide warmer had a cover made of perspex so that a cloud of moisture formed over the smears and moisture loss was minimised (Figure 6.5). When necessary, if evaporation of the reagent during the warming had been marked, additional pepsin reagent was applied after five minutes. The TS underwent a harsher pre-treatment proteolysis step than the PS. The pre-digestion solution contained 2.5 mg pepsin per ml 0.1 N hydrochloric acid and was applied for 30 minutes. The test and control areas were flooded with 350 µl of the proteolytic solution and incubated on the Fisher® slide warmer at 37 °C for 30 minutes. When necessary, additional pepsin reagent was applied, sometimes every five minutes. After the pre-digestion step, the PS and TS slides were dehydrated using graded ethanols, three washes for one minute each, and air-dried.

Step 3: Denaturation and Probing of Pap smears and tissue sections

Two drops of probe solution CHLT-DIG-1® were dropped onto cover slips and the slips were placed over the test areas (Figure 6.6) of the PS+, TS–, TS+, TS–, MC+ and TSC+ slides. By lowering the inverted slides onto the cover-slips, the cover-slips was picked up by capillary action of the probe solution. The cover-slips were placed so that the working area

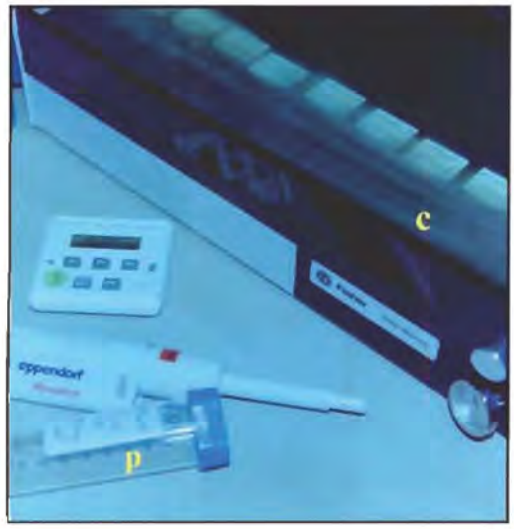


Figure 6.5. The Fisher® slide warmer had a perspex cover so that a pale cloud (c) of moisture formed over the smears and moisture loss was minimised. If evaporation of the pepsin (p) during the warming had been marked, additional reagent was applied after five minutes.

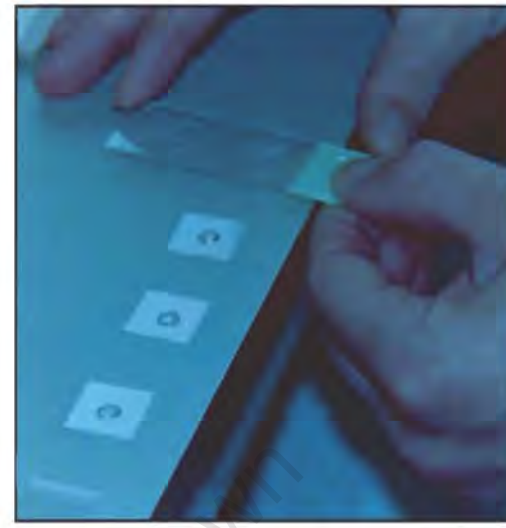


Figure 6.6. Two drops of the probe solution CHLT-DIG-1® were dropped onto 18 x 18 mm cover slips. The slips were placed over the appropriate test area on the slide. The slides were inverted and used to pick up the slips using surface tension.

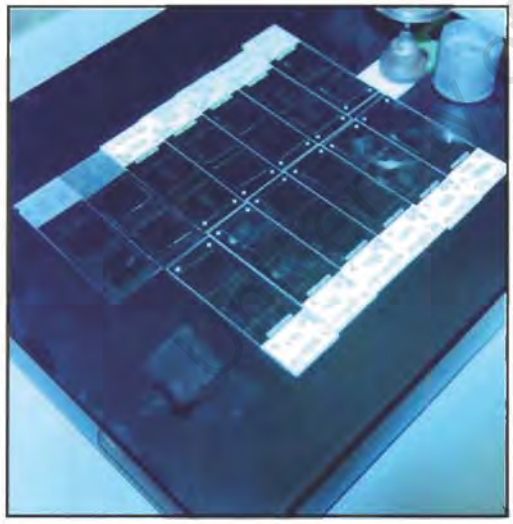


Figure 6.7. The slides with PS or TS were placed on a heating block for *precisely* three minutes at *exactly* 95°C to denature the target DNA. The Fisher® heating block could maintain a stable temperature. The number of slides per run was limited to 14 (here 13 were in place).

Figure 6.8. The temperature of the Fisher® heating block was monitored with a Reuger® surface thermometer. The sensor of the thermometer was placed on a glass slide (s) to obtain an accurate indication of the temperature attained by the slides being heated.

of the test section was flooded with the solution and covered by the cover-slip. Similarly, two drops of probe solution HPV-DIG-1® were placed over the test area of the PS- HPV and TS–HPV slides. On all slides, the negative control area was covered in the same way with PLD043® probe. The cover slips served to prevent drying out of probe solution during exposure of the slide to the high temperatures necessary during the step to denature target DNA. The slides with PS or TS were placed on a heating block for *precisely* three minutes at *exactly* 95°C to denature the target DNA (Figure 6.7). This Fisher® heating block could maintain a stable temperature that was monitored with a surface thermometer (Reuger®). The temperature sensor of the thermometer was placed on a glass slide to obtain an accurate indication of the temperature attained by the slides being heated (Figure 6.8).

Step 4: Hybridization

After the slides were removed from the heating block, they were immediately incubated at 37°C for 120 minutes to allow probe to anneal and hybridize with target DNA. To keep the slides moisturised during this process, the slides were placed in a specially designed covered tray (Kreatech). Tissue paper soaked in distilled water was placed under the slides that were supported on racks (Figure 6.9). After the hybridization step, the cover slips were removed by soaking the slides in TBS buffer (Figure 6.10) for 10 minutes. Although the cover slips frequently dropped off the slide once they were placed in the buffer solution, it was necessary to mechanically remove some using a needle. The slides were washed in three changes of TBS buffer for one minute each.

Step 5:Applying stringency conditions

Stringent conditions were applied to ensure that only specific Chlamydia/CHLT-DIG-1® hybrids and HPV/HPV-DIG-1® were retained in the smears and sections. Using the Kreatech dropper bottle, five or six drops of the differentiation reagent BC0013® were applied to each work area. The differentiation wash used comprised of 12 % formamide and 0.01 SSC pH 7.0, and was applied for precisely 15 minutes at exactly 37°C on the heating block. To keep the specimen flooded, additional differentiation reagent was applied when/if necessary during the process. Subsequently, slides were washed three times for one minute in TBS buffer.

Step 6: The detection system

The detection system used alkaline phosphatase (ALK-PHOS), α-digoxigenin (α-DIG), biotin (B) and nitro-tetrazolium blue (NBT). Two or three drops of ALK-PHOS-conjugated α-DIG/B (BCIP) component in the detection system were applied by Kreatech dropper bottle. The specimens were incubated for 30 minutes at 37°C on the heating block. Slides were soaked in TBS buffer three times for one minute, and then soaked in de-ionized water for one minute. Two or three drops of NBT/BCIP substrate were applied to each specimen (Kreatech dropper bottle) and the slides were incubated for 15 minutes in the dark (covered with a lid of the Kreatech moisture tray) at 37°C on the heating block. The excess substrate solution was tipped off the slides and they were washed in de-ionized water, three times for one minute each and air-dried.

Step 7: Mounting

Smears and sections were mounted using glycerol-gelatin heated to 95 °C on the heating block. The 18 x 18 mm cover slips were placed on the heating block and two drops of glycerol-gelatin was placed on the cover slip. The slides were warmed to 60 °C inverted, and brought down to pick up the hot cover slips by means of the surface tension in the drop of molten glycerol gelatin. The slides were placed momentarily on the hot surface while the cover slips were positioned precisely, one over each demarcated work area on the slide. The mounted slides were allowed to cool undisturbed. This technique of heating the cover slip and glycerol-gelatin to 95 °C prevented bubbles from forming in the mounting medium.

Laboratory technique

Although precise pipetting and time measurements were essential, once perfected, the hybridization technique was simple to perform and required six hours. The number of slides processed at a time was limited to a maximum of 14 (most runs comprised of ten or twelve slides). This was dictated firstly by the space available on the heating block (Figure 6.8). Secondly, each slide had to be placed upon and removed from the heating block individually. It was necessary to ensure that each slide was on the heating block (or the slide warmer) for exactly the correct time. Timing commenced when the first slide was placed on the block/warmer and the time taken to place the subsequent slides on the heating surface was regulated to an interval of 2 seconds per slide. The first slide was removed after the exact time required had elapsed and the subsequent slides had to be removed from the heating surface in the same order as they had been applied, at intervals of precisely 2 seconds.

To avoid burning the researcher's fingers, the slides were held at the frosted (label) end and placed onto the warmer, label end outwards, with the labeled area initially off the heating block. The demarcated test and control areas were immediately exposed to the hot surface. Slides were subsequently shifted to the centre of the block. The reverse process was used when the slides were removed. At all times the demarcated work areas were exposed to the hot surface for the full duration of the time required. This procedure, designed to ensure accurate control of the times that specimens were exposed to heat, required manual dexterity and took some practice.

Ninety separate tests were conducted on PS and a further 90 on TS. These tests included positive and negative controls, "housekeeping" controls and patient specimens regarded as Chlamydia positive or negative according to the morphologic criteria (Chapter 5). The tests were examined and interpreted blind and results subsequently evaluated. To enable 180 tests to be conducted, Kreatech supplied additional DISH kits for use at their laboratory in Amsterdam. No attempt was made by the management and staff at Kreatech to influence the outcome of the tests being performed there.

6.3.8 PCR TO TEST THE MORPHOLOGIC CRITERIA IN TISSUE SECTIONS AND TO FURTHER VALIDATE THE KREATECH DISH PROCEDURE.

The PCR testing was conducted by the researcher in the Cytology Laboratory at the George-August-University in Goettingen. The paired serial sections 6 & 7 of the positive and negative histology cases that had been subjected to DISH in Amsterdam were subjected to semi-nested PCR. If necessary the paired serial sections 8 & 9 were available for repeat testing.

The paired serial section numbers 6 and 7 of the 25 TS+ and the 10 TS- cases were subjected to PCR. The cases were tested in batches of between 7 and 9 cases. During testing, the cases were identified by their accession numbers only. All cases showing no Chlamydial amplification product in the first amplification cycle were subjected to the second cycle. In all, 61 PCR reactions were performed on test specimens and with controls, a total of 81 PCR reactions were conducted in ten days. The configuration of the tests performed is shown below (Table 6.7).

Table 6.7
PCR runs were conducted over ten days with between 7 and 9 reactions performed per run. A total of 101 reactions were performed. The second PCR sequence using the Sense 2 primer was conducted on 26 of the specimens that did not show reaction product after the first cycle. These 26 specimens included 16 TS+ and 10 TS -. Positive and blank (*aqua bi dest)* controls were also subjected to the PCR reaction.

Run	Controls		Specimens	Primer		No of Tests
	+	–		Sense 1	Sense 2	
1	1	1	6	x		8
2	1	1	6	x		8
3	1	1	6	x		8
4	1	1	6	x		8
5	1	1	6	x		8
6	1	1	5	x		7
Sub-total	6	6	35			47
7	1	1	6		x	8
8	1	1	6		x	8
9	1	1	7		x	9
10	1	1	7		x	9
Total	10	10	61			81

The Pap smears were not tested by PCR because material available for testing on any single patient was limited to one smear. Once the smear had been used for DISH, little or no material remained that was un-exposed to the Chlamydial probe. One possible solution was to re-use the probed cells by scraping them from the smear and extracting DNA for PCR. However, the DISH probe was directed at the cryptic Chlamydial plasmid and the sequence of the target DNA was regarded by Kreatech as a copyrighted trade secret. Re-use of the cells exposed the experiment to the risk of amplification of probe sequences and possible false positive results. Re-use of cells from PS would leave no reserve material for repeated DNA extraction if necessary for technical reasons. Further, the record of the DISH testing would be lost. Another alternative was to select different PS for PCR testing, but such an experimental design would negate the concept put forward by Black[1] of using an "expanded" gold standard on the same specimen.

It was the *morphologic criteria* that were being tested. Since the morphologic criteria for Pap smears and tissue sections were the same, it was decided that PCR testing of tissue sections alone was sufficient. Corroboratory testing in tissue sections would serve both to validate the DISH method and constitute the second component of the "expanded" gold standard. If there was significant concordance of the PCR and DISH results in tissue sections, there could be no reason to suggest that a similar concordance would not be obtained if Pap smears were also subjected to PCR.

6.3.8.1 EXTRACTION OF DNA FROM TISSUE SECTIONS

Introduction

The extraction of DNA from the tissue sections was probably the most critical step in the PCR validation process. The PCR test can only be as good as the DNA extract obtained. During initial training in the PCR method, un-mounted tissue sections were used to prepare DNA extracts.[19] In this method, sections were put into test tubes with 5 ml xylol to extract the paraffin wax. The tubes were incubated overnight at 55 °C in a hot water bath with a rocking platform for continuous agitation. The xylol was removed from the specimen with two washes of alcohol using the vortex mixer. The tissue was homogenised in a sterile microbiological tissue homogeniser. The process was lengthy and required a clean homogeniser for each specimen.

The tissue samples to be subjected to PCR were transported to Goettingen as un-stained dehydrated paraffin wax sections mounted on clean glass slides. Preparation of the tissue for DNA extraction from sections mounted on glass slides was also possible and proved to be a more practical method.[20] The xylol extraction took 30 minutes instead of overnight incubation. The sections were scraped from the slides with a clean scalpel blade and did not require homogenisation.

Step 1: Removal of paraffin wax

To remove the paraffin wax, the slide bearing the serial tissue sections numbers 6 & 7 from the 25 TS+ and 10 TS- were placed in glass histology racks. The sections were agitated in 3 changes of clean xylol for 10 minutes each. The xylol was removed by washing three times for 10 minutes in absolute ethanol. The sections were air-dried in an oven at 60° C for 10 minutes. The steps were meticulously applied to ensure that all of the paraffin and all of the xylol were removed since both compounds are known inhibitors of the PCR reaction.[17,19]

Step 2: Tissue digestion

The de-paraffinised, xylol-free tissue was scraped from the surface of the slides using a sterile surgical scalpel blade.[20] A new sterile blade was used for each specimen to prevent cross-contamination between specimens. The scrapings were placed in a 2 ml sterile capped Eppendorf cup® for the tissue extraction process. The fragmented tissue did not require further homogenisation. The DNA was extracted from the tissue fragments using the tissue protocol for the QIAamp® DNA Mini-kit[21] (Appendix H). The tissue (up to 25mg) was mixed with 180 µl ATL buffer (with detergent) and 20µml of the Protease K solution, both provided with the kit. The mixture was thoroughly mixed using a vortex mixer, then incubated at 56 °C for 90 minutes in a water bath with a rocking platform for continuous agitation. If complete lysis of the tissue was not obtained after 90 minutes, incubation was continued until no fragments of tissue remained.

Step 3: DNA extraction

After lysis of the tissue, the tube was centrifuged at 1200 rpm for one minute to bring fluid that had condensed on the lid of the tube down into the specimen. The next step was to suspend the tissue in 200 µl AL buffer[21] by thorough pulsed mixing on the vortex mixer for 15 seconds until the solution was homogeneous. If a white precipitate developed at this point, the mixture was incubated at 70 °C in a water bath with a rocking platform for continuous agitation until the

precipitate dissolved. Absolute ethanol 200 µl was added to the tissue sample and mixed by pulsed vortex for 15 seconds. The tube was centrifuged again at 1200 rpm for one minute to remove condensate from the lid of the tube. If a white precipitate formed at this point, all of the precipitate was processed in the next step.

The mixture was transferred using a micropipette and sterile tip to a QIAamp spin column® containing glass wool fibres and supported in a 2 ml collection tube. To avoid contamination of the sample, care was taken during the transfer and at all times in the subsequent process that the rim of the spin column was not wetted by the sample. The cap on the spin column was closed and the column within the collection tube centrifuged at 8 000 rpm for one minute. In the conditions provided by AL buffer and ethanol, tissue DNA remained adherent to the glass wool. The collection tube containing the buffer fluid and alcohol was discarded and the spin column containing the DNA extract was transferred to a clean 2ml collection tube.

Step 4: Washing the DNA

The spin column was carefully opened and 500 ml of QIAamp buffer AW1[21] was added by micropipette to wash the extracted DNA still adherent to the glass wool. Once more to avoid contamination, the rim of the spin column could not be wetted. To remove the washing buffer, the closed spin column in the clean collection tube was centrifuged at 14 000 rpm for three minutes. After centrifugation, the spin column was transferred to a clean collection tube and the collection tube containing the AW1 washing buffer was discarded. The cap of the spin column was carefully opened and a second wash using 500µl of buffer AW2[21] was added by micropipette, again avoiding contamination of the rim of the spin column. The cap was closed and the column and collection tube were centrifuged at 14 000 rpm for three minutes. The collection tube containing buffer AW2 was discarded. The spin column was placed in a clean collection tube and centrifuged again at 14 000 rpm for a further minute. Any residual AW2 buffer was discarded.

Step 5: Eluting the DNA

The QIAamp spin column® was finally placed in a 2ml collection tube, 200 µl of eluent buffer AE[21] added and the loaded column incubated at room temperature for 5 minutes. The spin column was centrifuged at 8 000 rpm for one minute and the fluid containing the DNA retained. The spin column was eluted the same way for a second time using a further 200 µl aliquot of buffer AE[21] in to a second 2ml collection tube. The two eluates were combined and stored at –20 °C.

6.3.8.2 THE PCR PROTOCOL

The PCR method in use at the Georg-August-University was a semi-nested procedure using three oligo-nucleotide primers selected for a DNA sequence in the cryptic plasmid common to *Chlamydia trachomatis* species.[22] The "semi-*nested*" method was used to improve the specificity and sensitivity of the test. When necessary, a second PCR cycle was performed on the amplicons from the first amplification, using a second primer specific for a shorter sequence occurring within the confines of the first sequence. However, in the circumstances where a second amplification is performed, manipulation of PCR products from the first PCR theoretically increases the chance of contamination. If a result was obtained on the first amplification cycle, the second PCR cycle was not necessary. At the time of making this decision,

the specimens were identified by their laboratory accession numbers only. The result of the previous DISH testing was not revealed until the outcome of the PCR had been determined and recorded.

Primers

The Chlamydial primer sequences were selected from the Chlamydial cryptic plasmid pCT.[23] The first open reading frame (ORF 1) codes for a dnaB-like protein "pCTT1" present in serotypes A-D *Chlamydiae*.[22,23] This bacterial plasmid is a constant part of the bacterial genome and is highly preserved with less than 1% nucleotide substitution.[24] On only three occasions have *Chlamydiae* without this plasmid been isolated.[23] Use of PCR primers for this plasmid confidently predicts detection of the full spectrum of *Chlamydia trachomatis* species.[25]

Chlamydial plasmid pCTT1 sequence

A portion of the published DNA ORF1 sequence[22] coding for the dnaB like protein by the cryptic Chlamydial plasmid "pCTT1" is shown below:

Chlamydial plasmid ORF1 pCTT1[22]

```
   1  cctatccgca aaatgtcctg attagtgaaa taatcaggtt gttaacagga tagcacgctc
  61  ggtatttttt tatataaaca tgaaaactcg ttccgaaata gaaaatcgca tgcaagatat
 121  cgagtatgcg ttgttaggta aagctctgat atttgaagac tctactgagt atattctgag
 181  gcagcttgct aattatgagt ttaagtgttc ccatcataaa aacatattca tagtatttaa
 241  atacttaaaa gacaatggat tacctataac tgtagactcg gcttgggaag agcttttgca
 301  gcgtcgtatc aaagatatgg acaaatcgta tctcgggtta atgttgcatg atgctttatc
 361  aaatgacaag cttagatccg tttctcatac ggttttcctc gatgatttga gcgtgtgtag
 421  cgctgaagaa aatttgagca atttcatttt ccgctcgttt aatgagtaca atgaaaatcc
 481  attgcgtaga tctccgtttc tattgcttga gcgtataaag ggaaggcttg atagtgctat
 541  agcaaagact ttttctattc gcagcgctag aggccggtct atttatgata tattctcaca
 601  gtcagaaatt ggagtgctgg ctcgtataaa aaaaagacga gcagcgttct ctgagaatca
 661  aaattctttc tttgatgcct tcccaacagg atacaaggat attgatgata aaggagttat
 721  cttagctaaa ggtaatttcg tgattatagc agctaggcca tctataggga aaacagcttt
 781  agctatagac atggcgataa atcttgcggt tactcaacag cgtagagttg gtttcctatc
 841  tctagaaatg agcgcaggtc aaattgttga gcggattgtt gctaatttaa caggaatatc
 901  tggtgaaaaa ttacaaagag gggatctctc taaagaagaa ttattccgag tggaagaagc
 961  tggagaaaca gttagagaat cacattttta tatctgcagt gatagtcagt ataagcttaa
1021 tttaatcgcg aatcagatcc ggttgctgag aaaagaagat cgagtagacg taatatttat
1081 cgattacttg cagttgatca actcatcggt tggagaaaat cgtcaaaatg aaatagcaga
1141  tatatctaga accttaagag gtttagcctc agagctaaac attcctatag tttgtttatc
1201  ccaactatct agaaaagttg aggatagagc aaataaagtt ccatgctttc agatttgcga
1261  gacagcggtc aaatagagca agacgcagat gtgatttgt
```

The sequence of the sense 1 primer was 5′-**aggcttgatagtgctatagc**-3′, and the sequence of the anti-sense primer was 5′-**ttatcatcaatatccttgtatcc**-3′. The sequence of the second (semi-nested) sense 2 primer was 5′-**ctattcgcagcgctagagg**-3′. The first primer combination yielded a 188 base pair (BP) reaction product and the semi-nested primer combination resulted in a 157 BP product.[22] The primers were commercially manufactured by MWG Biotech, Munich, Germany.

The sites on the genome that were selected for the primers are highlighted in colour. The sense 1 primer is shown in red, the anti-sense primer region in pink, and the sense 2 primer in blue. The sequence shown in purple is identical to the sequence (Figure 6.11) of the amplicon sequenced from the agarose gel to verify the specificity of the PCR procedure (*vide infra* **6.3.8.3**).

The sequence underlined is the 188 BP amplicon produced in the first amplification procedure. By convention, the sequence of the anti-sense primer is written 5′ to 3′ and is the sequence of the complementary strand. Thus, the sequence highlighted in pink below from the plasmid sequence is in the reverse order and is the complimentary sequence of bases to those of the primer itself. The primer sequence is reflected in black, and is read from right to left (5′ to 3′):

5′ **gg atacaaggat attgatgata a** 3′
3′ cc tatgttccta taactactat t 5′

The master mix

The PCR master mix was made with reagents supplied by Perkin-Elmer[26] and Pharmacia.[27] The mix included:

PCR buffer:	$MgCl_2$ 2.75mM, 500mM KCl, 100mM Tris-HCl, at pH 8.3, (Perkin-Elmer)	5.0 µl
dNTPs:	10µM of each (Pharmacia)	4.0 µl
Taq DNA polymerase:	1.5 units (Perkin-Elmer)	0.3 µl
Primer sense:	40 pM	0.4 µl
Primer anti-sense:	40 pM	0.4 µl
DNA extract:		10.0 µl
H_2O bi distilled (HPLC):		30.0 µl
Total volume:		50.0 µl

Controls

The positive controls used DNA extracted from Chlamydia cultured in McCoy cells. The presence of Chlamydia in the McCoy cells had been confirmed by EM (Figure 16.2). Negative controls were mixed with twice-distilled water replacing the DNA extract. A "housekeeping" or genomic control, was not used. The housekeeping control most widely used is for human β-globin. The stability of the bacterial plasmid DNA differs from the stability of eukaryotic human chromosomal DNA because the plasmid DNA is super-coiled. A positive PCR result for human β-globin or any other human DNA sequence would not establish that the PCR conditions had been appropriate for super-coiled plasmid DNA.

Amplification sequence

The chain reaction was conducted using a Perkin Elmer thermal-cycler (DNA Thermal Cycler 480). Following an initial denaturing step at 95° C for 7 minutes, DNA was amplified 40 times in an amplification series consisting of a 45 second melting step at 95° C, an annealing step at 55° C for 30 seconds and a synthesis step at 72° C for 60 seconds. The PCR was completed with an extension step at 72° C for 240 seconds. The PCR product was demonstrated by electrophoresis

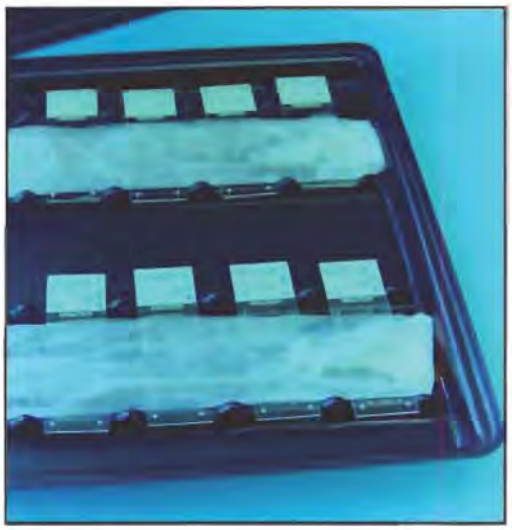


Figure 6.9. Slides were incubated for 120 minutes at 37°C to allow probe to hybridize with target DNA. The slides were placed in a Kreatech covered tray with tissue paper soaked in distilled water placed under the slides to keep the slides moisturised during incubation.

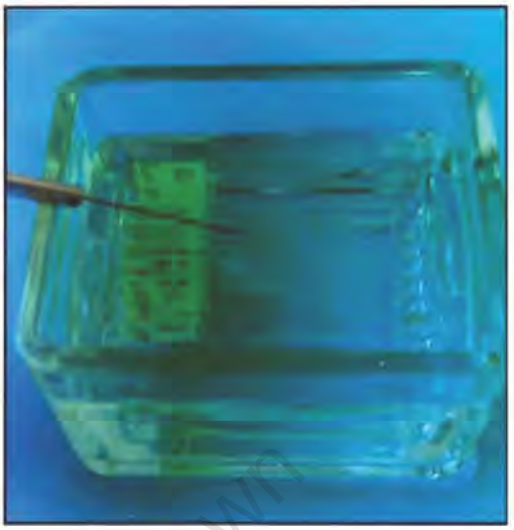


Figure 6.10. After hybridization, the cover slips were removed by soaking the slides in TBS buffer for 10 minutes. Once slides were placed in the buffer solution, the cover slips frequently dropped off unaided. It was sometimes necessary to mechanically remove the slips using a needle.

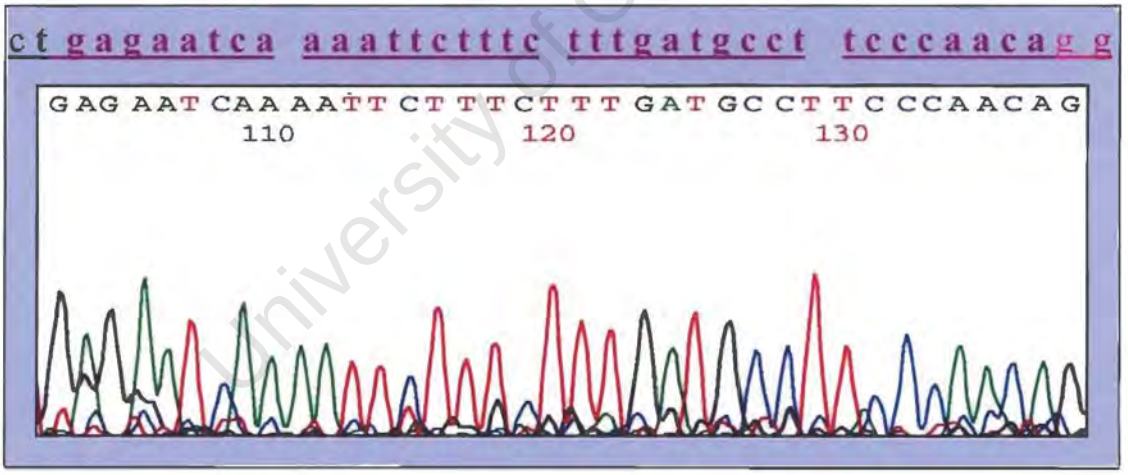


Figure 6.11. The sequence shown in purple above is taken from the portion of the published DNA ORF1 sequence[22] coding for the dnaB like protein by the cryptic Chlamydial plasmid "pCTT1." Below is the print out of the sequence of the amplicon determined by the DNA Analyser (Model ABI310, Applied Biosystems). The published sequence is identical to the sequence of the amplicon taken from the agarose gel. The specificity of the PCR procedure was verified.

induced by ethidium bromide in the gel and consequently bound to the DNA fragments. The purified PCR product of interest could be identified by comparison of its molecular weight with the migration ladder of DNA fragments of known molecular weight.

6.3.8.3 VALIDATION OF THE PCR METHOD

The method was initially set up using positive control Chlamydial DNA extracted from Chlamydial cultures in McCoy cells. The presence of *Chlamydiae* in the McCoy cells was verified by demonstrating the organism using EM (Figure 6.2). The extract was diluted 1: 100 000 and subjected to 40 cycles of PCR. The expected 188 BP product was detected by electrophoresis in agarose gel using ethidium bromide-induced fluorescence in UV light. Subsequently, a piece of agarose containing the purified PCR product was excised from the gel and placed in a clean Eppendorf reaction tube. The product was subjected to DNA sequencing (DNA Analyser Model ABI310, Applied Biosystems). The sequence of the PCR reaction product was confirmed to have the target sequence. A portion of the print-out from the sequencer shown previously (Figure 6.11) was identical to the anticipated sequence. The segment of the sequenced amplicon in Figure 6.11 lies between BP 100 and BP 130 of the amplicon, and reads:

"tgagaatca aaattctttc tttgatgcct tcccaacagg."

The same sequence is highlighted in purple in the DNA sequence for the cryptic Chlamydial plasmid pCTT1 (*vide supra* **6.3.8.2**):

ctgagaatca aaattctttc tttgatgcct tcccaacagg.

The DNA extract derived from the McCoy culture was used as a positive control in each of the PCR runs conducted during the testing of the specimens to corroborate the DISH results.

6.3.8.4 PROCEDURES & TECHNIQUES IN THE PCR LABORATORY

Since the PCR technique hugely amplifies the number of DNA copies, contamination of specimen by even one copy of exogenous DNA can cause a false positive result. Contamination of the PCR laboratory environment with vast numbers of copies of amplified DNA can be a serious problem. To address these issues, the principles of good laboratory practice in PCR were applied.[22]

Separate work areas

Four separate work areas were used with procedures performed in each work area precisely prescribed. Progression of the test material from one room to the next room was strictly controlled. The tests batches were taken through the steps of the PCR procedure in complete compliance with the prescribed sequence. Once a step in the procedure was commenced or had been completed, reaction tubes or their contents were never taken back to a room used for steps earlier in the sequence. The equipment and consumable supplies necessary for the procedures occurring in each room were kept in that room.

Room 1

DNA extraction from specimens occurred in room one. Here, care was taken to prevent cross contamination of DNA-containing material from one specimen to another. The
thermal cycling was also performed in this room. The closed Eppendorf reaction tubes undergoing thermal cycling were *never* opened in this room.

Room 2

Preparation of the master mix was performed in room two. Apart from oligonucleotide primers and purine and pyrimidine bases, no DNA-containing solutions were ever introduced into room two. In this room aliquots of the master mix were pipetted into the reaction tubes, mixed with water and the reaction tubes closed. Since a semi-nested technique was in use, reaction tubes for a possible second round of amplification were prepared at the same time. A third oligonucleotide primer was used (semi-nested) and the master mix for the second amplification sequence was stored in room one at –20°C until required.

Room 3

DNA was added to the reaction tubes in room three. Several techniques were used to reduce the possibility of DNA contamination occurring in this room (*vide infra*). Once the batch of reaction tubes had been prepared for amplification and closed, they were returned to work room one to be amplified.

Room 4

Separation of DNA fragments using electrophoresis, preparation of the agarose electrophoresis gels, exposure of the gels to UV light to induce fluorescence and photographic documentation of the outcome of the electrophoresis were performed in room four. Reaction tubes containing high numbers of DNA amplification products were only ever opened in room four.

Pipetting and handling techniques

Special attention was paid to pipetting and handling techniques in each work-room. Apart from the necessity for accurate, high precision pipetting, special handling techniques were used to prevent cross contamination between specimens, controls and amplified product. Autoclaved DNA-free micropipette tips with cotton plugs in the proximal (wide end) of the tip were used. The cotton plug prevented material being aspirated from the tip into the micropipette if air was inadvertently aspirated. They were picked up from their sterile container by inserting the micropipette into the wide end. The selected tip was tapped firmly against the bottom of the tip-container using the micro-pipette to ensure a secure seating of the tip on the micro-pipette was established before the tip was removed from the container. Once used, the tips were ejected from the micropipette using the built-in mechanical ejector. The tips were discarded into containers containing chlorine bleach that denatured any residual DNA in the tip. Tips were never touched or handled in any other way.

Handling reaction tubes

When opening reaction tubes, care was taken to prevent aerosolisation of any droplets on the under-surface of the lid. Further, since micro-volumes were used, loss of a single droplet from the reaction tube could significantly alter the dynamics of the reaction. Tubes could be handled differently in the different work-rooms. The functions of DNA extraction from specimens and thermal cycling were carried out in room one. To prevent contamination of specimens and DNA extracts, reaction tubes on the cycler were never opened in room one.

In room two, where the master mix was prepared, the contents of all reaction tubes was intended to be the same. One pipette tip could be used to dispense each reagent into all of the tubes. It was not necessary to open one reaction tube at a time. The reaction tubes were opened in the rack. To add a reagent, the tube was lifted from the rack in the left hand and brought to eye level for accurate pipetting technique. The micropipette was operated with the right hand. Once processed the tube was replaced in the rack. As each reagent was added a space in the rack between the previously processed tubes and the unprocessed tube served to avoid confusion. Again careful examination of a processed tube revealed more content than an unprocessed tube.

In room three, where different DNA solutions were added to reaction tubes and where cross contamination could occur, only one tube was opened at a time. While being processed, the tube was held between thumb and middle finger of the left hand, with the hinge of the lid placed on the side away from the index finger. The tip of the index finger was used to flip the lid up taking care to prevent aerosol formation. The right hand held and controlled the micropipette. Once processed, the tube was closed firmly and returned to the rack prior to the next being opened. Again confusion between processed and unprocessed tubes was prevented by keeping a space in the rack between the processed tubes and unprocessed tubes, and by comparison of the volume of content in the reaction tubes.

Contamination of negative controls with spurious DNA would negate the control function of that reaction tube. These were pipetted first. Similarly, contamination of either negative control tubes or specimen test tubes with DNA from the positive control would result in false positive tests and negate the function of the negative control. The positive control was therefore always processed last. The test specimens were processed after the negative control and before the positive control. This strategy served to avoid contamination of negative control with test material, and any other tubes with known positive material. Any reaction tube containing DNA solutions, especially positive control DNA, were always centrifuged prior to opening of the containers so that DNA was not present on the lid of the reaction tube. This step was necessary to prevent droplets containing DNA solution from being aerosolised when the tubes were opened.

In room three, each tube was processed using a new sterile tip. After the addition of DNA to the reaction tubes the DNA/master-mix combination (reaction mix) was stirred with the micropipette tip before the lid was closed. After the addition of DNA to each reaction tube, a drop of mineral oil was used to cover the surface of the reaction mix. The layer of oil served to prevent accumulation of steam and condensate on the lid of the reaction cup during the thermal cycling. The oil was dropped gently on to the top of the reaction mix using a micropipette. Care was taken to ensure that the

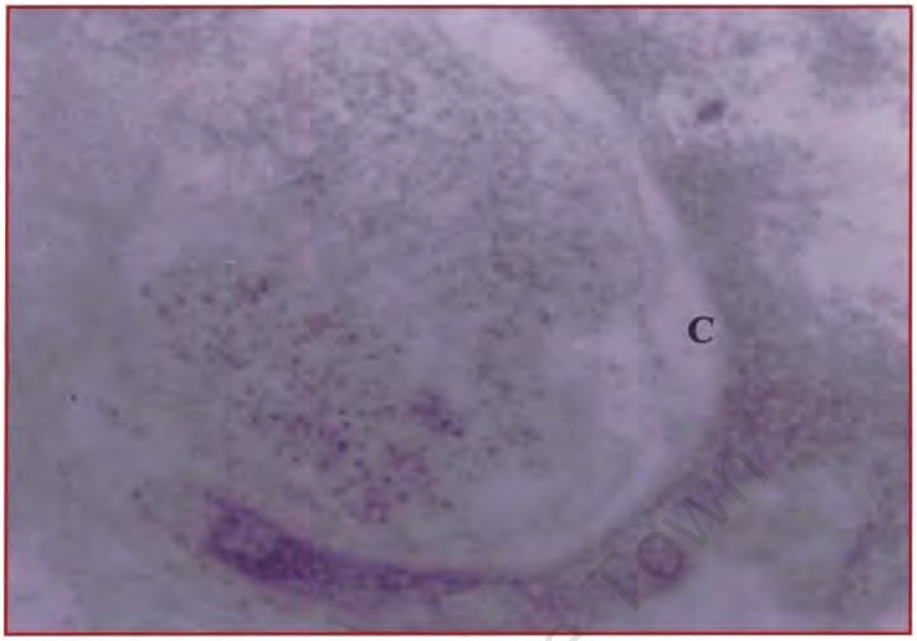


Figure 6.12. Electron micrograph of cells lifted from Pap smears showed poor preservation of cellular morphology. Cell membranes and organelles were identifiable but detail was not crisp. A possible Chlamydial vacuole (C) was identified but the content of the vacuole was degenerate. Although possibly Chlamydial, structures within the vacuoles could not be identified as Chlamydial with any degree of confidence.

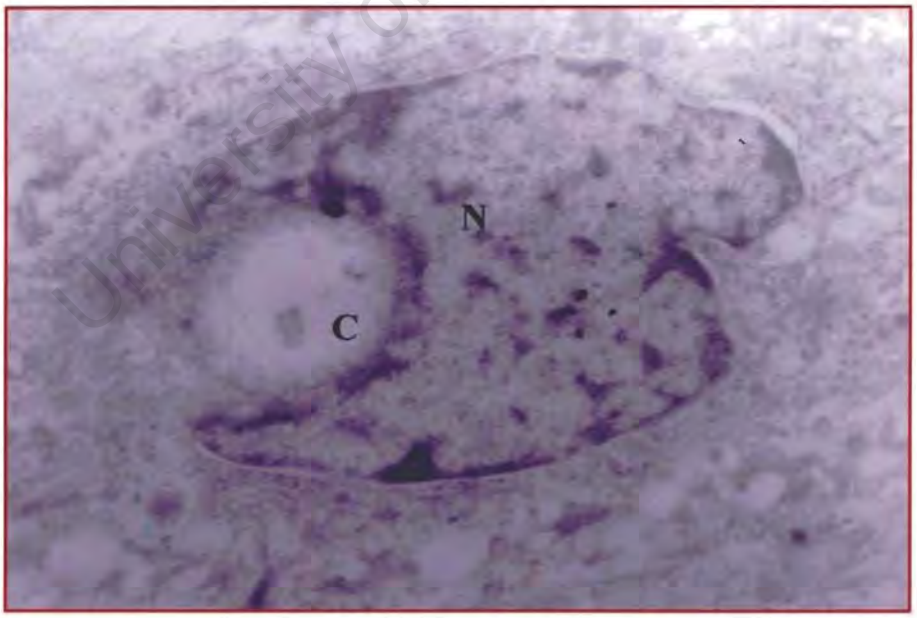


Figure 6.13. EM examination of ultra-thin sections from tissue blocks showed better preservation than that seen in cells lifted from Papanicolaou smears. The close relationship between the cell nucleus (N) with the Chlamydial vacuole (C) was similar to that shown in Swanson *et al.*[35] (Figures 3 & 4). Note the amorphous condensation lying centrally in the vacuole, similar to that seen in Figure 2 in the publication by Swanson *et al.*

micropipette tip did not touch the rim of the reaction tube during this process. After the addition of the oil layer, the reaction tubes were centrifuged to ensure layering of the oil over the aqueous phase.

After thermal cycling in room one, the reaction tubes were opened in room four. An aliquot of the reaction mixture was aspirated using a new tip for each specimen. The aliquot was carefully placed into the electrophoresis chambers in the agarose gel. Care was taken to prevent contamination of the gel or environment. The reaction tube was closed and the remaining mix stored at –20°C for one month. Fluorescent bands in the electrophoresis gels were photographed and the image printed immediately to record the result. When necessary, a piece of agarose gel containing the purified PCR product could be cut from the gel and subjected to DNA sequencing. Careful dissection technique was necessary to only remove gel containing the fluorescent band of interest. The excised gel and product was placed in a clean Eppendorf reaction tube. The purified PCR product of interest could be identified by comparison with the migration ladder of DNA fragments of known molecular weight. To prevent possible contamination of the lab by PCR products, gels were not retained after adequate photo-documentation had been made. The gels were discarded into waste disposal bags marked for incineration at a medical waste handling facility. Exposure of workers to the carcinogenic effects of ethidium bromide was also a consideration.

Protective clothing

During the entire process clean (sterile) latex gloves were worn. A new pair of gloves was used in each work room to prevent contamination of the controls and specimens by DNA from the operator's fingers. A different lab coat was worn in each room and lab coats were not transferred from room to room. The reaction tubes containing the mixes were carried from room to room in a clean rack. Care was taken to prevent contamination of the rack at all steps in the process. If a spill were to occur, the procedure was to decontaminate the room by thorough washing with bleach and alcohol solution. Alternative non-contaminated rooms in the laboratory would be designated for the affected work room. Care to avoid contamination of clothing, equipment, or the researcher's hands with ethidium bromide was necessary as the reagent is carcinogenic.

6.4 RESULTS

6.4.1 CULTURE OF *CHLAMYDIAE*

A total of 11 327 Pap smears was received in the practice during 1993. Amongst these, 74 cases (0.65 %) were identified as having features of infection by *Chlamydia trachomatis*. Thirteen of these 74 women (17.6 %) requested further testing to corroborate the morphologic diagnosis, and Chlamydial culture was offered to these patients. Seven patients (53.8 %) obtained a positive culture and six (46.2 %) a negative culture (Appendix G). The small numbers of tests precluded meaningful statistical analysis of this result. However, in this small group of patients, the correlation of cultures done because of a Pap smear finding was gratifying similar, if not better than the results achieved by other workers. [1,3,28-34]

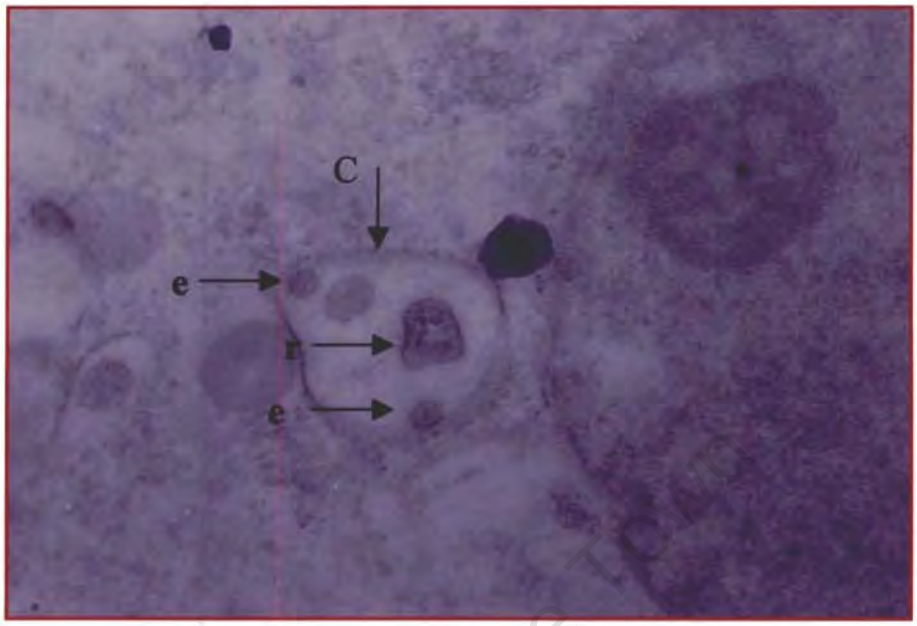


Figure 6.14. The inclusions within the vacuoles were identifiable as Chlamydial with reticulate (r), and elemental (e) bodies identified, similar to those seen in Figure 2 of Swanson *et al.*[35]

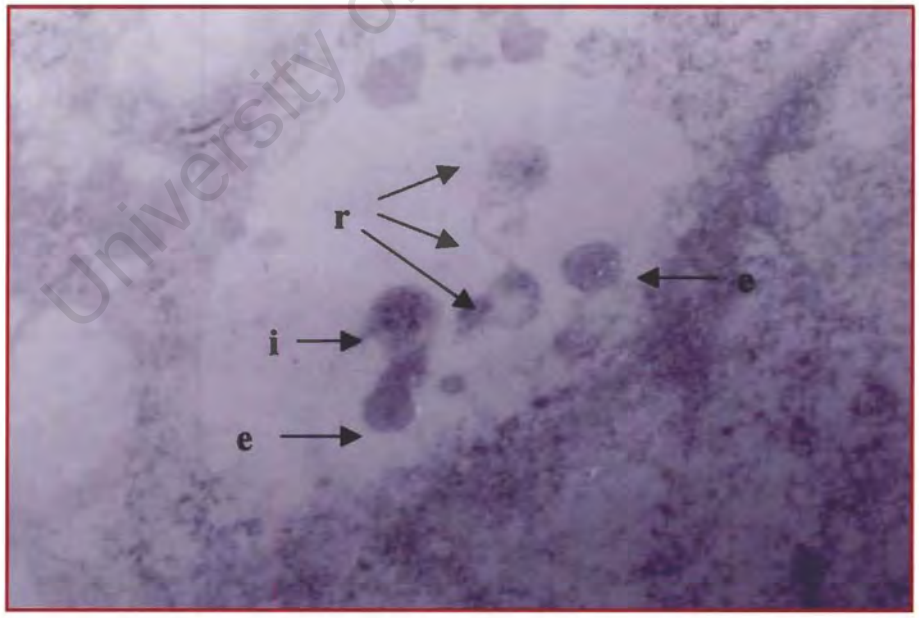


Figure 6.15. The tissue control block 7H 0139 showed Chlamydial elemental (e), intermediate (i) and reticulate (r) bodies.

6.4.2 FINDINGS USING ELECTRON MICROSCOPY

6.4.2.1 PAP SMEARS

Minute tissue fragments containing immature metaplastic squamous cells were successfully lifted from five Pap smears and after processing and ultra-microtomy examined under the electron microscope. The preservation of cellular morphology was poor. Cell membranes and organelles were identifiable but detail was not crisp (Figure 6.12). The Chlamydial vacuoles within the cells were identified but the content of the vacuoles was degenerate. Although the structures within the vacuoles were possibly Chlamydial in nature, it was not possible to identify Chlamydial organisms with any degree of confidence.

6.4.2.2 TISSUE SECTIONS

EM examination of ultra-thin tissue sections from nine tissue blocks showed better preservation than the material obtained from pap smears (Figure 6.13). Confident identification of Chlamydial organisms within vacuoles was possible in seven of the nine cases examined. The close relationship between the cell nucleus with the Chlamydial vacuole was similar to that shown in Figures 3 and 4 published by Swanson *et al*[35]

(Figure 6.13). The inclusions within the vacuoles were identifiable as Chlamydial with reticulate, intermediate and elemental bodies identified (Figure 6.14, 6.15), similar to those seen in Swanson *et al.*[35]

6.4.3 SETTING UP AND ESTABLISHING THE DISH METHOD

6.4.3.1 POSITIVE & NEGATIVE CONTROLS USING INFECTED MCCOY CELLS

The positive control slides with McCoy cells (MC+) infected with *Chlamydia trachomatis* stained positively when probed with the Kreatech Chlamydia-specific probe CHLT-DIG-1. Positive results were obtained using both the initial protocol P1 and the revised protocol P6. Positive staining was obtained with the reduced concentration of protease (0.1 mg/ml) and the reduced incubation time (10 minutes) for the pre-digestion step (Figure 6.16 A). When the procedure was applied using non-Chlamydial probe, PLD043, the known positive cell cultures did not stain (Figure 6.16 B).

6.4.3.2 ESTABLISHING POSITIVE CONTROL TISSUE FOR DISH (7H0139)

The tissue taken from hysterectomy specimen 7H0139 was shown to contain Chlamydial RB by EM at the University of Cape Town (Figure 6.15). Validation testing at Kreatech Laboratories showed positive staining of vacuolar inclusions within metaplastic squamous epithelium (Figure 6.17). During the validation of 7H0139, McCoy culture cells infected with *Chlamydia trachomatis* were used for the positive control. At Georg-August University, DNA extracted from serial sections of block 7H0139 was subsequently demonstrated to contain Chlamydial DNA by PCR. The control for this reaction was a DNA extract derived from Chlamydia-infected McCoy cells shown to contain Chlamydial inclusions by EM (Figure 6.2). Further, the PCR reaction product derived from 7H0139 was sequenced automatically using a DNA sequencer and shown to be homologous with the targeted sequence. Serial sections taken from block 7H0139 were used as a positive control for the DISH reaction.

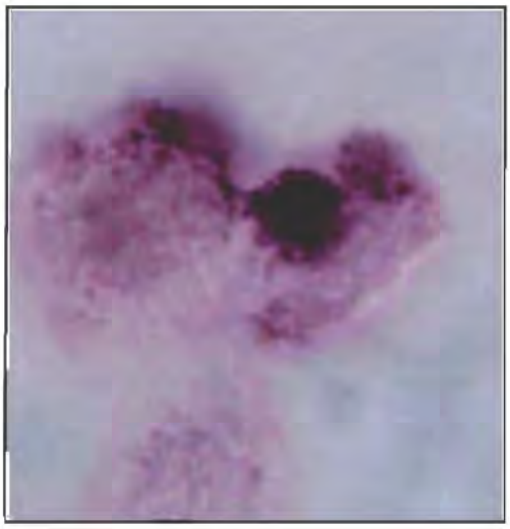


Figure 6.16 A. McCoy cells infected with *Chlamydia trachomatis* used for positive control Cells stained positively when probed with the Kreatech Chlamydia-specific probe CHLT-DIG-1. The background was clean and devoid of noise signal.

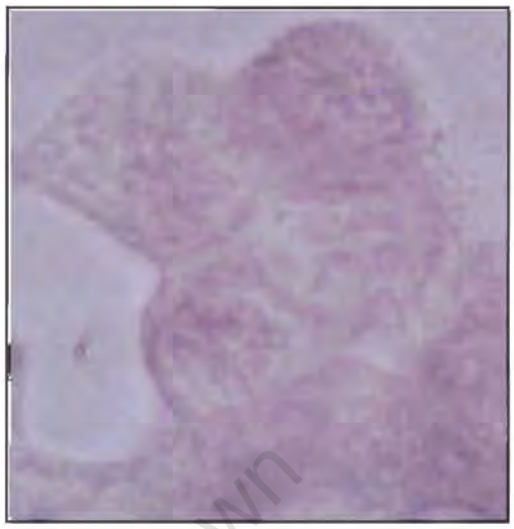


Figure 6.16 B. McCoy cells infected with *Chlamydia trachomatis* used as positive control. Cells did not stained positively when probed with the Kreatech non-Chlamydial probe PLD043.

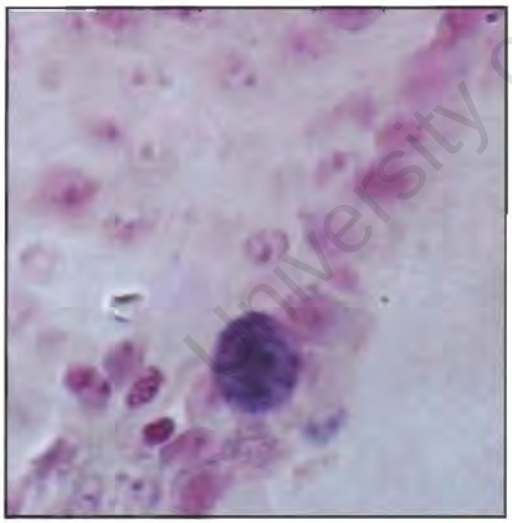


Figure 6.17. Tissue from hysterectomy specimen 7H0139 showed positive staining of vacuolar inclusions within metaplastic squamous epithelium. The background was clean and devoid of noise signal.

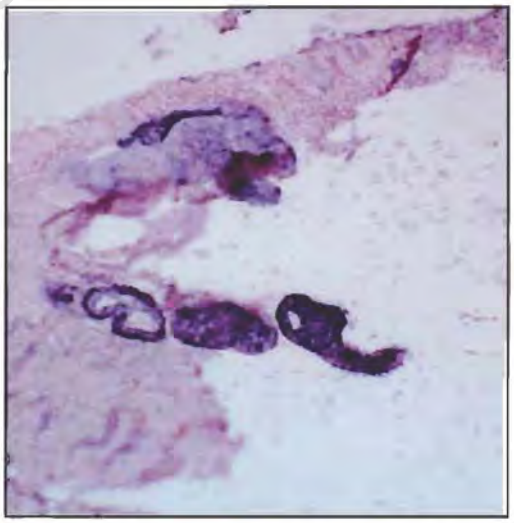


Figure 6.18. The technical quality of all tissue sections evaluated by the DISH method was acceptable. Where lifting of sections occurred, this was generally minimal and usually partial.

6.4.4 DISH TESTS

6.4.4.1 TECHNICAL EVALUATION OF PS AND TS QUALITY

The technical aspects of sections and smears subjected to the DISH staining process were assessed. Sections and smears were assessed for loss of tissue and for back ground staining (signal noise). The severity of the loss of cell groups in PS and tissue in TS was recorded and graded as absent, mild (+), moderate (++) or severe (+++) (Tables 6.8 & 6.9). Comparison of the background signal in the negative test area probed with PLD043 or HPV-DIG1 with the signal in the test area probed with CHLT-DIG-1 made it possible to assess background staining. The test areas generally showed very little background staining. When present, background staining was easy to identify and generally, it was possible to separate background from true positive staining. The background staining was graded as mild (+), moderate (++) or severe (+++) (Tables 6.8 &6. 9).

Pap smears

There was some loss of cellular material from most (if not all) of the Pap smear slides, but this was not excessive (Table 6.8). The loss of cellular material was obvious because cell groups of interest had been identified on the reverse side of the smear by scoring the glass with a diamond pencil. However, in all cases, sufficient cellular material remained for a proper interpretation of the DISH outcome. One PS- showed background staining that interfered with interpretation and was regarded as a possible false positive (*vide infra*). Unlike working with the tissue sections, when working with Pap smears, no spare material was available for repeated DISH testing when technical difficulties arose. Fortunately, technical difficulties did not necessitate rejection of any PS cases subjected to DISH.

Table 6.8

Twenty five PS+ and 10 PS- were subjected to DISH. 19 PS+ and 7 PS- showed mild loss of cellular material, 4 PS+ and 2 PS- showed moderate loss of cells while 2 PS+ and 1 PS- showed severe loss of cells. The Control smears (MC+, PS-HPV) showed similar rates of less cell loss. Background staining was mild in the majority of cases, but severe background staining was problematic in 1 PS-*. The outcome of DISH staining in all smears was interpretable.

Morphology of Smear	Number of Tests	Technical Quality of Smears					
		Cell loss			Background staining		
		+	++	+++	+	++	+++
PS +	25	19	4	2	19	3	1
MC +	4	1	2	1	4	0	0
PS –	10	7	2	1	5	3	2
PS– with other organisms	7	5	1	1	4	2	1
PS– sans other organisms	3	2	1	0	1	1	1*
PS–HPV/CIN	4	3	1	0	4	0	0
PS+ PS– PLD043	30	23	5	2	21	7	3
PS +, MC + HPV-DIG1	13	7	4	2	9	3	1

Tissue sections

The technical quality of sections in all TS cases evaluated by the DISH method was acceptable (Table 6.9). Where lifting of sections occurred, this was generally minimal and usually partial (Figure 6.18). Only one case showed lifting of sections severe enough to interfere with interpretation. Spare tissue sections were available and the paired serial sections 4 & 5 were used to successfully repeat the *in situ* hybridization. Two TS– and one TS+ showed marked background noise. However, the background signal interfered with interpretation of staining in only one TS–. This section showed some granular deposits that were difficult to interpret in both the negative control and the test areas. Serial sections 4 & 5 from this TS - case were subjected to repeat hybridization (*vide infra*).

Table 6.9

Twenty five TS+ and 10 TS- were subjected to DISH. There was no lifting of the sections in 14 TS+ and 8 TS– while 6 TS+ and 1 TS– showed mild loss of cellular material. 4 TS+ and 1 TS– showed moderate lifting of sections. Severe lifting of sections occurred in one of the TS+ (marked *) and a serial section was stained. The Control smears (C+, TS-HPV) showed essentially similar occasional tendency of sections to lift. One TS- (marked **) showed marked background staining that interfered with interpretation and a serial section was stained (marked **).

Morphology of Smear	Number of Tests	Technical Quality of Sections							
		Lifting of section				Background staining			
		–	+	++	+++	–	+	++	+++
TS +	25	14	6	4	1*	8	10	6	1
C +	4	2	1	1	0	1	3	0	0
TS –	10	8	1	1	0	3	2	3	2**
TS–HPV	4	3	1	0	0	2	1	1	0
TS + repeat*	1		1			1			
TS– repeat**	2	2				1	1		
TS+ TS– PLD043	39	15	8	5	2	6	22	2	0

6.4.4.2 CONTROLS

Negative control area in PS– & PS+

In Pap smears, assessment of the background staining in the negative test area was very useful. This assessment made it possible to clearly separate the staining of elemental bodies in the mucus streaks from non-specific background staining. In all but one of the cases tested, the demarcated negative control area on each test slide that had not been exposed to CHLT-DIG-1® probe (negative control area) did not stain with alkaline phosphatase. The case was a PS+ in which the negative control area had been probed with HPV-DIG1. Intra-nuclear and cytoplasmic signals were present, compatible with HPV infection.

Three PS+ and two PS- cases showed heavy background staining in the negative control area. In one of these PS - cases, the background signal had an impact on the interpretation of the DISH test as positive. Again, moderate background

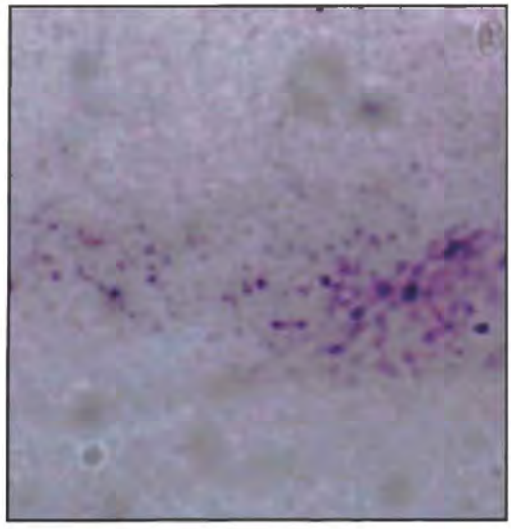


Figure 6.19A. One of the PS– cases showed moderate background noise signal. Fine dark granular deposits resembled the staining of EB were found in extra-cellular mucin streaks.

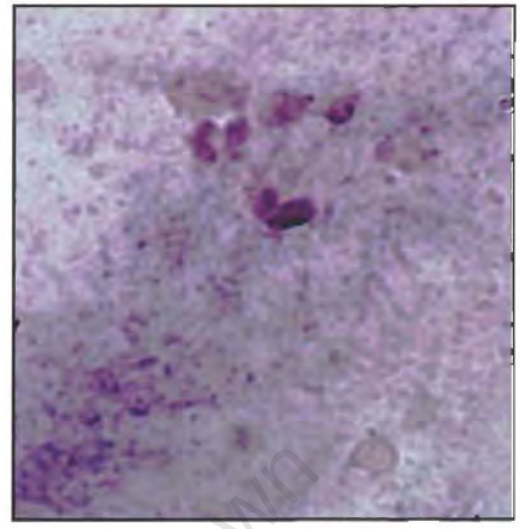


Figure 6.19B One PS– cases showed marked background noise signal. Coarse dark granular deposits resembled the staining of EB were found in extra-cellular mucin streaks.

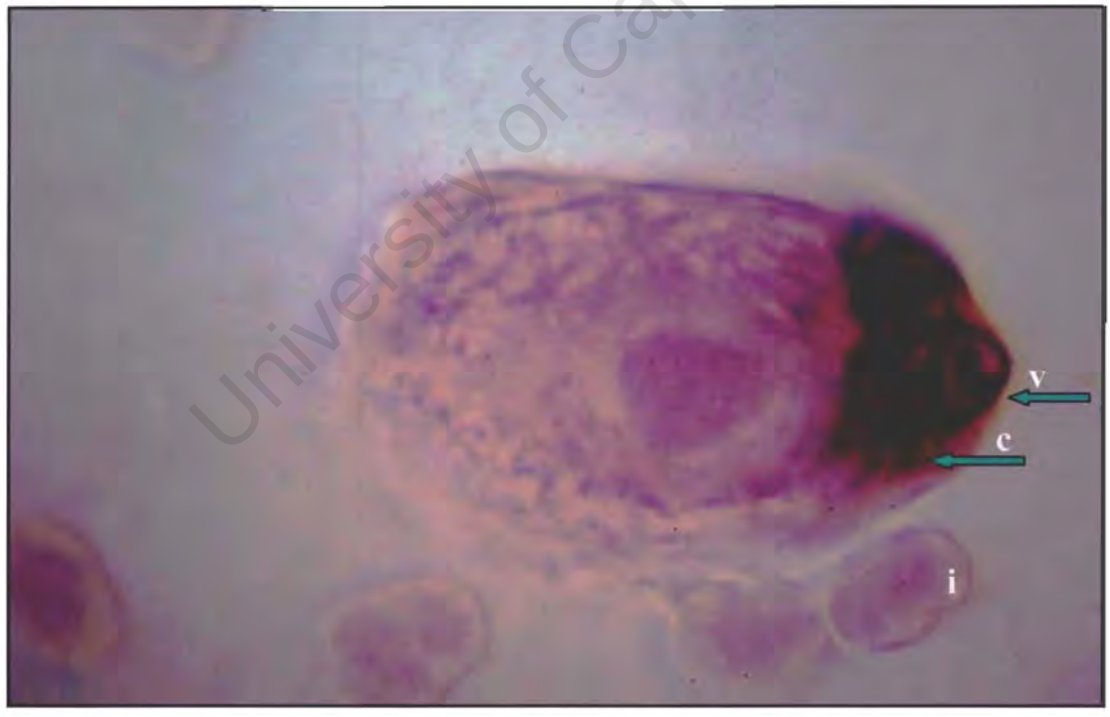


Figure 6.20. Chlamydial DNA-staining within vacuoles (v) in the cytoplasm (c) of metaplastic squamous cells. The background was clean and devoid of noise signal and inflammatory cells (i) were not stained. Twenty three of the 25 PS+ (92%) showed Chlamydial DNA-staining within vacuoles in the metaplastic squamous cells.

signal was present in the negative control area of three PS- and in one, this may have influenced the interpretation of the DISH.

Negative control PS–

Two PS- cases were interpreted as showing Chlamydial DNA in the test area probed with CHLT-DIG-1® (ie. the morphologic interpretations were falsely negative). One of these PS– cases showed marked background noise signal and in the other, background signal was moderate. Fine dark granular deposits were found in extra-cellular mucin streaks in the test area of these smears. These granular deposits resembled the staining of EB seen in PS+ cases (Figure 6.19A, 16.19B). Cell cytoplasm was not stained in either of these cases, and there were no intra-cellular inclusions. The negative control test areas of these slides were negative for granules but as noted, background signal in one was marked and moderate in the other. While these granules could have been regarded as artifactual and part of the background signal, it was not possible to exclude the presence of a few Chlamydial EBs in a morphologically unsuspected Chlamydial infection. Since only one smear was available for each cytology case, repeat staining to clarify the interpretation was not possible. In the calculations for the specificity of the DISH method, these cases were designated as possible false negative PS– morphologic selections (Table 6.10). Although repeat staining of false positive TS– cases (*vide infra)* showed that background staining could be associated with spurious granular deposits, it was not possible to say that the deposits case in these two TS– cases were necessarily also spurious.

Negative control TS–

One of the TS–sections showed granular deposits in the luminal mucus of the endocervical glands in the test area. There was severe background signal present in both the negative control and the test area sections. There was no staining of mucinous cell cytoplasm nor were there intra-cellular inclusions in the squamous epithelium. The staining possibly reflected the unpredicted presence of Chlamydial EB. However, these granules were regarded as artifactual and probably part of the background signal. Since it was possible to use serial sections, DISH tests were repeated on spare paired serial sections 4 & 5. The repeat staining showed mild background signal (Table 6.9) and the test area was negative for Chlamydial DNA. In the final outcome, all TS– were corroborated to be negative by DISH (Table 6.11).

Positive controls

Positive control McCoy cultures and the positive control tissue sections (7H0139) were appropriately positive in all cases when probed with CHLT-DIG1. The MC + probed with HPV-DIG1 did not generate signal. The positive staining was granular and was the characteristic dark purple/black colour associated with the ALK-PHOS, α-DIG/B, NBT/BCIP substrate (Figure 6.16, 6.17). The background was clean and devoid of noise signal in most cases. The presence of non-specific background staining was noted and graded as mild, moderate, or severe (Tables 6.8 & 6.9).

"Housekeeping" controls

The "housekeeping" controls showed morphologic features of HPV infection with or without CIN. All of the smears and tissue sections stained with HPV-DIG-1 showed nuclear and cytoplasmic staining, confirming the morphologic criteria

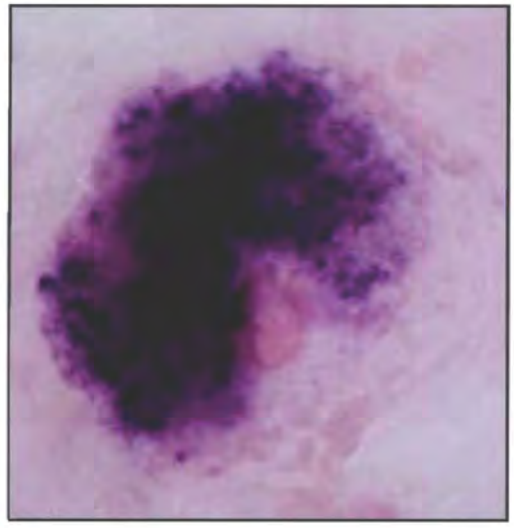


Figure 6.21. Abundant staining was seen in the cytoplasm of metaplastic squamous epithelial cells.

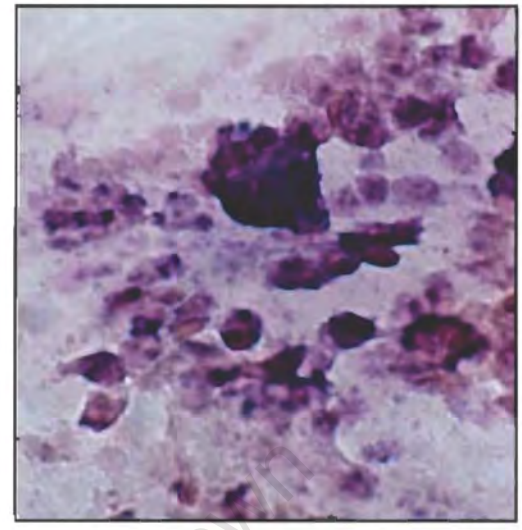


Figure 6.22. Cuboidal mucinous epithelial cells showed marked cytoplasmic staining, in some cases masking cellular detail to some extent.

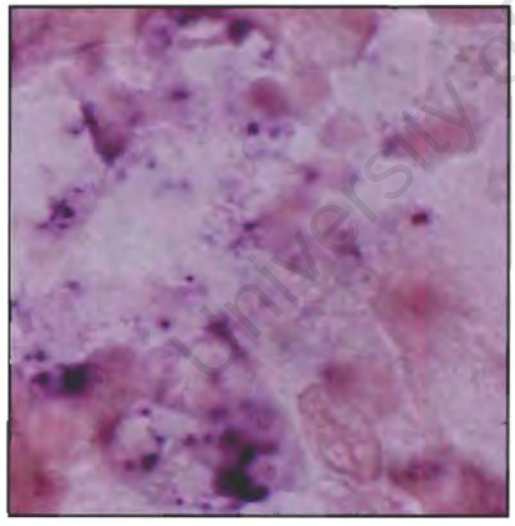


Figure 6.23. Fine granular staining was noted in endocervical mucus threads.

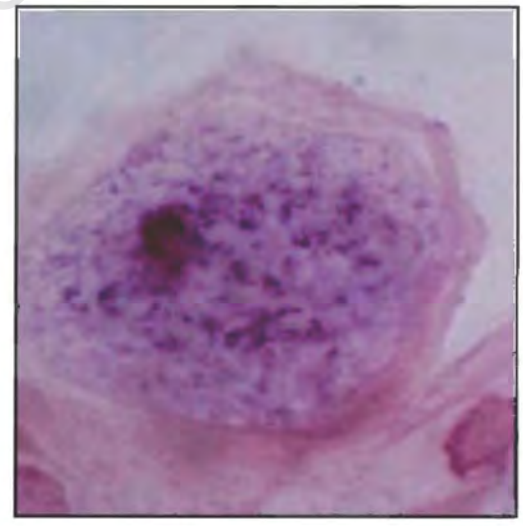


Figure 6.24. Chlamydial DNA was also found adherent to the surface of squamous cells, indicating the presence of numerous Chlamydial EB.

used to make the diagnosis of HPV infection. This result also confirmed that hybridization conditions had been successfully met during each of the DISH runs. The positive control MC+ Pap smears and the C+ tissue sections from 7H0139 did not stain for HPV. These latter results confirmed the specificity of the HPV and the Chlamydial probes.

6.4.4.3 RESULTS IN PAP SMEARS

Results using the *in situ* hybridization technique are shown below (Table 6.10). Known positive controls (MC+) were used with each batch of test slides and stained appropriately on each occasion (Figure 6.16A). Twenty three of the 25 PS+ (92%) showed Chlamydial DNA-staining within vacuoles in the metaplastic squamous cells (Figure 6.20). Abundant staining was also seen in the cytoplasm of metaplastic squamous (Figure 6.21) and cuboidal mucinous epithelial cells (Figure 6.22). Fine granular staining was noted in endocervical mucus threads (Figure 6.23). Chlamydial DNA was also found adherent to the surface of squamous cells (Figure 6.24). These extra-cellular signals were regarded as showing Chlamydial EB.

Table 6.10
Twenty three of 25 PS+ showed the presence of Chlamydial DNA (92%). Nine of 10 PS- (90%) did not show Chlamydial DNA. When PS - were combined with 14 PS-HPV/CIN as a group, 93% were negative for Chlamydial DNA. The correlation between morphologic diagnosis and DISH result was statistically significant to high degree ($p < 0.001$).

Morphology of Smear	Number of Tests	Chlamydia Positive	Chlamydia Negative	HPV Positive	Significance σ
PS+	25	23	2	1 of 9	$p < 0.0005$
MC+	4	4	0	0	
PS–	10	? 2	8	0	$p = 0.0255$
PS– sans other organisms	3	? 1	2	N/A	
PS– with other organisms	7	0	7	N/A	
PS–HPV/CIN	4	0	4	4	
PS– with PS–HPV/CIN	14	?2	12	4	$p < 0.001$
PS+ PS– PLD043	30	0	30	N/A	$p < 0.0001$

One of the two PS+ that did not show Chlamydial DNA did show signal in the negative control area probed with HPV-DIG1. The negative control area in the second PS+ without signal for Chlamydial DNA had been probed with PLD043 probe and showed no signal.

The eleven PS - with other infectious organisms present showed no signal for Chlamydial DNA (Table 6.10). The CHLT-DIG-1 probe did not cross react with the PS– cases showing HPV (4 cases), *Candida* sp. (2 cases), *Gardnerella* sp. (2 cases), Herpes simplex virus (1 case), *Neisseria* sp. (1 case) or *Trichomonas* sp (1 case). Of the four PS– cases that showed no evidence of other infectious organisms, three showed no signal. One showed some positive staining in mucus streaks (Figure 6.19). This staining did not appear to represent a cross reaction with DNA from another organism since in

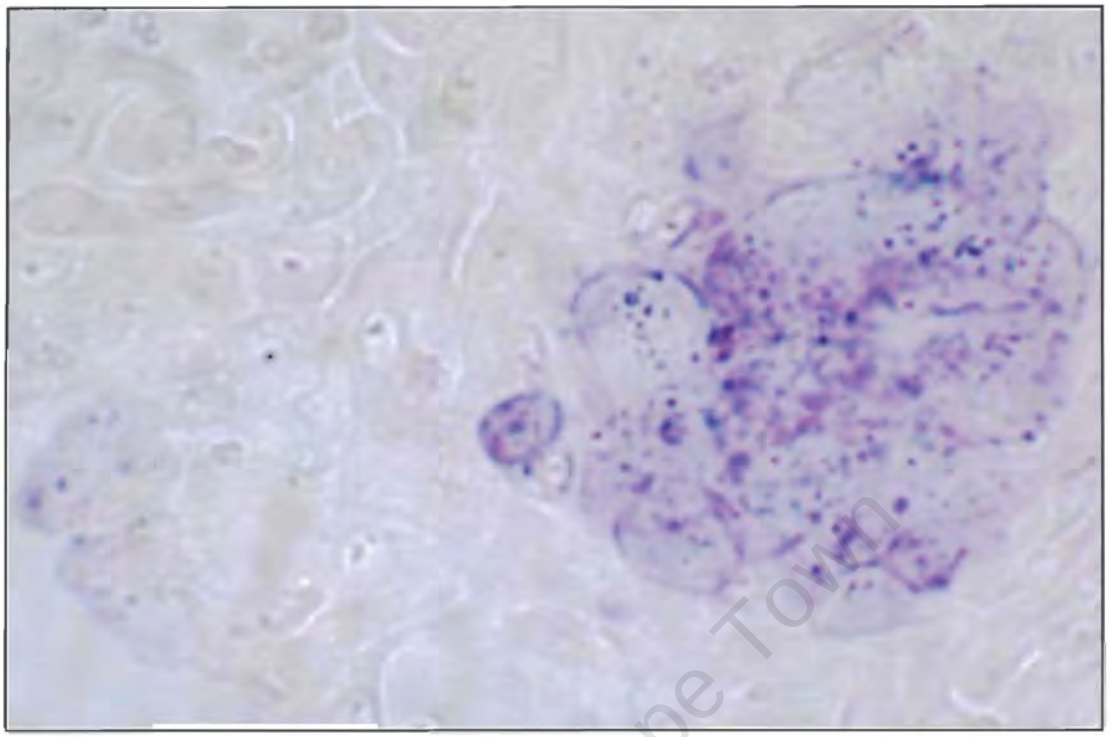


Figure 6.25. Tissue sections showed staining of Chlamydial DNA within vacuoles in the metaplastic squamous cells.

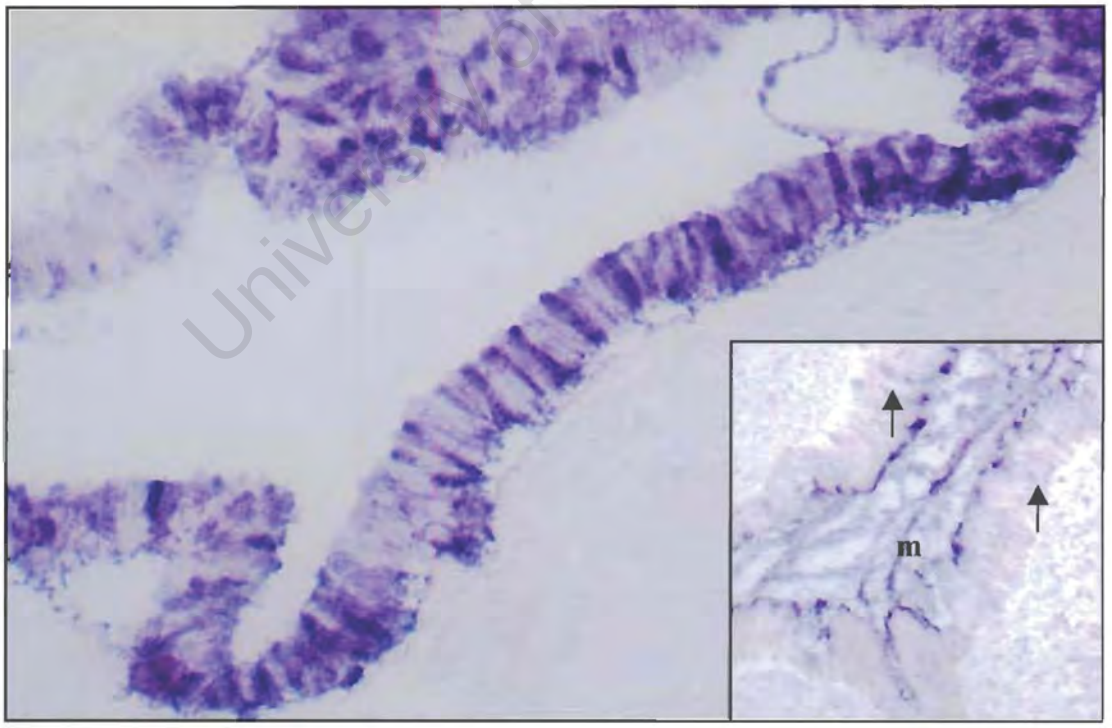


Figure 6.26. Strong staining was found in the cytoplasm of cuboidal mucinous epithelial cells lining endocervical glands. The background was clean. Insert: some cells showed scanty cytoplasmic staining (↑) and staining of EB in surface mucus (m) is present.

neither case, were other organisms identified. No other staining was found in these smears. No cells showed cytoplasmic positivity. This contrasted with the usual staining pattern found in PS+. The PS+ smears selected using the morphologic criteria for Chlamydial infection showed abundant cytoplasmic positivity in both mucinous cells and metaplastic cells. The few granules in the mucus streaks of this PS- smear could have represented Chlamydial EB but equally, may have been artifactual.

The presence of Chlamydial DNA confirmed in 23 of the 25 PS+ cases (92 %) selected by I.M was a statistically significant outcome with a p value of < 0.0005 (Table 6.10). However, the DISH method yielded one *possible* positive case amongst the negative controls. In this case, scanty DISH-positive material resembling EB was found within mucus streaks. Twelve of the 14 PS– cases (10 PS– and 4 PS HPV/CIN) showed no signal for Chlamydial DNA. Simple statistical analysis of this outcome was statistically significant ($p < 0.0005$) (Table 6.10).

6.4.4.4 Results in tissue Sections

The outcome of DISH staining for Chlamydial DNA in tissue sections is shown below (Table 6.11). The positive control sections (7H0139) used with each batch of tests stained appropriately on each occasion. Twenty three of the 25 TS+ (92%) showed staining of Chlamydial DNA within vacuoles in the metaplastic squamous cells (Figure 6.25) and staining was also seen in the cytoplasm of these cells. Strong staining was found in the cytoplasm of cuboidal mucinous epithelial cells lining endocervical glands (Figure 6.26).

Table 6.11
Twenty three of 25 TS+ showed the presence of Chlamydial DNA (92%). The correlation between morphologic diagnosis and DISH result was statistically significant to a high degree ($p < 0.005$). Thirteen (93%) of 14 TS- cases (TS– with TS- HPV), were negative for Chlamydial DNA. Nine of 10 TS- (90%) did not show Chlamydial DNA. When staining was repeated on the two TS- cases with marked background staining, reduction in background occurred but one of the 10 TS– (10%) remained as Chlamydia positive.*

Morphology of Section	Number of Tests	Chlamydia Positive	Chlamydia Negative	HPV Positive	Significance σ
TS+	25	23	2	N/A	$p < 0.0005$
C+ (7H0139)	4	4	0	N/A	
TS–	10	1 re-stained*	9	N/A	$p = 0.024$
TS– one case repeated	10	0	10	N/A	$p < 0.0001$
TS–HPV	4	N/A	4	4	
TS– with TS–HPV	14	? 1	13	4	$p < 0.0005$
TS+ PLD043	25	0	25	N/A	$p < 0.0005$
TS– PLD043	14	0	10	4	$p < 0.0005$

Background "noise signal" was mild in the majority of cases. All of the negative control sections (probed with PLD043) showed a relatively clean background (Table 6.9). Comparison of the background staining in the negative control section

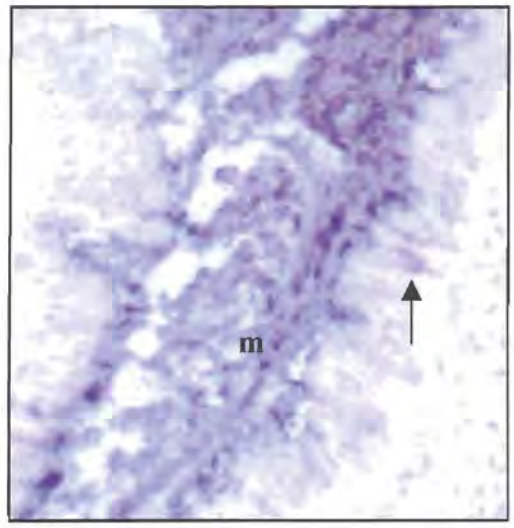


Figure 6.27. Abundant granular staining of EB is present in endocervical mucus (m) adherent to the surface of cells lining endocervical glands. Note the scanty intracellular staining present in some mucinous cells (↑).

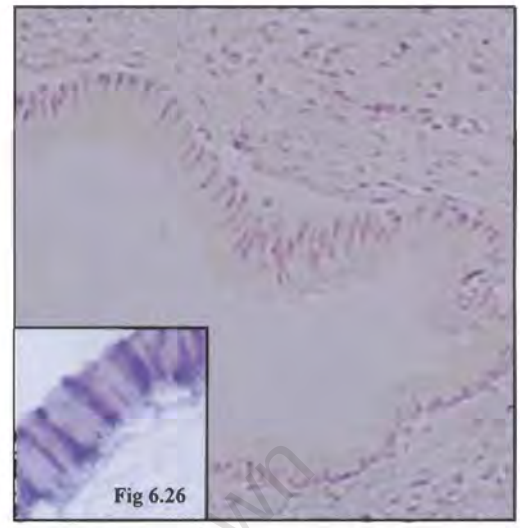


Figure 6.28. There was prominent staining of endocervical glands and their luminal content in test sections (Figure 6.26) but this was absent in all of the probed with PLD043 and TS– control sections probed with CHLT-DIG-1.

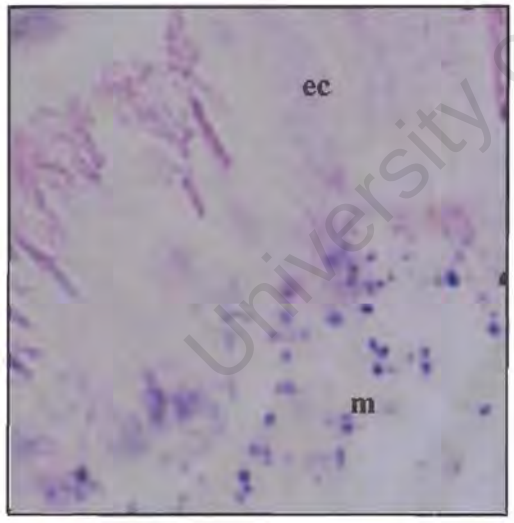


Figure 6.29. One case showed scanty granular positive staining in mucus (m) present in glandular lumina. The metaplastic and endocervical columnar mucinous cells (ec) did not show inclusions or cytoplasmic staining. Heavy background staining was present.

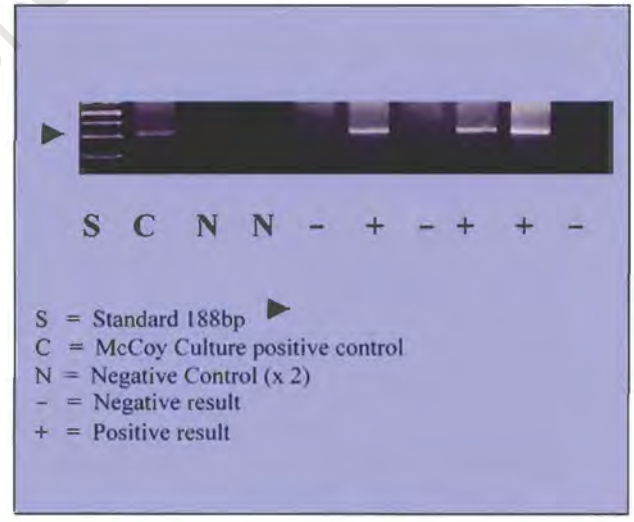


Figure 6.30. The when stained with ethidium bromide the DNA amplicons showed fluorescence induced by UV light. The electrophoresis result of the amplification product in agarose gel was recorded photographically using polar using Polaroid® images.

with the test section was very useful (Figures 6.26, 6.27). In particular, comparison of staining of the mucin within the lumina of endocervical glands and cytoplasm of endocervical glandular epithelium was very important. There was prominent staining of significant numbers of endocervical glands and their luminal content in test sections but this was absent in all of the PLD043 and TS– control sections (Figure 6.28). The granular staining of extra-cellular mucin was regarded as likely to be due to the presence of EB.

Two of the 14 TS– (5H 1884 & 6H 3001B) cases showed marked (+++) background staining that made interpretation difficult. One of these (5H 1884) showed scanty granular positive staining in mucus present in glandular lumina (Figure 6.29). The metaplastic cells and columnar mucinous cells did not show inclusions or cytoplasmic staining. The negative control test section on this slide was clean (Figure 6.28). Whether or not these granules were artifactual was uncertain, but the presence of some Chlamydial EBs in a morphologically unsuspected Chlamydial infection could not be excluded. The usual TS+ staining pattern was clear unequivocal cytoplasmic positivity in mucinous cells. Contrasted with usual TS+ positive results, the picture in this TS– case was very different. Since this case was one of two TS– (5H 1884 & 6H 3001B) that showed heavy background staining, both cases were re-tested on serial sections 4 & 5. The repeat test showed reduced background staining in both cases. However, the granules in the luminal mucus in serial section 2 of specimen 5H 1884 were also present in serial section 4. This specimen was reported as positive for Chlamydial DNA. On the other hand, in specimen 6H 3001B, despite the background staining, there was no signal in the luminal mucus in serial sections 2 or 4. It was concluded that 5H 1884 TS– (10%) should be regarded as a morphologic false negative (*vide infra* **6.5.4**).

Simple analysis by comparison of proportions showed that the significance of these results was of a high degree. The p value for the TS+ results was < 0.0005. For the non-Chlamydial controls, analysis of ten TS– cases combined with four TS– HPV cases showed a p value of < 0.0005 (Table 6.11).

6.4.4.5 SPECIFICITY AND SENSITIVITY OF DISH

The sensitivity of the tests was calculated according to the formula:

$$\text{Sensitivity} = 1 - \frac{\text{No false negative}}{\text{No true positives}} \times 100$$

The specificity of the tests was calculated according to the formula:

$$\text{Specificity} = 1 - \frac{\text{No false positives}}{\text{No true negatives}} \times 100$$

For the purposes of calculating the sensitivity and specificity of the DISH method, the morphologic prediction was regarded as the "true positive" or "true negative" outcome. Accepting the outlying negative control results as true, the specificity of the DISH method when applied to Pap smears was 92.0 % and the sensitivity was 85.8 % (Table 6.12). The results of the two PS– negative controls that showed scanty, equivocal granular deposits were included even though both showed marked background staining, and the full picture of positivity as seen in PS+ was not present.

Table 6.12
For the purposes of calculating the sensitivity and specificity of DISH when Pap smears were tested by the method, the morphologic prediction was regarded as the "true positive" or "true negative" result. The number of false negative results was 2 in 25 and the number of false positive results was 1 in 14.

DISH Result	Morphologic **True Positive**	Morphologic **True Negative**	Total
DISH Positive	23	2	24
DISH Negative	2	12	15 (14)
Total	**25**	**14**	**39 (38)**

$$\text{Sensitivity} = 1 - \frac{\text{No false negative}}{\text{No true positives}} \times 100 = 1 - \frac{2}{25} \times 100 = 1 - 0.080 \times 100 = 92.0\ \%$$

$$\text{Specificity} = 1 - \frac{\text{No false positives}}{\text{No true negatives}} \times 100 = 1 - \frac{2}{14} \times 100 = 1 - 0.142 \times 100 = 85.8\ \%$$

Similarly, the DISH method used on tissue sections showed a sensitivity of 92.0 % & a specificity of 92.9 % (Table 6.13).

6.4.4.6 Specificity and Sensitivity of morphologic criteria as confirmed by DISH

Using DISH as the "gold standard" for the purposes of analysis, the sensitivity and specificity of morphologic criteria as corroborated by the DISH method could be calculated. When the DISH results were regarded as the "true positive" or "true negative," the morphologic criteria when applied to Pap smears showed a sensitivity of 92.0 % and a specificity of 85.7 % (Table 6.14).

Table 6.13
For the purposes of calculating the sensitivity and specificity of the DISH method applied to tissue sections, the morphologic prediction was regarded as the "true positive" or "true negative" result. The number of false negative results was 2 in 25 and the number of false positive results was 1 in 14.

DISH Result	Morphologic **True Positive**	Morphologic **True Negative**	Total
DISH Positive	23	1	24
DISH Negative	2	3	11
Total	**25**	**14**	**39**

$$\text{Sensitivity} = 1 - \frac{\text{No false negative}}{\text{No true positives}} \times 100 = 1 - \frac{2}{25} \times 100 = 1 - 0.080 \times 100 = 92.0\ \%$$

$$\text{Specificity} = 1 - \frac{\text{No false positives}}{\text{No true negatives}} \times 100 = 1 - \frac{1}{14} \times 100 = 1 - 0.071 \times 100 = 92.9\ \%$$

Table 6.14

The sensitivity and specificity of the morphologic criteria, when applied to Pap smears, was calculated using the DISH method as the gold standard for "true positive" or "true negative" results. The number of false negative results was 1 in 24 and the number of false positive results was 2 in 15.

Morphologic Result	DISH **True Positive**	DISH **True Negative**	Total
Morphologic Positive	23	2	25
Morphologic Negative	2	12	14
Total	**25**	**14**	**39**

$$\text{Sensitivity} = 1 - \frac{\text{No false negative}}{\text{No true positives}} \times 100$$

$$= 1 - \frac{2}{25} \times 100$$

$$= 1 - 0.08 \times 100$$

$$= 92.0\ \%$$

$$\text{Specificity} = 1 - \frac{\text{No false positives}}{\text{No true negatives}} \times 100$$

$$= 1 - \frac{2}{14} \times 100$$

$$= 1 - 0.143 \times 100$$

$$= 85.7\ \%$$

Similarly, the morphologic criteria when applied in tissue sections showed a sensitivity of 95.8 % and a specificity of 86.7 % (Table 6.15).

Table 6.15

When calculating the sensitivity and specificity of the morphologic criteria applied to tissue sections, the DISH result was regarded as the "true positive" or "true negative" result. The number of false negative results was 0 in 27 and the number of false positive results was 2 in 14.

Morphologic Result	DISH **True Positive**	DISH **True Negative**	Total
Morphologic Positive	23	2	25
Morphologic Negative	1	13	14
Total	**24**	**15**	**39**

$$\text{Sensitivity} = 1 - \frac{\text{No false negative}}{\text{No true positives}} \times 100$$

$$= 1 - \frac{1}{24} \times 100$$

$$= 1 - 0.042 \times 100$$

$$= 95.8\ \%$$

$$\text{Specificity} = 1 - \frac{\text{No false positives}}{\text{No true negatives}} \times 100$$

$$= 1 - \frac{2}{15} \times 100$$

$$= 1 - 0.133 \times 100$$

$$= 86.7\ \%$$

6.4.5 PCR CORROBORATION

The amplification product when stained with ethidium bromide showed fluorescence induced by UV light (Figure 6.30). The result of the electrophoresis in agarose gel was recorded photographically using polar using Polaroid images. Sequencing of the amplification product confirmed the DNA to be Chlamydial (Figure 6.11). Of the 25 cases regarded as showing morphologic features of Chlamydial infection, 22 cases were confirmed by PCR ($p < 0.0005$). A single cycle PCR sequence was sufficient to detect 9 of the 25 TS+. The second amplification sequence using the semi-nested sense 2

primer was performed on the remaining 16 TS+ and 13 more cases were confirmed. Three of the TS+ could not be confirmed to contain Chlamydial DNA by PCR. All of the TS– were subjected to the two amplification cycles. Eight of the 10 TS– were negative for Chlamydial DNA after both PCR cycles. Two of the TS– tested positive by PCR, one after the first cycle and one after the second cycle. The outcome of the PCR is shown below (Table 6.16). The positive control MC+ specimens were all confirmed positive in a single cycle PCR sequence using either the sense 1 or the sense 2 primers. The negative controls (water blanks) were all negative.

Table 6.16
The PCR confirmed 22 of 25 TS+ cases to contain Chlamydial DNA ($p < 0.005$). Eight of the 10 TS– were confirmed negative for Chlamydial DNA by PCR ($p = 0.0255$). Positive control MC+ all produced a positive result in one cycle, 6 runs with the sense 1 primer and 4 runs with the sense 2 primer*.

Morphology of Specimen	No of Specimens	No positive for Chlamydia		No negative for Chlamydia (Sense 1 & 2)	Significance
		1st cycle (Sense 1)	2nd cycle (Sense 2)		
TS+	25	9	13	3	< 0.0005
TS–	10	1	1	8	< 0.0255
MC+	10	6	4*	0	< 0.0001
Blank	10	0	0	10	< 0.0001
Total	55	13	18	22	

When morphology was accepted as the true result, the PCR method had a sensitivity 88% and a specificity of 90% (Table 6.17).

Table 6.17
For the purposes of calculating the sensitivity and specificity of the PCR method, the morphologic prediction was regarded as the "true positive" or "true negative" result. The number of false negative results was 3 in 25 and the number of false positive results was 1 in 10.

PCR Result	Morphologic True Positive	Morphologic True Negative	Total
PCR Positive	22	2	23
PCR Negative	3	8	12
Total	25	10	35

$$\text{Sensitivity} = 1 - \frac{\text{No false negative}}{\text{No true positives}} \times 100$$

$$= 1 - \frac{3}{25} \times 100$$

$$= 1 - 0.12 \times 100$$

$$= 88\ \%$$

$$\text{Specificity} = 1 - \frac{\text{No false positives}}{\text{No true negatives}} \times 100$$

$$= 1 - \frac{2}{10} \times 100$$

$$= 1 - 0.2 \times 100$$

$$= 80\ \%$$

Testing the morphologic criteria where the PCR results were accepted as true, the sensitivity of the criteria was found to be 96% and the specificity was 73% (Table 6.18).

Table 6.18
When calculating the sensitivity and specificity of the morphologic criteria, the PCR results was regarded as the "true positive" or "true negative" results. The number of false negative results was 3 in 25 and the number of false positive results was 1 in 10.

Morphologic Result	PCR True Positive	PCR True Negative	Total
Morphology Positive	22	3	25
Morphology Negative	2	8	10
Total	**24**	**11**	**35**

Sensitivity = $1 - \frac{\text{No false negative}}{\text{No true positives}} \times 100$

$= 1 - \frac{1}{24} \times 100$

$= 1 - 0.042 \times 100$

$= 95.8\%$

Specificity = $1 - \frac{\text{No false positives}}{\text{No true negatives}} \times 100$

$= 1 - \frac{3}{11} \times 100$

$= 1 - 0.273 \times 100$

$= 72.7\ \%$

6.4.6 THE EXPANDED GOLD STANDARD (DISH & PCR TOGETHER)

The CDC has recommended use of two DNA detection methods to set a gold standard in the evaluation of testing for Chlamydial infection.[1] In determining the sensitivity and specificity of a detection method, identification of true positive and true negative cases using two DNA detection methods can be achieved in either of two ways (own observation). Firstly, an "expanded" gold standard can accept corroboration by *any one* of the two DNA tests. Alternatively a "combined" gold standard can require that *both* DNA tests corroborate the result. Using an "expanded" gold standard would improve the sensitivity of the gold standard since it is likely that the two DNA methods together would detect more cases than either test alone. On the other hand, the strict requirement for concordant results in the "combined" corroboration would improve the specificity of the gold standard. Both methods of determining true positive and true negative cases were applied (*vide infra*).

The results of corroboratory DNA testing of the cases selected on morphologic grounds as either Chlamydia positive (TS+) or negative (TS–) are shown below (Table 6.19). To enable comparison of the results of the two corroboratory tests in any single case, the results were tabulated showing the practice laboratory accession number of each case. The unique Kreatech numbers on the DISH results were matched with the names of the patients. The DISH results could then be re-assigned the blinded practice laboratory accession number of the specimen. The DISH results could then be matched with the PCR results, identified by the practice accession number.

TS + cases

Concordance of the morphology, DISH and PCR was obtained in 21 of the 25 cases. Of the 25 TS+ cases selected by light microscopy, 24 tested positive by at least one of the two DNA techniques. DISH detected Chlamydial DNA in 23 of the 25 (92 %) TS+ cases selected using the morphologic criteria (Tables 6.9 & 6.19). PCR confirmed the presence of Chlamydial DNA in 22 of 25 TS+ cases regarded as positive by LM (88 %) (Tables 6.16 & 6.19). Thirteen of the positive cases required two cycles of PCR (positive [2]) using semi-nested primers to detect the Chlamydial DNA. Two

of the three cases (5H 1016 C and 6H 1844 C) were shown to be positive by DISH and could therefore regarded as PCR discordant. One case (3H 3170 B) was negative by both DNA test methods and was regarded as a discordant morphology, false positive.

Table 6.19
The results of corroboratory DNA testing of the 25 TS+ and 10 TS– cases are shown for each case, identified by accession number. Thirteen of the positive cases required two cycles of PCR (positive [2]) using semi-nested primers to detect the Chlamydial DNA. Twenty one TS+ were confirmed by both DNA test methods. Another 3 TS+ were confirmed by at least one other DNA test. Only one TS+ was discordant with both of the DNA tests. Eight of the TS– were corroborated by both of the DNA test methods. Another TS–was corroborated by DISH only. Only one TS– tested positive by both methods. Nine of ten TS– and 24 of 25 TS+ were corroborated by one or other DNA method.

Sample by Accession No	Light Microscopy	DISH	PCR [1] or [2] cycles	Conclusion
3H 2616	Positive	Positive	Positive [2]	Concordant
3H 2943 D	Positive	Positive	Positive [1]	Concordant
3H 2944 C	**Positive**	**Negative**	**Positive [1]**	**Discordant DISH**
3H 2974	Positive	Positive	Positive [2]	Concordant
3H 3160 A	Positive	Positive	Positive [2]	Concordant
3H 3161 B	Negative	Negative	Negative [2]	Concordant
3H 3170 B	**Positive**	**Negative**	**Negative [2]**	**Discordant Morphology**
3H 3177 D	Positive	Positive	Positive [2]	Concordant
3H 4198 C	Positive	Positive	Positive [1]	Concordant
4H 2302 C	Negative	Negative	Negative [2]	Concordant
4H 2308 D	Negative	Negative	Negative [2]	Concordant
4H 2649	Negative	Negative	Negative [2]	Concordant
4H 2988 A	Positive	Positive	Positive [2]	Concordant
4H 3515 A	Positive	Positive	Positive [1]	Concordant
4H 3861 B	Positive	Positive	Positive [2]	Concordant
4H 3862 A	Positive	Positive	Positive [1]	Concordant
4H 4228 A	Positive	Positive	Positive [2]	Concordant
5H 0860 C	**Negative**	**Negative**	**Positive [1]**	**Discordant PCR**
5H1015 A	Positive	Positive	Positive [1]	Concordant
5H 1016 C	**Positive**	**Positive**	**Negative [2]**	**Discordant PCR**
5H 1884	**Negative**	**Positive**	**Positive [1]**	**Discordant Morphology**
5H 4897	Positive	Positive	Positive [2]	Concordant
5H 5177 C	Negative	Negative	Negative [2]	Concordant
5H 6200 D	Positive	Positive	Positive [2]	Concordant
5H 6407 C	Negative	Negative	Negative [2]	Concordant
5H 7164 A	Positive	Positive	Positive [2]	Concordant
5H 7935 C	Positive	Positive	Positive [2]	Concordant
6H 1637	Negative	Negative	Negative [2]	Concordant
6H 1844 C	**Positive**	**Positive**	**Negative [2]**	**Discordant PCR**
6H 1551 B	Positive	Positive	Positive [1]	Concordant
6H 2976 A	Positive	Positive	Positive [2]	Concordant
6H 3001 B	Negative	Negative	Negative [2]	Concordant
6H 4469 D	Positive	Positive	Positive [1]	Concordant
6H 5003	Positive	Positive	Positive [2]	Concordant
6H 7377A	Positive	Positive	Positive [1]	Concordant

TS- cases

Eight of the ten TS - cases selected by LM were confirmed negative by both DNA techniques. Nine of the ten TS- cases were negative by DISH. The other case (5H 1884) showed scanty signal in extra-cellular mucus, arguably extra-cellular EB, and was deemed positive. This case was subsequently shown to be positive by PCR also (Table 6.19). When the combined gold standard of PCR and DISH were applied to TS- case 5H 1884, the discordant morphology was regarded as a false negative. Nine of the TS- cases negative by light microscopy tested negative by at least one of the two DNA techniques. One DISH result, two morphologic results and three PCR results were discordant with respect to the other modalities.

6.4.6.1 SPECIFICITY AND SENSITIVITY OF THE MORPHOLOGIC CRITERIA

The strict (more specific) combined gold standard

The results obtained are summarised below (Table 6.20). To calculate the sensitivity and specificity of the morphologic diagnosis against the "combined" gold standard, the concordant DNA tests were regarded as "true" and non-concordant tests were excluded from the calculation. The sensitivity of the morphologic criteria measured against the exclusive expanded gold standard was 95.5 % and the specificity of the criteria was 88.9 %.

Table 6.20

The sensitivity and specificity of the morphologic criteria was calculated by comparison of the morphologic diagnosis with the "true diagnosis" derived from concordant DNA results. The number of false negative results was 1 in 22 and the number of false positive results was 1 in 9.

Morphologic Result	Concordant DNA True Positive	Concordant DNA True Negative	Discordant DISH	Discordant PCR	Total
Morphology +	21	1	1	2	25
Morphology –	1	8	0	1	10
Total	22	9	1	3	35

$$\text{Sensitivity} = 1 - \frac{\text{No false negative}}{\text{No true positives}} \times 100 = 1 - \frac{1}{22} \times 100 = 1 - 0.045 \times 100 = 95.5\%$$

$$\text{Specificity} = 1 - \frac{\text{No false positives}}{\text{No true negatives}} \times 100 = 1 - \frac{1}{9} \times 100 = 1 - 0.111 \times 100 = 88.9\ \%$$

The more sensitive "expanded" gold standard

Similar, slightly higher figures for the sensitivity (96.0%) and specificity (90.0%) of the morphologic criteria were obtained if corroboration by any one of the DNA methods was accepted as "true" (Table 6.21).

Table 6.21
In this model, a corroboratory PCR result &/or DISH result was regarded as the "true positive" or "true negative" outcome when calculating the sensitivity and specificity of the morphologic criteria. False results were those corroborated by neither method. The number of false negative results was 1 in 25 and the number of false positive results was 1 in 10.

Morphologic Result	PCR &/ or DISH True Positive	PCR &/or DISH True Negative	Total
Morphology Positive	24	1	25
Morphology Negative	1	9	10
Total	25	10	35

Sensitivity = $1 - \frac{\text{No false negative}}{\text{No true positives}} \times 100$

$= 1 - \frac{1}{25} \times 100$

$= 1 - 0.040 \times 100$

$= 96.0\%$

Specificity = $1 - \frac{\text{No false positives}}{\text{No true negatives}} \times 100$

$= 1 - \frac{1}{10} \times 100$

$1 - 0.100 \times 100$

$90.0\ \%$

6.4.6.2 SPECIFICITY AND SENSITIVITY OF THE DISH KIT

The specificity and sensitivity of the Kreatech Rembrandt Ultradig DISH® kit was calculated using concordant morphology and PCR results as a "combined" gold standard (Table 6.22). The DISH kit showed a sensitivity of 99.6% and a specificity of 100 %.

Table 6.22
In this model, a concordant morphology and PCR result was regarded as the "true positive" or "true negative" outcome when calculating the sensitivity and specificity of the DISH kit. The number of false negative results was 1 in 23 and the number of false positive results was 0 in 8.

DISH Result	Morphology & PCR True Positive	Morphology & PCR True Negative	Total
DISH Positive	22	0	22
DISH Negative	1	8	9
Total	23	8	31

Sensitivity = $1 - \frac{\text{No false negative}}{\text{No true positives}} \times 100$

$= 1 - \frac{1}{23} \times 100$

$= 1 - 0.0044 \times 100$

$= 99.6\%$

Specificity = $1 - \frac{\text{No false positives}}{\text{No true negatives}} \times 100$

$= 1 - \frac{0}{8} \times 100$

$= 1 - 0.000 \times 100$

$= 100.0\ \%$

6.4.6.3 SPECIFICITY AND SENSITIVITY OF THE PCR

Similarly, the specificity and sensitivity of the PCR test could be calculated using concordant morphology and DISH results as a "combined" gold standard (Table 6.23). The PCR showed a sensitivity of 95.5% and a specificity of 90 %.

Table 6.23
In this model, a concordant morphology and DISH result was regarded as the "true positive" or "true negative" outcome when calculating the sensitivity and specificity of the PCR. The number of false negative results was 1 in 22 and the number of false positive results was 1 in 9.

PCR Result	Morphology & DISH True Positive	Morphology & DISH True Negative	Total
PCR Positive	21	1	22
PCR Negative	1	8	10
Total	22	9	32

$$\text{Sensitivity} = 1 - \frac{\text{No false negative}}{\text{No true positives}} \times 100$$

$$= 1 - \frac{1}{22} \times 100$$

$$= 1 - 0.045 \times 100$$

$$= 95.5\%$$

$$\text{Specificity} = 1 - \frac{\text{No false positives}}{\text{No true negatives}} \times 100$$

$$= 1 - \frac{1}{9} \times 100$$

$$= 1 - 0.111 \times 100$$

$$= 88.9\ \%$$

6.5 DISCUSSION

6.5.1 AIM OF THE STUDY

The morphologic criteria for the diagnosis of Chlamydial infection developed during the study (Chapter 5) can be confidently applied in every-day routine microscopy. Using the rigorous "combined" gold standard recommended by the CDC,[1] the morphologic criteria were shown to be highly specific (95.5%). The more sensitive "expanded" gold standard showed a similar result (96.0%). The corroboratory testing showed that careful use of these criteria in either tissue sections or Pap smears can be a reliable means of diagnosing Chlamydial infection in the uterine cervix. The study showed that discovery of a Chlamydial infection in either of these types of specimen can and should be reported to the physician and the patient with confidence.

The purpose of the study was not to investigate the sensitivity of the Pap smear screening method. Pap smears or colposcopically directed cervical biopsies are not a sensitive screening method to detect Chlamydial infection in the general population.[*inter alia* 28] During the last two years of the study 22 045 Pap smears were screened and 1 141 cases showed evidence of Chlamydial infection (*vide supra* 5.4.1.1). The rate of detection was approximately 3.1%. The reported incidence of the infection in women in the USA is 3%.[33] Since there is no way to know the true incidence of Chlamydial infection in the population that was being screened, it is not possible to asses further the sensitivity of the Pap smear screening method for the detection of Chlamydial infection. The sensitivity calculations as applied to the testing of the criteria do not indicate a high sensitivity of the screening method *per se*.

6.5.2 COMPARISON WITH OTHER STUDIES

The high specificity of the morphologic criteria derived in this study far exceeds the apparent specificity of criteria used by other workers.[28,-31,36-51] However, this study is the only one where strictly defined criteria have been and tested by the extremely *sensitive* DNA methods that have recently become available. Previously, corroboratory testing has been

restricted to culture[31,36,37] or serologic methods.[48-52] Culture has been shown to be fraught with pitfalls resulting in low sensitivity.[38,39,44,45] Similarly, studies comparing Pap smear diagnoses with serologic detection methods[31,46, 48-52] have also been limited by the relatively low sensitivities of these methods.[1,40-43] These methods are no longer recommended by the CDC as appropriate gold standards.[1] Use of these relatively insensitive gold standards may have resulted in calculation of erroneously low specificity figures.

Failure of workers and opinion makers in the cytopathology discipline to recognise the limitations of these corroboratory tests has resulted in the widely held notion that morphologic diagnosis of Chlamydial infection is not reliable.[35]

One of the main differences between the criteria presently proposed and criteria used by many workers investigating the reliability of the criteria is the emphasis on finding Chlamydial inclusions in the appropriate cellular setting. In the present study, Chlamydial infection was associated with a characteristic inflammatory process and cellular changes, particularly in metaplastic squamous cells. These changes were frequently the clue that led to a successful search of Chlamydial inclusions. However, these changes alone were not accepted as diagnostic, and inclusions were regarded as an essential component of a constellation of changes. Further, strict adherence to the criteria defining the morphology of the inclusions, and differentiation of them from inflaminatory or degenerative inclusions was necessary. Features useful in separating nonspecific inflammatory or degenerative vacuolar changes were derived from the literature and carefully applied. Attention to these details required only a few minutes when examining a Pap smear or cervical biopsy, and enabled a reliable and confident morphologic diagnosis of Chlamydial infection.

6.5.3 THE ROLE OF CULTURE IN THE PRESENT STUDY

Seven of only 13 (54%) cases that were tested were corroborated by culture. When the pitfalls and shortcomings of the culture procedure were discussed with the submitting physicians, further testing was discontinued. The cost of these tests and the low sensitivity were important considerations in this decision. However, the majority of these physicians were reassured by the culture results. The consensus of opinion was that cases showing morphologic features of *Chlamydia trachomatis* infection should be reported as suggestive of a possible infection. One gynaecologist however, insisted that the changes should not be reported if found in smears from his patients. Accordingly, another pathologist in the practice was asked to examine smears from his patients. The possible consequence of not reporting suspicion of a potentially significant infection was an important consideration in this decision. This illustrates the need to resolve the dichotomy of opinion regarding the morphologic diagnosis of Chlamydial infection.

6.5.4 THE ROLE OF EM IN THE PRESENT STUDY

The use of EM to confirm the presence of Chlamydial organisms was not successfully used to corroborate infection in Pap smears because preservation of cellular detail after methanol fixation was found to be inadequate. The method was particularly useful in selecting the positive tissue control to be used for DISH testing of tissue sections. However, many tissue specimens were small biopsy fragments and the entire specimen was processed for routine histology. No reserve tissue was available for EM. Recovery of tissue from paraffin blocks for EM is possible but is time consuming and

preservation of morphology is usually not optimum (personal observation). The EM method, although fairly specific if preservation is good, is not sensitive and was not appropriate for corroboratory studies.

6.5.5 SELECTION OF THE CORROBORATORY TESTS FOR THE COMBINED GOLD STANDARD

One of the aims of this corroboratory study was to set up a reliable and supersensitive, highly specific DNA test for Chlamydial DNA in tissue specimens. DISH is highly specific because it combines location of the signal with characteristic morphologic features.[1] Development of a new signaling system (ULS) using a platinum linking molecule in the Kreatech Rembrandt Ultradig DISH[10] kit has improved the sensitivity of DISH ten-fold.[9-11] This method was selected for its high specificity and sensitivity to be one of the tests in a combined DNA gold standard. It proved to be an excellent DNA test.

It became clear that it was not possible to establish the DISH method in the Cape Town laboratory because of a lack of funding for the purchase of Kreatech DISH kits and suitable equipment. The most important item of equipment necessary was a heating plate that was large enough to conduct at least 15 tests at a time. The plate had to be capable of maintaining precise temperatures with sufficient stored energy to rapidly heat the glass slides without dropping its temperature. This problem was solved when Kreatech suggested that the investigator should conduct the DISH testing in Amsterdam. In exchange for use of their facilities, Kreatech Laboratories gained validation of their untried Chlamydial test and obtained a positive tissue control. The investigator did personally, all of the work done at Kreatech for this study. The management and staff at Kreatech Laboratories did not exert any pressure nor attempt in anyway to influence the outcome of the study.

Use of laboratory equipment, consumable reagents and the Ultradig DISH kits at Kreatech Laboratories, proved invaluable to the success of the DISH testing. Although the method was not established in Cape Town, some alterations to the protocol for DISH staining of Pap smears were derived in the initial battery of tests performed in Cape Town. These tests also served to identify the subbed slides best suited to the DISH protocol in tissue sections and to optimise the thickness of sections used.

The second DNA test selected for the combined gold standard was conventional PCR. The PCR method is widely accepted in the literature as both specific and sensitive *inter alia* [1,7,8,36,37,53] and is no longer regarded as an experimental tool only. The method is used widely in routine diagnostic testing. The opportunity to conduct the PCR tests in a well established reference laboratory circumvented the necessity of establishing and verifying the method in Cape Town. The methodology and technique was well established and operational in the Goettingen laboratory. The ability to sequence the end product of the PCR and prove the specificity of the reaction[19,20] was a particularly elegant refinement that made the test extremely valuable.

The limited amount of cellular material in the Pap smear specimens led to the decision that PCR would not be attempted on Pap smear material. This limitation was not regarded as a significant limitation of the study. The morphologic criteria

in Pap smears and tissue sections were essentially similar. The criteria as represented in tissue sections were interrogated by the second test in the combined gold standard. The PCR also served to validate the DISH testing. If there was concordance between the outcome of DISH testing and PCR testing in tissue sections, there was no reason to argue that the DISH results in Pap smears were less valid than those in tissue sections.

6.5.6 INTERPRETATION OF THE DNA TEST RESULTS

The PCR and DISH procedures were conducted in two different laboratories. The DISH staining was evaluated and the results recorded before the second round of PCR tests was performed. The DISH results were recorded by patient name and by a unique Kreatech experiment number. The practice laboratory accession number was used to identify specimens when the PCR tests were performed. The outcome of the DISH was blinded until all PCR tests were completed and interpreted.

There was very good concordance of the results. The results were in accord in all three modalities in 29 of 35 tests ($p < 0.0005$). The morphologic criteria were successfully corroborated by the DNA test methods with a high level of confidence ($p < 0.0005$). The two DNA methods showed concordant results in 31 of 35 tests. This was regarded as validation of the Kreatech Rembrandt Ultradig DISH® kit. This kit had not been previously verified for the detection of *Chlamydiae*. The results of the DISH method in Pap smears could be accepted as reliable.

There was discordance in only 6 of 35 specimens, two TS– and four TS+. Two of the specimens (one TS– and one TS+) showed discordance between morphology and combined DNA testing, indicating that these were probably assigned incorrect diagnoses based on morphologic appearance.

Negative controls for DISH and PCR

One PS– showed marked background staining that interfered with interpretation. There was uncertainty about the nature of extra-cellular granular deposits in mucus streaks. Similarly, one TS– with marked background staining showed granules in the luminal mucin without other signal for Chlamydial DNA. The severity of background staining in the test area probed with CHLT-DIG-1was assessed by comparison of the negative control area probed with either PLD043 or HPV-DIG1. Two TS– negative controls (5H 1844 and 6H 3001 B) showed marked background noise.

The problem of distinguishing low-level expression of target from non-specific background was emphasised by Wilkinson.[5] This author suggests that the problem can be solved by inclusion of a control in which a ten-fold excess of unlabelled nucleic acid identical to the probe is present during the hybridization. The unlabelled nucleic acid effectively competes with the probe for specific binding sites, removing the low-level specific signal, but not altering the background signal.[5] This technique does not find favour with some workers.[5a]

Background staining over the entire tissue section is usually due to non-specific binding of the probe.[5] Wilkinson noted that some tissues seem to be more "sticky" than others and stated that use of a different probe sequence or adjusting the

hybridization or washing conditions may resolve the problem. He indicated that certain probe sequences give higher background signal and use of a probe from a different region of the target gene may be necessary. Since the two TS– cases showing background staining affected the specificity calculations, such manipulations may have proved of interest. Unfortunately, in the study setting at Kreatech Laboratories, this was not an option. The non-hybridised control section recommended by Scott Young[54] revealed that the signal could be artifactual. The simple step of repeating the tests alleviated the problem to an extent.

Serial sections 4 & 5 from these two cases were subjected to repeat hybridization. Specimen 5H 1884 was shown to be Chlamydia positive in serial section 2 (first run) and in serial section 4 (second run). Subsequently, testing of these specimens with PCR showed Chlamydial DNA in 5H 1884 and resolved this problem. Case 5H 1884 was shown to be a morphological false negative. Returning to the Pap smear case, the few granules in the mucus streaks of the PS– smear could have been artifactual but they could equally have represented Chlamydial EB. The latter interpretation was accepted.

Re-staining of the two TS– cases helped to achieve the clarification of the diagnosis. The background was reduced in the second run but the apparent signal in question was still present in one of them in the second run. This particular TS– (5H1884) was interpreted as a false negative morphology (Table 6.19).

Positive controls DISH

Identification of a positive control for the DISH method required the use of EM, independent corroboration using DISH at the Kreatech Laboratories and PCR at the reference laboratory at Georg-August University. The PCR method used in the reference laboratory was validated when the reaction product was sequenced and was homologous to the targeted Chlamydial plasmid DNA sequence. The untested morphologic criteria were challenged for the first time when it became necessary to find a positive tissue control for DISH. This exercise proved to be a one case corroboratory study of the criteria.

Staining of endocervical mucinous cells in DISH

It was noted in the literature review that non-specific binding of primary or secondary antibody to tissue due to charged groups on mucins can cause non-specific staining.[55] These charged groups can be swamped with non-immune serum (bovine serum albumin or foetal calf serum) to prevent this non-specific staining. Non-specific staining due to aldehyde groups present in the tissue section can be blocked by sodium borohydride.[56] Use of either of these techniques may have served to demonstrate or reduce non-specific staining that was problematic in a minority of cases. However, the cases probed with non-Chlamydial probe PLD043 did not show staining of mucins, indicating that non-specific staining was not an issue.

Interpretation of discordant results

Failure of PCR conducted on formalin-fixed paraffin-embedded tissue to detect the target DNA can be due to inadequate extraction of DNA from the specimens or due to inhibition of the chain reaction by traces of xylol or paraffin wax in the DNA extract. The laboratory in Goettingen conducts PCR on tissue samples daily and the standard protocols are geared to deal with these pitfalls. It was evident that these potential problems did not arise in the majority of the specimens tested (Chlamydial DNA was found by PCR in 22 of 25 PS+). However, two TS+ cases (5H 1016C and 6H 1844 C) that showed signal for Chlamydial DNA by DISH were negative using PCR (Table 6.19). These cases could have been PCR false negatives due to inadequate extraction of DNA or the presence of inhibitory residues.

In the morphologic study, the similarity of the changes caused by the *Chlamydiae* and HPV was highlighted. This appeared to cause more of a problem in Pap smears. Criteria to separate the two were developed. To validate the criteria separating Chlamydial change from HPV effect, the protocol for DISH testing of Pap smears included probing TS+ for HPV. The negative control areas in seven of the PS+ were probed with HPV-DIG1 (the housekeeping gene) rather than the negative control probe PLD043. Since confusion between Chlamydial changes and HPV effect seemed a lesser problem in tissue sections, the protocol for DISH testing in TS+ did not include this refinement. One PS+ that did not show Chlamydial DNA by DISH in the test area showed signal in the negative control area probed with HPV-DIG1. It seemed that the morphologic changes in this smear were probably due to HPV not Chlamydia.

The results emphasised the association between Chlamydial and HPV infection and also highlighted the potential diagnostic problem of confusing the two. This observation may explain the occurrence of one TS+ in which the morphologic diagnosis was discordant with both DISH and PCR. It is possible that changes seen in this TS+ case were also due to HPV not Chlamydia. In retrospect, the DISH protocol for tissue sections should have included TS+ probed with HPV-DIG1. Serial sections of this case (and all of the others) are stored in the study archive and could be probed for HPV DNA in the future.

While three PCR test results were discordant with the other two modalities, only one DISH test was discordant with morphology and PCR. This suggests that of the two DNA testing methods, DISH is the more robust. Two of the three discordant PCR results were negative PCR that occurred in DISH confirmed TS+. It is possible that extraction of DNA in these two specimens was inadequate and/or inhibitory residues remained in the DNA extract.

6.5.7 FUTURE STUDIES

Investigation of possible non-specific staining of mucin

The strong staining of mucinous cells not containing obvious inclusions could possibly have been interpreted as falsely positive, although the negative controls did not indicate this. False positive staining can be eliminated by swamping sections with non-immune serum[55] and/or sodium borohydride.[56] An experiment dealing with this matter should be conducted in the future.

The relationship between ASCUS/CIN and Chlamydial infection

A study in smears and tissue sections investigating a possible association of ASCUS and CIN with Chlamydial infection is contemplated. This would compare cases showing dual pathology (CIN or ASCUS with Chlamydial infection) with cases showing single pathology (CIN or ASCUS or Chlamydia infection). Simultaneous probing for both Chlamydia and HPV on the same smear or section using different chromogens would assist greatly in such an investigation. Such a study may lead to criteria to assist in separation of atypia due to HPV and atypia due to Chlamydial infection. Investigation of such cases would begin to investigate a possible causal association between Chlamydial infection and HPV/CIN/cervical carcinoma. Some important answers about the possible aetiologic role of Chlamydia in intraepithelial neoplasia and malignancy may be forthcoming. The un-used the spare serial sections (4 & 5 and 8 & 9) have been kept in the study archive and are available for possible future use.

6.6 CONCLUSION

The morphologic criteria were corroborated in tissue sections by a highly specific and sensitive exclusive expanded gold standard. In these sections, the morphologic criteria showed a specificity of 96% and a sensitivity of 90%. The Kreatech DISH method was validated by PCR testing at an independent institution. Once validated, the DISH results in Pap smears were regarded as sufficient to corroborate the Pap smear diagnoses.

It was concluded that it is possible to diagnose *Chlamydia trachomatis* infection in Pap smears and in tissue sections of *cervix uteri* with a high degree of confidence (96% specific) approaching 100%. The morphologic diagnosis can be made when the characteristic intracellular inclusions are present in squamous metaplastic cells showing particular inflammatory and reactive cellular changes.

6.7 QUALIFYING STATEMENT

The necessity for extremely high specificity[1] (approaching 100%) in diagnosing sexually transmitted disease should be borne in mind when offering a diagnosis of Chlamydial infection. The possible consequences of such a diagnosis are considerable. For this reason, a morphologic finding of Chlamydial infection should probably be reported as *suggestive* of the infection. It may be appropriate to include a statement in the report noting that the specificity of the diagnosis exceeds 95% but that corroboratory testing may be clinically appropriate, using one or more molecular DNA detection methods on a specimen suitable for the intended test.

7. ACKNOWLEDGMENTS

TEACH:
Tæcan (Old English from a Germanic root meaning 'show'.) *Give systematic information to a person about a subject or skill, practice this professionally, to communicate, to enable persons.*[1]

I wish to acknowledge the many teachers who have shared their knowledge and wisdom with me. Sadly, most of them have passed on. The first and most important of my teachers were my parents Aidan and Valerie. One of the numerous lessons my late father taught me was from Rudyard Kipling's poem If, and I try still "to retain the common touch." My mother continues to expect the highest ethical and moral standards from all of her children.

There were many teachers during my school career, some of whom were successful. My maternal great uncle and school headmaster Geoff Lineham was one of those memorable few. Paddy Brett was another headmaster, mentor and teacher whose lessons find daily expression in my life. Dave Wright taught me that with perseverance I could excel, even in the mysteries of chemistry. He encouraged his students to attend to spiritual matters also. From Tubby Reynolds I learned a sense of self worth, a dedication to physical exercise and to respect my physical well-being. The most successful of all of my teachers was Dorothy Sibson who somehow taught me some mathematics and physics. More importantly, she taught me to think, to question and to explore all aspects of an issue thoroughly.

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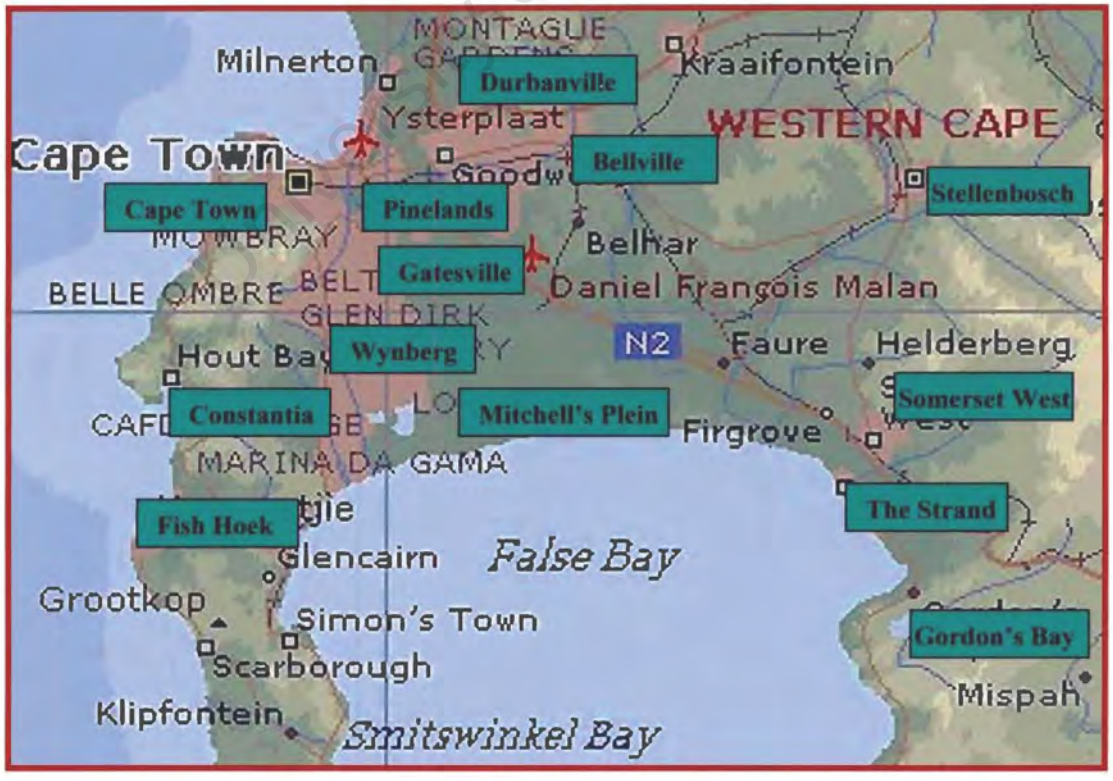
Appendix A: Geographic location of hospitals and clinics, drainage areas for the study.



APPENDICES

APPENDIX A

Geographic location of hospitals and clinics, drainage areas for the study

Atlantis:
Westfleur Medical Centre, Westfleur Circle.

Bellville:
Jan S Marais Clinic, Blanckenberg Street;
Louis Leipoldt Hospital, Broadway Road;

Brackenfell:
Boulevard Medical Clinic, Jeanette Street;

Cape Town:
City Park Hospital, Longmarket Street;

Claremont:
Claremont Surgical Clinic, Harfield Road;
Kingsbury Hospital, Wilderness Road;
Newlands Surgical Clinic, Main Road;

Constantia:
Constantiaberg Medi-Clinic, Burnham Street;

Durbanville:
Durbanville Private Hospital, Wellington Road;

Fish Hoek:
False Bay Hospital, 18th Avenue;

Gatesville:
Gatesville Medical Centre, Hazel Road, Rylands;

Goodwood:
Libertas Hospital, Voortrekker Road;
N1 City Hospital, Marinus Gerber Street;

Mitchell's Plein:
Mitchell's Plein Medical Centre;

Paarl:
Paarl Medi-City, Berlyn Street;
Paarl General Hospital, Hospital Street;

Panorama:
Panorama Medi-Clinic, Rothschild Boulevard;

Pinelands:
Vincent Pallotti Hospital, Alexandra Road;

Somerset West, Strand, Gordon's Bay:
Vergelegen Medi-Clinic, Main Street;
Hottentots Holland Hospital, Hospital Street;

Stellenbosch:
Stellenbosch Medi-Clinic, Cnr Safraan & Rokewood Street;

Worcester:
Medi-Clinic, Durban Road;

Wynberg:
Broad Road Medical Centre, Broad Road;
Wynberg Hospital, Darrol Brodie Street.

APPENDIX B

Papanicolaou Stain (1960)[5]

The progressive method of staining with haematoxylin was used, so that over-staining of the nuclei and cytoplasm in mucinous cells did not occur and subsequent differential extraction of haematoxylin with hydrochloric acid was not necessary. Graded alcohols were used to hydrate and dehydrate smears on the generally held assumption that distortion of cells is thus minimised.

The frequency of stain replenishment or replacement was monitored by microscopic checks and depended on the numbers of slides processed daily. Haematoxylin was replenished daily and was seldom discarded as this stain retains relatively constant staining characteristics. EA65 and OG6 were replaced at least every week, or when staining was dull or lost crisp contrast. Bluing solution was replaced, daily if not more frequently. Running water was used for water rinses. Alcohol rinses were changed after each use and the common practice of rotation of less contaminated alcohol was avoided. Absolute alcohol was changed daily and xylol was changed if slightly discoloured or if water contamination occurred, indicated by a milky appearance of the xylol.

Slides were stained manually in racks, dipped repeated in the rinses to remove excessive stain for crisp staining. The racks were tapped on a wad of paper toweling between stains and rinses to reduce contamination of reagents. The quality of staining was monitored daily and appropriate steps to maintain adequate nuclear detail and crisp cytoplasmic staining were taken.

Method:

1 Slides were hydrated through graded ethanol, 90%, 70%, 50%, distilled water, 10 dips each;
2 Nuclei were stained in Harris Haematoxylin, full strength with 4% acetic acid, 6 min.;
3 Slides were rinsed in running water;
4 Slides were blued in Lithium carbonate, 1 min.;
5 Slides were rinsed in running water;
6 Slides were rinsed in 50% ethanol;
7 Slides were dehydrated through graded ethanol, 70%, 90%, 100%, 100%, 10 dips each;
8 Cytoplasm was stained in EA-65, 3 min.;
9 Slides were rinsed in ethanol, 95%, 95%, 95%, 10 dips each;
10 Cytoplasm was stained in OG-6, 1 min.;
11 Slides were rinsed in ethanol, 95%, 95%, 95%, 10 dips each;
12 Slides were dehydrated in ethanol, 100%, 100%, 100%, 10 dips each;
13 Slides were cleared in 3 rinses of xylol, 10 dips each;
14 Slides were mounted and coverslip in Entalin® (3-aminopropyltriethoxysilane)

Solutions and stains:

Solutions were prepared using weight per volume, or volume per volume expressed as percentages. The weights of dye used in the EA-65 and OG-6 stains was based on the total dye content (TDC) printed on the label of certified dyes. The weight of dye actually used was calculated according to the percentage TDC. For example, if the TDC of OG-6 was 80%, for a 10% aqueous solution of OG-6, $^{10}/_{80}$ x 100 = 12.5 g were dissolved in 100 ml water.

Haematoxylin:

The Harris haematoxylin stain was used progressively in this method. Differential extraction using HCl was not necessary as staining time was reduced from 8 min. to 6 min. and prevented over staining of the cytoplasm. Preparation was as follows:

1 Haematoxylin 5g was dissolved in 50 ml ethanol;
2 Alum 100g $Al(NH_2)\ SO_4$ was dissolved in water, made up to 1 000 ml water, brought to the boil;
3 The solution was removed from the heat and 2.5 g HgO added as a ripening agent;
4 The solution was stirred until it became purple;
5 The flask was cooled in a water bath;
6 The solution was filtered and stored in a dark bottle. Shelf life was variable, months to years.

Eosin-Alcohol 65:

EA65 is a polychromatic cytoplasmic counter stain developed by Dr George Papanicolaou in 1942 and modified in 1954 and 1960.[158] The stain contains Light Green SF Yellow (Cl No. 42095), Bismarck Brown (CI # 21000), phosphotungstic acid in 95% ethanol. Aqueous stock solutions A and B are prepared and used to make the alcoholic stock solutions F and G for the making of EA-65 solution. [Solutions C,D and E not mentioned below, are variations

used in modified EA-65 used for specialised situations (eg staining cells on millipore filters)]. If Eosin Y (CI # 45380) is used, this stain dyes endocervical mucins pink. Eosin B (CI # 45400) stains endocervical mucins bluish. The stock solutions were prepared as follows:

1 Solution A: 2% aqueous TDC Light Green SF Yellow (CI # 42095);
2 Solution B: 10% aqueous TDC Bismarck Brown (CI # 21000);
3 Solution F: 1% alcoholic Light Green: 50 ml solution A in 950 ml 95% ethanol;
4 Solution G: 0.5% alcoholic TDC Bismarck Brown: 5 ml solution B in 95 ml 95% ethanol;
5 Solution H: 0.5% Eosin Y (CI # 45380): 5g Eosin in 1 000 ml 95% ethanol.

The EA-65 working stain (1 litre) was prepared as follows:

1 225 ml Solution F;
2 100 ml Solution G;
3 6 g phosphotungstic acid;
4 450 ml solution H;
5 225 ml 95% ethanol.

Orange G-6:
OG-6 is a polychromatic cytoplasmic counter stain that contains Orange G 6 (CI # 16230), 95% ethanol and phosphotungstic acid and was prepared (1 litre) as below:

1 50 ml 10 % aqueous TDC Orange G (CI # 16230);
2 950 ml 95% ethanol;
3 0.15 g phosphotungstic acid.

Graded alcohols:
Graded alcohols were prepared as follows:

Desired concentration (%)	Volume of water (ml)	Volume of 95 % ethanol (ml)
50	474	526
70	263	737
90	52	948

Lithium Carbonate:
Lithium carbonate was used as a bluing solution. A stock solution was prepared by dissolving 1.5 g $LiCO_3$ in 100 ml water. The working solution consisted of 30 drops of stock solution in 1 000 ml water.

APPENDIX C

Haematoxylin and Eosin stain

Haematoxylin is extracted from the heartwood of the tree *Haematoxylin campechianum* using hot water. The extracted is precipitated using urea. The precipitate is converted to the active satin hematein by oxidation. This may be done in air and light over a period of about four months ("ripening") and achieves a stable long lasting stain. Mayer's haematoxylin is oxidized using sodium iodate and Harris' haematoxylin using mercuric chloride. These reactions are instantaneous, but the tinctoral properties are poor. Hematein is anionic and has a poor affinity for tissue but stains acidic tissue (DNA and RNA) in the presence of a mordant. Aluminium, iron or tungsten salts and occasional lead salts are effective mordants. The stain and mordant form a complex. The cationic salt confers a net positive charge to the complex and allows the dye to bind to the anionic nuclei acids. It is usual to combine the mordant with the stains, to for alum haematoxylin, iron haematoxylin or tungsten haematoxylin. Pre-treatment of tissue with mordant before staining can be effective. Mayer's Haematoxylin uses aluminium potassium sulphate for mordant and confers a plumb red colour on nuclei. A regressive staining procedure is frequently used where the tissue sections are over stained then differentiated in weak acid alcohol to reduce excessive staining. The red staining is converted to a blue-black colour in weak alkali. Scott's Tap water, a weak bicarbonate solution, is frequently used.

Eosin is an effective cytoplasmic counterstain for alum haematoxylins. This dye stains different cell types and different types of connective tissue various shades of red and pink. Eosin is a xanthene dye obtainable as Eosin Yellow (CI 45380), Ethyl Eosin (CI # 45386) or Eosin B (CI # 45400). Eosin Y is soluble in water or alcohol and is widely used in a 0.5% or 1% solution in distilled water. Some differentiation of the stain results during a washing step in water. The addition of red dyes to eosin, example phloxine B is occasionally favoured to improve colour differentiation.

Method

1 De-wax sections in three washes of xylol
2 Hydrate sections through graded alcohols (100%, 95%, 70%) to running water
3 Stain in Mayer's Haematoxylin for 5 minutes
4 Wash in running water for 1minute
5 Differentiate in 1% acid alcohol about 1 or 2 seconds
6 Wash in water
7 Blue in Scott's Tap Water for 1 minute
8 Wash in running water for 2 minutes
9 Counterstain with Eosin for 1 minute
10 Wash in running water for 30 seconds
11 Dehydrate in graded alcohols (70%, 90%, 100%, 100%)
12 Clear in two washes of xylol for 5 minutes each
13 Mount using Entalin® and glass coverslips.

Solutions and stains

Solutions were prepared using weight per volume, or volume per volume expressed as percentages.

Mayer's Haematoxylin

Haematoxylin staining was regressive. The stain oxidized with sodium iodate and aluminium potassium sulphate used for mordant. The stain was prepared as follows:

1. Dissolve Haematoxylin 1g, potassium alum 50g and sodium iodate 0.2g d in 1000 ml distilled water, warm and stir, or leave over night;
2. Add 1g citric acid and 30 g anhydrous chloral hydrate to mixture and boil for 5 minutes;
3. Cool mixture, filter and use immediately. Store in a dark bottle and re-filter before use.

Eosin

Eosin Y 0.1% in distilled water was used without the addition of Phloxine.

APPENDIX D

Alcian blue periodic Schiff stain for mucins

The alcian dyes are cationic molecules with very high molecular weights (in excess of 1300). The alcian dyes form electrostatic bonds with tissue polyanions that contain either carboxyl or sulphate groups. The dye molecule is not able to combine with nucleic acid phosphates because of the large size of the molecule and the densely coiled structure of nucleic acids. Alcian blue stains acid mucins with high specificity and intensity and the reaction is permanent. Neutral mucins do not stain with alcian blue unless pre-treated with sulphuric acid or esterified by sequenced exposure to periodic acid and hydrochloric acid. Alcian blue is popularly used to stain acid mucins.

The periodic acid Schiff (PAS) reaction is an indicator for the presence of tissue carbohydrates (glycogen and mucins). Periodic acid cleaves C–C bonds by oxidation to form aldehydes from 1.2 glycols and the amino or alkylamino derivatives of 1.2 glycols. The aldehydes formed react with fuschin-sulphurous acid and subsequently combine with basic pararosaniline to form a distinctly magenta compound. The reaction stains neutral mucins magenta.

In combination Alcian blue and PAS stain both acid and neutral mucins. Acid and neutral mucins are clearly separated by this method and the stain is convenient to stain all mucins simultaneously.

Method

1 De-wax sections in three washes of xylol
2 Hydrate sections through graded alcohols (100%, 95%, 70%) to running water
3 Stain in Alcian blue for 5 minutes
4 Wash in water then in distilled water
5 Stain in 1% aqueous periodic acid for 5 minutes
6 Rinse well in distilled water
7 Stain in Schiff's reagent for 15 minutes
8 Wash in running water for 5 to 10 minutes
9 Lightly stain nuclei with Mayer's haematoxylin for 1 minute
10 Differentiate in 1% acid alcohol about 1 or 2 seconds if necessary
11 Wash in water
12 Blue in Scott's Tap Water for 1 minute
13 Wash in water for 30 seconds
14 Rinse in 100% alcohol
15 Clear in two washes of xylol for 5 minutes each
16 Mount using Entalin® and glass coverslips.

Solutions and stains

Solutions were prepared using weight per volume, or volume per volume expressed as percentages.

Alcian Blue

Dissolve 1g alcian blue in 100 ml 3% (v/v) Acetic acid.

Periodic acid solution

Dissolve 1 g periodic acid in 200 ml water.

Schiff's Reagent

1 Bring 200 ml distilled water to boiling point, remove from the heat and immediately dissolve 1 g of basic fuchsin in the hot water
2 Cool the solution to 50°C, add 2 g potassium metabisulphite and mix
3 Cool to room temperature and add 2ml concentrated HCl
4 Add 2g of activated charcoal, mix and stand overnight in the dark at room temperature (20°C)
5 Filter through # 1 Whatman filter paper to yield a clear or pale yellow solution
6 Store in a dark container at 4°C

APPENDIX E

Chlamydia Culture Protocol (Lancet Laboratories, Johannesburg).

Culture medium:
10% Minimal essential medium Eagle (EMEM) with EBSS and 25% µm Hepes Buffer

- 450 ml 10% EMEM,
- 50 ml foetal calf serum
- 10ml L Glutamine
- 500 µm Penicillin
- 500 µm Streptomycin
- 500 µm Fungizone

Maintenance medium:
2% EMEM with EBSS and 25% µm Hepes Buffer

Cell Culture Line:
McCoy cells.

Inoculation Procedure:
Use shell vial and make a good monolayer of cells:

- Make 1/40 dilution of cell line by trypsinzing 75 cm flask of cells.
- Suspend 1ml of 1/40 dilution into a shell vial trac bottle
- Incubate over night in 5% CO_2 incubator at 37°C
- Pour off medium from monolayer, add 40ml 10% EMEM and 1ml test culture in transport medium.
- Centrifuge at 4000 rpm for 60 minutes.
- Incubate for 2 hours in 5% CO_2 at 37°C to allow culture to equilibrate.
- Pour off test transport medium, add 1ml maintenance medium.
- Incubate in 5% CO_2 at 37°C for 48 hours.

Controls:
Run known negative and positive controls.

Passage Culture:
Blind passage culture for 48 hours, read if initial culture negative, inconclusive.

Detection method:

- Trypsinize culture and spin cells in a 5ml tube at 2000 rpm for 10 minutes
- Pour off supernatant and re-suspend in 1ml Hanks balanced salt solution
- Spin at 2000 rpm for 10 minutes, and repeat 5 times to remove all serum from cultured cells (serum may cause false positive results)
- After six washes, pour off most of the supernatant, mix culture cell sediment
- Spread 50mm cell sediment onto cell line slide and air dry
- Fix slide in acetone for 15 minutes, air dry.
- Stain slide using immuno-fluorescent anti-Chlamydial LPS & MOMP antibody method (CelLab)
- Put 50 µm stain onto fixed slide.
- Incubate in moisture chamber at 37°C in 5% CO_2 for 30 minutes
- Wash in PBS, air dry, mount and coverslip
- Examine for intra-cellular Chlamydial inclusions using fluorescent UV microscope

APPENDIX F

DNA *in situ* hybridization Protocol for Cytology Smears (Kreatech)

DIG/BIO-AP REMBRANDT® for Chlamydia trachomatis screening

PRETREATMENT of PARAFFIN SECTIONS	***INCUBATION TIME***
• de-wax in fresh xylene	2 x 10 min.
• soak in 100% ethanol and air-dry	5 min.
PRETREATMENT of CYTOLOGY SMEARS	
• soak-off coverslip in fresh xylene at 60°C	overnight.
• soak in 100% ethanol and air-dry	5 min.
PROTEOLYTIC TREATMENT	
• incubate *tissue sections* with 350 µl proteolytic reagent (2.5mg pepsin/ml 0.01 N HCl)	30 min. at 37°C
• incubate *cytology smears* with 350 µl proteolytic reagent (0.01 mg pepsin/ml 0.01 N HCl)	5-10 min. at 37°C
• discard excess proteolytic work solution	
• dehydrate slides in graded ethanol and air-dry	3 x 1 min.
HYBRIDIZATION of PROBE	
• apply 1-2 drop(s) Chlamydia-probe solution per specimen & cover with coverslip.	
• denature (heating block)	3 min. on 95°C
• hybridize (incubator)	2 hrs. at 37°C
• remove coverslips by soaking slides in TBS buffer	10 min.
WASHING	
• apply 5-6 drops differentiation reagent to each specimen (slide warmer)	15 min. on 37°C
• wash slides in TBS buffer	3 x 1 min.
DETECTION AND STAINING	
• apply 2-3 drops of AP-conjugated α-Digoxigenin/Biotin to each specimen (heating block)	30 min. at 37°C
• soak slides in TBS buffer	3 x 1 min.
• soak slides in deionized water	1 min.
• apply 2-3 drops of NBT/BCIP substrate to each specimen (slide warmer, in the dark)	15 min. at 37°C
• tap off excess substrate solution and wash slides in deionized water	3 x 1 min.
• mount sections in glycerol-gelatin for microscopical evaluation	

APPENDIX G

Chlamydia Culture Results (Lancet Laboratories, Johannesburg)

Patient	Lab No	Cyto Lab No	Date	Age	Race	Physician	Result
CH	CH8344	4C 7339; 4C8163	26/08/94	28	W	JD	Positive
EJvD	CH2831	4C7744	29/08/94	57	W	AJJB	Negative *
CM	CJ815	4C8486	02/09/94	36	C	AJJB	Positive
MB	CJ3420	4C9638	12/09/94	28	W	AJJB	Negative
LA	CJ5080	4C9938; 4C11237	16/09/94	25	W	KC	Positive
CB	CJ8020	4C9671	27/09094	32	W	RC	Negative
MM	CK5449	4C9745	20/10/94	41	C	AJJB	Negative
LdT	CK5448	4C9619	20/10/94	45	W	AJJB	Negative
ML	CL1584	4C10388	04/11/94	56	C	AJJB	Positive
EJ	CL2133	4C9893	07/11/94	35	W	SS	Positive
LB	CL2776	4C10795	09/11/94	47	C	AKL	Positive
CM	CL3154	4C10791	10/11/94	31	W	KC	Positive
YvZ	CP1680	5C1001	06/02/95	44	W	DS	Positive

* Patient had been treated for 3 days with iv Reverin (tetracycline) for pelvic sepsis, commencing 7 days prior to culture specimen taken.

No cases cultured	14	
No positive cultures	8	57%
No negative cultures	6	43%

APPENDIX H

Extraction of DNA from Tissue Sections for PCR (www.qiagen.com)

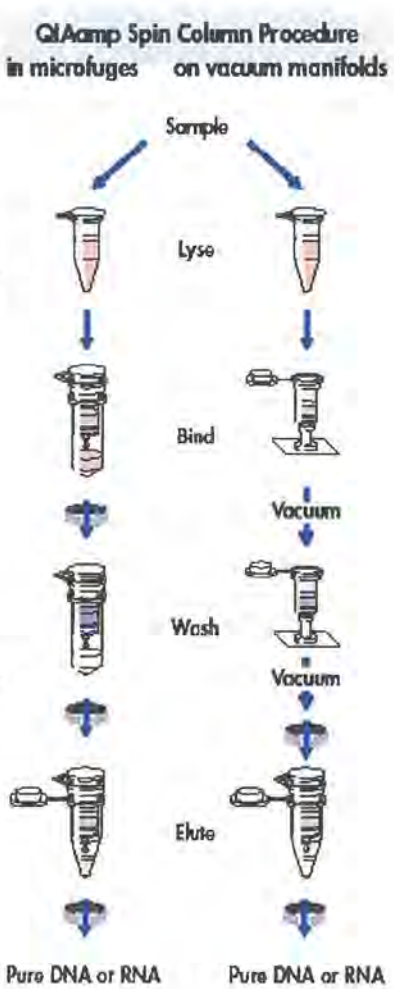
QIAamp DNA Mini Kit[2]

For isolation of genomic, mitochondrial, bacterial, parasite, or viral DNA

- Rapid isolation of high-quality, ready-to-use DNA
- No organic extraction or alcohol precipitation
- Consistent, high yields
- Complete removal of contaminants and inhibitors for reliable downstream applications

Format:	Mini spin columns
Sample sources:	Muscle, liver, heart, brain, bone marrow, and other tissues; swabs (buccal, eye, nasal, pharyngeal, and others), CSF, blood, body fluids, washed cells from urine
Sample size:	Up to 50 mg tissue or up to 200 µl fluid
Hands-on preparation time (after lysis):	20 minutes
Typical yield:	See table "Yields with the QIAamp DNA Mini Kit"
Elution volume:	50–200 µl

The QIAamp DNA Mini Kit is ideal for purification of DNA from most commonly used human tissue samples, such as muscle, liver, heart, and brain.



Principle

QIAamp Kits simplify isolation of nucleic acids from a wide variety of clinical samples with fast spin-column or 96-well-plate procedures. No phenol–chloroform extraction is required. Nucleic acids bind specifically to the QIAamp silica-gel membrane while contaminants pass through. PCR inhibitors such as divalent cations and proteins are completely removed in two efficient wash steps, leaving pure nucleic acid to be eluted in either water or a buffer provided with the kit. QIAamp technology yields genomic, mitochondrial, and bacterial DNA, total cellular RNA, and viral nucleic acids from many sample sources ready to use in PCR and blotting procedures.

No mechanical homogenization is necessary as the tissues are lysed enzymatically, and the convenient spin-column procedure means that hands-on preparation time is only 20 minutes. Samples can be processed using either a microcentrifuge or, if blood or other body fluids are being processed, using the QIAvac 24 or QIAvac 6S vacuum manifold. In addition, the rigorous lysis procedure employed makes the QIAamp DNA Mini Kit ideal for purification of genomic DNA from bacteria or parasites.

Procedure

Optimized buffers lyse samples, stabilize nucleic acids, and enhance selective nucleic acid adsorption to the QIAamp membrane. Alcohol is added and lysates loaded onto the QIAamp spin column or 96-well plate. Wash buffers are used to remove impurities and pure, ready-to-use DNA or RNA is then eluted in water or low-salt buffer. The entire process requires only 20 minutes of handling time for mini spin-column formats (lysis times differ according to the sample source). Processing time using high-throughput QIAamp 96 Kits is as little as 1 minute per sample.

Vacuum processing

With the QIAamp DNA Blood Mini Kit, the QIAamp DNA Mini Kit, and the QIAamp Viral RNA Mini Kit, blood and other body fluids can be processed by vacuum instead of centrifugation, for greater speed and convenience in nucleic acid purification. QIAamp mini spin columns are accommodated on the QIAvac 24 manifold using VacValves and VacConnectors, provided in the QIAamp Vac Accessory Set. VacValves should be used if sample flow rates differ significantly, in order to ensure consistent vacuum. Disposable VacConnectors are used to avoid any cross-contamination. Use of VacConnectors also allows these QIAamp spin procedures to be performed on QIAvac 6S with QIAvac Luer Adapters.

Table "Yields with the QIAamp DNA Mini Kit" shows typical DNA yields obtained from various tissue-sample types using the QIAamp DNA Mini Kit. QIAamp sample preparation technology is fully licensed, allowing QIAamp purified nucleic acids to be used in any molecular assay or other downstream application without risk of patent infringement.*

Yields with the QIAamp DNA Mini Kit

Sample		Yield	
		Total nucleic acids (µg)*	DNA (µg)†
Blood	(200 µl)	4–12	4–12
Buffy coat	(200 µl)	25–50	25–50
Cells	(107)	40–60	30–40
Liver	(25 mg)	60–115	10–30
Brain	(25 mg)	35–60	15–30
Lung	(25 mg)	25–45	5–10
Heart	(25 mg)	15–40	5–10
Kidney	(25 mg)	40–85	15–30
Spleen	(10 mg)	25–45	5–30

** Nucleic acids obtained without RNase treatment*

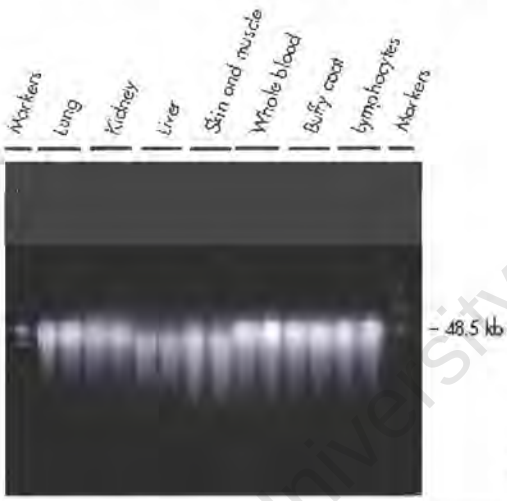
† Nucleic acids obtained with RNase treatment

Downstream applications

DNA purified using the QIAamp DNA Mini Kit is sized up to 50 kb (see figure "Purified Genomic DNA up to 50 kb"). DNA of this length denatures completely and has the highest amplification efficiency. QIAamp Kits are intended for general-purpose use in a number of diagnostic applications,* including:

- Viral research
- Bacterial research
- Fungal research
- Cancer research
- Human genetic testing research
- Paternity testing
- Forensic analysis

Purified Genomic DNA up to 50 kb



Size distribution of DNA prepared with QIAamp Kits from the indicated sources (3 µg per lane).

* QIAamp Kits are intended as general-purpose devices. No claim or representation is intended for their use to identify any specific organism or for clinical use (diagnostic, prognostic, therapeutic, or blood banking). It is the user's responsibility to validate the performance of QIAamp Kits for any particular use, since the performance characteristics of these kits have not been validated for any specific organism.

APPENDIX I

Abstract of the presentation made during the meeting of the Papanicolaou Society held during the XX International Congress of the International Academy of Pathology in Hong Kong in 1994.

Abstract:
Knight BK
"Identification by DNA in situ hybridization of *Chlamydia trachomatis* in Papanicolaou smears of the uterine cervix." Int J Surg Pathol 1995, 2 Suppl: Abstract 106.

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IDENTIFICATION BY DNA *in situ* HYBRIDIZATION OF *Chlamydia trachomatis* IN PAPANICOLAOU SMEARS OF THE UTERINE CERVIX

Bryan Knight, University of Cape Town, South Africa

Chlamydia trachomatis infects 3% of females and causes about 40% of cases of cervicitis seen at STD clinics in the USA. The infection may present as a muco-purulent cervicitis, but most women harbour sub-clinical disease. Thus infection may be discovered unexpectedly. Criteria for the diagnosis by Papanicolaou smear have been well described. Chlamydia culture is neither simple, sensitive nor cost-effective and the sensitivities of direct immuno-fluorescence and ELISA assays are not fully satisfactory. Consequently, correlation between cytology and these methods is poor and has resulted in wide-spread doubt about the validity of morphologic diagnosis. However, some immunologic and electron microscopic studies have confirmed that Chlamydial vacuoles seen in Papanicolaou smears are indicative of *C. trachomatis* infection.

This study reports the results of *in situ* DNA hybridization techniques on Papanicolaou smears using a commercially available kit (Kreatech Labs, Amsterdam). *Chlamydia trachomatis*-specific DNA was demonstrated in both positive controls and test smears. Elemental Bodies and Reticulate Bodies in characteristic vacuolar intra-cellular inclusions were positively stained. It is concluded that using simple morphologic criteria, confident diagnosis can be made of *Chlamydia trachomatis* infections on routine Papanicolaou smears.

APPENDIX J

Abstract of the presentation made during the XIV International Congress of the International Academy of Cytology in Amsterdam, The Netherlands in 2001.

Abstract:
Knight B, Janssen P, Schaapveld R & Houthoff H.
"Morphologic criteria for the diagnosis of *Chlamydia trachomatis* infection corroborated by DNA *in situ* hybridization using the new Universal Linkage System (ULS®)."
Acta Cytol. 2001, **45**: 151 (Suppl).

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DNA IN SITU HYBRIDIZATION FOR CHLAMYDIA TRACHOMATIS USING THE NEW UNIVERSAL LINKAGE SYSTEM

Bryan Knight [1]; Patrick Janssen [2]; Roel Schaapveld [2].

1. Anatomical pathology, University of Cape Town, South Africa;
2. Kreatech Diagnostics, Amsterdam, the Netherlands.

Objective: In the light of recent advances in DAN technology, corroboration of cytologic criteria for the diagnosis of *Chlamydia trachomatis* infection using DNA testing as a gold standard seemed appropriate.
Method: A new method for labelling DNA probes, known as the Universal Linkage System (ULS®) was used to demonstrate Chlamydial DNA in Papanicolaou smears. This non-enzymatic system uses a platinum derivative as an intermediary between the nucleic acid probe and the non-isotopic marker, digoxigenin. Signal was obtained using alkaline--phosphatase/NBT/BCIP. Twenty five Papanicolaou smears showing morphologic features suggestive of *Chlamydia trachomatis* infection and ten smears that did not show these changes were tested. Smears were randomly assigned to four separate batches stained on four consecutive days and screened blind.
Results: Twenty three of 25 positive smears showed Chlamydial DNA-staining within vacuoles in the metaplastic squamous cells. Staining was also seen in the cytoplasm of both metaplastic squamous and cuboidal mucinous epithelial cells. Fine granular staining of the elementary particles present in endocervical mucous threads was abundant. One of the 10 smears that was negative for Chlamydial inclusions showed some positive staining in mucous streaks and several metaplastic cells. In all of the 35 cases tested, a demarcated area on each slide that had not been exposed to probe (negative control) did not stain. Known positive controls with each batch of slides and stained appropriately.
Conclusion: DNA *in situ* hybridization confirms that reliable morphologic diagnosis of *C. trachomatis* can be made using Pap smears.

Seminar XIII: Molecular Biology in Cytology: May 30: 1400 – 16.00: Oral presentation: 12 min.

APPENDIX K

Abstract of the presentation made during the meeting of the American Society of Clinical Pathology held in Washington, DC 2002.

Abstract:
Knight BK, Janssen P, Schaapveld R & Schlott T.
"Histologic diagnosis of Chlamydia trachomatis infection in the uterine cervix confirmed by two DNA techniques."
Am J Clin Pathol, 2002, **118:** 339 –340.

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HISTOLOGIC DIAGNOSIS OF *CHLAMYDIA TRACHOMATIS* INFECTION IN THE UTERINE CERVIX CONFIRMED BY TWO DNA TECHNIQUES.

Bryan Knight, [1] M Med Path (Anat), FIAC; Patrick Janssen, [2] BSc; Roel Schaapveld, [2] PhD; and Thilo Schlott, [3] PhD.

1. University of Cape Town, Cape Town, South Africa;
2. Kreatech Diagnostics, Amsterdam, The Netherlands;
3 Georg-August University, Goettingen, Germany.

Chlamydia trachomatis infects 3% of females in the United States and causes 40% of cases at sexually transmitted disease clinics. Sub-clinical in most women, infection is associated with menstrual irregularities, pelvic pain, infertility, risk of ectopic pregnancy, cervical intraepithelial neoplasia, and possibly cervical carcinoma. *Chlamydiae* are unique bacteria taking two forms: elemental bodies (EBs) and reticulate bodies (RBs). EBs are metabolically inactive; RBs are reproductive within intraepithelial vacuoles. Variable in size, Chlamydial vacuoles contain eosinophilic or basophilic inclusions visible by microscopy in smears of exfoliated cells. Diagnosis of inclusions on Papanicolaou (Pap) smears was advocated years ago but is not described in the histology literature.

We histologically examined 2,682 surgical specimens, including hysterectomies, cervical biopsies, polypectomies, cone biopsies and hot loop resections. Features for the recognition of Chlamydial infection in sections were anticipated by extrapolation of the Pap smear morphologic features. Serial sections from 20 positive and 7 negative cases were tested for Chlamydial DNA by *in situ* hybridization (DISH) and polymerase chain reaction (PCR).

Chlamydial infection with inclusions was found in 257 cases. These showed inflammation, usually lymphocytic with plasma cells, and sometimes neutrophils or lymphoid follicles. All showed immature squamous metaplasia with "reactive" nuclear changes. Intracellular Chlamydial vacuoles ranged from 1 μm to 15 μm in size and were found in metaplastic cells. Chlamydial vacuoles showed intravacuolar inclusions of four types: "targetoid eosinophilic," "targetoid basophilic," "nebular" with numerous coccoid bodies, or "festooned" with large irregular inclusions. Using DISH, Chlamydial DNA was localized within vacuolar inclusions in 95% of cases (100 % specificity, 78 % sensitivity). Using PCR, Chlamydial DNA was detected in 80% of cases (89% specificity and 83% sensitivity). Confirmation by both methods was possible in 75% of cases.

Two independent molecular techniques confirmed histologic diagnoses of Chlamydial inclusions.

APPENDIX L

Processing of tissues for Electron Microscopy

All processing and handling of tissue and reagents done under fume hood, operator required to wear gloves, handle tissue and sections with forceps. Specimens carefully labelled and steps recorded on laboratory processing form.

Pop-off technique for Papanicolaou smears

Procedure

1 Locate cells of interest and mark the back of the slide using a diamond pencil
2 Soak slides in xylol until coverslips drop off unassisted
3 Wash in two changes xylol for ten minutes each
4 Hydrate smears through graded alcohols (100%, 95%, 70%) to running water
5 Wash slides in phosphate buffer solution
6 Post-fix with 2% osmium tetroxide (OsO_4) for 1 hour
7 Wash in phosphate buffer solution
8 Dehydrate in graded alcohols (70%, 90%, 100%, 100%)
9 Wash in two changes of acetone
10 Cover the slide with a mixture of Spurs resin/propylene oxide 1:1 for 15 minutes
11 Tip slide to drain resin
12 Cover the slide with a mixture of Spurs resin/propylene oxide 3:1 for 15 minutes
13 Tip slide to drain resin
15 Cover the slide with two changes of Spurs resin for 15 minutes each
16 Fill an embedding capsule to the brim with Spurs resin, place in a rack
17 Place the slide face down with demarcated cells on the surface of the filled resin capsule
18 Allow the Spurs resin to set overnight in an incubator at 70°C
19 Run cold tap water over the slide/capsule, slide should dislodge with ease
20 Cut ultra-thin sections, taking care not to cut the cells away while trimming the block
21 Place a drop of saturated uranyl acetate solution on a sheet of dental wax
22 Place grid, shiny surface down into the drop of uranyl acetate, stain for 2 minutes
23 Wash grid well in two changes of ultra-pure water
24 Dry on blotting paper
25 Place a drop of lead nitrate solution on a sheet of dental wax
26 Place grid, shiny surface down into the drop of lead nitrate, stain for 2 minutes
27 Wash grid well in two changes of ultra-pure water
28 Dry on blotting paper, store section in clean dry petri dish
29 Examine ultra-thin section in electron microscope, photograph cells of interest.

Processing of tissue blocks

Procedure

1 Fix block of tissue 2 x 2 x 4 mm in 2.5% gluteraldehyde for 2 – 4 hours at 4°C
2 Wash in phosphate buffer solution over night
3 Store tissue indefinitely in phosphate buffer solution at 4°C
4 Wash in distilled water
5 Post-fix with 2% osmium tetroxide (OsO_4) for 1 hour at room temperature (20°C)
6 Wash in distilled water
7 Stain in 0.5% uranyl acetate in 80% acetone
9 Wash in 90% acetone for 15 minutes
10 Wash in 100%acetone for 30 minutes
11 Impregnate tissue in Spurs resin/acetone 1:1 for 90 minutes
12 Impregnate tissue in Spurs resin for 60 minutes at 70°C, repeat once
13 Embed in embedding capsule with Spurs resin, incubate at 70°C over night
14 Run cold tap water over the capsule, block should dislodge with ease
15 Trim the block, cut several semi-thin sections, place on glass slide in a droplet of water
16 Heat slide on hotplate at 60°C until drop evaporates
17 Pipette filtered 1% Toluidine Blue onto sections
18 Continue to heat until golden rim develops at the edges of drop
19 Remove from heat, wash with distilled water, blot dry
20 Examine by light microscopy to locate tissue cells of interest

21 Trim the block, cut ultra-thin sections using a glass knife, lift from water trough onto shiny side of a copper EM grid
22 Place a drop of saturated uranyl acetate solution on a sheet of dental wax
23 Place grid, shiny surface down into the drop of uranyl acetate, stain for 2 minutes
24 Wash grid well in two changes of ultra-pure water
25 Dry on blotting paper
26 Place a drop of lead nitrate solution on a sheet of dental wax
27 Place grid, shiny surface down into the drop of lead nitrate, stain for 2 minutes
28 Wash grid well in two changes of ultra-pure water
29 Dry on blotting paper, store section in clean dry petri dish
30 Examine ultra-thin section in electron microscope, photograph cells of interest.

Solutions, stains and reagents

Gluteraldehyde 2.5%
Stock solution of 25% Gluteraldehyde kept at 4°C
Working solution 2.5% Gluteraldehyde, 100 ml stock made up to 1000 ml with phosphate buffer solution kept at 4°C prior to use, good for 30 days.

Phosphate buffer solution (0.1M, pH 7.2)
Solution A: prepare 0.2M NaH_2PO4 by dissolving 27.68g in 1000 ml distilled water, keep at 4°C
Solution B: prepare 0.2M Na_2HPO4 by dissolving 28.38g in 1000 ml distilled water, keep at 4°C
Mix 280 ml solution A and 720 ml solution B, mix well, adjust pH to 7.2, keep at 4°C

Osmium tetroxide (Palades)
Solution A: prepare stock veronal acetate buffer: mix 14.7g sodium barbitol (veronal) with 9.7g sodium acetate in 500 ml distilled water
Solution B: stock osmium tetroxide (2%): mix 1g (ampoule) OsO_4 with 50 ml distilled water
Solution C: prepare 0.1N HCl: add 8.6 ml HCl to 1000 ml distilled water
Palades solution: mix 12.5 ml solution A, 31.25 ml solution B, 12.5 ml solution C & 6.25 ml distilled water, keep at 4°C

Spurs resin (kit Taab)
Add the bottle Spurs resin and the vial of accelerator in to the bottle of hardener, mix well, store at –20°C

Uranyl acetate
Prepare saturated uranyl acetate in ultra-pure water, store in a dark bottle at 4°C
Centrifuge 1 ml uranyl acetate in an Eppendorf cup at 5000 rpm for 15 minutes prior to use.

Lead Nitrate
Store Lead Nitrate at 4°C
Centrifuge 1 ml lead nitrate in an Eppendorf cup at 5000 rpm for 15 minutes prior to use.