THE DEVELOPMENT AND EVALUATION OF A POLYMERASE CHAIN REACTION ASSAY FOR THE DIAGNOSIS OF *MYCOBACTERIUM TUBERCULOSIS* INFECTIONS

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ABSTRACT

The diagnosis of *Mycobacterium tuberculosis* by conventional techniques is associated with a number of problems which include lack of sensitivity (microscopy) and prolonged incubation periods (laboratory culture). For these reasons various "rapid detection" methods have been developed but none of these meet all the requirements of sensitivity, specificity and rapidity. DNA probes, although rapid, only have a sensitivity similar to that of Ziehl-Neelsen stain.

The polymerase chain reaction (PCR) has been shown to exhibit extreme sensitivity and specificity for a wide variety of infectious agents including virus, bacteria and protozoans. There have also been a few promising reports of PCR assays for *M.tuberculosis* and *M.leprae.*

This thesis deals with the development of a PCR assay in which a repetitive 336 bp fragment is amplified from *M.tuberculosis* DNA. The assay is extremely sensitive (less than 10 organisms) and is entirely specific for *M.tuberculosis*. The assay was assessed in a comprehensive trial in which PCR was compared with conventional detection techniques in pleural fluids from 84 patients. The results of this trial demonstrate that PCR is more sensitive than laboratory culture and indicate that PCR can be used for the routine diagnosis for *M.tuberculosis* infections.

PUBLICATIONS

Some of the work contained in this thesis can be found in the following publications:

De Wit D, Steyn LM, Shoemaker S, Sogin M (1990). Direct detection of *Mycobacterium tuberculosis* in clinical specimens by DNA amplification. J Clin Microbiol **28:2437-2441**

De Wit D, Maartens G, Steyn LM (1991). A comparative study of the polymerase chain reaction and conventional procedures for the diagnosis of tuberculous pleurisy. Under review New Engl J Med

Copies of these articles can be found in the back cover of this thesis.

LIST OF ABBREVIATIONS

bp	:	Base pair
^{o}C	:	Degrees Celcius
D	:	Dalton
dATP (A)	:	Deoxyadenosine triphosphate
dCTP (C)	:	Deoxycytidine triphosphate
dGTP (G)	:	Deoxyguanosine triphosphate
dTTP (T)	:	Deoxythymidine triphosphate
DNA	:	Deoxyribonucleic acid
EDTA	:	Ethylenediaminetetra - acetic acid
fg	:	Femtogram
g	:	Gram
kb	:	Kilobase pair
kD	:	Kilodalton
M	:	Molar
mg	:	Milligram
ml	:	Millilitre
mM	:	Millimolar
ng	:	Nanogram
nM	:	Nanometre
nt	:	Nucleotide
^{32}P-dCTP	:	dCTP radioactively labelled with phosphorus-32
pg	:	Picogram
U	:	Unit
ug	:	Microgram
V	:	Volt
V/V	:	Volume per volume
W/V	:	Weight per volume

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CHAPTER 1

LITERATURE REVIEW

1.1 *MYCOBACTERIUM TUBERCULOSIS*

1.1.1 Historical Aspects

Tuberculosis is a disease of great antiquity and has afflicted man for many centuries. Possibly the earliest evidence of tuberculosis was that of a skeleton unearthed from a Neolithic burial ground near Heidelberg in Germany which showed fusion of spinal vertebrae that was almost certainly caused by tuberculosis. Carbon dating showed that the affected patient died more than 6000 years ago (Collins, 1989). Clear evidence of bone tuberculosis has been found in Egyptian remains that date back to around 3400 B.C., and the well preserved mummy of Nesperehan showed evidence of Potts disease. Ancient skeletal remains unearthed in one part of Egypt show so much evidence of tuberculous destruction that it is thought there might have been a large sanatorium for the treatment of tuberculosis (Collins, 1989).

Some 4000 years ago the disease is known to have existed in the land between the Tigris and Euphrates rivers and during the reign of King Hammurabi (about 2200 B.C.) there was an ancient prayer against tuberculosis (Collins, 1989). Hippocrates wrote an account of the clinical features of both pulmonary and spinal tuberculosis in 400 B.C. and had a temple of healing on the island of Cos in the Agean (Grange 1984b).

The Bible makes reference to possible tuberculous disease on a number of occasions: Moses excluded hunchbacked members of priestly line from officiating at the altar (Leviticus 21:20); a wasting disease

killed many of the Israelites in the exodus from Egypt under Moses after they complained about the monotonous diet of manna (Psalms 106:15); the warriors of the King of Asyria were smitten by a wasting disease (Isiah 10:16).

A mummified child of the Nazca culture (about 700 A.D.) was discovered in Southern Peru. It had clear evidence of Potts disease; a psoas abscess and tuberculosis of the lung, pleura, liver and kidney. Tubercle bacilli were also demonstrated in the lung, liver and kidney (Allison *et al.*, 1973).

It is not known when tuberculosis reached Western Europe but there is abundant evidence in medical and non-medical writings (e.g. Charles Dickens). From these accounts, it appears that the disease ravaged Europe during the 16th Century and although waxing and waning, remained a serious problem till after the Second World War. It was thought that the disease was brought to the Americas by European explorers but the Peru find appears to contradict this.

When tuberculosis reached South Africa is not entirely clear. Paola da Gama (older brother of Vasco) reached Southern Africa in 1497 and died of tuberculosis in the Azores in 1499. In the years that followed many sailors, settlers and "consumptives" from Europe came to South Africa, the latter seeking a cure in a more favourable climate. This importation of the disease, together with the crowded conditions associated with the establishment of the mining industry during the late 19th and early 20th century, probably started the epidemic of tuberculosis which continues to the present.

Although tuberculosis had afflicted man for centuries, the nature of the disease was only established in the latter half of the nineteenth century. In 1865, Francois Villemin demonstrated that extracts of diseased human and bovine tissue when injected subcutaneously into guinea pigs and rabbits, caused disease in the recipients (Des Pres and Goodwin, 1985). These experiments demonstrated for the first time the transmissiblity of tuberculosis. In 1882, Robert Koch was finally able to demonstrate the tubercle bacillus in diseased tissue and showed that it was the cause of tuberculosis. This discovery was made possible by the use of special staining techniques and the recognition that the tubercle bacillus required much longer incubation times than the known bacteria of the time. Robert Kochs work was and must still be regarded as one of the greatest achievements of experimental medicine (Koch, 1932. English translation of original article).

1.1.2 **Epidemiology**

Tuberculosis causes more morbidity and mortality than any other infection in the world, due mainly to the high prevalance of the disease in the densely populated developing countries. There are an estimated 20 million active cases in the world (1983) and the mortality due to the disease is approximately 3 million annually (Stead and Dutt, 1983).

Most epidemics of infectious diseases can be measured in weeks or months but a tuberculosis epidemic is measured in decades. Grigg (1958), has described a theoretical tuberculosis epidemic curve and described three separate categories of disease (Figure 1.1): i.e. mortality (elimination of susceptibles), morbidity (disease in the more resistant), and contacts (infection without disease). All three

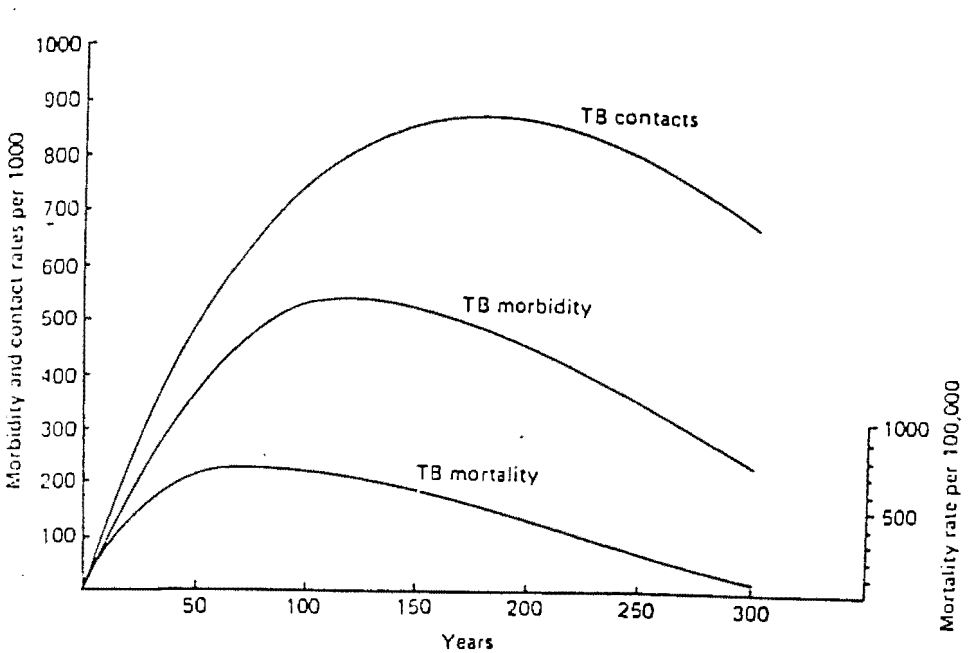


Figure 1.1: Epidemic Wave of Tuberculosis. A theoretical curve of the development of tuberculosis in a community. Three curves are defined and all show an ascending limb and a more prolonged descending limb (Grigg, 1958)

curves peak at 50 to 100 year intervals and run a course over 300 years. These curves imply that tuberculosis is a cyclic disease and as such is a self-limiting phenomenon. This is supported by the fact that the mortality rate of tuberculosis was well on the decline before the introduction of BCG vaccination and Isoniazid (INH) therapy (see Figure 1.2). This may reflect the gradual development of resistance to the organism in a previously susceptable population (Kass, 1971, Comstock, 1982).

In England, it is estimated that the present epidemic began in the sixteenth century, reached its peak at about 1780 and since then has been slowly declining. The peaks of Western Europe, Eastern Europe and North America occurred about 1800, 1880 and 1900 respectively (Stead and Dutt, 1983).

South Africa, together with many other under-developed countries, is at present experiencing an epidemic which probably began at about the turn of the century (Collins, 1982). The annual morbidity rates for South Africa since the disease became notifiable in 1920 are shown in Figure 1.3. Projection of the curve backwards supports the theory that the present epidemic had its origin at the beginning of the 20th century. The marked increase in notification rate that begins from about 1950 is not considered natural but was partly due to the implementation of numerous mobile X-ray units which were used throughout South Africa as well as routine X-ray screening of mine recruits since 1942 to 1947. It does, however, appear that the peak of the epidemic has passed. In comparison with the cycles in Europe which spanned two centuries, the epidemic in South Africa appears to have been dramatically

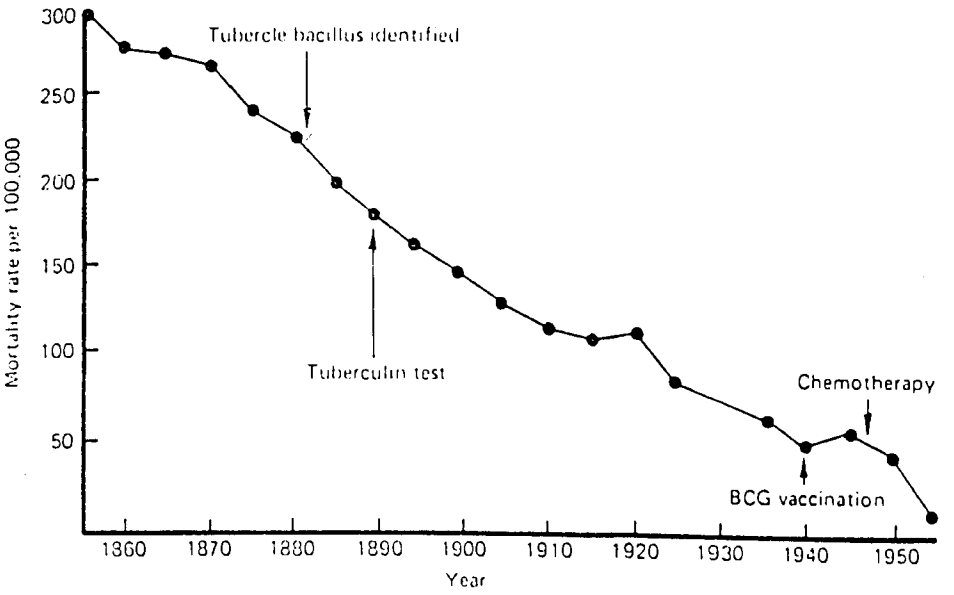


Figure 1.2: Natural Decline of Tuberculosis in a Community. Mean annual morbidity figures of pulmonary tuberculosis in England and Wales show the gradual decline of tuberculosis prior to the advent of BCG and INH (Kass, 1971).

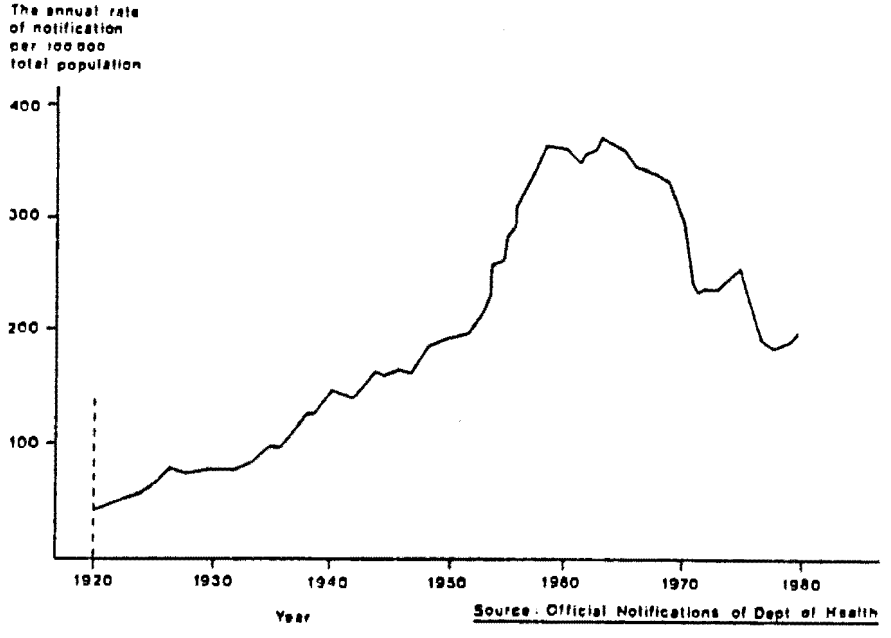


Figure 1.3: The Epidemic Curve in South Africa. The annual notification rate of all forms of tuberculosis in South Africa from 1921 to 1980 (Collins, 1982).

shortened, and this must undoubtedly be due to the introduction of INH in the 1950's and to a lesser extent to the introduction of BCG vaccination in 1941. The dramatic effect of INH on mortality rates in South Africa is shown in Figure 1.4.

The mortality and morbidity data are published annually by health authorities in most countries. In developed countries, this data is usually considered to be fairly accurate. In developing countries such as South Africa, however, most data must be considered as rough estimates only (Botha and Bradshaw, 1985; Yach, 1987). This is due to numerous factors such as the continual migration of the population, uncertainty of population numbers, under-registration and misclassification of deaths and cases, inadequate case finding and case holding and insufficient funding and resources. In spite of these problems, a number of authors have attempted to evaluate the South African morbidity and mortality data using available statistics (Benatar, 1982; Kleeberg, 1982; Yach, 1987; Collins, 1982). The rates of registered deaths per 100 000 population for tuberculosis were in 1980: 30 for Blacks, 38 for Coloureds, 2 for Whites and 6 for Asians. The figures were similar for 1984 (Yach, 1987). The annual notified case rate per 100 000 population in 1980 was 250 for Blacks, 326 for Coloureds, 13 for Whites and 86 for Asians. This annual notified case rate, however, probably only represents the tip of the iceberg and figures of 1500 per 100 000 in the Transkei and Ciskei (Kleeberg, 1982) are probably a more accurate reflection of the "infected pool".

It is well established that patients who produce sputum in which acid fast bacilli can be demonstrated by microscopy are the principle

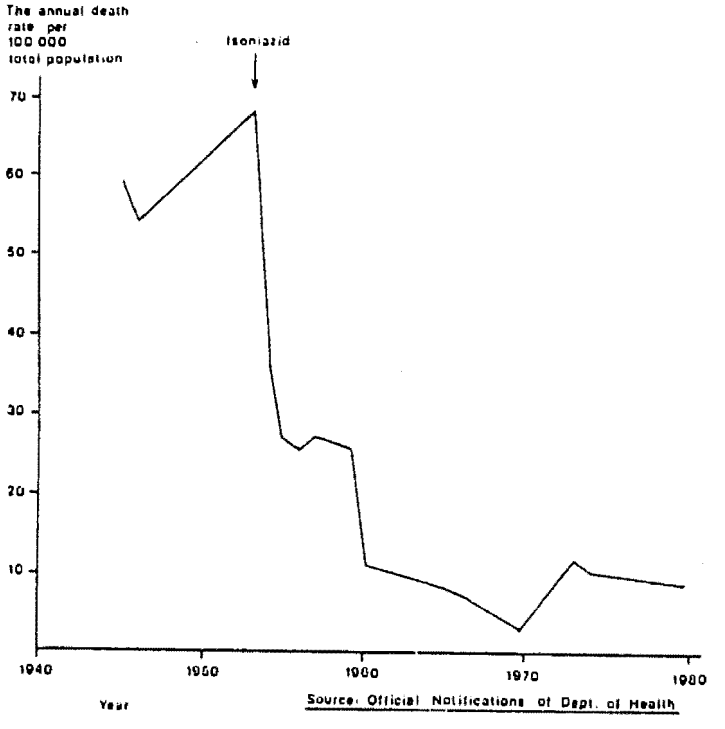


Figure 1.4: The Effect of INH on the South African Epidemic Curve. The annual death rate for all forms of tuberculosis in South Africa from 1945 to 1980 (Collins, 1982).

source of the disease and also that malnutrition and overcrowding is associated with increased susceptibility to disease (Des Pres and Goodwin, 1985). It is not surprising, therefore, that the highest mortality and morbidity occur in those areas where overcrowding and poor socio-economic conditions prevail. Recently it has been suggested that hereditary factors may also explain the high prevalence in these areas (Stead *et al.*, 1990). Stead's data indicate that Black populations are more readily infected by *M.tuberculosis* than White populations, but whether this difference is due to specific hereditary factors or just a reflection of exposure time and development of resistance within a population, is debatable.

Conditions causing immunosuppression have been identified as increasing the risk of tuberculosis (Williams *et al.*, 1976) and the Human Immunodeficiency Virus (HIV) is known to cause immunosuppression. There is a strong association between tuberculosis and HIV infection and tuberculosis is now a recognised complication of HIV infection (Reider and Snider, 1986; Sunderam *et al.*, 1986). Whether the increase of *M.tuberculosis* infections in HIV infected patients represents new infection or reactivation in an immuno-compromised host is still debatable. Recent evidence however, (Selwyn *et al.*, 1989) suggests that tuberculosis in patients infected with HIV most often results from the reactivation of latent tuberculosis.

The association of HIV and tuberculosis in developing countries is extremely disconcerting. In patients with tuberculosis the HIV positivity rate ranges from 17-54% and tuberculosis is found at presentation in approximately one third of patients infected with HIV (Harries, 1990). It is possible that, even in this era of advanced

medical technology, a bacterium identified in 1882 and a virus isolated a century later could still together cause one of the most devastating epidemics the world has experienced.

1.1.3 Taxonomy

The discovery of the tubercle bacillus by Koch was made possible by the use of the acid fast stain as well as the preparation of appropriate culture media and the recognition that visible growth may only occur after 4 to 6 weeks of incubation. With this new knowledge it soon became apparent that there were acid fast bacteria that did not cause tuberculosis. These non-tuberculous Mycobacteria were considered to be saprophytic. This assumption was based on the observation that these organisms were not pathogenic in guinea pigs and therefore, when they occured in human tissue, they were labelled contaminants (Brem, 1909; Pinner, 1935). It was only in the mid-1950's with the development of effective chemotherapy for *M.tuberculosis* and with more widespread culturing of biologic fluids with positive acid-fast smears that researchers demonstrated that there were persons with tuberculosis-like diseases from whom only non-tuberculous Mycobacteria could be isolated and that these organisms were the causal agents of the disease (Buhler and Pollak, 1953; Timpe and Runyon, 1954). Finally, with the statement by Runyon in 1959 that "the guinea pig no longer sits alone on the throne of decision as to pathogenicity of acid-fast bacilli for man", most workers recognised the human pathogenicity of the non-tuberculous Mycobacteria. Since then the recognition of new pathogenic and non-pathogenic species increased rapidly to the point where there are now more than 54 species in the genus Mycobacterium.

For purposes of classification, *M.tuberculosis* and *M.leprae* were usually kept separate from the non-tuberculous Mycobacteria. The first workable classification of the non-tuberculous Mycobacteria was introduced by Runyon in 1959. This classification was developed mainly from the morphological features (pigmentation and growth rates) of 120 clinical isolates of non-tuberculous Mycobacteria (Table 1.1). The basic concept of this classification remained for many years and newly identified Mycobacteria were simply added to Runyon's groups (Table 1.1). However, it is now preferable to identify mycobacterial organisms by species rather than by group since each species may have its own cultural, biochemical, genetic and clinical characteristics. Because of close similarities, there is, however, some controversy that the species standing is appropriate for some of the Mycobacteria.

For example, *M.avium* and *M.intracellulare* are so similar that they are difficult to differentiate by routine chemical tests. Only DNA relatedness studies (Baess, 1979) and DNA probes (Drake *et al.*, 1987) have demonstrated that these are in fact different organisms. It has, therefore, been suggested (Good, 1985) that species that resemble each other biochemically and pathogenically be considered as complexes e.g. *M.avium* complex (MAC) includes *M.avium* and *M.intracellulare*. A similar problem of taxonomy applies to members of the *M.tuberculosis* complex. Several variants of *M.tuberculosis* have been described and there remains considerable debate as to whether each or some of these variants should have separate species status or belong to an o7 3 *M.tuberculosis* complex. The six described variants are the following:

Runyon 1959		Good 1979
Group I		
Photochromogens (slow-growing)	"yellow bacilli" *M.kansasii*	*M.kansasii* *M.marinum* *M.simiae*
Group II		
Scotochromogens (slow-growing)	"orange bacilli"	*M.scrofulaceum* *M.szulgai* *M.xenopi* *M.gordonae* *M.flavescens*
Group III		
Non-photochromogens (slow-growing)	"Battey type"	*M.avium-intracellulare* *M.ulcerans* *M.malmoense*
Group IV		
Rapid growers	"*M.fortuitum*" Norcardia	*M.fortuitum* *M.chelonei* *M.smegmatis* *M.phlei*

Table 1.1: Classification of the 'anomymous' Mycobacteria. This clasification excludes the major pathogens *M.tuberculosis* and *M.leprae*

1. The classic human variety.
2. The Asian variety. This appears to have its origin in Southern India but occurs among Asians resident in other countries, including Britain.
3. The African variety I. This corresponds to *M.africanum* of the type isolated mainly in patients from West Africa.
4. The African variety II. This corresponds to *M.africanum* of the type isolated mainly from patients from East Africa.
5. The classic bovine variety, synonymous with *M.bovis*.
6. Bacille Calmette-Guerin (BCG). This vaccine variety can be designated *M.bovis*BCG.

The Vole or murine bacillus (*M.microti*) has also been included by some (Tsukamura, 1966) in the *M.tuberculosis* complex because its cultural characteristics resemble those of the tubercle bacillus. This variant however is not pathogenic for man.

The 6 variants of *M.tuberculosis* can be distinguished by simple biochemical tests as shown in Table 1.2 and should perhaps by these criteria be accredited species status. Species status has in fact been proposed for *M.tuberculosis*, *M.africanum* and *M.bovis* (Skerman *et al.*, 1980).

Furthermore, restriction fragment analysis of DNA has been utilised to clearly distinguish the various members of the *M.tuberculosis* complex (Collins and de Lisle, 1985). However, Stanford and Grange (1974), using antigenic analysis techniques showed that all these variants form a single species with some degree of intraspecific variation.

Species	Variant	TCH	Nitratase	Oxygen preference	Pyrazinamide	Cycloserine	Niacin
M.tuberculosis	Classical human	R	+	A	S	S	+
	Asian human	S	+	A	S	S	+
M.africanum	African I	S	-	M	S	S	V
	African II	S	+	M	S	S	V
M.bovis	Classical bovine	S	-	M	R	S	-
	BCG	S	-	A	R	R	-

TCH, Thiophen-2-carboxylic acid hydrazide; S, Sensitive; R, Resistant; A, Aerobic; M, Microaerophilic; V, Variable.

Table 1.2: Species and variants of tubercle bacilli

Workers using other techniques such as numerical taxonomy (David *et al.*, 1978; Tsukamura, 1966), skin test susceptibility (Magnusson, 1981), and DNA hybridisation (Baess, 1979), strongly support this concept. Furthermore, tuberculosis caused by any of the variants is indistinguishable in man and differentiation has no bearing on treatment. For practical purposes, therefore, it would appear to be more useful to group members of the *M.tuberculosis* complex into one species (with recognition of intraspecies variation) and to leave further differentiation to systematists.

1.1.4 Morphology

M.tuberculosis is an obligate parasite predominantly infecting humans. Other primates and mammalian species in close contact with humans, especially domestic dogs and cats, can also be infected.

M.tuberculosis is an aerobic, non-spore forming, non-motile bacillus. The bacteria appear as straight or slightly curved rod-shaped organisms, with more or less parallel sides and rounded ends, usually 1-4 um x 0.3-0.6 um in size and are often arranged in small groups. Although the acid-fast rods are the predominant morphological types, some authors have considered that there may be other intermediary forms contributing to a more complicated life cycle. In the period following Kochs discovery of the tubercle bacillus, numerous workers described the presence of granular forms which sprouted into bacillary forms. In 1907 Much also reported these granules and has received the recognition for their discovery (Sweany, 1928). Coccal forms which elongate into rods have also been described in the Mycobacteria (Csillag, 1964; Chang and Anderson, 1969) as have mycelial forms (Brieger and Glauert, 1953). Other authors have considered that some

of these variants are products of degeneration or artefacts arising during specimen preparation (Yegian and Porter, 1944). Cell wall defective forms of Mycobacteria have also been described (Mattman, 1970) and these forms can be demonstrated by treatment of *M.tuberculosis* with certain chemical agents (Imaeda *et al.*, 1968; Willet and Thacore, 1966), but there is no evidence that these forms are viable or capable of either reversion or multiplication.

The most challenging publication on the issue of variant forms of *M.tuberculosis* is that by Khomenko (1987). He describes the presence of ultra fine, non-acid fast forms of the tuberculous agent in cavitatary pulmonary disease. The proportion of these forms increased during chemotherapy and they were shown to revert to typical bacterial forms after culture in liquid media. He suggests that these forms may represent the main way in which *M.tuberculosis* persists under unfavourable conditions (e.g. chemotherapy). These forms would do much to explain our failure to control tuberculosis despite the use of effective antibacterial agents. The existence of similar non-acid fast bodies in *M.leprae* has also been described (Barksdale *et al.*, 1973). Furthermore, the findings of numerous authors concerning the transmissibility of Sarcoid (Mitchell and Rees, 1969) and Crohns disease (Mitchell and Rees, 1970) as well as the isolation of variably acid fast, pleomorphic organisms from Crohns tissue could also be explained by a more complicated mycobacterial life cycle (Burnham *et al.*, 1978; Graham *et al.*, 1987).

1.1.5 The Cell Wall

Electron microscopy shows that Mycobacteria possess a relatively thick (20 nm) cell wall (Fig. 1.5). The inner peptidoglycan layer resembles

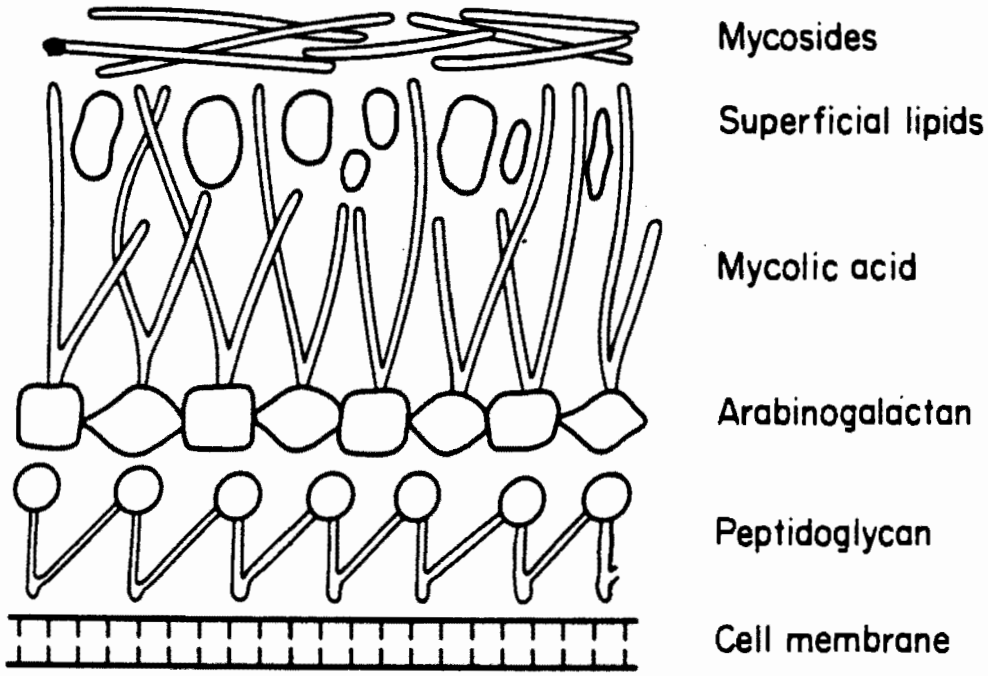


Figure 1.5: Diagrammatic Representation of the Mycobacterial Cell Wall (From Grange, 1984c)

that of other gram positive bacteria. Outside the peptidoglycan is a layer of branching arabinogalactan to which mycolic acids are attached. This layer is attached to the peptidoglycan through phosphodiester bonds. A third layer consists of ribbon-like fibrils which extend from the matrix of the intermediate layer and consist of peptido glycolipids termed mycosides.

Chemical Composition of Cell Wall

The mycobacterial cell wall contains up to 60% lipid. This compares to 20% for "lipid-rich" cell walls of gram negative organisms (Goren and Brennan, 1979). There are many different lipids in the mycobacterial cell wall, the principle ones being: mycolic acids; glycolipids; mycosides; phospholipids; sulphalipids; as well as many straight and branched chain fatty acids.

The mycolic acids are B hydroxy acids substituted at the alpha position with a moderately long aliphatic chain (Goren and Brennan, 1979). The mycolic acids vary in structure from genus to genus and differ mainly in the lengths of the main and side chains. They can be used to distinguish the Mycobacteria from Corynebacteria and Norcadia (Alshamaony *et al.*, 1976). The number of carbon atoms of mycolic acids has also been used to speciate members of the Mycobacteria (see section 1.2.5).

The mycosides are superficially situated peptidoglycolipids consisting of fatty acids linked to an oligopeptide to which various sugar groups are attached. They appear on electromicroscopy as ribbon-like fibrils wrapped around the bacilli (Goren and Brennan, 1979). Numerous functions have been attributed to the mycosides; i.e. determining

colonial morphology; antigenicity; conferring virulence (Goren and Brennan, 1979); and even enabling the mycobacteria to survive within vacuoles in macrophages (Draper and Rees, 1973).

Phospholipids occur in the cell membrane and cell wall and include cardiolipids, phosphotidyl ethanolamine and phosphatidylinositol mannosides. The sulpholipids, which include cord factor, are fatty acids linked to trehalose. The fatty acids probably do not occur in the free form and are found in the form of esters and amides as part of the other lipids.

1.1.6 Mycobacterial Antigens

A vast number of mycobacterial antigens have been described and it is probable that the only limit to the identification of individual antigens or epitopes is the effort which an investigator wishes to expend.

Mycobacterial antigens can be classified or investigated according to the following four properties or functions:

1. Chemical structure: both protein (Daniel and Janicki, 1978) and glycolipid (Goren, 1982) antigens have been described.
2. The nature of the immune response they illicit: cell mediated (Lamb and Rees, 1988) or humoral (Grange, 1984a).
3. Cellular location and function: surface capsule (Goren, 1982); secreted antigens (Young, 1988); stress proteins (Young, 1988) and intracellular survival (Lowrie, 1988).
4. Antigenic distribution within the genus can be used to assist speciation (Grange, 1984b).

From this classification, it is clear that there is room for a diversity of interests within the field of antigen characterisation. Later in this thesis, I will consider the relevance of immunodiagnosis for *M.tuberculosis*, therefore, only those antigens which have been utilised for this purpose (i.e. protein antigens mainly) will be discussed. In 1890, Robert Koch first reported the preparation of a product which he named "old tuberculin" (O.T.). It consisted of a concentrated sterile filtrate of autolysed, heat-killed, liquid cultures of *M.tuberculosis*. This material was used for skin tests on patients, but non-specific reactions to O.T. were soon recognised. Seibert, therefore, further purified filtrates by repeated precipitation with 50% ammonium sulphate and termed the product "purified protein derivative" (PPD) (Seibert and Glen, 1941). Further attempts to purify PPD by alcohol and acetic acid precipitation (Seibert, 1949) resulted in 4 protein and 2 polysaccharide fractions. These fractions all contain a number of antigens and have been well characterised (Daniel and Janicki, 1978).

Many investigators have preferred to work with extracts of whole bacterial cells or of the bacterial cell wall, and these can be prepared by a variety of procedures, including mechanical rupture of the cell by grinding, pressure and sonication (Janicki *et al.*, 1976). It has been demonstrated that there are no gross qualitative differences between the antigenic composition of cell extracts and culture filtrates (Daniel *et al.*, 1975).

In the last two decades more advanced fractionation techniques have been used. The methods used and the antigens purified by these methods are summarised in Table 1.3.

Finally, the advent of B cell hybridoma technology provided the means for producing monospecific antibody which could be used to characterise the complex antigen mixtures found in bacterial extracts. The antigens recognised by these monoclonal antibodies were characterised at a W.H.O. workshop in June 1985 (Engers, 1986) and will be discussed later.

1.1.7 Genetic Characteristics

The size of the genome of *M.tuberculosis* is similar to most bacteria (e.g. *E.coli* 2.5 x 10^9 daltons) and has been reported as 3.13 x 10^9 daltons (Baess and Mansa, 1978); 2.5 x 10^9 daltons (Bradley, 1972) and 2.0 x 10^9 (Imaeda *et al.*, 1982).

The guanine cytosine (GC) content of the genome is high, i.e. 63% to 67% (Baess and Mansa, 1978; Imaeda *et al.*, 1982; Wayne and Gross, 1968b). The GC content and deoxyribonucleic acid relatedness of Mycobacteria has been extensively studied in order to improve taxonomy (see Section 1.2.8).

The rare bases, 6-methylaminopurine and 5-methylcytosine, have been reported in mycobacterial DNA (Dunn and Smith, 1958; Vanyushin *et al.*, 1968; Srivastava *et al.*, 1981). The role of these rare bases in bacterial DNA is not entirely understood. It has been suggested that the presence of 5-methyl cytosine may be associated with mycobacterial virulence (Srivastava *et al.*, 1981).

PURIFICATION OF MYCOBACTERIAL ANTIGENS

REFERENCE	METHOD	PRODUCTS
Kniker and La Borde, 1964	DEAE - cellulose ion exchange chromatography	Protein and polysaccharide-rich antigenic fractions. Not antigenically pure (As assessed by serologic technique).
Chaparas and Baer, 1964	Molecular exclusion. chromatography.	Protein and polysaccharide-rich antigenic fractions. Not antigenically pure.
Janicki *et al.*, 1972	Paper curtain electro-phoresis	Protein and polysaccharide-rich antigenic fractions. Not antigenically pure.
Rozman *et al.*, 1968	Acrylamide gel electro-phoresis	Small quantities purified antigenic proteins.
Yoneda and Fukui, 1965 Daniel and Ferguson, 1970 Kuwabara S, 1975	Serial ammonium sulfate precipitation, gel filtration, ion exchange chromatography, zonal electrophoresis.	Highly purified antigenic proteins with low yields
Azuma *et al.*, 1968 Yamamura *et al.*, 1965	Alkaline extraction, ethanol precipitation, exchange chromatography	Highly purified antigenic arabinogalactan and arabino-mannon: non-antigenic mannan and glucan.
Daniel, 1974 Daniel and Misaki, 1976	Concanavalin A affinity chromatography	Purified antigenic arabino-galactan and arabinomannan.
Daniel and Anderson, 1977 Daniel and Anderson, 1978	Immuno absorbent affinity chromatography	Highly purified protein Antigen 5 and Antigen 6
Thongkrajai *et al.*, 1989	Immuno absorbent affinity chromatography	Purified Mycobacterial Antigen (PMA)

Table 1.3

Ribosomal RNA is much more abundant than chromosomal DNA and could possibly be a useful diagnostic tool (see Section 1.2.8). Analysis of ribosomal RNA from many organisms has shown that although there is a great deal of phylogenetic conservation there are also several very variable regions (Gutell *et al.*, 1985). Studies indicate that there is species variability in mycobacterial rRNA genes (Cox *et al.*, 1986) and therefore, the potential for specific diagnostic probes. A feature of mycobacterial ribosomal RNA genes is that while there are multiple copies of rRNA genes in most bacteria, the slow growing Mycobacteria (e.g. *M.tuberculosis*) have only one copy per genome, and faster growing species such as *M.smegmatis* and *M.phlei* have only two copies (Bercovier *et al.*, 1986).

Plasmids are not often found in *M.tuberculosis*. A plasmid has been extracted from a pathogenic *M.tuberculosis* strain (Srivastava *et al.*, 1981) and a "satellite" DNA band observed from a lysate of *M.tuberculosis* (Crawford and Bates, 1979). Among the other Mycobacteria, however, plasmids appear to be more common, notably members of the *M.avium, M.intracellulare, M.scrofulaceum* (MAIS) complex. In this group plasmids have been used as epidemiological markers (Meissner and Falkinham, 1986), and they have been associated with virulence (Crawford and Bates, 1986) and antibiotic resistance (Franzblau *et al.*, 1986).

Bacteriophages capable of lysing Mycobacteria are well described (Grange, 1984c). Lysogeny has also been demonstrated in many species of Mycobacteria. Many Mycobacteria infected with temperate phages are not truly lysogenic. Instead of being integrated into the host chromosome the phage genome appears to exist independently within the

bacterium, like a plasmid. The establishment of lysogeny may modify the activity of enzymes, modify antigenic structure or change colonial morphology (Grange 1984c). The ability of certain phages to lyse certain strains of mycobacterial species has previously been used for epidemiological studies. This methodology is however not routinely used today.

1.1.8 Pathogenesis and Host Responses to *M.Tuberculosis*

Tuberculosis remains the classic example of a disease that is controlled almost entirely by cell mediated immunity. Infection is associated with a rich antibody response to *M.tuberculosis* but no role for this in the host defence against disease has been demonstrated. The immune mechanisms that protect the individual against tuberculosis are extremely complex. Only a broad outline and features of the immune response which are particular to this thesis (e.g. histological lesions, association with malignancy, skin testing) will be discussed. A more detailed account can be found in a recent review (Wiegeshaus *et al.*, 1989).

In the first few weeks after inhalation the host has no defence against tuberculosis infection and the bacilli can multiply freely within alveolar spaces or intracellularly after phagocytosis by alveolar macrophages. The macrophage may process the bacterial antigens and present them to T-lymphocytes which in turn (upon antigen recognition) proliferate and secrete mediators (lymphokines) that influence other T-lymphocytes, macrophages and B-lymphocytes.

When the population of activated lymphocytes reaches a certain size, cutaneous delayed reactivity to tuberculin (PPD) or tissue

hypersensitivity (TH) develops. At about the same time enhanced microbicidal activity or cell mediated immunity (CMI) develops. Both TH and CMI develop between 6-14 weeks after exposure and it remains a matter of dispute whether TH and CMI are endpoints of the same immunologic sequence or if they are simply parallel and closely associated events.

The CMI/TH response is seen histologically as three types of tuberculous lesions. First there is the classic productive or proliferative lesion consisting of aggregates of histocytes that undergo metaplasia to form epithelioid cells. These cells are seen as large macrophages with a vesicular or euchromatic nucleus and often have an epithelial-like appearance. Sometimes the cell membranes fuse and Langhaans, multinucleated, giant cells are formed. When the cells are arranged in a small sphere the structure is termed a tubercle or a granuloma. The productive lesion is thought to occur when antigen load is small and the CMI is high. It is a very successful tissue reaction with respect to containment of the infection.

Secondly, there is the inflammatory or exudative lesion consisting of vasodilatation, oedema, fibrinous exudate and an influx of all elements of the leukocyte series including polymorphonuclear cells, monocytes and lymphocytes. Epithelioid cells and giant cells are sparse and some tissue necrosis may be present. This lesion indicates that both TH/CMI and antigen levels are high.

Thirdly, there are the lesions containing a homogenous, amorphous mass resembling cheese, called caseous necrosis, that occurs in the centre of either the exudative or proliferative lesions. The environment

within this lesion is not conducive to mycobacterial growth but is, however, unstable and demonstrates a tendency to liquefaction and discharge and therefore promotes mycobacterial spread.

If any of these lesions are demonstrated in tissue sections, in the absence of acid fast organisms, from a patient with clinical evidence of tuberculosis, they may be considered to be highly suggestive of tuberculosis and chemotherapy is often instituted based on these findings. It must be pointed out, however, that the granuloma is not pathognomonic of *M.tuberculosis* infection and is also observed in many other chronic infections including brucellosis, syphilis, coccidiomycosis, other mycobacterial disease, foreign body reactions and mycoses. The clinical manifestations and prognosis of tuberculosis depends not only on the host response but also on the virulence factors of the organism. Although much work has focussed on the demonstration of toxic substances isolated from the tubercle bacillus, it is doubtful whether such substances are directly associated with virulence. The virulence of *M.tuberculosis* is, however, almost certainly linked to the ability of the bacterium to survive within macrophages. This is due partly to their ability to prevent fusion of the tubercle entrapping phagosomes and lysosomes which contain microbiocidins and hydrolytic enzymes.

Pulmonary tuberculosis usually occurs by the inhalation of infectious droplets which are small enough to avoid entrapment by the bronchial mucocillary apparatus and to reach the terminal air spaces. Bacilli are ingested by macrophages but can resist this attack initially. As

more macrophages and lymphocytes accummulate, some cells degenerate and a local area of pneumonitis may develop. Infected macrophages carry bacteria to regional lymph nodes where a focus of infection is again established. This initial primary pulmonary focus of infection together with regional lymph node involvement is termed the Ghon complex.

The outcome of the primary focus depends largely on immune status of the individual (i.e. previous infection or vaccination) which may be further modulated by various intercurrent events, such as chronic illness, poor nutrition, corticosteroid therapy and the presence of malignancy. In those patients with no CMI, mycobacterial growth is uninhibited and rapid progressive pulmonary involvement or haematogenous spread may occur. At the other immunological extreme in a patient with a well established CMI, the initial focus may be well contained with no evidence of bacterial spread. The foci may remain under control for years, but may "reactivate" at a much later stage due to the development of intercurrent events (as discussed above) leading to the development of disease.

Tissue hypersensitivity (TH) can occur at any site of infection due to the presence of mycobacterial antigens. The same TH can be elicited at any site where mycobacterial antigen is inoculated, and this principle forms the basis of the tuberculin skin test. Usually 0.1 ml of PPD containing 5 Tuberculin Units is injected intracutaneously into the volar aspect of the forearm and the size of the subsequent induration is measured 48-72 hours later. Induration of greater than 10 mm is usually considered positive.

However, certain conditions (infections including HIV, vaccinations, chronic renal failure, severe protein malnutrition, malignancy, steroid) can result in false negative results and one must interpret the test with caution should the patient have one of these conditions. A state of anergy can usually be demonstrated by the injection of a ubiquitous antigen (e.g. Candida) in the contralateral forearm.

False positive reactions can also occur due to the presence of non-tuberculous Mycobacteria but reactions to these organisms are usually less than 10 mm.

The tuberculin test remains a useful test to document the presence of TH to *M.tuberculosis* infection if the above factors are taken into consideration (Dickensheets, 1989).

1.1.9 **Tuberculous Pleural Effusions**

A description of the pathogenesis, clinical symptoms and signs of pulmonary tuberculosis and its complications, as well as extrapulmonary tuberculosis, is beyond the scope of this thesis and is well described in standard texts (Des Pres and Goodwin, 1985). This review will be limited to a discussion of tuberculous pleural effusion, the diagnosis of which is the subject of this thesis (Chapter 7 and 8).

The pleural space is approximately 10-20 um in width and lies between the parietal and visceral pleura. It contains 0.1-0.2 ml/kg of pleural fluid in the normal individual. Normal pleural fluid is clear, colourless with protein concentration of 1.5 gm/dl. There are approximately 1500 cells/µl with a predominance of monocytic cells. Small numbers of lymphocytes, macrophages and mesothelial cells may

also be found but polymorphonuclear cells are rare and no red blood cells are present.

The parietal pleural is responsible for both influx and exit of pleural fluid. The pleural fluid is formed from a low protein filtrate of the systemic circulation through the parietal mesothelial cells. The fluid and protein is drained directly via parietal pleura stomata which are 2-12 um openings situated between the mesothelial cells and communicate with lymphatic vessels.

Influx and exit of liquid and protein of the pleural space is balanced so that a constant volume and protein concentration exists in the normal state. If this balance is disturbed, fluid will accumulate. There are six mechanisms responsible for the accumulation of pleural fluid (Sahn, 1988; Sahn, 1989):

1. An increase in the hydrostatic pressure in the microvascular circulation, which may be due to increased systemic venous pressure;

2. A decrease in microvascular oncotic pressure (e.g. due to a low plasma albumin);

3. Decreased pressure in the pleural cavity as occurs with the collapse of the lung;

4. Increased permeability of the microvascular circulation due to inflammation;

5. Impaired lymphatic drainage due to malignancy, fibrosis or chronic inflammation.

6. In the presence of ascites, fluid can move into the pleural fluid via diaphragmatic lymphatics and is driven by the pressure gradient between the two cavities.

Pleural fluids can be divided into transudates or exudates based on the composition of the fluid. Exudates have at least one and transudates have none of the following features: pleural fluid/serum protein ratio > 0.5; pleural fluid/serum LDH ratio > 0.6; pleural LDH level greater than two thirds of the serum upper limit of normal. Furthermore exudates tend to have higher leukocyte counts than transudates. Transudates are usually due to imbalances in hydrostatic and oncotic pressure or from the movement of fluid from the peritoneum. Exudates are caused predominantly by pleural inflammation or impaired lymphatic drainage, resulting in a protein leak or a decrease in protein removal from the pleural cavity.

Pleural effusions occur in tuberculosis by the rupture of a subpleural focus into the pleural space. *M.tuberculosis* reaches the subpleural site by direct extension of the primary disease or by hematogenous dissemination. The effusion develops on an immunological basis. Tuberculin protein or live bacilli interact with sensitised T-lymphocytes with the resultant liberation of lymphokines which may alter the permeability of the pleural vasculature and affect the activity of mononuclear phagocyte and pleural fibroblast. This results in an outpouring of fluid and cells from the pleural surfaces. As chronicity of tuberculosis develops, a block of lymphatics by fibrosis may occur and comprise fluid drainage.

The clinical presentation of tuberculous pleural effusions covers a spectrum from an acute illness similar to bacterial pneumonia to an indolent disease first suspected following a chest radiograph. A non-productive cough and pleural chest pain are seen in 75-80% of patients and are the two most common presenting symptoms at presentation (Berger and Mejia, 1973).

The chest x-ray usually shows a small to moderate pleural effusion. Co-existent parenchymal disease on the ipsilateral side is seen in about one thirds of patients (Berger and Mejia, 1973).

The PPD skin test is negative in 30% of patients on admission, but becomes positive in most patients by 6-8 weeks after presentation. The probable explanation for a negative PPD in patients with tuberculous pleural effusion is that in the acute phase of the disease, circulating mononuclear cells suppress the sensitised T-lymphocytes in the peripheral blood and skin, but not in pleural fluid (Ellner, 1978).

Tuberculous pleural fluid is a serous exudate with a total protein content almost always greater than 4.0 gm/dl and frequently greater than 6 gm/dl. The total leukocyte count is generally < 5000/µl with a predominance of lymphocytes (90-95%) but with a polymorphonuclear predominence in the first few days of the effusion. A paucity of mesothelial cells is also characteristic of tuberculous pleural effusions. The pleural fluid glucose is most often normal and only reaches levels below 40 mg/dl in 20% of patients. The pleural fluid pH is in the range 7.00 to 7.29.

The definitive diagnosis of tuberculous pleural effusion is dependent on bacteriological and histological findings. The contribution that each or combinations of these procedures make has been the subject of numerous studies, some of which are summarised in Table 1.4. Acid-fast bacilli are seen in the smears of pellets of centrifuged pleural fluid in less than 10% of cases (Sahn, 1988). The data in Table 1.4 indicate that culture of *M.tuberculosis* from the pleural fluid varies from 12-47%. Pleural biopsy is the best test for establishing the diagnosis and a histological diagnosis can be made in 63-71% of cases. A combination of biopsy culture and histology of biopsy results in detection of 80-95% of cases.

The greater efficiency of detection of *M.tuberculosis* by examination of the biopsy is consistent with the fact that the major site of infection is in the pleura. Aspiration of fluid without biopsy is technically much easier and can be achieved in most cases with a simple hypodermic syringe. However, the low yield of positive results by aspirate alone renders this approach unsatisfactory.

The contribution of other methods such as immunodiagnostic assays and adenosine deaminase will be discussed in sections 1.2.4 and 1.2.6.

1.2 LABORATORY DIAGNOSIS OF *MYCOBACTERIUM TUBERCULOSIS*

1.2.1 Laboratory Culture

The culture of the Mycobacteria is used in most mycobacteriology laboratories as the definitive test for detection and identification. Before considering the available culture media a few variables, which are peculiar to and may affect the efficiency of culture of

DIAGNOSIS OF TUBERCULOUS PLEURAL EFFUSION

Reference	Number of patients with tuberculosis	Number (Percentage) Positive for *M.Tuberculosis*			
		Effusion Culture	Biopsy Culture	Biopsy Histology	Biopsy Culture & Histology
Levine *et al.*, 1970	21	10 (47%)	16 (76%)	15 (71%)	20 (95%)
Berger and Mejia, 1973	49	11/45 (24%)*	11/17 (65%)*	29/42 (69%)*	
Scharer and McClement, 1968	40	9 (23%)	22 (55%)	25 (63%)	32 (80%)
Scerbo *et al.*, 1971	49	6 (12%)	12/16 (75%)	35 (71%)	

* Not all effusions and biopsies were studied

TABLE 1.4: A comparison of microbiological and histological procedures for the detection of *M.tuberculosis* in pleural effusions

M.tuberculosis need to be discussed: i.e. centrifugal force; growth rate and decontamination.

Usually only small volumes (about 100 µl) of a bacterial suspension can be satisfactorily spread on a standard agar plate and concentration of bacteria in clinical specimens (usually by centrifugation) is therefore standard laboratory practice. Due to their waxy coat, Mycobacteria have a low specific gravity and therefore, require high centrifugal forces to achieve sedimentation. It has been demonstrated that a centrifugal force of between 3000-4000g for 20 minutes is needed to recover 80% of the Mycobacteria in the pellet (Ratnam and March, 1986). It is, therefore, important that this parameter is well monitored in the mycobacteriology laboratory.

The generation time for *M.tuberculosis* is slow and varies from 15-24 hours (David, 1973; Gutierrez-Vazquez, 1956). Most other bacteria, however, may divide every 40-60 minutes. This disproportionate rate of growth often results in the accummulation of metabolic waste products derived from the rapidly growing bacteria, making the culture medium unsatisfactory for mycobacterial growth. A variety of decontamination procedures have, therefore, been developed and these are listed in Table 1.5.

The rationale for these decontamination procedures is that the high lipid content of the mycobacterial cell wall is relatively resistant to alkali and acids, therefore, these procedures will eliminate common bacterial flora while maintaining the viability of the Mycobacteria.

DECONTAMINATION PROCEDURES USED IN MYCOBACTERIOLOGY LABORATORIES

PROCEDURE	COMMENT
2% NaOH 2% NaOH + Dithiothreitol 2% NaOH + N-acetyl-2-cysteine (NALC)	NaOH provides gentle decontamination of specimens Reserved for specimens rapidly transported to laboratory. Mucus is liquified by dithiothreitol and NALC. Time of exposure to NaOH is critical.
4% NaOH 4% NaOH + Dithiothreitol	Useful for grossly contaminated specimens. Time of exposure to NaOH is absolutely critical.
Benzalkonium chloride (Zephiran) + NALC Benzalkonium chloride (Zephiran) and 13% trisodium phosphate	Gentle decontamination. Time of exposure not crucial.
13% trisodium phosphate	Very gentle decontamination for fresh uncontaminated specimens.
1% cetyl-pyridium chloride + 2% NaCl	For decontamination prior to sending to laboratory e.g. mailing or if long delays anticipated.
4% sulphuric acid	For decontaminating urines.
5% oxalic acid	A harsh decontaminent useful if *Pseudomonas aeruginosa* is present.

Table 1.5

However, the decontaminating agents can themselves be harmful to the Mycobacteria and this is most often related to the time of exposure of the agent to the mycobacterium (see Figure 1.6).

Many different culture media have been devised for growing the tubercle bacillus but relatively few of these media are in use today. The media can be divided into 3 groups: egg based; agar based and liquid media. Furthermore, various combinations of antibiotics can be added to these media to prevent growth of contaminating bacteria i.e. selective media. These media are summarised in Table 1.6.

Of the egg based media, the various modifications of Lowenstein-Jensen are the most popular. The original media contained starch but this has been omitted in subsequent modifications as it is not necessary for mycobacterial growth. Most of the egg based media contain malachite green, in varying concentrations, for the suppression of growth of other organisms. Glycerol enhances growth of *M.tuberculosis* but may be inhibitory to bovine strains.

The agar based media (Middlebrook 7H10 + 7H11) are transparent and can easily be prepared in culture plates. Visualisation of the characteristic colonies of *M.tuberculosis* and detection of contamination is therefore easier. These media are, however, used mainly for identification and sensitivity testing and seldom for primary isolation. The addition of sodium selenate to 7H11 medium has been shown to enhance the growth of *M.tuberculosis* (Jaques *et al.*, 1981).

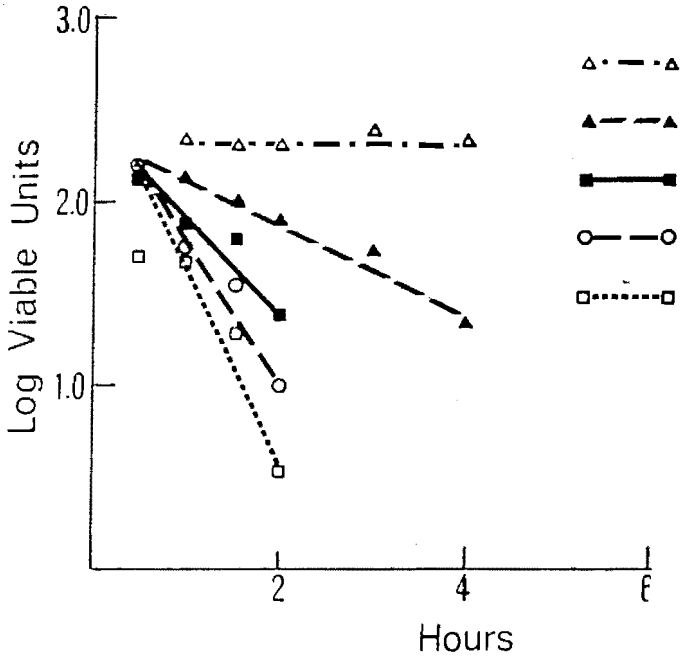


Figure 1.6: Death Rates of Tubercle Bacilli with Various Decontamination Procedures. Z-AC = Zephiran-N-acetyl-L-cysteine; Z-TSP = Zephiran-trisodium phosphate; NaOH 2%-AC = 2% NaOH + N-acetyl-L-cysteine; 3% and 4% NaOH contain no acetylcysteine. (From Krasnow and Wayne, 1966)

MEDIUM	COMPONENTS	INHIBITORY AGENT	COMMENTS
EGG BASED			
Lowenstein-Jensen	Defined salts, whole eggs, glycerol, potatoe flour	Malachite Green 0.025g/100 ml	Original Media
American Thoracic Society	Whole eggs plus additional egg yolk, potatoes, glycerol	Malachite Green 0.02g/100 ml	Less inhibitory. For uncontaminated specimens
Petragnani	Whole eggs, glycerol, milk	Malachite Green 0.52g/100 ml	Increased inhibitor concentration for heavily contaminated specimens
Stonebrink	Defined salts, pyruvate, whole eggs	Malachite Green 0.2g/100 ml	For bovine strains
AGAR BASED			
Middlebrook 7H10	Defined salts, glycerol, vitamins, cofactors, albumin, oleic acid, dextrose	Malachite Green 0.0025g/100 ml	
Middlebrook 7H11	As 7H10 plus sodium casein hydrolysate	Malachite Green 0.0025g/100 ml	
Middlebrook Selenate 7H11	As 7H11 plus sodium selenate	Malachite Green 0.0025g/100 ml	
FLUID MEDIA			
Kirchner	Defined salts, Glycerol Horse serum	Phenol Red	
Dubos and Davis	Defined salts, Tween 80, Casein Hydrolysate Bovine albumin	NIL	
Sulas Medium	Defined salts, Glycerol	Malachite Green 0.02g/100 ml Penicillin 10 U/ml	
Middlebrook 7H9 Broth	Defined salts, Tween 80, vitamins, co-factors, albumin, oleic acid, dextrose.	NIL	For subculturing mycobacteria and for preparation prior to sensitivity testing.
SELECTIVE MEDIA			
Lowenstein-Jensen Gruft		Penicillin, naladixic acid, malachite green	
Middlebrook Selective 7H11		Carbenicillin, polymixin B, trimethoprim, amphotericin B, malachite green	
Mycobactosel Lowenstein-Jensen		Lincomycin, cyclohexi-mide, naladixic acid, malachite green	

Table 1.6

The liquid media are most useful in that they are inexpensive and can support a larger primary inoculum than solid media. To allow for even larger inocula Ives and McCormick (1955) prepared a double strength Sulas medium so that an equal amount of specimen fluid could be added. This method was superior to other fluid and solid media for the isolation of *M.tuberculosis* from pleural fluids. Various antibiotics may also be added to these liquid media to control growth of other organisms.

Selective media usually contain a variety of antimicrobial agents (Table 1.6) to prevent the growth of contaminating bacteria that may not be inhibited by the decontaminating procedure. The use of these antibiotics does not however preclude the need for decontamination of specimens.

Most laboratories use a combination of media to achieve optimal recovery of Mycobacteria.

The culture of Mycobacteria is usually regarded as the "gold standard" by which any new technique is compared. Laboratory culture is extremely sensitive and can detect less than 10 organisms per inoculum (Hobby *et al.*, 1973). Furthermore, analysis of the cultured organism by a variety of biochemical tests allows accurate identification.

Generation rates for *M.tuberculosis* vary from 15-24 hours (David, 1973; Gutierrez-Vazquez, 1956) with visible growth occuring only after 12-42 days of incubation. The slow growth rate is the major drawback of conventional culture methods due mainly to the fact that therapeutic decisions need to be made within the 24 hours admission period. A further disadvantage of culture methods is that viable

organisms are necessary and a bacteriological diagnosis may be missed should the decontamination procedure be too harsh or if the patient has received treatment prior to specimen collection. It is for these reasons that numerous "rapid diagnostic" techniques have been developed and these shall be discussed later in this chapter.

Identification of Mycobacteria

Once colonies of *Mycobacteria* have become visible, a further 3 to 4 weeks incubation is usually required to produce a lawn of mycobacterial growth which is sufficient for all the biochemical tests necessary for species identification. The specific tests and schemes used to identify mycobacterial isolates vary between laboratories. The scheme used at Groote Schuur is shown in Table 1.7.

It is not within the scope of this thesis to discuss the identification of all the Mycobacteria but only to highlight those tests which identify and differentiate those organisms within the *M.tuberculosis* complex.

Niacin is formed as a metabolic by-product of all Mycobacteria and most species possess an enzyme that converts free niacin to niacin ribonucleotide. *M.tuberculosis*, however, lacks this enzyme and niacin accummulates and can be detected by a colormetric test based on the reaction between niacin and cyanogen bromide (Runyon *et al.*, 1959; Konno, 1956). This test is used by many laboratories to differentiate *M.tuberculosis* from all other Mycobacteria. However, some *M.bovis*, some *M.africanum*, some *M.simiae* and some *M.avium* strains are also niacin positive while some classic human *M.tuberculosis* variants are niacin negative (Tsukamura, 1974).

Table 1.7

SPECIES	Acid Fast	Niacin	Growth Rate	Temp: 25°C	30°C	37°C	45°C	Pigment: light	dark	Nitrate:	Tween 80	Arylsulfatase: 3 days	14 days	Urease	NaCl tolerance	Mac. agar	Catalase	
M.tuberculosis (INH Sens)	+	+	R	-	+	+	-	-	-	+	±S	-	-	+	-	-	<45	*INH Resistant Catalyse Neg.
M.africanum	+	-	S	-		+	-	-	-	±		-	-					
M.bovis	+	-	S	-		+	-	-	-	+	+S	-	+	+	-	-	<45	
M.ulcerans	+	-	S	-	+	-	-	-	-	-	-	-		-	-		>45	
M.kansasii	+	-	S	+	+	+	-	+	-	+	+	-	+	+	-	-	>45	
M.marinum	+	w	S	+	+	-	-	+	-	-	+	-	+	+	-	-	<45	
M.simiae	+	+	S	+	+	+	-	+	-	-	-S	-	+		-		>45	
M.asiaticum	+	-	S	+	+	+	-	+	-	-	+	-		-	-		>45	
M.scrofulaceum	+	-	S	+	+	+	-	+	+	-	-		+	+	-	-	>45	
M.szulgai	+	-	S	+	+	+	-	+*	+*	+	+S	-	+	+	-	-	>45	*Scotochrom 37° Photochrom 25°
M.gordonae	+	-	S	+	+	+	-	+	+	-	+	-	+	-	-	-	>45	
M.flavescens	+	-	M	+	+	+		+	+	+	+	-		+	+	-	>45	
M.xenopi	+	-	S	-	-	+	+	±	±	-	-	±	+	-	-	-	<45	
M.avium	+	-	S	±		+	+	-	-	-	-	-	-	-	-	-	<45	
M.intracellulare	+	-	S	±		+	-	-	-	-	-	-	+	-	-	-	<45	
M.gastri	+	-	S	+	+	+	-	-	-	-	+	-	+	+	-	-	<45	
M.haemophilum	+	-	S	+	+	-	-	-	-	-	-	-		-	-	-	Ø	Iron Dependent
M.malmoense	+	-	S	+	+	+	-	-	-	-	+S	-		v	-	-	>45	
M.terrae	+	-	S	+	+	+	-	-	-	+	+	-	-	-	-	v	>45	
M.trivale	+	-	S	+	+	+	-	-	-	+	+	±	+	-	+	-	>45	
M.paratuberculosis	+		S	+	+	+	-											Mycobactin Dependent
M.fortuitum	+	-	R	+	+	+	-	-	-	+	v	+	+	+	+	+	>45	
M.chelonei	+	v	R	+	+	+	-	-	-	-	v	+	+	+	±	+	>45	
M.phlei	+	-	R	+	+	+	+	-	+*	+	+	-	±	+	+	-	>45	*Salmon Pink
M.smegmatis	+	-	R	+	+	+	+	±	-	+	+	-		+	+	-	>45	

Catalase = Semiquantitative Test
w = Weak
v = Variable
Tween 80 S = Positive after 10 days

M.tuberculosis is resistant to thiophen-2-carboxylic acid hydrazide (TCH) and the bovine strains are sensitive (Harrington and Karlson, 1966). More recently, however, it has been established that certain human strains are also sensitive to this compound (Yates and Collins, 1979) and this invalidates the use of the test for identifying bovine strains. Any strain that grows on TCH, however, may safely be regarded as a classical human variant and further tests need be done only on strains that are sensitive to TCH. These tests include nitratase activity, cycloserine and pyrazinamide sensitivity and oxygen preference and have been discussed in section 1.1.3.

1.2.2 Microscopy

The Mycobacteria have a unique staining characteristic i.e. binding the stain carbolfuchsin so tightly that it resists destaining with strong decolourising agents such as alcohol and strong acid. This acid fastness has in the past been attributed to a number of cell wall components but it now appears that the most likely explanation is that complexes are formed between the dye and the mycolic acid residues which then traps the dye and renders the organism acid fast (Goren and Brennan, 1979). The stain is not entirely specific for the Mycobacteria as fungal and bacterial spores as well as some *Norcardia* are also acid fast.

Two procedures are commonly used for acid fast staining: carbolfuchsin methods including Ziehl-Neelsen and Kinyoun staining procedures; and fluorochrome methods using either auramine 0 or auramine-rhodamine dyes.

Various researchers have contributed to the present day Ziehl-Neelsen stain and it would be more accurate to call it the Ehrlich-Ziehl-Rindfleisch-Neelsen stain (Bishop and Neumann, 1970). The procedure is simple and rapid and visualisation can be performed with an ordinary light microscope. The fixed smears are stained with carbolfuchsin by gentle heating to allow penetration of the stain and then decolourised by acid alcohol followed by counterstaining.

A cold staining procedure, used when heating slides is technically difficult (e.g. field work) was introduced by Kinyoun, who however, disclaims any credit for the procedure, saying that all credit belongs to Uhlenhuth (Kinyoun, 1915). The method has, however, retained his name.

Various other staining procedures have also been used. These are essentially the same as the traditional Ziehl-Neelsen methods but the carbolfuchsin solution contains dimethyl sulphoxide or Tween 80 (Collins *et al.*, 1985).

Fluorescent arylmethane dyes have also been used to detect acid fast bacilli. The dyes used are auramine 0 or a combination of auramine and rhodamine B. The staining technique is identical to the Ziehl-Neelsen procedure. Smears are treated with a phenolic solution of the dye or dyes but are not heated and are then decolourised with acid or acid-alcohol. The counterstain may be either potassium permanganate, which suppresses non-specific fluorescence or a fluorescent dye of a different colour.

Carbolfuchsin stained slides must be scanned with an oil immersion objective which greatly restricts the area of the slide that can be

viewed in a given period of time. In comparison, fluorochrome-stained Mycobacteria appear bright yellow against a dark background permitting the slide to be scanned under lower magnification. However, fluorescent techniques have the disadvantage that fluorescent microscopes are required. Some authors (Ritterhof and Bowman, 1945; Collins *et al.*, 1981) consider that fluorescent methods give rise to false positive results due to the presence of naturally occuring fluorescent particles. Others have, however, disputed this (Holst *et al.*, 1959; Kubica, 1980).

Bulk staining of slides has often been advocated in order to save time and materials but with this methodology there is the possibility that acid fast bacilli may float from one side to another and hence give false positive results. A number of authors have, however, indicated that this does not occur (Clancey *et al.*, 1976; Fodor, 1984) and bulk processing should be considered in laboratories where large numbers of smears are processed.

False positive results can also occur by a variety of other technical problems e.g. re-used slides, contaminated blotting paper, scratches on slides retain fuchsin and may look like acid fast bacilli, and fluorescent artefacts in storage or demineralised water (Collins *et al.*, 1981).

The sensitivity of carbolfuchsin and fluorochrome methods is similar (Kubica, 1980). It has been demonstrated that detection of Mycobacteria by microscopy requires the presence of 5000 to 10000 bacilli per ml of sputum (Yaeger *et al.*, 1967; Hobby *et al.*, 1973) and staining procedures are therefore rather insensitive. However in

those patients with active disease there is good correlation of microscopy with culture and microscopy therefore provides a good first line detection system for active disease. On the other hand, in those patients with minimal or less active disease, the correlation (i.e. smear +ve culture +ve) may only be 60 to 70% (Sommers, 1979; Kim *et al.*, 1984).

Acid fast stains may also be used to follow the response to drug therapy. During treatment, sputum culture and smears usually become negative at about the same time (i.e. within 1 month of commencement of treatment), but in a proportion of patients (20%) the culture may become negative approximately 2 weeks before the smear (Kim *et al.*, 1984) indicating that the bacilli are injured sufficiently to prevent replication but not to prevent binding of the stain. The time of conversion to culture and smear negativity is variable and depends upon extent of the disease. The majority of patients who show the smear positive culture negative phenomenon during treatment are those who have advanced cavitary disease and those with the inclusion of rifampicin in the treatment protocol (Kim *et al.*, 1984; Dominguez and Vivas, 1977). Results of culture may only become available 3-6 weeks after the specimen collection and the sputum smear therefore provides an immediate objective measure of response to therapy. Should the number of organisms in the smears fail to decline after therapy the possibility of drug resistance must be considered.

1.2.3 **Radiometric Culture**

In 1969 Deland and Wagner developed a technique for the detection of microbial growth and metabolism by measuring the conversion of substrate labelled with carbon-14 to $[^{14}C]\ O_2$ with an ion chamber

device. Modification of this technique is now used routinely for detection of microbial growth in blood cultures.

Radiometric detection of Mycobacteria was first described in 1975 (Cummings *et al.*, 1975) and its usefulness in clinical specimens described in 1977 (Middlebrook *et al.*, 1977).

The procedure is simple: An aliquot of the specimen is inoculated into a bottle containing Middlebrook 7H12 medium supplemented with ^{14}C-labelled palmitic acid (Bactec 12A). An antibiotic mixture of polymixin B, amphotericin B, naladixic acid, trimethoprim and azlocillin is reconstituted with polyethylene stearate (a growth promoting substance for many Mycobacteria) and added to the bottle. Thereafter, $[^{14}C]O_2$ is measured daily and the culture is examined microscopically and subcultured if a significant growth index is demonstrated.

The clinical usefulness of the radiometric method was assessed in several trials, using a wide variety of specimen types including sputa, urine, tissue, CSF and gastric washings. The findings of some of these trials are summarised in Table 1.8. It would appear that the detection efficiency (sensitivity) of the radiometric method is comparable with that of conventional culture methods. However, the advantage of this method lies in the much faster recovery times (2-3x), which has obvious clinical and therapeutic implications.

Although radiometric culture methods can rapidly detect mycobacterial growth, further identification may require up to six weeks for completion. A major advance, therefore, was made when it was demonstrated that the radiometric technique can also be adapted to

RADIOMETRIC DETECTION OF MYCOBACTERIA IN CLINICAL SPECIMENS

REFERENCE	SMEAR POSITIVE SPECIMENS					SMEAR NEGATIVE SPECIMENS				
	No. of specimens	Species	No. isolated (time taken)			No. of specimens	Species	No. isolated (time taken)		
			Total	Radiometric	Conventional			Total	Radiometric	Conventional
Parker *et al* 1984	11	Mtb Other	10 1	9 (7) 1 (3)	8 (18) 1 (20)	987	Mtb Other	11 38	10 (20) 35 (1-18)	8 (28) 8 (5-30)
Roberts *et al* 1983	463	Mtb Other	254 116	245 (8,3) 105 (5,2)	232 (19,4) 104 (17,8)					
Takahashi & Foster 1983						* 5375	Mtb Other All myco-bacteria	143 131	121 106 (12,8)	122 93 (21)
Fadda and Roe 1984						483	Mtb	20	20 (10,9)	19 (22,5)
Morgan *et al* 1983						2165	Mtb Other	14 57	12 (13,7) 39 (4-17)	13 (26,3) 50 (18,42)

* NOT STATED WHETHER SMEAR POSITIVE OR NEGATIVE

Mtb = *M.tuberculosis*

Table 1.8

differentiate the *M.tuberculosis* complex from other Mycobacteria by use of the NAP test (Siddiq *et al.*, 1984). NAP (pNitro-a-acetylamino-b-hydroxypropiophenone) is an inhibitor of the *M.tuberculosis* complex but not of other Mycobacteria. The test requires, on average, an additional 5 days once growth has been documented radiometrically.

A number of studies (Snider *et al.*, 1981; Vincke *et al.*, 1982) compared drug susceptibility from cultures obtained by radiometric and conventional methods and found that there was good correlation. The use of the radiometric method for combined primary recovery and drug susceptibility testing was, however, first demonstrated by Roberts and colleagues (1983). After a positive growth index is demonstrated, the test drug can be added and the change in growth index measured. Using this procedure, Roberts demonstrated an overall agreement of 95-100% between the two methods. The average additional time for drug susceptibility results ranged from 4.2 to 6.9 days for the combined method and 13.7 to 21 days for the conventional methods.

Culture positive mycobacteraemia due to *M.tuberculosis* was reported in the 1930's (Jensen, 1935; Shapiro, 1932) and the radiometric detection of mycobacteraemia was first shown when Landau and colleagues (1980) isolated from two immunosuppressed patients, *Mycobacterium chelonei* subsp *abscesses* using standard aerobic BACTEC bottles (6B). Because of the intracellular location of pathogenic Mycobacteria the lysing of peripheral white cells before inoculation into appropriate media needed to be considered. A lysis centrifugation blood culture technique had previously been described for the detection and isolation of bacteria and fungi from blood (Dorn and Smith, 1978). With this method blood is inoculated into a double-

stoppered, evacuated tube (Isolator: E I du Pont de Nemoun and Co., Inc.) containing sodium polyethanol sulfonate, EDTA and the lysing agent Saponin. After centrifugation, the supernatant fluid is discarded and the pellet plus residual fluid inoculated into the appropriate media.

M.chelonei was the first Mycobacterium detected by this procedure (Fojtasek and Kelly, 1982) followed by the detection of *M.tuberculosis* bacteraemia in 1985 (Kiehn *et al.*, 1985). Since then a number of authors have demonstrated that mycobacteraemia due to *M.tuberculosis* or other Mycobacteria can be detected by using this lysis procedure combined with the radiometric technique (Gill *et al.*, 1985; Kiehn and Cammarata, 1986).

In order to circumvent the need for a separate isolator, Johnson Laboratories introduced the 13A medium. This medium was compared with the isolator system and shown to have the same sensitivity and slightly faster detection times (Witebsky *et al.*, 1988). A major advantage of this system is that less manipulation is required, resulting in less exposure to possible HIV positive samples from patients with associated mycobacteraemia.

In order to further improve the sensitivity and speed of the radiometric system, analysis of the incubated bottles by ELISA (Friedman *et al.*, 1989) and by nucleic acid probes (Ellner *et al.*, 1988) have been reported. Ellner and colleagues, using acommercially available probe (Gen. Probe), documented a reduction in time of detection and also the elimination of biochemical tests of isolates giving a positive reaction with one of the probes. Friedman and

colleagues (1989) using a commercially available anti-BCG antibody documented detection of *M.tuberculosis* within 10 days.

1.2.4 Immunodiagnostic Assays

1.2.4.1 Serological Assays

Serologic diagnosis of infections is widely used in medicine. Because of the problems associated with conventional detection techniques for *M.tuberculosis* numerous serologic techniques have been employed. However, the major limitation with this approach is that most serologic assays are not specific for *M.tuberculosis*, probably, as a result of low level antibody against environmental mycobacterial antigens. Even sera from healthy Tuberculin negative infants have antibodies which react with antigens of *M.tuberculosis* (Bardana *et al*, 1973).

The first report of a serodiagnostic assay (agglutination) for *M.tuberculosis* was in 1898 (Arloing, 1898) only 16 years after Koch's identification of the tubercle bacillus. Since that time, immunodiffusion (Parlett and Youmans, 1959), passive hemagglutination (Takahashi and Ono, 1961), whole cell agglutination (Riska *et al.*, 1980), enzyme linked immunosorbent assay (Nassau *et al.*, 1976b), fluorescent antibody (Bhardwaj *et al.*, 1980) and solid-phase radioimmunoassay (Nassau *et al.*, 1976) have been used. Because enzyme-linked immunosorbent assay (ELISA) does not require sophisticated instrumentation and is relatively inexpensive, this technique has been the most widely used serodiagnostic method.

Early workers used crude bacillary antigens for serodiagnostic assays. Table 1.9 summarises some of these results and the data indicate that both lack of specificity or lack of sensitivity is a major problem.

Tuberculin purified protein derivative (PPD) is a relatively crude antigen preparation derived from heat-killed cultures of *M.tuberculosis* and has been studied extensively as an antigen in ELISA serodiagnostic tests (Table 1.10). The results of these assays again show poor sensitivity and specificity. Only in the study of Koshino is there satisfactory specificity. There is no correlation between results and concentration or source of PPD.

These crude antigens, prepared from *M.tuberculosis* or *M.bovis*BCG contain many antigens that are identical or similar to antigens of the non-tuberculosis Mycobacteria. The non-specific results obtained using these crude antigens are therefore probably due to low levels of antibody against environmental mycobacterial antigens. Further purification of antigens can be achieved by various methods (see section 1.1.6) and some of these antigens have been used in clinical trials (Table 1.11).

Antigen 5 is the best characterised and most widely used antigen. This antigen was purified by immunoabsorbent affinity chromatography, has a molecular weight of 35 000 daltons, gives a single precipitation arc in immunoelectrophoresis and has a carbohydrate content of about 10 per cent (Daniel and Anderson, 1978). Workers showed high *in vitro* specificity (Daniel *et al.*, 1979) and good clinical disease correlation (Table 1.11) for Antigen 5. More recently it has become apparent that this antigen contains two non-specific epitopes which

TUBERCULOSIS ELISA RESULTS USING CRUDE BACILLARY ANTIGENS

REFERENCE	ANTIGEN	PATIENTS WITH TB		CONTROL PATIENTS	
		ELISA POSITIVE	ELISA NEGATIVE	ELISA POSITIVE	ELISA NEGATIVE
Nassau *et al* 1976b	Mtb filtrate	26	20	1	47
	Mtb filtrate	37	9	4	44
Grange *et al* 1980	BCG sonicate	73	27	1	29
Kardjito *et al* 1982	BCG sonicate	82	25	4	139
Grange and Kardjito 1982	BCG sonicate	136	64	1	49
Jagganath *et al* 1983	Mtb sonicate	35	36	12	75
Garcia-Ortigoza and Gutierrez-Velazquez 1982	BCG sonicate	39	11	0	50
Benjamin *et al* 1984	Mtb filtrate	15	12	7	38
Kiran *et al* 1985	Mtb sonicate	47	3	1	29
	Mtb filtrate	47	3	1	29

Mtb = *M.tuberculosis*

MODIFIED FROM: Daniel and Debanne, 1987

Table 1.9

TUBERCULOSIS ELISA RESULTS USING PURIFIED PROTEIN DERIVATIVE

REFERENCE	PPD CONCENTRATION	PATIENTS WITH TB		CONTROL PATIENTS	
		ELISA POSITIVE	ELISA NEGATIVE	ELISA POSITIVE	ELISA NEGATIVE
Kalish *et al* 1983	Parke Davis 200 µg/ml	11	7	4	115
Zeiss *et al* 1984	Parke Davis 200 µg/ml	14	7	27	99
Tandon *et al* 1980	RT-23 1 000 µg/ml	45	21	1	24
Gupta *et al* 1983	RT-23 1 000 µg/ml	49	17	1	24
Viljanen *et al* 1982	Connaught 10 µg/ml	31	4	10	77
Pan *et al* 1983	PPD 10 µg/ml	105	17	2	90
Kiran *et al* 1985	Weybridge 10 µg/ml	40	10	3	27
Koshino *et al* 1984	PPD 100 µg/ml	13	2	0	7
Balestrino *et al* 1984	PPD 10 µg/ml	24	38	15	76
Daniel *et al* 1985	PPD 10 µg/ml	13	28	4	55

MODIFIED FROM: Daniel and Debanne, 1987

Table 1.10

TUBERCULOSIS ELISA RESULTS USING PURIFIED AND SEMI-PURIFIED ANTIGENS

REFERENCE	ANTIGEN	PATIENTS WITH TB		CONTROL PATIENTS	
		ELISA POSITIVE	ELISA NEGATIVE	ELISA POSITIVE	ELISA NEGATIVE
Benjamin & Daniel 1982	Antigen 5	42	8		
	Antigen 5	17	8	7	78
Balestrino *et al* 1984	Antigen 5	55	31	0	91
Ma *et al* 1986	Antigen 5	73	11	0	30
Daniel *et al* 1985	Antigen 5	20	21	1	58
Alde *et al* 1989	Antigen 5	18	3	0	19
Stroebel *et al* 1982	Antigen 6	15	1	0	21
Reggiardo *et al* 1980	SAG A_1	42	4	1	89
	SAG B_1	34	12	0	90
	SAG C1	26	20	2	88
Reggiardo & Vazquez 1981	SAG A_1	63	11	3	141
	SAG B_1	39	35	0	144
	SAG C1	35	39	3	141
Krambovitis 1986	Plasma membrane Antigen	94	6	6	180
Thongrajai *et al* 1989	Purified mycobacterial antigen	49	5	8	88

MODIFIED FROM: Daniel and Debanne, 1987

Table 1.11

are probably carbohydrate in nature, as well as one species specific epitope which is shared with Antigen 6 (Oldes *et al.*, 1987).

M.tuberculosis Antigen 6 was prepared by immunoabsorbent affinity chromatography (Daniel and Anderson, 1977). Stroebel and colleagues (1982) used Antigen 6 in clinical trials and showed good sensitivity and specificity. However, subsequent studies were less satisfactory and indicated that both Antigen 5 and PPD gave better results than Antigen 6 (Kiran *et al.*, 1985).

Three mycobacterial glycolipids extracted from *M.bovis*BCG and purified by silicic acid chromatography were found to be serologically active (Reggiardo and Middlebrook, 1975). Clinical trials using these serologically active glycolipids (SAGs) as antigens, indicate high specificity but low sensitivity.

Krambovitis (1986) using *M.tuberculosis* $H_{37}RV$ used sodium dodecyl sulphate in his antigen extraction technique and further purified a plasma membrane antigen by ion retardation chromatography. The results of one study indicate that sensitivity and specificity are not ideal (Table 1.11).

The results obtained with these chemically pure antigens suggest that species specificity may not rest with whole antigens but rather with specific epitopes. With the advent of hybridoma technology monoclonal antibodies directed at specific epitopes could be used and characterised with regard to species cross-reactivity.

In order to standardise nomenclature of the numerous monoclonal antibodies that became available, an international workshop, held in

1985, studied 33 monoclonal antibodies which were submitted by different laboratories (Engers, 1985).

Table 1.12 lists the monoclonal antibodies submitted, the results obtained for mycobacterial specificities using different techniques and the molecular sizes and nature of the antigen recognised by the monoclonal antibodies. The specificity patterns of the monoclonal antibodies for the different Mycobacteria were divided into four categories i.e.:

1. *M.tuberculosis* specific;
2. *M.tuberculosis complex* specific (*M.tuberculosis*, *M.bovis* BCG and *M.africanum*);
3. Limited cross-reactivity (reacting with a restricted number of other Mycobacteria);
4. Broad cross-reactivity (reacting with a majority of strains tested).

None of the monoclonal antibodies were found to be specific for *M.tuberculosis* when all techniques were taken into account. Six of the monoclonal antibodies tested showed a specificity restricted to the *M.tuberculosis* complex. Three of these reacted with a 14 kDa (Workshop No. 1, 4 and 20) antigen and another 3 with a 38 kDa (Workshop No. 15, 21 and 23) antigen. These monoclonal antibodies were also used as probes to detect phage in an *M.tuberculosis* λgtII recombinant DNA library which expressed antigens recognised by the monoclonal antibodies. The results suggested that certain monoclonal antibodies react with the same target antigen but not with the same epitope on that antigen e.g. monoclonal antibodies 1, 4 and 20 react with different epitopes on the 14 kDa protein. Although this workshop

CHARACTERISATION OF MONOCLONAL ANTIBODIES TO MYCOBACTERIA SUBMITTED TO THE INTERNATIONAL WORKSHOP

Monoclonal Antibody			Mycobacterial specificity by				Molecular size (kDa)	Nature of antigen
Workshop no.	Supplier Designation	Immunoglobulin subclass	ELISA-RIA	Dot Blot	IFA	Western SGIP		
1	F 23-49	IgG2a	Mt-C	Mt-C	Mt-C	Mt-C	14	Protein
2	25 D 4		CR-B	CR-B	Neg.	Neg.	Unknown	Unknown
3	SA 12	IgG2a	CR-L	CR-L	CR-L	CR-L	12	Protein
4	F 24-2	IgM	Mt-C	Mt-C	Mr-C	Mt-C	14	Protein
5	25 E 3		CR-B	CR-B	CR-B	CR-B	Unknown	Unknown
6	MT 107 A 4/5	IgM	CR-B	CR-B	CR-B	CR-B	Unknown	Unknown
7	F 29-29	IgGa2	Weak	CR-B	Weak	CR-L	40	Protein
8	18 B 10		Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
9	MT 107 F5/ C5	IgM	CR-B	CR-B	CR-B	CR-B	20-80	Protein (?)
10	F 29-47	IgM	CR-L	CR-L	CR-L	CR-L	19	Protein
11	51 A	IgG2a	CR-L	CR-L	Mt-C	CR-L	71	Protein
12	HY T 6	IgG	CR-L	CR-L	CR-L	CR-L	19	Protein
13	WTB 78-A1	IgG1	CR-L	CR-L	CR-L	CR-L	65	Protein
14	TB-C-14	IgG2a	Weak	CR-B	CR-L	Neg.	Unknown	Unknown
15	WTB 72-A6	IgG1	CR-L	Mt-C	Neg.	Mt-C	38	Protein
16	66	IgG2a	CR-L	CR-L	CR-L	CR-L	19	Protein
17	SAID2DI	IgG1	CR-B	CR-B	CR-B	CR-B	23	Protein
18	TB-C-15	IgG2a	Neg.	Neg.	Neg.	Neg.	Unknown	Unknown
19	WTB 23-A1	IgG1	CR-L	CR-L	CR-L	CR-L	19	Protein
20	WTB 68-A2	IgG1	Mt-C	Mt-C	Neg.	Mt-C	14	Protein
21	HY T 28	IgG	Mt-C	Mt-C	Neg.	Mt-C	38	Protein
22	TB-C-15	IgG2a	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
23	WTB 71-A2	IgG2b	Mt-C	Mt-C	Neg.	Mt-C	38	Protein
24	TB-C-14	IgG2a	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
25	TB-C-14	IgG2a	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
26	TB-C-15	IgG2a	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
27	TB-C-13	IgG2a	Weak	Mt-C	Neg.	Weak	19	Protein
28	TBI B6 F4	IgM	Neg.	Neg.	Neg.	Neg.	Unknown	Unknown
29	TBI B2 H2	IgG3	Weak	CR-B	Neg.	Neg.	23	Protein (?)
30	TBI A 11 C3	IgA	Weak	Mt-a	Neg.	Neg.	Unknown	Unknown
31	SA2 D5 H 4	?	CR-L	CR-L	Neg.	CR-L	65	Protein
32	SA2 A1 A5	IgM	CR-B	CR-B	CR-N	Weak	20-80	Unknown
33	ML 11 H 9	IgG1	CR-L	CR-L	CR-L	CR-L	65	Protein

Mt-a, specific for *M. tuberculosis*; Mt-C, *M. tuberculosis* complex specific; CR-L, Limited cross-reactivity; CR-B, Broad cross-reactivity; Weak, Weak activity
See text for details

(Modified from Engers, 1985)

Table 1.12

set out to standardise nomenclature authors continue to use their own designation and as such there still appears to be confusion in this field.

Recombinant DNA technology has also been used to obtain pure antigen. A 64-65 kDa protein from *M.bovis* has been produced but several epitopes of this protein show marked cross-reactivity with many bacteria. Only certain epitopes (TB78) are specific for *M.tuberculosis* (Thole *et al.*, 1987).

In order to measure the antibody titres directed against single epitopes a solid-phase antibody competition test (SACT) was developed (Hewit *et al.*, 1982). This test is based on the inhibition of binding of labelled monoclonal antibodies to antigen by antibodies present in the test sera and therefore permits the use of monoclonal antibodies without purification of the appropriate antigen.

By using SACT a number of authors were able to evaluate some of these monoclonal antibodies in patients with tuberculosis and some of these results are shown in Table 1.13. From these data it would certainly appear that monoclonal antibodies do not offer any advantage over previous serological tests in terms of sensitivity. All studies, however, report 100% specificity due to the appropriate placing of the cut-off titre.

Serodiagnosis of *M.tuberculosis* using ELISA is certainly a rapid, convenient and inexpensive test. However, sensitivity is poor, and the assay can only accurately detect *M.tuberculosis* in smear positive patients. Specificity is poor if crude or purified antigens are used but can be improved if monoclonal antibodies are employed.

DISEASE CORRELATIONS USING MONOCLONAL ANTIBODIES

MONOCLONAL ANTIBODY CODE	% OF SMEAR POSITIVE PATIENTS WITH POSITIVE TITRES		
	Hewit *et al* 1982	Hoeppner *et al* 1987	Ivanyi *et al* 1983
TB 78	20	53	-
TB 71	29	74	-
TB 23	49	76	-
TB 68	51	80	-
TB 72	63	83	74
TB 72 and TB 23	68	88	-
TB 72 and TB 68	71	93	-
TB 72, TB 23 and TB 68	71	95	

NOTE: Cut-off selected to give 100% specificity

Table 1.13

1.2.4.2 **Immunodiagnosis of *M.tuberculosis* in Body Fluids**

Due to the devastating nature of *M.tuberculosis* meningitis and to the difficulty in obtaining a microbiological diagnosis, a considerable effort has been made to develop a sensitive and specific immunological test for the diagnosis of this disease. A number of different techniques have been employed for detection of antigen, antibody or both in the CSF (Table 1.14).

The results of a number of the studies look extremely promising particularly in terms of specificity. In tuberculous meningitis, however, an extremely sensitive diagnostic test is required and this criteria is not met by these immunological assay i.e. in most of the studies immunological assays correlated well with bacteriologically proven cases but in those instances where there was only a presumptive diagnosis, only a small proportion were positive by the immunological assay.

The value of ELISA for the diagnosis of tuberculous pleural effusion has not been well defined. Dhand and colleagues (1988) used a double antibody in the solid phase. They studied pleural fluids from 36 patients and reported positive results in 12 from 15 tuberculous patients, 12 from 12 cancer patients and 1 patient from 9 with miscellaneous causes of pleural effusion. The data indicate that non-specific reactions are common with this assay.

Ramkisson and colleagues (1988) however, used a competition ELISA with anti-BCG antibody and showed positive results in 5 from 5 tuberculous pleural fluids and 10 from 10 tuberculous ascitic fluids. 10 control ascitic fluids were all negative.

IMMUNODIAGNOSIS IN TUBERCULOUS MENINGITIS

REFERENCE	TECHNIQUE	NUMBER TEST POSITIVE				
		PROVEN TB	PRESUMPT. TB	TREATED TB	OTHER NEUROL. DIS.	CONTROLS
Sada *et al* 1983	ELISA-Ag	4/4	9/12	-	0/16	-
Bal *et al* 1983	ELISA-Ag	7/7	1/1	-	1/32	-
Hernandez *et al* 1984	ELISA-Ab	20/20	-	-	0/70	-
Coovadia *et al* 1986	ELISA-Ab	7/37	-	-	3/63	-
Krambovitis *et al* 1984	LATEX-Ag	17/18			1/134	
Chandramuki *et al* 1985	Haemaggl-Ag	22/25	47/64		6/128	
Kadival *et al* 1987	RIA-Ag	15/19	-	2/17	2/56	
Watt *et al* 1988	ELISA-Ag+Ab	15/29		-	4/83	0/15
Wu *et al* 1989	ELISA-Ag	5/5	-	0/6	0/9	0/134
Ramkisson *et al* 1988	ELISA-Ag	10/10	-	-	1/24	0/25
Donald & Cooper 1987	ELISA-Ag	22/22			6/31	

Table 1.14

Samuel and colleagues (1985) examined both pleural and ascitic fluid for *M.tuberculosis* antigen and for antibody to *M.tuberculosis*. Antigen was detected in 12 from 41 cases of tuberculous peritoneal or pleural fluid while antibody was detected in 20 cases. No antibody or antigen was detected in 38 control patients. Although this study suggests good specificity, the lack of sensitivity precludes this assay as a diagnostic test.

1.2.5 Chromatographic Techniques

The chemical detection and differentiation of Mycobacteria has been an attractive possibility since the demonstration by Anderson of the uniqueness and complexity of mycobacterial cell wall lipids (Anderson, 1943).

Various chromatographic techniques have been employed in an attempt to separate the lipids or other cell components and so to define characteristic profiles which could be used to differentiate and detect the various Mycobacteria.

As early as 1954, column chromatography and detection by infrared spectroscopy was used to characterise mycobacterial species and to distinguish bovine from human strains (Smith *et al.*, 1954). Later, in several studies thin layer chromatographic analysis of lipids was used to differentiate many types of non-tuberculous Mycobacteria (Jenkins *et al.*, 1972; Szulga *et al.*, 1966). This procedure was further adapted by differentiating mycobacterial species incubated with [^{35}S] methionine by the patterns of radioactive lipid spots (Tsukamura and Mizuno, 1975).

It was, however, not until the introduction of gas liquid chromatography (GLC) that good discrimination between the different Mycobacteria could be achieved. The gradual improvements seen in GLC of the Mycobacteria are related to improvements in the extraction and derivatisation of specimens as well as the development of more sensitive detection systems.

Pyrolysis GLC of whole cells was successfully employed in a series of studies by Reiner and colleagues to classify and identify many mycobacterial strains (Reiner, 1965; Reiner, 1967; Reiner *et al.*, 1969a; Reiner *et al.*, 1969b; Reiner *et al.*, 1971).

It was, however, felt that better discrimination would be achieved by initial extraction of lipid followed by GLC. This was first achieved by Lucchesi who showed that Mycobacteria could be differentiated by GLC of methylated lipid extracts (Lucchesi *et al.*, 1967). Later work by Thoen and colleagues demonstrated good differentiation of Mycobacteria by characteristic branched chain fatty acids profiles (Thoen *et al.*, 1971a; Thoen *et al.*, 1971b; Thoen *et al.*, 1972). The lipid extraction procedures were complex and a single step extraction of whole cell hydrolysates was at the time seen as a major advance (Ohahsi *et al.*, 1977). With this procedure these workers were able to distinguish mycobacterial species fairly rapidly and also demonstrate a unique chromatographic peak for *M.tuberculosis*.

Tisdall and colleagues (1979) developed an identification scheme using specific chromatographic patterns from 128 stock strains. In a later study of 335 routine mycobacterial isolates, they showed good correlation of the chromatographic patterns with conventional

biochemical profiles (Tisdall *et al.*, 1982). A further study of 110 isolates using a modification of the previous methods also showed good correlation with biochemical tests (Mayal, 1985).

As mycolic acids make up a large proportion of the lipid content of the cell wall, various attempts have been made to analyse the cleavage products of mycolic acids (Kaneda *et al.*, 1986; Guerrant *et al.*, 1981; Valero-Guillen *et al.*, 1985 and Lambert *et al.*, 1986). Although these results appear promising the instability of myolic acids at high temperatures and their high boiling points may make these compounds unsuitable for routine GLC analysis.

All the previously mentioned studies were performed on laboratory isolates. This is an obvious disadvantage as most GLC methods require well grown cultures of Mycobacteria and this may take a further 2-3 weeks after detection of the organism. The usefulness of GLC for direct detection of Mycobacteria in clinical specimens had not yet been achieved. This is not surprising as there are numerous variables which would undoubtedly influence the GLC patterns i.e. the complexity of the body fluids and factors which control or influence its composition; the variables introduced by specimen collection; transport; extraction and derivatisation and finally the analysis and interpretations of complex data generated by such investigations. One would therefore, require a compound unique to the organism or patterns very different to those of the control fluids and this would require a large number of clinical specimens both normal and mycobacterial in order to establish these differences. Furthermore, in order to detect the small amount of compound in the body fluid more sophisticated detector systems would have to be used.

Despite these problems, Brooks and colleagues (Craven *et al.*, 1977) using electron capture gas-liquid chromatography (EC-GLC) demonstrated an indolic type of compound, 3-(2′-ketohexyl) indoline (KHI) in the cerebrospinal fluid of patients with acute tuberculous meningitis. The compound was not present in cerebrospinal fluids of patients with cryptococcal and aseptic meningitis. In a subsequent clinical study (Brooks *et al.*, 1977) of 46 CSF specimens (17 tuberculous, 15 crytococcal, 14 viral) these authors confirmed the above findings and also noted the gradual disappearance of KHI during therapy. A further study (Brooks *et al.*, 1980) of 260 CSF specimens (34 tuberculous) these authors demonstrated the presence of KHI in cases of acute tuberculous meningitis but noted its absence in treated patients and in those in whom the tuberculous meningitis was of a more chronic nature. The study also demonstrated the detection of specific GLC profiles in meningitis of different aetiologies including tuberculous meningitis. It was therefore possible to diagnose tuberculous meningitis in the absence of KHI. The identification of specific GLC-profiles was also used in a study of pleural effusions, (Brooks *et al.*, 1978) and the authors were able to differentiate between effusions caused by congestive heart failure, *M.tuberculosis* and some other bacteria.

Probably the greatest advance in the chromatographic detection of *M.tuberculosis* has been the detection of tuberculostearic acid by gas liquid chromatography.

Tuberculostearic acid (10-methyloctadecanoic acid) was first isolated from *M.tuberculosis* in 1929 (Anderson and Chargaff, 1929). It has been demonstrated in some mycobacterial species and also in a limited

number of other members of the *Actinomycetales* such as *Norcadia* and *Actinomyces*, but is not normally present in human tissues. In *M.tuberculosis* the fatty acid amounts to 10% of the total fatty acid content (wt/wt) (Odham *et al.*, 1979).

Using the highly sensitive technique of gas chromatography and mass spectrophotometry (GC-MS) with selected ion monitoring (SIM), Odham and colleagues (1979) were able to detect tuberculostearic acid in the sputum of a small number of patients with pulmonary tuberculosis. The same procedure was used to detect tuberculostearic acid in the CSF of one patient with tuberculous meningitis (Mardh *et al.*, 1983).

It was, however, not until 1987, that a large trial was undertaken to accurately determine the sensitivity of this procedure (French *et al.*, 1987a). The results are shown in Table 1.15 and would indicate that the procedure is at least as sensitive as laboratory culture for the detection of *M.tuberculosis* in sputum.

Another study in which cerebrospinal fluids were examined (French *et al.*, 1987b) also indicate the extreme sensitivity of this procedure (Table 1.16).

A further trial using bronchial aspirates and bronchoalveolar lavage specimens from patients who either failed to produce sputum or whose sputa were smear negative, also showed very good results with this assay (Table 1.17; Pang *et al.*, 1989).

DIRECT DETECTION OF TUBERCULOSTEARIC ACID IN SPUTUM BY GC-MS/SIM

SPECIMEN GROUP	NO. OF SPECIMENS	GC-MS/SIM	
		POSITIVE	NEGATIVE
M.tuberculosis			
Smear-positive, culture-positive	39	39	0
Smear-positive, culture-negative	0	0	0
Smear-negative, culture-positive	66	63	3
Smear-negative, culture-negative	200	1	199
Controls	100	0	100

from French *et al.*, 1987a

Table 1.15

DETECTION OF TUBERCULOSTEARIC ACID IN CSF BY GC-MS/SIM

SPECIMEN GROUP	NO. OF SPECIMENS	GC-MS/SIM	
		POSITIVE	NEGATIVE
Smear/Culture positive	8	8	0
Smear/Culture negative with radiological evidence of active pulmonary tuberculosis	5	5	0
Suspected tuberculous meningitis	9	8	1*
Non-tuberculous meningitis	87	1+	86

* Possibly cerebral lupus and not tuberculous meningitis

\+ Neonate on amikacin

from French *et al.*, 1987b

Table 1.16

DETECTION OF TUBERCULOSTEARIC ACID IN BRONCHOSCOPIC ASPIRATES AND LAVAGE

SPECIMEN GROUP	NO. OF SPECIMENS	GC-MS/SIM	
		POSITIVE	NEGATIVE
Pulmonary TB confirmed (by Stain/Culture/Tissue Histology)	29	26	3
Active TB excluded	11	2	9
Controls (e.g. Cancer Bronchectasis)	13	0	0

from Pang *et al.*, 1989

Table 1.17

Tuberculostearic acid was also detected without mass spectroscopy using a sensitive pulsed electron capture detection system (Brooks *et al.*, 1987). Although only a few specimens were used, detection of tuberculostearic acid in CSF and serum was achieved.

The high sensitivity of this procedure makes it a very promising technique for the rapid diagnosis of *M.tuberculosis* in clinical specimens. There are, however, a number of disadvantages:

1. Tuberculostearic acid is also present in mycobacteria other than tuberculosis and also in other micro-organisms belonging to the order Actinomycetales;
2. Amikacin contains substances that produce ions which can be interpreted as tuberculostearic acid (false positive in study by French *et al.*, 1978b);
3. The equipment is extremely costly and complex;
4. Because of the nature of GC analysis only 10 specimens can be analysed in an 8hr working day (French *et al.*, 1987a) and therefore, the procedure is not suitable for routine work.

To overcome the problem of non-specificity a further adaptation namely negative ion mass spectrometry was introduced (Larsson *et al.*, 1987). With only limited specimen numbers the authors claim superior sensitivity and selectivity compared with GC-MS.

It has also been suggested (French *et al.*, 1987a) that specificity may be enhanced by analysing C_{32} mycoserosic acid together with tuberculostearic acid. The former acid is specific for *M.tuberculosis*, *M.bovis*, *M.africanum* and *M.kansasii* and is not found in the other Mycobacteria. However, although more specific the

sensitivity of C_{32} mycoserosic acid detection is poor compared with that of tuberculostearic acid.

1.2.6 Adenosine Deaminase

Adenosine deaminase (ADA) catalyses the conversion of adenosine to inosine in the purine catabolic pathway. ADA is found in most tissues but enzyme activity is particularly high in T-lymphocytes (Sullivan *et al.*, 1977). Furthermore, enzyme activity increases during T-lymphocyte maturation and activity is highest in the cortical thymocyte stage. Further maturation to peripheral lymphocytes is associated with a decrease in ADA activity (Barton *et al.*, 1979). Consistent with the high ADA levels in T-lymphocytes is the observation that ADA activity is increased in the body fluids of patients who have infections which are associated with T-lymphocyte stimulation and proliferation e.g. typhoid fever (Galanti *et al.*, 1981), rickettsia (Piras *et al.*, 1982) and tuberculosis (Piras *et al.*, 1973). The study by Piras and colleagues (1973) shows increased ADA activity in patients with tuberculous meningitis and these authors suggested that it may be a useful diagnostic marker.

Since this initial observation, various trials have been conducted to assess the reliability of ADA activity as a diagnostic marker in various body fluids (Table 1.18).

Most of the studies on pleural, peritoneal and pericardial effusions indicate excellent sensitivity and specificity for the ADA assay. A few authors (Niwa *et al.*, 1985; Van Klempema *et al.*, 1987 and Maartens and Bateman, 1990) however suggest that this assay is not a good diagnostic test for tuberculous effusions. The value of ADA

ADENOSINE DEAMINASE ACTIVITY IN BODY FLUIDS

REFERENCE	BODY FLUID	NUMBER OF PATIENTS		CUT OFF VALUE Units/l	SENSITIVITY	SPECIFICITY
		TB	CONTROLS			
Piras *et al* 1978	Pleural	21	33		1.00	1.00
	Peritoneal	-	42	30		
Blake and Berman 1982	Pleural	82	120	30	0.89	0.98
	Peritoneal and Pericardial	4	15	30	1.00	1.00
	CSF	24	110	6	0.83	0.98
Ocana *et al* 1983	Pleural and Peritoneal	48	173	45	1.00	0.97
Malan *et al* 1984	CSF	30	Aseptic 24 Bacterial 42	6	1.00 No significant difference between bacterial and tuberculous meningitis	1.00
Niwa *et al* 1985	Pleural	28	30	30	0.78	0.86
Martinez-Vazquez *et al* 1986a	Pericardial	3	53	30	1.00	1.00
Martinez-Vazquez *et al* 1986b	Peritoneal	10	56	35	1.00	0.96
Ocana *et al* 1986	Pleural	38	25	50	1.00	0.97
Ribera *et al* 1987	CSF	21	213	9	1.00	0.99
Van Keimpema *et al* 1987	Pleural	5	90	44	0.80	0.91
Strankinga *et al* 1987	Pleural	10	76	53	1.00	0.87
Voigt *et al* 1987	Peritoneal	41	41	32.3	0.95	0.98
Maartens and Bateman 1990	Pleural	61	48	45	0.77	0.83

Table 1.18

activity in CSF is even less well defined. Malan and colleagues (1984) were not able to distinguish between tuberculous and bacterial meningitis. Furthermore these authors also noted that CSF ADA is increased in miscellaneous neurological conditions such as brain abscess, central nervous system leukaemia, craniopharyngioma, inclusion body myositis, cryotococcal meningitis and post measles encephalitis. The studies of Ribera and colleagues (1987) and Blake and Berman (1982) however demonstrate good sensitivity and specificity for this assay in CSF.

In order to improve the specificity of the ADA assay in CSF an assessment of the CSF to plasma ADA ratio was determined in patients with aseptic, bacterial and tuberculous meningitis (Donald *et al.*, 1986). This ratio, however, was not able to distinguish tuberculous meningitis from bacterial meningitis.

Adenosine deaminase activity also decreases with antituberculous therapy (Blake and Berman, 1982; Ribera *et al.*, 1987) and may, therefore, be a good marker of therapeutic efficiency.

The cause of the increased ADA activity in tuberculous effusions is not entirely clear. It is known that there is a high percentage of T-lymphocytes in tuberculous effusions, but this does not correlate with the level of ADA (Ocana *et al.*, 1983; Martinez-Vazquez *et al.*, 1986b). One explanation is based on the observation that the activity of the enzyme correlates with the stage of T-cell maturation rather than the number of lymphocytes i.e. in tuberculous effusions immature, active T-lymphocytes, with high ADA activity may be present (Ocana *et al.*, 1983).

1.2.7 **Bromide Partition Ratio**

In 1929 Walter described a method for estimating bromide levels in blood and CSF and showed that in normal subjects there was a blood brain barrier for bromide. The partition of bromide between the blood and CSF could be expressed as a bromide partition ratio (BPR) which in normal subjects fell within the range of 2.9 to 3.5. He also showed that this ratio fell in tuberculous meningitis and neurosyphilis. The method of bromide estimations was later modified (Hunter, 1953) and with this improved methodology Taylor and colleagues (1954) reported a normal BPR of 2.6. These authors also demonstrated a fall in the BPR in tuberculous meningitis and their results together with subsequent studies are shown in Table 1.19. These results indicate that a BPR of less than 1.6 correlates well with tuberculous meningitis, although false positive results are noted.

A later study (Wiggelinkhuizen and Mann, 1980) demonstrated the usefulness of the BPR in patients without bacteriologically proven tuberculous meningitis (Table 1.20). The finding of false positive results in patients with bacterial meningitis suggests that the test is not specific for tuberculosis.

Furthermore, a low BPR has also been reported in neurosyphillis, multiple sclerosis, congenital hypothyroidism and mumps meningitis (Wiggelinkhuizen and Mann, 1980).

Mann and colleagues (Mann *et al.*, 1982) compared the BPR with ADA activity in tuberculous meningitis and found the BPR marginally more accurate than CSF ADA.

STUDIES OF THE BROMIDE PARTITION RATIO

REFERENCE	MENINGITIS	NUMBER OF PATIENTS	
		BPR < 1.6	BPR > 1.6
Taylor *et al* 1954	Tuberculous	30	2
	Other	3	33
Smith *et al* 1955	Tuberculous	42	3
Nicol and Fawns 1958	Tuberculous	6	0
	Other	2	18
Mandal *et al* 1972	Tuberculous	4	0
	Other	0	15

Table 1.19

BPR IN PATIENTS WITHOUT BACTERIOLOGICALLY PROVEN TUBERCULOUS MENINGITIS

	Bacteriologically Proven Tuberculous meningitis	Highly Probable Tuberculous Meningitis e.g. proven Pulmonary TB	Probable Tuberculous meningitis Clinical Suspicion only	Tuberculous meningitis excluded
No. of patients	13	8	30	32
BPR < 1.6	12	6	30	4*
BPR > 1.6	1	2	0	28

* Pyogenic bacteria isolated from three CSFs.
The fourth false positive occurred in a child with encephalo-opthalmic dysplasia

From Wiggelinkhuizen and Mann, 1980

Table 1.20

Coovadia and colleagues (1986) compared the BPR, ADA activity and ELISA (Ag5) and found the BPR to be the most reliable test in the early differentiation of tuberculosis from other causes of meningitis.

The measurement of the bromide partition ratio is now straightforward and involves the intravenous or oral administration of ^{82}Br-ammonium bromide solution followed by an equilibration period of 24 hours (IV) or 48 hours (oral). Serum and CSF specimens can then be collected and radioactivity measured.

It appears that a drop in the bromide partition ratio is relatively specific for tuberculous meningitis. A clue to the cause of this apparent specificity is given by early workers (Taylor and Smith, 1954; Smith *et al.*, 1955). These authors injected tuberculin into the CSF of patients with a positive tuberculin test and observed low bromide partition ratio identical to that observed in tuberculous meningitis. They, therefore, suggested that the specific blood-CSF barrier defect is in some way a function of a hypersensitivity response.

1.2.8 Nucleic Acid Analysis and Detection

One of the first applications of nucleic acid technology in mycobacterial detection and differentiation was the study of genome size, guanine-cytosine (GC) composition and relationships between Mycobacteria based on deoxyribonucleic acid reassociation.

All the Mycobacteria were shown to have genome sizes of between 3-5 x 10^9 daltons with the closely related *M.tuberculosis*, *M.bovis* and *M.bovis*BCG having smaller genomes than the other Mycobacteria (Baess and Mansa, 1978).

All the Mycobacteria have high GC contents of between 66-70% and belong to the high GC subdivision of gram positive bacteria, which includes Actinomycetes such as Streptomyces and Nocardia. Studies of GC content confirmed that Corynebacteria and Mycobacteria should be separate genera and that the Nocardia and Mycobacteria are more closely related (Hill, 1966). Further studies based on GC content indicated a bimodal clustering in terms of GC content among the different Mycobacteria, but the separation did not correspond to the division of the species into slow and rapid growers (Wayne and Gross, 1968b).

It soon became apparent that one could determine relatedness amongst Mycobacteria by the degree of DNA reassociation. By using DNA from one or two mycobacterial species as standards numerous researchers published relatedness based on percentage homology with the "standard" DNA. Bradley (1973) showed marked overlap of Nocardia with Mycobacteria while numerous other authors indicated variable degrees of relatedness among the various Mycobacteria (Baess, 1982; Gross and Wayne, 1970; Baess and Bentzon, 1978, Bradley, 1972 and Imaeda *et al.*, 1982).

Another approach for differentiation of Mycobacteria was to establish species or strain specific binding patterns of restriction digests of genomic DNA. Patel and colleagues (1986) were able to demonstrate specific banding patterns in nine mycobacterial species and were also able to differentiate *M.tuberculosis* strains based on the banding patterns. Collins and De Lisle (1987) were able to differentiate BCG by specific restriction fragment patterns. Shoemaker and colleagues

(1986) differentiated 15 strains of *M.tuberculosis* by restriction o7 3 enzyme patterns of *Mbo*I digests of genomic DNA. Profiles of the plasmids found in the *M.avium - M-intracellulare - M.scrofulaceum* (MAIS) complex have been used as epidemiological markers and from these results the authors concluded that MAIS in natural aerosols may contribute significantly to human colonisation and disease (Meissner and Falkinham, 1986). Crawford and Bates (1986) isolated plasmids from 26 strains of *M.avium* complex and suggested that plasmids may be associated with virulence. The association of virulence with plasmids in *M.avium* was also demonstrated by Gangadharam and colleagues (1988). Furthermore, an association between the presence of plasmids and antibiotic resistance in *M.avium* complex has also been demonstrated (Franzblau *et al.*, 1986).

Because of the uniqueness of the DNA from a particular organism, various researchers set about to identify specific DNA probes which would hopefully also be more sensitive than other established methods of diagnosis. Shoemaker and colleagues (1984) using radiolabelled total *M.tuberculosis* DNA were able to detect 10^{-4} ug of *M.tuberculosis* DNA. Given that the size of the *M.tuberculosis* genome is approximately 3×10^9 daltons (Baess *et al.*, 1978; Bradley, 1972) this amount of DNA represents approximately 2×10^4 organisms. This total genomic DNA did not react with a wide range of non-mycobacterial DNA but did cross-hybridise with other Mycobacteria. Roberts and colleagues (1987) used whole chromosomal probes for both *M.tuberculosis* and *M.avium* and were able to detect as few as 10^4 organisms and correctly identified the species in 93% of mycobacterial cultures grown on agar plates.

A commercially available, I^{125} labelled, single stranded DNA probe o7 3 complementary to the ribosomal RNA of the *M.tuberculosis complex*, *M.avium* and *M.intracellulare* respectively, has become available (Gen-Probe Corp., San Diego, California). These probes have been primarily used for the identification of Mycobacteria once isolation has been accomplished. This identification may be particularly useful in the case of the *M.avium* complex where *M.avium* and *M.intracellulare* are difficult to differentiate by conventional means. The results of 3 studies (Table 1.21) indicate high sensitivity and specificity and identification can be achieved within 2 hours.

Pao and colleagues (1988) constructed a plasmid DNA library from a *Bam*HI digest of *M.tuberculosis* DNA. They identified two clones which were able to detect 50 pg of *M.tuberculosis* DNA (10 000 cells) and which also hybridised with 49 out of 50 *M.tuberculosis* clinical isolates. The clones showed no cross-hybridisation with non-mycobacterial organisms and of the Mycobacteria other than *M.tuberculosis*, the clones only showed cross-hybridisation with *M.avium* - *M.intracellulare* and *M.xenopi*. Analysis of 341 uncultured clinical specimens showed that 76 out of 84 culture positive specimens were positive using these probes but that 58 culture negative specimens were also positive. Many of these cultures came from patients with clinical features suggestive of tuberculosis.

Patel and colleagues (1989) constructed a plasmid library from a *Sau*3A1 digest of *M.tuberculosis*. Three clones were identified which hybridised strongly with a number of *M.tuberculosis* strains, weakly with DNA from other Mycobacteria and not at all with *E.coli* or human DNA. Smaller fragments from one clone were subcloned and these

STUDIES ASSESSING GEN-PROBE

REFERENCE	SPECIES	NO. TESTED	NO. POSITIVE WITH PROBE FOR:		
			M.avium	*M.intracellulare*	*M.tuberculosis*
Drake *et al* 1987	*M.avium* complex	134	94	40	Not determined
	M.tuberculosis	22	-	-	Not determined
	Other Mycobacteria	14	-	-	Not determined
Kiehn *et al* 1987	*M.avium* complex	56	45 (7)*	4 (7)*	0
	M.tuberculosis	11	0	0	11
	Other Mycobacteria	9	0	0	0
Musial *et al* 1988	*M.avium* complex	114	53	57	0
	M.tuberculosis	102	2	0	102
	M.bovis	10	0	0	10
	Other Mycobacteria	123	0	0	0

* Positive with both *M.avium* and *M.intracellulare* probe

Table 1.21

recombinant plasmids hybridised with DNA from *M.tuberculosis*, *M.bovis* and *M.bovis* BCG but not with other mycobacterial DNA. These probes were able to detect 800 pg of *M.tuberculosis* DNA (4.5×10^5 organisms).

Eisenach and colleagues (1988) selected three clones from a DNA library prepared from a *Mbo*I digest of *M.tuberculosis* DNA. These three clones hybridised only with members of the *M.tuberculosis* complex. Each of the three DNA fragments was repeated multiple times (10-16) in the *M.tuberculosis* chromosome. It was felt that the repetitive nature of the clone would greatly enhance the sensitivity of the probe. However, each probe was only able to detect 2-4 ng of DNA (10^6 bacilli).

Although these probes can achieve rapid detection of *M.tuberculosis* the sensitivity achieved by the best candidate (Pao *et al.*, 1980) is similar to that of the Ziehl-Neelsen stain and therefore the use of DNA probes for the direct detection of Mycobacteria in clinical specimens is not practical. This is in contrast to the detection of mycobacterial DNA in clinical specimens by the polymerase chain reaction (PCR) (Brisson-Noel *et al.*, 1990; Shankar *et al.*, 1990; Shankar *et al.*, 1991; Eisenach *et al.*, 1990; Patel *et al.*, 1990; Woods and Cole, 1990). The PCR technique and relevant studies will be discussed in the following section.

1.3 POLYMERASE CHAIN REACTION

1.3.1 Introduction

The polymerase chain reaction (PCR) is an *in vitro* method for the enzymatic synthesis and amplification of specific DNA sequences. The

concept of PCR was first introduced as early as 1971 (Kleppe *et al.*, 1971). This early reference has, however, only recently received attention, but the last paragraph of this paper clearly describes a mechanism almost identical to PCR:

"The principles for extensive synthesis of the duplexed tRNA genes which emerge from the present work are the following. The DNA duplex would be denatured to form single strands. This denaturation step would be carried out in the presence of a sufficiently large excess of the two appropriate primers. Upon cooling, one would hope to obtain two structures, each containing the full length of the template strand appropriately complexed with the primer. DNA polymerase will be added to complete the process of repair replication. Two molecules of the original duplex should result. The whole cycle could be repeated, there being added every time a fresh dose of the enzyme. It is however, possible that upon cooling after denaturation of the DNA duplex, renaturation to form the original duplex would predominate over the template primer complex formation. If this tendency could not be circumvented by adjusting the concentrations of the primers, clearly one would have to resort to the separation of the strands and then carry out repair replication. After every cycle of repair replication, the process of strand separation would have to be repeated. Experiments based on these lines of thought are in progress".

It was 14 years later, however, that the PCR methodology was introduced (Mullis *et al.*, 1986; Mullis and Faloona, 1987) and it was initially used to amplify specific B globin genomic sequences for the diagnosis of sickle cell anaemia (Saiki *et al.*, 1985).

The principle of PCR is illustrated and described in Figure 1.7.

Because products synthesised in one cycle serve as a template in the next, the increase in specific fragment is exponential. The theoretical amplification can therefore be expressed as 2^n, where n is the number of cycles performed. In practice, however, the extent of amplification is $(1 + x)^n$ where x is the fractional efficiency of each cycle. Amplification of 10^5 and 10^6 are usually achieved in a 30-cycle procedure.

The ability to amplify specific DNA sequences by such an order of magnitude represents a major advance in molecular biology and this is reflected in the large number of applications of PCR since its introduction 5 years ago.

1.3.2 Components and Parameters

Although PCR appears to be a relatively simple procedure, it is in fact a complex and incompletely understood biochemical brew and because of the large number of components, parameters and biochemical interactions, it is convenient to examine each separately and attempt to assess how it may affect the total reaction.

1.3.2.1 DNA Polymerase

The initial PCR studies were performed with DNA polymerase I, Klenow fragment (Mullis and Faloona, 1987; Saiki *et al.*, 1985). Because the enzyme lacked stability at denaturing temperatures of 95°C fresh enzyme was added at the beginning of each extension step. As the temperature optimum for this enzyme was 37°C extension at this low temperature allowed for significant non-specific binding of oligonucleotide primers (Saiki *et al.*, 1988a) and hence non-specific

POLYMERASE CHAIN REACTION

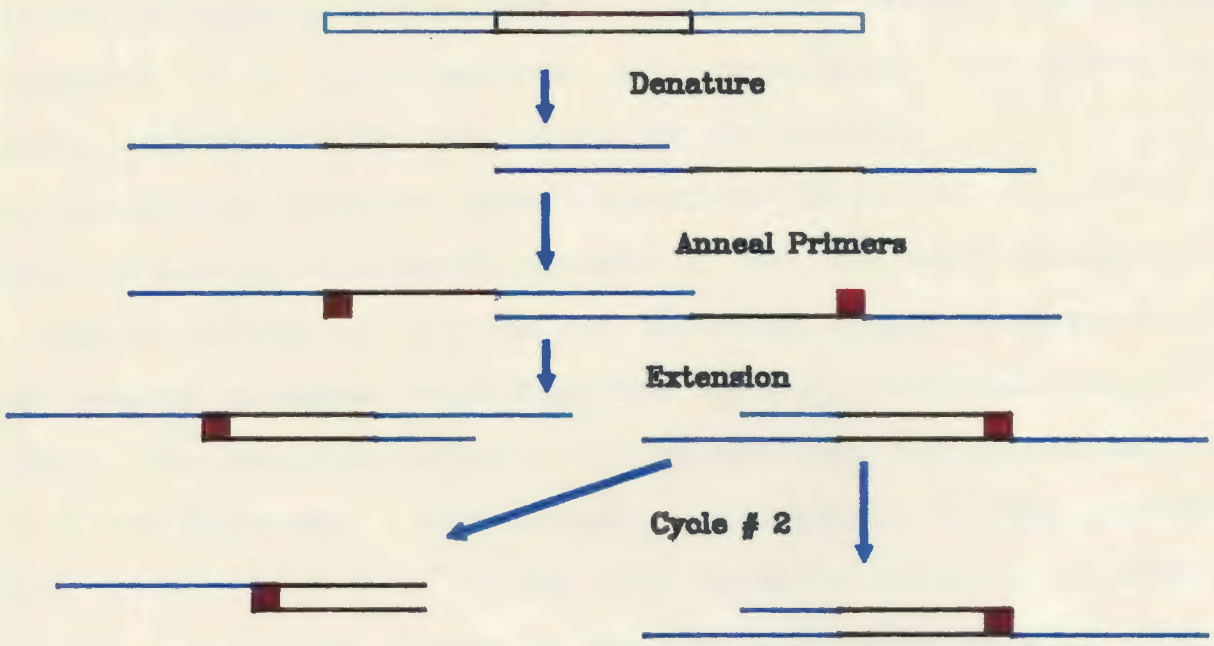


Figure 1.7: Double stranded DNA with target sequence (▭) is shown at top of diagram. Separation of the strands (Denaturation) is achieved by heating the strands to 95°C. Primers (▬) are then annealed to complementary sequences of the target sequence by lowering the temperature to 55°C. Extension of the primers occurs via a DNA polymerase and is achieved by raising the temperature to 70°C. Denaturation, primer annealing and extension represent one cycle. At the end of cycle I the newly synthesized strands are longer than the target sequence but by the end of cycle 2 correct target sequence length is being synthesised. Thirty such cycles are usually performed in a standard PCR assay.

synthesis of amplification products. These disadvantages were soon overcome when the thermostable DNA polymerase from *Thermus aquaticus* (*Taq* polymerase) was introduced (Saiki *et al.*, 1988).

Strains of *Thermus aquaticus* were first isolated from thermal springs in Yellowstone Park (Brock and Freeze, 1969) and are now known to be inhabitants of aquatic thermal environments both natural and man-made. The organism is a gram-negative, non-sporulating, non-motile rod; successful enrichment requires incubation at 70-75^{o}C.

DNA polymerase was purified from *T.aquaticus* (Chien *et al.*, 1976) and was shown to have a temperature optimum of 80^{o}C and to be stable at 93-95^{o}C. The pH optimum was 8.0 and the molecular weight as determined by sucrose density gradient centrifugation and gel filtration was 63 000 to 68 000. The specific activity of the purified *Taq* polymerase was 2 000 to 8 000 units/mg. More recent purifications of *Taq* polymerase (Gelfand, 1989) yielded an enzyme with specific activity of 2000 000 unit/mg and a molecular weight on SDS polyacrylamide gels of about 95 000. A molecular weight of 93 910 has been inferred, based on DNA sequence information (Lawyer *et al.*, 1989).

The effect of temperature on the activity of *Taq* polymerase is shown in Figure 1.8. The skewed curve shows significant activity at lower temperature, optimum activity at 80^{o}C and hardly any activity above 90^{o}C. The activity expressed as processivity rate (i.e. number of nucleotides incorporated per second) is also optimum at 75-80^{o}C (150 nt/sec) as shown in Table 1.22.

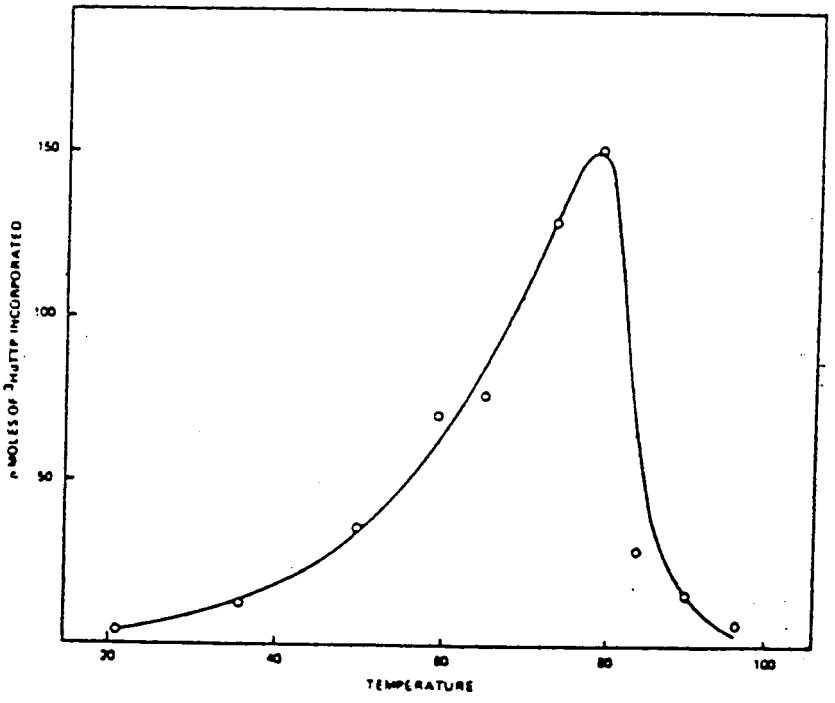


Figure 1.8: The Effect of Temperature on the Activity of *Taq* Polymerase. The incorporation of [^{3}H]dTTP into DNA was measured at different temperatures (from Chien *et al.*, 1976).

The effect temperature has on the stability of the enzyme has been investigated (Gelfand, 1989). He showed that after incubation for 130 minutes at 92°C there was still 50% residual activity. In addition, there was 50% residual activity after incubation at 95°C for 40 minutes or 97°C for 6 minutes. Therefore, if 30 cycles are performed with a denaturation step of one minute at 95°C per cycle there should still be sufficient activity remaining at the end of the reaction. At higher temperature, however, the efficiency of the reaction would be reduced.

RATE	TEMPERATURE	REFERENCE
150 nt/sec	75-80°C	Gelfand, 1989
60 nt/sec	70°C	Innes *et al.*, 1988
24 nt/sec	55°C	"
1.5 nt/sec	37°C	"
0.25 nt/sec	22°C	"

Table 1.22: EFFECT OF TEMPERATURE ON PROCESSIVITY RATE

Early workers (Chien *et al.*, 1976) demonstrated the absolute requirement of *Taq* polymerase for the divalent cation Mg^{2+}. The concentrations of this cation and its relationship with deoxynucleotide triphosphates (dNTP) will be discussed in section 1.3.2.3. The effect of KCl, gelatin, salt solutions and Dimethyl sulphoxide (DMSO) on *Taq* polymerase will be discussed in the same section.

Taq polymerase does not contain 3′-5′ exonuclease, proofreading activity (Chien *et al.*, 1976; Tindall and Kunkel, 1988) and this

taken together with the decrease in stability of the DNA duplex at higher temperatures one would expect a significant increase of incorrectly incorporated nucleotides. *Taq* polymerase produces single-base substitution errors at a rate of 1 for each 9 000 nucleotides and frameshift errors at a frequency of 1 per 41 000 nucleotides (Tindall and Kunkel, 1988). By adjusting dNTP and Mg^{2+} concentration more recent studies have shown a mutation rate of less than 1 per 81000 nucleotides (Goodenow *et al.*, 1989) and less than 1 per 100 000 nucleotides (Fucharoen *et al.*, 1989). In comparison, the Klenow fragment shows a misincorporation frequency which is 6 to 8-fold less than *Taq* polymerase (Tindall and Kunkel, 1988). There is only a two-fold increase in misincorporation rate by *Taq* polymerase for reactions performed at 70°C versus 55°C. It is not entirely clear how non-proofreading *Taq* polymerase can maintain such high fidelity. Experimental evidence indicates that *Taq* polymerase extends a mismatched primer template significantly less efficiently than a matched primer template and this may be related to a binding cleft on the polymerase which "fits" matched pairs better than mismatched pairs (Petruska *et al.*, 1988).

Whether a proofreading exonuclease exists *in vivo* but has been dissociated from the *Taq* polymerase during purification remains to be determined.

Taq DNA polymerase has some DNA synthesis dependent, strand replacement, 5′-3′ exonuclease activity (Gelfand, 1989). This does not appear to have any effect on PCR reactions. *Taq* polymerase shows considerable amino acid sequence homology with *E.coli* Polymerase I (Lawyer *et al.*, 1989). Significant similarity is shown in the domains

containing the 5′-3′ exonuclease activity. This data may explain the observed exonuclease functions. More recently the *Taq* polymerase gene has been cloned and expressed in *E.coli* (Lawyer *et al.*, 1989) and the cloned gene product is commercially available (Ampli *Taq*. Cetus Corporation).

DNA polymerases from a number of other thermophilic organisms have also been purified and partially characterised (Kaboev *et al.*, 1981; Ruttiman *et al.*, 1985; Klinezak *et al.*, 1986). Possibly some of these polymerases will have advantages over *Taq* polymerase, but this still has to be demonstrated.

1.3.2.2 Oligonucleotide Primers

The primers are probably the most important reaction component in determining the success or failure of an amplification reaction. The selection, concentration and the quality of primers can be considered separately.

The selection of efficient and specific primers remains somewhat empirical and only the following guidelines have been offered by various authors (Saiki, 1989; Saiki *et al.*, 1988b): primers should have a random base distribution and the GC content should be similar to that of the target fragment; stretches of polypurines, polypyrimidines and unusual sequences should be avoided; avoid sequences with significant secondary structure, particularly at the 3′ end of the primer (certain computer programmes are available to predict these structures); avoid primer complementarity.

With reference to the last point listed above, primers with a two to three base overlap at the 3′ end will anneal to each other and can

produce a primer-dimer effect (Saiki, 1989). This artefact is seen in particular when many cycles of amplification are performed on a sample containing very few initial copies of template. The primer dimer is a double stranded fragment which is close in size to the sum of the two primers. It appears to occur when one primer is extended by the polymerase over the other primer but the exact mechanism by which this occurs is not completely understood.

The length of primers is an important consideration, as short primers can give rise to non-specific amplification. Primers of between 20 and 30 bases will give adequate specificity and longer primers are not considered necessary.

If primer concentration is too high one may expect problems of increased non-specific binding and also primer dimer effect. The concentration must also not be too low so that primer exhaustion occurs: 0.2 μM to 1 μM of primer is usually used per reaction.

Primer quality does not appear to be of great importance. Primers can be used directly from the synthesizer but the norm is to deprotect for six hours and then ethanol precipitate directly from ammonium hydroxide. Further purification is not considered necessary.

1.3.2.3 PCR Buffer

Components and component concentrations vary between authors and manufacturers and are largely determined by the particular characteristics and requirements of the primer/template combination.

The various components that have been incorporated in PCR buffers will be considered in the following paragraphs.

The magnesium concentration in particular appears to have a marked effect on PCR efficiency and specificity. Generally, excess Mg^{2+} will result in accumulation of non-specific amplification products and insufficient Mg^{2+} will reduce the yield of amplification product. The DNA polymerase is sensitive to the concentration of magnesium ion i.e. 2.0 mM MgCl is optimal for *Taq* polymerase activity, whereas higher concentrations are inhibitory with 40% to 50% inhibition at 10 mM $MgCl_2$ (Lawyer *et al.*, 1989). It is the free magnesium ion concentration that is important for *Taq* polymerase activity. Since magnesium ions bind in a 1:1 molar ratio with dNTPs, any change in the dNTP concentration can markedly effect the free magnesium concentration. The Mg^{2+} requirement will also vary between different primer/template/nucleotide/enzyme combinations and, therefore, the optimal Mg^{2+} concentration should be determined for each combination.

Modest concentrations of KCl can enhance the rate of DNA synthesis by *Taq* polymerase by 50% to 60% with an apparent optimum at 50 mM KCl. Innes and colleagues (1988) suggest that KCl can be ommitted. At a concentration of 75 mM KCl the activity of *Taq* polymerase can be significantly inhibited.

Gelatin is usually added as an enzyme stabiliser but evidence indicates that this component may also be ommitted in certain reactions (Innes *et al.*, 1988). Deoxynucleotide triphosphates are usually present at concentrations of 50 to 200 mM each. At these concentrations, dNTPs are certainly not

limiting and there is sufficient to synthesise 6.5 μg to 25 μg of DNA. Higher concentrations may promote base misincorporations by the

polymerase (Petruska *et al.*, 1988). The relationship between free Mg^{2+} and the dNTP concentration has been discussed and both components should always be adjusted simultaneously. Ten percent DMSO was added to early buffer systems but most authors now omit DMSO and some have shown that DMSO can be slightly inhibitory to *Taq* polymerase and decrease the overall yield of amplification product (Table 1.23).

DMSO CONCENTRATION	TAQ POLYMERASE ACTIVITY
< 1%	100%
10%	53%
20%	11%

Table 1.23: Effect of DMSO Concentration on *Taq* Polymerase Activity (From Gelfand, 1989)

Several investigators have observed that the inclusion of 10% DMSO facilitates certain PCR reactions, but it is not clear which parameters are affected. The DMSO may reduce secondary structure of the DNA template, it may affect the melting temperature (Tm) of the primers, alter the thermal activity profile of *Taq* DNA polymerase or the degree of product strand separation achieved at a particular denaturation temperature.

Various salt solutions have different effects on the *Taq* polymerase activity. For example, 50 mM ammonium chloride has a mild inhibitory effect on *Taq* polymerase while 50 mM ammonium acetate has no effect and 50 mM sodium chloride has a mild stimulatory effect on enzyme

activity. β-mercaptoethanol was used in early buffers but does not appear to have any beneficial effect and is no longer used.

Besides the components mentioned above, other chemicals have been used experimentally in an attempt to enhance the efficiency of the reaction. The effect of these components on *Taq* polymerase activity is summarised in Table 1.24.

COMPONENT	CONCENTRATION	ACTIVITY
ETHANOL	< 3%	100%
	10%	110%
UREA	< 0.5 M	100%
	1.0 M	118%
	1.5 M	107%
	2.0 M	82%
DIMETHY FORMAMIDE	< 5%	100%
	10%	82%
	20%	17%
FORMAMIDE	< 10%	100%
	15%	86%
	20%	39%
SDS	0.001%	105%
	0.01%	10%
	0.1%	< 0.1%

Table 1.24 Effect of Various Components on *Taq* Polymerase Activity (from Gelfand, 1989)

1.3.2.4 Cycling Parameters

As indicated in Figure 1.7, PCR is usually performed by incubating the reaction mixtures at 3 temperatures.

The denaturing temperature is usually 90°C to 95°C. This step is critical and it is important that the reaction reaches a temperature at which complete strand seperation occurs. On the other hand high temperatures and prolonged denaturation time will decrease the *Taq* polymerase activity.

The upper limit of the annealing temperature depends on the "melting temperature" (Tm) of the primers. The Tm is related primarily to the length and GC content of the primers and can be calculated with the use of well-known equations (Sambrook *et al.*, 1989). At temperatures much lower than the Tm non-specific binding and therefore non-specific amplification will occur. The annealing temperatures must therefore be selected within the constraints of these two variables.

Although GC content plays a major role in determining optimum annealing temperature there are also probably other minor factors. This is illustrated in Table 1.25 where the relationship of GC content and optimum annealing temperatures are not exactly linear.

If short primers (12-15 bases) or primers with mismatched bases (degenerate primers) are used then the annealing temperature can be much lower e.g. 40°C.

Because of the larger molar excess of primers present in a reaction mix, hybridisation occurs almost instantaneously and long annealing times are not required.

NO. OF PRIMERS PAIRS TESTED	GC CONTENT	OPTIMUM ANNEALING TEMP
9	< 60%	60°
1	60%	60°
1	62.5%	60°
1	60%	65°
1	67.5%	65°
2	65%	70°
1	67.5%	70°

Table 1.25: Effect of GC Content on Optimum Annealing Temperature (from Kim and Smithies, 1988)

Extension temperatures should be as close as possible to the temperature optimum for *Taq* polymerase. Although *Taq* polymerase has significant activity at lower temperatures, extension temperatures around 70°C are necessary if maximal efficiency is required.

The extension time is dependent on the length of the target being amplified. At 70°C the extension rate has been calculated to be greater than 60 nucleotides/sec (Innes *et al.*, 1988). One minute is, therefore, certainly sufficient for target sequences up to 3 kb.

Certain variations in the standard 3 temperature cycle can be used under certain conditions, i.e.:

- when the Tm of the primers is high, the annealing and extension step can be performed at the same temperature (Kim and Smithies, 1988). This simplifies the procedure and may further improve specificity;

- the extension step can also be eliminated if the target is 150 bases or less. Due to the significant polymerase activity at lower temperatures, complete extension can occur during transition from annealing to denaturation.

The time taken for change from one temperature to another (ramp time), does not appear to be important other than shortening the cycle time. However, if short or degenerate primers are used one can anneal at temperatures of 40°C and then gradually heat to 70°C, thus allowing sufficient synthesis in the transition period before the possible denaturing conditions for the extension temperatures are reached.

It is important to note that it is the sample temperature which is critical and not that of the heating block or waterbath. The sample temperature is routinely measured in most of the newer commercially available thermal cycling machines. If this facility is not available, the test sample temperature should be determined during a test amplification.

1.3.3 PCR Automation

The polymerase chain reaction is based on the process of thermal cycling and this is illustrated and described in Figure 1.7. Thermal cycling performed manually is a tedious process and therefore, much effort and money has been expended in developing automated thermal cycling machines.

Before the advent of the heat stable *Taq* polymerase, automated machines also had to have built-in pipetting devices for the dispensing of the Klenow fragment of *E.Coli* DNA polymerase I. Such a machine was used by the Cetus corporation (Oste, 1989) and consisted

of two temperature controlled aluminium blocks, one of which held uncapped reaction tubes and the other uncapped Klenow fragment solutions. The reaction block was controlled by two waterbaths, one at 94°C and the other at 37°C, while the enzyme block was held constant at 4°C. An automated pipetting device introduced enzyme into the appropriate tubes after each denaturation step.

However, with the advent of *Taq* polymerase, PCR was greatly simplified. There was greater interest in the technique and, therefore, the need to develop more sophisticated machinery. Numerous thermal cycling machines were developed commercially but because of high cost a large number of custom machines were developed. The details of some of these have been published.

Two possible approaches can be used in the design of a thermal cycling machine: the temperature stations can be stationary and the samples moved between them or the samples remain in the same physical position and the temperature around the sample can be changed.

In the first type the degree of sophistication can vary greatly and some devices will be described. In one department at our Medical School, a machine was developed where a robotic arm moved tubes between two temperature controlled blocks (denaturation and extension) with a time in air representing the annealing temperature (personnel communication).

In another system three temperature controlled waterbaths are arranged in a circle and the sample arm moves in a circular path from one bath to the next. There is no vertical displacement of the sample arm and

tubes tilt as they pass over the rim of each temperature station (Oste, 1989).

The most sophisticated of this type of system is that described by Foulks and colleagues (1988). They modified a Histokinette which is a conventional tissue embedding instrument which has been employed in histopathology departments for many years. A circular mechanical arm which holds the samples and pivots about a central axis, sequentially dips the specimen rack into three thermostatically controlled waterbaths, completely submerging the tubes. The temperature profiles, obtained by thermo-couples within a reaction tube indicate that there is very little temperature lag between bath changes and that the ramp times are very fast.

Although these type of devices are simple and cheap there are numerous disadvantages; most of these devices require a large amount of laboratory space, a precious commodity in todays molecular biology laboratory; the control of the rate of temperature change in samples is impossible during transport through air; fine tuning of temperatures may be a lengthy process as waterbaths and temperature blocks are usually slow to equilibrate.

The approach of stationary samples and changing temperatures has been the most popular approach and is used by all commercially available machines. This type of machine falls into three basic categories:

1. Heating and cooling the samples by fluids.
2. Heating by electric resistance and cooling by fluid or fan.
3. Heating by electric resistance and cooling by semiconductors.

An example of the first type has been described by Rollo and colleagues (1988). The reaction tubes are held in a central sample chamber. Water from either a 100^{o} or a 40-70^{o} waterbath (ie. a two temperature cycle) is pumped through this central chamber. Solenoid valves and pumps are controlled by suitable timing devices so that the water within the central chamber is sequentially and repeatedly changed. Temperature profiles indicated accurate and rapid temperature changes but these profiles were obtained by a thermocouple in the reaction vessel and not in a reaction tube and therefore may not be an accurate reflection of the reaction temperature.

Another example of this approach is that described by Kim and Smithies (1988.) The samples were held in a reaction block in which criss-crossing channels were machined to allow good heat transfer between the water and the block. The sample block was connected to three thermostatically controlled water baths. Water was driven through the block by pumps. Solenoid valves, which controlled water flow, and pumps were controlled by a simple repetitive timer.

Heating by electric resistances and cooling by water has been used by Techne Incorporated for their PHC-2 Dri Block. Heating times are relatively slow (specifications indicate ramp rates of 48^{o}/minute but personal experience disagrees with this data) and cooling is controlled by the water flow rates. Because of the long ramp times actual running time is about twice the actual thermal cycling time. The machine is equipped with sophisticated software so that numerous programmes can be entered and a digital display allows immediate examination of various parameters of the reaction (eg. temperature, cycles performed, ramp rate and what segment of the cycle the sample

is in). The cooling cycle relies on tap water and the machine is therefore, not truly self-contained. There is a facility for the introduction of chilled water (from a refrigeration unit) and tubes may, therefore, be left at 4°C after the run.

A machine has been developed at our Medical School which uses a small graphite block, through which a current is passed to heat the block. The block is cooled by tap water running through the block. The machine is compact and temperature responses are good (personnel communication).

Instruments which use tap water to cool the block do, however, present some disadvantages. Most of them require a fairly high minimum water pressure in order to prevent backpressure and if this minimum pressure is not reached, pressure may build up in the tubing and this may result in some water induced damages in the laboratory. Residual tap water will always stagnate inside the block at the end of the experiment resulting in progressive corrosion. Furthermore, large quantities of "laboratory water" is washed down the drain and this may be ecologically unacceptable in some countries. Furthermore, if only tap water is used, subambient temperatures cannot be achieved. The ability to reach 4°C after the experiment, improves safekeeping of the specimen until they are retrieved by the operator. Some machines may therefore, use refrigerated water which then also has the advantage of shortening cooling ramp times (Oste, 1989).

Hybaid has developed a commercially available machine which is cooled by a small fan. The ramp times for this machine and therefore the operating times are extremely long. The machine may be operated in a

cold room to decrease cooling ramp times. However, this machine is probably the least expensive of the commercially available machines and is completely programmable.

The most sophisticated of these machines are those that use electrical resistance for heating and semiconductors for cooling. Cetus Corporation introduced the first of these machines in 1985 to 1986 (Oste, 1989). This machine used semiconductors (Peltier devices) for both heating and cooling the sample holding block. Peltier devices allow heating if a current is passed in one direction and allow cooling if passed in another direction. The problem with this mechanism is that the Peltier devices progressively wear at their junctions and need replacement at 6-monthly intervals. The cooling rate of these machines was also slow and this approach was subsequently abandoned.

The present machines marketed by Cetus Corporation are compact, have highly sophisticated software, are totally self-contained, can reach subambient temperatures and are the most expensive machines available. I have not had the pleasure of using one of these machines and I am unaware of the exact mechanism of heating and cooling, although this must be achieved through some type of semi-conductor.

Collasius and colleagues (1989) developed their cyclotherm instrument which can be built for one thousand dollars (US). Both heating and cooling are achieved through Peltier elements. The problems of stress on the Peltier junctions has been overcome by the use of thermal energy conducting glue. The machine is programmable, self-contained and can reach subambient temperatures.

Finally, a machine has been described which is entirely different to any of those described (Wittwer *et al* 1988). Samples are sealed in glass capillary tubes and held in a closed aluminium chamber. Hot air is then blown through the chamber and the temperature controlled by a thermocouple situated in the path of the hot air. Cooling is achieved by introducing room air. The temperature responses are very good and the machine is very simple and cheap to build.

I am sure that there are numerous other machines both commercially available and custom made.

A few general points should be considered. One is the method by which temperatures are measured and thus the accuracy and reproducibility of the set temperatures. This problem applies mainly to sample holding blocks. Two problems related to temperature measurement are the homogeneity of cooling and heating across the whole block and the physical fit between the wells and the microfuge tubes containing the samples. These are important engineering considerations. Because of these problems, the use of a thermocouple in a "dummy" sample in one of the wells, will record only the temperature of that specific well and this may be different to the other samples if the block is not homogenous. These thermocouples also consistently report lower than actual temperatures due to their significant thermal mass (Oste, 1989). The ideal approach, therefore, is to have sensors continously monitoring the block temperature and feed the data into an algorithm that will mimic the actual sample temperature and instruct the control software to take corrective action by heating according to needs.

A further point is the use of liquid paraffin to prevent condensation. This requires one extra step (ie. removal) after PCR and it would be advantageous to avoid this. In totally submersable systems, however, no condensation occurs and although less sophisticated in other respects, these machines do offer this advantage.

Because of automated thermal cycling machines, the PCR reaction itself has become a routine procedure. The bottlenecks in the procedure now are the pre- and post-PCR steps. This problem has already been adressed by the use of a machine which utilises a microtitre plate for the PCR reaction (Oste, 1989). All sample preparation could, therefore, be performed by an automated liquid handling machine and this may also be adapted to post-PCR analysis by allowing easy withdrawal of sample aliquots for subsequent reactions, eg. gel electrophoresis and sequencing.

1.3.4 Preparation of DNA for PCR Analysis

There are many methods that have been applied for the preparation of DNA for PCR analysis and these have recently been reviewed (Higuchi, 1989a). However, due to the nature of the mycobacterial cell wall these procedures are all too gentle for mycobacterial lysis and will therefore, not be described.

Some general points concerning DNA preparation from clinical material need to be considered. The extraction procedures should include as few steps as possible so as to limit the possibility of contamination (see section 1.3.5). The extraction procedure should be as efficient as possible. This is particularly important when DNA is extracted from clinical specimens which may contain very few copies of target

DNA. The problem of inhibitors of PCR is well recognised and the purity of the DNA preparation may be more important than the yield of DNA. A number of authors have documented the presence of inhibitors in the DNA preparations. If cells are directly lysed in the reaction vessel the amount of DNA amplified is definitely limited by the amount of cell debris (Saiki *et al.*, 1988b). Furthermore as little as 1 µl of whole blood in a 100 µl reaction is inhibitory and it is believed that the porphyrin compounds which are released from haem are responsible (Higuchi, 1989). De Franchis and colleagues (1988) documented an inhibitor in samples of DNA extracted from human blood. The DNA failed to amplify but remained a good substrate for restriction analysis. They were not able to ascribe the inhibitor to any specific compound but described a method (boiling the sample with subsequent spinning through a Sephadex G50 column) which removed the inhibitor. Lo and colleagues (1989b) have demonstrated the presence of inhibitors in DNA extracts of paraffin embedded tissue. They showed that one tissue slice gave optimal amplification and increasing the number of slices proportionately decreased the amplification yield.

Most DNA extraction procedures require that cells first be lysed and this is most often achieved by detergents. Proteolytic enzymes may then be used to digest wall components and proteins such as histones which remain tightly bound to the DNA. This will usually be followed by extraction with organic solvents to remove protein followed by precipitation of the nucleic acids by ethanol. This general approach is used by most workers for the extraction of DNA from Mycobacteria. However, due to the nature of the cell wall, numerous modifications

particularly with regard to cell lysis have been introduced and some of these will be considered below.

A large number of investigators use a method which was introduced by Mizuguchi and Tokunage (1970). These authors added glycine to actively growing cells, so interfering with cell wall synthesis and thus rendering the cells more susceptible to subsequent lysis by sodium dodecyl sulphate (SDS). They obtained a yield of 0,67 mg of DNA per gram of wet cells. Later, numerous other authors substituted cycloserine for glycine (Crawford and Bates, 1979; Imaeda *et al* 1982; Shoemaker *et al* 1986; Roberts *et al* 1987; Eisenach *et al* 1986).

Pao and colleagues (1988) reported the incubation of cells with lysozyme with subsequent lysis by SDS. Patel and colleagues (1986) utilised organic solvents to first extract lipoidal components from the outer membrane followed by treatment with lysozyme and SDS. They obtained 2 mg of *M.tuberculosis* DNA per gram of wet cells.

Wayne and Gross (1968a), aerated the bacterial cells vigorously for 3 days and then obtained lysis by abruptly subjecting the cells to anaerobic conditions followed by exposure to sodium deoxycholate. They obtained a yield of 0,67 mg of DNA per gram of wet cells.

Baess (1974) mechanicaly disrupted the cells by passing the cells through a French pressure cell press at a pressure of 14 000 psi. Yields of 1 mg/gm of wet cells were obtained.

The methods described above have all been used for the extraction of DNA from laboratory grown cultures of *M.tuberculosis* and not for DNA extraction from clinical specimens. Many of the methods described,

particularly those that require pre-incubation of viable cells with substances such as glycine, cycloserine and lysozyme are obviously not practical for isolation of DNA in clinical samples.

Very few workers who have described either radiolabelled probes or PCR techniques for the detection of mycobacterial DNA have done so using clinical specimens. The methods used for extraction of mycobacterial DNA from clinical specimens is, therefore, not well documented. Pao and colleagues (1988) report the successful use of their probe on DNA extracts prepared from sputa. They cite the method of Mizuguchi and Tokunga as the procedure used for DNA extraction but it is difficult to understand how a method that reqires pre-incubation with glycine can be used for extraction of DNA in clinical specimens. None of the other radiolabelled probes described in section 1.2.8 have been used for the detection of DNA in clinical specimens. Of the PCR methods described for the detection of *M.tuberculosis* (see section 1.3.6.2) only two use the procedure for the detection of *M.tuberculosis* in clinical specimens. Brisson-Noel and colleagues (1989) lysed the bacteria by incubating the samples in 0.1 mol/l NaOH, 2 mol/l sodium chloride and 0.5% sodium deoxycholate for 15 minutes at 95^{o}C. Shankar and colleagues (1991) extracted DNA from cerebrospinal fluids using 0.1 mol/l NaOH, 1.0 mol/l NaCl and 0.5% SDS. They were able to detect *M.tuberculosis* in 65% of cases of suspected tuberculous meningitis while laboratory culture was only able to detect 12% of cases.

1.3.5 Contamination in PCR Assays

The extreme sensitivity of the PCR technique also gives rise to its greatest drawback i.e. false positive results due to contaminating DNA. The contaminating DNA can arise from many sources: sample to

sample contamination; carry over of DNA from a previous amplification; shedding of skin cells or aerosols generated by vortexing, pipetting or centrifugation under vacuum.

Various procedures have been suggested in an attempt to minimise false positive results. Meticulous laboratory technique is a prerequisite for successful PCR work and no decontamination procedure can substitute for this.

To prevent carry over of amplified DNA sequences, pre and post PCR procedures should be performed in separate rooms or containment units. All buffer solutions used for PCR and sample preparation should be autoclaved prior to use. Autoclaving under conditions that provides bacterial decontamination degrades DNA to very low molecular weight fragments (Kwok and Higuchi, 1989) and thus renders the DNA unsuitable for PCR. DNA may also be degraded by UV irradiation. Recently it has been demonstrated that buffers, deoxytriphosphates, oligonucleotide primers or *Taq* polymerase can be decontaminated by UV irradiation (Sarkar and Sommer, 1990). It is, however, difficult to explain how UV irradiation destroys target DNA sequences but does not damage the oligonucleotide primers.

All reagents used for preparation of the PCR assay should be dispensed and stored in aliquots. This reduces the number of repeat samplings from one tube and also minimises cost should only one aliquot become contaminated.

Pipettes are a potential source of DNA contamination. When pipetting volumes greater than 500 μl (e.g. DNA extraction) disposable pipettes

should always be used. Micropipettes have to be used for pipetting smaller volumes. The shaft of a conventional air displacement micropipette is exposed to aerosols produced during suction of samples into and out of the disposable tip and is therefore a likely vehicle for carrying over DNA. In order to overcome these problems positive displacement pipettes have been advocated for use in all PCR procedures. The conventional micropipettes can, however, be used if they are assigned to only one specific procedure which does not involve transfer of DNA. They could therefore be used for preparation of PCR assays prior to addition of DNA. Conventional micropipettes which are in general laboratory use may also be used for post PCR procedures (e.g. electrophoresis) if re-amplification is not contemplated.

Contamination during preparation of the PCR assay can be minimised by pre-mixing of reagents and then dispensing the required volume into the appropriate number of reaction tubes. This minimises the number of sample transfers and the chances of sporadic contamination. A negative (no DNA) control should be pipetted last from each pre-mixture. DNA should be added to the tubes last with a positive displacement pipette and each tube should be capped before proceeding to the next sample.

The selection of controls is critical for proper evaluation of results. If DNA is prepared from clinical specimens a "no DNA" extraction control should be included for each batch of specimens. A "no DNA" reagent control should also be included for every PCR assay and is best prepared by using the last aliquot from a pre-mixture of PCR components as described above.

A number of general considerations should also be mentioned i.e. change gloves frequently, quick spin tubes before opening them, uncap and close tubes carefully to prevent aerosols and minimise sample handling.

1.3.6 Applications of PCR

PCR has allowed scientists to synthesis large amounts of specific DNA from complex mixtures of templates. This has had a tremendous impact on both research methodology and the study of disease processes.

1.3.6.1 Research Applications

Numerous routine procedures such as restriction enzyme analysis and preparation of DNA probes, require laborious techniques (cloning etc.) to produce sufficient material for analysis. This problem is now circumvented by PCR. Besides the obvious advantages of having more DNA available, there are numerous specific research applications which have been developed, some of which are summarised in Table 1.26.

1.3.6.2 Medical Applications

PCR methodology has had a tremendous impact in the diagnosis and elucidation of pathogenetic mechanisms of human disease.

Genetic Disease

It is in the field of genetic disorders that PCR and molecular biology has met its greatest challenge. Mutations are extremely varied, even within a specific genetic disorder and can range from point mutatins and frameshifts to small and large deletions. Mutations may be homozygous, heterozygous or X-linked, each type presenting different molecular diagnostic problems. In several genetic disorders the gene

RESEARCH APPLICATIONS OF PCR

APPLICATION	METHODOLOGY	REFERENCE
Direct sequencing of PCR product.	Direct sequencing of double stranded product using denaturing conditions and one primer.	Gyllenstein 1989
	Production of single stranded template by asymetric PCR	Gyllenstein & Erlich 1988
	Incorporation of T7 polymerase promotor sequence in one primer, amplification transcription and sequencing.	Stoflet *et al* 1988
	Biotinylation of one primer, amplification denaturation and separation of strands by avidin column.	Gyllenstein 1989
Modification of Target DNA	Incorporation of restriction enzyme site for subsequent cloning.	Scharf *et al* 1986
	Incorporation of GC-rich regions for "clamping" DNA.	Sheffield *et al* 1989
	Creation of base substitutions, insertions and deletions.	Higuchi 1989b Valhette *et al* 1989
	Production of chimeric DNA sequences	Horton *et al* 1989
Detection of Gene Expression (i.e. mRNA)	Initial synthesis of cDNA by reverse transcriptase followed by PCR.	Kawasaki and Wang 1989
Construction and Screening of cDNA libraries	Production of sufficient cDNA for library construction.	Tung *et al* 1989 Belyvasky *et al* 1989
	Incorporation of restriction enzyme site or phage promotor sequences in cDNA. Allows for direct cloning or sequencing.	Rasmussen *et al* 1989
	Use of PCR to isolate a specific gene with limited protein sequence information.	Tung *et al* 1989
Inverse PCR	Allows amplification of unknown DNA that flanks the known core regions.	Ochman *et al* 1989 Silver and Keerikatte 1989

Table 1.26

has not even been located and information must be obtained through linkage studies.

Several different approaches have therefore, had to be used to deal with these varied problems. Some of the approaches used will be briefly outlined.

If a point mutation exists several approaches can be utilised. Normal and/or mutant primers can be synthesised so that a one base pair difference in the template can be selected by either the mutant or normal primer. Absence or presence of specific product will determine if there is a mutation or not. This approach is the simplest as it allows direct analysis of the product by agarose gel electrophoresis. Probably the most innovative modification of this approach is the use of mutant and normal primers labelled with yellow or red fluorescent dyes (Chehab and Kan, 1990). Amplified product was either yellow (homozygous), red (normal) or orange (heterozygous).

However, the single base change in the template may not be sufficient to provide a large enough annealing temperature differential for the different primers and both normal and mutant sequences may be amplified. One attempt to overcome this problem is by allowing the difference in the primers to occur close to the 3′ end of the primer. It has been observed that the closer the mismatch is to the 3′ end of the primer the more refractory the primer is to the template. This method has been termed Amplification Refractory Mutation System (ARMS) (Newton *et al.*, 1989a). Another approach for detection of point mutations is to amplify both normal and mutant sequences and detect the differences by allele specific probes, high stringency washing,

restriction enzyme analysis or direct sequencing of amplified DNA.

Detection of deletions can be achieved by examination of restriction fragment length polymorphisms, or direct sequencing of amplified DNA. A novel approach is by using numerous primer pairs which "cover" a number of regions in the gene. The absence of one specific product indicates the presence of a deletion. This procedure, termed multiplex PCR can be used for the detection of deletions in large genes (e.g. Duchenes Muscular Dystrophy. Chamberlain *et al.*, 1988).

A further problem is that of a genetic disorder in which the gene has not yet been located. PCR can be used to amplify sequences which are known to be linked to the genetic disorder.

The number of genetic disorders in which PCR has been used is very large and only a list of some are given in Table 1.27.

HLA Gene Polymorphisms

The demonstration of the extensive allelic diversity of HLA class II loci has been greatly facilitated by PCR (Erlich and Bugawan, 1989; Bugawan *et al.*, 1988). The characterisation of these loci has made possible the following: precise HLA typing necessary for organ transplantations; the study of genetic susceptibility to diseases such as diabetes mellitus (Todd *et al.*, 1987; Horn *et al.*, 1988), pemphigus vulgaris (Sinha *et al.*, 1988; Scharf *et al.*, 1988), coelic disease (Bungawan *et al.*, 1988; Howell *et al.*, 1988) and pauciarticular juvenile rheumatoid arthritis (Erlich and Bungawan, 1989); accurate individual identification for forensic analysis (von Beroldingen *et al.*, 1989) and detailed evolutionary and phylogenetic studies (Erlich and Bungawan, 1989).

GENETIC DISORDERS IN WHICH PCR METHODOLOGY HAS BEEN APPLIED

Sickle cell anaemia	Saiki *et al* 1985 Chehab and Kan 1990
β Thalassemia	Wong *et al* 1987 Cai *et al* 1989
Phenyketonuria	Di Lella *et al* 1988 Sommer *et al* 1989
Hemophilia A	Kogan *et al* 1987 Sarkar *et al* 1989
Hemophilia B	Denton *et al* 1988 Winship *et al* 1989
Alpha-1 Antitrypsin deficiency	Abbott *et al* 1989 Newton *et al* 1988
Duchennes muscular dystrophy	Chamberlain *et al* 1988
Lesch-Nyhan Syndrome	Gibbs *et al* 1989 (a), 1989 (b)
Huntingtons Disease	McIntosh *et al* 1989
Apolipoprotein mutations	Collins *et al* 1988 Tennyson *et al* 1989 Weisgraber *et al* 1988
Lebers hereditary optic neuropathy	Singh *et al* 1989
Cystic fibrosis	Williams *et al* 1988 (a) Highsmith *et al* 1989 Williams *et al* 1988 (b) Feldman *et al* 1988 Newton *et al* 1989
Diabetes Mellitus	Horn *et al* 1988
Antithrombin III	Wu *et al* 1989
Hereditary fructose intolerance	Cross *et al* 1990

Table 1.27

Both individual identification and phylogenetic studies may also be analysed by PCR at other polymorphic loci such as mitochondrial DNA (Higuchi *et al.*, 1988) and length polymorphisms exemplified by the VNTR (variable number of tandem repeat) loci (von Beroldingen *et al.*, 1989).

Detection of Residual Disease

In some haematological malignancies characterised by specific chromosomal abnormalities (e.g. Chronic Myeloid Leukaemia (CML) and Follicular Lymphoma) PCR has been used for the detection of residual disease after therapy. The Philadelphia chromosome is characterised by a translocation between chromosome 9 and 22 and is present in 95% of patients with CML. This unique translocation has been utilised by PCR methodology to detect residual disease in peripheral blood and bone marrow cells (Lee *et al.*, 1988). Bone marrow transplantation is at present the only curative treatment for CML but there still remains considerable debate concerning the use of T-cell depleted versus non-T-cell depleted marrow transplantation. Several authors have used PCR to detect residual disease following both T-cell and non-T-cell depleted marrow transplantation but the results have been conflicting (Morgan *et al.*, 1989; Bartram *et al.*, 1989; Gabert *et al.*, 1989). Some patients with Acute Lymphoblastic Leukaemia (ALL) also carry the Philadelphia chromosome. By using PCR and specific probes, the translocation in ALL can be distinguished from CML (Kawasaki *et al.*, 1988).

A 14 to 18 chromosomal translocation occurs in 95% of patients with low grade follicular lymphoma and less frequently in diffuse large cell lymphoma. This unique abnormality has been detected by PCR in

peripheral blood and bone marrow cells after chemotherapy but could not be detected by morphological examination or Southern blot analysis (Lee *et al.*, 1987). This work has been extended to the detection of the translocation in paraffin embedded tissue (Pezzella *et al.*, 1989) and to detect extranodal progression (Cunningham *et al.*, 1989; Price *et al.*, 1989).

Oncogenes and Malignant Disease

The polymerase chain reaction has greatly facilitated the study of the incidence of oncogene mutations in numerous malignant diseases. Of these the *ras* oncogenes have been found in 90% of adenocarcinomas of the exocrine pancreas, in 50% of adenocarcinomas of the colon and 50% of follicular lymphomas of the thyroid and in a smaller proportion of numerous other tumours (Bos, 1989). Although PCR has simplified the analysis of *ras* mutations, the precise role of these oncogenes in human tumours remains obscure.

Prenatal Sexing

Sexing of the pre-implantation embryos can also now be performed by amplification of Y chromosome specific sequences (Handyside *et al.*, 1989). Y chromosome specific sequences can also be amplified from peripheral blood cells of pregnant women from as early as 9 weeks gestation (Lo *et al.*, 1989b). These approaches may be invaluable to couples at risk of transmitting X-linked genetic disease.

Infectious Diseases

It is in the field of infectious diseases, however, that PCR has had a major impact. The ability to detect a few copies of the DNA of a particular pathogen and the fact that the amplification is targeted to

a particular DNA sequence make PCR a highly sensitive and specific diagnostic tool. PCR has also proved very useful in studying the pathogenesis of certain infectious diseases as well as in establishing links between certain disease processes and possible infective causes. The contribution made by PCR for the diagnosis and study of some infectious diseases is summarised in Table 1.28. As can be seen from the table, PCR has been applied to a wide range of viruses and to a few bacteria and parasites and has proven to be a highly sensitive and specific molecular tool.

Recently there have been reports of the use of PCR for the detection of *M.leprae* and *M.tuberculosis*. Woods and Cole (1989) described a PCR assay for the detection of *M.leprae* DNA in armadillo liver, mouse footpads and in human biopsy material. They amplified two target sequences: a 714 bp fragment encoding part of the 65 Kd antigen, and a 372 bp fragment which is part of an *M.leprae* specific repetitive sequence. Although cross-reactivity with other Mycobacteria was not assessed, the assay was able to detect the amount of DNA equivalent to that found in 10^2 cells.

Plikaytis and colleagues (1990) amplified a 347 base pair fragment encoding part of the groEL gene of *M.leprae*. By using a nested primer approach, they were able to detect the amount of DNA equivalent to that found in one organism. Cross-reactivity with a few other non-tuberculous Mycobacteria was, however, reported.

Hance and colleagues (1989) amplified a 383 bp fragment encoding part of the 65 Kd mycobacterial antigen from *M.tuberculosis*, *M.bovis*, *M.avium*, *M.paratuberculosis* and *M.fortuitum*. After amplification,

Table 1.28

USE OF PCR IN INFECTIOUS DISEASE

MICRO-ORGANISM	CONTRIBUTION OF PCR	REFERENCE
HIV I	Detection of viral nucleic acid sequences in peripheral blood and tissue culture	Ou *et al* 1988 Kwok *et al* 1987 Hart *et al* 1989
	Detection of HIV in paraffin embedded tissue	Shibata *et al* 1989
HIV I	Detection prior to generation of antibodies i.e. seronegative window or those seronegative but at risk.	Loche and Mach 1988 Edwards *et al* 1989 Imagawa *et al* 1989 Pezzella *et al* 1989
HIV I	Screening of neonates from seropositive mothers.	Rogers *et al* 1989 Laure *et al* 1988 De Rossi *et al* 1988
HIV I	Confirmation that HIV I proviral sequences are present mainly in CD4 cells.	Hufert *et al* 1989
HIV I and II	Determining virus type present (HIV I or II)	Rayfield *et al* 1988
HIV I	Analysis of gene function and regulation	Arrigo *et al* 1989
HTLV I and II	Identification of HTLV I DNA in patients who have Adult T-cell leukaemia.	Kwok *et al* 1988 Kinoshita *et al* 1989
	Confirming infection in seropositive and seronegative individuals.	Kwok *et al* 1989
	Typing the virus present i.e. type I or type II	Kwok *et al* 1988 Kwok *et al* 1989 Erhlich *et al* 1989
	Documenting HTLV I infection in those presenting with symptoms dissimilar to classic Adult T-cell lymphoma.	Duggan *et al* 1988
HTLV I	Documenting presence of HTLV I DNA sequences in patients with chronic progressive myelopathy and tropical spastic paraparesis.	Bhavagati *et al* 1988 Bangham *et al* 1988 Kwok *et al* 1989
HTLV II	Documentation of high prevalence of HTLV II amongst IV drug abusers.	Lee *et al* 1989
CMV	Detection of CMV DNA sequences in tissue culture, peripheral blood and urine.	Hsia *et al* 1989 Jiwa *et al* 1989 Cassol *et al* 1989 Olive *et al* 1989
CMV	Diagnosis of congenital CMV infection. tissue.	Demmler *et al* 1988
Hepatitis Viruses	Detection of Hepatitis B DNA in serum of seropositive patients.	Kaneko *et al* 1989a Kaneko *et al* 1989b Ulrich *et al* 1989
	Detection of Hepatitis B DNA in hepatocellular carcinoma.	Lo *et al* 1989

Table 1.28 continued

MICRO-ORGANISM	CONTRIBUTION OF PCR	REFERENCE
	Detection of Hepatitis B DNA in paraffin embedded tissue.	Lo *et al* 1989
	Detection of Hepatitis B DNA in normal and chronic liver disease patients with HB core antibodies only.	Sumazaki *et al* 1989
	Demonstration of mother-to-infant transmission of Hepatitis B virus by detection of DNA in colustrum.	Mitsuda *et al* 1989
	Detection of Hepatitis B in normal and chronic Hepatitis B patients with no serological markers for Hepatitis B.	Thiers *et al* 1988
Hepatitis C	Detection of Hepatitis C in some post transfusional non-A, non-B Hepatitis patients.	Weiner *et al* 1990
	Detection of Hepatitis C DNA in donor blood.	Garson *et al* 1990
Human Papilloma Virus	HPV DNA virus has been detected in: cervical Ca and metastasis in both fresh and paraffin embedded tissue.	Claas *et al* 1989 Dallas *et al* 1989
	in cervical smears of patients with normal cytology;	Young *et al* 1989 Melchers *et al* 1989a
	in dysplasia of cervix i.e. CIN I - CIN III;	Cornelissen *et al* 1989 Shibata *et al* 1988
	in normal cervical tissue;	Shibata *et al* 1988
	in other anogenital Ca e.g. anal, vulvar, penile, vaginal;	Kiyabu *et al* 1989
	in normal mucosa, benign tumours and malignant tumours of the oral cavity;	Maitland *et al* 1989
	in urine of males with metal condylomata accuminata;	Melchers *et al* 1989b
	Premalignant and malignant lesions of the conjunctiva.	McDonnel *et al* 1989
	PCR has also been used to differentiate HPV types as an epidemiological tool or assessment of those with more oncogenic potential.	Most of above references specify type.
Parvovirus B19	Development of a PCR assay which is more sensitive than hybridisation techniques.	Salimens *et al* 1989a
	Detection of B19 DNA in the tissues of a case of intra-uterine death.	Salimens *et al* 1989b
Herpes Simplex	Detection of HSV DNA in paraffin embedded puch biopsies of skin lesions.	Cao *et al* 1989
	Demonstration of HSV gene expression (mRNA) during experimental latent infection.	Lynas *et al* 1989a Lynas *et al* 1989b
	Diagnosis of HSV encephallitis.	Rowley *et al* 1990

Table 1.28 continued

MICRO-ORGANISM	CONTRIBUTION OF PCR	REFERENCE
BK and JC Virus	Detection of viral DNA in urine: from known positive immunosuppressed patients, from healthy negative patients, from negative bone marrow recipients, from brain tissue of a patient with multifocal leukoencephalopathy.	Arthur *et al* 1989
Epstein Bar Virus	Detection of EBV DNA in salivary gland biopsies and peripheral blood lymphocytes from patients with Sjogren's syndrome.	Saito *et al* 1989
Rhinovirus	Detection of Rhinovirus DNA in nasal washings of infected patients.	Gamma *et al* 1989
Human B-lymphotropic Virus (HBLV or HHV-6)	Detection of HBLV DNA in a high proportion of lympho proliferative disorders and peripheral blood of AIDS patients.	Buchbinder *et al* 1988
Enterotoxigenic *E.coli*	Development of a sensitive PCR assay which is specific for bacteria producing a heat labile toxin. Detection of the DNA in all stool specimens positive by, and one negative by bioassay.	Olive 1989
Clostridium difficle	Detection of *Clostridium* difficle DNA in stools by PCR.	Wren *et al* 1990
Legionella pneumophilia	Development of a specific and sensitive PCR assay. DNA detected after seeding water with *L.pneumophilia*.	Starnbach *et al* 1989
Chlamydia	Development of a sensitive PCR assay specific to a *Chlamydia trachomatis* serovars.	Dutilh *et al* 1989
	Production by PCR of an oligonucleotide probe which may have a level of detection greater than culture.	Dean *et al* 1989
Trypanosoma cruzi	Specific and sensitive amplification of a repetitive element from *T.cruzi* DNA. No detection of DNA sequences in blood samples from 2 patients known to be infected with *T.cruzi*.	Moser *et al* 1989a
Typanosoma brucei and *Trypanosoma congolense*	Specific and sensitive amplification of repetitive elements from DNA of *T.brucei* and *T.congolense*. Detection of DNA from animals infected with these parasites.	Moser *et al* 1989b
Toxoplasma gondii	Specific and sensitive amplification of a 35-fold repetitive sequence of *T.gondii* DNA.	Burg *et al* 1989
Pneumocyotis carinii	Detection of *Pneumocyotis carinii* DNA in alveolar lavage specimens which were negative by silver staining techniques.	Wakefield *et al* 1990
Mycobacteria	Discussed in text	

using three oligonucleotide probes they were able to distinguish *M.tuberculosis* and/or *M.bovis*, *M.avium* and/or *M.paratuberculosis* and *M.fortuitum*. The procedure was extremely sensitive and the equivalent of 3-60 Mycobacteria could be detected. In a subsequent study (Brisson-Noel *et al.*, 1989) this assay was used to detect *M.tuberculosis* in a variety of clinical specimens (sputum, gastric aspirates, lymph node biopsies and an abscess aspirate).

Shankar and colleagues (1990) amplified a 240 bp fragment encoding part of the MPB 64 protein. The assay was specific for members of the *M.tuberculosis* complex but could not distinguish members of this complex. The investigators were also able to detect *M.tuberculosis* DNA in a variety of clinical specimens (sputum, urine and CSF). In a subsequent study these authors (Shankar *et al.*, 1991) compared PCR with conventional laboratory techniques in 85 (34 tuberculous and 51 other) cerebrospinal fluids. PCR was able to detect *M.tuberculosis* in 65% of cases of suspected tuberculous meningitis while laboratory culture was only able to detect 12% of cases.

Eisenach and colleagues (1990) amplified a 123 bp fragment which is repeated at least 12 times in the chromosomes of *M.tuberculosis*. The assay was specific for, but could not distinguish between members of the *M.tuberculosis* complex. Sensitivity was excellent (1 organism) but detection of *M.tuberculosis* DNA in clinical specimens was not assessed.

Patel and colleagues (1990) described a PCR assay for *M.tuberculosis* in which low annealing temperatures were used (37^{o}C and 55^{o}C). Amplification of DNA from *M.tuberculosis*, *M.bovis*, *M.bovis*BCG,

M.kansasii, *M.avium* and *M.intracellulare* yielded amplification products of varying sizes. One of these fragments was specific for *M.tuberculosis* and was used as a probe to distinguish *M.tuberculosis* from other Mycobacteria. Sensitivity was excellent (1 organism) but again detection of *M.tuberculosis* DNA in clinical specimens was not assessed.

1.4 Discussion and Aims of the Study

Tuberculosis has been and still remains a major health problem throughout the world and therefore much time and effort has been expended to find a sensitive, specific and rapid diagnostic test. However, of the tests described, very few meet all these diagnostic requirements. PCR is certainly sensitive, specific and rapid and initial PCR assay for *M.tuberculosis* appear very promising.

The aim of this study was to develop a diagnostic test for *M.tuberculosis* infections based on the detection of *M.tuberculosis* DNA. Two approaches were envisaged:

1. the development of a DNA probe which would be more sensitive than previously described probes;

2. the development of a sensitive and specific PCR assay which would be used for the direct detection of *M.tuberculosis* DNA in clinical specimens.

The development of the DNA probe will be described in Chapter 3 and the PCR assay in Chapter 4 to 8.

CHAPTER 2

MATERIALS

2.1 PLASMID p36

Dr SA Shoemaker formerly of the Eleanor Roosevelt Institute for Cancer Research prepared a partial DNA library of *M.tuberculosis* by cloning DNA, partially digested with the restriction enzyme *Mbo*I, into the *Bam*HI site of the vector pBR322. One of the recombinant clones, p36 hybridised with a number of bands (about 6) generated by restriction enzyme digests of *M.tuberculosis* DNA, indicating that there may be multiple copies of the cloned sequences present in the bacterial genome.

The repetitive nature of p36 made this recombinant clone a potential candidate for a diagnostic probe, and Dr LM Steyn therefore, proceeded to further characterise p36. A restriction enzyme map of p36 was prepared by cutting the plasmid with various restriction enzymes and resolving the fragments according to size by agarose gel electrophoresis. p36 was shown to contain an insert of mycobacterial DNA of 5.7 kb and the restriction map is shown in Figure 2.1.

Further subcloning of p36 and hybridisation with *M.tuberculosis* DNA digests identified the *Mbo*I/*Sst*II fragment of approximately 1.2 kb as the fragment responsible for the multiple banding pattern. 956 bp of this *Mbo*I/*Sst*II fragment was sequenced and the sequence is shown in Figure 2.2.

(○) BamH1; (⊘) EcoR1; (▼) Mbo1; (▲) Kpn1;
(▽) Pst1; (◆) Sal1; (●) Sma1; (◇) Sst1;
(△) Sst11. (——) Insert DNA; (- - -) vector DNA

Figure 2.1: Restriction Map of p36

```
  1  CGTTCTGGCC CTCCAGCAGC CCGCCGGCGC CACCGACGCC
 41  GACGCTGCCA GTCCCGGCTT TGCCAAATCG CGTTGCCGCC
 81  GTTGCCGCCC TGGCCCGTCA GCCAGGCGTT GCCGCCGTTA
121  CCGCCGCTAC CACCCGTTTG CCTGCTATCG TGGCACCGCC
161  GTCGCCGCCG TTGCCGCCCT CGCCGACCAA CCCGGCCTTG
201  CCGCCCACGC CGCCGTTTCC GCCGTCGCNG TTGTCGCTCT
241  CCCCGCCGGC GCCGCCGGCG CCGCCGGAAC CGTAGAACGT
281  GCCGGCGTTA CCGCCGGCCC CGCCGCGACC ACCACTTAGG
321  TACCGGTGCC GCCGCTGCCG CCGGCGCCGC CAGAACCGAC
361  CAACCCGCCG ATACCGCCGT CGCCGCCGTC GCCGCCGAAC
401  CCGCCGAACC CGCCGGCTCC GCCGGCCCCA CCGGTGCCGT
441  ATAGCAGGCC GGCATTACCG CCGTGACCAC CGTGGCCAGC
481  GACAGTGGCG ACTCCGCCTG CACCGCCGGC CCCGCCCGAG
521  CCGAAGACCG TGCCGCCGGC GCCGCCCGCG CCGCCGTCGC
561  CGCCGGCAGC ACTGCCGAAG CCACCGTGCC CGCCGGCCCC
601  GCCGACGCCA AACAGCCCGC CGCTGCCGCC GTCGCCGCCG
641  GTCCCACCGG TGTCGCCGCA AGCCACCGAC GCCGCCCGAG
681  CCGCCGCCCG GGCCGAACAG CCCGCCGGCC CCGCCAGCCC
721  CGCCGGTCCC ACCGGTGTCG CCGAAGCCGC CGGCGCCGCC
761  GGCACCGCCG GCGCCGAACA GCCCACCGAC CCCGCCCGCA
801  CCGCCGTTGC CACCGGCGGC CTGGGCGCCG CCGCCGAGGG
841  CGGCGCCGCC TACGCCGCCG GCCCCGCCCG CGCCGAACAG
881  CCCACCGGCG CCGCCGGCCC CGCCGGCACC CCCAGCGGCA
921  TCGAAGAAAG GTGGTGAACC CGCCGTTGCC ACCGGA
```

Figure 2.2: Partial Sequence of the *Mbo*I/*Sst*II Fragment of p36. The 366 bp fragment extends from a *Kpn*I restriction enzyme site (◆) at base 323 to a *Sma*I restriction site (*) at base 689.

The sequence reads from about 300 bp from the *Mbo*I site, through the *Kpn*I, *Sma*I and the *Sst*II site. The fragment is extremely GC-rich (>80%) and contains many direct repeat sequences. The high GC content and direct repeats made sequencing technically very difficult and this was particularly problematical around the *Kpn*I site. There are no open reading frames of greater than 600 nucleotides present in the sequence and it therefore, appears unlikely that this DNA fragment encodes a functional protein. Furthermore, subsequent experiments indicated that this fragement was not transcribed and the function of this element thus remains unknown. A 366 bp *Kpn*I *Sma*I fragment was used in this study (Chapter 3). The sequence of the *Mbo*I/*Sst*II fragment is shown in Figure 2.2.

2.2 PREPARATION OF MYCOBACTERIAL DNA

A type culture strain of *M.tuberculosis* H37RV was obtained from the diagnostic Medical Microbiology Laboratory at Groote Schuur Hospital, while strains of *M.avium*, *M.fortuitum*, *M.gordonae*, *M.intracellulare*, *M.kansasii*, *M.marinum* and *M.scrofulaceum* were obtained from the Tuberculosis Research Institute (Pretoria). *M.bovis* BCG (Tokyo strain) was obtained from the State Vaccine Institute, (Pinelands, Cape Town) and was cultured on Sautons broth, while the other mycobacterial strains were cultured on Lowenstein-Jensen medium. The bacteria were harvested and the DNA was extracted as follows: the cells were suspended in 2 ml of TES buffer (Tris-HC1, pH 8.5 (10 mM); EDTA, (1 mM); NaCl (150 mM), and heated at 70^{o}C for 30 minutes. The cells were lysed by the addition of 10% SDS (1.5% final concentration) and an equal volume of buffered phenol. The lysates were mixed at 37^{o}C for 3 hours on an orbital shaker to ensure complete lysis and to

enhance the extraction of proteins and lipids. The lysates were centrifuged (15 minutes in a Beckman microfuge) and the aqueous supernatants were extracted twice with an equal volume of chloroform: iso-amylalcohol (24:1 V/V). The DNA was precipitated by the addition of an equal volume of polyethylene glycol (PEG 6000, Sigma) in 2.5 M NaCl at room temperature. The DNA was harvested 15 minutes after the addition of the PEG by centrifugation for 15 minutes in a Beckman microfuge. The DNA pellet was washed twice with 70% ethanol and dried in a Speedivac concentrator (Servant) and finally dissolved in sterile, distilled water at a final concentration of 0.1-0.3 µg/ul. This procedure gave yields of 50-300 µg of DNA per gram of wet cells with an absorbance ratio (260 nm to 280 nm) of greater than 1.75. The yield of DNA is not as high as described for other extraction procedures (see Chapter 1) but the minimal protein contamination made this "pure" DNA ideal for PCR assays.

2.3 VECTORS AND HOST CELLS

pUC19 and JM109 cells were received from Professor F Robb, Department of Microbiology, University of Cape Town, Rondebosch.

2.4 ENZYMES, CHEMICALS, SOLUTIONS AND MEDIA

All reagents used were of high quality and suitable for use in molecular biology experiments. The reagents and suppliers are listed in Appendix A.

CHAPTER 3

ISOLATION, CHARACTERISATION AND CLONING OF A 366 bp FRAGMENT FROM p36

3.1 INTRODUCTION

The repetitive nature of the *Mbo*I/*Sst*II fragment of p36 made this fragment an ideal candidate for a prospective DNA probe for the detection of *M.tuberculosis*. It was felt that a smaller fragment would maximise specificity and a 366 bp *Kpn*I, *Sma*I fragment was therefore, selected for further study (see Figure 2.1 and 2.2). The aim of this chapter was, therefore, to describe the purification of this fragment, ascertain whether it was part of the repetitive element of p36, assess its potential as a diagnostic probe and clone the fragment in order to facilitate subsequent studies.

3.2 PREPARATION OF p36

Preparation of competent JM109 cells, transformation of these cells with p36 and subsequent large scale plasmid extraction and purification are described in Appendix B1, B2 and B3.

3.3 ISOLATION OF THE 366 FRAGMENT

3.3.1 Restriction Enzyme Digestion

For the digestion of p36 with *Kpn*I and *Sma*I the following proportionate conditions were used: 20 µg of plasmid, 1 µl *Kpn*I (90 units/µl), 1 µl *Sma*I (90 units/µl), 2 µl, React 4 buffer (BRL) and sterile distilled water to a final volume of 20 µl. This digestion mixture was incubated at 37°C for one and a half hours and the reaction terminated by placing the reaction tube on ice and thereafter storing it at 4°C.

3.3.2 Agarose Gel Electrophoresis and Electroelution

To this digestion mixture was added 2 µl 10 x Stop buffer (50% v/v glycerol, 100 mM Na_2 EDTA pH 8, 1% w/v SDS, 0.1% w/v bromophenol blue, 0.1% cyanol). This DNA digest was electrophoresed as detailed in Appendix B4 and the DNA fragments were visualised by ethidium bromide staining. The desired 366 bp fragment was cut from the gel and the DNA eluted by means of a biotrap (Schleicher and Schuell, Appendix B5). Thereafter, the DNA was concentrated, dried and reconstituted to give a final concentration of 300 ng/µl of fragment. An aliquot of this DNA preparation was electrophoresed in order to determine purity and recovery. The amount of DNA in the gels was determined by the intensity of the ethidium bromide staining and 80 - 90% recovery was calculated after electroelution and concentration.

3.4 REPETITIVE NATURE OF THE 366 bp FRAGMENT

3.4.1 Digestion and Electrophoresis of Chromosomal DNA

M.tuberculosis DNA (0.5 µg/µl) was digested with *Bam*HI. The composition of the digestion mixture was as follows: 100 µl DNA, 2 µl *Bam*HI, 18 µl React 3 buffer (BRL) and 60 µl sterile distilled water. The mixture was incubated at 37°C for 1 hour. The efficiency of the digestion, was then ascertained by a checking gel and thereafter the digestion mixture was electrophoresed in a preparative 7% agarose gel and a voltage of 20 volts was applied for 20 hours. After electrophoresis, the DNA was transferred to a Hybond-N-membrane (Amersham) as described by Southern (1975) (Appendix B6).

3.4.2 Hybridisation of the Chromosomal DNA Digest with Radiolabelled 366 bp Fragment

Three hundred nanograms of purified 366 bp fragment was radiolabelled by Nick Translation (Appendix B7) and hybridised to the *Bam*HI digest of *M.tuberculosis* DNA on the Hybond-N-membrane (Appendix B8). Thereafter, autoradiography of the membrane was performed (Appendix B9).

3.5 ASSESSMENT OF THE 366 bp FRAGMENT AS A DNA PROBE

Chromosomal DNA (0.5 µg/µl) was serially diluted in sterile distilled water to give concentrations of 10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl and 1 pg/ul (all 20 ul volume). These solutions were heated for 10 minutes at 100°C. 1 µl of each of these DNA solutions was removed, made up to 200 µl with sterile distilled water and applied directly to Hybond-N-membrane using a Slot Blot apparatus (Schleicher and Schuell. SRC072/0. Minifold II). The DNA was fixed to the membrane by UV irradiation and thereafter hybridised with the gel purified radiolabelled 366 bp fragment as described in Appendix B7 and B8.

3.6 CLONING OF THE 366 bp FRAGMENT

3.6.1 Insert and Vector Preparation and Ligation

Two methods of cloning the 366 bp fragment from p36 in pUC19 were attempted i.e.:

i) **Shotgun Cloning**

pUC19 and p36 were digested separately with *Kpn*I and *Sma*I as described in 3.3.1 after which the digestion mixtures were heated at 65°C to destroy the enzyme. Four different molar ratios of p36 and pUC19 were then prepared to determine the optimum ratio for ligation. The ratios of p36 to pUC19 were 20:1, 10:1, 5:1, 3:1 and 1:1, approximately 10 ng

of pUC19 being used in each reaction. As ligation controls, pUC19 linearized with *KpnI* and *SmaI* as well as pUC19 linearized with *SmaI* or *KpnI* were ligated. The reactions mixtures (10 µl final volume) included: 1 µl of 10 x ligase buffer (660 nM Tris-HCl, pH 7.2, 10 mM EDTA, 100 mM $MgCl_2$, 100 mM dithiothreitol, 1 mM ATP), 1 µl of T_4 DNA ligase (1 unit), p36 DNA, pUC19 DNA and sterile distilled water.

All reactions were incubated at 15°C for 4 hours after which 5 µl was removed and the remaining 5 µl was incubated for a further 18 hours. A thermostatically controlled "ambient" temperature waterbath was used for all ligations (Techne. Tempette TE-8A).

ii) **Cloning with Gel Purified 366 bp Fragment**

The 366 bp fragment was purified as described in 3.3.2. The purified fragment and pUC19, digested with *KpnI* and *SmaI* were mixed in molar ratios (fragment: pUC19) of 4:1 and 1:1 (approximately 100 ng of pUC19 was used in each reaction) and ligated as described above together with the appropriate controls.

3.6.2 Transformation

Competent JM109 cells, stored at -70°C, were thawed slowly (30 minutes) on ice before transformation. Five microlitres of each ligation mixture was added to 200 ul of competent cells and placed on ice for at least 40 minutes. As a transformation control, circular pUC19 (10 ng) was also added to competent cells.

The cells were then heat-shocked at 42°C for 2 minutes after which they were incubated with 1 ml of pre-warmed Luria broth at 37°C for 1 hour for expression of the B lactamase gene encoding ampicillin resistance. The cell suspension was spread onto Luria agar plates

containing ampicillin, X-Gal and IPTG. After the liquid had been absorbed, the plates were incubated at 37°C overnight in an inverted position. The plates were examined and transformation efficiency, success of ligation and the presence of recombinants (white colonies) was assessed.

3.6.3 Extraction and Analysis of Plasmid DNA

Plasmid DNA was extracted from the recombinant colonies according to the method in Appendix 10 (Birnboim and Doly, 1979), and finally reconstituted in 100 µl of sterile distilled water. Ten microlitres of the DNA extract was digested with *Kpn*I and *Sma*I and the DNA fragments separated on a 1.2% agarose gel. Circular pCU19 and pUC19 linearised with *Kpn*I and *Sma*I were included as molecular weight markers.

Colonies containing the desired recombinant plasmid were subjected to large scale plasmid preparation as described in Appendix B3. The recombinant plasmid containing the 366 bp fragment will hereafter be referred to as p366.

3.7 RESULTS AND DISCUSSION

The transformation control yielded 1.3 x 10^8 colonies/µg of pUC19. Yields of 1 x 10^7 to 2 x 10^8 colonies/µg of plasmid are indicative of well prepared competent cells and good transformation methodology (Hanahan, 1985).

Large scale plasmid extraction and purification by isopycnic gradient centrifugation yielded 52 µg of p36 and 48 µg of p366 and the absorbance ratio (260:280) was 2.0 indicating good yields of pure DNA.

Complete digestion of p36 was achieved using the conditions described. Lane 3 of Figure 3.3c shows a *Kpn*I, *Sma*I digest of p36 and shows a slow migrating band consisting of the vector DNA and a 2.4 Kb portion of p36, 2 large fragments (1.6 Kb and 1.3 Kb) and the smaller 366 bp fragment. This pattern is consistent with the restriction enzyme map of p36 (Figure 2.1).

Figure 3.1 is an autoradiograph following transfer of the digested *M.tuberculosis* DNA and hybridisation with the radiolabelled 366 bp fragment. High stringency washes were used following hybridisation (see Appendix B8) to ensure that there was only hybridisation with homologous sequences. There at least 6 well defined bands which indicated that the 366 bp fragment is repeated at least this number of times in the chromosome and is therefore part of the repetitive element contained in p36.

The autoradiograph in Figure 3.2 shows a signal only when the 366 bp fragment is hybridised with 1 ng or greater of chromosomal DNA. This sensitivity is similar to that described for other *M.tuberculosis* probes (see section 1.2.8) and the repetitive nature of this probe therefore does not appear to confer increased sensitivity. Eisenach and colleagues (1988) also used a repetitive element as a probe and reported a similar sensitivity.

In all cases of both 'shotgun' cloning and the use of the gel purified fragment, recombinant colonies were observed. Ligation controls were always successful (1×10^7 to 1×10^8 colonies/µg of pUC19). However, after the examination of 100-150 recombinant colonies, none were found which contained the 366 bp fragment within pUC19.

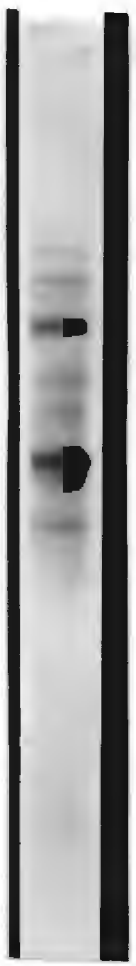


Figure 3.1: Repetitive Nature of the 366 bp Fragment

Autoradiograph of *M.tuberculosis* chromosomal DNA digested with *Bam*HI and hybridised with the 366 bp fragment. The DNA fragments were separated by agarose gel electrophoresis and transferred onto Hybond-N-membrane prior to hybridisation.

Figure 3.2: Assessment of the 366 bp Fragment as a Probe Autoradiograph following hybridisation of ten-fold dilutions of *M.tuberculosis* DNA with the radiolabelled 366 bp fragment

In one shotgun cloning experiment, however, using a ratio of p36 to pUC19 of 20:1, there were a number of colonies which contained the 366 bp fragment in the vector pBR322. This is demonstrated in Figure 3.3. Figure 3.3a shows circular and linear pUC19 as well as plasmid digests (*Kpn*I/*Sma*I) from 5 recombinant colonies, one of which (lane 7) shows an insert of 366 bp. Digestion of this plasmid with only *Kpn*I and only *Sma*I does not generate the 366 bp fragment (Figure 3.3b). In Figure 3.3b and c, the insert is shown to have the same mobility as the 366 bp fragment of p36. The mobility of the vector of this recombinant plasmid is, however, slower than linear pUC19 (see Figure 3.3a) indicating that pUC19 cannot be the vector in this instance. Furthermore, the vector appears to have a similar mobility to both circular and linear pBR322 (Figure 3.3c). One must conclude that a deletion has occured in p36 with only the 366 bp fragment remaining. However, from the restriction enzyme map of p36 (Figure 2.1), it becomes apparent that a single *Kpn*I *Sma*I fragment cannot recircularise p36 if cut with *Kpn*I and *Sma*I due to the fact that both sticky ends of p36 will contain *Kpn*I sites. One possible explanation is that there is an unmapped *Sma*I site very close to the upstream *Kpn*I site and therefore may not be detected when p36 is digested with *Kpn*I and *Sma*I. Another possible explanation is that two 366 bp fragments have been inserted in a head to tail fashion.

I have not attempted to prove either of these two explanations as this information was not essential for performing the PCR experiments in the following chapters. In Chapter 5 the successful amplification of a 336 bp which is contained within the 366 bp fragment is described using this recombinant plasmid as the target DNA. This further

Figure 3.3: Cloning of the 366 bp Fragment

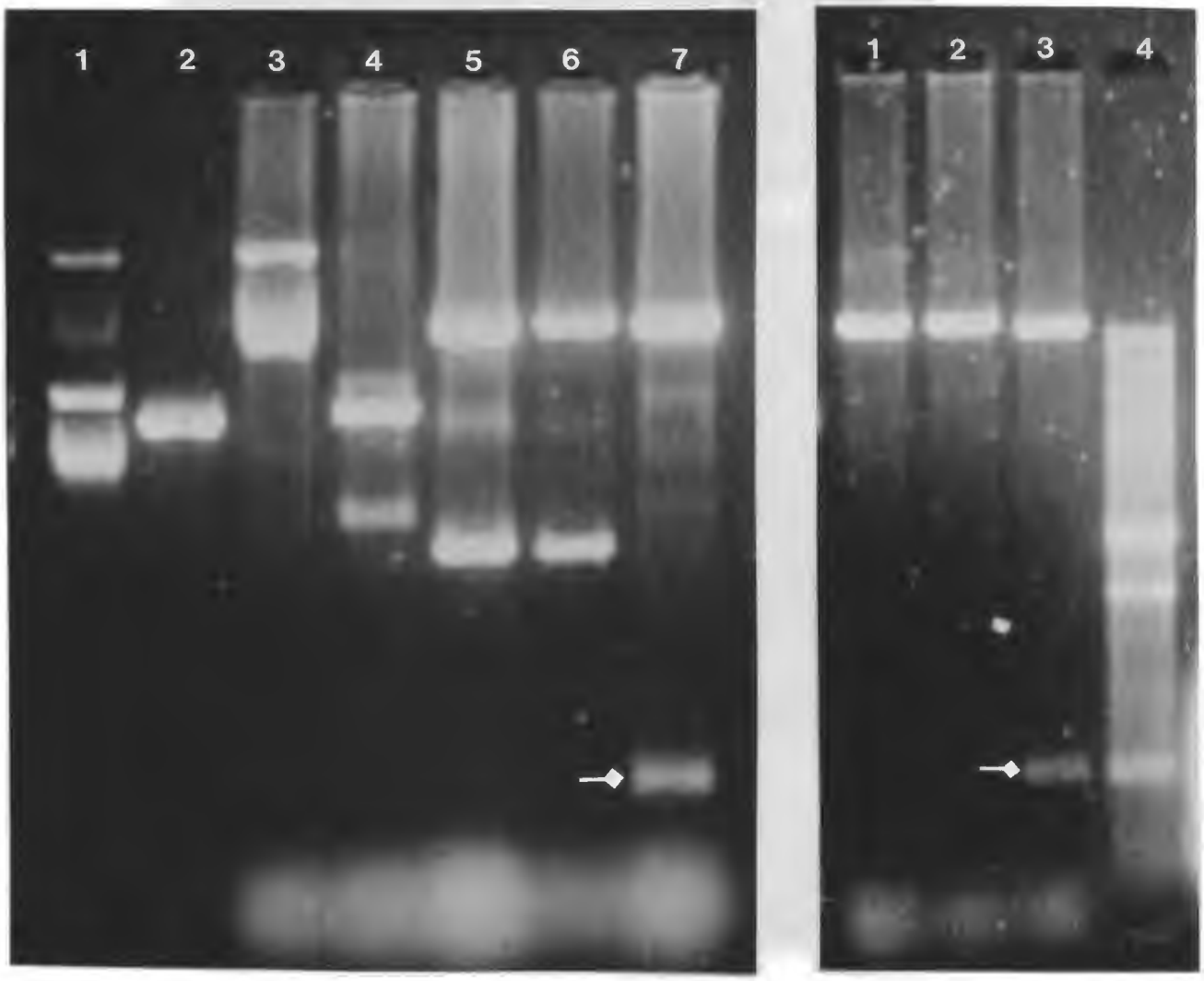


Figure 3.3a: Lane 1: Undigested pUC19; Lane 2: pUC19 linearised with *Kpn*I and *Sma*I; Lane 3 to 7: *Kpn*I/*Sma*I digests of plasmid extracts from 5 recombinant colonies. Lane 7 shows the 366 bp fragment (arrow).

Figure 3.3b: Recombinant plasmid seen in Lane 7 of Figure 3.3a digested with: *Kpn*I only - Lane 1; *Sma*I only - Lane 2; *Kpn*I and *Sma*I - Lane 3; Lane 4 shows a *Kpn*I/*Sma*I digest of p36 and demonstrate that the insert has the same mobility as the 366 bp fragment of p36 (arrow)

Figure 3.3: Cloning of the 366 bp Fragment

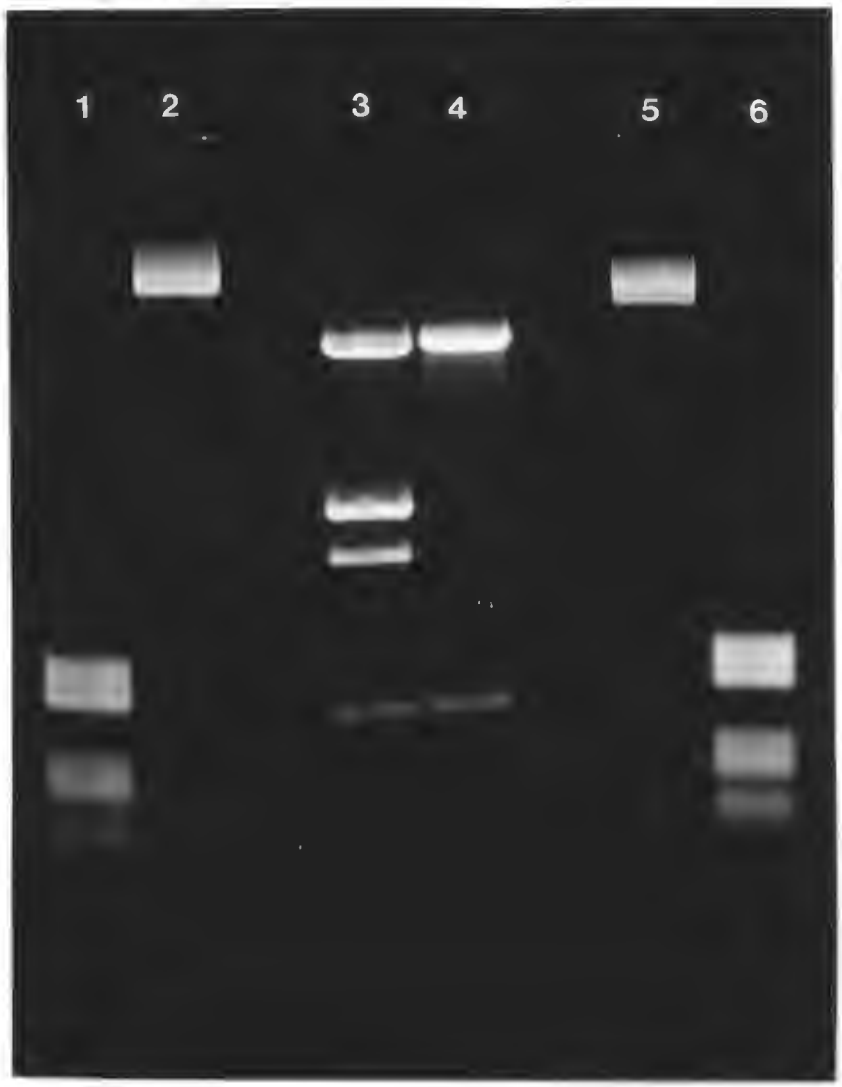


Figure 3.3c: Confirmation that the 366 bp fragment inserted in pBR322. Lane 1: Molecular weight marker (*Hae*III digest of pBR322); Lane 2: undigested p36; Lane 3: p36 digested with *Kpn*I and *Sma*I; Lane 4: Purified p366 digested with *Kpn*I and *Sma*I; Lane 5: Undigested purified p366; Lane 6: Molecular weight marker (*Hae*III digest of pBR322)

confirms that the desired fragment was inserted in the plasmid and therefore this plasmid provides a convenient source from which large quantities of specific DNA (336 bp fragment) could be generated (by PCR) and thereafter be used for making DNA probes.

CHAPTER 4

IDENTIFICATION OF THE OPTIMUM PRIMER ANNEALING SITE FOR PCR

4.1 INTRODUCTION

The 366 bp fragment was chosen as the target sequence for PCR and two sets of primer annealing sites were selected within this target sequence. The sequences of both sets of primers as well as the primer annealing sites are shown in Figure 4.1. All primers were synthesised by Beckman (SA). Primer set A consisted of two 17 mer oligonucleotides binding adjacent to the *Kpn*I and *Sma*I restriction enzyme site. Amplification with this set of primers should yield a 366 bp product. However, amplification was largely unsuccessfull and therefore a second set of primers (set B) was synthesised. Because Dr LM Steyn experienced difficulty sequencing near the *Kpn*I site, the primer annealing site was moved 25 bases towards the *Sma*I site. The other primer annealing site was moved 6 bases towards the *Kpn*I site. Furthermore, in order to improve specificity, the primers were increased to 25 bases and the bases at the 3′ end were thymine or adenine. Amplification using this set of primers should yield a 336 bp product.

4.2 PREPARATION OF PCR COMPONENTS

4.2.1 Buffer Preparation

The buffer system used was that described by New England Biolabs for use with their enzyme. A 10 x stock buffer was prepared with the following: 166 mM Ammonium sulphate, 670 mM Tris-HCl (pH 8.8), 67 mM $MgCl_2$, 100 mM B Mercaptoethanol, 47 uM EDTA. The buffers were prepared with sterile distilled water and sterilised by filtration.

```
   1  CGTTCTGGCC CTCCAGCAGC CCGCCGGCGC CACCGACGCC
  41  GACGCTGCCA GTCCCGGCTT TGCCAAATCG CGTTGCCGCC
  81  GTTGCCGCCC TGGCCCGTCA GCCAGGCGTT GCCGCCGTTA
 121  CCGCCGCTAC CACCCGTTTG CCTGCTATCG TGGCACCGCC
 161  GTCGCCGCCG TTGCCGCCCT CGCCGACCAA CCCGGCCTTG
 201  CCGCCCACGC CGCCGTTTCC GCCGTCGCNG TTGTCGCTCT
 241  CCCCGCCGGC GCCGCCGGCG CCGCCGGAAC CGTAGAACGT
 281  GCCGGCGTTA CCGCCGGCCC CGCCGCGACC ACCACTTAGG
 321  TACCGGTGCC GCCGCTGCCG CCGGCGCCGC CAGAACCGAC
 361  CAACCCGCCG ATACCGCCGT CGCCGCCGTC GCCGCCGAAC
 401  CCGCCGAACC CGCCGGCTCC GCCGGCCCCA CCGGTGCCGT
 441  ATAGCAGGCC GGCATTACCG CCGTGACCAC CGTGGCCAGC
 481  GACAGTGGCG ACTCCGCCTG CACCGCCGGC CCCGCCCGAG
 521  CCGAAGACCG TGCCGCCGGC GCCGCCCGCG CCGCCGTCGC
 561  CGCCGGCAGC ACTGCCGAAG CCACCGTGCC CGCCGGCCCC
 601  GCCGACGCCA AACAGCCCGC CGCTGCCGCC GTCGCCGCCG
 641  GTCCCACCGG TGTCGCCGCA AGCCACCGAC GCCGCCCGAG
 681  CCGCCGCCCG GGCCGAACAG CCCGCCGGCC CCGCCAGCCC
 721  CGCCCGGTCCC ACCGGTGTCG CCGAAGCCGC CGGCGCCGCC
 761  GGCACCGCCG GCGCCGAACA GCCCACCGAC CCCGCCCGCA
 801  CCGCCGTTGC CACCGGCGGC CTGGGCGCCG CCGCCGAGGG
 841  CGGCGCCGCC TACGCCGCCG GCCCCGCCCG CGCCGAACAG
 881  CCCACCGGCG CCGCCGGCCC CGCCGGCACC CCCAGCGGCA
 921  TCGAAGAAAG GTGGTGAACC CGCCGTTGCC ACCGGA
```

Figure 4.1a: Partial sequence of the *MboI*/*SstII* Fragment of p36

The 366 bp fragment extends from a *KpnI* restriction site (◆) at base 323 to a *SmaI* restriction site (*) at base 689. The primer binding sites are underlined (Set A ———— Set B - - - - - -). *AvaI* restriction sites are also shown (↓).

Figure 4.1b: Primer sequences

PRIMER SET A

Primer 1 5′-CGGTGCCGCCGCTGCCG-3′

Primer 2 5′-GGGCGGCGGCTCGGGCG-3′

PRIMER SET B

Primer 1 5′-GCCAGAACCGACCAACCCGCCGATA-3′

Primer 2 5′-GCGGCTCGGGCGGCGTCGGTGGCTT-3′

Dimethyl sulphoxide and bovine serum albumin were not included in the stock buffer but were added separately to the reaction mixes.

4.2.2 Preparation of Oligonucleotide Primers

The concentration of the primers was determined by measuring absorbance at 260 nm. The primers were then either used directly or were further purified. Purification of primers was performed by polyacrylamide gel electrophoresis. A 20% polyacrylamide (1% bis) and 8 M urea gel was prepared in 1 x NNB. Prior to pouring, the gel was warmed to 37^{o}C, degassed and 400 µl ammonium persulphate and 30 µl TEMED added to 100 ml of polyacrylamide. Prior to seperation of the oligonucleotides the gel was pre-electrophoresed at 1100 volts for 1 hour.

Urea (200 mg) was added to each of the oligonucleotide primers (150 µg in 200 µl water) and the solution heated at 95^{o}C for 2 minutes. The samples were loaded onto the gel and electrophoresed at 1400V for 5 hours. Two marker dyes (bromophenol blue and xyelene cyanol) were loaded into wells separate from the oligonucleotide. Electrophoresis was continued until these dyes were 16-18 cm apart. The oligonucleotides migrate between the two dyes. The gel was then placed on fluorescent plate (PEI Cellulose F Plate) and the DNA visualised by the UV shadow.

The oligonucleotide band was cut out, the gel slice was cut into very small fragments and the oligonucleotides were eluted with 2 changes (5 ml each) of TE buffer at 4^{o}C for 16 hours with shaking.

The oligonucleotides were further purified by reverse phase column chromatography (C-8 'Bond-Elut' column. Analytichem International).

Washes (1 ml) and elutions (1 ml) were 'pushed' through the column under very low nitrogen pressure.

Prior to loading the sample, the column was washed twice with methanol, twice with water, twice with 70% acetonitrile and twice with 50 mM ammonium acetate. Ammonium acetate was added to the purified oligonucleotides to a final concentration of 50 mM and this solution was loaded onto the column. The column was washed twice with 50 mM ammonium acetate and once with 1 mM ammonium acetate. The oligonucleotides were then eluted with 50% acetonitrile (four fractions) and the column "stripped" with 2 washes of 70% acetonitrile. The optical density of all fractions was determined to verify elution. The fractions containing the oligonucleotides were dried and resuspended in 50 mM Tris-HCl pH 8 and the concentrations determined by spectrophotometry.

4.2.3 Preparation of Deoxynucleotide Triphosphates

Approximately 2 mg of each deoxynucleotide triphosphate (dNTP) was dissolved in 200 µl of Tris-HCl pH 7.4. An aliquot (5 µl) of this solution was diluted with 2 ml of 10 mM Tris-HCl pH 7.4. In the case of dCTP the dilution was made with 0, 01N HCl. The absorbance of these dilutions was measured at the optimal wavelength for the particular dNTP and the exact concentration was calculated by using the appropriate extinction coefficient. The concentrations were adjusted with Tris-HCl to 10 mM dNTP for stock solutions which were stored at -70°C. The wavelength and extinction coefficient used are indicated in the following table.

dNTP	Max Absorbance	Extinction Coefficient
dATP	259 nm	15.2 at pH 7
dGTP	253 nm	13.7 at pH 7
dTTP	267 nm	9.6 at pH 7
dCTP	280 nm	13.1 at pH 2

4.2.4 DNA Templates

p366, p36 and *M.tuberculosis* chromosomal DNA were all used at varying concentrations (1 pg to 1 µg) as templates for PCR.

4.2.5 *Taq* Polymerase

Three batches of enzyme supplied by New England Biolabs and 2 batches supplied by Cetus Corporation were used.

4.3 POLYMERASE CHAIN REACTION

4.3.1 Standard Methodology

The reaction mixes were prepared separately in 1.5 ml microfuge tubes and had the following final concentrations: 16.6 mM Ammonium sulphate, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 10 mM β Mercaptoethanol, 4.7 µM EDTA, 10% DMSO, 170 µg/ml BSA, 33 µM each dNTP and 300 ng each oligonucleotide primer. DNA and sterile distilled water were added to make a final volume of 100 µl. The reaction mix was heated to 100°C for 10 minutes, placed on ice and 2.5 units of *Taq* polymerase was added.

Thermal cycling was performed manually. The mixtures were first heated at 95°C for 1 minute, cooled at room temperature for 2 minutes, (during this cooling period, the tubes were centrifuged in a microfuge

to remove the condensate on the walls and top of the tube) and then heated at 70^{o} for 2 minutes. 30 cycles were usually performed.

4.3.2 Variations of the Standard Method

Reactions were also performed in siliconised glass capillary tubes (300 µl volume). The 100 µl reaction mixture was drawn into the capillary tube by a modified syringe and the ends sealed in a flame.

Several variations in the temperature cycling were attempted. The denaturing time was extended to 2 minutes, the annealing temperature was varied between room temperature and 70^{o}C and the extension temperature ranged from 60^{o}C to 70^{o}C.

Liquid paraffin was layered on top of the reaction mix to avoid evaporation.

4.3.3 Analysis of PCR products

After thermal cycling, the DNA in the reaction tube was concentrated with 1 tenth volume of 4 M LiCl and 2.5 volumes of absolute ethanol, the DNA pellets were washed with 70% ethanol, dried and reconstituted in 30-40 ul of sterile distilled water.

In most cases, half of this solution was applied to a 3% agarose gel and after electrophoresis, the products were examined by ethidium bromide staining. In those reactions where amplification products were observed, the DNA was transferred to Hybond-N-membranes and hybridised with the radiolabelled 366 bp fragment as described previously.

4.3.4 Verification of the PCR Product

Amplification of the 366 bp fragment (using primer set A) was largely unsuccessful and verification of amplified products was therefore not attempted. In order to confirm that a PCR product was in fact the desired 336 bp fragment (using primer set B) use was made of the fact that there is an *Ava*I restriction enzyme site approximately in the middle of the 336 bp fragment (Figure 4.1). There is also an *Ava*I restriction enzyme site within the downstream primer binding site. Digestion of the 336 bp fragment will therefore yield three fragments of 167 bp, 160 bp and 9 bp respectively. Amplified 336 bp fragment was cut from a gel, electroeluted and digested with *Ava*I. This digest was analysed by agarose gel electrophoresis and after separation and DNA transfer the fragments were hybridised with labelled 366 bp fragment with the labelled 366 bp.

4.4 RESULTS AND DISCUSSION

All attempts to amplify the 366 bp fragment (primer set A) were unsatisfactory. Neither the standard method, the variations of the standard method the use of purified oligonucleotide primers, the use of large amounts of template (100 ng of *M.tuberculosis* DNA) or the use of plasmid DNA (p366 or p36) gave satisfactory results.

Some of the better results are shown in the ethidium bromide stained gel and the autoradiograph in Figure 4.2. In lane 3 and 4 are the amplification products obtained using 100 ng of *M.tuberculosis* DNA using England Biolabs enzyme (lane 3) and Cetus Corporation enzyme (lane 4). It appears from this experiment that amplifications performed with Cetus enzymes were more efficient. These findings were confirmed on numerous occasions and for this reason only Cetus enzyme

was used in all subsequent experiments. Lane 5 shows the amplification products obtained from 10 ng of p36. From both *M.tuberculosis* DNA and p36 a fragment which has a similar mobility to the 366 bp fragment and which hybridises with the 366 bp fragment is amplified. However, other fragments are also amplified. Furthermore, a faster migrating fragment which also hybridises with the 366 bp fragment appears to be more efficiently amplified than the 366 bp fragment.

If one considers that the 100 ng of DNA was used for these amplifications it is apparent that very little product is generated and amplification is not very efficient.

Adjustment of assay conditions did not result in more efficient or more specific amplification. It was felt that the problems were related to the target sequence. There are numerous inverted and direct repeats within the p36 fragment (see section 2.1) and perhaps the failure of amplification may be due to the formation of secondary structures in the single stranded DNA. Such structures if involving the binding site may prevent primer annealing or may affect extension if involving non-priming sequences. Rather than attempt to solve the problem, it was decided to use another set of primers (set B).

With primer set B it was very soon apparent that the desired amplification of the 336 bp fragment was being achieved. Successful amplification occurred with the standard method using unpurified oligonucleotides and with all DNA templates (p36, p366 and chromosomal DNA). Amplification was also achieved using a wide range of annealing temperatures and this shall be fully described in Chapter 5. Figure

4.3 shows the amplified product and confirms that the PCR product is in fact the desired fragment. Digestion of the PCR product with *Ava*I results in a DNA fragment with a mobility corresponding to the size of 160-167 bp. This finding is consistent with the position of the *Ava*I restriction enzyme site as shown in Figure 4.1.

Successful amplification of the 336 bp fragment confirms the previous evaluation that the binding sites of primer set A were not ideal for primer binding; possibly due to the formation of secondary structures in the single stranded DNA.

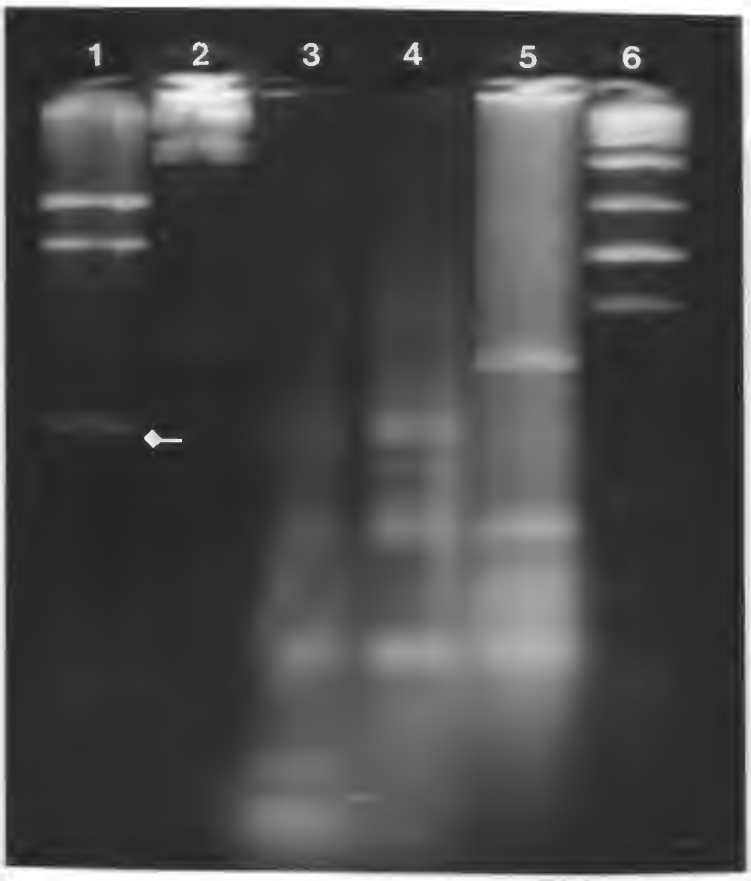


Figure 4.2a:

Ethidium bromide stained 3% Agarose gel Amplification products using the 366 bp fragment as the target sequence. Lane 1: p36 digested with *Kpn*I and *Sma*I (366 bp fragment arrowed).
2: Molecular weight marker (*Hind*III digest of phage lambda).
3 & 4: Products obtained from 100 ng of *M.tuberculosis* DNA using New England Biolabs enzyme (Lane 3) and Cetus enzyme (Lane 4).
5: Products obtained from 10 ng of p36.
6: Molecular weight marker (*Eco*RI digest of a *Hind*III "E" fragment of Variola cloned into pAT153. Personal communication)

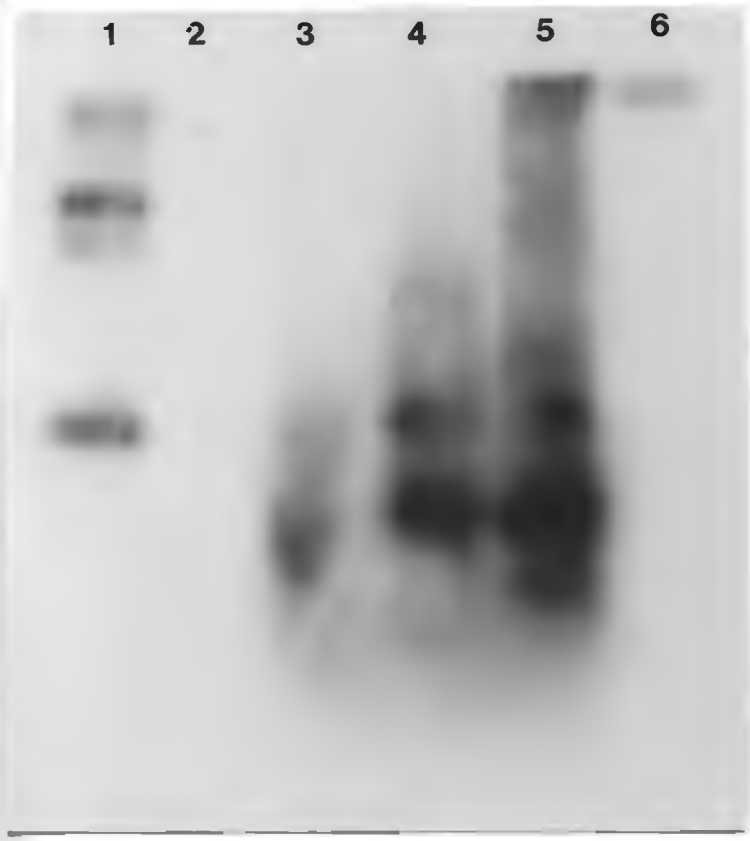


Figure 4.2b:

Autoradiograph following transfer of DNA and hybridisation with the 366 bp radiolabelled probe. Lanes 1 to 6 as in 4.2a

Figure 4.3: Verification of the 336 bp Amplified Product.

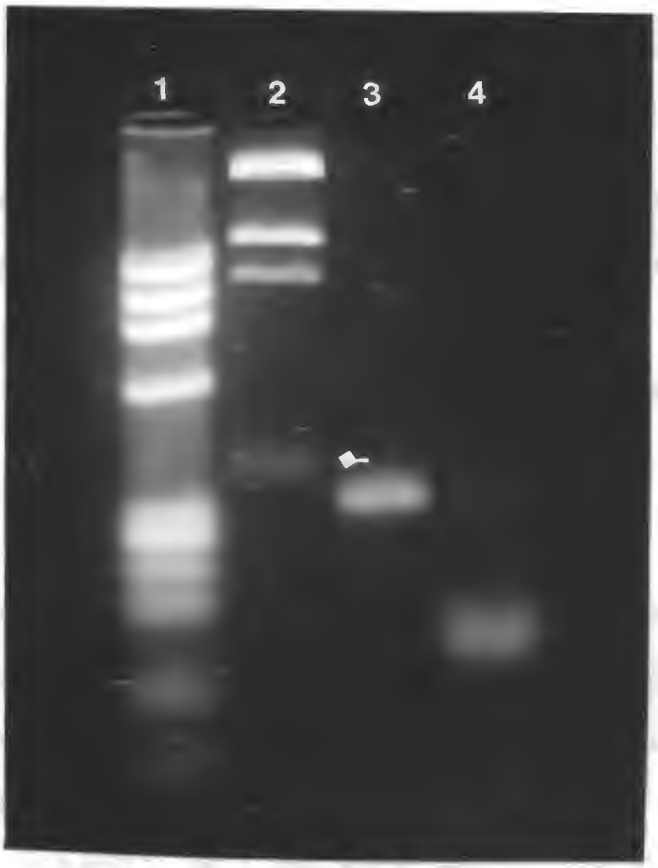


Figure 4.3a: 3% Agarose gel
Lane 1: Molecular weight marker (*Hae*III digest of ØX174).
Lane 2: *Kpn*I/*Sma*I digest of p36 (366 bp fragment arrowed).
Lane 3: Amplified 336 bp product.
Lane 4: 336 bp fragment digested with *Ava*I.

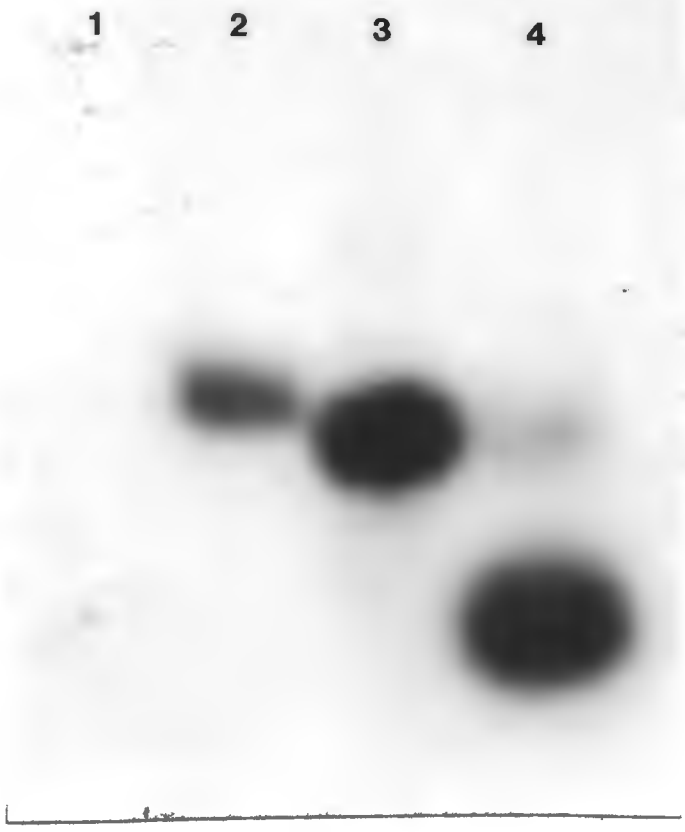


Figure 4.3b: Autoradiograph following transfer of DNA and hybridisation with the 366 bp fragment.

Lanes 1 to 4 as in Figure 4.3a

CHAPTER 5

OPTIMISATION AND CHARACTERISATION OF THE PCR ASSAY

5.1 INTRODUCTION

In Chapter 4 the identification of a set of oligonucleotide primers which generate a 336 bp product was described. In this chapter optimisation of the PCR assay using these primers will be described as well as an assessment of the sensitivity and specificity of the assay.

5.2 OPTIMIZATION OF THE PCR

5.2.1 Standard Buffer Systems

Initially the buffer system used in Section 4.2 was utilised except that bovine serum albumin was replaced by gelatin. A 10 x buffer was prepared so that the final reaction mixture was as follows: 16.6 mM ammonium sulphate, 67.0 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 10 mM β Mercaptoethanol, 6.7 μM EDTA and 0.01% gelatin. Dimethyl sulphoxide was added separately to the reaction mix to a final concentration of 10% V/V. The buffer system described by Cetus Corporation was also used. Dimethyl sulphoxide was however, also added to the reaction mixture. The final component concentrations used in the reaction were as follows: 10% dimethyl sulphoxide, 50 mM KCl, 10 mM tris-HCl pH 8.3, 1.5 mM $MgCl_2$ and 0.01% gelatin.

In both the buffer systems 200 μmols of each dNTP, 1-2 μmoles of each oligonucleotide primer were used. Chromosomal DNA (1 fgm to 10 ng) were added to the appropriate reaction tubes and the mixtures heated to 95°C for 10 minutes. The tubes were then placed on ice and 2.5 units *Taq* polymerase added to each tube.

Thirty thermal cycles were then performed. Initially thermal cycling was performed manually but was later performed in a custom-made thermal cycling machine (see Chapter 6).

5.2.2 Thermal Parameters

These experiments were conducted using the New England Biolabs buffer system. 1 ng of *M.tuberculosis* DNA was added to each reaction tube and the tubes were heated to 95°C for 10 minutes. The denaturation step was performed at 95°C for 1 minute in all cases. The annealing step was 1 minute but the effect of increasing temperatures was assessed i.e. 35°C, 45°C, 55°C, 70°C and 75°C. The extension step was performed in all cases at 70°C for two minutes. Where the annealing temperature was 70°C, thermal cycling was as follows: 95°C for 1 minute and 70°C for two minutes. Thirty cycles were performed in all cases.

5.2.3 The Effect of Dimethyl Sulphoxide

Dimethyl sulphoxide was omitted from the PCR buffer. *M.tuberculosis* chromosomal DNA (1 ng) was added to each reaction mixture. Thereafter the tubes were heated to 95°C for 10 minutes, enzyme added and then subjected to 30 cycles of PCR (95°C for 1 minute and 70°C for 2 minutes).

5.2.4 $MgCl_2$ Concentration

These experiments were conducted using the Cetus Corporation buffer (with dimethyl sulphoxide) except that the $MgCl_2$ concentrations were varied i.e. 0.5 mM, 1.5 mM, 3.0 mM, 6.0 mM and 9.0 mM. The $MgCl_2$ used was supplied as a 1 M solution (Sigma). *M.tuberculosis* chromosomal DNA (100 pg) was used in each reaction. The reaction tubes were

heated to 95°C for 10 minutes, enzyme added and subjected to 30 cycles of PCR (1 minute at 95°C and 2 minutes at 70°C).

5.2.5 *Taq* Polymerase from Different Manufacturers

In Chapter 4, there was an indication that there was variation in quality of enzyme preparations. This was now assessed in a optimum PCR assay. *M.tuberculosis* DNA (100 pg) was used in each reaction. New England Biolabs enzyme (3 batches) and Cetus enzyme (2 batches) were assessed (2.5 units for each reaction).

5.3 SENSITIVITY AND SPECIFICITY

Serial 10-fold dilutions of stock chromosomal *M.tuberculosis* DNA (0.5 µg/µl) were made. 100 pg, 10 pg, 1 pg, 100 fg and 10 fg of DNA were added to separate reaction mixtures (Cetus buffer). Thereafter the tubes were heated to 95°C for 10 minutes, enzyme was added and 30 cycles of PCR performed (95°C for 1 minute and 70°C for 2 minutes).

Ten nanograms of chromosomal DNA from each of the following Mycobacteria were added to separate reaction tubes: *M.avium*, *M.bovis*BCG, *M.fortuitum*, *M.gordonae*, *M.intracellulare*, *M.kansasii*, *M.marinum* and *M.scrofulaceum*. The reaction mixtures were thereafter treated as described above.

Thirty micrograms of DNA from *M.avium*, *M.gordonae*, *M.intracellulare*, *M.kansasii*, *M.marinum*, *M.scrofulaceum* and Acinetobacter was also subjected to PCR using the following thermal cycling profile: 1 minute, 95°C; 1 minute 50°C and 1 minute 70°C. The objective was to assess if similar sequences to the 336 bp fragment could be detected if low stringency annealing was used.

5.4 ANALYSIS OF PCR PRODUCTS

After amplification the reaction mixtures were placed on ice and the DNA was concentrated with 1 tenth volume of 3 M ammonium acetate and 2.5 volumes of absolute ethanol. The DNA pellet was washed with 70% ethanol, dried and dissolved in 30 ul sterile distilled water. In all cases (unless otherwise stated in diagram text), 15 ul was separated on a 3% agarose gel as previously described. DNA bands were visualised by ethidium bromide staining and UV transillumination.

DNA was transferred to Hybond-N-membranes and then hybridised with a labelled 366 bp probe followed by autoradiography.

5.5 RESULTS AND DISCUSSION

Successful amplification occured using both manual or automatic thermal cycling and also with the use of either buffer system.

The effect of increasing annealing temperatures is shown in Figure 5.1. The ethidium bromide stained gel (Figure 5.1a) shows a number of distinct amplification products as well as background smearing when temperatures of 35^{o}C, 45^{o}C and 55^{o}C are used. At 70^{o}C, however, only the desired 336 bp fragment is observed. When a 75^{o}C annealing temperature is used the yield is markedly decreased as observed by the fainter 336 bp band. The smaller DNA fragments noted when annealing temperatures of 35^{o}C, 45^{o}C and 55^{o}C may represent primer dimer (see section 1.3.2). Hybridisation with radiolabelled 366 bp fragment (Figure 5.1b) indicates binding mainly to the 336 bp fragment but also to some of the other PCR products when low annealing temperatures are used. At low annealing temperatures binding of the primer can occur to non-homologous target sequences which results in the amplification

Figure 5.1: Effect of Primer Annealing Temperature on Amplification of *M.tuberculosis* DNA

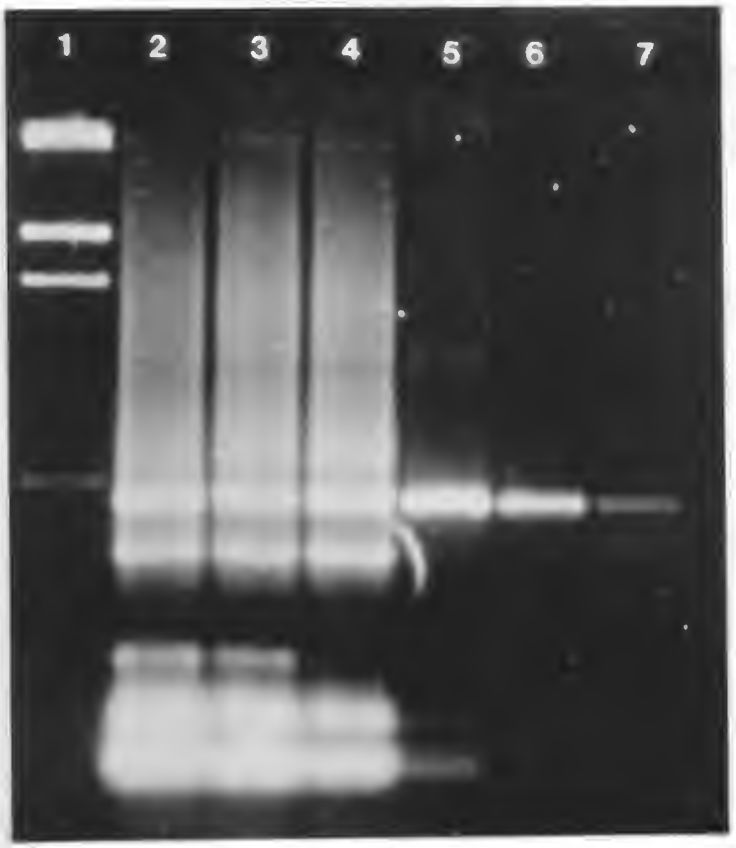


Figure 5.1a: 3% Agarose Gel
Lane 1: *Kpn*I/*Sma*I digest of p36.
Lanes 2, 3, 4, 5, 6: 1 ng of *M.tuberculosis* DNA amplified using an annealing temperature of 35°C, 45°C, 55°C, 70°C and 75°, respectively.
Lane 7: 20 pg of *M.tuberculosis* DNA amplified with an annealing temperature of 70°C

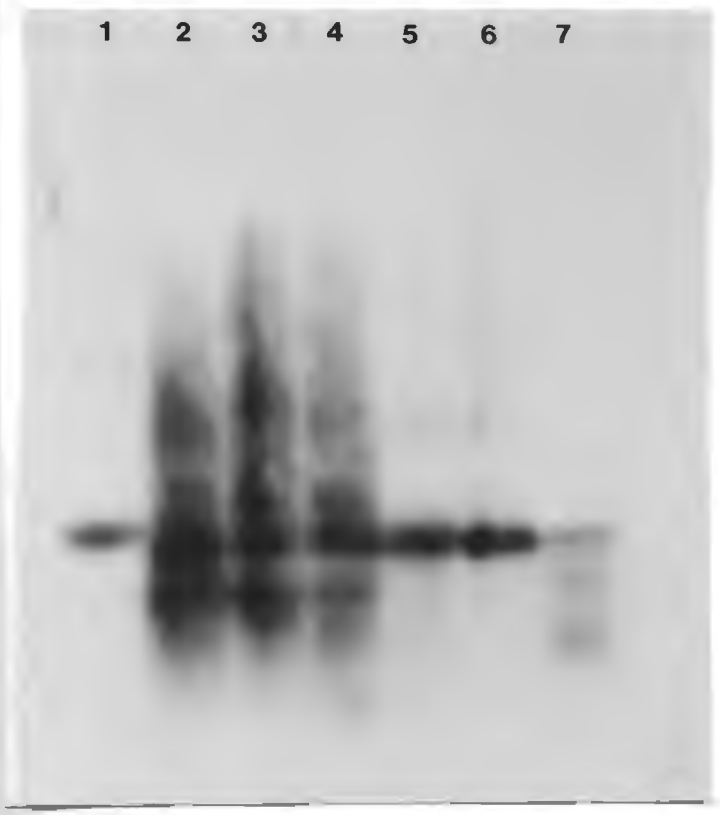


Figure 5.1b:

Autoradiograph following transfer of DNA and hybridisation with the 366 bp fragment

Lanes 1 to 7 as in Figure 5.1a

of 'non-specific' products. At 70°C, however, primer binding only occurs with the specific binding site resulting in the generation of the single desired product. At 75°C the temperature is too high and possibly inhibits primer binding resulting in a decreased product yield. All subsequent thermal cycling was hereafter performed with a 1 minute 95°C denaturing step, followed by a 2 minute 70°C annealing and extension step.

Dimethyl sulphoxide was absolutely essential for successful amplification. No amplification was achieved if DMSO was omitted from the buffer system. The reason for the DMSO requirement in some PCR assays (Gelfand, 1989) is not well understood. DMSO may affect, Tm of the primers, the thermal activity profile of the *Taq* polymerase or the degree of strand separation achieved at a particular temperature.

The effects of $MgCl_2$ can be seen in Figure 5.2a and 5.2b. Amplification only occurs if concentrations of 1.5 mM to 3.0 mM are used. The magnesium concentration range appears to be limited as no amplification at all occurs with lower or higher $MgCl_2$ concentrations. A point to be noted is that successful amplification was achieved using the New England Biolabs buffer in which a 6.7 mM $MgCl_2$ concentration is used. This appears inconsistent with the above results but may be explained by the fact that crystalline $MgCl_2$ was used for preparation of the New England Biolabs buffer. Due to the hygroscopic nature of this substance (it always appears wet) perhaps the amount of $MgCl_2$ was incorrectly weighed and a concentration closer to 3 mM was actually used. For this reason, all subsequent buffers were made from the 1 M stock solution as supplied by Sigma.

Figure 5.2: Effect of $MgCl_2$ Concentration on Amplification

Figure 5.2a: 3% Agarose gel

Lane 1: 0.5 mM $MgCl_2$

Lane 2: 1.5 mM $MgCl_2$

Lane 3: 3.0 mM $MgCl_2$

Lane 4: 6.0 mM $MgCl_2$

Lane 5: 9.0 mM $MgCl_2$

100 pg of *M.tuberculosis* DNA was used as a template in each case. The bright staining band (arrow) may represent primer dimer but the reason for its presence in this instance is not clear.

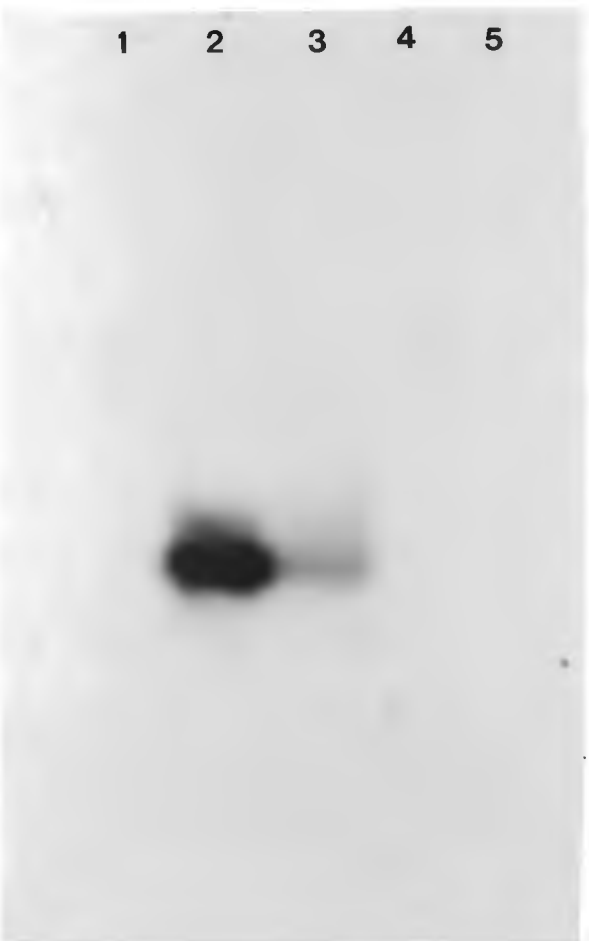


Figure 5.2b: Autoradiograph following transfer of DNA and hybridisation with a radiolabelled internal (primer site not included) 105 bp fragment of the 366 bp fragment (See Chapter 8 for 105 bp fragment preparation.)

Amplification using enzyme supplied by New England Biolabs was largely unsuccessful. Amplification could be achieved if a large quantity of template DNA was used (100 ng chromosomal DNA). However, only faint hybridisation signals were obtained if less than this amount of DNA was used. On the other hand the use of Cetus enzyme resulted in excellent amplification and this enzyme was therefore used throughout this study. The reason for the difference in these enzymes in this study remains unexplained. It may be due to enzyme quality as a result of manufacturer purification or to a disruption of the cold chain during delivery. Numerous other independent researchers at our Medical School also experienced difficulty with the New England Biolabs enzyme and the cold chain theory therefore appears unlikely.

Figure 5.3 demonstrates the effect of decreasing the amount of chromosomal DNA. The ethidium bromide stained gel indicates that when greater than 10 pg of DNA is used, the 336 bp product can be visualised. Transfer of the DNA to a Hybond-N-membrane followed by hybridisation with the radiolabelled probe and autoradiography indicates that when 10 fg of DNA is used the 336 bp product can be detected. This amount of DNA represents 1 to 10 strands of chromosomal DNA (i.e. 1 to 10 organisms). This is extremely sensitive and is consistent with other published PCR procedures (see section 1.3.6) and makes this assay suitable for the detection of *M.tuberculosis* directly in clinical specimens.

As PCR products generated from less than 10 pg of DNA cannot be visualised by ethidium bromide stained gels, hybridisation techniques have to be employed and this may take 2 to 3 extra days. The use of

Figure 5.3: Assessment of Sensitivity

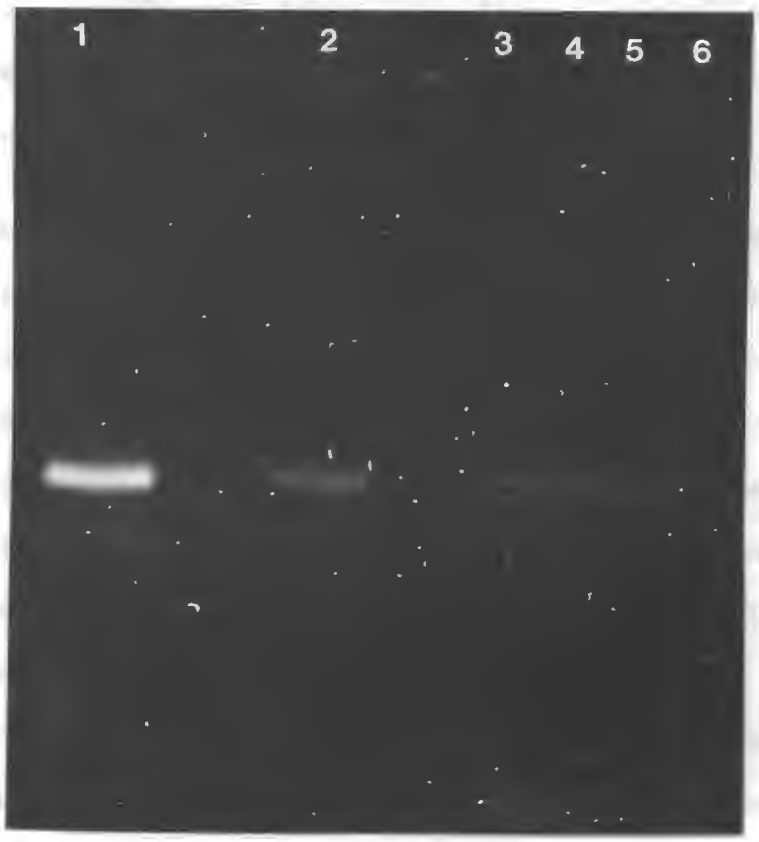


Figure 5.3a: 3% Agarose gel
M.tuberculosis DNA used as template in each case.

Lane 1: 100 pg

Lane 2: 10 pg

Lane 3: 1 pg

Lane 4: 100 fg

Lane 5: 10 fg

Lane 6: Negative control

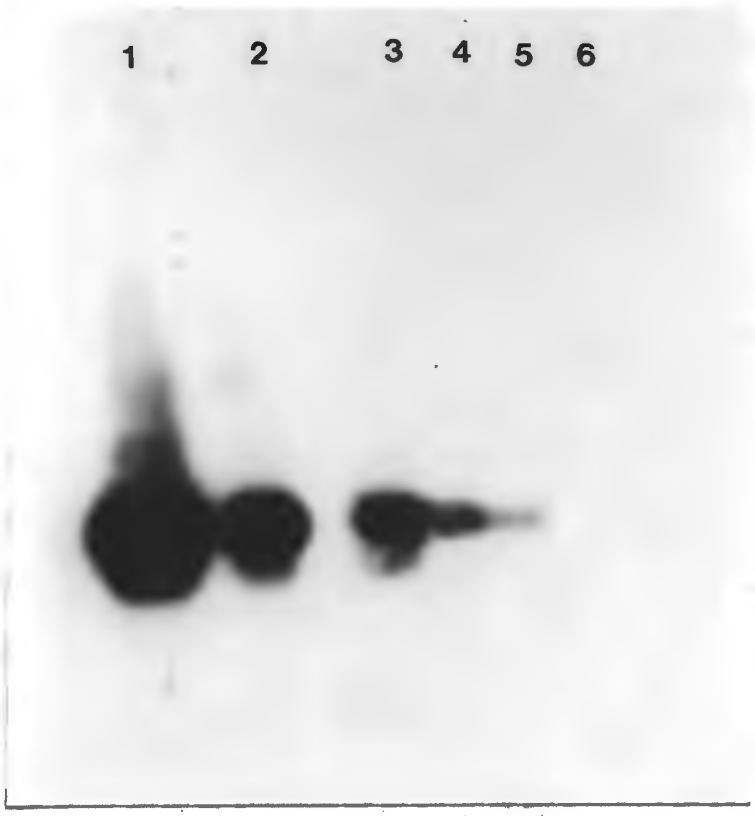


Figure 5.3b:
Autoradiograph following transfer of DNA and hybridisation with the 366 bp fragment.

Lanes 1 to 6 as in Figure 5.5a

"nested" primers may allow more efficient amplification (Plikaytis *et al.*, 1990) and therefore direct visualisation of products on agarose gels. Amplification with "nested" primers is therefore underway in our laboratory and initial results appear promising.

When an annealing temperature of 70°C is used the 336 bp fragment is only generated from *M.tuberculosis* DNA (Figure 5.4). With the exception of *M.bovis*BCG no amplification products were detected with the non-tuberculosis DNA by either agarose gel electrophoresis or by hybridisation. Furthermore it must be noted that the amount of starting DNA used for the non-tuberculous Mycobacteria is 10^3 times more than that used for *M.tuberculosis* (10 ng vs 10 pg). A band with a mobility corresponding to the size of 260 bp was present in the *M.bovis*BCG assay. It is therefore possible to distinguish the amplification products obtained from *M.tuberculosis* and *M.bovis*BCG in this case. The ability to distinguish between members of the *M.tuberculosis* complex has only been reported in one of the published PCR assays (Patel *et al.*, 1990). However, at this stage only one variant (*M.bovis*BCG) has been tested and PCR analysis of other variants needs to be further evaluated.

When low annealing temperatures are used, the 336 bp fragment is also not amplified from other mycobacterial DNA (Figure 5.5). The ethidium bromide stained gel indicates that although amplification products are generated from non-tuberculous mycobacterial DNA at low annealing temperature none of the products hybridise with the 366 radiolabelled probe (Figure 5.5b). This would indicate that not even sequences that are very similar to the 336 bp fragment are present in the genome of the non-tuberculous Mycobacteria. The characterisation of the PCR

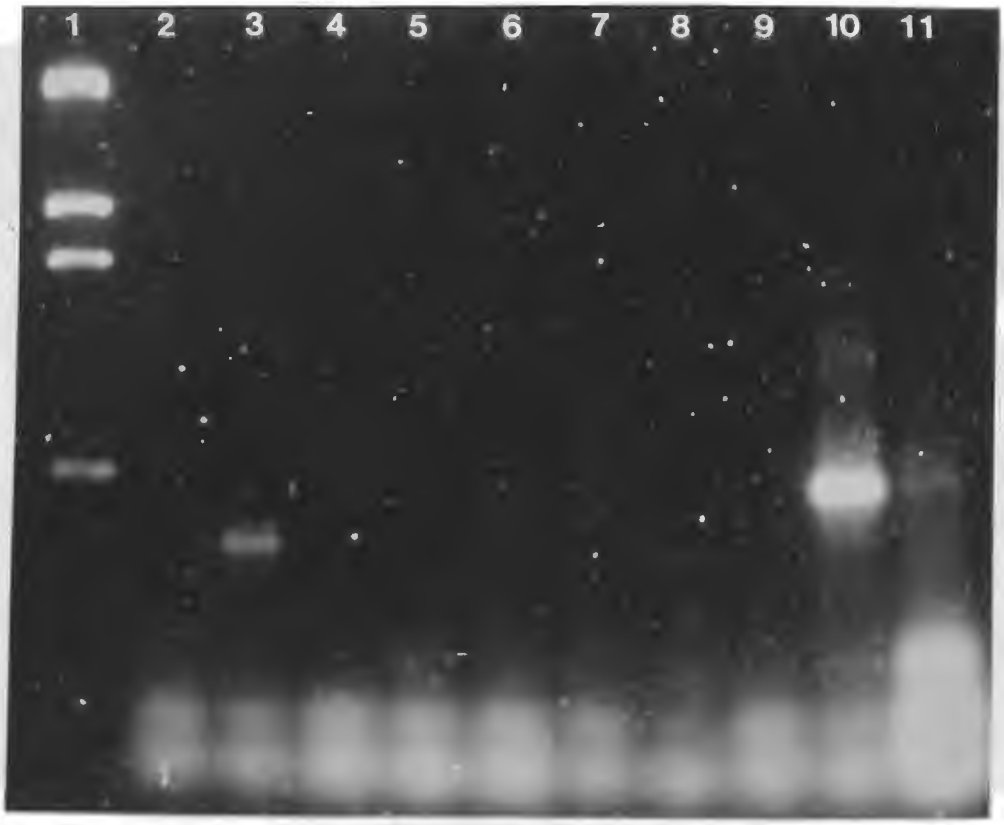


Figure 5.4a: 3% Agarose gel showing amplification products from Mycobacterial DNA (10 ng) when an annealing temperature of 70°C was used. Lane 1: p36 digested with *Kpn*I and *Sma*I Lane 2: *M.avium* Lane 3: *M.bovis*BCG Lane 4: *M.fortuitum* Lane 5: *M.gordonae* Lane 6: *M.intracellulare* Lane 7: *M.kansasii* Lane 8: *M.marinum* Lane 9: *M.scrofulaceum* Lane 10: *M.tuberculosis* (10 pg of chromosomal DNA). Lane 11: *M.tuberculosis* (1 pg chromosomal DNA)

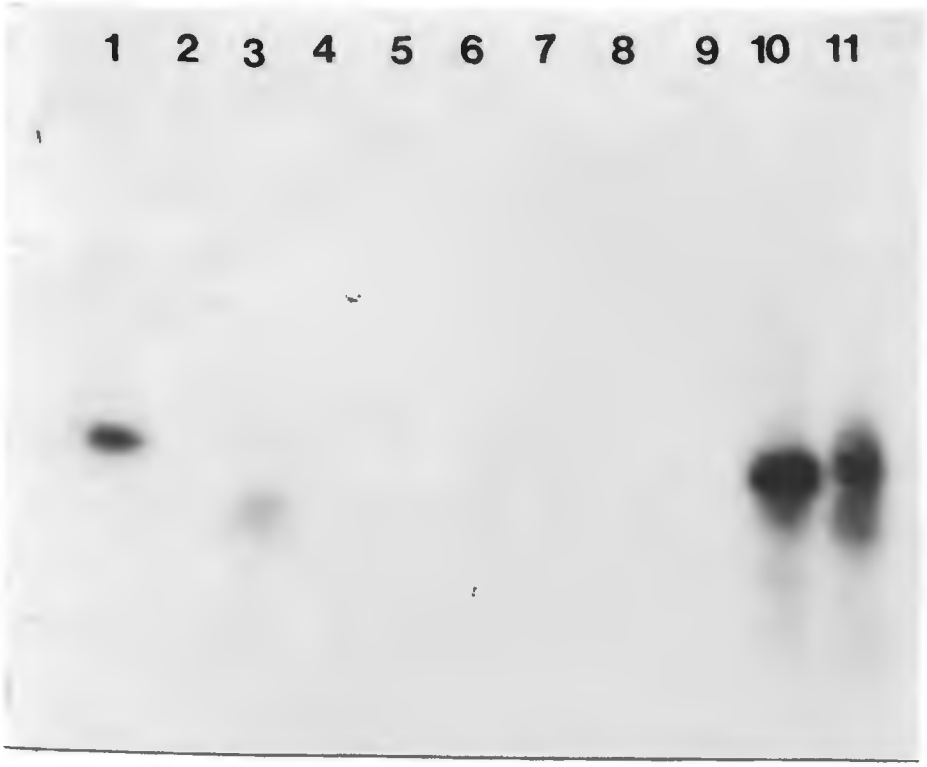


Figure 5.4b: Autoradiography following transfer of DNA and hybridisation with the 366 bp fragment.

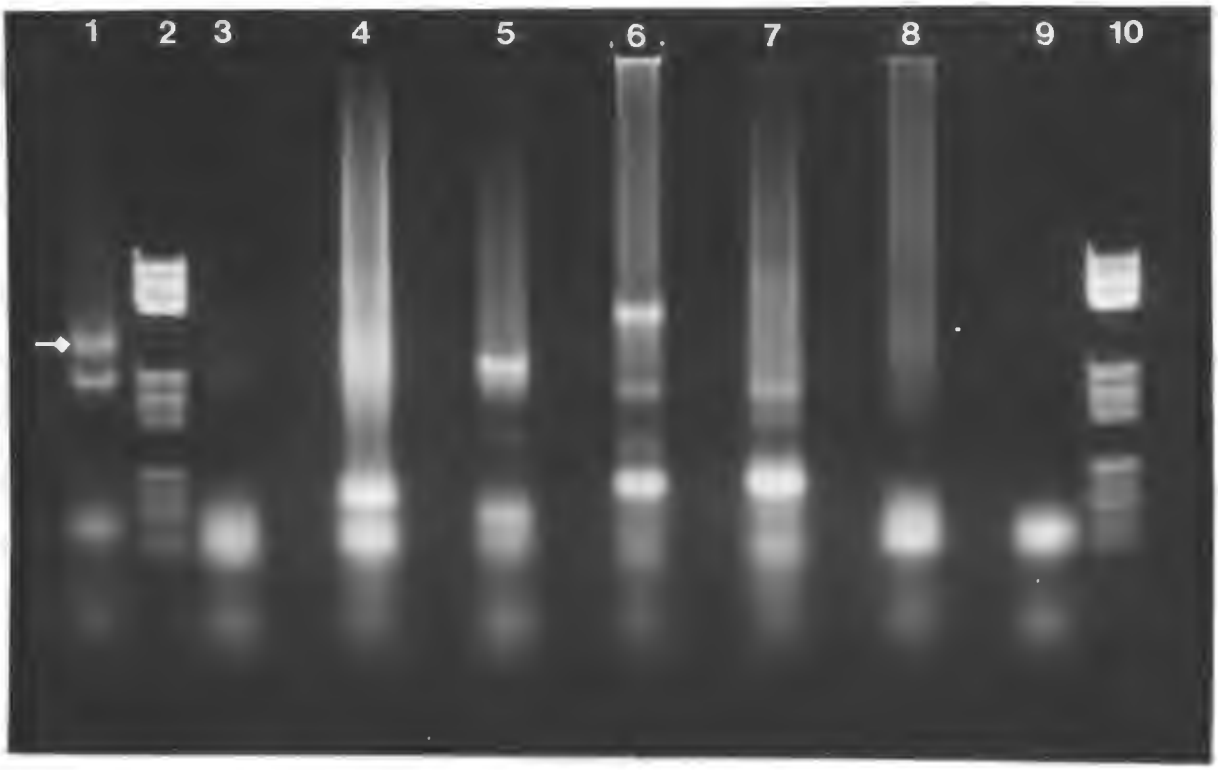


Figure 5.5a: Ethidium bromide stained gel of material obtained after amplification of Mycobacterial DNA using an annealing temperature of 50°C. Lane 1: *M.tuberculosis* (arrow indicates 336 bp fragment); Lane 2: Marker (*Hae*III digest of pBR322); Lane 3: *M.avium*; Lane 4: *M.gordonae*; Lane 5: *M.intracellulare*; Lane 6: *M.kansasii*; Lane 7: *M.marinum*; Lane 8: *M.scrofulaceum*; Lane 9: *Acinetobacter*; Lane 10: Marker (*Hae*III digest of pBR322);

Figure 5.5b: Autoradiography following transfer of DNA and hybridisation with the radiolabelled 366 bp fragment.

Lanes 1 to 10 as in Figure 5.5a

products generated at low annealing temperatures and the utilisation of this observation as a PCR technique for the detection of non-tuberculous Mycobacteria is described elsewhere (de Wit, 1990).

The absolute specificity of this PCR assay makes this procedure ideal for the amplification of *M.tuberculosis* DNA from clinical specimens. Extracts of DNA from these specimens will almost certainly include DNA from a wide variety of sources, including perhaps closely related environmental species.

CHAPTER 6

DEVELOPMENT AND TESTING OF A THERMAL CYCLING MACHINE

6.1 INTRODUCTION

Manual thermal cycling is extremely tedious and the need for an automated thermal cycling machine is essential. Funds were not available for a commercial machine and it was therefore decided to build a custom made PCR machine.

The concept, design and development of the machine used in this thesis was the result of input from numerous people who have all been duly acknowledged. The building of this machine was already well under way before the first published reports of non-commercial machines became available (see section 1.3.3). Since then, however, numerous custom made machines have appeared in the literature and perhaps our design might have been different had this data been available.

6.2 MACHINE DESIGN

A design based on the concept of stationary samples and variable water temperatures was used. The design is best demonstrated by a schematic diagram (Figure 6.1) and a photograph of the machine (Figure 6.2). A central sample chamber was connected by an inlet and outlet circuit to each of three thermostatically controlled waterbaths. The water flow in each circuit was controlled by solenoid valves and a pump, sited on the inlet hose of each circuit which pumped water through the sample chamber. The solenoid valves and the pump on each circuit were controlled by timer switches.

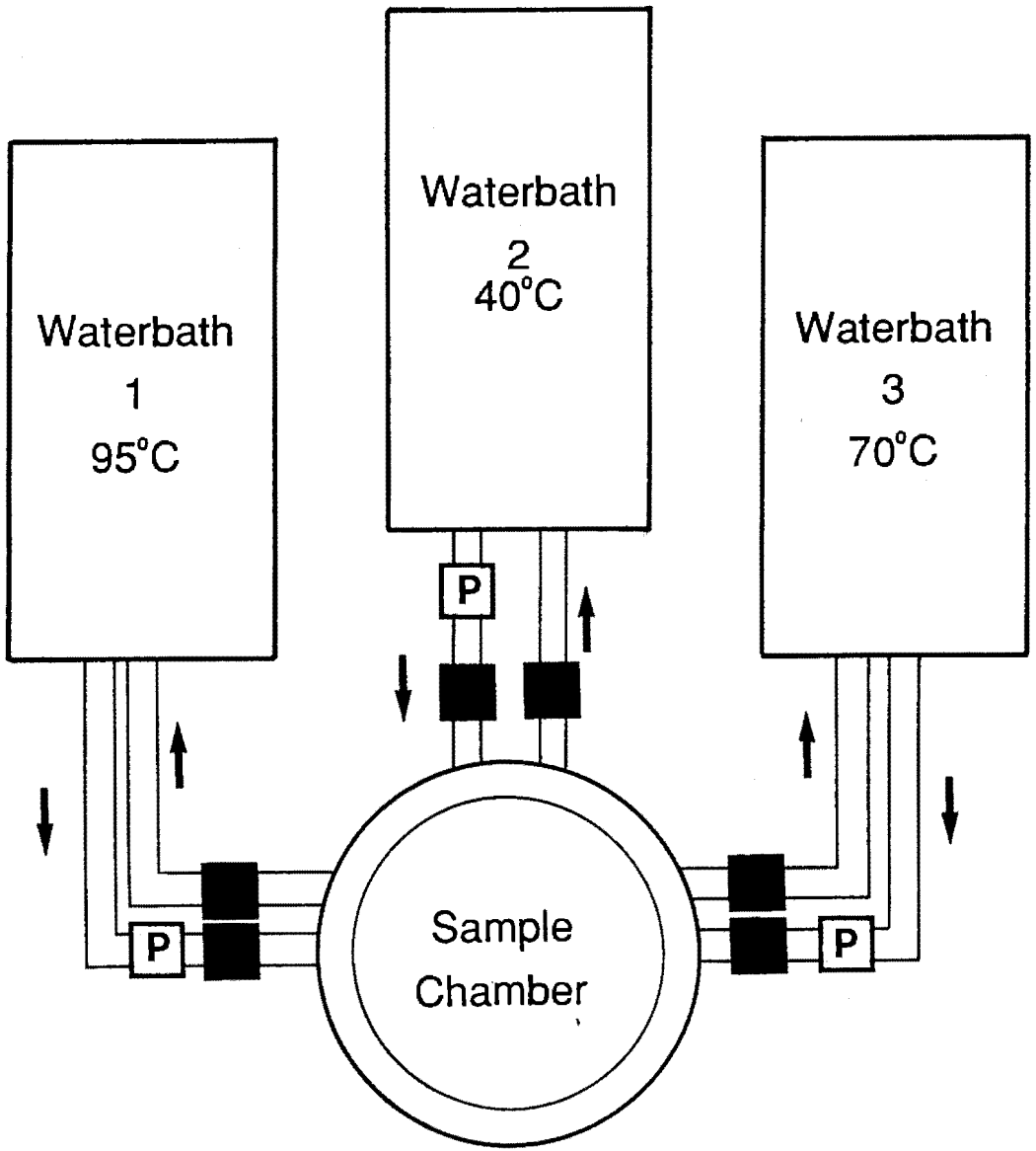


Figure 6.1 Schematic Prepresentation of Thermal Cycling Machine
→Direction of water flow ■Solenoid valves [P] Pump

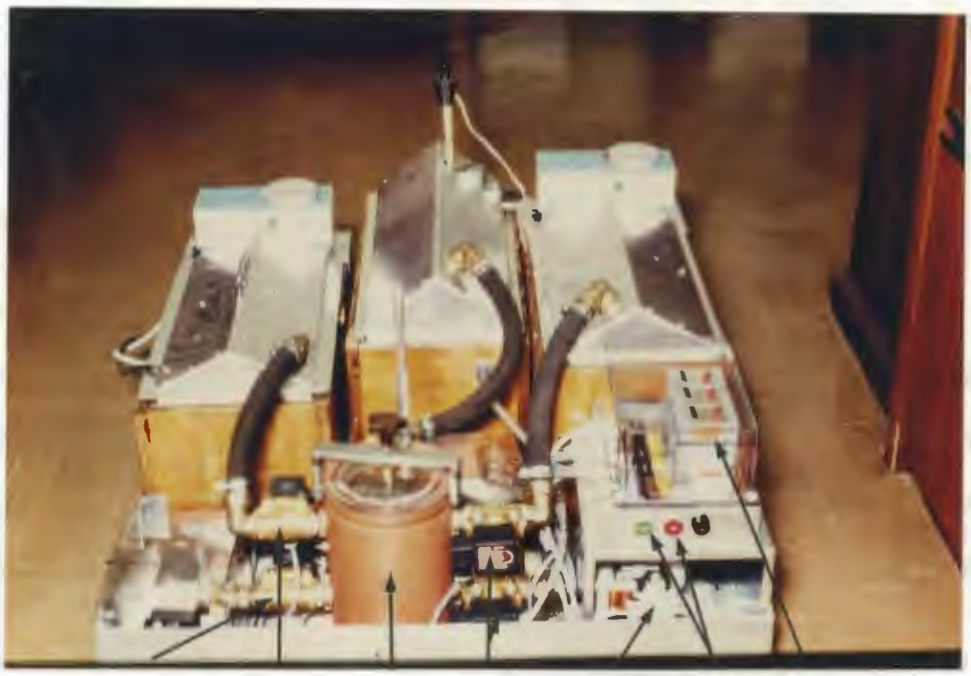


Figure 6.2: Thermal Cycling Machine

1. Inlet of circuit I
2. Outlet of circuit 1
3. Sample chamber
4. Solenoid valves
5. Pump
6. On and off Switch
7. Timers

6.3 MACHINE COMPONENTS

Sample Chamber: A cross-sectional view of the chamber is seen in Figure 6.3. The chamber was made of a polypropylene rubber tube which was welded to a polypropylene base. This material proved to be a good insulator and was also durable and could be easily machined i.e. drilling holes etc. The lid was made of clear perspex and was secured to the chamber by a metal clamp. A hole was drilled through the lid so that a thermometer could monitor the water temperature in the chamber.

The **sample holder** (Figure 6.4) was designed to hold 32 1.5 ml Eppendorf tubes and was made of perforated steel plates so that water, under pressure, could easily flow past the tubes. The sample holder was so designed that it could be assembled and the reaction tubes inserted at 4°C prior to placing in the sample chamber.

Reaction tubes: Because of possible leakage with standard Eppendorf tubes, 1.5 ml tubes with a screw cap and 'O' ring were used (Eppendorf Cat. no: 3810).

Waterbaths: These were standard waterbaths made of aluminium and were housed in a wooden jacket, which provided good insulation. The lids were insulated on the inside with foamalyte. The 70°C waterbath and the 40°C waterbath were thermostatically controlled. (Tecam TE-7 Tempette, Laboratory and Scientific Equipment Co. (PTY) Ltd.). The "95°C" waterbath was heated to boiling temperature by 2 x 1 kWatt emersion elements. When the water from the boiling bath was pumped through the sample chamber the temperature in the sample chamber was 95°C - 96°C.

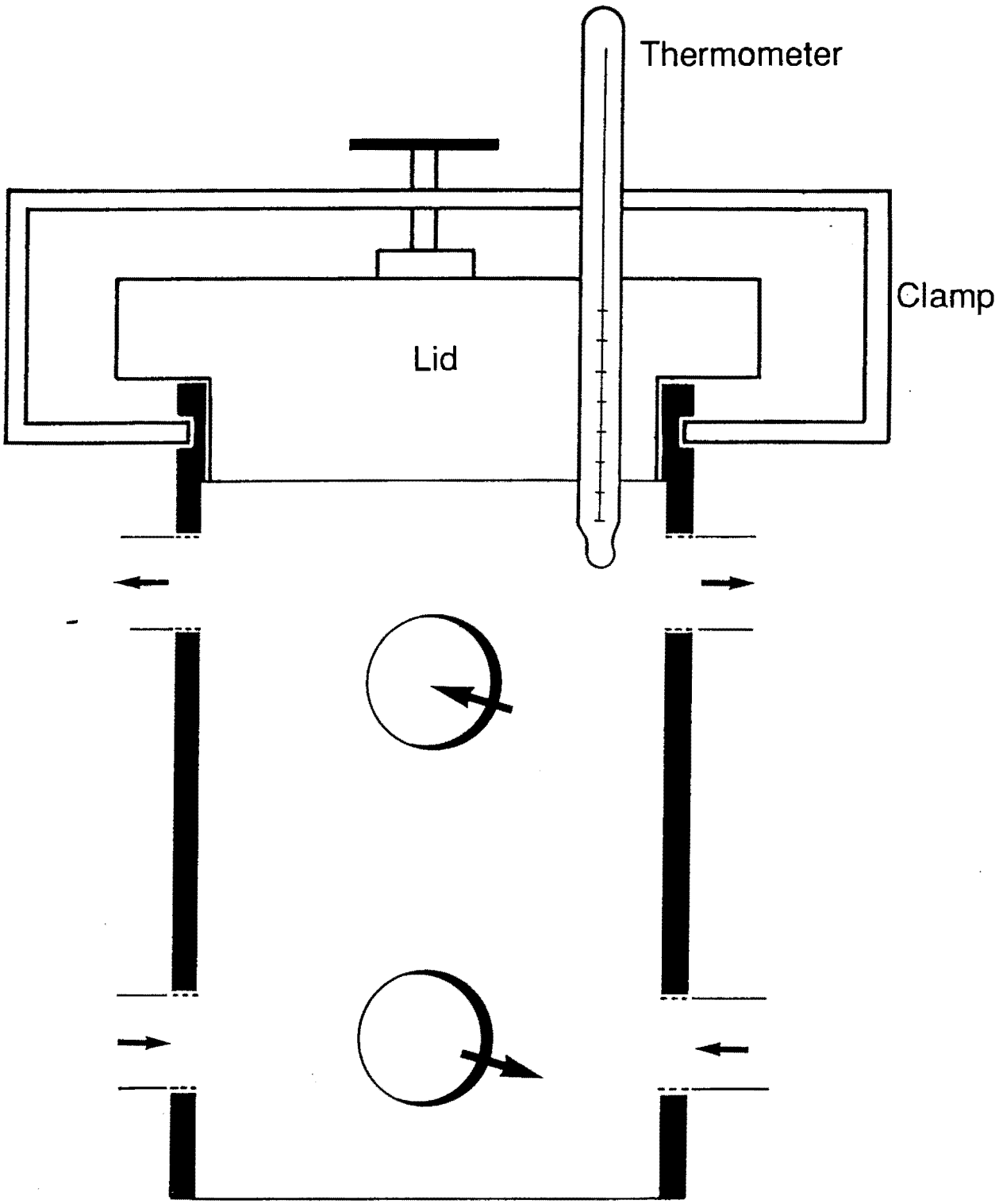
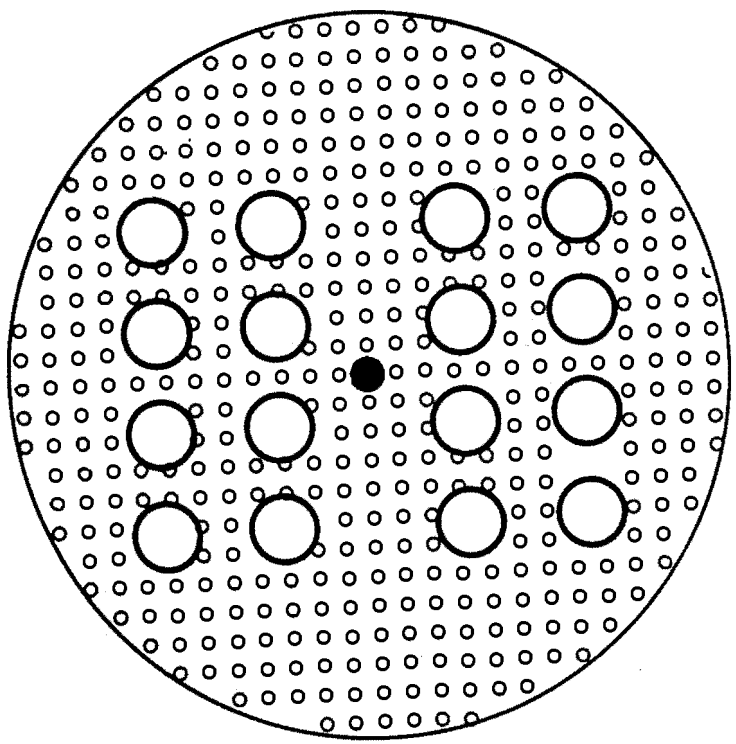
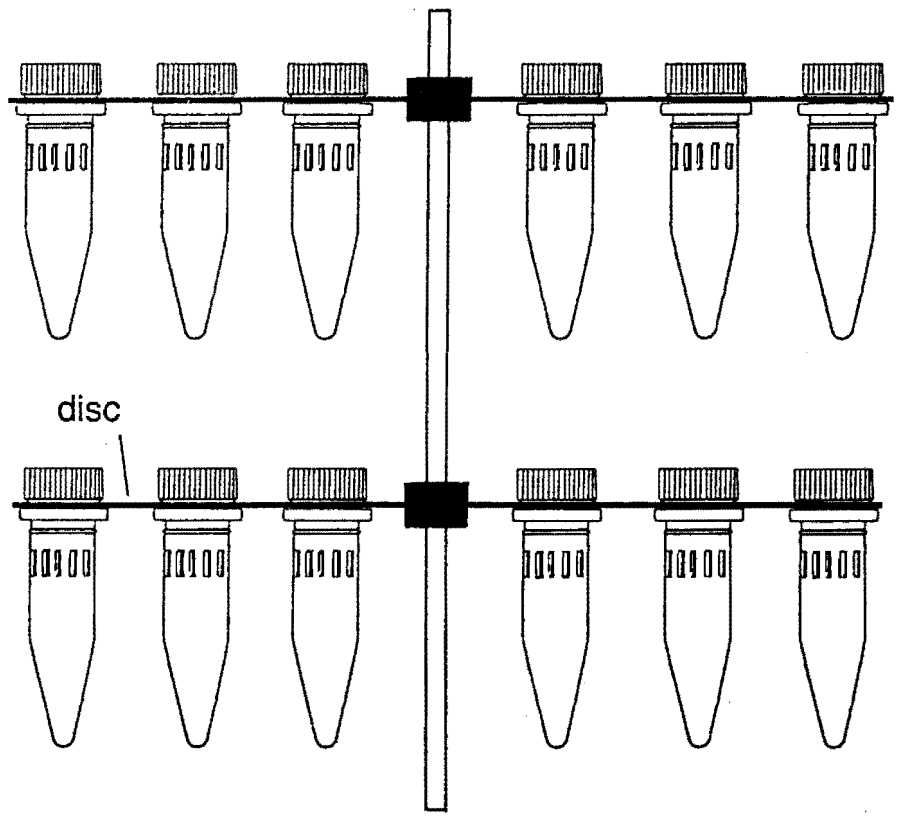


Figure 6.3 Cross-sectional View of Sample Chamber
(→ ← Direction of water flow)

Figure 6.4 Sample Holder



6.4a Sample Holder Disc.
Each disc can hold 16 tubes. Smaller perforations allow water flow



6.4b Side View of Sample Holder

Electronic timers were supplied by ATW. Two identical timers were used for circuits 1 and 2 (Catalogue number ATE1-01). A slightly different timer (Catalogue number ATE1-02) was used for circuit 3 (see circuit diagram).

These timers could be adjusted for four different time ranges (0.5 - 6 secs; 5 - 60 secs; 50 - 60 secs; 5 - 60 minutes).

Solenoid valves were supplied by ASCO (Catalogue number WPSC 8210 D95). These are normally closed valves of brass construction and are specifically designed to withstand fluid temperatures of 100^{o}C.

Pumps: These were Hoover washing machine pumps and were able to withstand temperatures of 96^{o}C to 98^{o}C.

Relay: This was supplied by Sprecher and Schuh. Catalogue number C54.22.Z.

Electrical Circuit: The circuit comprised of a stop and start switch connected to a relay and 3 time switches which each controlled 2 solenoid valves and a pump. A circuit diagram best demonstrates the layout (Figure 6.5).

6.4 MACHINE TESTING

The machine was designed to perform a 3 temperature cycle. However, it was soon evident that only a two temperature cycle was needed for amplification of target sequences from *M.tuberculosis* DNA i.e. 95^{o}C for 1 minute and 70^{o}C for 2 minutes. This two temperature profile was obtained by allowing the water in waterbath 1 to boil, waterbath 2 to reach 40^{o}C and waterbath 3 to reach 70^{o}C. Water was pumped from bath 1 for 1 minute followed by a 5 second 'flush' from waterbath 2 and

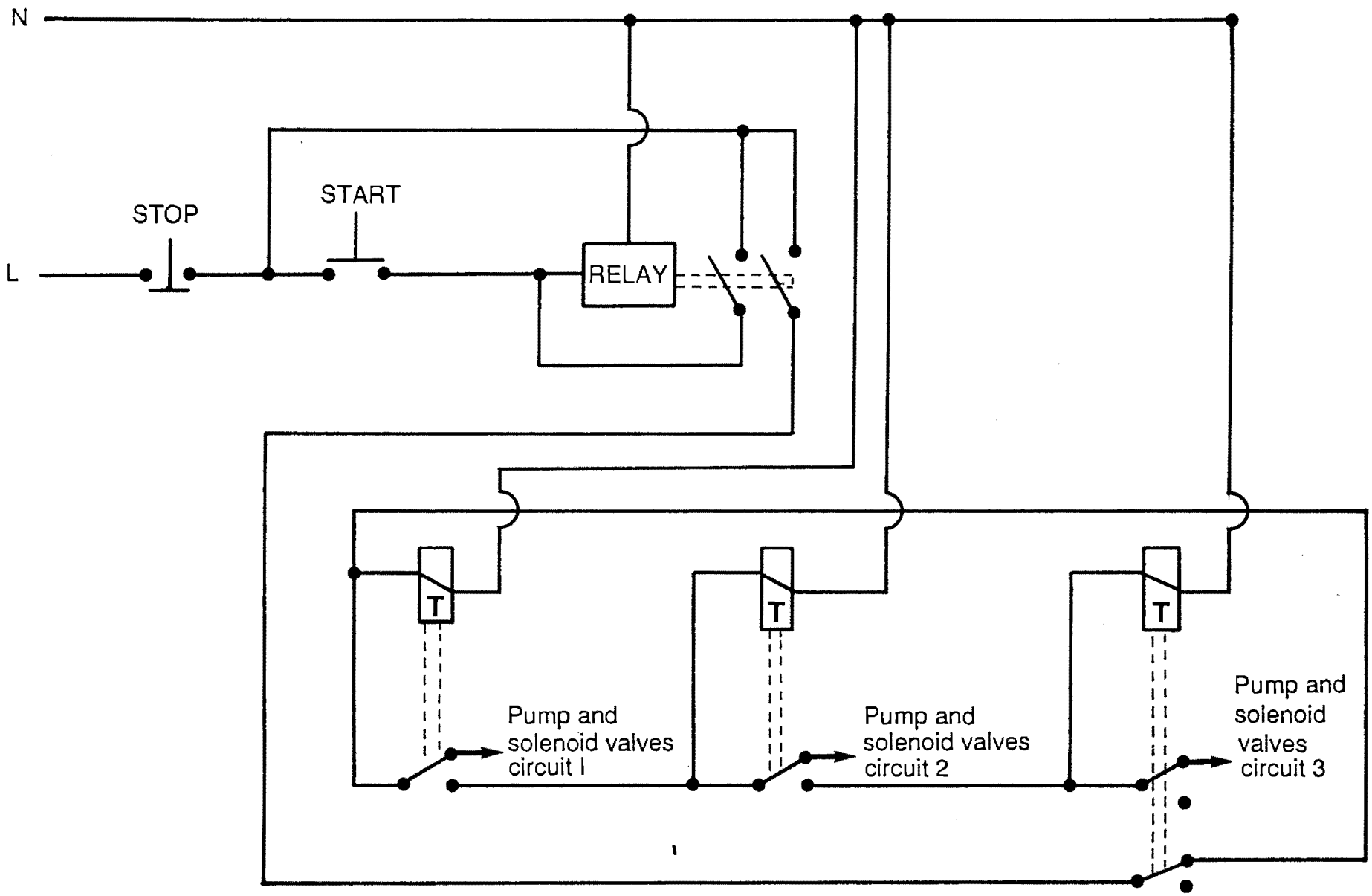


Figure 6.5 Circuit Diagram
T=Timer N=Neutral L=Live

then 1 minute and 55 seconds from waterbath 3. The flushing from waterbath 2 resulted in a very rapid decrease in temperature.

The temperature profile of the water in the sample chamber, as monitored by the sample chamber thermometer, is shown in Figure 6.6, together with the temperature profile of the sample temperature (see below). The temperature changes i.e. ramp times are very rapid and therefore no time had to be allowed for these changes. The sample chamber water temperature was used to monitor all routine PCR assays.

To ensure that the waterbath temperature was a reflection of the temperature within the sample tube, a special modification was made in the lid of the sample chamber (Figure 6.7). A thermistor probe coupled to a Wheatstone bridge with output to a chart recorder was used to directly monitor the temperature within the reaction tube. The temperature profile (Figure 6.6) indicates that denaturing and annealing-extension temperatures are accurate and that ramp times are very rapid. The samples only reach maximum denaturing temperature (96^{o}C) after 30 seconds, but results indicate that this is sufficient for denaturation. Once it had been established that the sample was reaching the desired temperature, the hole for the thermistor probe was sealed and all further temperature monitoring was performed by observation of the sample chamber temperature.

6.5 DISCUSSION

This machine was first used in October 1988 and is still being used routinely in the Molecular Mycobacteriology laboratory. Only minor maintenance has been required.

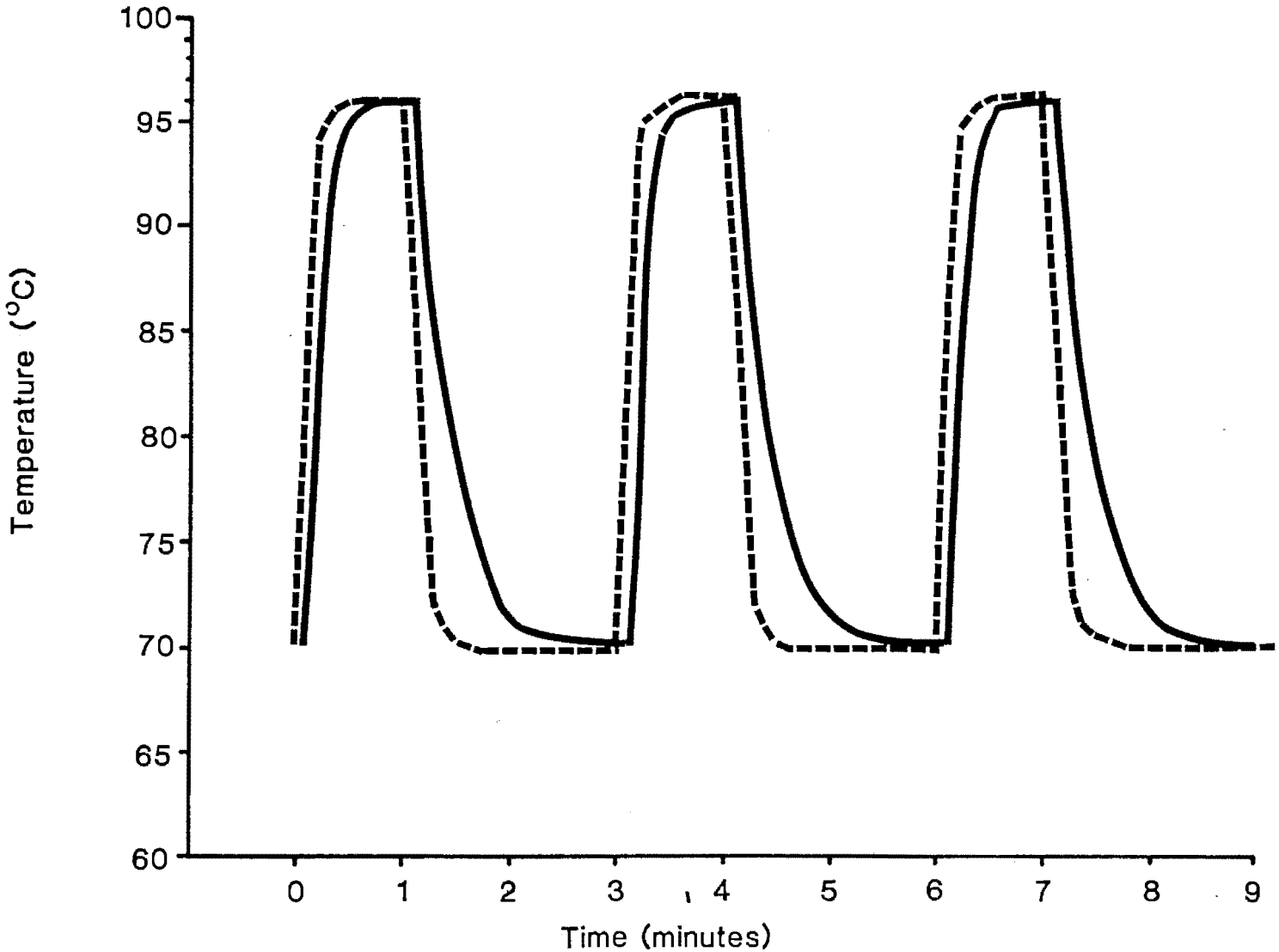


Figure 6.6 Temperature Profile of Sample Chamber (- - -) and Reaction Mixture (———)

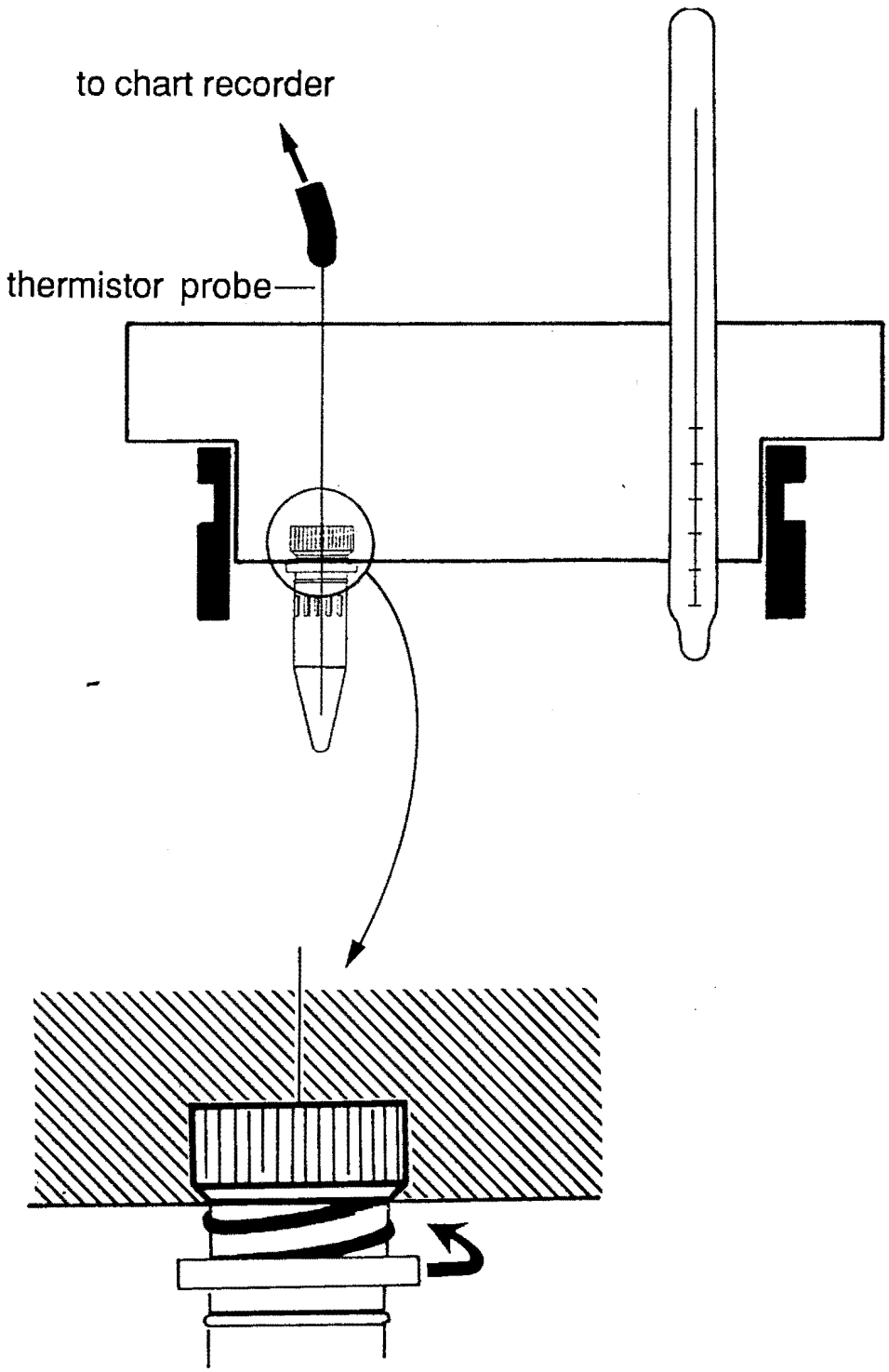


Figure 6.7 Modification to Allow Direct Measurement of Sample Temperature. Lid of eppendorf tube was fixed into sample chamber lid. The reaction tube with reaction mixture was then screwed into lid.

The temperature responses are extremely rapid and therefore no time needs to be included in the cycle to allow for ramp times.

A further advantage of this machine is that due to total submersion of the tubes no condensation occurs on the top and sides of the tube and there is therefore no need to overlay the sample with oil, as is required for most commercial machines.

The machine is, however, rather large and utilises considerable laboratory space. Another possible disadvantage is that 1 and a half hours are required for the waterbaths to reach the desired temperatures. However, the preparation of 16-32 samples can take almost this amount of time, so there should be no 'dead' time if the machine is switched on just prior to sample preparation.

CHAPTER 7

PREPARATION OF DNA FROM CLINICAL SPECIMENS

7.1 INTRODUCTION

At the time of this study pleural fluids were being collected for a trial to compare different conventional diagnostic tests for *M.tuberculosis* (Maartens and Bateman, 1990). These fluids were later made available for PCR (Chapter 8). Most of the clinical material available, therefore, were pleural fluids and the extraction procedures and problems described below refer to these specimens. The extraction procedure was easily adapted to other specimens and these variations will be described later in this chapter.

The method of SDS and phenol extraction described for the preparation of *M.tuberculosis* DNA in section 2.2 is the basic technique used for the preparation of DNA from clinical specimens. For the preparation of DNA from pleural fluids, a number of specific factors needed to be considered. The first consideration is that of the nature of the sample, i.e. centrifuged pellet or unspun pleural fluid. It had to be established whether the supernatant fluid carried sufficient chromosomal DNA to warrant processing. If this was so then the second consideration was that of the volume of fluid which could be conveniently handled. The high protein concentration of these exudates was a further consideration and this factor became more problematical as the volume of fluid was increased.

The collection of pleural fluids and pleural biopsies used in this chapter are described in greater detail in Chapter 8.1. For these

initial pilot studies, fluids and biopsies which showed strong growth on laboratory culture were chosen.

Because of the known problems of contamination (Chapter 1.3.5), great care was taken during specimen handling. A totally disposable system of plastic pipettes and tubes was developed and this is described in detail in the standard procedure (7.3.5).

7.2 ASSESSMENT OF SUPERNATENT FLUID

Two millilitres of pleural fluid from two patients was centrifuged at 4000 rpm for 15 minutes (Heraeus Christ, Labofunge GL). The supernatant fluids (2 ml) were extracted three times with 2 ml of phenol and of 2 ml of chloroform:isoamylalcohol (24:1); centrifugation being performed at 4000rpm for 10 minutes between extractions (HN-SII Centrifuge. IEC). The aqueous phase was removed and added dropwise with stirring to an equal volume of 20% PEG 6000 (Sigma) in 2.5 M NaCl and the mixture was left to stand at room temperature for 15 minutes. The DNA was then concentrated by centrifugation in a Beckman microfuge (about 6 tubes per specimen) for 15 minutes. The pellet was washed with 70% ethanol, dried (SpeedVac Concentrator. Savant) and reconstituted in 40 µl of sterile distilled water. Twenty microlitres was subjected to PCR and the products were analysed by electrophoresis and hybridisation with a radiolabelled probe.

The centrifuged pellets of the pleural fluids were extracted as described in the Standard Method (7.3.5).

7.3 EXTRACTION AND PURIFICATION OF DNA

In section 7.2 it was established that extraction of DNA from total fluids was warranted (see results). In this section the various

procedures employed to extract DNA from these exudates will be described. The use of proteinase K for protein digestion, the problem of inhibitors and the precipitation of DNA with PEG will be assessed. Finally from these experiments a standard method of DNA extraction and purification has been developed and is described (7.3.5).

7.3.1 The Use of Proteinase K in Pleural Fluids

The high protein content of pleural fluids is always problematical and a proteinase (proteinase K) was therefore used in an attempt to overcome this problem.

To 2 ml of 3 pleural fluids 200 µl 10% SDS, 200 µl 0.5 M EDTA and 1 mg Proteinase K were added. The mixture was incubated at 37°C for 1 hour. Thereafter, 2 ml of 10% SDS and 2 ml phenol were added and the tubes incubated for 3 hours at 37°C with shaking. After extraction the tubes were centrifuged and the supernatant fluids were removed and treated with phenol/chloroform/isoamyl alcohol, as described in 7.2. One tenth volume 4 M, LiCl and 2.5 volumes absolute ethanol were added to the aqueous phases and the DNA was pelleted by centrifugation.

The pellet was washed twice with 70% ethanol, dried and reconstituted in 40 µl sterile distilled water. 20 µl of this material was subjected to PCR and the products were analysed by electrophoresis, hybridisation and autoradiography.

7.3.2 Use of Proteinase K in Pleural Biopsies

In order to assess the effect on PCR of proteinase K without the problems of high protein content, pleural biopsies were treated with and without proteinase K.

Five pleural biopsies were made up to 600 µl in TES buffer. 100 µl 10% SDS, 60 µl 0.5 M EDTA and 77 µg of proteinase K were added and the mixtures were incubated at 37°C for 1 hour.

Thereafter the DNA was extracted by the addition of 600 µl 10% SDS and 600 µl phenol and incubated at 37°C with shaking for 3 hours. After extraction the tubes were centrifuged in a microfuge for 10 minutes and the supernatant fluids treated twice with phenol/chloroform/isoamyl alcohol. An equal volume of 20% PEG in 2.5 M NaCl was added dropwise to the aqueous phase and the DNA was pelleted by centrifugation in a microfuge.

The pellets were washed, dried and reconstituted in 40 µl sterile distilled water. Twenty microlitres was subjected to PCR and the products analysed by electrophoresis, hybridisation and autoradiography.

A further 3 pleural biopsies were made up to 500 µl of TES. To these were added 500 µl 10% SDS and 500 ml phenol. The tubes were incubated with shaking at 37°C for 3 hours. After extraction the DNA was processed as described above (7.3.2).

7.3.3 Removal of Inhibitors

To confirm if there were inhibitors in the proteinase K treated specimen, an attempt was made to remove these using the method, described by de Franchis and colleagues (1988) which is discussed in section 1.3.4.

Sephadex G50 was prepared in 67 mM Tris-HCl buffer (pH 8.0) and sterilised by autoclaving. One millilitre syringes were prepared and filled with Sephadex 650 as described in Appendix B7.

Twenty microlitres of the DNA extracts prepared from 3 proteinase K treated pleural biopsies were made up to 100 ul in 67 mM Tris-HCl buffer and heated at 95^{o}C for 10 minutes and then placed on the column and centrifuged at 1500 rpm for 5 minutes.

The eluate was collected and the DNA concentrated by precipitation with PEG as described earlier (7.3.2). The pellet was washed with 70% ethanol, dried and reconstituted in 20 ul H_2O and subjected to PCR. The products were analyzed by agarose gel electrophoresis and hybridisation.

7.3.4 Efficiency of Ethanol and Polyethylene Glycol Precipitation of DNA

The method of preparation of mycobacterial DNA involves a final precipitation step using PEG. We have found in the past that this results in DNA preparation which have very little protein contamination (260:280 greater than 1.75. Section 2.2). During the course of DNA extraction from clinical specimens, I also observed that larger pellets (possibly protein containing) were obtained if precipitation was performed with alcohol as compared with PEG. Also occassionally alcohol precipitated DNA from pleural fluid extracts could not be amplified by PCR. In view of the known problems with inhibitors in PCR methodology it was felt that the purist possible DNA was desirable and PEG precipitation therefore appeared to be the most suitable. However, the yield of DNA from clinical specimens is also

important and this DNA study was therefore undertaken to assess if there was a marked difference in DNA precipitation efficiency between PEG and alcohol.

M.tuberculosis DNA (1.2 µg) was labelled by Nick Translation as previously described (Appendix B7). One and one half picograms of labelled DNA was then added to 200 µl of sterile distilled water in each of 12 microfuge tubes. Four tubes were not treated further and were used as controls. To 4 tubes 1 tenth volume of 4 M LiCl and 2.5 volumes of absolute ethanol were added and the solutions were placed at -20°C overnight. Two hundred microlitres of 20% PEG in 2.5 M NaCl was added dropwise to another 4 tubes and the solutions were left to stand at room temperature for 15 minutes. To assess the effect of washing the DNA pellets, 2 tubes from both the PEG and the ethanol precipitation were washed twice with 70% ethanol while the other 2 tubes were not washed. All 8 tubes were then dried (SpeedVac Concentrator. Savant) and the pellets reconstituted in 200 µl of sterile distilled water.

All twelve tubes were placed directly into scintillation vials and counted in a Beta counter (Beckman L5800).

7.3.5 Standard Procedure

The procedure was based on the results of the previous sections and was used for extraction of DNA from all clinical specimens unless otherwise stated.

Extractions of DNA and protein were performed in 10 ml plastic tubes (Griener). Addition of all reagents and transfer of fluid was performed with 5 ml disposable bulb pipettes (Liquipette TM 3 ml.

Weil Organisation). All reagents were prepared using water from a laboratory where no mycobacterial work was being done and were autoclaved prior to use.

To 2 ml of fluid in a 10 ml plastic tube 2 ml of 10% SDS and 2 ml of phenol was added. Reagents were added with a 5 ml disposable pipette. The mixture was incubated at 37°C with shaking for 3 hours. After extraction the tubes were centrifuged for 10 minutes at 4000 rpm (HN-SII Centrifuge. IEC). The supernatant fluid was extracted 3 times with an equal volume of phenol and an equal volume of chloroform/isoamyl alcohol (24:1). The aqueous phase was added dropwise to an equal volume of 20% PEG in 2.5 M NaCl and left at room temperature for 15 minutes. The DNA was then pelleted by centrifugation in a microfuge for 15 minutes, washed with 70% ethanol, dried and reconstituted in 40 µl sterile distilled water. PCR was done on 20 µl and the products were analysed by agarose gel electrophoresis and hybridisation.

7.4 EXTRACTION OF DNA FROM VARIOUS CLINICAL SPECIMENS

In section 7.2 and 7.3 DNA extractions of pleural fluids and pleural biopsies are described and PCR was shown to be successful if the standard extraction and purification method (7.3.5) was employed (see results). To assess whether this extraction technique could be applied to various other biological fluids, CSFs (4), pericardial aspirates (5) and a lung biopsy were also analysed. DNA was extracted from 2 ml of each CSF and each pericardial aspirate. A further 2 ml aliquot of each CSF and each pericardial aspirate was examined by microscopy and bacteriological culture. The lung biopsy was first cut

into small fragments and thereafter the DNA extracted by the standard method (7.3.5).

7.5 RESULTS AND DISCUSSION

7.5.1 Assessment of Supernatent Fluid

The results of these experiments are summarised in Table 7.1. PCR was performed on DNA extracts of the supernatant fluid and the pellet of two pleural fluids. In both pleural fluids the 336 bp fragment was successfully amplified from both the supernatant fluid as well as the extracted pellet. The supernatant fluids were not subjected to a lysis procedure and the amplified products must therefore have been generated from free DNA. In any infectious process there must be a significant turnover of organisms so it would be reasonable to assume that bacterial degradation products (e.g. DNA) are released in significant quantity into the pleural fluid.

Due to its waxy coat the tubercle bacillus has a lower buoyant density than most bacteria and is therefore not easily pelleted by centrifugation. It is well established that even if 3000 to 4000 g for 20 minutes are used, culturable *M.tuberculosis* can still be recovered in the supernatant fluid (Ratnam and March, 1986). For this reason and because of the presence of free DNA, it is imperative that uncentrifuged fluids should be used for the preparation of DNA for PCR.

7.5.2 Use of Proteinase K

The results of these experiments are summarised in Table 7.1. In all three pleural fluids where proteinase K was used no PCR products were generated. Furthermore, no PCR products were generated from five

EXPERIMENT	SPECIMEN TYPE	NUMBER OF SPECIMENS	PCR RESULT
Assessment of Supernatant Fluid (7.2)	Pleural Fluid Supernatant	2	2 +ve
	Pleural Fluid Pellet	2 (same specimens as above)	2 +ve
Use of Proteinase K (7.3.1 and 7.3.2)	Pleural fluid (proteinase K used)	3	3-ve
	Pleural biopsy (proteinase K used)	5	5-ve
	Pleural biopsy (proteinase K not used)	3	3+ve
Removal of Inhibitors (7.3.3)	Pleural biopsy (3 of above 5 in which proteinase K was used)	3	3+ve

Table 7.1: Summary of Results of Sections 7.2, 7.3.1, 7.3.2 and 7.3.3

All pleural fluids and pleural biopsies used showed good growth of *M.tuberculosis* on conventional media

pleural biopsies which has been treated with proteinase K. However, in 3 pleural biopsies where no proteinase K was used, the 336 bp product was amplified in each instance. Furthermore, 3 of the proteinase K treated pleural biopsies were subjected to gel filtration (Sephadex G50) and the 336 bp fragment was successfully generated from the eluate.

Although proteinase K successfully digested these protein rich fluids and therefore made extraction more manageable, it appeared that the use of this enzyme resulted in inhibitors which co-purify with the DNA. It is unlikely that there is residual proteinase K in the DNA extract which could inactivate the *Taq* polymerase. In the extraction procedure phenol would remove the protein and furthermore the DNA extract is heated to 95^{o}C for 10 minutes prior to the addition of *Taq* polymerase and PCR; a procedure which would denature proteinase K. It is possible that small peptides may be responsible for this inhibition. Proteinase K, however, is a well recognised constituent of DNA extraction procedures and inhibition of the PCR reaction by proteinase K has not previously been described. The nature of these proteinase K generated inhibitors in this study still therefore remains unanswered.

7.5.3 Polyethylene Glycol and Alcohol Precipitation

These experiments were conducted in order to assess the DNA precipitation efficiency of alcohol and PEG and results are shown in Table 7.2. From this small study it appears that the recovery of DNA after alcohol precipitation is greater than after PEG precipitation (90% cf 60%). Washing with 70% ethanol does not appear to effect the DNA recovery.

SPECIMEN	COUNTS	MEAN	PERCENT RECOVERY
Controls	101		
	113		
	236		
	140	147.5	
Alcohol precipitation	181		
	117		
Alcohol precipitation	125		
and ethanol washes	106	132.3	90%
PEG precipitation	79		
	73		
PEG precipitation and	71		
washes	119	87.5	60%

TABLE 7.2: Polyethylene Glycol and Alcohol Precipitation

Although the recovery was less with PEG precipitation, it was still felt that 60% recovery was adequate. It is, however, theoretically possible that if only a few copies of the desired DNA are present, these may not be precipitated. However, the extreme sensitivity of the PCR assay (as determined by the pleural fluid study in Chapter 8) would indicate that for practical purposes the DNA recoveries obtained by PEG precipitation are adequate.

7.5.4 **Extraction of DNA from various Clinical Specimen**

The results of PCR and conventional techniques for detection of *M.tuberculosis* in pleural fluids, pleural biopsies, cerebrospinal fluids, pericardial aspirates and a lung biopsy are shown in Table 7.3. In one CSF AFBs were demonstrated by microscopy and *M.tuberculosis* was grown by laboratory culture. In the 3 other CSFs *Streptococcus pneumoniae* was demonstrated by microscopy and by laboratory culture. In one pericardial aspirate *M.tuberculosis* was grown on laboratory media and in 4 others no diagnosis was established. Microscopy of the lung biopsy showed AFBs and culture yielded *M.tuberculosis*. PCR was positive in all specimens in which *M.tuberculosis* was detected and negative in those in which *M.tuberculosis* was not detected.

From this data it is clear that the 336 bp product can be amplified from a wide variety of clinical specimens. Furthermore there is no amplification in 3 CSFs (*Streptococcus pneumoniae* meningitis) and in 4 pericardial aspirates (unknown diagnosis) in which *M.tuberculosis* culture was negative. Thus there is no non-specific amplification and there does not appear to be problems of contamination with exogenous DNA.

Specimen type (number)	Number of specimens positive for *M.tuberculosis*			Number of specimens negative for *M.tuberculosis* (acid-fast stain and culture)
	Acid-fast stain	Bacterial culture	PCR	
Pleural fluid (2) (from 7.2)	0	2	2	0
Pleural biopsies (3) (from 7.3.2)	0	3	3	0
CSF (4)	1	1	1	3
Open lung biopsy (1)	1	1	1	0
Pericardial aspirates (5)	1	1	1	4

TABLE 7.3: Comparison of Different Detection Methods for *M.tuberculosis* in Clinical Specimens

It would appear that the assay is at least as sensitive as acid fast stain and judging from the pleural fluids and pleural biopsies perhaps as sensitive as laboratory culture. However, to adequately ascertain whether PCR has a lower level of detection than laboratory culture further investigations are required.

A stock solution of viable tubercle bacilli can be serially diluted and the dilutions examined by PCR and by laboratory culture. Such an experiment can be used to determine the efficiency of the extraction process and to compare PCR and laboratory culture. However, as the stock solution and dilutions are not true biological fluids, with all the inherent problems of protein content and inhibitors, such an approach may not be a true reflection of the sensitivity of the PCR assay for the detection of *M.tuberculosis* in body fluids.

A true comparison of laboratory culture and PCR can only be achieved by a comprehensive, well-controlled clinical trial. The selection of body fluid for such a trial is however very important. It may be anticipated that some PCR positive results may not be confirmed by laboratory culture and therefore these results could be regarded as false positives (contamination) or true positives. One therefore requires confirmatory evidence from other sources. Shankar and colleagues (1991) performed a trial (85 specimens) on CSFs. Only four of 34 cases of suspected tuberculous meningitis were confirmed by laboratory culture of the organism. The remainder were determined to be tuberculous on the basis of clinical data and other laboratory tests (ELISA, CSF chemistry). The interpretation of clinical data is often rather subjective and it would therefore be desirable to obtain evidence of *M.tuberculosis* from other sources.

A direct comparison of PCR and conventional laboratory techniques could however be made on pleural fluids and culture and histology of the biopsy could be used as confirmatory evidence. Such an approach would not be possible with other body fluids e.g. CSF and pericardial aspirates.

The details of such a trial using pleural fluids is described in the following chapter.

CHAPTER 8

PCR ANALYSIS OF PLEURAL FLUIDS

8.1 PATIENTS AND SPECIMEN HANDLING

Eighty four adult patients with pleural effusions were entered into a prospective study which was conducted in the 6 month period - December 1988 to June 1989. The patients were randomly selected from admissions to the general medical wards as well as patients from the radiotherapy wards with suspected malignant effusions. Aspiration of the fluid and biopsy of the pleura were performed using an Abrams needle.

Pleural fluid (10 ml) was centrifuged at 4000 rpm for 15 minutes (Heraeus Christ, Labofuge GL) and a Ziehl-Neelsen stain was performed on the pellet. The remainder of the pellet was divided into three equal parts and inoculated onto a Lowenstein-Jensen slope, a Stonebrink slope and into a Kirchner medium. These conventional media were incubated at 37^{o}C and were checked weekly for up to eight weeks for bacterial growth. Colonies were tested for Niacin production (Difco. Bact TB Niacin test strips) to identify them as *M.tuberculosis*.

A further 10 ml of fluid was centrifuged and the pellet was inoculated into a Bactec 12B bottle to which a standard antibiotic cocktail (polymyxin, amphotericin B, nalidixic acid, trimethoprim and azlocillin) was added. The medium was incubated at 37^{o}C for 3 days and thereafter the radiometric growth index was assessed once weekly for up to 8 weeks of incubation. Cultures with a positive growth index were examined for the presence of *M.tuberculosis*. In 39 of the

specimens, 5 ml of pleural fluid was directly inoculated into Bactec 13A medium and then incubated as above. The pleural fluid was analysed for protein content, lactate dehydrogenase (LDH) activity and adenosine deaminase (ADA) activity. Cytological analysis was also performed. Fluid (2 ml aliquots) was also stored at -20^{o}C for later PCR analysis.

Biopsies sent for mycobacterial culture were crushed in a Ten Broeck grinder and inoculated onto the conventional media and thereafter examined weekly for bacterial growth. Biopsies sent for histological analysis were considered positive for tuberculosis if granuloma, caseation or acid fast bacilli were demonstrated.

8.2 PREPARATION OF A 105 bp PROBE

In the previous chapters a 366 bp probe was used for hybridisation. This probe, however, includes the primer sequences and therefore hybridise with the primers. In order to avoid any background hybridisation signals and to make interpretation easier it was felt that a probe not containing the primer sequences would give better results. Furthermore it was anticipated that at a later stage the PCR products would be analysed by a dot blot method and the presence of primers in the dot would make interpretation extremely difficult.

The 336 bp fragment sequence was analysed for restriction enzyme sites which would generate a suitable internal sequence. *Ban*I was considered to be the most suitable candidate and digestion with this enzyme generates a 105 bp fragment (not including the primer sequences) and 2 other fragments, 146 bp and 85 bp, respectively. *Ban*I sites are shown in Figure 8.1.

Figure 8.1: Partial Sequence of the *MboI*/*SstII* Fragment of p36

```
  1  CGTTCTGGCC CTCCAGCAGC CCGCCGGCGC CACCGACGCC
 41  GACGCTGCCA GTCCCGGCTT TGCCAAATCG CGTTGCCGCC
 81  GTTGCCGCCC TGGCCCGTCA GCCAGGCGTT GCCGCCGTTA
121  CCGCCGCTAC CACCCGTTTG CCTGCTATCG TGGCACCGCC
161  GTCGCCGCCG TTGCCGCCCT CGCCGACCAA CCCGGCCTTG
201  CCGCCCACGC CGCCGTTTCC GCCGTCGCNG TTGTCGCTCT
241  CCCCGCCGGC GCCGCCGGCG CCGCCGGAAC CGTAGAACGT
281  GCCGGCGTTA CCGCCGGCCC CGCCGCGACC ACCACTTAGG
321  TACCGGTGCC GCCGCTGCCG CCGGCGCCGC CAGAACCGAC
361  CAACCCGCCG ATACCGCCGT CGCCGCCGTC GCCGCCGAAC
401  CCGCCGAACC CGCCGGCTCC GCCGGCCCCA CCGGTGCCGT
441  ATAGCAGGCC GGCATTACCG CCGTGACCAC CGTGGCCAGC
481  GACAGTGGCG ACTCCGCCTG CACCGCCGGC CCCGCCCGAG
521  CCGAAGACCG TGCCGCCGGC GCCGCCCGCG CCGCCGTCGC
561  CGCCGGCAGC ACTGCCGAAG CCACCGTGCC CGCCGGCCCC
601  GCCGACGCCA AACAGCCCGC CGCTGCCGCC GTCGCCGCCG
641  GTCCCACCGG TGTCGCCGCA AGCCACCGAC GCCGCCCGAG
681  CCGCCGCCCG GGCCGAACAG CCCGCCGGCC CCGCCAGCCC
721  CGCCGGTCCC ACCGGTGTCG CCGAAGCCGC CGGCGCCGCC
761  GGCACCGCCG GCGCCGAACA GCCCACCGAC CCCGCCCGCA
801  CCGCCGTTGC CACCGGCGGC CTGGGCGCCG CCGCCGAGGG
841  CGGCGCCGCC TACGCCGCCG GCCCCGCCCG CGCCGAACAG
881  CCCACCGGCG CCGCCGGCCC CGCCGGCACC CCCAGCGGCA
921  TCGAAGAAAG GTGGTGAACC CGCCGTTGCC ACCGGA
```

The 366 bp fragment extends from a *KpnI* restriction site (◆) at base 323 to a *SmaI* restriction site (*) at base 689. The target for DNA amplification is a 336 bp fragment extending from base 349 to base 684. The primer binding sites (25 bp each) are underlined. The *BanI* restriction sites are also indicated (↓) and digestion of the target fragment with this enzyme will yield an 85 bp, a 105 bp and a 146 bp fragment.

The 336 bp fragment was first amplified from p366 using the Cetus buffer system described in section 5.2. After electrophoresis, the 336 bp fragment was cut from the gel and purified by electroelution. Four micrograms of the 336 bp fragment were then digested with *Ban*I in the following digestion mixture: 12 μl DNA, 6 μl*Ban*I (10 units/μl, 4 μl buffer (Boehringer. Buffer A) and 18 μl sterile distilled water. The mixture was incubated at 50^{o}C for two and a half hours after which the fragments were separated by agarose gel electrophoresis and purified by electroelution. The 105 bp fragment was then radiolabelled as previously described (Appendix B7).

8.3 **ANALYSIS BY PCR**

Pleural fluids (2 ml) were numbered and processed in batches of 8-16 specimens which included a negative control (extraction control). The extraction and purification procedures as well as the steps taken to minimise contamination, are as described in the standard method in section 7.3.5. DNA preparations were finally dissolved in 40 μl sterile distilled water. The reaction mixture for PCR was also prepared in batches with enough reagent for 8-16 assays. Aliquots of 80 μl were dispensed into separate reaction tubes prior to adding the sample DNA. The DNA preparations (20 μl) or sterile distilled water (20 μl. Reagent control) were heated to 95^{o}C for 10 minutes, cooled on ice and then added to the reaction mixture to give a final volume of 100 μl. The reaction mixtures contained 10% dimethyl sulphoxide, 50 mM KCL, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μMoles of each dNTP, 1 - 2 μMoles of each of the oligonucleotide primers and 2.5 units of *Taq* polymerase (Cetus).

Thermal cycling (30 cycles) at 95°C for 1 minute and at 70°C for 2 minutes was then performed in the custom-made machine described in Chapter 6. Following amplification, the DNA was concentrated by alcohol precipitation and dissolved in 30 µl of sterile distilled water. Fifteen microlitres was then analysed by agarose gel electrophoresis followed by transfer of DNA and hybridisation with the radiolabelled 105 bp probe. Transfer of DNA for all specimens in this trial was performed by a modified alkaline transfer method (Reed and Mann, 1985. Appendix B6). These authors claimed that the alkaline transfer of DNA gave better resolution and a ten-fold increase in sensitivity of subsequent hybridisation analysis. The results of the PCR were recorded without knowledge of the culture results. When all analyses were complete the data were compared.

8.4 -RESULTS

PCR, using p366 as template, yielded the desired 336 bp fragment (Figure 8.2a). Restriction enzyme digestion of the 336 bp fragment with *BanI* resulted in the 3 expected fragments (146 bp, 105 bp and 85 bp) which together with the gel purified 105 bp fragment are demonstrated in Figure 8.2a. An autoradiograph of the hybridisation of the 336 bp fragment, the *BanI* digest and the 105 bp fragment with the radiolabelled 105 bp fragment are shown in Figure 8.2b. The *BanI* digest is incomplete (Lane 3) as evidenced by the strong signals from some larger fragments. There is also cross-hybridisation with the 146 bp and the 85 bp fragment. The probe is, however, pure as indicated by the single strong signal in Lane 4.

Figure 8.2: Preparation of a 105 bp Fragment

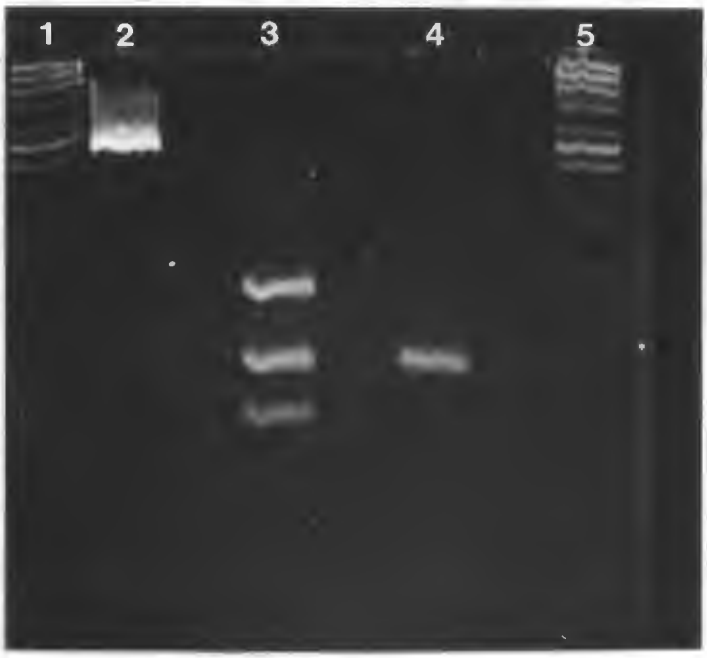


Figure 8.2a: 4% ethidium bromide stained gel

Lane 1: Molecular weight marker (*Hae*III digest of pBR322). Lane 2: Gel purified 336 bp fragment. Lane 3: *Ban*I digest of 336 bp fragment. Lane 4: Gel purified 105 bp fragment. Lane 5: Molecular weight marker (*Hae*III digest of pBR322).

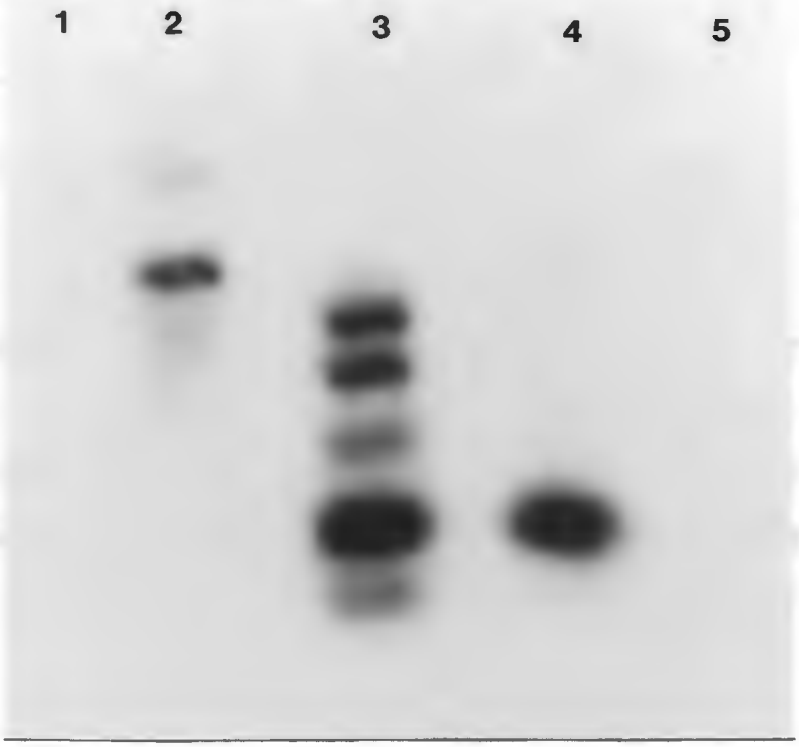


Figure 8.2b: Autoradiograph following transfer of DNA and hybridisation with the radiolabelled 105 bp fragment.
Lanes 1 to 5 as in 8.2a

An example of the PCR analysis of 7 pleural fluids is shown in Figure 8.3. In only 10 of the pleural fluids was the 336 bp fragment identified following ethidium bromide staining and UV transillumination of the agarose gel; autoradiography being necessary for detection of the fragment in the majority of cases. The autoradiographs alone were used to identify positive tests. The primers are not present in the autoradiographs due to the use of a probe directed against the central region of the amplified product.

The 84 patients were divided into 5 groups according to the results of conventional laboratory detection methods and clinical data i.e. Group I: *M.tuberculosis* detected by culture of pleural fluid; Group II: *M.tuberculosis* detected by culture of the pleural biopsy (Group I excluded); Group III: histological evidence of *M.tuberculosis* (Group I and II excluded); Group IV: clinical or extrapulmonary evidence of active tuberculosis; Group V: no evidence of active *M.tuberculosis* infection. In none of the pleural fluids could acid fast bacilli be detected by Ziehl-Neelsen staining. The full results of culture, histology (groups I to III) and clinical diagnosis (groups IV and V) of each patient together with the PCR results are given in Table 8.1 and are summarised in Table 8.2. In groups IV and V, in which no laboratory evidence of *M.tuberculosis* could be demonstrated, *M.tuberculosis* needed to be actively looked for (group IV) or excluded (group V). For this reason a thorough assessment of each patients history and clinical details was made and is presented in the form of clinical summaries in Appendix C. The conclusions reached in those summaries are adequately documented in Table 8.1.

Figure 8.3: PCR analysis of Pleural Fluids

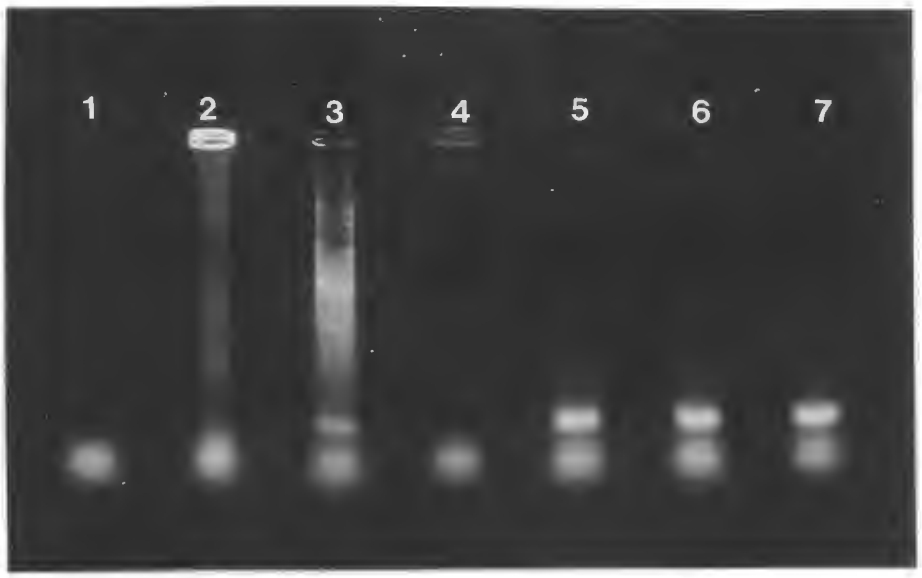


Figure 8.3a: Ethidium bromide stained gel of products obtained after amplification of DNA extracts from 7 selected pleural fluids. In most cases no products can be visualised. Only in 10 cases can the 336 bp fragment be seen (none in this selection). In some cases (Lanes 2 and 3) smears or vague bands are obtained.

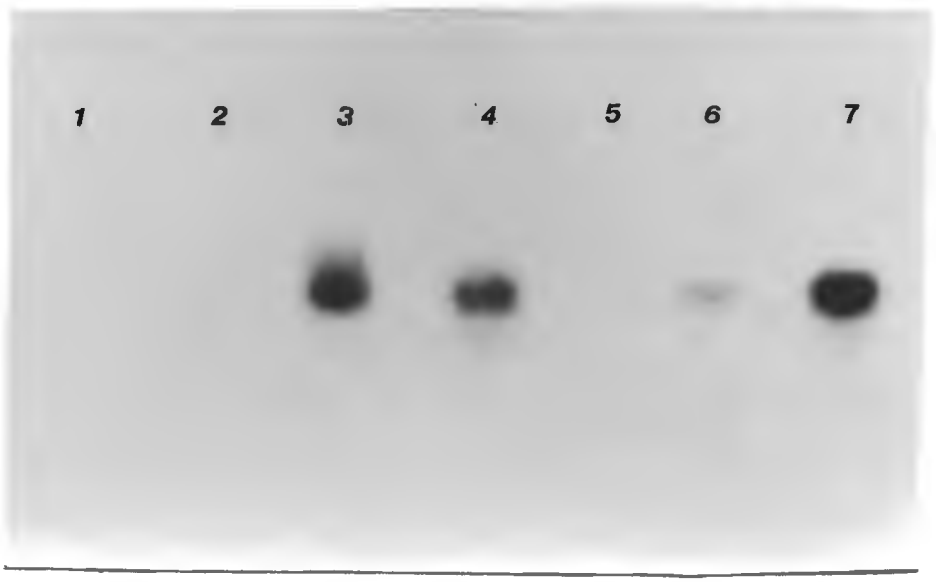


Figure 8.3b: Autoradiograph following transfer of DNA and hybridisation with the radiolabelled 105 bp fragment. Positive signals are obtained in Lane 3, 4, 6 and 7 and represent the 336 bp fragment.

Table 8.1

COMPARISON OF PCR WITH CONVENTIONAL PROCEDURES

	PATIENT NUMBER	FLUID CULTURE	BIOPSY CULTURE	HISTOLOGY		PCR
				AFB	GRANULOMA/ CESEATION	
GROUP I	1	+	+	+	+	+
	2	+	+	-	+	+
	3	+	+	-	+	+
	4	+	+	+	+	+
	5	+	+	-	+	-
	6	+	+	+	+	+
	7	+	+	-	+	+
	8	+	+	+	+	+
	9	+	+	+	+	+
	10	+	+	+	+	+
	11	+	+	-	+	+
	12	+	+	-	-	+
	13	+	No biopsy for culture	No pleura		+
	14	+	-	-	-	+
	15	+	+	-	+	-
	16	+	+	+	+	-
	17	+	+	+	+	+
	18	+	+	+	+	+
	19	+	+	-	+	+
	20	+	-	+	+	+
	21	+	-	-	+	+
	22	+	-	No pleura		+
	23	+	+	No pleura		+
	24	+	-	-	-	-
	25	+	+	-	+	+
	26	+	+	+	+	+
	27	+	+	+	+	+
	28	+	+	-	+	+
Group II	29	-	+	+	+	+
	30	-	+	-	+	+
	31	-	+	+	+	+
	32	-	+	+	+	+
	33	-	+	No pleura		+
	34	-	+	+	+	+
	35	-	+	+	+	+
	36	-	+	+	+	+
	37	-	+	+	+	+
	38	-	+	+	+	+
	39	-	+	-	+	+
	40	-	+	-	+	+
	41	-	+	+	+	+
	42	-	+	+	+	+
	43	-	+	+	+	-
Group III	44	-	-	-	+	-
	45	-	-	-	+	-
	46	-	-	+	+	+
	47	-	-	-	+	+
	48	-	-	-	+	-

Table 8.1 continued

	PATIENT NUMBER	CLINICAL DETAILS	PCR
Group IV	49	Laboratory culture of *M.tuberculosis* from an aspirated anterior chest lump and a supraclavicular lymph node	+
	50	Bilateral cervical and axillary lymphadenopathy. Casseous material seen in aspiration biopsy of one node	-
	51	No laboratory evidence of *M.tuberculosis*. Clinical features highly suggestive of tuberculosis	+
	52	As in 51	+
	53	As in 51	-
Group V	54	Carcinoma of the bronchus. No evidence of active tuberculosis. Previous history of tuberculous meningitis	+
	55	Metastatic breast carcinoma	+
	56	Metastatic breast carcinoma	+
	57	Metastatic carcinoma. Unknown primary	+
	58	Metastatic breast carcinoma	+
	59	Metastatic carcinoma. Unknown primary	+
	60	Adenocarcinoma of the lung	-
	61	Metastatic squamous carcinoma	-
	62	Metastatic tonsilar carcinoma	-
	63	Carcinoma of the lung	-
	64	Metastatic carcinoma of the cervix	-
	65	Metastatic carcinoma of the breast	-
	66	Squamous carcinoma of the lung	-
	67	Metastatic carcinoma. Unknown primary	-
	68	Metastatic Hodgkins lymphoma	-
	69	Metastatic breast carcinoma	-
	70	Metastatic carcinoma of the lung	-
	71	Metastatic carcinoma. Unknown primary	-
	72	Poorly differentiated carcinoma	-
	73	Parapneumonic pleural effusion	-
	74	Parapneumonic pleural effusion	-

Table 8.1 continued

	PATIENT NUMBER	CLINICAL DETAILS	PCR
Group V	75	Parapneumonic pleural effusion	-
	76	Empyaema	-
	77	Eosinophilic pulmonary infiltrate	-
	78	Amoebic liver abscess complicated by pleural effusion	-
	79	Congestive cardiac failure	-
	80	Congestive cardiac failure with an exudative effusion. CT scan of chest shows foci of calcification in lung and hilar nodes. Highly suggestive of old tuberculosis	+
	81	Pneumothorax	-
	82	Undiagnosed. Suspected malignancy	-
	83	Undiagnosed. Suspected malignancy	-
	84	Undiagnosed. Suspected malignancy	-

GROUP	TOTAL NO. OF PATIENTS	FLUID CULTURE POSITIVE	BIOPSY CULTURE POSITIVE	HISTOLOGICAL EVIDENCE	PCR +ve
I	28	28	22	22	24
II	15	0	15	14	14
III	5	0	0	5	2
IV	5	0	0	0	3
V	31	0	0[i]	0	7
Total	84	28	37	41	50

[i] As these patients were strongly suspected of having malignant effusions, biopsy was not performed in 5 cases.

Table 8.2: Comparison of PCR with Conventional Procedures

The fluid culture procedures included the use of conventional culture media and Bactec 12B and 13A media. No comparison of these culture methods is made as this is described elsewhere (Maartens and Bateman, 1990). The results of fluid protein, LDH and ADA activity and cytological analyses are described and analysed elsewhere (Maartens and Bateman 1990). This data can, however, be found in the clinical summaries in Appendix C.

Of the 84 patients, 53 (1-53) had laboratory or clinical evidence of active *M.tuberculosis* infection and were placed on antituberculosis treatment. In 52.8% (28/53) *M.tuberculosis* was detected by culture of the pleural fluid, 69.8% (37/53) by culture of the biopsy and 77.3% (41/53) by histology of the biopsy. PCR detected *M.tuberculosis* in 81.1% (43/53) of these patients. Statistical analysis reveals that the 81% yield by PCR was significantly higher than the 52.8% for fluid culture ($p<0.01$ by Mc Nemar's test; 95% confidence intervals of the difference in yield, 12%-44%). It was similar to that of pleural biopsy histology ($p=0.8$; 95% confidence intervals of the difference in yield, -11% to 19%) and not significantly better than the 69.8% yield of the biopsy culture ($p=0.18$; 95% confidence intervals of the difference in yield, -2% to 24%). *M.tuberculosis* was also detected in 6 patients with malignant effusions, one of whom previously had tuberculous meningitis (Patient 54). A further patient in Group V (Patient 80) had an exudative effusion and evidence suggestive of previous pulmonary tuberculosis. The PCR assay has a specificity for active disease of 0.78, a positive predictive value of 86% and a negative predictive value of 70.6%. Culture of fluid has a negative predictive value of 55.4%.

DISCUSSION

The efficiency of detection of *M.tuberculosis* in pleural fluids by conventional methods (52.8%) is within the range of 25-70% described in similar studies (see section 1.1.9). The efficiency of detection of *M.tuberculosis* in pleural fluids by PCR (81.1%) is significantly greater than pleural fluid culture and also appears to be superior to culture of the biopsy (69.8%).

The greater sensitivity of PCR as compared with culture results not only from the inherent sensitivity of the method but also from the mechanism of formation of tuberculous effusions. Because pleural effusions in tuberculosis can occur on an immunologic basis (Sahn 1988), there may be relatively few bacteria located in the pleura and none present in the aspirated fluid resulting in a negative laboratory culture. On the other hand, the effusions may contain *M.tuberculosis* degradation products, including DNA, which can be detected by PCR.

There are other factors which need further consideration. The first concerns the 4 PCR negative results within Group I. The presence of inhibitors or the high protein content of tuberculous pleural fluids may be responsible for inhibition of the assay. Inhibition of the PCR assay is well described (see section 1.3.4). Unfortunately in this study there was no remaining specimen and removal of inhibitors by gel filtration (Sephadex G50) was therefore not possible. It should be noted that the aspirate volume was smaller for the PCR assay than for culture and larger sample volumes may have resulted in positive assays in these cases.

A second problem is that of the positive results in patients without active tuberculosis. There were 6 patients with malignant effusions who had positive PCR results. Of these, a patient with carcinoma of the bronchus did not appear to have active tuberculosis at the time of this study. He did, however, have a history of previous tuberculous meningitis and in view of the known sensitivity of PCR, this result may represent the detection of latent, disseminated tuberculosis. A seventh patient in Group V had congestive cardiac failure, but since his pleural effusion was exudative, this suggests an infective etiology. Also, the chest CT scan of this patient was strongly suggestive of previous tuberculosis. For the purpose of this study, these positive PCR results were regarded as false positives. The prevalence of tuberculosis in our community needs to be taken into account however. The official, annual, notified case rate is 350 per 100 000 (Yach, 1987) but due to the uncertainty of case finding and under notification, a figure of 1 500 per 100 000 is probably a more accurate reflection (Kleeberg, 1982). It is feasible that these false positives may represent latent infections or even reactivation due to the immunosuppressive nature of the underlying carcinoma.

Another explanation is that these results represent DNA contamination, a constant problem with PCR method (see section 1.3.5). This is unlikely as strict procedures were employed to eliminate this problem, and extraction and reagent controls were always negative. Subsequent to this study a number of procedures have been described or suggested which may help to eliminate potential sources of contamination. These include direct inoculation of fluid at the bedside; UV illumination of buffers prior to PCR analysis (Sarkar and Sommer, 1990); the use

of nested primers; or the amplification of 2 or more separate target sequences in the same assay.

If our hypothesis that latent organisms are responsible for the positive PCR results within the non-tuberculous group is correct, then PCR may be of greatest value in populations with a low prevalence of tuberculosis. The presence of latent infection raises a number of theoretical and practical issues such as whether all patients in whom PCR is positive require anti-tuberculous therapy. The prophylactic treatment of patients without evidence for active disease may be acceptable since many may have latent tuberculosis and may develop active disease as a result of other conditions such as advanced malignancy.

This study has demonstrated that PCR is more sensitive than current diagnostic methods but the place of PCR in the diagnosis of tuberculous pleural effusions in high prevalence areas requires further definition.

CHAPTER 9

CONCLUSIONS

The well known problems associated with conventional detection techniques for the detection of *M.tuberculosis* stress the importance of establishing new, rapid and sensitive detection methods. In this thesis a PCR assay for *M.tuberculosis* has been described which fulfills these requirements. The assay is extremely sensitive and can detect the amount of chromosomal DNA present in less than 10 organisms. This sensitivity is compatible with other published PCR assays for *M.tuberculosis*. The assay was shown to be entirely specific for *M.tuberculosis* and could also distinguish *M.bovis*BCG. The assay is also extremely rapid. Extraction and PCR can be performed in 1 day but further hybridisation and autoradiography required a further 2 days. The assay has been used for a wide spectrum of clinical specimens and is therefore very adaptable. The extreme sensitivity demonstrated using purified DNA was confirmed in a comprehensive pleural fluid study where the PCR assay was shown to be more sensitive than laboratory culture.

In this study a number of problems and relevant issues were encountered and these shall be briefly discussed.

A 366 bp fragment which is repeated several times in the chromosome of *M.tuberculosis* was chosen as the target sequence for PCR. However, it was clearly demonstrated that certain regions of this target sequence were suitable for primer annealing but that others were not. It is believed that the GC rich nature and the presence of direct and inverted repeat sequences within the target may be responsible for

inadequate primer binding, due to the formation of secondary structures in the single-stranded (denatured) DNA.

The optimisation of the PCR assay (Chapter 4) highlighted a number of features possibly related to the GC rich DNA. DMSO was absolutely essential for successful amplification. It has been observed that the inclusion of 10% DMSO facilitates certain PCR assays (Gelfand, 1989) but it is not clear which parameters of PCR are affected. DMSO may affect the Tm of the primers, the thermal activity profile of the *Taq* polymerase or the degree of strand separation achieved at a particular denaturation temperature. However, of the PCR assays described for the Mycobacteria (see section 1.3.6.2) none use DMSO in the buffer system and this requirement therefore appears to be a property of the sequence chosen in this assay.

The optimum primer annealing temperature for the PCR assay was 70°C. At lower temperatures there was non-specific binding of primers resulting in non-specific product while at higher temperatures (75°C) PCR was less efficient. The non-specific binding is most likly due to the presence of similar sequences in the *M.tuberculosis* chromosome while the ability to anneal at 70°C is due to the high Tm of the GC rich primer sequences. High annealing temperatures also allow for a much simplified two-step temperature cycle. The use of high annealing temperatures is also described for other *M.tuberculosis* and *M.leprae* PCR assays. Plikaytis and colleagues (1990) use a two-step cycle (94°C and 68°C) for the *M.leprae* assay and Eisenach and colleagues (1990) use a high annealing temperature but a three temperature cycle (94°C, 68°C and 72°C). Other authors use lower annealing temperatures

(50°C Hance *et al.*, 1989 and 55°C Woods and Cole, 1989) and are able to generate specific PCR product. Patel and colleagues (1990) however use annealing temperatures of 37°C or 50°C and demonstrated the presence of a number of non-specific PCR products.

Experiments dealing with the extraction and purification of DNA from clinical specimens (Chapter 7) resulted in a number of important observations. The fact that free DNA is present in the supernatant fluid is an essential consideration when any PCR work is being considered. This may be particularly relevant in partially treated patients or if harsh decontamination procedures are used. The increased sensitivity of PCR over laboratory culture (Chapter 8) is partly due to the detection free DNA by PCR.

The problem of inhibitors also remains a problem, particularly when extraction of DNA from clinical specimens was performed. Proteinase K was excluded from the standard procedure because experimental evidence indicated the presence of inhibitors if this enzyme was used. Furthermore PEG was used for DNA precipitation prior to PCR because evidence indicated that this resulted in "pure" DNA. However, even using these precautions, the four false negative results seen in patients with positive fluid culture (Chapter 8) are possibly due to inhibitors. A practical problem possibly associated with the extraction procedure and the presence of inhibitors is the inability to visualise PCR products on ethidium bromide stained gels in the majority of cases (Chapter 8). This may be due to the fact that the amount of starting DNA is extremely low (i.e. less than 1 pg) but may also be due to the fact that amplification is not optimum. A clue to the latter possibility is that when a two-step nested primer approach

is used, as little 50 fg of purified *M.tuberculosis* DNA can be detected by agarose gels but if this method is used on DNA extracts of clinical samples hybridisation still needs to be employed (personal communication). The problem of inhibitors of PCR is an issue which still requires further investigation.

Chapter 8 deals with the comparison of PCR with conventional detection techniques in a comprehensive clinical trial. The conclusions of this study indicate that PCR is more sensitive than laboratory culture. However, due to the presence of PCR positive results in patients without active tuberculosis the use of this assay for routine diagnostic work still requires further definition. This study was conducted in a region of South Africa which has a particularly high prevalence of *M.tuberculosis* infections and perhaps the PCR positive results may be due to the reactivation of latent disease in patients with advanced malignancy. For this reason PCR may be more useful in low prevalence areas or in body fluids which are further removed and selectively protected from the primary pulmonary focus. For example, involvement of the CSF requires haematogenous spread and crossing of the blood brain barrier.

Because of the extreme sensitivity of PCR this assay can possibly be utilised for the elucidation of a number of diagnostic and pathogenetic problems. PCR is an ideal candidate for the detection of *M.tuberculosis* in peripheral blood. The preparation of DNA from isolated peripheral monuclear cells and subsequent analysis by PCR can be contemplated. The detection of *M.tuberculosis* DNA in peripheral blood of patients with suspected tuberculosis would be a very convenient "serological" test. Such studies are at present underway

in our laboratory and initial observations indicate that *M.tuberculosis* can be detected in the peripheral blood of patients with suspected tuberculosis and also in healthy "patients" who have family members with active tuberculosis (personal communication).

Another possible application of the technique is for establishing historical evidence of the disease. PCR could be used on bones or possibly even fossilised bones. Workers at our Medical School have recently discovered in Namibia, bones from a group of Bushmen which show evidence of tuberculosis. Dating indicates that these Bushmen lived before the turn of the 20th century and detection of *M.tuberculosis* DNA by PCR in these bones could alter theories about the origins of tuberculosis in Southern Africa.

PCR may be used for investigating the pathogenesis of disease. One area where this is particularly pertinent is the question of latent disease and other forms of *M.tuberculosis*. This problem has again recently been highlighted by Khomenko (1987) with the observation of other forms of *M.tuberculosis* in treated patients. With PCR we now surely have the molecular tools to solve this question and hopefully thereafter to offer curative treatment.

A number of granulomatous disorders such as Crohns disease, sarcoidosis and ulcerative colitis have long been considered by some to have a mycobacterial aetiology (For review. Chiodini, 1989). If the reason for the inability to conclusively detect *M.tuberculosis* in diseased tissue is due to other forms of the organism or only a few organisms, then PCR can be used to solve this problem. However, these disorders may be due to known or unknown non-tuberculous Mycobacteria

and the specific *M.tuberculosis* PCR assays would therefore not be suitable for their detection. A genus specific PCR assay is therefore required. Such a PCR assay has recently been described (de Wit, 1991) and uses the same primers described in this thesis but utilises a lower annealing temperature (50^{o}C) and can distinguish *M.tuberculosis*, *M.avium-intracellulare* and *M.kansasii*. The use of this assay for the investigation of granulomatous diseases is being considered.

APPENDIX A

A1 Enzymes and Kits

*Ava*I	Boehringer
*Bam*HI	BRL
*Ban*I	Boehringer
*Hind*III	Boehringer
*Kpn*I	Boehringer
Proteinase K	Boehringer
*Sma*I	Boehringer
T_4 DNA ligase	Boehringer
Taq Polymerase	New England Biolabs and Cetus Corp.
Nick Translation Kit (No. 5000)	Amersham

A2 Chemicals, Reagents and Other Materials

Acetic Acid	BDH
Acetonitrile	BDH
Acrylamide	BDH
Agarose (SeaKem GTG)	FMC Bioproducts
Ammonium Acetate	Merck
Ammonium persulphate (APS)	Sigma
Ammonium sulphate	BDH
Ampicillin	Beechams
ATP	Sigma
Blotto (milk powder)	Carnation
Boric acid	Merck

Bovine serum albumin	Boehringer
Bromophenol blue	Sigma
Calcium Chloride	Merck
Ceasium chloride	Boehringer
Chloroform	BDH
Chromotography paper 3mm	Whatman
Deoxynucleotide triphosphates	Boehringer
Dimethyl sulphoxide	Merck
Ethylenediaminetetraacetic acid (EDTA)	Boehringer
Ethidium bromide	Merck
Ethanol	Merck
Fixer (AMFIX)	Maybaker
Formamide	BDH
Gelatin	BDH
Glucose	BDH
Glycerol	Merck
Hardener ('S' Type)	Maybaker
Hybond N	Amersham
Hydrochloric acid	Merck
8-Hydroxyquinolone	Merck
ITPG	BRL
Isoamyl alcohol	Merck
Isopropanol	BDH
Lysozyme	Boehringer
Lithium Chloride	Sigma
Magnesium chloride	Sigma
Magnesium sulphate	Merck

Manganous chloride	BDH
Methanol	Merck
2-Mercapto ethanol	Merck
3-[N-Morpholino] propanesulfonic acid (M.O.P.S.)	Sigma
Phenol	BDH
Polyethylene glycol	Sigma
Potassium acetate	Fluka
Potassium chloride	Merck
Sephadex G50	Pharmacia
Sodium chloride	Merck
Sodium citrate	BDH
Sodium dodecyl sulphate (SDS)	Sigma
Sodium deoxycholate	Hopkin and Williams
Sodium hydroxide	BDH
Sterile distilled water	BDH
Sucrose	BDH
TEMED	Sigma
Tris (hydroxymethyl) amino methane (Tris)	BDH
Triton	BDH
Urea	BDH
X-Gal	BRL
X-ray film (curix RPI)	Agfa
X-ray film developer (Ilford Phenisol)	

A3 Media and Solutions

Luria agar:
10 g trytone
5 g yeast
10 g NaCl
12 g Difco agar
pH 7.2
Total volume 1 litre

Luria broth:
10 g tryptone
5 g yeast
10 g NaCl
pH 7.4
Total volume 1 litre

Both agar and broth were sterilised at 115^{o}C for 15 minutes and stored at room temperature.

Lowenstein-Jensen and Stonebrink agar slopes and Kirchner liquid medium were prepared by the Department of Medical Microbiology, University of Cape Town.

Phenol: Phenol was melted at 57^{o}C. 8-Hydroxyquinolone was added to a final concentration of 0.1%. Repeated extractions with an equal volume of 1 mM Tris pH 8 were performed until the pH of the aqueous phase exceeded 7.6. 2-Mercaptoethanol was added to a final concentration of 0.2% and the phenol was stored under 0.1 M Tris pH 8 at 4^{o}C.

Ampicillin (25 mg/ml) was stored at -20^{o}C in aliquots and used at a final concentration of 25 ug/ml.

A4 Suppliers

Sigma	:	P O Box 14508, St Louis MO63178, USA
Merck	:	Frankfurter Strasse 250, D-6100, Darmstadt, Germany
BDH	:	Poole, England
Bio-Rad Labs	:	32nd and Griffin Ave, Richmond, California, 39804
FMC Corp	:	5 Maple Street, Rocklands, Maine, USA
Schleicher & Schuell	:	D-3354 Dassel, West Germany
Spectrum Medical Industries	:	60916 Terminal Annex, Los Angeles 90054
Hopkin & William Ltd	:	Chadwell Heath, Essex, England
Boehringer Mannheim GmbH	:	Biochemica, P O Box 310, 120, D-6800, Mannheim 31, West Germany
Pharmacia, Fine Chemicals	:	Uppsala, Sweden
Amersham	:	Little Chalfont, Buckinghamshire, England
BRL	:	Cowley Mill Trading Estate, Longbridge Way, Uxbridge, Middlesex, England

APPENDIX B

COMMONLY USED TECHNIQUES IN MOLECULAR BIOLOGY

B1 **PREPARATION OF COMPETENT CELLS** A single JM109 colony grown on a minimal medium was inoculated into 10 ml of modified Luria broth (Luria broth with $MgSO_4$ 10 mM, MgCl 10 mM and glucose 20 mM). The cells were incubated for approximately 18 hours at 37°C with aeration (1.8 - 2 x 10^2 rpm. Labline Orbit Environment Shaker). The cells were cooled on ice for 30 minutes and 2 ml of the culture mixed with 100 mls of pre-warmed modified Luria broth. The optical density (at 600 nm) of the mixture was adjusted to between 0.02 - 0.03 with additional Luria broth. Fifty millilitres of this mixture was placed in a 500 ml conical flask and incubated at 37°C for 1 to 2 hours with shaking (2.5 - 3.0 x 10^2 rpm) during which time the optical density was periodically measured. The optical density at 600 nm was allowed to reach 0.5. The culture was then placed on ice for 10 to 15 minutes and then dispensed into sterilised corex tubes and centrifuged at 2000 rpm for 12 minutes at 4°C in a JA20 rotor (Beckman Centrifuge).

After centrifugation the combined pellets were suspended in 33 mls of resuspension solution 1 (100 mM KCL, 50 mM $MnCl_2$, 30 mM potassium acetate (pH 7.5), 10 mM $CaCl_2$ and 15% glycerol). The cell suspension was centrifuged as above and the cells were suspended in 8 mls of resuspension solution 2 (10 mM M.O.P.S., 10 mM KCL, 75 mM $CaCl_2$, 15% glycerol and pH adjusted to 6.8).

The cells were then incubated on ice for 15 minutes and 200 µl aliquots were dispensed into plastic vials and snap frozen with alcohol and dry ice. The cells were stored at -70°C.

B2 TRANSFORMATION OF JM109 CELLS WITH PLASMID

Competent JM109 cells, stored at -70°C, were thawed slowly for 30 minutes on ice before transformation. One microlitre of a p36 or p366 solution (20 ng/µl) was added to 200 ul of competent cells and placed on ice for at least 40 minutes. As a transformation control circular pUC19 (10 µg) was added to competent cells. The cells were then heat shocked at 40°C for 2 minutes after which they were incubated with 1 ml of pre-warmed Luria broth at 37° for 1 hour for expression of the B lactamase gene encoding ampicillin resistance. Aliquots (50 µl or 100 µl) of the suspensions were then spread onto Luria agar plates containing ampicillin. After the liquid had been absorbed the plates were inverted and incubated at 37°C overnight.

Competence of the JM109 cells was assessed from the pUC19 transformation and the plates with colonies from the p36 or p366 transformation were wrapped in Saran wrap and stored at 4°C.

B3 LARGE SCALE PLASMID EXTRACTION

The method used for preparing large quantities of plasmid DNA was a modification of that described by Greenaway and Dale (1983). A single bacterial colony was picked from a Luria-agar plate containing ampicillin and inoculated into 10 ml of Luria broth containing ampicillin. This was incubated at 37°C with shaking for approximately 4 hours.

All 10 ml of the culture was inoculated into 200 ml of pre-warmed Luria broth (containing ampicillin). To maximize surface aeration the broth was divided into four 500 ml flasks (i.e. 50 ml in each) and incubated at 37^{o}C with shaking for 15 hours.

The cells were harvested by centrifugation at 6000 rpm for 15 minutes at 4^{o}C in a JA-10 rotor. All culture supernatant was removed.

The cells were suspended in 6 ml of 25% sucrose in 0.05 M Tris HCl pH 8.0 and transferred to a polypropylene centrifuge tube. The cells were maintained on ice.

Five to ten milligrams of powdered lysozyme was added to the cell suspension which was subsequently mixed by swirling on ice for 5-10 minutes. Six millilitres of 0.25 M Na_2 EDTA pH 8 was added followed by swirling on ice for 5 minutes.

Ten millilitres of Triton/DOC solution (1% Triton 100, 0.4% Sodium deoxycholate in 0.01 M Tris, 0.001 M Na_2 EDTA, pH 8) was rapidly added from a 10 ml pipette. Even mixing was ensured by drawing up and expelling three times. The mixture was left on ice for 20 to 30 minutes.

Cell debris and chromosomal DNA was pelleted by centrifugation at 10000 rpm for 2 hours at 4^{o}C in a JA20 rotor. Because the bacterial chromosome is attached to the cell membrane it will be pelleted together with the membrane in a detergent solution.

The cleared lysate (supernatant) was decanted into a plastic container, the volume made up to 10 ml with Triton/DOC solution and kept chilled prior to isopycnic gradient centrifugation.

Plasmid Purification by Isopycnic Gradient Centrifugation

Isopycnic gradient centrifugation is the separation of macromolecules according to their densities. For DNA separation a caesium chloride (CsCl) solution of 1.6 g/ml density is made.

9.5 g of CsCl was dissolved in the 10 ml of DNA solution (0.95 g/ml) and 0.2 ml of ethidium bromide (10 mg/ml) was added. The solution was transferred to polyallomer tubes which were heat sealed and centrifuged in a VTi65 rotor at 50 000 rpm for 15 hours at 20^{o}C.

The DNA attained an equilibrium position in the tube at a level corresponding to its density and was visualized under an ultraviolet light of 375 nm wavelength. Usually two bands were visible, the higher band corresponding to chromosomal and nicked plasmid DNA, and the lower band corresponding to covalently closed supercoiled plasmid DNA. The difference in density is due to the different amounts of ethidium bromide intercalation between supercoiled DNA (plasmid) and chromosomal DNA.

The plasmid was eluted through the side of the tube by puncturing both the top and the side of the tube. This procedure avoided the RNA contamination which occurs if elution takes place through the bottom of the tube.

Removal of Ethidium Bromide and Excess CsCl

Ethidium Bromide was removed by adding an equal volume of isoamyl alcohol, vortexing the tube and separating the two phases by a two-minute centrifugation step. The upper organic layer was discarded and the procedure was repeated until there was no pink colour visible in either phase.

The plasmid preparation was then dialysed in TE buffer (0.1 M Tris, 0.01 M EDTA pH 8). The buffer was changed twice at four hourly intervals and then left at 4°C overnight. After removing the DNA solution from the dialysis bags, 400 µl aliquots were dispensed into Eppendorf tubes and 1 tenth volume 4 M LiCl and 2.5 volumes of absolute alcohol were added. The DNA was stored in this precipitated form at -20°C.

For further use aliquots were removed from -20°C, centrifuged for 15 minutes in a microfuge, washed twice with 70% ethanol and resuspended in sterile distilled water. To determine the plasmid yield and purity, an aliquot was scanned from 220 nm to 300 nm on a Beckman DU-40 spectrophotometer.

B4 AGAROSE GEL ELECTROPHORESIS

Agarose gels (0.7%, 1.2%, 3% and 4%) were made by dissolving the appropriate amount of agarose in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). This was done in a microwave oven. Twenty microlitres of a stock solution of ethidium bromide (10 mg/ml) was added to 50 ml of dissolved agarose and the gel poured approximately 3 mm thick with the slot former already in position. Once the gel had set, the slot former was removed and the gel placed in a tank of TAE buffer containing ethidium bromide (100 µl stock per 200 ml TAE). After the samples were loaded into the wells a potential difference of 80 volts was usually applied for 1 and a half to 2 hours. After electrophoresis the fragments could be immediately visualised and the gel photographed. If necessary, the desired fragment was cut from the gel.

B5 **ELECTROELUTION**

A "Biotrap" together with membranes were obtained from Schleicher and Schuell and was used for all elutions of DNA from gels. The Biotrap was assembled according to manufacturers instructions.

The Biotrap was placed in an electrophoresis tank containing Tris-borate buffer (0.045 M Tris-borate, 0.001 M EDTA). The gel slice containing the desired DNA fragment was placed in the apparatus and a potential difference of 200 V was applied for 1 hour, after which the polarity was reversed for 20 seconds. The voltage was switched off, the DNA solution removed from the appropriate chamber and concentrated by precipitation with 1 tenth volume 3 M ammonium acetate and 2.5 volumes of absolute ethanol. The DNA pellet was washed twice with 70% ethanol, dried (SpeedVac Concentrator. Savant) and reconstituted in sterile distilled water.

B6 **DNA TRANSFER TO HYBOND-N-MEMBRANES**

Using a Neutral Transfer Buffer

DNA separated by agarose gel electrophoresis was transferred onto a Hybond-N-membrane by a modified method of Southern (1975). The DNA was first cut by shaking the gel in 0.25 M HCl for 20 minutes. The DNA was then denatured by shaking the gel in an excess volume (500 ml) of denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 30 minutes. This was repeated once. Neutralizing buffer (1.5 M NaCl, 0.5 M Tris-HCl ph 7.2, 0.001 M Na_2 EDTA) for 30 minutes. This was repeated twice. Thereafter, the DNA was transferred to a Hybond-N-membrane in a neutral transfer buffer (3 M NaCl, 0.2 M sodium citrate pH 7.0). The transfer was allowed to proceed overnight.

After transfer, the Hybond-N-membrane was air-dried, wrapped in Glad Wrap and UV-illuminated for 10 minutes (DNA side down) to irreversibly bind the DNA to the membrane.

Using an Alkaline Transfer Buffer

DNA separated by agarose gel electrophoresis was transferred onto a Hybond-N-membrane by a modified method of Reed and Mann (1985). The DNA in the gel was denatured by shaking the gel in an excess volume (500 ml) of denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 minutes. Thereafter the gel was placed in 500 ml of tranfer solution (1.5 M NaCl, 0.2 M NaOH) for 5 minutes. The DNA was then transferred to the Hybond-N-membrane in the transfer solution. The transfer was performed overnight and thereafter the Hybond-N-membrane air dried, wrapped in Glad Wrap and UV illuminated for 10 minutes.

B7 RADIOLABELLING DNA BY NICK TRANSLATION

Radiolabelling of DNA was performed in all cases with a Nick Translation Kit (Amersham No. N5000). Approximately 300 ng of the purified 366 bp or 105 bp fragment was used, to which was added 10 µl nucleotide buffer solution, 5 µl dCTP (α-^{32}P) and sterile distilled water to make the volume up to 45 µl. Finally 5 µl enzyme solution containing DNA polymerase I was added. The mixture was incubated at 15^{o}C for 2-3 hours in an "ambient" temperature waterbath (Techne Template TE-8A). The reaction was terminated by the addition of 5 µl xyelene cyanol dye. Forty-five microlitres of Nick Translation buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.1% SDS) was added to give a final volume of 100 µl.

The probe was then purified by separation in a Sephadex G50 spun column: A piece of siliconised glass wool was inserted into a 1 ml syringe. A small rubber ring was pushed down on top of the glass wool. The syringe was filled with Sephadex G50 (equilibrated in Nick Translation Buffer) and placed in a plastic tube and centrifuged at 1500 rpm for 5 minutes. The sample (100 µl) was loaded and the syringe centrifuged for 5 minutes. The sample was collected in a microfuge tube.

Before hybridisation the probe was denatured by heating it for 10 minutes at 100°C.

B8 **HYBRIDISATION**

To reduce non-specific binding of the probe to the membrane, the Hybond-N was placed in 40 ml of prehybridisation solution (0.25% Blotto, 6 x SSC), in a sealed plastic bag for at least 2 hours at 42°.

The fluid was then discarded and 20 ml of hybridisation solution (6 x SSC, 0.5% SDS, 50% Formamide), and the probe was added to the membrane. The bag was sealed and incubated with gentle shaking at 42°C overnight.

After hybridisation the membrane was washed to remove non-specific binding of the probe to the membrane. Two 45 minutes washes were performed with 500 ml of washing solution 1 (0.05 Blotto, 2 x SSC, 0.1% SDS) at room temperature. A further two washes were performed at 55° with 500 ml of washing solution II (0.1% SDS, 0.1 x SSC). The membrane was then sealed in a plastic bag and autoradiographed.

B9 **AUTORADIOGRAPHY**

An X-ray film (Curix RPI Agfa) was exposed to the membrane for a varying length of time (3 hours - 2 days) at -70°C. Under illumination of a red light only the film was developed for 3 minutes (Iford Phenisol). It was then placed in a 2% acetic acid stop bath for 1 minute and finally in fixer (AMFIX) containing hardener for 3 minutes. The film was then washed under tap water for approximately 15 minutes and air-dried.

B10 **EXTRACTION OF PLASMID DNA**

The method used for DNA preparation was a modification of the Birnboim and Doly procedure (1979). Single white colonies from the appropriate transformation plates were incubated with shaking at 37°C overnight in 4 ml of Luria broth containing ampicillin. An aliquot (1.5 ml) was centrifuged for 10 minutes in a microfuge. The pellet was resuspended in 180 µl of 50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl pH 8.0. Five to ten milligrams of powdered lysozyme was added to the tube, the contents mixed by vortexing and then incubated for 5 minutes at room temperature to allow the cells to lyse.

Four hundred microlitres of 0.2 M NaOH, 1% SDS was added, the tube was gently vortexed and left on ice for 5 minutes. The solution cleared at this stage as protein and DNA denatured and dissolved. Chromosomal DNA and protein were precipitated with 300 µl potassium acetate (3 M K, 5 M acetate) for 10 to 60 minutes on ice.

After centrifugation for 15 minutes 450 µl of isopropanol was added to 750 ul of the supernatant fluid and the plasmid DNA and RNA was pelleted by centrifugation for 5 minutes in a microfuge. The pellet

was washed in 70% ethanol and the pellet then dried in a vacuum. The DNA was dissolved in 200 µl TE plus 0.15 M NaCl. To this was added 200 µl buffered phenol and the contents vortexed. Two microlitres of chloroform/isoamylalcohol (24:1) were added, the tube vortexed and the phases separated by centrifugation for 2 minutes.

The upper aqueous phase was carefully removed and to it was added 2 and a half volumes of absolute ethanol. After 5 minutes incubation in dry ice (-70°C) the tube was spun for 5 minutes, the DNA pellet washed with 70% ethanol, vacuum dried and dissolved in 100 µl of sterile distilled water.

APPENDIX C

DETAILS OF PATIENTS IN GROUP IV AND V

C1 INTRODUCTION

There was no laboratory evidence of *M.tuberculosis* in patients 49 to 84 and therefore clinical evidence needed to be actively looked for (group IV) or actively excluded (group V). A thorough assessment was therefore made of each patients history and clinical data and is presented here in the form of clinical summaries.

No attempt is made to analyse statistically the pleural fluids chemistry data (i.e. ADA, LDH, Protein). This is described elsewhere (Maartens and Bateman, 1990). In brief, this ADA study demonstrated that the test had a specificity of 0.83 and sensitivity of 0.77. A value of 45 u/l was chosen as the cut-off value. Due to this low specificity, ADA levels in the present study are used only as confirmatory evidence and only in one case (patient 51) when the level was very high, was treatment commenced on the basis of the ADA result.

C2 CLINICAL SUMMARIES OF PATIENTS 49 TO 84

PATIENT 49

This 76 year old black male was admitted for investigation of a right-sided pleural effusion. He had a 2 month history of a productive cough, pleuritic chest pain and loss of weight. Examination revealed supraclavicular lymphadenopathy and a fluctuant mass over the anterior chest.

Investigations:

Chest X-ray: Right-sided pleural effusion. Ill-defined nodular opacities in both upper zones suggestive of tuberculosis.

Pleural fluid chemistry: LDH 197, Protein 47, ADA 71 u/l.

Pleural fluid cytology: No malignant cells. A lymphocytic reaction was present.

Pleural biopsy: Fibrous tissue with skeletal muscle and some organising fibrinous pleurisy.

Mantoux: Reactive (10 x 15 mm).

Aspiration of supraclavicular lymph node and anterior chest wall mass: Laboratory culture of *M.tuberculosis* from both sites.

In view of the positive culture results from the extrapulmonary sites the patient was commenced on anti-tuberculous treatment.

PATIENT 50

A 76 year old black female who presented with a 3 week history of a cough productive of white sputum, right-sided pleuritic chest pain, loss of weight and night sweats. Examination revealed bilateral cervical and axillary lymphadenopathy.

Investigations:

Chest X-ray: Large right-sided pleural effusion. Areas of pulmonary calcification and a diffuse interstitial infiltrate.

Pleural fluid chemistry: LDH 110, Protein 43, ADA 110

Pleural fluid cytology: Lymphocytic reaction.

Pleural biopsy: Organising fibrinous pleurisy.

Mantoux: Reactive (size not recorded)

Lymph node culture: Negative for *M.tuberculosis*

Lymph node histology: Two foci of caseous necrosis with a mild surrounding granulomatous response.

The patient was placed on anti-tuberculous treatment but returned 1 year later for investigation of the persistent right-sided pleural effusion. All investigations were repeated and were negative for *M.tuberculosis*.

PATIENT 51

A 65 year old black male with a two week history of malaise, loss of weight, dyspnoea, productive blood streaked cough and pleuritic chest pain.

Investigations:

Chest X-ray: Right-sided pleural effusion.

Pleural fluid chemistry: LDH 120 u/l, Protein 54 g/l, ADA 156 u/l

Pleural fluid cytology: Lymphocytic and mesothelial reaction.

Pleural biopsy: Necrotic inflammatory exudate with organising inflammatory granulation tissue.

Echocardiography: cardiomyopathy.

The patient was treated for cardiac failure and because of the markedly raised ADA as well as a suggestive history he was commenced on anti-tuberculous therapy. The patient was thereafter seen regularly in the Outpatients Department with some resolution of the pleural effusion.

PATIENT 52

A 50 year old black male who presented with a two week history of a dry cough, drenching night sweats, malaise and loss of weight. One of his six children was on anti-tuberculous treatment. Examination showed evidence of right-sided cardiac failure and a right-sided pleural effusion.

Investigations:

Chest X-ray: Large right-sided and small left-sided pleural effusion with no evidence of active tuberculosis.

Pleural fluid chemistry: LDH 348 u/l, Protein 69 g/l, ADA 55 u/l

Pleural fluid cytology: Lymphocytic and mesothelial reaction.

Pleural biopsy: Skeletal muscle and fibrofatty tissue.

Mantoux: Reactive (20 x 20 mm)

Transketolase: 20 umol/hr/gram Hb. (Reference range 15-20)

The patient was considered to have beri-beri although this was not chemically confirmed. He was treated with multivitamins but when he returned 5 months later (lost to follow-up) with no resolution of symptoms and after repeat negative pleural fluid aspiration, he was commenced on anti-tuberculous therapy.

PATIENT 53

A 51 year old coloured male who presented with a short history of intermittent chest pain associated with fever and night-time sweating. Examination revealed a right-sided pleural effusion which on drainage, was haemorrhagic.

Investigations:

Chest X-ray: Pleural effusion. After drainage, upper lobe volume loss with fibrotic shadowing was noted.

Pleural fluid chemistry: LDH 436 u/l, Protein 49 g/l, ADA 43 u/l

Pleural fluid cytology: Lymphocytic and mesothelial reaction.

Pleural biopsy: Reactive mesothelial cells.

Due to the moderately raised ADA levels and the lack of evidence of malignancy the patient was commenced on anti-tuberculous treatment.

PATIENT 54

A 65 year old white male with a background of heavy cigarette smoking presented with a 6 month history of recurrent episodes of mild haemoptysis, increasing dyspnoea, loss of weight and night sweats. In 1978 he was treated for tuberculous meningitis. On the same admission in 1978 he also had a chronic loculated empyaema but microbiological and histological investigation did not demonstrate *M.tuberculosis*.

Investigations:

Chest X-ray: Large left-sided pleural effusion. Collapse of left upper lobe and apical pneumothorax.

Bronchoscopy: Vascular tumour seen at end of left main bronchus. Brushings and biopsy were non-contributory.

Pleural fluid chemistry: LDH 228 u/l, Protein 48 g/l, ADA 19 u/l

Pleural fluid cytology: Non-malignant cells.

Pleural biopsy: Mainly fibrous tissue with some reactive mesothelial tissue and no evidence of malignancy.

The patient was commenced on palliative chemotherapy with regular drainage of effusion.

PATIENT 55

A 40 year old coloured female previously diagnosed (1987) with carcinoma of the left breast. She was treated with chemotherapy but in mid-1988 she developed brain metastases and received palliative radiotherapy. On this occasion she presented with dyspnoea and a productive cough and examination revealed a large left-sided pleural effusion.

Investigations:

Chest X-ray: Large left-sided pleural effusion.

Pleural fluid chemistry: ADA 17 u/l. No LDH or protein.

Pleural fluid cytology: Not performed.

Pleural biopsy: Metastatic carcinoma.

The patient returned home with palliative treatment.

PATIENT 56

A 57 year old coloured lady with a 1 year history of swelling of the right breast and a 1 week history of dyspnoea and a cough productive of white sputum. Clinical assessment indicated a stage T4 N2 carcinoma of the right breast.

Investigations:

Chest X-ray: Large right-sided pleural effusion. Underlying pathology difficult to assess.

Pleural fluid cytology: Malignant cells compatible with metastatic adenocarcinoma.

Pleural fluid chemistry: LDH 148 u/l, Protein 37 g/l, ADA 90 u/l.

The patient was given morphine and died at home.

PATIENT 57

A 70 year old coloured man who presented with a three week history of increasing dyspnoea and right-sided chest pain. Examination revealed bilateral cervical and axillary lymphadenopathy and a right-sided pleural effusion.

Investigations:

Chest X-ray: Large right-sided pleural effusion. Rib erosion.

Pleural fluid chemistry: LDH 1170 u/l, Protein 51.3 g/l, ADA 23 u/l.

Pleural fluid cytology: Numerous malignant cells.

Pleural biopsy: Metastatic carcinoma.

The patient died at home one month later.

PATIENT 58

A 66 year old white female with known breast carcinoma who presented with a right-sided pleural effusion.

Investigation:

Chest X-ray: Large right-sided pleural effusion.

Pleural fluid chemistry: LDH 1586 u/l, Protein 48 g/l, ADA 40 u/l

Pleural fluid cytology: A lymphocytic reaction.

Pleural biopsy: No evidence of malignancy. Skeletal muscle and fibroadipose tissue.

CT scan: Infiltration of pleura by tumour.

The effusion was drained twice and reaccumulated on each occasion. The patient died 2 months later.

PATIENT 59

A 68 year old white female who presented with nausea and vomiting, non-productive cough and loss of weight. Examination revealed hard right supraclavicular lymphadenopathy and right pleural effusion. Previously investigated (approximately 10 years ago) for "pleurisy" and all investigations for tuberculosis were negative.

Investigations:

Chest X-Ray: Cardiomegally. Paratracheal lymph nodes. Right mid-zone opacity. Right pleural effusion.

Pleural fluid chemistry: LDH 19 u/l, Protein 24 g/l, ADA 2 u/l

Pleural fluid cytology: Lymphocytic and mesothelial reaction.

Supraclavicular lymph node biopsy: Undifferentiated metastatic carcinoma.

The patient refused treatment and died at home 1 month later.

PATIENT 60

A 46 year old black male with a background of 15 years of employment in an asbestos factory presented with right upper quadrant and chest pain. Found to have cholecystitis and cholecystectomy was performed. While in the ward noted to have a small pleural effusion.

Investigations:

Chest X-ray: Small left-sided pleural effusion.

Pleural fluid chemistry: LDH 427 u/l, Protein 63 g/l, ADA 35 u/l

Pleural biopsy: Adenocarcinoma

The patient was given radiotherapy.

PATIENT 61

A 64 year old coloured male who presented with a 10 day history of dyspnoea and central chest pain.

Investigations:

Chest X-ray: Right-sided pleural effusion.

Pleural fluid chemistry: Protein 50 g/l, ADA 20 u/l, No LDH

Pleural fluid cytology: Highly atypical squamous cells.

Pleural biopsy: Metastatic carcinoma. Probably squamous in origin.

The patient was given palliative treatment.

PATIENT 62

A 69 year old white male who was diagnosed with carcinoma of the tonsil with nodal involvement in December 1988 and given radiotherapy. He presented on this occasion with a 1 month history of dyspnoea and orthopnoea.

Investigations:

Chest X-ray: Small right-sided pleural effusion.

Pleural fluid chemistry: ADA 8 u/l. No LDH or Protein.

Pleural fluid cytology: Not performed.

Pleural biopsy: Reactive mesothelium. No evidence of malignancy.

Cervical node biopsy: Metastatic Carcinoma

The patient was discharged with palliative treatment.

PATIENT 63

An 85 year old white female on treatment for a previously diagnosed carcinoma of the lung. Presented on this occasion with dyspnoea, hoarse voice, pleuritic chest pain and non-productive cough.

Investigation:

Chest X-ray: Massive left-side effusion.

Pleural fluid chemistry: LDH 166 u/l, Protein 36 g/l, ADA 10 u/l

Pleural fluid cytology: Normal mesothelial cells with no malignant cells.

Pleural biopsy: Not performed.

The patient deteriorated while in the ward and died suddenly 3 days after admission.

PATIENT 64

A 47 year old coloured female treated for carcinoma of the cervix in 1986. She presented on this occasion with dyspnoea, loss of appetite and left-sided pleuritic chest pain.

Investigations:

Chest X-ray: Small right-sided pleural effusion. No evidence of metastatic carcinoma.

Pleural fluid chemistry: LDH 439 u/l, Protein 52 g/l, ADA 10 u/l

Pleural fluid cytology: Malignant cells noted compatible with metastatic carcinoma of the cervix.

Pleural biopsy: Not performed.

The patient died 3 months later.

PATIENT 65

A 65 year old white female who was previously diagnosed (1985) with carcinoma of the breast and recently developed bony metastases. On this admission she presented with a left-sided pleural effusion.

Investigations:

Chest X-ray: Left pleural effusion.

Pleural fluid chemistry: ADA 19 u/l. No LDH and Protein.

Pleural fluid cytology: Clusters of degenerate atypical cells.

Pleural biopsy: Not performed.

The patient was discharged with palliative treatment.

PATIENT 66

A 68 year old black male who presented with a 3 months history of a productive cough, increasing shortness of breath and right-sided chest pain.

Investigations:

Chest X-ray: Large right-sided pleural effusion with some underlying opacification of the right lower zone.

Pleural fluid chemistry: LDH 967 u/l, Protein 64 g/l, ADA 50 u/l

Pleural fluid cytology: Malignant cells, compatible with a poorly differentiated squamous carcinoma.

Pleural biopsy: No evidence of malignancy. Fibrous tissue with some necrosis.

The patient deteriorated, was treated with morphine and died soon after.

PATIENT 67

A 76 year old coloured male in whom a T1 carcinoma of the bladder was fulgarated in 1974. He presented on this occasion with dehydration, dyspnoea and loss of weight and appetite.

Investigations:

Chest X-ray: Large right-sided pleural effusion with some suggestion of collapse of the right lung.

Pleural fluid chemistry: Protein 43 g/l, ADA 30 u/l, No LDH

Pleural fluid cytology: Malignant cells compatible with metastatic carcinoma.

Pleural biopsy: Metastatic carcinoma set within a desmoplastic stroma.

The patient was referred for radiotherapy but died soon afterwards.

PATIENT 68

A 76 year old white female diagnosed with a stage 4 low grade Hodgkins lymphoma in January 1989. Since then the disease progressed rapidly. Metastases were demonstrated in the stomach and mediastinal and supraclavicular nodes. She presented on this occasion with dyspnoea.

Investigations:

Chest X-ray: Large left-sided pleural effusion. Opacification at left base was noted after drainage.

Pleural fluid chemistry: LDH 637 u/l, Protein 27 g/l, ADA 13 u/l

Pleural fluid cytology: No malignant cells seen.

Pleural biopsy: On one edge of the biopsy was a cellular infiltrate compatible with lymphoma.

The patient presented one month later with evidence of a gastrointestinal perforation and died a few hours after admission.

PATIENT 69

A 68 year old coloured female diagnosed with carcinoma of the breast in 1977 and subsequent evidence of metastatic disease (in a thyroidectomy scar). She presented on this occasion with increasing dyspnoea.

Investigations:

Chest X-ray: Large right-sided and smaller left-sided pleural effusion. Ill-defined opacification related to the region of the horizontal fissure.

Pleural fluid chemistry: LDH 128 u/l, Protein 48 g/l, ADA 25 u/l

Pleural fluid cytology: Malignant cells compatible with metastatic breast carcinoma.

Pleural biopsy: Not performed.

She was transferred to a hospice for terminal care.

PATIENT 70

A 65 year old coloured female who presented with haemoptysis and mild weight loss.

Investigations:

Chest X-ray: Right-sided pleural effusion. "Cut-off" in the right lower lobe bronchus.

Pleural fluid chemistry: LDH 3845 u/l, Protein 60 g/l, ADA 44 u/l

Pleural fluid cytology: Lymphocytic reaction.

Pleural biopsy: Fibrous tissue, organising thrombus and granulation tissue. No evidence of malignancy.

Fine Needle Aspiration of Right Axillary Lymph Node: Undifferentiated squamous carcinoma.

With the diagnosis of carcinoma of the lung with metastases the patient was discharged for terminal care.

PATIENT 71

A 54 year old white male who present with right hip pain, dyspnoea, non-productive cough and left-sided pleuritic chest pain.

Investigations:

Chest X-ray: Left-sided pleural effusion and probable lymphangitis carcinomatosa.

Pleural fluid chemistry: LDH 1233 u/l, Protein 47 g/l, ADA 49 u/l

Pleural fluid cytology: Malignant cells, compatible with adenocarcinoma.

Pleural biopsy: Poorly differentiated adenocarcinoma.

He was given palliative radiotherapy for his right hip and sent to a hospice for terminal care.

PATIENT 72

A 42 year old coloured male who presented with a 2 month history of a non-productive cough, dyspnoea and loss of weight.

Investigations:

Chest X-ray: Large right-sided pleural effusion with cystic changes in the right apex.

Pleural fluid chemistry: LDH 7390 u/l, Protein 74 g/l, ADA 41 u/l

Pleural fluid cytology: Groups of highly atypical, degenerate cells very suspicious of malignancy.

Pleural biopsy: Poorly differentiated carcinoma.

The patient was referred to the Radiotherapy Department for treatment.

PATIENT 73

A 79 year old black female who was previously investigated for an interstitial lung disease ("hut" lung). She presented on this occasion, with a 2 week history of increasing dyspnoea, vague body aches and production of foul smelling sputum.

Investigations:

Chest X-ray: Consolidation of the right upper lobe with right-sided pleural effusion.

Pleural fluid chemistry: LDH 862 u/l, Protein 42 g/l, ADA 18 u/l

Pleural fluid cytology: No malignant cells seen.

Pleural fluid biopsy: Organising fibrinous pleurisy and reactive mesothelium.

Mantoux: Negative

The patient was treated with intravenous antibiotics and showed a good recovery. Diagnosis: Pneumonia with parapneumonic pleural effusion.

PATIENT 74

A 71 year old white male who presented with dyspnoea, productive cough and left-sided pleuritic chest pain.

Investigations:

Chest X-ray: Right lower lobe collapse with pleural effusion.

Pleural fluid chemistry: LDH 7995, Protein 46 g/l, ADA 75 u/l

Pleural fluid cytology: Lymphocytic reaction

Pleural biopsy: Fibrous reaction with reactive mesothelium and organising acute inflammatory exudate.

The patient was commenced on antibiotics and showed a slow, gradual response. Diagnosis: Collapse consolidation with pleural effusion.

PATIENT 75

A 64 year old black female with a background of osteoarthritis and non-steroidal anti-inflammatory use presented with a perforated peptic ulcer. During her post-operative period, she developed acute tubular necrosis and a left-sided basal pneumonia.

Investigations:

Chest X-ray: Left basal consolidation and effusion.

Pleural fluid chemistry: LDH 242 u/l, Protein 52 g/l, ADA 8 u/l

Pleural fluid cytology: Not performed.

Pleural biopsy: Reactive, non-specific process with organising fibrinous exudate.

The patient responded well to antibiotics and her ongoing problems are related to chronic renal failure and gastric ulcer. Diagnosis: Basal pneumonia with pleural effusion.

PATIENT 76

An 18 year old coloured male with epilepsy presented initially with a 5 day history of right-sided chest pain and a non-productive cough. Chest x-ray indicated a pneumonia and was treated with antibiotics but returned 1 week later due to an unsatisfactory response.

Investigations:

Chest X-ray: Right pleural effusion with patchy consolidation of the right middle lobe suggestive of an abscess.

Pleural fluid chemistry: LDH 4380 u/l, Protein 122, no ADA

Pleural fluid cytology: Not performed.

Pleural biopsy: Mixed chronic inflammatory reaction.

Aspiration of abscess: *Bacteroides*

Formal thoracotomy and drainage was performed and antibiotics were continued, and a dramatic recovery followed. Diagnosis: Empyaema.

PATIENT 77

A 21 year old black female who presented with a 3 day history of dyspnoea, pleuritic chest pain and a cough productive of mucoid sputum. Three other patients who live in the same dwelling also presented with similar symptoms.

Investigations:

Chest X-ray: Bilateral soft alveolar infiltrates and a right pleural effusion.

Pleural fluid chemistry: LDH 263 u/l, Protein 36 g/l, ADA 10 u/l

Pleural fluid cytology: No malignant cells.

Pleural biopsy: Reactive mesothelium and an inflammatory and fibrinous exudate.

Full blood count: Haemoglobin 10 g/dl, WCC 33.2, Platelets 103

Differential count: Eosinophils 66%, Neutrophils 21%, Lymphocytes 11%

A diagnosis of pulmonary infiltrative eosinophilia was made and the patient was successfully treated with steroids.

PATIENT 78

A 23 year old black male with a 1 month history of right-sided stabbing chest pain, loss of weight and night sweats. He was pyrexial, jaundiced and had a 3 cm hepatomegally.

Investigations:

Chest X-ray: Right-sided pleural effusion.

Pleural fluid chemistry: LDH 419 u/l, Protein 52 g/l, ADA 19 u/l

Pleural fluid cytology: Lymphocytic reaction.

Pleural biopsy: Reactive mesothelial cells with an acute inflammatory exudate.

Ultrasound Liver: 2 abscess. "Anchovy paste" aspirated under ultrasound guidance.

The patient was treated for amoebiasis and discharged for follow-up at the Day Hospital. Diagnosis: Amoebic liver abscess complicated by pleural effusion.

PATIENT 79

A 76 year old black male with a 3 month history of dyspnoea and night sweats who presented with severe bronchospasm.

Investigations:

Chest X-ray: Cardiomegally with a right-sided pleural effusion and ill-defined opacification in the right lower zone.

Pleural fluid chemistry: LDH 197 u/l, Protein 32 g/l, ADA 9 u/l

Pleural fluid cytology: Not performed.

Pleural fluid biopsy: Reactive mesothelium and fibrinous pleurisy.

A diagnosis of congestive cardiac failure was made and the patient was treated with bronchodilators and diuretics with a notable clearing of the effusion.

PATIENT 80

A 70 year old coloured male presented with a right hemiplegia due to a small thalamic infarct. On examination, he was found to have bilateral pleural effusions and hepatomegally.

Investigations:

Chest X-ray: Bilateral effusions suggestive of cardiac failure.

Pleural fluid chemistry: LDH 402 u/l, Protein 38 g/l, ADA 25 u/l

Pleural fluid cytology: Lymphocytic reaction.

Pleural biopsy: Organising fibrinous pleurisy.

CT scan chest: Calcification in the right apex and fibrotic changes in both upper lobes. Also calcification in paratracheal and hilar nodes.

The patient was considered to have congestive cardiac failure and was placed on anti-failure therapy. Follow-up 6 months later however only showed a mild decrease in the effusions.

PATIENT 81

A 25 year old coloured male who developed a sudden onset of severe left-sided chest pain and on examination was shown to have a tense pneumothorax. A chest drain was inserted and blood stained fluid was drained.

Investigations:

Chest X-ray: Pneumothorax with effusion.

Pleural fluid chemistry: LDH 482 u/l, Protein 48 g/l, ADA 10 u/l

Pleural fluid cytology: Lymphocytic reaction

Pleural biopsy: Chronic inflammatory reaction.

The fluid reaccumulated after a few days but the patient left the hospital and was lost to follow-up. Diagnosis: Pneumothorax of unknown cause.

PATIENT 82

A 56 year old black female who presented with a history of dyspnoea, right-sided chest pain, mild weight loss and some night time sweating.

Investigations:

Chest X-ray: Large left-sided and smaller right-sided pleural effusion.

Pleural fluid chemistry: LDH 5758 u/l, Protein 62 g/l, ADA 19 u/l

Pleural fluid cytology: Lymphocytic and mesothelial reaction.

Pleural biopsy: Fibrous tissue with organising fibrinous pleurisy.

Open biopsy: Fibrous plaques

Mantoux: Non-reactive.

Although investigations for tuberculosis were all negative, the patient was placed on anti-tuberculous treatment, as this was a possible reversible condition. Six months later however the patient returned with some deterioration of his condition. No diagnosis could be made and malignancy was suspected.

PATIENT 83

A 69 year old black male with a prior history of asbestos exposure was admitted with right-sided pleuritic chest pain, a cough productive of white sputum and loss of weight.

Investigations:

Chest X-ray: Large right-sided pleural effusion.

Pleural fluid chemistry: LDH 239 u/l, Protein 48 g/l, ADA 25 u/l

Pleural biopsy: Non-specific fibrinous inflammation. Also a seperate fibrous area consistent with a fibrous plaque. No asbestos bodies were seen.

Mantoux: Reactive (no record of size)

Although malignancy (possibly mesothelioma) was suspected, the patient was placed on anti-tuberculous treatment as this was a possible reversible condition. The patient was however lost to follow-up.

PATIENT 84

A 40 year old black female who presented with a 5 day history of a productive cough, right-sided chest pain, loss of weight and night time sweating.

Investigations:

Chest X-ray: Right-sided pleural effusion.

Pleural fluid chemistry: ADA 61 u/l, LDH and protein not performed

Pleural biopsy: Non-specific inflammation.

The patient was discharged for follow-up of results but did not return and was lost to follow-up.

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