CHARACTERIZATION OF AVIPDXVIRUSES

FOR USE IN RECOMBINANT VACCINES

by

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Submitted in fulfilment of the requirements for the degree of M. Sc. in the Department of Medical Microbiology at the University of Cape Town.

FEBRUARY 1992

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ACKNOWLEDGEMENTS

Firstly, I would like to thank the Department of National Health and Population Development, who afforded me the opportunity to read for my M.Sc. degree in Medical Microbiology.

I wish to express my sincere thanks to:

Professor K.R. Dumbell, my supervisor for all assistance and encouragement.

Dr. W. Katz for co-operation and encouragement during this study.

Dr. L. Stannard for her help with the electron microscopy studies and the photographs contained herein.

To all the members of the "Plasmid Unit" and the Department of Virology (0.C.T.) for their support and assistance during this study.

Finally I would like to thank my family for the support, strength and endurance needed during the duration of this study.

I humbly thank the Almighty God for Divine Inspiration to persevere in completing this task:

TO YOU ALONE, 0 LORD TO YOU ALONE,
AND NOT TO US, MUST GLORY BE GIVEN
BECAUSE OF YOUR CONSTANT LOVE AND FAITHFULNESS.
(Psalms 115:1)

ABSTRACT

Pox viruses have been demonstrated in over 60 types of wild and exotic birds as well as domestic birds. Avipox viruses have been isolated and characterised from fowls, quails, canaries, parrots and lovebirds.

This work describes the first isolation of a poxvirus from Jackass penguins *(Spheniscus dermersus)* and the characterisation of the virus as a separate species of penguinpox virus.

A penguin was brought into the South African National Foundation for the Conservation of Coastal Birds (SANCCOB) with lesions around the eyes. Virus was cultured from scrapings of the lesions and a characteristic avipox virus was found. A year later an avipox virus was isolated from two other penguins. These three penguinpox isolates were found to be very similar viruses, only differing in the terminal fragments of their genomes.

The virus was identified by electron microscopic, histological and morphological studies. The electron microscopy studies revealed a structure typical of poxviruses with a bi-lipid membrane and surface tubules. A feature seen, but not found on

orthopox viruses, was a large fatty coat in which the penguinpox virus was embedded. This coat presented several problems during the purification of the virus. The coat aggregated the virus into agglomerations which precipitate out during the centrifugation steps using the purification method developed for orthopox viruses. This decreased the amount of virus. The use of an organic solvent, Arklone X, was added at the initial grinding stage and this dissolved the fatty coat allowing the virus to be purified from the cellular debris.

The histological studies revealed A-type inclusions characteristic of avipox viruses and this confirmed the penguinpox virus to be an avipox virus as the cross-sections of the pocks on choric-allantoic membranes showed a viral infection similar to that of fowlpox virus. The morphology of the pock was examined. However, as the penguin isolate is a fresh field isolate no conclusion can be drawn on the basis of pock morphology alone.

Facilities were not available for the extensive serological, cross-typing, pathology and cross-protection studies in various species of birds, and it was therefore decided to do a restriction analysis of DNA genomes of the representative avipox viruses. Molecular biological studies were only conducted on fowlpox virus.

Three reference strains of avipox viruses were used: of fowlpox, canarypox and quailpox. These three viruses and the penguinpox isolate were purified and the DNA extracted. The DNA was subjected to digestion by restriction endonucleases and electrophoresed on agarose gels. The separation of the different sized DNA fragments produced restriction profiles unique to each virus. Examination of these restriction profiles revealed that each of the four viral genomes examined were different.

As comparison, a local isolate of fowlpox virus from a domestic fowl was made and the genome was shown to be similar to the characterised fowlpox virus. An isolate was made from a wild turtle dove which revealed a virus with a restriction fragment profile different to the other viruses.

In light of the avipox viruses being used as recombination vaccines in mammalian hosts, attempts were made to locate the thymidine kinase gene in each of the avipox viruses. Preliminarily studies indicate that the avipox virus growth cycle in mammalian cells is terminated after DNA replication. This means that the antibodies produced in response to a recombinant avipox virus results from early transcription.

ABBREVIATIONS

A	adenosine
Amp	Ampicillin
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATV	Activated Trypsin Versene
bp	base pair
	cytidine
CAM	chick chorioallantoic membrane
CEF	chick embroyic fiboblasts
$CR1^2$	centimeter squared
CPE	cytopathic effect
°C	degrees Celicus
DEEM	Dulbecco's Modified Eagles's Essential Medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic-acid
Etd Br	ethidium bromide
FCS	foetal calf serum
	guanosine
	gravitational force
Gen	Gentamycin
HF	human fibroblast
kb	kilobase

	molar
MEM	Eagles Minimum Essential Medium
mg	milligram
ml	millilitre
mm	millimeter
mM	millimolar
	normal
Neo	Neomycin
ng	nanogram
nm	nanometer
np	radioactive isotope of phosphorous
PBS	phosphate buffered saline
pC	picoCurie
Pen	Penicillin
pfu	pock forming unit
pm	picomoles
PNS	antibiotic solution containing Pen, Neo and Strep
RNA	ribonucleic acid
RNAse	ribonuclease A
SANCCOB	South African National Foundation for the Conservation of Coastal Birds
SDS	sodium dodecyl sulphate
Strep	Streptomycin
	thymidine
TE Buffer	TRIS-EDTA buffer
TE Cells	tortosie embryonic cells

TK	thymidine kinase
	units
Ag	microgram
Al	mircolitre
UV	ultra violet
V/cm	volts per centimeter - potenial difference
v/v	volume per volume
w/v	weight per volume

SECTION I

INTRODUCTION - PLAN OF THIS THESIS

A general introduction to the poxvirus genera and more specifically the avipox viruses is presented in Chapter 1. The various wild bird isolates and presumptive typing of those isolates are discussed.

The general methods section, Section II (Chapters 2, 3 and 4), follows. This section covers the methods used for inoculation and harvesting of CAMs of chick embryos, the tissue culture methods and finally, DNA analysis.

Section III covers the first objective of the study, the isolation of an avipox virus from penguins and from a bantam and a turtle dove. It includes evidence and confirmation of the pox-like penguin isolate as an avipox virus.

The second objective of the study discussed in Section IV relates to the typing of the penguinpox isolate based on genome restriction mapping. A brief investigation was conducted to determine the location of the TK gene in the various avipox viruses used in the study.

Section V descibes the prospect of using avipox viruses as a recombination vehicle for the preparation and manufacture of veterinary mammalian vaccines.

Finally, Section VI, the conclusion, details the assessments of findings contained in this study and outlines the prospects of further research into avipox viruses.

CHAPTER ONE

PDX VIRUS DISEASES OF BIRDS

Pox viruses that have been isolated from poultry and wild birds and that are serologically related to each other have been classified in the genus : AVIPDX (Fenner, et al., 1987). The most important and most widely studied is fowlpox virus.

1.1: FOWLPDX : A TROUBLESOME DISEASE

The fowlpox virus causes a serious disease of poultry which has affected chickens for centuries. It was mentioned as early as 1873 when Bollinger described the pathology of fowl pox (cited in Kirmse, 1969). The disease occurs worldwide and results in economic loss, though this is significantly reduced by immunization with attenuated fowlpox and pigeon pox vaccines (Fenner et al., 1987).

Fowlpox virus causes two types of diseases, according to the route of infection. The most common form is characterised by small papules or lesions which normally develop on the face and non-feathered areas of the birds. These include the comb, wattles, around the beak, the legs and the feet. The lesions become yellowish and form a dark scab. There may be secondary bacterial infections, but poultry normally recover from the disease within three weeks. This picture is referred to as the

dry form of the disease.

The second form of the disease results from infection of the birds by aerosol inhalation. The infection is termed the wet form of the disease as it involves the mucous membranes of the mouth, pharynx, larynx and sometimes spreads to the trachea. This form is also known as the "diphtheritic" form of fowlpox as the lesions coalesce into a necrotic pseudomembrane which causes death by asphyxiation.

The lesions caused by fowlpox virus on CAMs are larger and flatter than those of vaccinia virus (5 to 7 mm as against 3 -4 mm) (Mayr, et al., 1972).

Electron microscopic studies of fowlpox virons revealed a structure similar to vaccinia virus though the actual size of the fowlpox virus is larger than vaccinia virus at 390 x 260 nm compared to 360 x 240 nm (Mayr, et al., 1972 and Hyde, et al., 1965).

Both fowlpox and vaccinia viruses are characterized by having A-Type cytoplasmic eosinophilic inclusions. The fowlpox inclusions are also lipophilic (Mayr, et al., 1972).

The inclusion bodies of fowlpox virus are extremely hardy,

resisting hypotonic treatment, repeated freezing, thawing and grinding, and extraction with a variety of organic solvents (Randall and Gafford, 1962). The chemical studies done by Randall and Gafford on viral inclusions, revealed that 50 % of the virus inclusions were extractable lipid. There were significant amounts of DNA and RNA found although the authors were not clear as to the amount present.

1s21_8111XLAR **DISEASES FOUND IN WILD AND EXOTIC BIRDS**

Avipox infection has been described in captured wild birds of over 60 species.

A partial list (Table 1.1) is given below of some birds found infected with avipoxvirus with the corresponding references:

Table 1.1: A partial list of birds infected with avipox viruses.

Avian Host	Reference
Laysan finch	Warner, 1968
Diederick Cuckoo	Markus, 1974
Masked Weaver	Markus, 1974
Cape sparrows	Markus, 1974
Australian magpie	Harrigan, et al., 1975
Magpie-lark	Annuar, et al., 1983
Silvereyes	Austin, et al., 1973
Manx shearwaters	Nuttall, et al., 1985
Great tit	Holt and Krogsrud, 1973
Sparrows	Giddens, et al., 1971
Weavers	Giddens, et al., 1971
Golden eagle	Moffatt, 1972
Cherag falcon	Thiele, et al., 1979
Sandhill cranes	Simpson, et al., 1975
Junco	Kirmse, 1966

None of the viruses isolated from wild birds have been studied in any detail. The above authors were satisfied to identify the virus as a poxvirus, usually on the basis of morphological evidence alone.

Pox virus infection has also been a problem with captive exotic birds such as canaries and parrots (Burnett, 1936 and Boosinger, et al., 1982).

An avipox virus has been isolated from lesions on a rhinoceros, but was not shown to be capable of infecting mammalian tissue (Mayr and Mahnel, 1966).

1.3: THE SPREAD OF AVIPDX VIRUSES DISEASES

Avipox viruses in the wild have been found to be spread by arthropod vectors, such as mosquitoes and biting flies, the hippoboscids (Markus, 1974 and Warner, 1968) sometimes with devastating effects. Mosquitoes were able to infect susceptible birds 2 months after feeding on a sick bird and infectious virus has been isolated from mosquitoes caught in the wild (French and Reeves, 1954). No multiplication of avipox virus in mosquitoes has been reported to date, which would mean that only mechanical transmission of the virus occurs. Though the work of French and Reeves suggest that virus may survive for unexpectedly long periods in mosquitoes.

Warner (1968) describes the effects of introducing avian diseases, which have been responsible for extinction of much of the avifauna of the Hawaiian Islands. The author notes that at the time of the discovery of the Hawaiian Islands by Cook in 1778

birds inhabited all parts of the islands from seashore to the upper limits of the vegetation. Avipox viruses introduced in 1826, probably by mosquitoes spread rapidly and resulted in great mortality. During an investigation in 1958, it was discovered that certain bird species, previously found throughout all altitudes on the islands were restricted to heights above that at which mosquitoes live.

Avipox infection has been found in migratory birds such as quail and finches. This could result in avipox viruses being distributed widely throughout the world.

Avipox virus infections have also been reported in raptor species such as falcons and eagles.

Orthopoxviruses are all closely related but some specialization has occurred in different ecological and geographical situations, resulting in distinguishable "species" such as cowpox, camelpox, monkeypox and racoonpox. Avipoxviruses have not been studied to a similar degree. Some speciation must have occurred as there are different groups, such as psittacine pox, quailpox and fowlpox which do not confer cross-protection against each other (Tripathy, et al., 1973).

1.4: CLASSIFICATION OF THE AVIPDX VIRUSES

The avipox viruses form part of the poxvirus family which has been divided into two smaller subfamilies:
Chorodopoxviridae - the pox viruses which infect vertebrates and Entomopoxviridae - the pox viruses which infect insects. A short table of the various poxvirus genera with the prototype member for each is given below (Moss, 1990):

Table 1.2: The Family Poxviridae

Subfamilies	Genera	prototype member
Chorodopoxviridae	Orthopoxvirus	vaccinia virus
	Parapoxvirus	orf
	Avipoxvirus	fowlpoxvirus
	Capripoxvirus	sheeppox
	Lepripoxvirus	Myxoma virus
	Suipoxvius	swinepox virus
	Molluscipoxvirus	molluscum contagiosum
	Yatapoxvirus	tanapoxvirus
Entomopoxviridae	A	Melontha melontha
	B	Amsacta moori
	C	Chironimus luridus

The fowlpox virus as the prototype virus of the avipox virus has been the most widely studied of the viruses.

1.5: CLASSIFICATION WITHIN THE AVIPDX VIRAL GROUR

The types of avipox viruses studied to date include fowlpox, canarypox, pigeonpox, turkeypox, psittacinepox, starling pox and juncopox (Tripathy, et al., 1973). To date comparative studies of avipoxviruses have been limited to the serological relationships of the viruses, host range studies, cross-protection tests and neutralisation tests. Serological and cross-protection studies have shown that pigeon pox is closely related to fowlpox (Tripathy, et al., 1973 and Winterfield and Reed, 1985).

Cross-protection studies have shown that quailpox and psittacinepox are not antigenically similar (Tripathy, et al., 1973) nor do antisera protect against fowlpox or pigeonpox (Winterfield and Reed, 1985).

The study conducted by Tripathy, et al., (1973) was based on serological tests using cross-neutralization, complement-fixation and agar-gel precipitation tests. These authors have also shown no cross-reaction between quail and psittacine pox.

1.6: MOLECULAR STUDIES OF AVIPDX VIRUSES

Fowlpox is the only avipox virus which has been studied to any extent by molecular biology techniques. It has been shown that the viral genome is vastly different from that of orthopox viral genomes (Muller, et al., 1977).

Miller, et al., 1979 performed several restriction digests of fowlpox and estimated the genome size as 230 kb. A restriction map of the genome was, constructed by Coupar, et al., 1990. This map confirmed Muller's work. Some DNA sequencing has been done, (Boyle and Coupar, 1988 and 1990, Binns, et al., 1987, Tomley, et al., 1988 and Drillien, et al., 1987), which showed some homology to vaccinia viral DNA and that the gene organization was similar to that of vaccinia. The thymidine kinase (TK) gene has been identified and sequenced (Boyle and Coupar, 1986; Boyle,et als, 1987, Drillien, et alt, 1988 and Binns, et al., 1988). Curiously this gene appeared to have been translocated to a different region of the fowlpox genome compared to that in vaccinia.

1.7: THIS COMPARATIVE STUDY

Amongst the 60 species of wild bird avipox virus isolates, no virus has been isolated or lesions seen on penguins to date.

This study describes the isolation of a pox virus from several Jackass penguins *(Spheniscus demersus).* In order to study these

isolates in relation to other avipox viruses it was not practical to undertake any cross-protection tests, though limited study of the lesions on CAMs were undertaken.

It was decided to analyze the viral DNA of the penguinpox isolates in relation to other avipox viruses. At the commencement of this study, the only available published information related to fowlpox virus. Consequently studies of the DNA of fowlpox, pigeon, canarypox and quailpox viruses were undertaken. Since this began, two studies have appeared in the literature comparing the DNA of quail and fowlpox (Schnitzlien, et al., 1988 a and b and Ghildyal, et al., 1989).

This work was extended to include a comparison of other avipox viruses isolated within the Cape Peninsula. There were isolations from a domestic barnyard fowl, (Hackett or local fowlpox virus) and from a turtle dove (turtle dove isolate) caught in the wild.

SECTION II

CHAPTER TWO

VIRAL GROWTH METHODS

2.1: MATERIALS

Fertilized eggs of domestic hens *(Gallus domesticus)* were supplied by the Golden Grove Poultry Farm, Cape Town, South Africa.

The reference strains of fowlpox, canarypox, turkeypox, quailpox and pigeonpox viruses were obtained from Professor A. Mayr of the University of Munich, Germany.

The blood agar plates, the Luria agar, tryptone broth and the carbol fushsin stain were supplied by the Department of Medical Microbiology, University of Cape Town (U.C.T.).

The antibiotics (discs and powder) were obtained from Mast Industries, Merseyside, United Kingdom; Glaxo Pharmaceuticals, Wadeville, South Africa; and Oxoid Pharmaceuticals, Basingstoke, United Kingdom.

The stains of haematoxylin and eosin were supplied by Unilab Saarchem, Krugersdorp, South Africa.

2.2. METHODS USED FOR GROWTH OF VIRUS ON CAMS.

2.2.1. PREPARATION OF CHORIO-ALLANTOIC MEMBRANES (CARR)

The method of preparation of CAMs was adapted from Westwood et al. (1957) and McCarthy and Dumbell (1961). The embryonated eggs were first candled to determine the age of the chick. Ideally the embryonated egg should be between 11 and 13 days old. The presence of the air-sac, found on the blunt end of the egg, and a region in the vicinity of the main blood vessels on the dorsal side of the egg was determined and marked. A small hole was made at the markings with a spring-loaded bore, just powerful enough to break the shell. The hole at the air-sac was enlarged by pushing the nib of a witch pen into it. A mixture of petroleum jelly and paraffin wax, melted beforehand, was placed over the dorsal hole. Using the nib of the witch pen, an area was cleared above the hole by making a "well" into which a drop of physiological saline (Appendix A) could be placed. The nib of the pen was carefully positioned so as to break the shell membrane and create an air-lock to allow the saline to penetrate between the two layers of the membranes of the egg. A rubber suction bulb was used to extract all the air from the air-sac. The egg was re-candled to ensure that the CAM had dropped leaving the newly formed air-sac above it.

The eggs were incubated at 36,5°C for 2 hours to allow the

membranes to equilibrate.

2.2.2. INOCULATION OF CAMS

The prepared CAMs were inoculated with 0,1 ml of the viral material, using a 1 ml syringe fitted with a 22 gauge needle. The hole through which the inoculum was introduced, was sealed with the mixture of petroleum jelly and wax. The egg was slowly rotated five to six times to ensure an even spread of the inoculum, and incubated at 36,5°C.

2.2.3. HARVESTING OF MEMBRANES

The eggs were harvested four days post-inoculation in the following manner:

Each egg was cut open along its midline using a sterile pair of scissors. The contents of the egg was discarded into a bucket containing Biodan disinfectant. The upper half of the shell contained the CAM.

The membrane which adhered to the shell was trimmed, so that only the infected area was retained. Using sterile forceps, the membrane was carefully peeled away from the shell, washed in physiological saline, and examined for the presence of any focal lesions.

The membranes were placed in pre-cooled glass bottles, a third filled with glass beads. One ml of 4 mM McIlvains pH 7.4 buffer (Appendix A) was added for each membrane. A paper towel was wrapped around the glass bottle which was then shaken for 1 to 1,5 minutes. The paper towel prevented the contents from warming up rapidly, and allowed detected of any leakage from the bottle seal during the shaking process.

The bottles were centrifuged for 6 to 8 minutes at 110 g in a Sigma 301K centrifuge (Sigma, St. Louis, United States of America) with a swing out rotor cooled to 4°C. The supernatant was harvested and this was regarded as the viral stock. Stocks of the viruses were made in 80 % glycerol (Appendix A). Working stocks were stored at -20°C and a permanent stock at -70°C.

2.2.9. GROWTH OF REFERENCE VIRUSES.

Five reference viruses were obtained for the identification and comparative study of the penguinpox virus isolate. They were: canarypox virus, fowlpox virus, pigeonpox virus, quailpox virus and turkeypox virus. The four last-mentioned viruses were obtained in a freeze-dried form. The virus pellets were resuspended in sterile P.B.S. and diluted 100-fold before inoculating CAMs as previously described (Chapter 2.2.2.).

Professor Dumbell supplied the canarypox virus as an 80% glycerol stock. This sample was diluted 1000-fold in sterile P.B.S. and used to inoculate CAMS as described in Chapter 2.2.2.

The CAMs were incubated for 4 days at 36,5°C and then harvested as previously described (Chapter 2.2.3.). The membranes were examined for the presence of any focal lesions. Photographs of these lesions are shown in Chapter 6.3.2.

CHAPTER THREE

METHODS USED IN BIOLOGICAL COMPARISONS

3.1: MATERIALS USED

The cell lines MDBK and MDCK and the fertilised eggs of the Leopard tortoise *(Geochelone pardalis)* were supplied by the Veterinary Research Institute at Onderstepoort, Pretoria, South Africa.

The cell lines CV1, RK13 and HeLa and the primary HF cells were supplied by the Department of Virology, Medical School, U.C.T.

The Foetal Calf Serum (FOS) was supplied by the State Vaccine Institute, Cape Town, South Africa.

The media for tissue culture growth, the Eagle's Minimum Essential Medium with Earle's salts (MEM) and the enriched medium of Dulbecco's Modified Eagle's Essential Medium (DMEM) were obtained from Highveld Biologicals, Pretoria, South Africa.

The tissue culture flasks and multi-well plates were supplied by Falcon, United States of America; Nunc, Roskilde, Denmark and Sterilin, Hounslow, United Kingdom. The carbol fuchsin

stain was supplied *by* the Department of Medical Microbiology, U.C.T.

The antibiotic powders of Penicillin, Streptomycin and Neomycin were obtained from Glaxo Pharmaceuticals, Wadeville, South Africa and Scherag, Isando, South Africa.

Difco Laboratories, Detriot, United States of America supplied the trypsin powder. The stain Bisbenzimid H33258, Fluorochrom was supplied by Riedel-De Haen AG Seelze, Hanover, Germany.

3.2: METHODS USED FOR VIRUS GROWTH IN TISSUE CULTURE.

3.2.1. CELL LINES USED FOR VIRUS PROPAGATION.

The cell lines used in the viral propagation experiments were: CV1, HeLa, RK13, MDBK and MDCK cell lines.

The CV1 cell line is a continuous fibroblast-like cell line derived from a kidney of an adult male African Green Monkey *(Cercopithecus aethiops)* (ATCC Catalogue, reference CCL 70).

The HeLa cell line is a continuous epithelial-like cell line derived from a carcinoma of the cervix of a 31-year-old female. (ATCC Catalogue, reference CCL 2.2).

The RK13 cell line, initiated from kidney cells of a 5-week-

old rabbit, is a continuous epithelial-like cell line. (ATCC Catalogue, reference CCL 37).

The MDBK is a continuous cell line obtained from a kidney of a normal, heathy steer *(Bos taurus).* (ATCC Catalogue, reference CCL 22).

The MDCK cell line originates from dog kidney cells. (ATCC Catalogue, reference CCL 34).

3.2.2. PREPARATION OF PRIMARY CELLS.

The tortoise embryonic (TE) and the chick embryonic fibroblast (CEF) cells were primary cells, prepared in the following manner:

The CEF cells were prepared from 8-day-old chick embryos, and the TE cells were prepared from 10-month-old tortoise embryos. The embryo was carefully removed from the egg by breaking the shell, and then decapitated. The body of the embryo was cut into 2 to 3 mm pieces. These were placed into a sterile glass bottle containing 4 ml of Activated Trypsin Versene (ATV) buffer (Appendix A), and incubated on ice for 6 to 24 hours. The ATV buffer was removed and the digested sample was incubated at 37°C for 30 minutes. Tissue culture media with 10% FCS was added and the con-

tents gently agitated until the tissue dispersed completely. A cell count was made (Chapter 3.2.4.) and 106 cells per ml of medium were seeded into each flask (Freshney, 1987).

The HF cells are primary cells obtained from the lung tissue of human foetus and were supplied by the Department of Virology, Medical School, U.C.T.

3.2.3. GROWTH AND MAINTENANCE OF TISSUE CELL CULTURES.

All the cells were grown in medium containing 10% FCS. The CV1 and HeLa cell lines and the HF and CEF cells were grown in MEM supplemented with 3 mM sodium bicarbonate and an antibiotic solution made up of Penicillin (100 Units/ml), Streptomycin (100 mg/ml) and Neomycin (100 mg/ml) (PSN solution - Appendix A).

The cells MDBK, RK13, MUCK and TE were grown on the enriched medium of DMEM supplemented with the above antibiotic solution.

The cell monolayers were grown either in tissue culture flasks or in multi-well dishes. The confluent cell monolayers were maintained with the appropriate medium containing the supplements described above, but with 4% FCS in the medium.

Confluent cell monolayers were trypsinized off the flask as follows:

The supernatant fluid was carefully aspirated and the flask rinsed with PBS and thereafter with ATV. A second volume of ATV was added to the flask, incubated at 37°C for 5 to 10 minutes to allow the cell monolayer to dislodge itself from the surface of the culture vessel. The cells were mono-dispersed by gentle agitation and re-seeded into appropriate culture vessels.

3.2.4. CELL COUNTING

To establish the number of cells/ml for seeding the appropriate tissue culture vessel, a cell count was made. The cells were pelleted in a swing-out rotor at 800 g for 10 minutes at ambient temperature. The supernatant was discarded and the cell pellet re-suspended in 10 ml of 10 % FCS MEM. A volume of 0,1 ml was removed and an equal volume of trypan blue (Appendix A) added to this volume. (The trypan blue stains dead cells blue whereas living cells exclude the stain). The suspensions were mixed, and a sample was removed and placed on a haemocytometer (Neubauer, Brand, Blaubrand, Germany). The living cells were counted in four different squares. The average of these readings was multiplied by the dilution factor and then by the factor of 104. This factor is obtained

from the area and the depth of the haemocytometer on which the cells were counted. This calculation gives the amount of cells/ml. The cell suspension was re-centrifuged, re-suspended in the appropriate amount of medium and seeded in the appropriate tissue culture vessel.

CHAPTER FOUR

4.1. MATERIALS

The endonuclease restriction enzymes Damn I, Bc1 II, Eco RI, Hind III, Pst I, Sal I, Sma I, Xba I and Xho I with the appropriate buffers were supplied by Amersham International, Amersham, United Kingdom; Bethesda Research Laboratories, Gaithersburg, United States of America and Boeringer Mannheim, Mannheim, Germany.

Amersham International, Amersham, United Kingdom, supplied the Polynucleotide kinase and the Hybond N+ membrane.

The Proteinase K and the blocking agent were supplied by Boeringer Mannheim, Mannheim, Germany, and the Ribonuclease A enzyme were supplied by Sigma Chemicals, St Louis, United States of America.

The Department of Biochemistry, U.C.T. supplied the gamma labelled 32E-ATP and the 18-mer oligonucleotide.

4.2.: METHODS

4.2.1. DNA PRECIPITATIONS

DNA was precipitated after purification by phenol and chloroform extractions. The phenol extractions involved the addition of an equal volume of phenol (Appendix A) to the DNA suspension. These were mixed by gentle inversion. The suspension was centrifuged at 800 g for 5 minutes to allow the organic and aqueous phases to separate. The aqueous phase was harvested. This procedure was repeated on the aqueous phase.

The aqueous phase was measured and an equal volume of chloroform-isoamylalcohol (24:1 v/v) was added. These two solutions were mixed and centrifuged at 800 g for 5 minutes as above. The aqueous phase was retained and the process repeated. The volume of the aqueous phase was measured.

Sufficient 1 M Na-acetate solution was added to a final concentration of 0,3 M and the DNA precipitated by the addition of 2,5 volumes of ice-cold absolute ethanol. The DNA was allowed to precipitate completely overnight at -20°C.

The phenol was added to removed the protein, especially nucleases which would degrade any DNA present. The chloroform-isoamylalcohol extractions were done to remove residual phenol which causes DNA degradation on long exposure.

4.2.2. RESTRICTION ENDONDCLEASE DIGESTIONS.

The restriction endonuclease digestions were performed as recommended by the supplier. The correct salt concentration was carefully monitored in the buffer as enzyme activity would be inhibited and the rate of digestion retarded if incorrect. Also certain enzymes (e.g. Eco RI) would produce star activity at incorrect salt concentrations.

Digestions of avipox viral DNA, where the actual size of the genome was not accurately known, were incubated for two to three hours at 37°C in a water-bath. The Pst I digests were incubated overnight for 18 hours at 37°C in a humidified incubator to minimize the rate of evaporation.

All reactions were terminated in one of two ways:

A: The DNA samples were heated to 75°C for 15 minutes after which a 1/6 volume of a 6 times concentrated loading buffer (50% v/v glycerol, 1% w/v SDS, 0,1% w/v bromophenol blue and 0,1% w/v cyanol) was added.

13: A 1/10 volume of a 10 times concentrated stop buffer (50% v/v glycerol, 1% w/v SDS, 100 mM Na2EDTA pH 8, 0,1% w/v bromophenol blue and 0,1% w/v cyanol) was added to the reaction mixture.

Fragments generated from the digestions were separated by agarose gel electrophoresis and visualised by ethidium bromide (Etd Br) staining.

4.2.3. GEL ELECTROPHORESIS OF DNA FRAGMENTS.

Gels of 0,8% or 1% w/v agarose were prepared by dissolving agarose in TAE buffer (0,04 M Tris-acetate and 0,001 M Na2EDTA). The agarose was melted in a microwave oven and cooled to 56°C in a water-bath. With the slots for wells already in position horizontal gel slabs, 5 mm thick, were poured. Once the gel set, it was placed in a tank filled with TAE buffer.

The samples were loaded onto the gel. An initial potential difference of 4 V/cm was applied across the gel for 30 minutes, which allowed the DNA fragments to migrate into the gel. The potential difference was decreased to 1,6 V/cm and electrophoresis at ambient temperature continued for 18 to 20 hours.

Higher resolution of the large molecular weight bands (25 kilobase pairs, kb, and above) were attained when the gel was electrophoresed for 18 to 20 hours at 4°C, and the potential difference increased to 2 V/cm.

The DNA in the agarose was visualized by staining with Etd Br,

at a concentration of 1 to 2 gg/ml, for 30 minutes. If the gel was overstained, it could be destained in distilled water for 30 minutes.

Ethidium bromide intercalates with the DNA and fluoresces in the presence of a 300 nm ultra-violet light source. A Fotodyne Incorporated DNA transilluminator, which contained two 300 nm lamps as well as a ultra-violet glass filter, was used to induce fluorescence and also to absorb extraneous emissions from the light source. This is illustrated in Figure 4.2.1

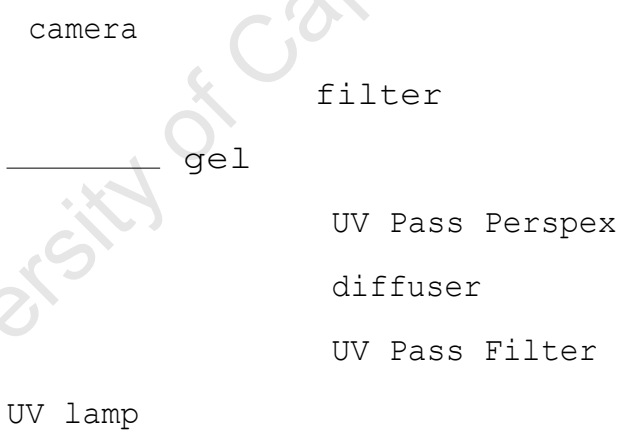

Figure 4.2.1: A schematic diagram of the UV-transilluminator.

Gels satisfactarily stained were photographed on polaroid film, and/or negative black and white film.

4.2.4. HYBRIDIZATIONS

The DNA template was transferred (see below) onto an immobile, permanent membrane, Hybond N+. The radioactive DNA probe, which hybridised to the membrane-bound DNA on the membrane, was carefully washed and the membrane applied to an autoradiograph film.

4.2.4.1. DNA TRANSFER TO HYBOND N+.

After separation on an agarose gel by gel electrophoresis, DNA was transferred onto an immobile membrane, Hybond N+, by two modifications of the Southern blotting method (Southern, 1975). Both methods proved equally efficient for the transfer of DNA molecules.

4.2.4.2. ALKALI TRANSFER METHOD OF REED AND MANN (1985)

The agarose gel, after electrophoresis, was transferred to a solution containing 0,25 **M** HC1 for 20 minutes. The gel was rinsed in distilled water and placed into 0,4 **M** NaOH. The volume used for each wash was 250 to 300 ml of the appropriate solution.

The capillary blot was assembled as follows:

The blotting solution, 0,4 M NaOH, was poured into a dish containing four Bijou bottles. They supported a glass plate which served as a platform. Three sheets of

Whatmann 3 MM paper, saturated in blotting solution, covered the platform and acted as a wick on which the gel was placed upside down eliminating air bubbles. One sheet of Hybond N+ and three sheets of Whatmann 3 MM paper (same size as the gel) were placed on the gel. The Hybond N+ membrane was carefully placed on the gel, ensuring that no air bubbles were trapped between them. No transfer of DNA would occur in an air bubble.

Paper towels were placed onto the Whatmann 3MM paper. Plastic wrap was placed around the perimeter of the gel to prevent transfer of buffer directly from the wick onto the paper towels. A second glass plate was placed on the paper towels and an one kilogram weight placed on the plate. The layout of the blotting procedure is illustrated in Figure

Transfer occurred overnight at ambient temperature. The apparatus was carefully dismantled and the membrane removed after the positions of the slots were marked on it. The membrane was rinsed briefly in 2 times concentrated SSC buffer (Appendix A) with gentle agitation.

The membrane was either placed in hybridisation buffer or stored at 4°C in 6 times concentrated SSC buffer.

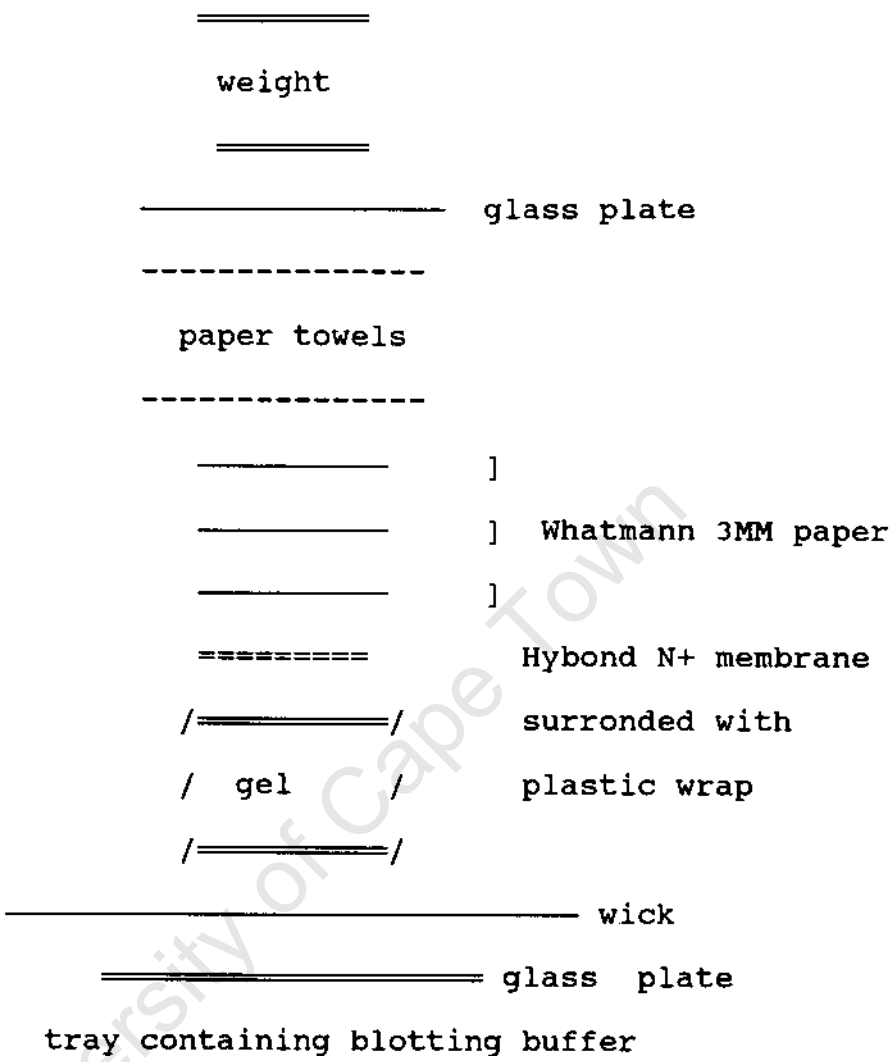

Figure 4.2.2: A schematic diagram of the apparatus for the alkali transfer method.

4.2.4.3. DRY BLOT METHOD OF SOUTHERN AND SUMMERS (1980).

After electrophoresis, the agarose gel was transferred to a solution of 0,25 M HCl with gentle agitation for 20 minutes. The gel was rinsed in distilled water. It was immersed in a denaturing buffer consisting of 1,5 **M** NaCl and 0,5 M NaOH for 20 minutes, agitating gently. The gel was rinsed with distilled water and transferred to the blotting buffer consisting of 0,02 M NaOH and 1 M ammonium acetate for 40 minutes. A sheet of Hybond N+ and three sheets of Whatmann 3MM paper of exactly the same size as the gel, were pre-wet in the blotting buffer.

The transfer was set up as follows:

A piece of plastic wrap was laid on a flat surface. The gel was placed upside down on it. The Hybond N+ membrane, covered by the three sheets of Whatmann 3 MM paper, was placed on the gel. Air-bubbles trapped between the gel and the membrane were dislodged. Paper towels were placed on the Whatman paper. A glass plate, on which rested a one kilogram weight was placed on the towels. A diagram of the apparatus is illustrated in Fiaure 4.2.3.

The DNA transfer to the membrane continued for 20 hours.

After transfer, the DNA was fixed to the membrane by placing

it on 0,4 M NaOH pre-wet Whatman 3 MM paper for 30 to 60 minutes. The membrane was placed in hybridisation buffer or stored at 4°C in 6 times concentrated SSC buffer.

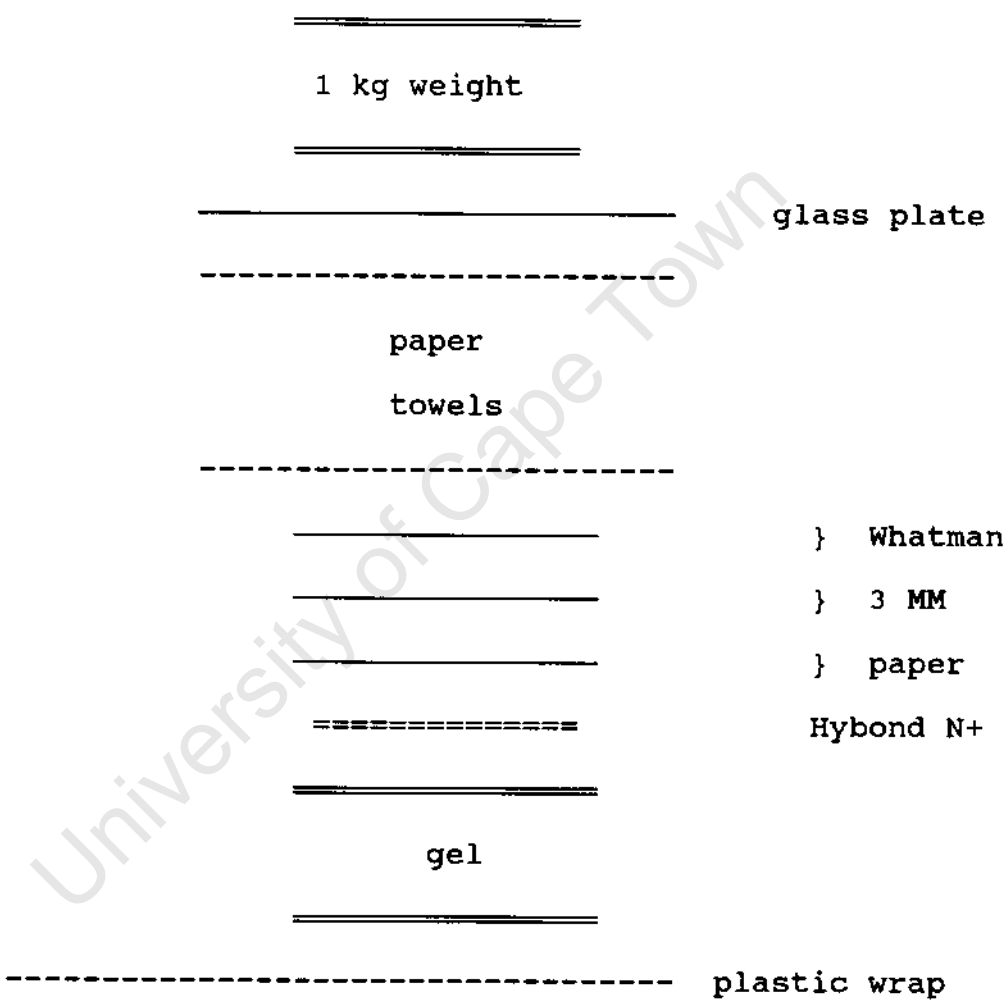

Figure 4.2.3: The dry-blot method of Southern and Summers.

4.2.4.4. HYBRIDIZATIONS.

Hybridizations were preformed as described by Johnson et al., 1984. The membrane was pre-hybridized in a buffer containing 0,25% non-fat milk powder (w/v) in 6 times concentrated SSC for 6 - 18 hours at 42°C in a water bath. The pre-hybridization process prevented any non-specific binding of the probe to the membrane. The labelled probe was denatured by heating above 95°C for 5 minutes, immediately cooled on ice and added to the pre-hybridization buffer. Low stringency conditions were applied and no formamide was added to the hybridization buffer. Hybridization was conducted overnight at 20 - 25°C.

After hybridization, the blot was washed in decreasing salt concentrations and at increasing temperature during which the unhybridized probe was removed. After each wash the blot was autoradiographed to ensure that the washing process was not too stringent. The washes were:

Two washes of 30 minutes each of 6 times concentrated SSC containing 0,1% SDS (w/v) and 0.25% (w/v) non-fat milk powder at room temperature.

Two washes of 30 minutes each of 2 times concentrated SSC, containing 0,1% SDS (w/v) and 0,25% (w/v) non-fat milk powder at room temperature.

Two washes of 30 minutes each of 2 times concentrated SSC and containing 0,1% SDS (w/v) at 30°C.

Two washes of 15 minutes each of 2 times concentrated SSC containing 0,1% SDS (w/v) at 37°C.

One wash of 10 minutes of 2 times concentrated SSC containing 0,1% SDS (w/v) at 42°C. This wash removed an 18-mer oligonucleotide probe which had bound both specifically and non-specifically.

4.2.4.5. AUTORADIOGRAPH

The blot, after washing, was wrapped in plastic wrap and exposed to autoradiograph film (X-ray film) for periods ranging from 30 minutes to 18 hours in an autoradiograph cassette. The film was developed. Depending on the result obtained, the blot could be re-exposed for several days at -70°C.

The X-ray film was developed for 3 - 5 minutes. The film was washed in stop buffer (2% acetic acid v/v) for 1 minute. It was fixed and hardened for 5 minutes. The film was finally washed in running tap water for 15 to 20 minutes and air-dried.

4.2.4.6. RE-USE OF BLOTS.

Because the DNA is irreversibly bound to the Hybond N+ membrane, blots could be re-used if the membranes were stored in 6 times concentrated SSC buffer. The probe could be removed by immersing the blot into a boiling solution of 0,5% SDS (w/v) and slowly cooling to room temperature. The removal of the radioactive probe was confirmed by autoradiographing the blot for 18 hours. The blot was only re-used when no radioactivity could be detected on the film.

4.2.5. SIZING OF BANDS USING KNOWN MOLECULAR SIZE MARKERS

The sizes of DNA fragments were estimated by comparing the migration distance, in an agarose gel, of the unknown fragments with those of fragments of known size in the same gel during the same electrophoretic run.

DNA from the bacteriophage lambda, restricted with the enzyme Hind III, was used as size markers. The migration distance of the known fragments were plotted (x-axis) against the logw of their molecular size in kb (y-axis) to form a standard curve (Figure 4.2.4.). The distance of migration of the unknown fragments was measured and their sizes calculated by extrapolation from the standard curve. Fragments larger than 20 kb are not easliy resolved on agarose gels and their sizes cannot be estimated.

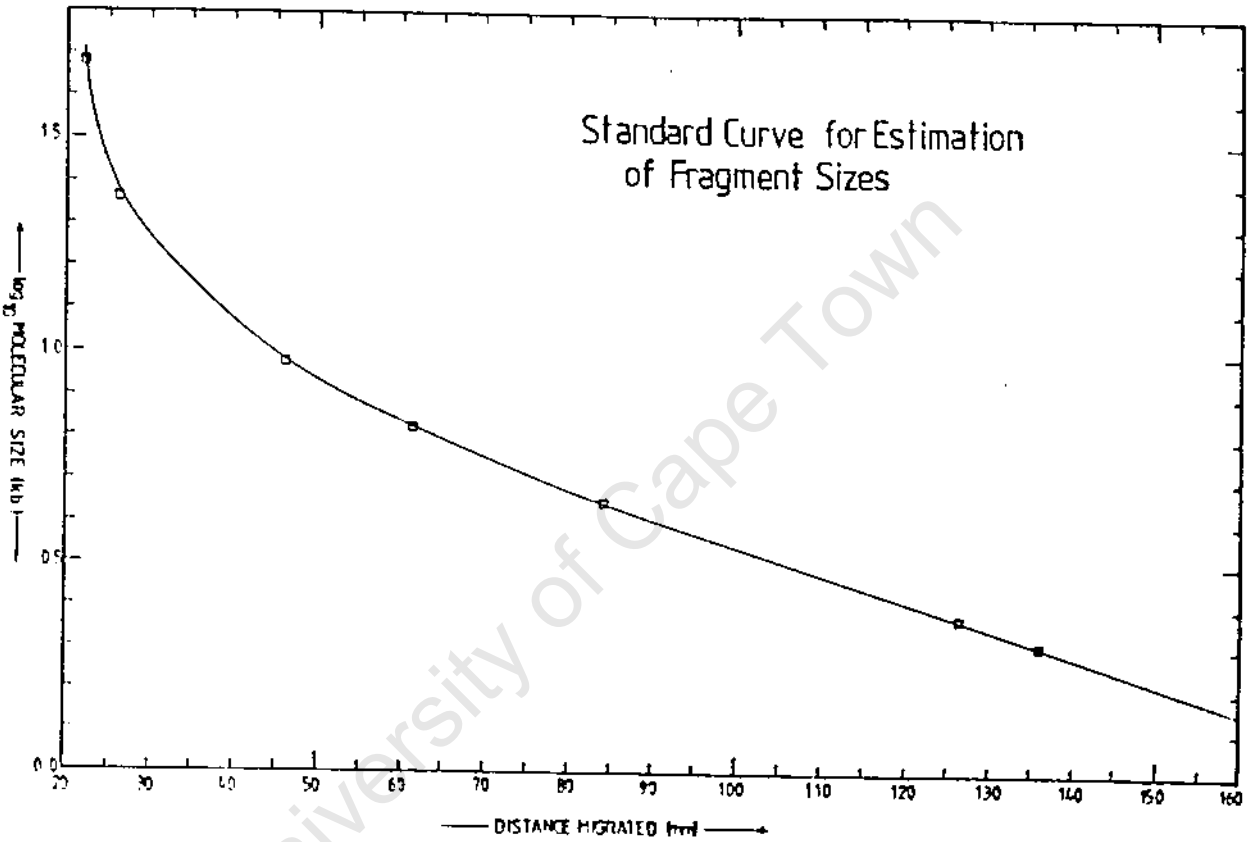

Figure 4.2.4.: The standard curve using lambda DNA digested with Hind III for the molecular size estimations of unknown DNA fragments.

SECTION III

gRAPTER EIGHT ISOLATION OF AN AVIPDX VIRUS FROM IMPORTED PARROTS

CHAPTER FIVE

ISOLATION OF FIRST PDX-LIKE VIRUS FROM PENGUINS

5.1: OCCURRENCE OF LOCAL AVIPDX VIRUSES

Jackass penguins *(Spheniscus demersus)* are found along the Southern African coastline especially in the colder waters around Cape Town and Saldanha Bay. (Refer to Figure 5.1.1.) A popular breeding ground for these birds is Robben Island,but the alarming decline in the penguin population poses a threat to the balance of the ecological system along the coastline.

Penguins found injured or coated with oil along the Cape Peninsula coastline are sheltered at the South African National Foundation for the Conservation of Coastal Birds (SANCCOB) Centre in Milnerton, north of Cape Town. Some penguins develop pox-like lesions on the bare skin round the eyes after they have been at SANCCOB for a few days. It was from lesions such as these that specimens were taken for this study. Furthermore, some of the penguins which had developed pox lesions became fatally ill with what appeared to be bronchopneumonia. It appears that the incidence of pox-like infections in penguins is seasonal.

Figure 5.1.1.: A photograph showing a penguin resident at SANCCOB.

5.2.: MATERIALS AND METHODS

5.2.1. COLLECTION AND PREPARATION OF SPECIMEN

A lesion (illustrated in Figure 5.2.1) was sampled from a Jackass penguin in October, 1988, by cutting off the skin on and around the affected area with a sterile scalpel. The scrapings were placed in Bijou bottles containing transport medium (Appendix A) and were taken to the laboratory, at ambient temperature, for processing.

The scrapings were dounced aseptically in a Tenbroek grinder in a sterile laminar flow hood. Sterile McIlvain's pH 7,4 buffer (Appendix A) was added to the grinder, at 1 ml per scraping. The ground material was diluted ten-fold in phosphate buffered saline (PBS, Appendix A) containing the following antibiotics Penicillin (200 Units/ml), Streptomycin (0,2 mg/ml) and Neomycin (0,2 mg/ml). The diluted material was inoculated onto previously prepared CAMs of 12-day-old chick embryos (Chapter 2.2.1. and Chapter 2.2.2).

5.2.2. VIRAL ISOLATION

Membranes harvested (Chapter 2.2.3.) three days post-inoculation showed the presence of focal lesions; however, the presence of these lesions was completely overshadowed by contaminating bacteria. Membranes harvested after four days showed more focal lesions but there was a higher degree of bacterial

contamination and, at this stage, the chick embryos had died.

Figure 5.2.1.: A photograph showing a Jackass penguin. The lesion is indicated by a black arrow.

The membranes harvested four days post-inoculation were placed into universal bottles which were a third filled with glass beads of an average diameter of 5 mm. Mcilvainls buffer was added to the bottles at 1 ml per membrane. The bottles were shaken vigorously for 1 to 2 minutes, to release the virus into the hypotonic solution, and kept on ice for one hour, after which they were centrifuged for 8 to 10 minutes at 600 g in a Sigma 301K swing-out rotor (Sigma, St Louis, United States of America). The supernatant fluid was retained and the bottles containing the membranes were discarded.

The antibiotic concentration in the diluent was increased ten-fold to Penicillin (2000 U/m1), Streptomycin (2 mg/ml) and Neomycin (2 mg/ml). The supernatant fluid, called Passage 1, was used as the viral stock.

The viral stock (Passage 1) was serially diluted with the more concentrated antibiotic solution to 104, 102 and 104. Two sets of previously prepared CAMS (Chapter 2.2.1.) were then inoculated with each dilution as described above (Chapter 2.2.2.), and incubated at 36,5°C for 3 days and for 4 days respectively.

The membranes were harvested and examined. The virus was released from the membrane by grinding and low speed

centrifugation, and the supernatant was labelled as Passage 2. Before any further passaging, an antibiotic sensitivity test was conducted on Passage 2 material.

5.2.3. ANTIBIOTIC SENSITIVITY STUDY.

To obtain single bacterial colonies, the viral stock was streaked out on a 2% blood agar plate (Appendix A) and incubated overnight at 37°C. Bacterial colonies could be divided into three groups on the basis of their morphology. Single colonies from each group were picked off the blood agar plate and suspended in tryptone broth (Appendix A). The suspended colonies were then plated separately on the inner halves of 3 Luria agar plates (Appendix A), using an antibiogram wheel (Mast Industries, Merseyside, United Kingdom). As controls a highly resistant E. coil strain was plated on the outer half of one of the agar plates and a highly sensitive strain of E. coli was plated on the two other plates. Various antibiotic discs were placed at intervals around the interface between the two organisms. The antibiotics and the concentration of the discs are tabulated in Table 5.2.1. The plates were incubated overnight at 17°C and examined for growth of the organisms the following day.

Table 5.2.1: The concentrations of the antibiotics used in the antibiotic sensitivity study.

Antibiotic	Concentration in µg/ml
Amikacin	10
Amoxycillin	10
Ceftriaxone	30
Chloramphenicol	10
Gentamicin	10
Kanamycin	30
Neomycin	30
Tobramycin	10
Trimethoprim	1,25

5.2.4. GROWTH OF FOCAL LESIONS IN PRESENCE OF GENTAMICIN.

Gentamicin (Gen) at a concentration of 400 µg/ml, was added to the diluent PBS (Appendix A). The Passage 2 stock was diluted and inoculated onto CAMs and incubated at 36,5'C for three and four days respectively.

The virus was passaged sequentially as described above (Chapter 5.2.1) for three further passages in the presence of the diluent containing the four antibiotics (Pen, Strep, Neo and Gen) to produce a bacterial-free viral stock.

5.2.5. VIRAL PURIFICATION BY SINGLE POCK PASSAGE.

Before any further studies could be conducted, a pure homogeneous viral stock had to be obtained originating from a single pock. The viral glycerol stock was serially diluted in the range from 1 to 10-6 and each dilution was injected onto a CAM. The CAMs were incubated for 4 days at 36,5°C and processed as follows:

A "nest" of paper towel was placed in a petri-dish and into which the egg was rested. A sterile blunt-ended forceps was used to pierce through the shell, which was removed to reveal the infected CAM. A single pock, located at the highest dilution (10-6), wasexcised from the CAM and crushed in a sterile pestle and mortar. PBS

diluent was added and the suspension was inoculated onto three freshly prepared CAMs, which were incubated and used for viral harvesting as described in Chapter 5.2.1. The viral stock from this single pock stock was used for further experiments.

5.3: RESULTS

5.3.1. COLLECTION OF SPECIMEN

The lesion on the penguin was examined by a veterinary surgeon who suggested that scrapings of the lesion be investigated for the presence of any infectious agent. The penguins could have been exposed to the agent at SANCCOB, or it could have resulted from a latent infection caused by stress experienced on admission to this centre.

5.3.2. ISOLATION OF VIRUS

The membranes harvested and examined were found to be heavily contaminated with bacteria but focal lesions (probably viral) could still be seen.

5.3.3. ANTIBIOTIC SENSITIVITY STUDY

Bacteria from each of the three morphological colonies, were all shown to be Gram negative.

Results of the antibiotic susceptibility study are tabulated

in Table 5.3.1. The resistance or sensitivity of the three different colonies isolated was determined by the presence or absence of growth on the plate. Two E.coli strains were used as controls.

Table 5.3.1: The results of the antibiotic susceptibility study of the three isolated colonies.

Antibiotic	Concentration in µg/ml	Colony 1	Colony 2	Colony 3
Amikacin	10	R*	R	R
Amoxycillin	10	S*	S	R
Ceftriaxone	30	S	S	S
Chloramphenicol	10	S	S	S
Gentamicin	10	S	S	S
Kanamycin	30	R	R	R
Neomycin	30	R	R	R
Tobramycin	10	S	S	R
Trimethoprim	1,25	R	R	R

Where * S represents antibiotic sensitivity and R represents antibiotic resistance.

All three isolated colonies were sensitive to Gentamicin, Ceftriaxone and Chloramphenicol but not to Penicillin, Streptomycin and Neomycin.

5.3.4. VIRAL GROWTH IN GENTAMYCIN

The viral material (Passage 2) was grown in a solution containing 400 *mg/m1* Gen and the antibiotics Pen, Strep and Neo. The membranes were harvested and examined after 4 days incubation at 36,5°C revealed few contaminating bacteria.

The viral material was passaged sequentially for three passages to produce a bacterial-free suspension. This was confirmed by streaking the viral supernatant, Passage Six, on a blood agar plate and incubating at 37°C. Examination of the plate after 18, 42, 66 and 90 hours showed no bacterial colonies.

This viral material was used to prepare a fresh stock of uncontaminated virus. The viral supernatant was passaged, harvested and placed into glass bottles containing 80% glycerol in McIlvains buffer. This stock was stored in two aliquots, one a working stock at -20°C and the other for long term storage at -70°C. Because the lesions were more clearly visible after incubation for four days, this period was adopted for the propagation of viral material.

5.3.5. PURIFICATION OF VIRUS BY SINGLE POCK PASSAGE

A single pock was located on the membrane inoculated with the highest dilution (le) of the viral material. The pock was harvested, crushed, diluted with diluent and inoculated onto freshly prepared CAMs. These were harvested after 4 days incubation at 36,5°C and 2 stocks were prepared as described above (Chapter 5.3.4.).

5.4: CONCLUSION

An infectious agent was isolated from the lesion of a Jackass Penguin. This agent was isolated on CAMs of 12-day-old chick embryos and found to be resistant to Pen, Strep and Neo. Antibiotic sensitivity tests were conducted on contaminants that were found to be sensitive to Gen (used at 400 mg/m1). Neither filtration nor centrifugation was used to remove the bacteria because it had not yet been established that the infectious agent was a virus. The growth on CAMs, after the exclusion of bacteria (Chapter 5.3.4), confirmed the presence of a virus.

CHAPTER SIX

IDENTIFICATION OF PENGUINPDX VIRUS

6.1: IDENTIFICATION OF AVIPDX VIRUSES

Avipox viruses have been identified by electron microscopy studies (Randall, et al., 1964), by cross-protection studies (Boosinger, et al., 1982), by microscopic examination of the infected material (Harrigan, et al., 1975) and by inoculation of avian hosts (Chung and Spradbrow, 1977, and Boosinger, et al., 1982).

The electron microscopy studies and the microscopic examination of infected material were conducted on the penguinpox isolate. The methods and results are described below.

Host range and cross-protection studies could not be conducted because the extensive isolation facilities required for the study were unavailable.

6.2: METHODS USED

6.2.1. ELECTRON MICROSCOPY OF VIRUS.

The viral material was purified by centrifugal sedimentation into a I M sucrose cushion at 15 000g in a IEC B20-A centrifuge (IEC/Damon, Needham Heights, United States of America) for one hour at 4°C. The pellet in sucrose was resuspended in

McIlvains buffer and pelleted at 15 000 g without a sucrose cushion. After two further centrifugation steps the purified virus was negatively stained with phosphotungstic acid at pH 6,2 and examined in the electron microscope. The virus was photographed by Dr L Stannard of the Department of Medical Virology (Medical School, UCT).

6.2.2. MACROSCOPIC EXAMINATION OF POCKS ON CAMS

The macroscopic appearance of pox-like virus from penguins was compared with that of other known avipox viruses, as well as with that of orthopox viruses. Other avipox viruses available for comparison included fowlpox virus, pigeonpox virus, canarypox virus, turkeypox virus and quailpox virus. Each of the viral stocks was suitably diluted and inoculated on CAMs.

6.2.3. HISTOLOGY OF POCKS ON CAMS.

In order to confirm the identification of the virus, the type of lesion produced by the virus on CAMs was studied. Histological cross-section studies were performed on the virus grown on CAMs.

The pox-like virus was diluted suffeiciently to ensure single pocks on CAMs. The CAMS were harvested at two, three and four days post-inoculation, and instead of being processed as described in Chapter 5.2.5., where harvested and the membrane

placed on a soft piece of cardboard and fixed by immersation in Zenker's base (Appendix A) for 30 minutes. The excess base was washed off with running tap water and the membrane stored in this manner. For histological studies, the fixed membranes were enclosed in a plastic cassette. The membranes were embedded in paraffin wax and longitudinal cross-sections were cut by staff in the Department of Histology (Medical School, UCT).

Differential tissue staining was carried out according to the methods of Culling (1963) and Drury and Wallington (1976). The sections were first de-waxed in xylol for one hour at room temperature, washed for 30 seconds in decreasing concentrations of ethanol (absolute ethanol, 96% and 70% ethanol), rinsed in water and immersed in Iodine alcohol (Appendix A) for five minutes, rinsed again in water, bleached with a 5% sodium-thiosulphate solution for five minutes and washed in water for five minutes. These sections were stained in haematoxylin (Appendix A) for five minutes and rinsed thoroughly under running water. The tissues were differentiated by placing the sections in a 1% acid-alcohol solution (Appendix A), rinsed under running water and placed in a Scotts water solution (Appendix A) for 30 - 60 seconds before rinsing under running water. The sections were counterstained in eosin (Appendix A) for five minutes, washed under running water and

were dehydrated for 30 seconds in increasing concentrations of ethanol (70%, 96% and three washes of absolute ethanol). The sections were placed in xylol and mounted in DFX mounting fluid.

6.2.4. SUSCEPTIBILITY OF CEF CELLS TO VIRUS GROWTH COMPARED WITH MAMMALIAN CELLS.

Various mammalian cell lines (CV1, RR 13, MDCK and MDBK cells), mammalian primary cells (HF), CEF cells and TE cells were grown and maintained as described in Chapter 3.2.

Confluent cell monolayers, grown in 24-well plates or in 25 cm2 cell culture flasks, were infected with various dilutions of the virus as follows:

The viral stock was first diluted to 100 pock forming units per ml (pfu/ml) in MEM diluent (Appendix A) as determined by a titration conducted on CAMS. The supernatant fluid from a newly confluent cell monolayer was carefully aspirated and the viral inoculum of 1 ml per 5 cm2 was added. (This was enough to just cover the whole cell sheets). The viral suspension was allowed to adsorb to the cells for one hour at 37°C, thereafter the inoculum was removed and maintenance MEM with 5% FCS was added. The cells were incubated for 5 days at 37°C, with daily examination for any changes to the cell layer.

After the fifth day the supernatant was harvested and stored at -20°C. The cells were fixed with a strong carbol fushsin solution for 30 - 60 seconds (Appendix A) by covering the cell layer with the fixative, then washed by flooding the plate with water, and left to dry. The stored supernatant cutlure fluid harvested was used to re-inoculate fresh confluent cell monolayers.

6.3: RESULTS AND DISCUSSION

6.3.1. ELECTRON MICROSCOPY OF ISOLATE

Photographs taken during the electron microscopy studies are shown as Figures 6.3.1, 6.3.2., 6.3.3. and 6.3.4. Several observations were made on examination of these photographs.

Figure 6.3.1. at a magnification of 60 000 times shows three virus particles with typical poxviral morphology, and similar to fowlpox viruses as seen by Randall and Gafford, (1961). Two intact virions are brick-shaped and measure approximately 130 nm wide and 150 nm long. They display characteristic surface tubules. One virus parlice is slightly collapsed and has been penetrated by the negative stain to reveal an apparent bi-lipid membrane.

Figure 6.3.2. depicts a higher magnification (of 80 000 times) of a typical poxvirus encased within a large lipid-like sac.

Figure 6.3.3. shows a virus particle similar to that in Figure 6.3.2. but the lipid-like sac has been ruptured and appears as an empty bag on the side of the virus particle (shown by arrow). The bi-lipid membrane (associated with pox viruses) can be clearly seen. The surface tubules, present on previous photographs, are not evident in this figure.

Figure 6.3.4. at a magnification of 80 000 times, shows a freely-lying virus particle (without its lipid sac) on which the surface tubules are clearly visible.

From the electron microscopic examination, the size, shape and morphological components of the virions confirms that the penguin virus isolate is a pox virus. This is consistent with the appearance of the lesion seen on penguins and with the morphology of the pocks produced on CAMs.

The length of the incubation period on CAMs as well as the virus morphology (size, shape and presence pf surface tubules and bi-lipid membrane) are factors common to all pox viruses. The morphological similarities between the penguin poxvirus and published photographs of fowlpox (Holt and Krogsrud, 1973 and Randall et al., 1964) is further evidence that the penguin isolate is a pox virus.

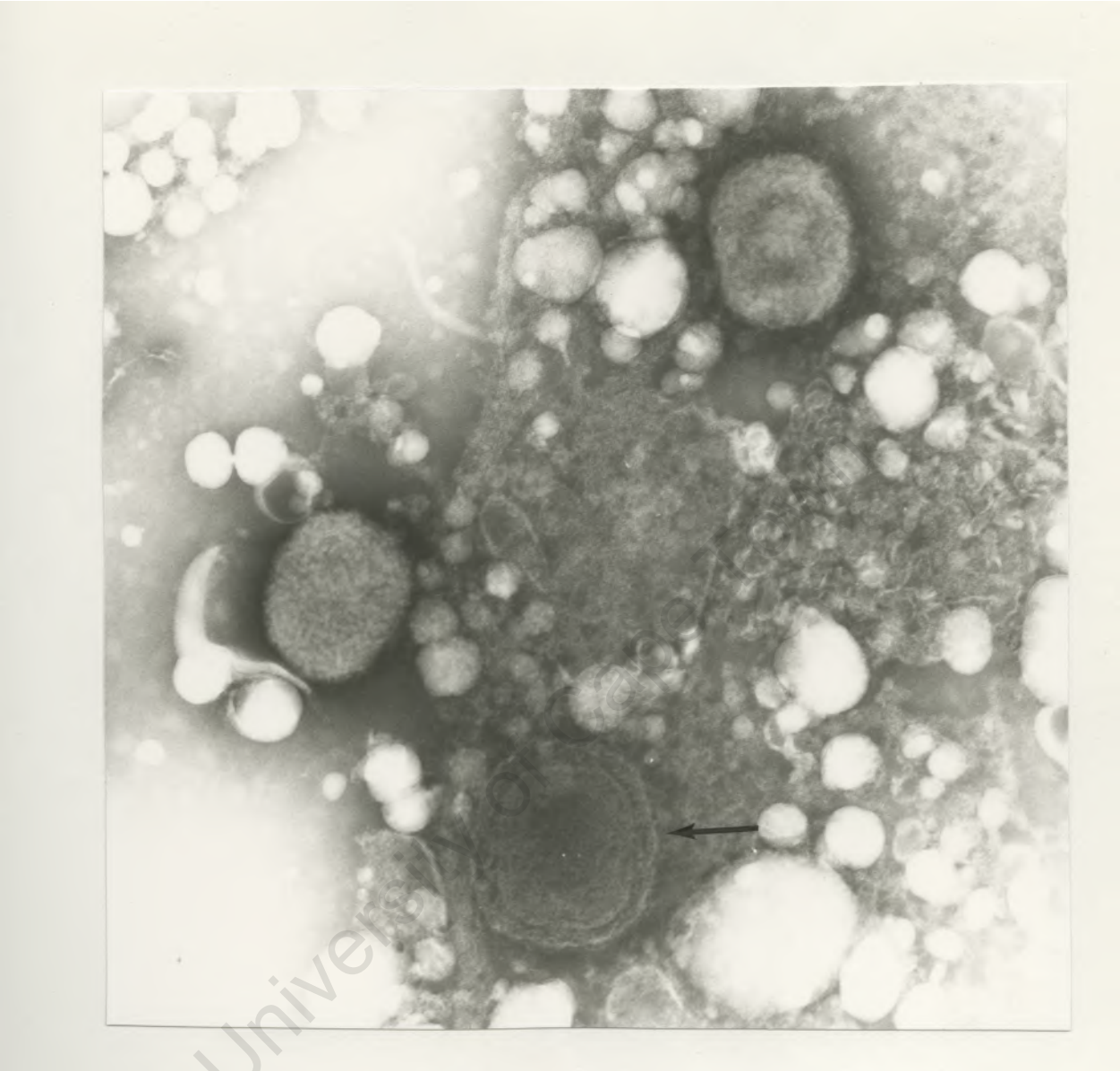

Figure 6.3.1.: A photograph showing three virus particles, negatively stained. Two particles are intact and display distinctive surface tubules. The third particle (arrow), has collapsed to reveal the inner component.

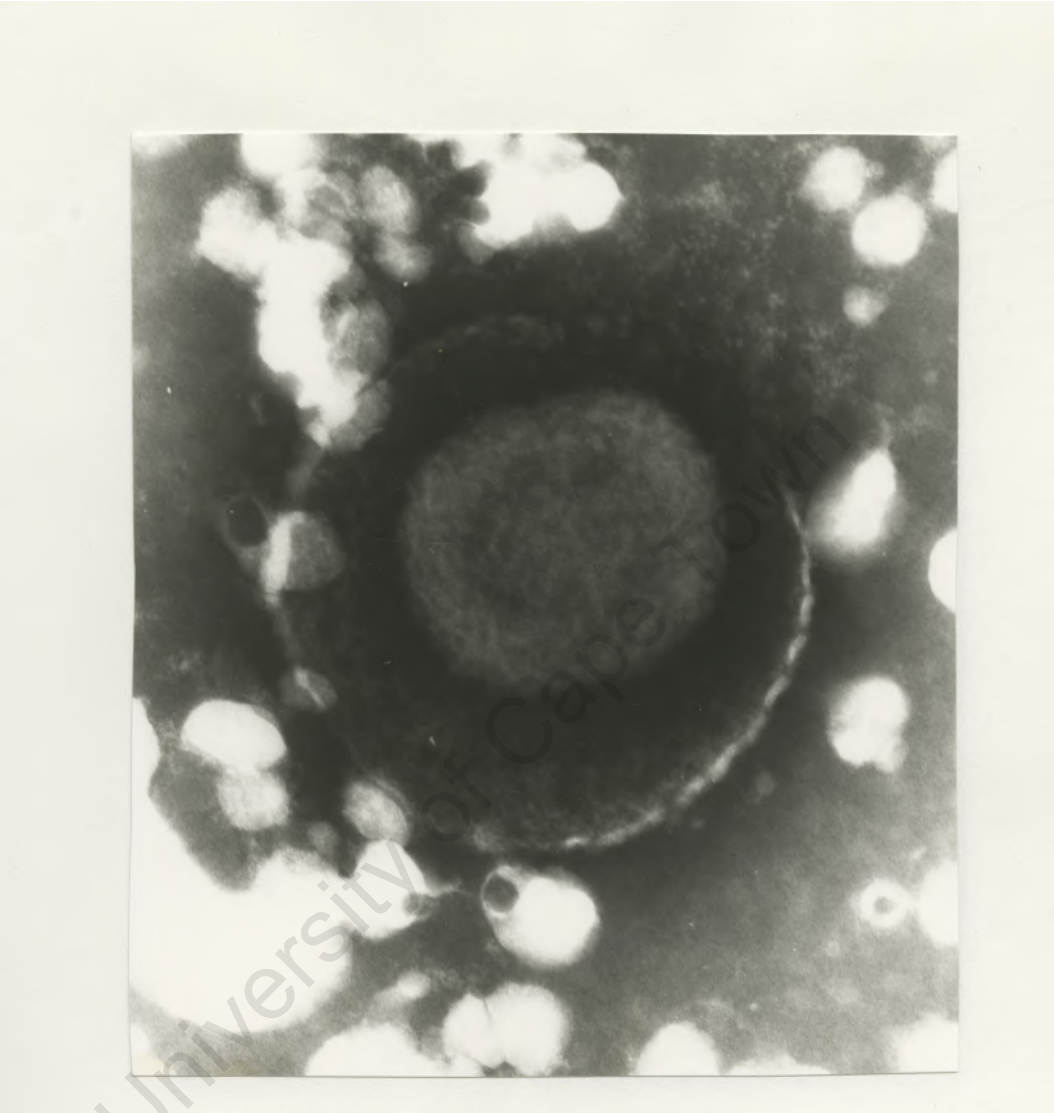

Figure 6.3.2.: The virus particle seen at a magnification of 80 000 times showing the lipid-like casing and the surface tubules on the surface of the particle.

Figure 6.3.3.: A virus particle emerging from a broken lipid sac (arrow) can be seen. No surface tubules are visible, but a bi-lipid membrane similar to that of poxvirusesis discernible. (Magnification is 80 000 times).

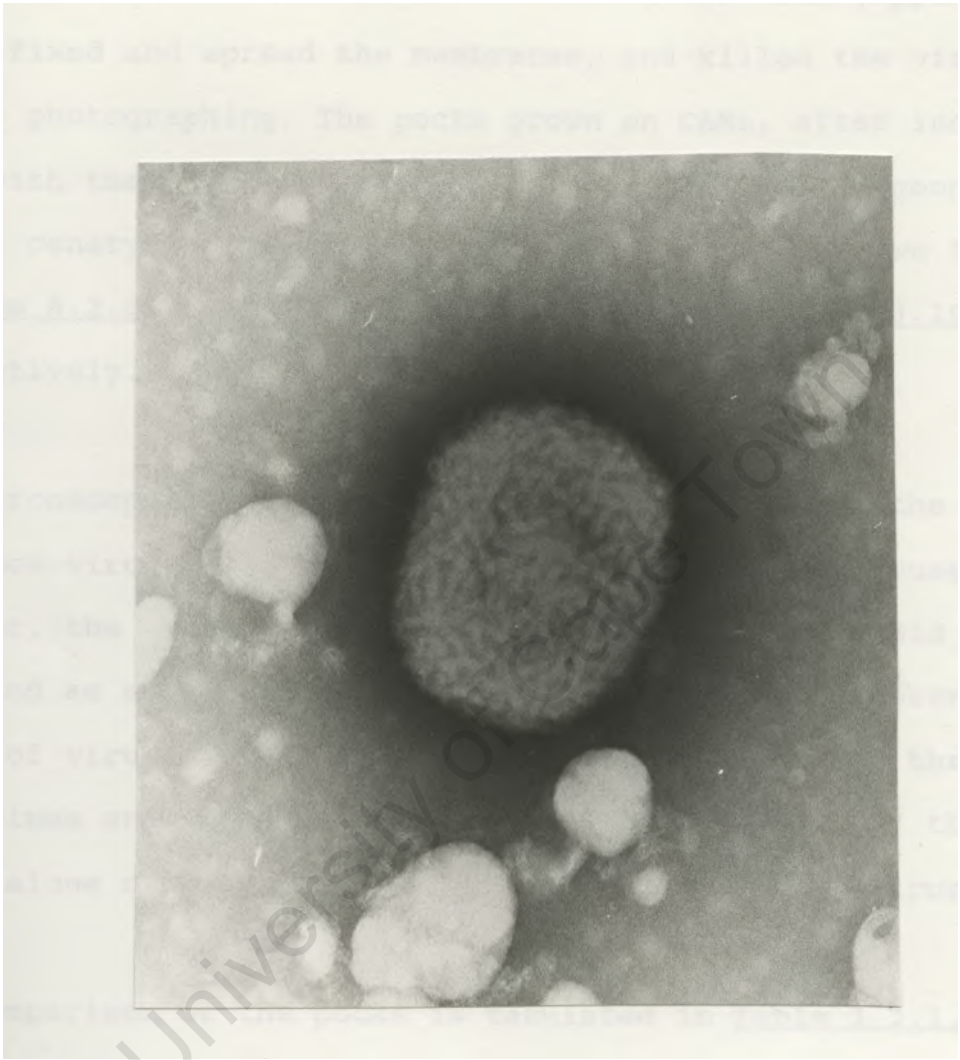

Figure 6.3.4.: A fre-lying a virus particle showing distinctive surface tubules. (Magnification 80 000 times)

6.3.2. MACROSCOPIC APPEARANCE ON CAMS

The membranes were harvested after 4 day's incubation at 36,5°C. The membranes were washed in 5 % formalin (Appendix A) which fixed and spread the membranes, and killed the viruses before photographing. The pocks grown on CAMS, after inoculation with the penguin pox isolate, fowlpox virus, pigeonpox virus, canarypox virus and turkeypox viruses are shown in Figures 6.3.5., 6.3.6., 6.3.7., 6.3.8. 6.3.9. and 6.3.10. respectively.

The marcoscopic appearence of the pocks produced by the penguin pox virus is different from the other avipox viruses. However, the penguin pox virus isolate is a fresh field isolate and as such the pock morphology might look different to those of viruses which have been passaged many times through cell times and on CAMs. The macroscopic appearance of the pocks alone cannot be a basis to differentiate the viruses.

The comparison of the pocks is tabulated in Table

Table 3.3.1: A morphological comparison of pocks produced on CAMS.

Virus	First described	Appearance of pocks	Relative size (mm)
penguin isolate	this study	grey-white, some ulceration	1,3
fowlpox	Mayr (1963)	opaque, capillary haemorrhage	3,2
pigeonpox	Mayr (1963)	grey, slightly ulcerated.	2
canarypox	Burnet et al., (1933)	grey-white, ulcerated, hollow or dense centre, capillary haemorrhage	2,6
quailpox	Rinaldi et al.,(1972)	opaque grey, ulcerated, hollow or dense centre	2
turkeypox	Mayr (1963)	grey, ulcerated, capillary haemorrhage	1

Figure 6.3.5.: Pocks of penguin isolate grown on CAMs.

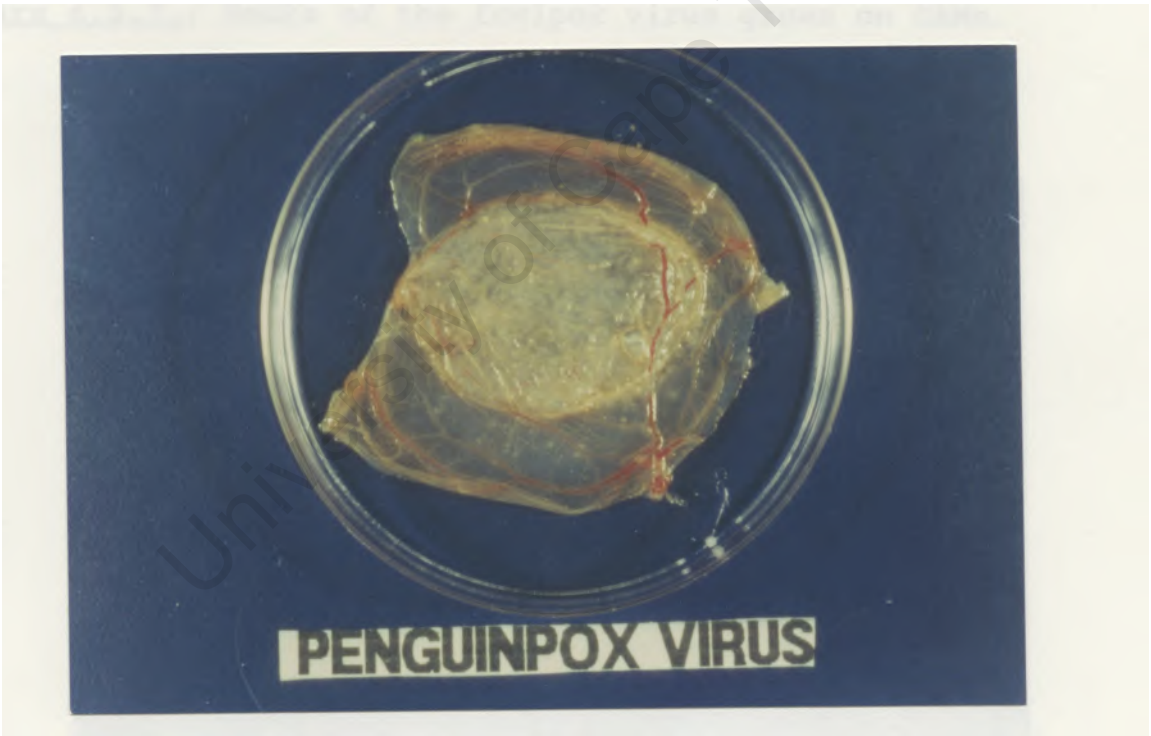

Figure 6.3.6.: Pocks of penguin virus grown to confluence on CAMs.

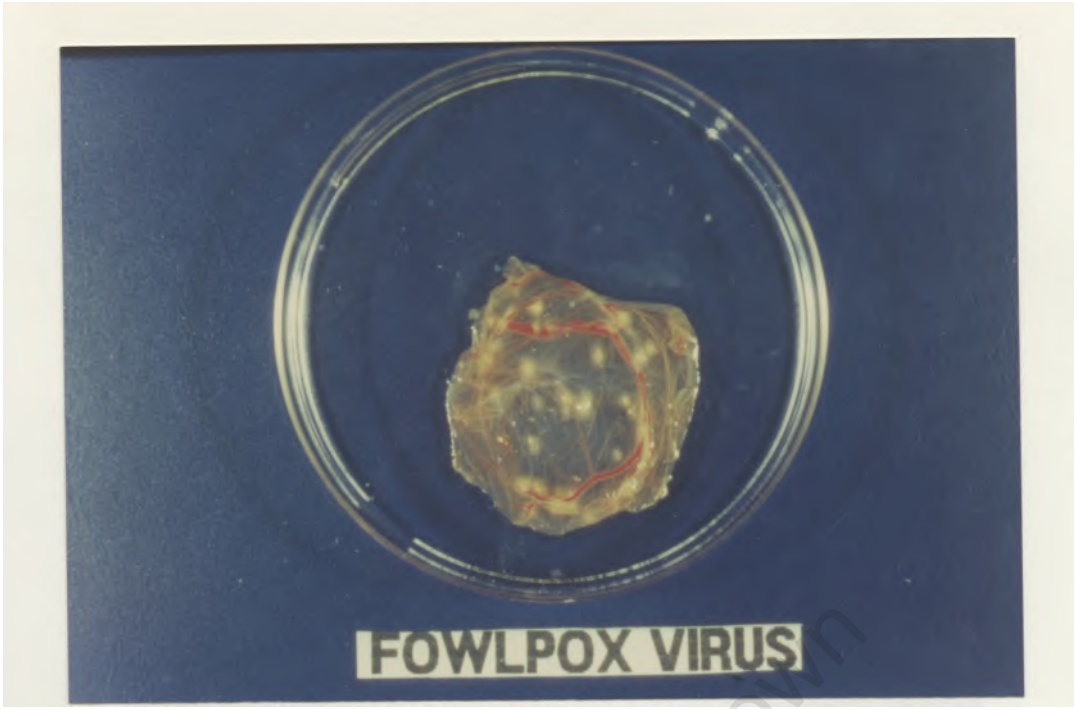

Figure 6.3.7.: Pocks of the fowlpox virus grown on CAMs.

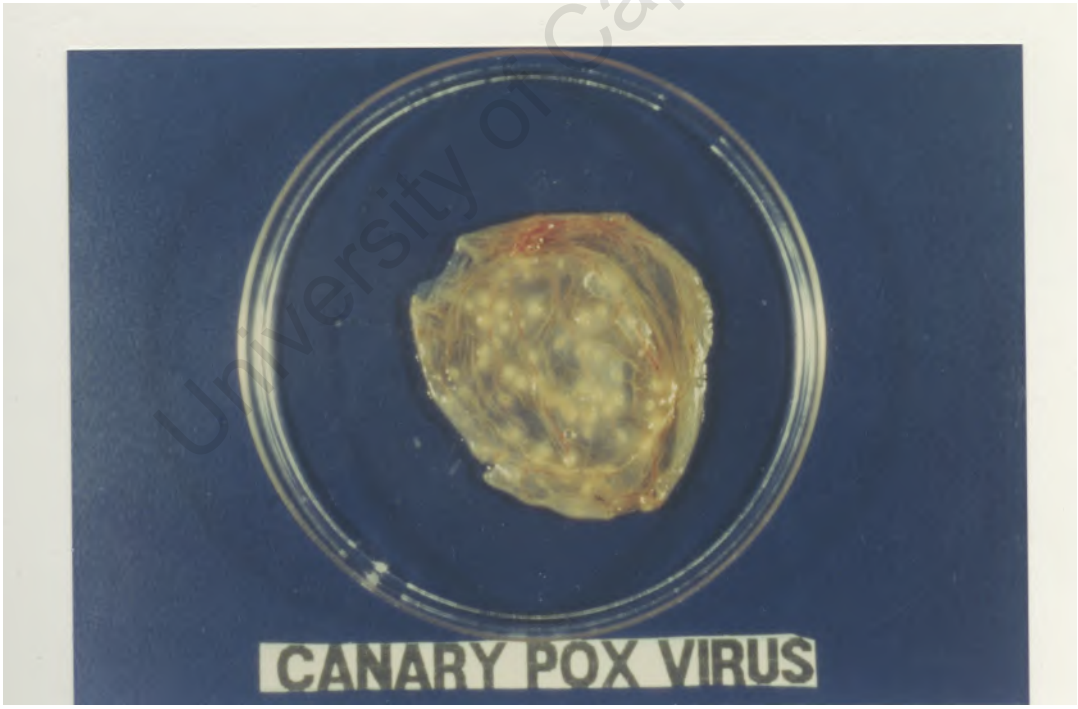

Figure 6.3.8.: Pocks of the canarypox virus grown on CAMs.

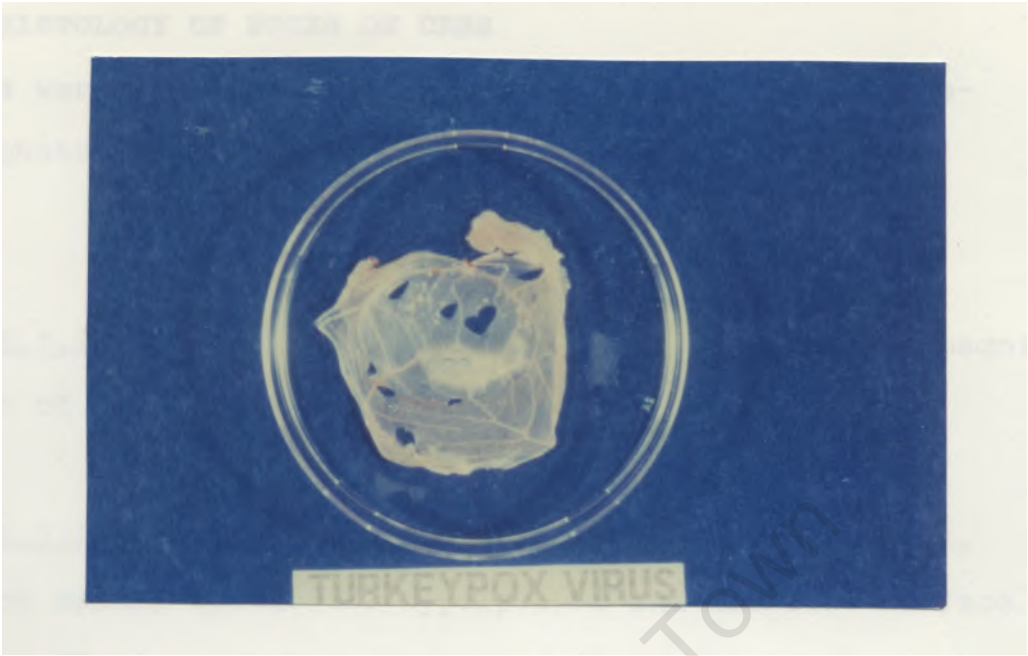

Figure 6.3.9.: Pocks of the turkeypox virus grown on CAMs.

Figure 6.3.10.: Pocks of pigeonpox virus grown on CAMs.

6.3.3. HISTOLOGY OF POCKS ON CAMS

Sections were examined under a light microscope and appropriate photographs taken as Figures 6.3.11., 6.3.12. and

Figure 6.3.11. shows a normal, uninfected membrane at a magnification of 40 times.

Figure 6.3.12. shows a section through the edge of a pock, where the marked epithelial hyperplasia and necrotic surface layer are distinguishable. Leucocytosis is evident from the cross-section of a large blood-vessel and some inflammatory cells can be seen infiltrating the deeper layers of the hyperplastic epithelium.

Figure 6.3.13. shows the ballooned cytoplasm of the epithelial cells in the pock. Diffuse, eosinophilic cytoplasmic inclusion material was evident on examination under the microscope, but cannot be distinguished in this photograph.

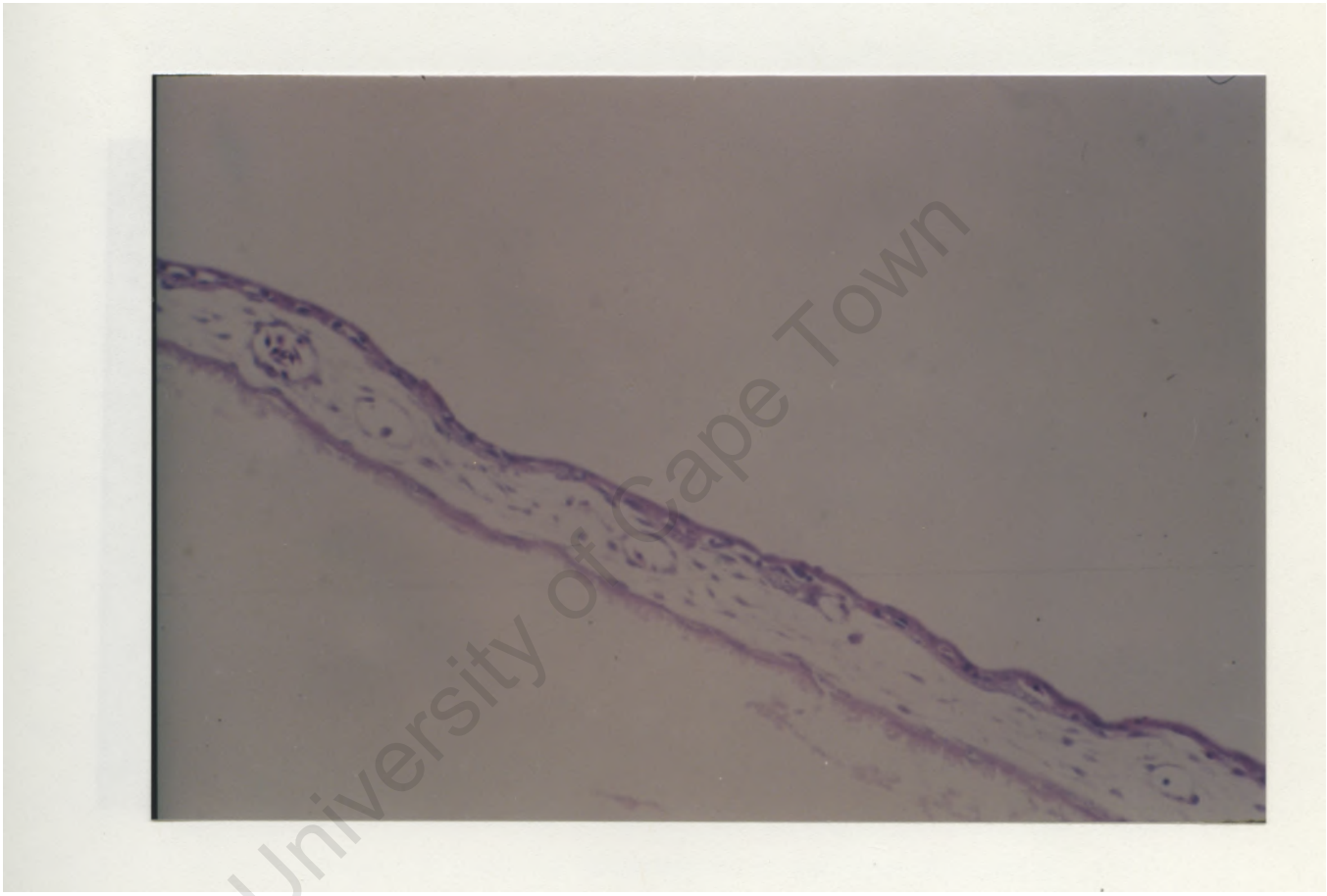

Figure 6.3.11.: An uninfected membrane at a magnification of 40 times.

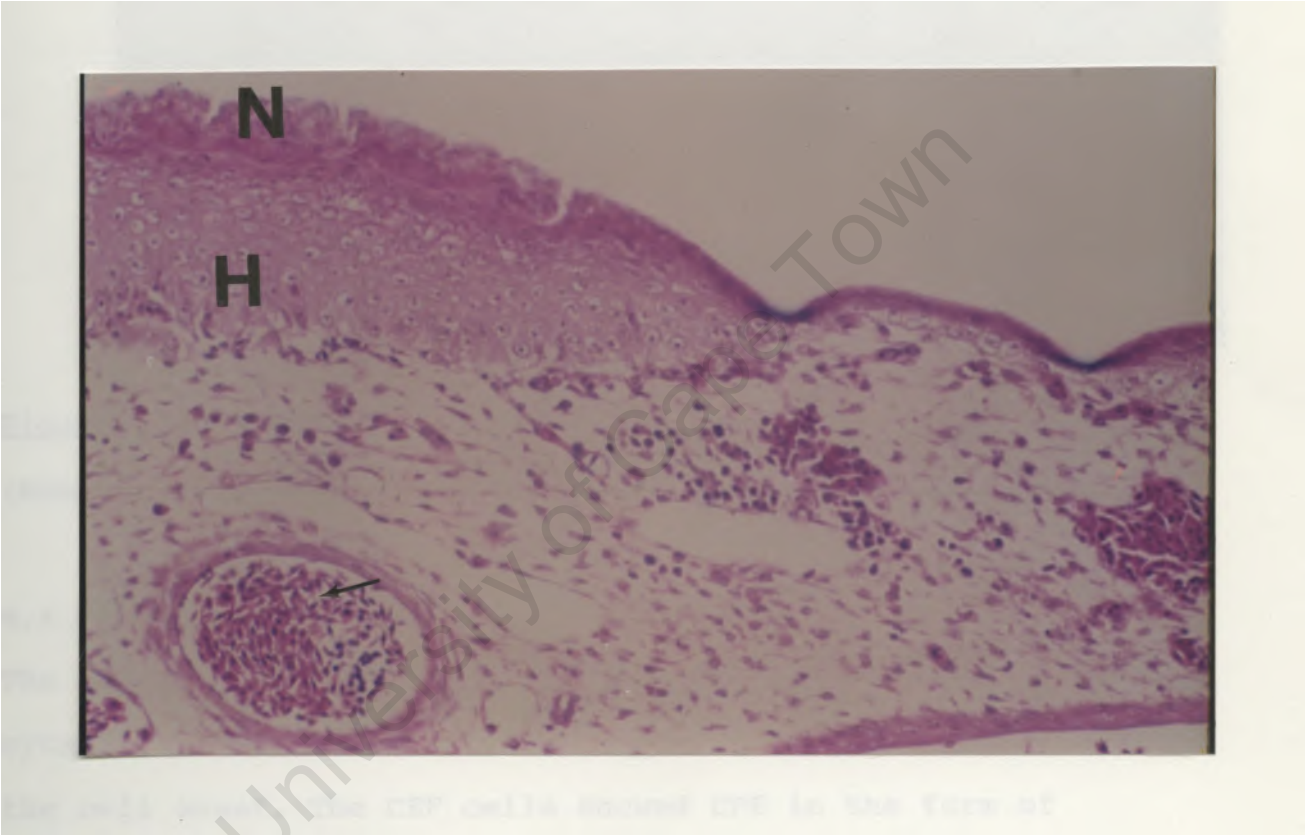

Figure 6.3.12.: A cross-section through an edge of a pock showing epilhelial hyperplasia (H) beneath a necrotic surface layer (N). Inflammatory cells are indicated by arrows. (Magnification of 120 times).

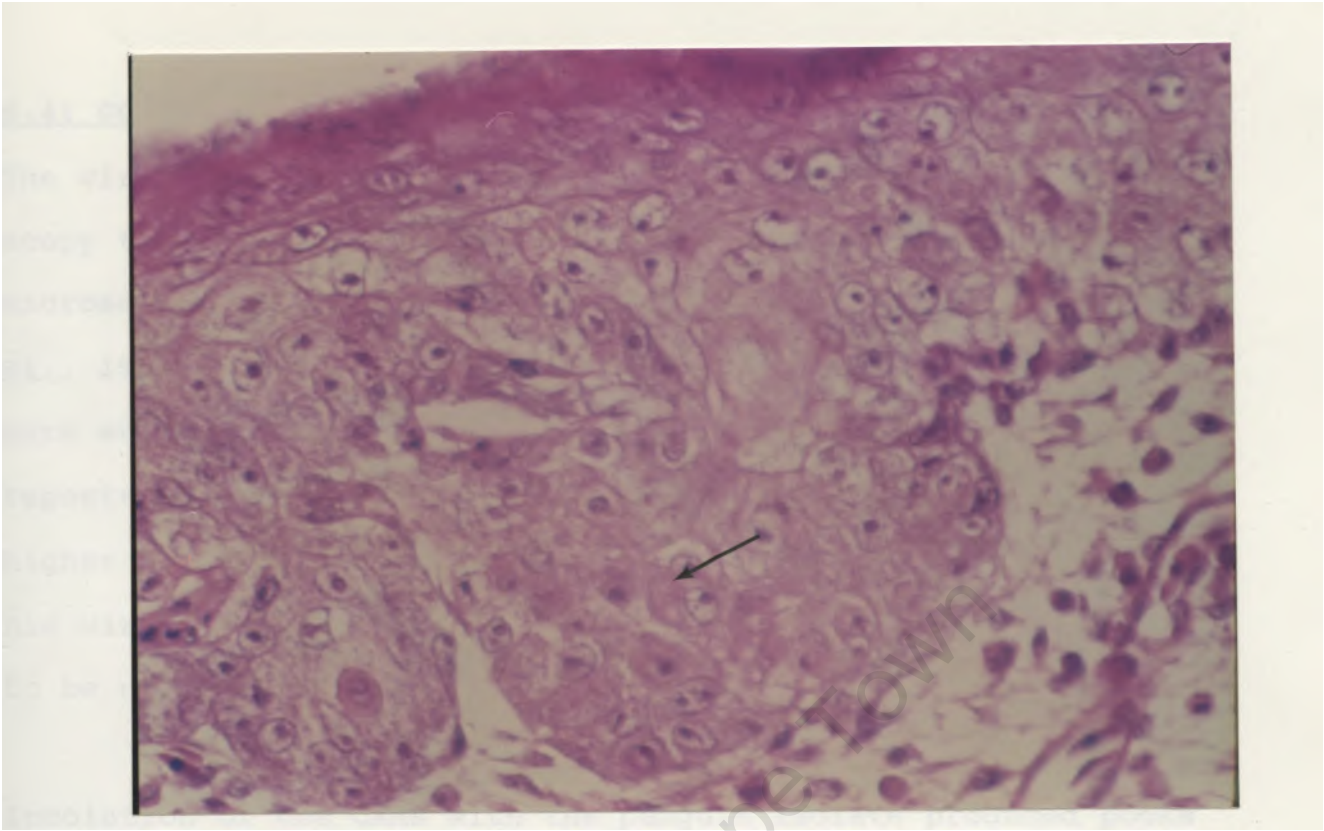

Figure 6.3.13.: Ballooned cytoplasm of the epithelial cells (shown by arrows) within a pock. (Magnification 800 times).

6.3.4. SUSCEPTIBILITY OF CEF CELLS

The cell sheet stains dark red whereas the plaques, pox virus cytoplasmic effects (CPE), were visible as clear regions in the cell sheet. The CEF cells showed CPE in the form of syncytia forming after three days post infection, and plaques after the fourth day. None of the other cell lines showed CPE even after five days, and after 5 sequential passages no CPE could be found in any other of the cell lines tested.

6.4: CONCLUSION

The virus isolated from penguins was shown by electron microscopy to be a poxvirus. The structures found by electron microscopic resembled those reported for fowlpox (Randall, et al., 1966) and vaccinia viruses. In addition, the particles were encased in a large lipid-like capsule. This has not been reported before although Randall et al, (1962) reported a higher amount of lipid present in fowlpox virus than in vaccinia virus. Further investigation of these capsules will have to be undertaken before definitive conclusions can be made.

Incolation of the CAMs with the penguin isolate produced pocks which were similar to, bu smaller than, the pocks found with other avipox viruses. The new isolate needs to be adapted to grow in these artificial circumstances before a definite species differentiation can be based on pock morphology.

Histological examination of pocks revealed enlarged infected cells showing degeneration. These cells also contained eosinophilic inclusions, known as Bollinger bodies (Tripathy, et al., 1983) or A-type inclusion bodies. Avipox viruses are said to produce both A- and B-type inclusions. The B-type inclusion bodies are seen in the cytoplasm of infected cells, but only at very high magnification. These results provide additional evidence that the penguinpox isolate is an avipox

virus.

Confirmation that the pox-like isolate described in this study is an avipox virus, came from the susceptibility of primary chick embryonic fibroblasts to this virus and its inability to replicate in any non-avian cell line. The inability of the virus to replicate in mammalian cells, but with full growth potential in chich embryo cells, also suggests that the isolate is an avian virus.

Due to its similarity to fowlpox virus, it was concluded that the virus isolated from penguins was a member of the avipox virus genus. The next step in this investigation was to ascertain the degree of similarity with other avipox viruses.

CHAPTER SEVEN

ISOLATION OF OTHER AVIAN PDX-LIKE VIRUSES

7.1: ISOLATION OF LOCAL VARIETIES OF AVIAN PDX-LIKE VIRUSES

It was possible that the penguinpox isolate represented a generally distributed South African avipox virus rather than a virus specific to penguins. For comparative purposes, alternate avipox viruses were isolated from different species of birds in this region.

7.2: SECOND ISOLATION OF PDX-LIKE VIRUSES FROM JACKASS PENGUIN

7.2.1. METHODS

7.2.1.1. SPECIMEN COLLECTION AND PREPARATION

A year after the first isolations, two more isolates of a pox-like virus were obtained from different Jackass penguins.

Unlike the first isolation, where the lesion was crusted and closed, these lesions, occurring around the eyes of both birds, were discharging. Specimens were obtained by swabbing the discharge and transporting it to the laboratory to be kept at 4°C for further culture and examination.

Viruses were isolated using the same procedure as previously described (Chapter 5.2.2) except that McIlvains buffer was added to the swabs, and no grinding was necessary.

7.2.2. RESULTS

7.2.2.1. ISOLATION OF VIRUS

Isolation of these viruses were as previously described in Chaptul_5_s_asa. In addition Ampicillin (Amp), at 200 mg/m1 was added to the PBS diluent after an antibiotic susceptibility test had been conducted on the first passage of the virus. The viruses were also pock-purified and glycerol stocks were made and stored at -20°C and -70°C.

7.3.1. METHODS

7.3.1.1. COLLECTION AND PREPARATION OF SPECIMEN

On a local farm, several bantams (2./at) were found to have lesions around their eyes and mouth. One of these birds was sacrificed and the lesions excised. This sample was labelled "Hackett fowlpox" after the veterinary surgeon who provided the excised lesions.

Viral isolation procedures were carried out on this lesion as previously described in Chapter 5.2.1. except that it was ground in a pestle and mortar.

7.3.2. RESULTS

7.3.2.1 ISOLATION OF VIRUS

The CAM membranes were harvested, examined, and an agent, assumed to be a local variety of fowlpox, was isolated.

Photographs of thepocks grown on CAMs after inoculation with local pox-like virus (Hackett fowlpox) are shown as Figure 7.3.1. The pocks are seen to be small and slightly haemorrhagic. This local isolate was compared to the reference strain fowlpox virus shown in Figure 6.3.7. (Mayr, 1963). A difference in morphology between the pocks of the two strains of fowlpox virus is evident.

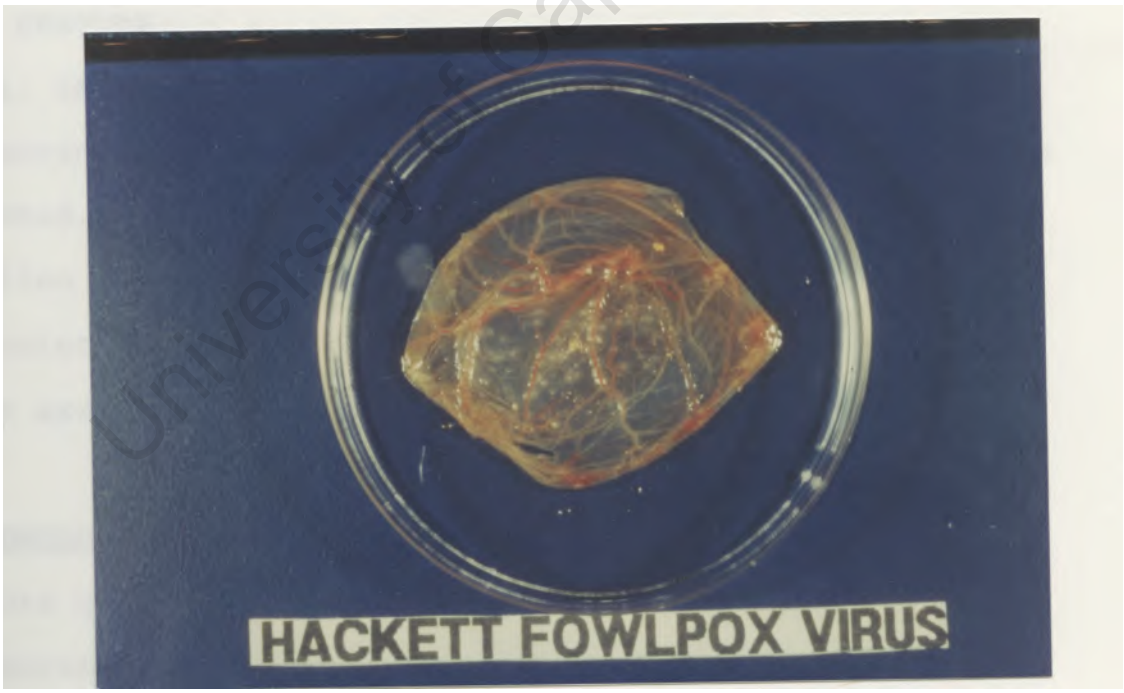

Figure 7.3.1: Pocks of the "Hackett fowl isolate" (The local fowlpox isolate) grown on CAMS for 4 days at 36,5°C.

7.4: ISOLATION OF AVIPDX VIRUS FROM A TURTLE DOVE

7.4.1. METHODS

7.4.1.1. COLLECTION AND PREPARATION OF SPECIMEN

An injured immature turtle dove *(Streptopelia* was found in Newlands, a suburb in Cape Town. There were 2 wart-like lesions present on the bird, one on the eyelid and one on the commissure of the beak.

Both lesions were harvested and treated as previously described in Chapter 5.2.1, but an extra antibiotic, Amp, at 200 mg/ml, was added to the PBS diluent.

7.4.2. RESULTS

7.4.2.1. ISOLATION OF VIRUS

The membranes were harvested, examined and focal lesions were cultivated. They were small, hollow pocks, showing little ulceration and no capillary haemorrhage. Glycerol stocks of this isolate were made and stored at -20°C and at -70°C for further examination and comparative studies.

7.5: CONCLUSION

The pocks grown from penguin isolates two and three had the same macroscopic morphology as those produced by the first penguin isolate. The appearance of the pocks was not sufficient evidence to confirm or deny the same species of virus.

The pocks from the local fowl isolate and the turtle dove isolate differed from the penguin isolates and from one another.

The pock morphology of the local fowlpox virus was compared to the reference fowlpox virus. They were found to be vastly different (Refer to Figures 6.3.7. and 7.3.1). The fact that the local fowl isolate is a fresh field isolate, whereas the established reference strain of fowlpox virus had been passaged for several generations in cells and on CAMs, is probably the reason for the difference. Pock morphology cannot be regarded as a basis for differentiation of the species of virus, and genomic typing must be done on fresh isolates.

CHAPTER EIGHT

ISOLATION OF A PDX-LIKE VIRUS FROM IMPORTED PARROTS

8.1: PARROTPDX (PSITTACINE) ISOLATES.

Parrot pox (psittacine pox virus) is rife in parrots imported into South Africa. It presents a problem in quarantine areas and is an economic drain. A recombinant vaccine, using the psittacine pox virus isolated from one such bird, was envisaged to overcome this problem. For this purpose, it was atempted to identify the location of the thymidine kinase (TK) gene of the psittacine pox virus.

8.2.: METHODS

8.2.1: ISOLATION OF A PDX-LIKE VIRUS FROM PARROTS

Lesions, from each of three different parrots held in quarantine at the Veterinary Institute at Onderstepoort, were excised, frozen, and transported to Cape Town.

Lesions were obtained from the tongue, intestine and liver of one parrot; from the intestine and tongue of a second; and from the larynx of a third parrot. Only the lesion and the area immediately around it were excised. The material was ground to a paste in a pestle and mortar. The PBS diluent (Appendix A) containing the antibiotics Penicillin (2000 units/ml), Streptomycin (2 mg/ml), Neomycin (2 mg/ml),

Gentamycin (400 gg/ml) and Ampicillin (200 pg/m1) were added to the paste.

The ground paste and diluent were inoculated onto previously prepared CAMs (Chapter 2.2.1 and 2.2.2). The CAMs were incubated for 4 days at 36,5°C and harvested as described in Chapter 2.2.3. The membranes were placed in sterile universal bottles containing glass beads with McIlvains pH 7,4 buffer, and shaken to release the virus into the hypotonic solution. The viral material was sequentially passaged until free of bacteria which was confirmed by plating on 2% blood agar plates. The plates were incubated overnight at 37°C and examined for bacterial growth on four consecutive days.

8.2.2. SINGLE POCK GROWTH

A single pock was obtained for further studies and treated as described in Chapter 5.2.3..

8.2.3. PURIFICATION AND DNA EXTRACTION OF PSITTACINE VIRAL ISOLATE.

The parrotpox viral isolates were purified as described in Chapter 9.4.1. and the DNA was extracted as described in Chapter 10.

8.3: RESULTS

8.3.1. ISOLATION OF PDX-LIKE VIRUS FROM PARROTS

The membranes were examined after 4 days incubation at 36,5°C for the presence of any focal lesions. Those infected were all positive for viral growth. The lesions on the membranes were fine and minute and resembled those produced by the penguin isolate (Figure 6.3.5.). The results of the growth and subsequent labelling are tabulated in Table 8.3.1.

Bacterial contaminants were present, although fewer than previously seen in the penguin, local fowl and turtle dove isolates. The use of the antibiotic solution described above (Chapter 8.2.1.) eliminated all bacteria; this was proved by streaking the viral suspension on agar plates, and examining the plate after 18 and 42 hours.

Table 8.3.1.; The results of virus growth from parrot specimens.

Parrot No.	Samples obtained	Viral growth (+ve/-ve)	Grown further as
1	intestine	+ve	NG
	liver	-ve	NG
	tongue	+ve	PP1
2	intestine	-ve	NG
	tongue	+ve	PP2
3	larynx	+ve	PP3

(+ve = growth on CAMs; -ve = no growth on CAMs; PP = parrot pox isolate; MG = no further investigation).

8.3.2. SINGLE POCK STOCK

The stock was stored in glycerol at -20°C and -70°C.

8.3.3. DNA EXTRACTION

The DNA obtained was stored in ultra-pure water at 4°C until used.

8.4 CONCLUSIONS

The virus isolated from parrots wproduced pocks similar in morphology to those produced by the penguin isolates.

A lower yield of virus was obtained from a more concentrated sample which suggests either that the parrot pox virus grew slower on CAMs, or that the extraction process was not appropriate for the parrot pox isolation.

SECTION IV

CHAPTER TWELVE IDENTIFICATION OF THYMIDINE RINASE GENE IN AVIPDX VIRUSES

CHAPTER NINE

EXTRACTION OF PDX VIRUSES FROM INFECTED TISSUE

9.1: EXTRACTION OF PDX VIRUSES

Joklik (1962) reported a method for the extraction and purification of orthopox viruses from rabbit skin and CAMs. This method involves shaking the tissue with glass beads, buffer and a fluorocarbon, followed by differential centrifugation and finally sedimentation through a density gradient.

Burnett, et al., (1936) described difficulties in extracting fowlpox virus from CAMs due to the loose, fatty nature of the inclusions in which the virus was embedded. Randall, et al., (1964) developed a lengthy method of purification of the fowlpox virus which involved 16 rounds of differential centrifugation.

Since cell culture techniques have become the principal means of growing poxviruses, the extraction procedure has been simplified. A purification method for orthopox viruses was demonstrated by Esposito, et al., (1978). In this method cells are lysed with detergent at a concentration too low to lyse the nuclear membrane. After sedimentation of the nuclei, the viruses liberated from the cytoplasm are concentrated and purified by differential and gradient centrifugation.

To date, two studies, have compared quailpox virus and fowlpox virus found in the Southern States of America (Schnitzlein, et al., 1988 and Ghildyal, et al., 1989). The method of purifying viruses used in those studies was that of Esposito, et al., (1981) which involved the extensive use of a permanent avian cell line, a quail cell line called QT-35 for growing the viral material. During this study it was not possible to obtain sufficient penguin pox virus from cell cultures, so infected CAMs were used as a source of viral material.

9.2. BASIC PURIFICATION METHOD.

The initial method used for the purification of penguinpox viruses was a procedure, developed in this department for orthopox viruses, which was a modification of the method developed by Joklik (1962).

Flurocarbons were omitted and purification relied principally on differential sedimentation as described below.

9.2.1. METHODS

A dilution of the 80% glycerol stock virus was prepared to ensure a confluent spread of pocks on the CAMs after incubation at 36,5°C for 4 days. The membranes were harvested as described in Chapter 2.2.3, washed in physiological saline, and placed in chilled, sterile universal glass bottles con-

taining glass beads. Confluent membranes were combined with 9 ml of cold McIlvains' pH 7,4 buffer in each bottle containing 6 membranes. All solutions used were pre-cooled on ice and membranes kept as cold as possible to reduce the autolytic enzyme activity of the virus. All centrifugation steps were conducted at 4°C for the same reason.

Bottles were wrapped in paper towels to prevent undue warming and were vigorously shaken for 1 to 2 minutes, then balanced and centrifuged for 8 to 10 minutes at 110 g. The supernatant was aspirated into sterile bottles and stored on ice. A second volume of McIlvains' buffer was added to the bottles and the process was repeated. The supernatant fluids were pooled and incubated on ice for 1,5 hours, clarified by centrifugation at 1200 g for 15 minutes and supernatant fluid harvested and layered onto a cushion of 36 % sucrose (w/v in TE pH 9 buffer) (Appendix A). The virus was centrifuged at 15 000 g for 60 minutes. The supernatant was discarded, and the yellow-coloured viral pellet was retained and re-suspended in a smaller volume of 1 to 2 ml of TE pH 9 buffer. The viral suspension was layered onto a density gradient consisting of two volumes of 10 % Dextran T10 (w/v in TE buffer pH 9) and one volume of 36 % sucrose. The virus was centrifuged in the gradient at 15 000 g for 60 minutes.

The supernatant fluid was discarded and the viral sample re-suspended in a small volume (less than 1 ml) of TE pH 9 buffer. If the viral sample was still yellow in colour, the centrifugation procedure was repeated until the viral sample was a milky white colour. The final volume in which the virus was re-suspended was carefully noted and recorded. The purified virus was kept at 4°C overnight and the DNA extraction (Chapter 10) was continued the following day.

Whenever purified virus had to be used for growth experiments, the viral pellet was re-suspended in McIlvains' buffer and stored in aliquots of 0,2 ml at -70°C.

Low speed centrifugation and clarification steps were conducted in sealed centrifuge tubes in a Sigma 301K centrifuge (Sigma Products, Saint Louis, United States of America). High speed centrifugations were done in a swing-out rotor in either an IEC B20-A Centrifuge (Damon/IEC Division, Needham Heights, United States of America) or a Beckman L7-55 ultracentrifuge (Beckman, Irvine, United States of America).

9.2.2. RESULTS

The purification procedure was evaluated by a titration of samples taken at various stages during the process, but tvery poor yields of infectious virus were obtained. The final viral

pellet obtained by this procedure did not yield DNA suffiently pure quantity to be used for futher experiments. The basic purification method seemed to be inadequate for penguin pox virus.

9.3. EVALUATION OF BASIC PURIFICATION.

9.3.1. METHODS

The basic purification process was re-evaluated using an orthopox virus (buffalo isolate 81/85), and a canarypox virus. Samples were removed at various stages for virus titrations.

The samples were taken at the following stages:

Sample 1 - removed 1 ml of the pooled supernatants after the first low speed centrifugation steps.

Sample 2 - removed 1 ml after the first clarifying step at 1200 g.

Sample 3 - removed 1 ml after the virus was centrifuged through the density gradient. The viral pellet of the buffalo isolate was milky white and dissolved easily, whereas the viral pellet of the canarypox, although milky white, did not dissolve as easily and was very gelatinous.

Virus was titrated on CAMs and 2 CAMs per dilution were inoculated. The CAMs inoculated with buffalo pox were incubated for three days and with canary pox for four days.

The membranes were harvested as described previously (Chapter 2.2.4), but rinsed in 5 % formalin (v/v in distilled water) instead of physiological saline. The membranes, containing formalin inactivated viruses, were spread out in a petri-dish and examined for the presence of pocks.

9.3.2. RESULTS

The results of the titration are tabulated in Table 9.3.1.

Table 9.3.1: Results of the titration of different sub-species of pox viruses using the basic purification process. All the results are given as pfu/ml

Sample	Canarypox virus	Buffalopox virus
1	$7,50 \times 10^3$	$2,12 \times 10^7$
(Removed after the first low speed centrifugation step)		
2	$8,52 \times 10^3$	$2,79 \times 10^7$
(Removed after the clarifying spin at 1200g)		
3	$5,40 \times 10^3$	$2,89 \times 10^9$
(Removed from the viral pellet obtained at the end of the purification process)		

These results indicate that the canarypox virus was being lost in the purification procedure, whereas the orthopox virus was being concentrated. It therefore appeared that the basic purification process (involving differential centrifugationa and sucrose gradients) was inadequate for the purification of avipox viruses. Consequently future isolation attempts employed the use of organic solvents.

9.4. MODIFICATION OF JOKLIK'S METHOD.

W.Joklik (1962) described the use of an organic solvent (Gentron) in conjunction with a mechanical process to disrupt the CAMs for the extraction of the virus. This was followed by centrifugation of the virus particles into a sucrose cushion, and subsequent banding in a sucrose density gradient.

A similar solvent called Arklone X was used in this study (1,1,2 - trichloro - 1,2,2 - trifluoroethane).

9.4.1. METHOD

The membranes were prepared, inoculated with canarypox virus and harvested as described in Chapter 2.2. Nineteen membranes were divided into five sets with each set treated as follows:

Set 1: Control. Three membranes were placed in a universal bottle containing glass beads and 3 ml of cold McIlvains buffer was added. The membranes were shaken for 2 minutes and centrifuged for 10 minutes at 600 g. The supernatant was harvested and the process repeated and the supernatants pooled. A sample was removed for titration.

Set 2: Four membranes were shaken, as before in a universal bottle containing glass beads with 4 ml of

cold McIlvains buffer and 1 ml of cold Arklone-X added. The membranes were shaken for 2 minutes, centrifuged for 10 minutes at 600 g and the supernatant harvested. The process was repeated and the supernatants were pooled. A sample was removed for titration.

Set 3: Four membranes were placed into a universal bottle containing glass beads with 4 ml of cold McIlvains buffer added to the bottle. The membranes were shaken for two minutes, centrifuged for 10 minutes at 600 g. The supernatant was collected and the process was repeated. The supernatants were pooled and placed into an universal bottle containing glass beads. Two ml of cold Arklone-X were added to the supernatant fluid and the mixture was shaken for a further 2 minutes, centrifuged for 10 minutes at 600 g and the supernatant harvested. A sample was removed from the second supernatant fluid for titration purposes.

Set 4: Four membranes were ground to a paste in a pre-cooled mortar and pestle. (The mortar and pestle were cooled at -20°C for one hour before use.) The membranes froze immediately on introduction to the

pestle and mortar. The paste was transferred into a universal bottle containing glass beads with 4 ml of cold McIlvains buffer and shaken for 2 minutes and centrifuged for 10 minutes at 600 g. The supernatant fluid was retained and a sample removed for titration.

Set 5: Four membranes were treated as in Set 4 above. Two ml of cold Arklone-X were added during the grinding process. The paste was removed and placed into a universal bottle containing glass beads with 4 ml of cold McIlvains buffer. The paste was shaken for 2 minutes and centrifuged for 10 minutes at 600 g. The supernatant was harvested and a sample was removed for titration.

Supernatants were stored at -70°C for further processing.

9.4.2. RESULTS

Serial ten-fold dilutions of the five samples were inoculated onto prepared CAMs and incubated for 4 days. Discrete pocks were counted and the results are shown in Table 9.4.1.

Table 9.4.1.: The results of the titration to determine the effect of an organic solvent on the yield of virus.

Sample from set number	Amount of virus given in pfu/ml
One (Control, 3 membranes shaken hypotonic solution)	$1,95 \times 10^{8}$
Two (4 Membranes shaken in presence of Arklone in hypotonic solution)	$2,688 \times 10^{10}$
Three (4 Membranes shaken in hypotonic solution, centrifuged. Supernatant harvested and shaken with Arklone and centrifuged)	$1,95 \times 10^{9}$
Four (4 Membranes ground, shaken in hypotonic solution and centri - fuged)	$6,72 \times 10^{9}$
Five (4 Membranes ground with Arklone, shaken with glass beads in hypotonic solution and centrifuged)	$1,0368 \times 10^{10}$

9.5. DISCUSSION

In order to establish the optimal conditions for the extraction of penguin pox virus from infected CAMs, various methods were investigated.

Initially, the basic purification method was used. Infected membranes were lysed in hypotonic solution and virus particles recovered by repeated differential centrifugation and sucrose density gradient separation. Although this method of purification was shown to be very successful for orthopox viruses, (buffalo pox used in this study), the yield was very poor. It is suspected that the lack of success with avipox viruses was caused by two factors: (a) incomplete release of virus particles from the membranes and (b) aggregation of virus particles as a result of their high lipid content; a feature which is exacerbated at low temperatures.

Randall *et al* (1964), comment on the fact that fowlpox virus cannot be separated easily due to aggregation of the virus particles. They suggest that the utilisation of sucrose gradients might improve the separation of the virus particles and increase the yield of virus particles. Sucrose gradients where not successful for purifying avipox viruses in this study. The authors of that article also comment on the high lipid content of the fowlpox virus and suggest that the virus might be

inactivated by organic solvents.

It was decided to assess the effects of organic solvents on the purification of an avipox virus, namely canarypox virus. Incorporation of the fluorocarbon Arklone-X, greatly enhanced the yield of infectious canarypox virus. For best results, it was necessary to add the solvent before the initial clarifying centrifugation. Grinding of the membranes in the presence of Arklone-X provided no added advantage. The membranes were therefore shaken with the Arklone-X present as this had the fewer manipulation steps and therefore fewer stages for the virus to lyse.

The optimum purification procedure for the avipox viruses in this study was shaking the intact CAMs with glass beads in the presence of Arklone-X in the hypotonic solution, McIlvains buffer. This procedure was used to purify all avipox viruses used in the typing study.

CHAPTER TEN

DNA EXTRACTION

10.1: DNA EXTRACTION OF AVIPDX VIRUSES

In 1962 Randall, et al., confirmed the viral genome of fowlpox virus to be DNA and that the purified virions contain DNA with a higher A + T % (64 %) than the host chicken DNA (58 - 60 . The DNA was extracted from a pure virus pellet obtained from several low speed centrifugation steps followed by 3 high speed centrifugation steps. The viral DNA was extracted with perchloric acid, washed with ethanol and dried under liquid nitrogen.

In 1966, Randall, et al., published a paper where the process of DNA extraction from fowlpox virus followed a method proposed for Shope papilloma virus. This involved the use of a detergent, sodium lauryl sulphate to lyse the virus, which was deproteinized through several cycles of shaking with chloroform-butanol. The DNA was precipitated by the addition of absolute ethanol.

The purification method of Esposito, et al., (1978), utilized a detergent to lyse the virions prior to the extraction of DNA. A method similar to Esposito was adapted for the extraction of orthopox viral DNA using the detergent Na-n-lauryl

sarcosinate at a concentration of 2 %. An alternive detergent, SDS, was also used. The lysis of the virions was followed by enzymatic digestion, and followed by phenol and chloroform extractions.

10.2: METHODS

10.2.1. DNA EXTRACTION

An equal volume of lysis buffer (Appendix A) was added to the purified viral suspension. The lysis buffer contains Na n-lauryl sarcosinate and B-mercaptoethanol. The Na n-lauryl sarcosinate has the advantage over 5DS in that it can be stored at 4°C (whereas the SDS can only be used at room temperature) and thus limits the action of protease degradation. The B-mercapto ethanol, a reducing agent, can only be added prior to use as it is unstable at low concentrations.

The viral suspension was lysed at room temperature for 10 to 15 minutes, Proteinase K was added to a final concentration of 100 mg/ml, and incubated at 56°C for 1,5 hours. Enzyme and protein debris were removed by phenol and chloroform-isoamylalcohol extractions as described in Chapter 4.2.1. The DNA was precipitated with ethanol after the volume of the aqueous phase was measured. A 1 M Na-acetate solution was added to give a final concentration of 0,3 M. The DNA was precipitated by the addition of 2,5 volumes of ice-cold abso-

lute ethanol and storage overnight at -20°.

The DNA was pelleted at 1600 g for one hour at 4°C in a fixed angle Beckman JA-20 rotor, followed by washing with 70 % ethanol and recentrifuging for one hour at 1600 g. The DNA pellet was dried under vacuum in a Speedivac drier (Eppendorf, Germany) for 15 minutes, re-suspended in 500 Al of RNAse buffer (Appendix A) and allowed to dissolve overnight at 4°C.

The preparation of DNA was treated with RNAse A at 37°C for one hour to remove any contaminating RNA. RNAse A was added to a final concentration of 25 µg/ml. The RNAse enzyme was removed by phenol and chloroform extractions as described in Chapter 4.2.1. After the addition of Na-acetate and absolute ethanol, the DNA was precipitated as described previously. The sample was centrifuged at 1600 g for one hour at 4°C followed by washing with 70 % ethanol and centrifugation at 1600 g for one hour at 4°C. The pellet was dried in a Speedivac. The DNA was re-suspended in ultra-pure water and allowed to dissolve at 4°C overnight. Before any restriction analysis could be undertaken the concentration of DNA had to be determined.

10.2.2. DETERMINATION OF DNA CONCENTRATION

The DNA concentration was determined as follows:

(i) The concentration of DNA was estimated using a

Beckman Spectrometer DU-40 (Beckamn, Irvine, United States of America). Three gl of the DNA solution was diluted 1 in 100 in distilled water, to obtain a final volume of 300 gl. A distilled water blank was used for calibration. The DNA solution was placed in a quartz cuvette and a reading was obtained at 260 nm. This value was multiplied by a factor of 50, and then by the dilution factor, to give the concentration of DNA in gg/ml (Maniatis et al., 1982). A reading at 280 nm was also taken and, when divided into the reading taken at 260 nm, provided an indication of the RNA and protein contamination of the sample.

(ii) The concentration of DNA could also be estimated by electrophoresis and comparison of the DNA sample with a known concentration of DNA, normally 1 gg of lambda DNA cut by endonuclease restriction enzyme Hind III. The DNA sample was digested as described in Chapter 4.2.2. The known and unknown concentrations of DNA were electrophoresed as described in Chapter 4.2.3. After staining with ethidium bromide, the intensities of the bands were compared. The concentration of the sample DNA was assessed by comparison with a band of equal or equivalent intensity.

10.2.3. DIGESTION OF AV/PDX VIRAL DNA.

The viral DNA was subjected to restriction endonuclease digestion with Hind III and electrophoresed overnight as described previously (Chapter 4.2). The agarose gel was stained and photographed after electrophoresis.

10.3: RESULTS

10.3.1. DIGESTION OF AVIPDX VIRAL DNA

The gel was stained and photographed (Figure 10.3.1.)

The restriction fragments are clearly visible on the photograph of the gel.

Ther were no fragments identical for all three viruses. On visual insoection the electrophorises profile of the penguin pox virus (Lane 2) differed signfantly from the fowl pox (Lane 3) and canary pox (Lane 4 and 5) viruses after the digestion with Hind III. These differences were signficant because the duplicate smaple of canary pox virus in Lanes 4 and 5 gave identical electrophoresis patterns.

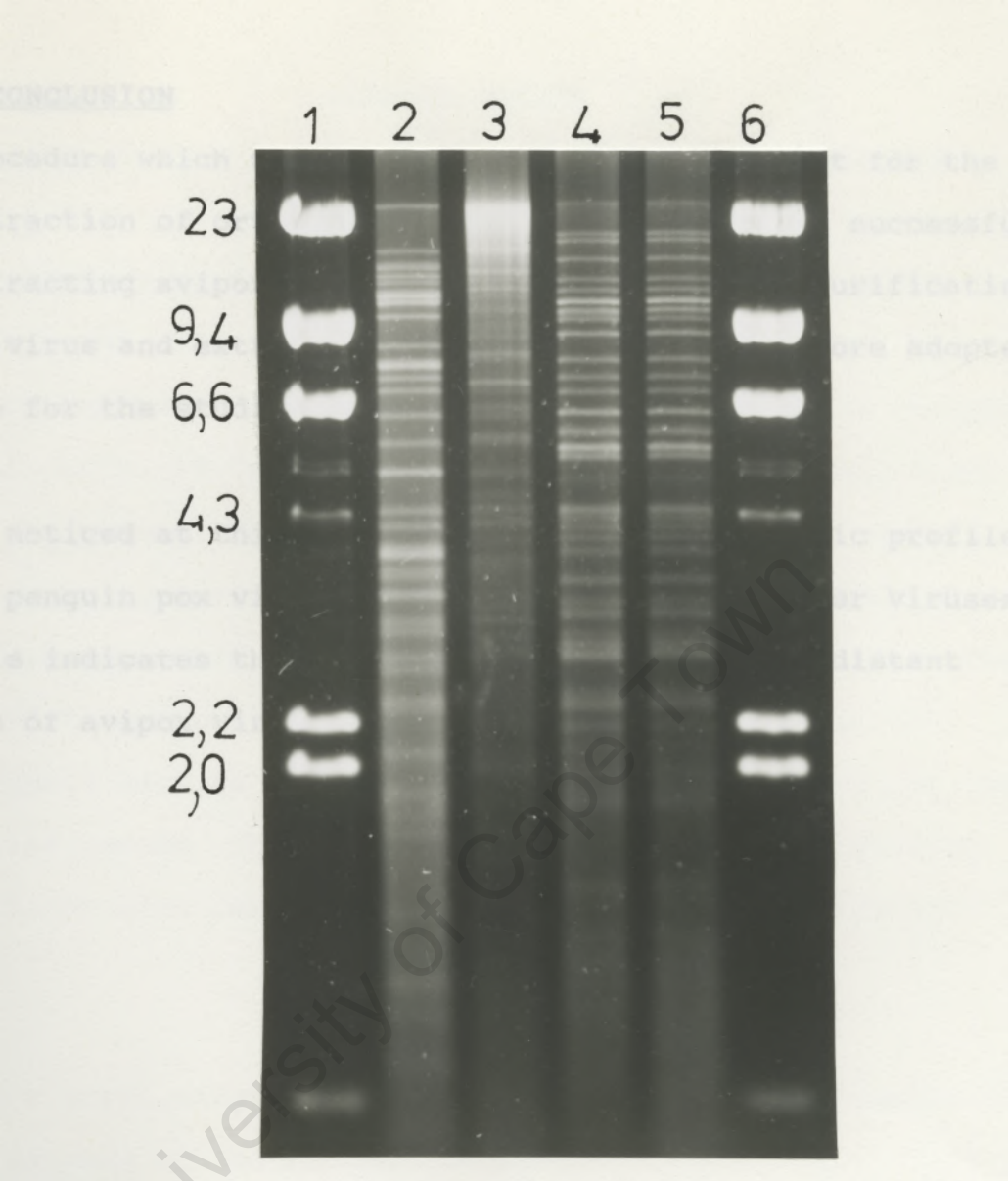

Figure 10.3.1.: A 0,8% agarose gel showing various avipox DNA after digestion with Hind III.

Lane 1: Lambda DNA; lane 2 : penguinpox isolate; lane 3 : fowl pox; lanes 4 and 5 : canary pox and lane 6 : Lambda DNA.

(The Lambda DNA was used as a size marker as described in Chapter 4.2.5.). The gel was electrophoresed for 18 hours at 2 V/cm.

10.4. CONCLUSION

The procedure which was developed in this department for the DNA extraction of orthopox viruses, was found to be successful for extracting avipox viral DNA. These methods of purification of the virus and extraction of the DNA were therefore adopted for use for the studies of avipox viral DNA.

It was noticed at this stage that the electrophoretic profile of the penguin pox virus was different from the other viruses and this indicates that the penguin pox virus is a distant species of avipox virus.

CHAPTER ELEVEN

TYPING OF AVIPDX VIRUSES BY DNA RESTRICTION ANALYSIS

11.1: TYPING OF GENOMES BY RESTRICTION ENDONUCLEASE ANALYSIS

Each enzyme used in this study recognises and cuts DNA at specific base pairs characteristic for that particular enzyme and will cut viral DNA at those specific sites if they exist within the genome. The DNA restricted in this manner would produce varying amounts of different sized fragments which would normally, although not always have different sizes. The number of times that a particular sequence appears in the genome would dictate the number of fragments produced for that particular genome. These fragments can be differentiated on the basis of size during electrophoresis within an agarose gel.

There are three stages used in the analysis and the comparison of DNA genomes by restriction mapping.

STAGE ONE:

This is a simple comparison of the patterns produced, due to the size distribution of the fragments, after digestion and agarose gel electrophoresis. Closely related genomes are 'likely to show a number of fragments of similar size but these do not necessarily have the same sequence.

STAGE TWO:

After digestion into small fragments, a reasonably accurate estimation of the size of the intact genome can be calculated by adding the approximate sizes of the component fragments.

STAGE THREE:

By using double digestion with different restriction enzymes and cross-hybridisation, the linkage order of the fragments can be ascertained. This same method can also be used to determine the restriction map of the genome.

11.2: TYPING OF PDX VIRUSES BY DNA RESTRICTION MAPPING

Typing of pox viruses on the basis of the fragments generated by endonuclease restriction digestion of viral DNA has been used for the orthopox viruses (Mackett and Archard 1979; Esposito, et al., 1978; Esposito and Knight 1985; Midler, et al., 1977). Fragment-size profiles clearly distinguish the different species of orthopox viruses and can also be used for intra-species typing.

Although orthopox viruses with similar restriction profiles frequently offer cross-protection in vaccinated animals (Tripathy, et al., 1973 and Bossinger, et al., 1982) the same cannot be said for the avipox viruses.

This study on the restriction fragment profiles of avipox viruses was undertaken for two reasons:

(i) to determine the relationship between the different avipox viruses, and

(ii) to type the penguinpox genome.

Restriction fragment profiles of fowlpox virus have been published (Muller, et al., 1977) but at the start of this study the technique had not been used for comparative studies of avipox viruses. Since the commencement of this study comparisons of DNA of fowlpox and quailpox viruses have appeared (Schnitzlien, et al., 1988 and Ghilyal, et al., 1989)

11.3: COMPARISON OF PENGIUNPDX ISOLATE WITH REFERENCE VIRUSES.

The penguinpox isolate was typed by comparing its DNA with established reference avipox viral DNA.

Previously in Chapters 9 and 10 the methods of purification of the viruses and the extraction of the DNA were reported. DNA was obtained from three of the reference viruses, and from the penguinpox isolate.

11.3.1. METHODS

11.3.1.1. RESTRICTION ENDONUCLEASE DIGESTION

The DNA of the three reference viruses, and the penguinpox isolate, were subjected to restriction endonuclease digestion with various enzymes. The procedure followed in each case was described previously (Chapter 4.2.2.).

The enzymes used were:

BamH I, Hind III, Pst I, Sal I and Xho I

11.3.1.2. GEL ELECTROPHORESIS

The fragments obtained after digestion of DNA were subjected to agarose gel electrophoresis as described in Chapter 4.2.3.. The agarose gels were electrophoresed for 18 hours at 4°C, stained and photographed.

Bacteriophage Lambda DNA was digested with the restriction enzyme Hind III and used with all the restriction digests as a molecular size marker. The sizes of the bands are recorded in Appendix D (Oliver and Ward, 1985).

11.3.2. RESULTS AND DISCUSSION

11.3.2.1. RESTRICTION ENDONUCLEASE DIGESTION

The photographs are shown as Figures 11.3.1., 11.3.2., 11.3.3., 11.3.4., and 11.3.5. The size of the viral genomes could not be determined due to the large size of some of the fragments in each digest.

11.3.2.2. GEL ELECTROPHORESIS

These results are divided into thre considerations:

(i) These fragments were difficult to resolve in agarose gels;

(ii) The comparison of the four different nNAs digested with one restriction enzyme showed few fragments with the same mobility;

(iii) Because the band intensity of the fragments is a factor of the amount of intercalation of the ethidium bromide, the intensity diminishes as the fragments become smaller. This means that if a smaller fragment were more fluorescent than a larger fragment, the former would present more than one fragment.

Bam HI digest:

The digestion of the various avipox viruses with the restriction enzyme Bam HI clearly differentiates between the different viruses (Figure 11.3.1.). The penguinpox isolate and the fowlpox and canarypox viruses produce many high molecular

sized fragments having similar mobilities. By comparing the intensity of the stain, several bands appear to be doublets or triplets. The quailpox virus appears to have more Bam HI sites than the other three viruses, as larger sized fragments were found in the restriction profiles of the penguinpox, fowlpox and canarypox viruses.

Hind III digest:

The digest of all four samples with Hind III (Figure 11.3.2) produced four distinct restriction profiles for the different viruses showing that the viral genomes were different. In these profiles there are a few bands with the same mobility. The gel shown in Figure 11.3.2. the DNA bands are sharply defined and now clearly separated than in the gel in Figure 10.3.1.. In the later the bands are more diffuse and it would seem that fragments whose separate electrophoretic mobitilies can be seen in Figure 11.3.2. are merged in Figure 10.3.1.. Throughout this work difficultly was experienced in getting avipox DNA preparations which restricted easliy and showed clearly separated fragments. Penguin pox preparation in Figure 11.4 also shows some merging of bands. Further evidence of this is the greater variation in intensity of band staining which can be seen in Figure 11.4.1.. These difficulties were experienced more with the recent isolated strains than with the well established strains of fowlpox, canarypox and

quailpox viruses.

Pst I digest:

The digest of the four samples with Pst I (Figure 11.3.3.) produced different restriction profiles. This further emphasises that the penguinpox isolate is a different virus to the other viruses tested. Unfortunately there appears to be DNA degradation visible as a smear on the upper part of the quailpox digest. No definite conclusion can therefore be made about similar sized fragments. There is a very high molecular sized fragment in the fowlpox, penguinpox and canarypox viruses. It appears that this fragment could be undigested DNA, especially if one considers the intensity of the stain taken up by that particular band.

Sal I digest:

Sal I produced different restriction profiles for each of the four viruses (Figure 11.3.4.). The majority of the fragments of the fowlpox, penguinpox and canarypox viruses were very large and not well separated, leaving a few fragments of smaller size available for comparison. The quailpox, however, is cut frequently by this enzyme which is evidenced by restriction profile containing many small fragments. In these digests there was evidence of co-migrating fragments, a fragment of approximately 6,6 kb was present in all of the 4

viruses and two fragments each approx. 5 kb in size was present in penguin, canary and fowl pox viruses. These co-migrating fragments may represent a part of the genome which is conserved amoung avipox viruses and this could be further tested by cross-hybridisation experiments.

Xho I digest:

The digest of the enzyme Xho I also produced distinct restriction profiles for the different viruses. As noted for the Sal I, digest the quailpox virus was cut at greater frequency than the other viruses. This means that there are more Xho I recognition sites in the quailpox genome than in the other genomes.

The overall assumptions that can be drawn from these digests are (i) The four viruses investigated are all different with regarded to their restriction profiles;

(ii) the penguinpox isolate is different and could be a novel virus; and

(iii) the quailpox virus appears to be very dissimilar to the other viruses.

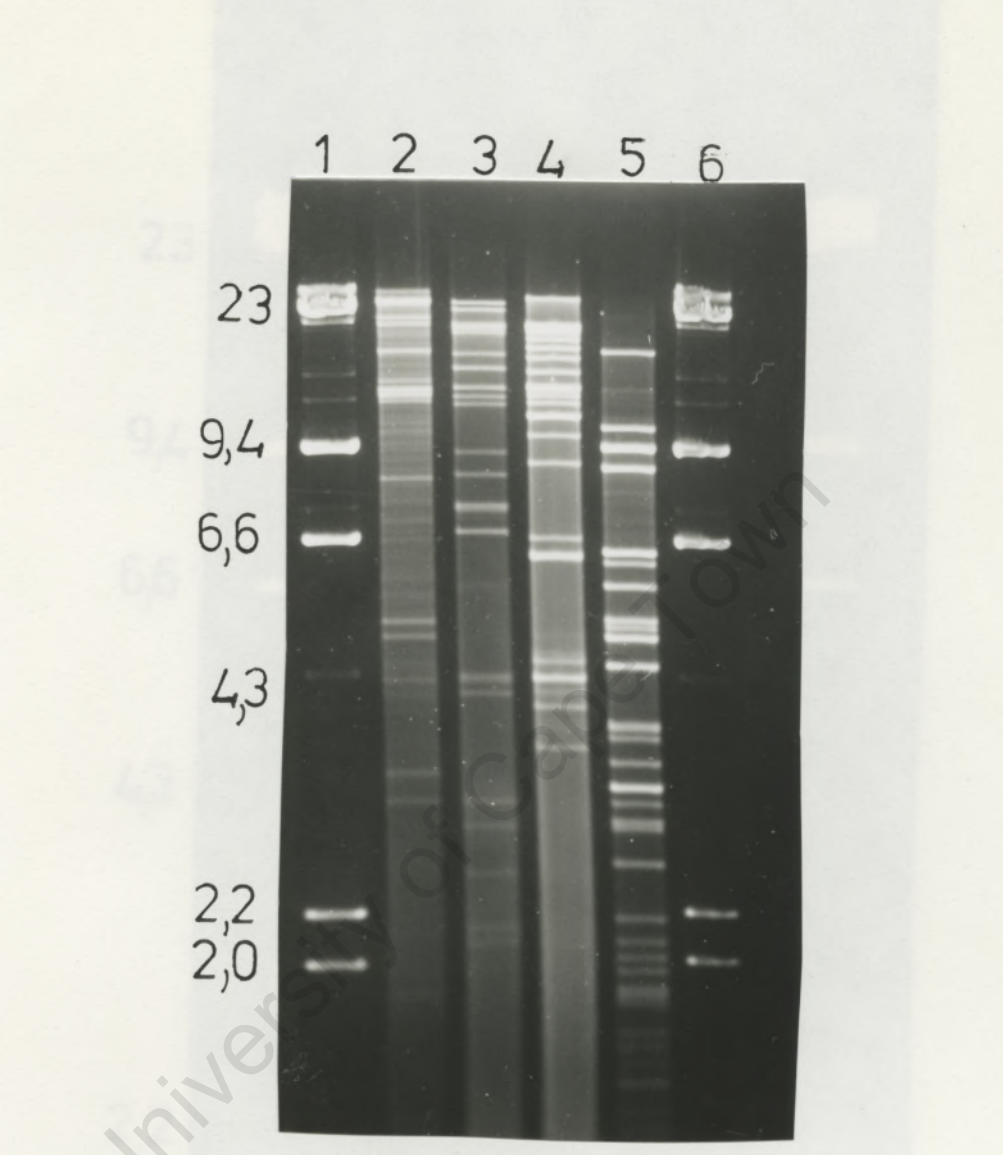

Figure 11.3.1.: BamH I restriction profiles of avipox viral DNA lanes 2 to 5. Lane 1 : Lambda DNA; lane 2 : penguinpox isolate; lane 3 : fowlpox virus; lane 4 : canarypox virus; lane 5 : quailpox virus and lane 6 : Lambda DNA.
The 0,8% agarose gel was electrophoresed at 2 V/cm for 18 hours.

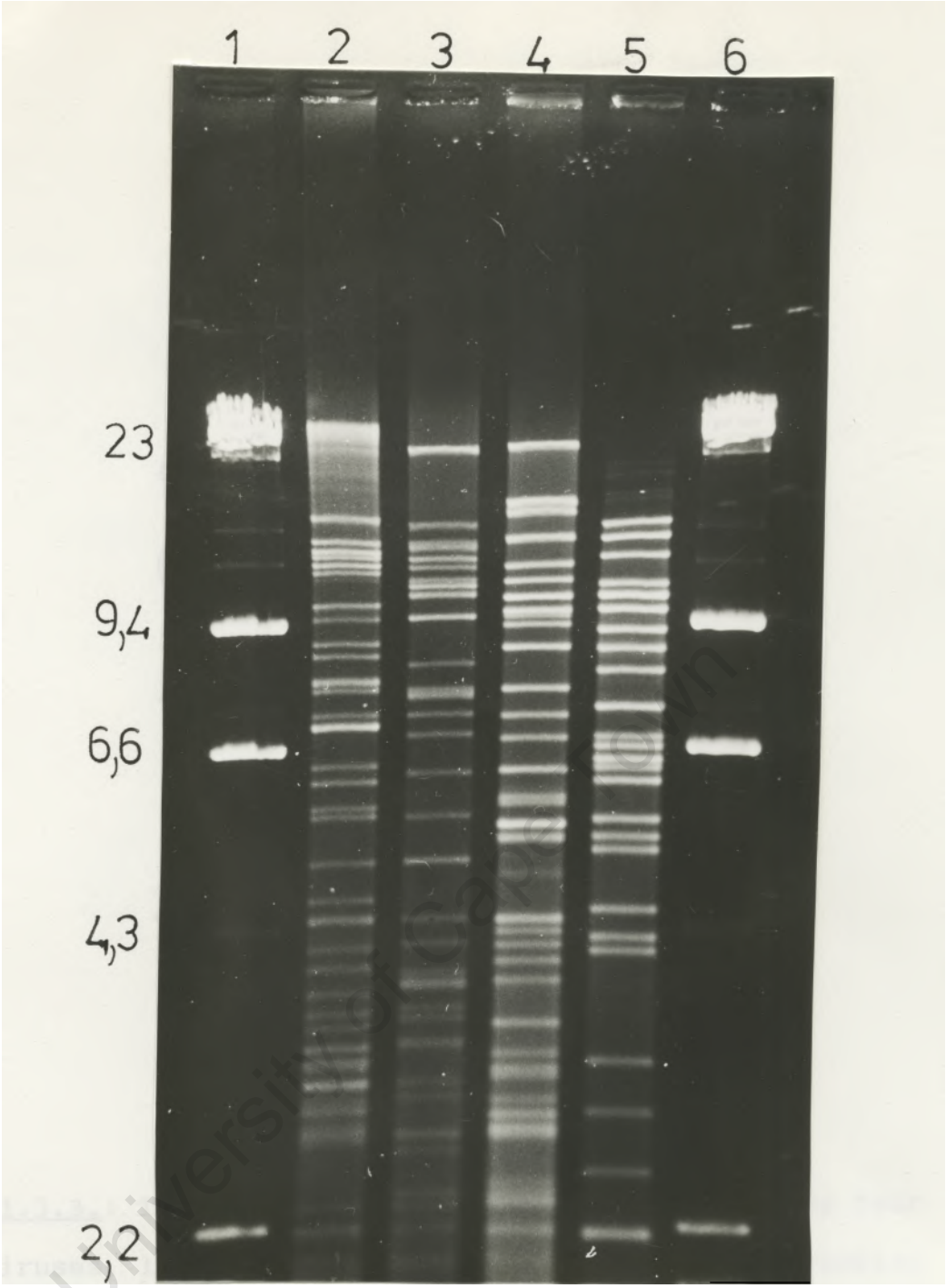

Figure 11.3.2.: Hind III restriction profiles of avipox viral DNA lanes 2 to 5. Lane 1 : Lambda DNA; Lane 2 : pengiunpox isolate; lane 3 : fowlpox virus; lane 4 : canarypox virus; lane 5 : quailpox virus and lane 6 : Lambda DNA.
The 0,8% agarose gel was electrophoresed for 18 hours at 2 V/cm.

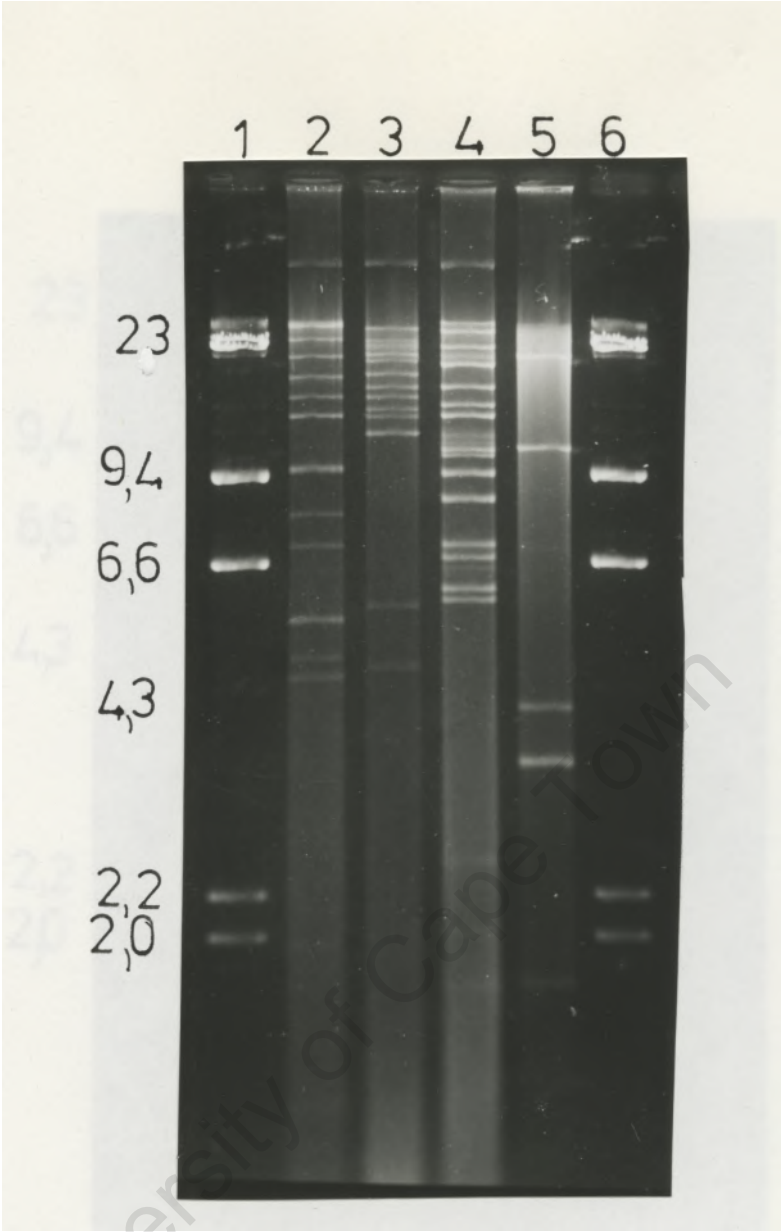

Figure 11.3.3.: The electrophoretic profiles produced by four avipox viruses, lanes 2 to 5, digested with Pst I restriction enzyme. The fragments were separated on a 0,8% agarose gel at 2 V/cm for 18 hours.

Lane 1 : Lambda DNA; lane 2 : pengiunpox isolate;

lane 3 : fowlpox virus; lane 4 : canarypox virus;

lane 5 : quailpox virus and lane 6 : Lambda DNA.

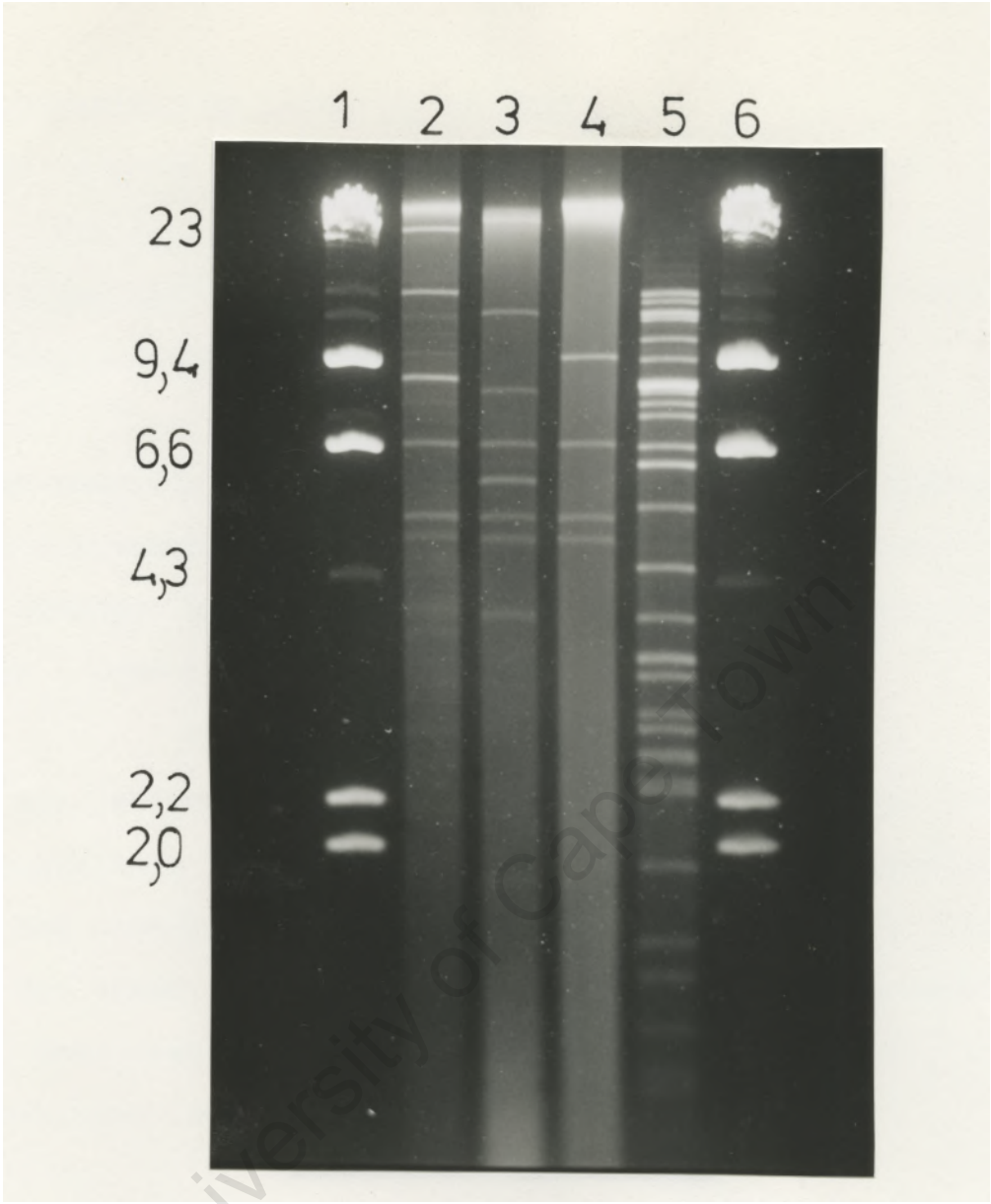

Figure 11.3.4.: The comparison of the restriction profiles produced by four avipox viral genomes, lanes 2 to 5, upon digestion with the restriction enzyme Sal I and separated on a 0,8% agarose gel at 2 V/cm for 18 hours.

Lane 1 : Lambda DNA; lane 2 : penguinpox isolate; lane 3 fowlpox virus; lane 4 : canarypox virus; lane 5 : quailpox virus and lane 6: lambda DNA.

Figure 11.3.5.: The comparison of the restriction profiles of the four avipox viruses, lanes 2 to 5, upon digestion with the enzyme Xho I and separated on a 0,8% agarose gel at 2 V/cm for 18 hours.

Lane 1 : Lambda DNA; lane 2 : penguinpox isolate; lane 3 fowlpox virus; lane 4 : canarypox virus; lane 5 : quailpox virus and lane 6 : Lambda DNA.

11.4: COMPARISON OF LOCAL AVIPDX VIRUSES.

Profiles of penguinpox, fowlpox, quailpox and canarypox viruses with each of the five endonucleases confirmed that the four viruses had distinctive different genomes with little or no evidence of conserved regions.

The next question to be addressed was whether the penguinpox isolate represented a casual infection (perhaps located in or around SANCCOB) with an avipox virus native to Southern Africa but with a wide host range.

Two further avipox isolates were made. One isolate came from a pox disease in a local poultry farm, and this was investigated to see how closely it resembled the standard strain of fowlpox virus. The other isolate from a wild turtle dove (a common bird in this area) was examined for possible resemblance to the penguinpox isolate.

11.4.1. METHODS

11.4.1.1. RESTRICTION ENDONUCLEASE DIGESTION

The DNA of the three viruses were subjected to restriction endonuclease digestion with various enzymes.The procedure followed in each case was that described in Chapter 4.2.2.

The various enzymes used were:

Hind III, Pst I and Xho I.

11.4.1.2. GEL ELECTROPHORESIS

The digests were subjected to agarose gel electrophoresis as described in Chapter 4.2.3. The agarose gels were electrophoresed for 18 hours at 4°C at 2 V/cm, stained and photographed. These photographs are shown in Figures 11.4.1., 11.4.2. and 11.4.3.

11.4.2. RESULTS AND DISCUSSION

11.4.2.1. RESTRICTION ENDONUCLEASE DIGESTIONS

The viral DNA of bacteriophage Lambda was digested with the restriction enzyme Hind III and used as a molecular size marker for all the restriction profiles obtained. The digestion reactions were conducted for 3 hours at 37°C.

11.4.2.2. GEL ELECTROPHORESIS

Hind III digest:

The restriction profiles after digestion with Hind III (Figure 11.4.1.) showed each of the three viral isolates producing large numbers of bands, a number of which had the same mobility.

Pst I digest:

The restriction profiles after digestion with Pst I (Figure 11.4.2.) clearly show the differences between the three avipox viruses. All three viruses produced high molecular size bands (with sizes is beyond the range of resolution of the agarose gel), but the penguinpox isolate was the only virus which produced bands with readily determinable sizes. When comparing Pst I fragments with those produced by Hind III (Figure 11.4.1.), there are hardly any bands of similar molecular size.

Xho I digest:
The restriction profiles produced by Xho I (Figure 11.4.3.) also reveal differences, with the profile of the penguinpox isolate having different sized bands when compared to the other local avipox viruses.

The Pst I and the Xho I digest of turtle dove isolate and the local fowl pox isolate produced few bands of low molecular weight it is not clear at present whether relatively few cuts made by theses enzymes represent the scarce distrubution of the corresponding restriction sites of whether these DNA prepartions were partly inhibitory to the enzymes. To cover this, a later digest was continued for much longer than the normal practice but without increasing the number of bands illustrated. Nevertheless the comparsions that can be made of

the local avipox viruses confirms the assumption that the penguinpox isolate is a different virus from the other two local avipox viruses. Therefore, the penguinpox isolate is not a is a species of virus endemic in the Cape Peninsula as the restriction profiles show that the three local avipox viral isolates are very different viruses. The local fowlpox (Hackett) isolate is shown in these profiles to be similar to the international reference strain of fowlpox virus.

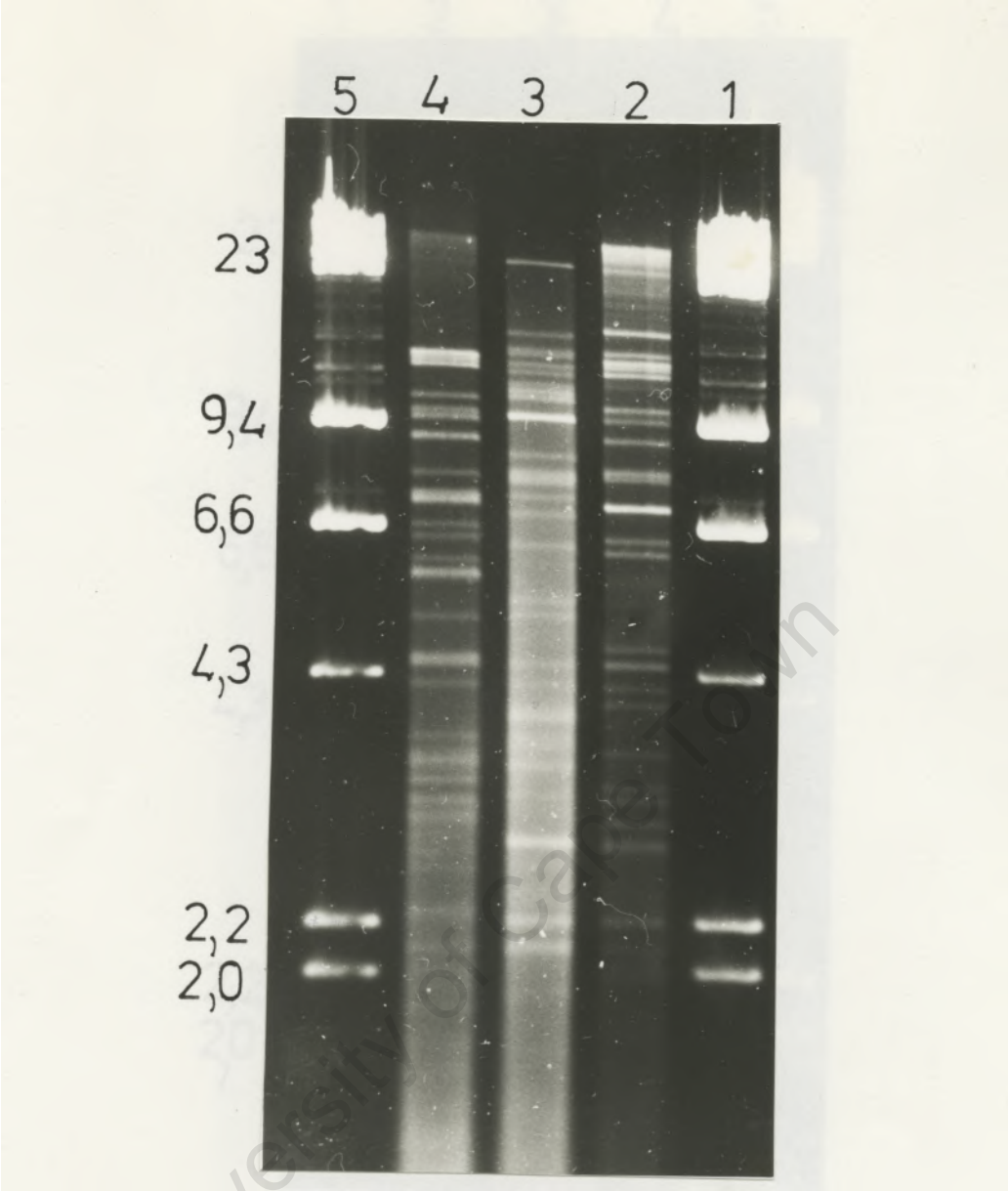

Figure 11.4.1.: The electrophoretic profiles produced by the three local avipox viral DNA, lanes 2 to 4, digested with the restriction enzyme Hind III. The fragments were separated on a 0,8% agarose gel at 2 V/cm for 18 hours.

Lanes 1 and 5: Lambda DNA; lane 2 : penguinpox isolate;

lane 3 : local fowlpox isolate ; lane 4 : turtle dove isolate.

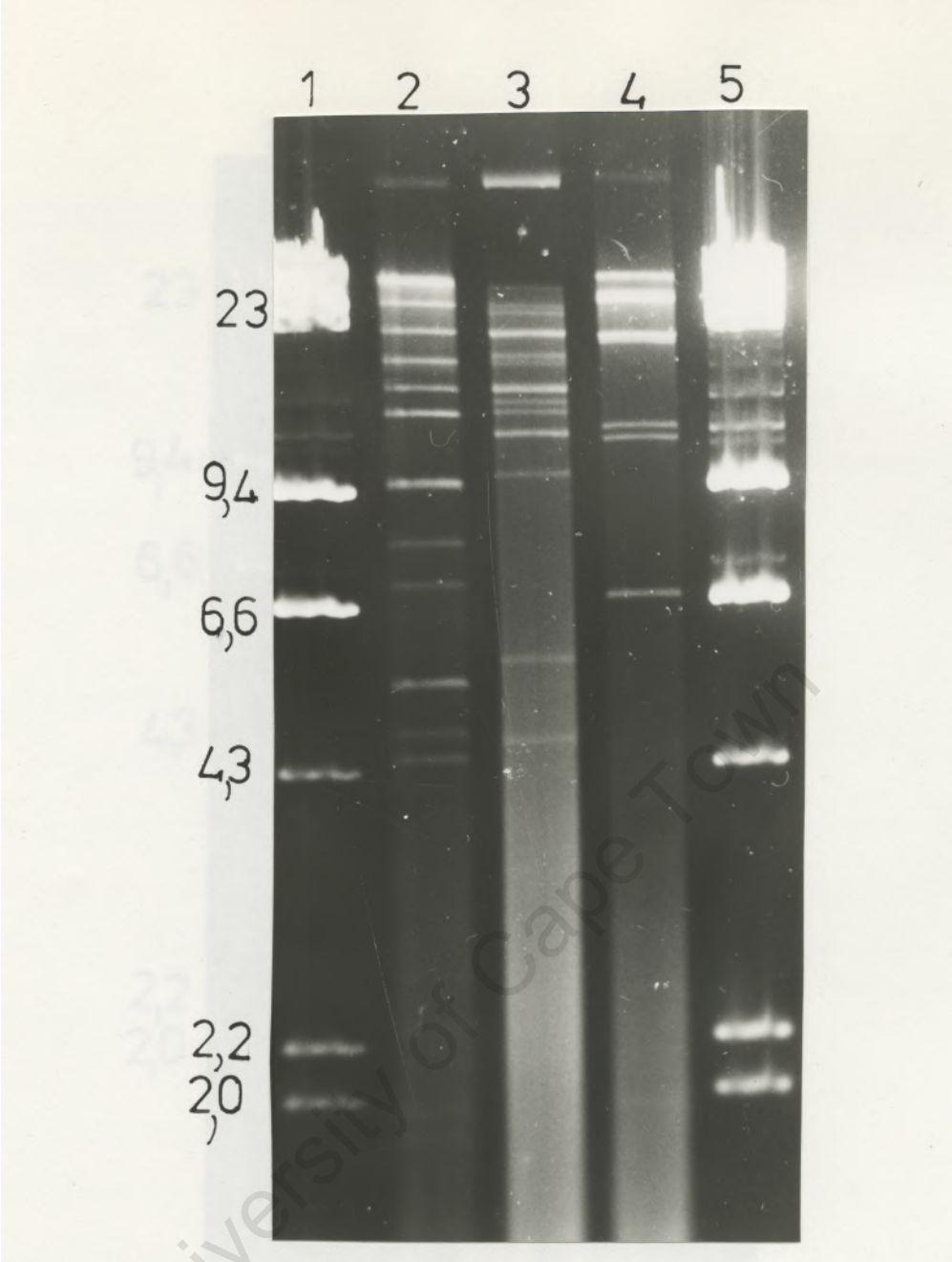

Figure 11.4.2.: The comparison of the restriction profiles produced by the three local avipox viruses, lanes 2 to 4, upon digestion with the restriction enzyme Pst I and separated on a 0,8% agarose gel at 2 V/cm for 18 hours.
lanes 1 and 5 : Lambda DNA; lane 2 : penguinpox isolate;
lane 3 : local fowlpox isolate; lane 4 : turtle dove isolate.

Figure 11.4.3.: Xho I restriction profiles of three local avipox viral genomes, lanes 2 to 4.

lane 1 : Lambda DNA; lane 2 : penguinpox isolate; lane 3: local fowlpox isolate and lane 4 : turtle dove isolate. The fragments were separated on a 0,8% agarose gel electrophoresed at 2 V/cm for 18 hours.

11.5: COMPARISON OF DIFFERENT PENGUINPDX VIRAL ISOLATES.

As penguinpox differed from the two local isolates of avipox viruses, the next question was whether repeated isolates from penguins would show identical, or closely related genomes - suggesting a genuine penguinpox infection and not a casual cross-infection. Two penguinpox viral isolates were made a year later.

11.5.1. METHODS

11.5.1.1. RESTRICTION ENDONUCLEASE DIGESTION

The viral DNA of the three penguinpox isolates was extracted after viral purification (Chapter 9) . The DNA was subjected to three restriction endonuclease digestions as outlined in chapter 4.2.2.

11.5.1.2. GEL ELECTROPHORESIS

The fragments obtained after digestion were subjected to electrophoresis on a 0,8% agarose gel at 2 V/cm for 18 hours at 4°C, stained and photographed as described in Chapter 4. The restriction enzymes used were Bam HI, Pst I and Xho I.

11.5.2. RESULTS AND DISCUSSION

11.5.2.1. RESTRICTION PROFILES OBTAINED ON AGAROSE GELS.

Digestions of the three isolates with Bam HI, Pst I and Xho I are shown in Figures 11.5.1, 11.5.2, and 11.5.3. It is clear

from these three pictures that the isolates with minor differences in fragment profiles, are closely related. The differences are most clearly seen in the Pst I digest. Here the J band in Penguin Number 1 has no equivalent sized band in Penguin Number 2 and 3. In Penguin Number 2 and 3, the J bands are identical and larger than the J band in Penguin Number 1. These is no band in the Penguin Number 1 digest equivalent in size to the J band in Penguin 2 and 3. There is also a suggestion in Penguin Number 3 the I Band is more intense. A possible explanation would be a variation in the length of the terminal fragments - a phenomenon well recognised in the orthopox viruses. This possibility was tested by a DNA snap-back analysis experiment to identify the terminal fragments (Section 11.6.).

The similar profiles produced by the three penguinpox isolates in comparison to the vastly different profiles of the reference avipox viruses, confirms that the penguinpox isolates are the same virus.

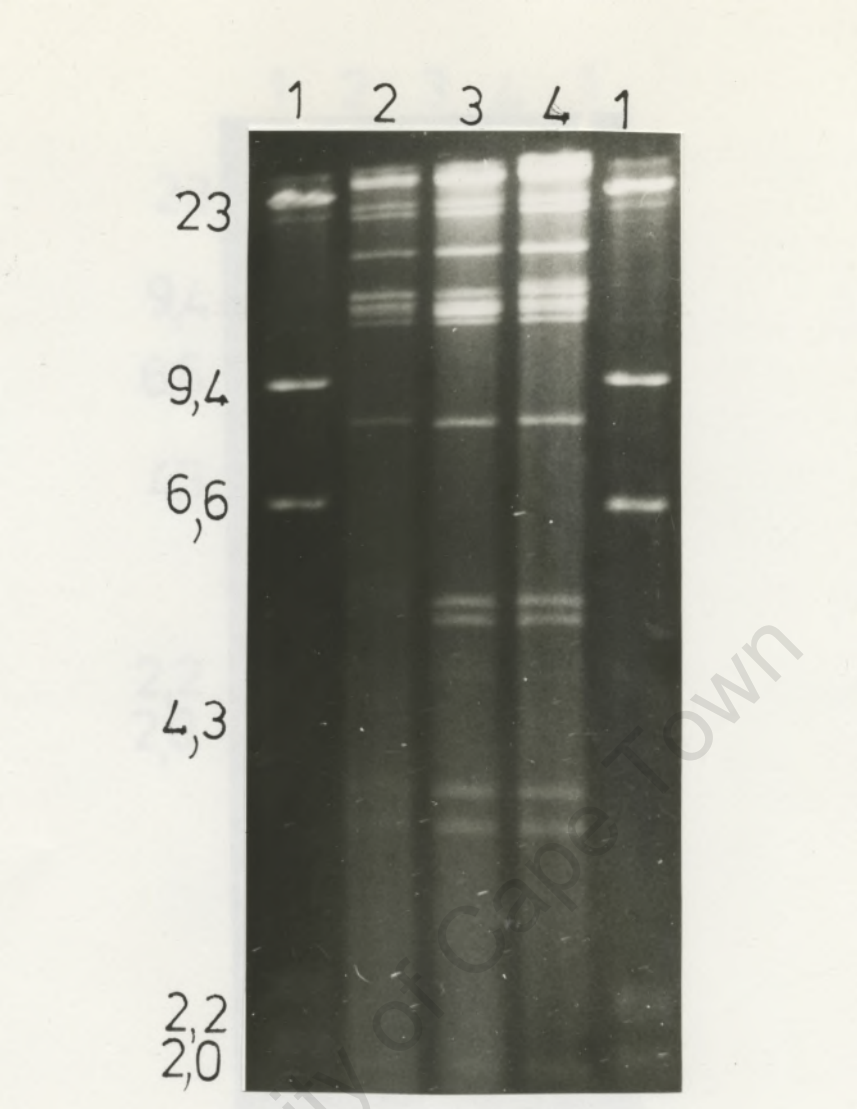

Figure 11.5.1.: The comparison of the restriction profiles produced by the three penguinpox isolates, lanes 2 to 4, after digestion with the restriction enzyme BamH I and separated on a 0,8% agarose gel at 2 V/cm for 18 hours.

Lane 1: Lambda DNA;

lane 2 penguinpox isolate Number 1;

lane 3 : penguinpox isolate Number 2;

lane 4 : penguinpox isolate Number 3.

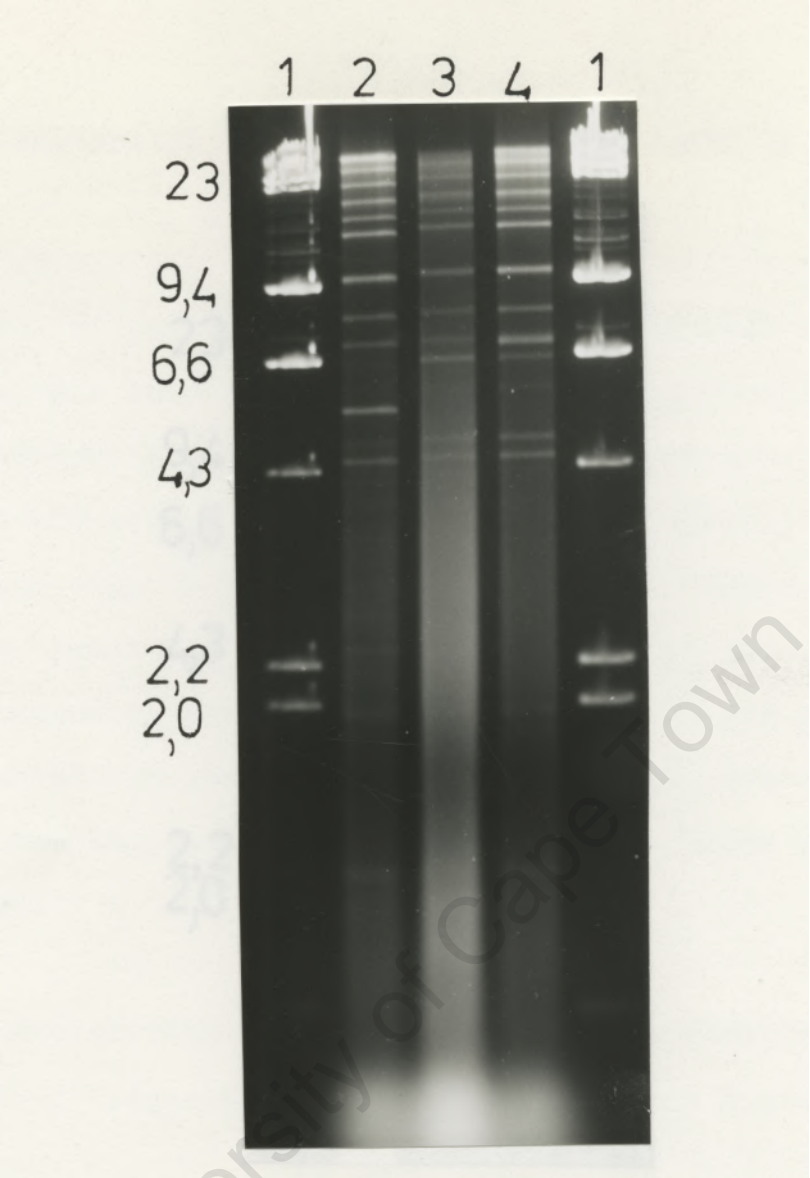

Figure 11.5.2.: The electrophoretic profiles produced by the three penguinpox isolates, lanes 2 to 4, after digestion with the restriction enzyme Pst I and separation on a 0,8% agarose gel at 2 V/cm for 18 hours.

Lane 1: Lambda DNA;

lane 2: penguinpox isolate Number 1;

lane 3: penguinpox isolate Number 2 and

lane 4: penguinpox isolate Number 3.

Figure 11.5.3.: The comparison of the restriction profiles of the three penguinpox isolates, lanes 2 to 4, upon digestion with the restriction enzyme Xho I and separated on a 0,8% agarose gel at 2 V/cm for 18 hours.

Lane 1: Lambda DNA;

lane 2: penguinpox isolate Number 1;

lane 3: penguinpox isolate Number 2 and

lane 4: penguinpox isolate Number 3.

11.6: DETERMINATION OF END FRAGMENTS OF GENOMES OF PENGUINPDX ISOLATES.

11.6.1. METHODS

The genomes of pox viruses have covalently closed ends which form a hairpin loop at the ends of the genomes called terminal loops (Baroudy, et al., 1982). When restriction digest is denatured the single strands representing internal fragments can diffuse away from each other. Denaturing the terminal fragments results in two single strands still joined at one end. Consequently when reannealing conditions are applied the single strands of the two terminal fragments re-associate more rapidly than the other fragments and this enables them to be identified.

11.6.1.1 RESTRICTION DIGESTIONS AND GEL ELECTROPHORESIS

The terminal fragments were identified in the following experiment.:

The viral genomes were digested with an appropriate enzyme. The linear fragments obtained were denatured by heating above 95°C for five minutes. The fragments were snap-cooled on ice. The linear DNA fragments with the cross-linked termini could re-nature after cooling where-as the linear fragments might not re-anneal with the correct fragment.

Duplicate digests of the penguinpox isolates Number 1 and 3 were restricted with the restriction enzyme Pst I. The reactions were allowed to run to completion. One reaction mixture of each virus was terminated as described in Chapter 4.2.2. The other reaction mix of each penguinpox isolate was denatured by heating the samples above 95°C for five minutes. The samples were immediately cooled on ice. The resulting samples were electrophoresed on an agarose gel, stained after 18 hours and photographed.

11.6.2. RESULTS AND DISCUSSION

The agarose gel is shown in Figure 11.6.1.. The strong bands which were found after denaturation in lanes 2 and 4. They correspond in postion on the non-denatured DNA (lanes 1 and 3). The extra 5 KB fragment found in Penguin Number 1 (lane 1) and the 6 kb fragment of increased density in Penguin Number 2 (lane 3) are the terminal fragments. It is therfore concluded that there are minor differences between the penguin isolates taken a year apart.

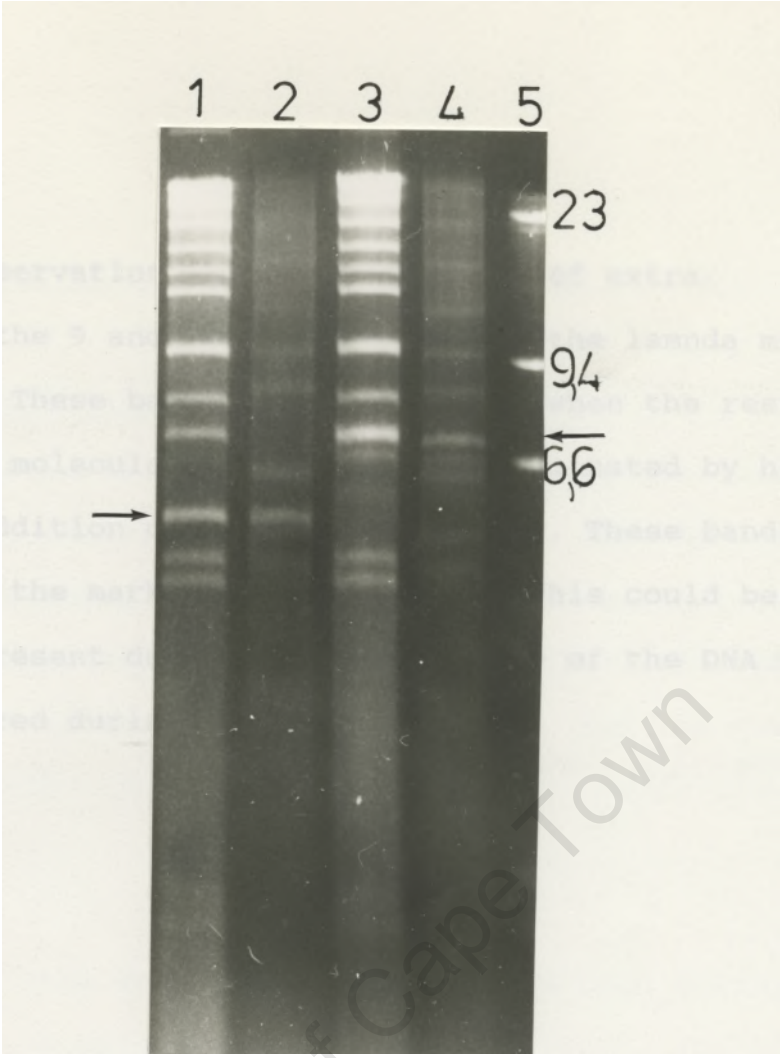

Figure 11.6.1.: Denatured penguinpox isolate DNA Pst I digests were electrophoresed in a 0,8% agarose gel for 18 hours together with non-denatured Pst I digests of the penguinpox isolate DNA.

Lane 1:penguinpox isolate Number 1 - non-denatured;

Lane 2:penguinpox isolate Number 1 - denatured;

lane 3: penguinpox isolate Number 2 - non-denatured;

lane 4: penguinpox isolate Number 2 - denatured and

lane 5: lambda DNA. The terminal fragments under discussion is marked by arrows on either side.

Comment:

A constant observation was the occurrence of extra bandsbetween the 9 and 23 kb fragments in the lamnda molecular size markers. These bands always appeared when the restriction digest of the molecular size maker was terminated by heating followed by addition of the loading buffer. These bands never appeared when the marker was not heated. This could be due to concatemers present during the manufacture of the DNA which became denatured during heating.

CHAPTER TWELVE

IDENTIFICATION OF THYMIDINE KINASE GENE IN AVIPDX VIRUSES.

12.1: THYMIDINE KINASE GENE IN PDX VIRUSES.

Thymidine kinase (TK) is an enzyme involved in the nucleotide salvage pathway (Black and Hruby 1990). The importance of this enzyme is underscored by its conservation throughout a divergent group of organisms including human, mouse, chicken and a variety of viruses including herpesviruses, and the pox viruses (vaccinia, monkeypox, Shope fibroma and fowlpox viruses).

The location of the TK gene of vaccinia has been established by Weir et al., (1982) and Hruby et al., (1983). The gene is encoded by an open reading frame of 531 nucleotides in the Hind III J fragment. Although the TK gene has been found to be non-essential for the growth of pox viruses (Mackett, et al., 1982; Panicali and Paoletti 1982), the virulence of the virus is decreased in the absence of a functional TK gene.

This gene is one of the major areas where foreign genes, examples - Hepatitis B virus surface antigen and influenza haemagglutinin gene (Smith, et al., 1983 a and b), have been inserted into the genome of the vaccinia virus. TK negative and positive mutants can be selected for by different tech-

niques (Mackett, et al., 1982; Panicali and Paoletti, 1982) and this facilitates the use of the TK gene as a marker for insertions. A preliminary study was made to identify digests of avipoxviruses in which the TK gene was located in small, clonable fragments, with a view to subsequent possible work on avipox recombinants.

12.1.1. IDENTIFICATION OF TA GENE OF FOWLPDX AND QUAILPDX VIRUSES.

The sequence of the fowlpox viral TK gene was published in three papers by different groups, namely, Boyle, et al., (1987); Binns,et al., (1987), Boyle, et al., (1988) and Drillien, et al., (1987). The sequences of the gene was identical in each investigation, although differences were found within the flanking regions of the gene. The TK gene of fowlpox virus was located by homology studies with vaccinia virus. The gene was cloned and sequenced. In contrast, the TK gene employing quailpox virus was located using a rapid technique of an end-labelled degenerate oligonucleotide probe. The probe represents a conserved consensus sequence found in a variety of TK genes (Schnitzlein, et al., 1988). This method overcame the difficulties encountered in homology studies using the vaccinia virus where, although the flanking regions of both vaccinia and fowlpox viruses were the same, as the TK gene of fowlpox was found to be in a different region of the

genome. The oligonucleotide probe eliminates false positive results which can occur when the flanking regions show up in homology studies despite the absence of the TK gene.

12.2: CONSTRUCTION OF OLIGONUCLEOTIDE PROBE

The regions which flank the TK gene in vaccinia virus have similar sequences in the same region of the fowlpox virus genome. However, in fowlpox virus the TK gene is not found between the "flanking" sequences but is translocated to another part of the genome (Drillien, et al., 1987). Consequently, when looking for the TK gene in other avipox viruses, it was necessary to used a probe specifically directed to part of the TK gene cloning sequence. The oligonucleotide originally used in this study to determine the position of the TK gene in the avipox viral group was

GG(A/G/T/C)CCCATGTT(T/C)TC(A/G/T/C)GG,

created by Upton and McFadden (1986) for the identification of the TK gene of the Shope fibroma virus. This oligonucleotide represents a conserved region in the 3' portion of the vaccinia virus, mouse and human TK gene.

Another consensus sequence in the centre of the TK gene (amino acid positions 105 to 112, included) of four pox viruses and two vertebrates was published (Boyle, et al., 1987). This sequence was found to be similar in TK genes of fowlpox,

variola, vaccinia, and monkeypox viruses, as well as in humans and chickens.

The sequence was reverse translated from the amino acid sequence (DEAQFFLD) into the nucleotide sequence of

5'GA(T/C)GA(A/G)GC(A/G/T/C)CA(A/G)TT(T/C)TT(T/C)(T/C)T(A/G/T/C)GA(T/C)3'.

The 18-mer oligonucleotide of

5'(A/G)AA(A/G)AA(T/C)TG(A/G/T/C)GC(T/C)TC(A/G)TC3'

was constructed and designed to complement with the underlined portion of the consensus sequence.

12.3: METHODS

12.3.1. END LABELLING OF PROBE

The oligonucleotide was labelled with gamma-32P, using a polynucleotide kinase enzyme.

Fifty pm of DNA (oligonucleotide) were heated above 95°C for five minutes (to remove any secondary structure which could occur in the single-stranded molecule of DNA). The DNA was cooled immediately by placing the sample on ice for ten minutes. Eighteen units of bacteriophage T4 polynucleotide kinase enzyme with 10 times concentrated kinase buffer (Appendix A), 100 pC gamma-ATP and water to 50 gl were added. The solution

was mixed and centrifuged 5 seconds in a microfuge. The sample was incubated at 37°C for 30 minutes.

The reaction was terminated by adding 25 41 of phenol (Appendix A) and 25 41 of chloroform-isoamylalcohol (24:1 v/v) to the solution. The solutions were mixed and centrifuged for 2 minutes to separate the two phases. The aqueous phase was retained and 10 Al stop buffer (Appendix A, 10 times concentrated) was added. The radio labelled probe was separated from the unincorporated radio-activity in a Sephadex column. The Sephadex G50 sepharose, which had been equilibrated (Appendix A) in nick translation buffer (0,15 M NaCI; 0,01 M EDTA; 0,1% w/v SDS in 0,05 M TRIS-HC1 pH 7,5) was allowed to settle. A sterile glass bead of 1 mm diameter was placed in a sterile Pasteur pipette. The sephadex still containing the nick translation buffer, was packed into the pipette.

The solution containing the mixture of labelled probe, free radio-active bases and the stop buffer (Appendix A) was applied to the packed column. Fractions of 100 Al were eluted and collected in microfuge tubes. Once the first of the dyes had been eluted from the column, the radioactivity of each fraction collected was monitored using a Geiger counter (Mini Instruments). The first peak contained the radio labelled probe . These five samples were pooled and used as a probe for

hybridisation experiments. Unincorporated label eluted after the probe and was discarded. The probe was stored at -20°C until use.

Before use in hybridisation reactions, the probe was heated above 95°C for five minutes to remove any secondary structures and immediately cooled on ice for ten minutes.

(At all times radioactive reagents were handled with gloves and the reactions and manipulations were conducted behind a perspex shield. A lead apron was used to shield the body during the transport of radioactivity not enclosed in a lead container.)

12.3.2. DIGESTION AND ELECTROPHORESIS OF DNA FRAGMENTS.

The genomic DNA of the avipox viral genomes were digested with the endonuclease restriction enzymes, Bam HI, Bgl II, Eco RI, Hind III, Pst I, Sma I, Xba I and Xho I.

The reactions were conducted as described previously (Chapter 4.2.2.). Digestion reactions were stopped by heating the solutions at 75°C for 15 minutes then adding loading buffer (Appendix A) to the reaction mixes.

These digestions were electrophoresed on a 0.8% agarose gel

using TAE buffer for 18 hours at 2 V/cm at room temperature. The gel was stained with ethidium bromide and photographed. The DNA fragments were visualised as described in Chapter 4.2.3.

12.3.3. HYBRIDISATION OF PROBE TO GENOMES

The DNA was transferred to Bybond N+ membrane (Amersham, UK) by the dry blot method described in Chapter 4.2.4.3.

The hybridisation was carried out as described by Johnson et al (1984) and set out in Chapter 4.2.4.4.

The hybridization and pre-hybridization buffers were made using the blocking agent from Boehringer Mannheim. The buffer consisted of: 5% (w/v) blocking agent, 0,2% (w/v) SDS, 10% (w/v) Dextran in 5 times concentrated SSC. Fifty ml of solution was sufficient for one hybridisation reaction.

The membrane was pre-hybridised at 42°C for 18 hours. The probe was denatured at 95°C for 5 minutes and cooled on ice. The pre-hybridisation buffer was removed from the hybridisation cassette, the probe added to it and both returned to the cassette.

The membrane was hybridised overnight at 20-25°C. With sequen-

tial washing steps the unbound probe was washed off the membrane. A washing step was conducted and the membrane autoradiographed for 18 hours at -70°C. The film was developed and examined before the next washing step was conducted.

The washing steps were conducted as follows:

(i) Two thirty minute washes consisting of 6 times concentrated SSC containing 0,1% w/v SDS and 0,25% w/v fat-free milk powder at 25°C.

(ii) Two thirty minute washes consisting of 2 times concentrated SSC containing 0,1% w/v SDS and 0,25% w/v fat-free milk powder at 25°C.

(iii) Two thirty minute washes consisting of 2 times concentrated SSC containing 0,1% w/v SDS at 25°C.

(iv) Two thirty minute washes consisting of 2 times concentrated SSC containing 0,1% w/v SDS at 30°C.

(v) Two thirty minute washes consisting of 2 times concentrated SSC containing 0,1% w/v SDS at 37°C.

(vi) One fifteen minute wash consisting of 2 times concentrated SSC containing 0,1% w/v SUS at 42°C.

12.3.4. IDENTIFICATION OF TK GENE IN AV/PDX VIRUSES.

The location and the sequence of the TK gene within the avipox virus subgroup is an indicator of the diversity of the sub-group. For this reason the other avipox viruses were probed with the same 18 mer oligonucleotide. The location and sequence analysis of the TK gene of the reference viruses of fowlpox and quailpox were compared to the position of the TK gene already published (Schnitzlein, et al 1988).

The viral particles were purified and the DNA was extracted from each of the following: parrotpox, fowlpox, penguinpox, quailpox and canarypox viruses and the turtle dove isolate.

The membranes were probed with the oligonucleotide constructed in Chapter 12.2. Two membranes were placed in the same hybridisation cassette at one time. The same radio-labelled oligonucleotide probe was re-used thrice. The probe was heated above 95°C for 5 minutes followed by cooling on ice for 10 minutes.

12.4: RESULTS

12.4.1. OLIGONUCLEOTIDE END-LABELLING

The determination of the location of the TIC gene relied on the labelling of the oligonucleotide, the hybridisation of the labelled oligonucleotide to a corresponding sequence in the

avipox viral genome and the visualization of that hybridisation reaction.

12.4.2. GEL ELECTROPHORESIS OF AVIPDX GENOMES

The fragment profiles obtained are shown as Figures 12.4.1.(A), 12.4.2.(A), 12.4.3.(A), 12.4.4.(A), 12.4.5.(A) and 12.4.6.(A).

12.4.3. HYBRIDISATION OF OLIGONUCLEOTIDE

The gels were blotted and the labelled oligonucleotide was hybridised to each of the membranes which were autoradiographed and developed and are shown as Figures 12.4.1.(B), 12.4.2.(B), 12.4.3.(B), 12.4.4.(B), 12.4.51B) and 12.4.6.(B).

12.4.4. AUTORADIOGRAPHS AND RESULTS OF OTHER AVIPDX VIRUSES

Each membrane was washed and autoradiographed and X-ray film developed after overnight exposure at -70°C.

Parrotpox virus:

The restriction endonuclease profiles for parrotpox isolate can be seen in Figure 12.4.1.(A). The autoradiograph obtained after hybridisation is shown as Figure 12.4.1.(B).

The band corresponding to 2.2 kb (size estimated from the

Lambda Hind III size markers electrophoresed in lanes 1 and 8) of the Hind III restriction endonuclease enzyme digest contained the TK gene. This band was the only one in the Hind III digest to bind to the probe and the size was the best suited for cloning for further study.

The viruses which have been compared in Chapter 10, the fowlpox, quailpox , canarypox and penguinpox viruses were shown to have totally dissimilar restriction profiles, and therefore not the same virus.

The identification of the restiction fragment containing the TK gene was determined for these viruses.

Fowlpox virus:
The photograph of the agarose gel and the autoradiograph are shown as Figure 12.4.2.(A). and Figure 12.4.2 (B).

The fowlpox TK gene was found to be located on a 4,5 kb fragment of the Bgl II digest. This fragment size is slightly smaller than the fragment of 5.5 kb the gene was found on by Boyle and Coupar (1986). There is a smaller bandin this digest where the probe has bound, however, the signal is not as strong as the higher band. There is also more than one signal in the Xba I digests. The fragment containing the TK gene

would be the stronger positive signal, however this must still be proved. On the basis of this preliminary study, our strain of fowlpox virus differs from strains used by Boyle and Coupar (1986) where the gene was located on a larger fragment of a different digest. The Eco RI digestion of fowlpox virus used in this present study shows the TK gene to be located on a fragment of approximately 9 kb in size.

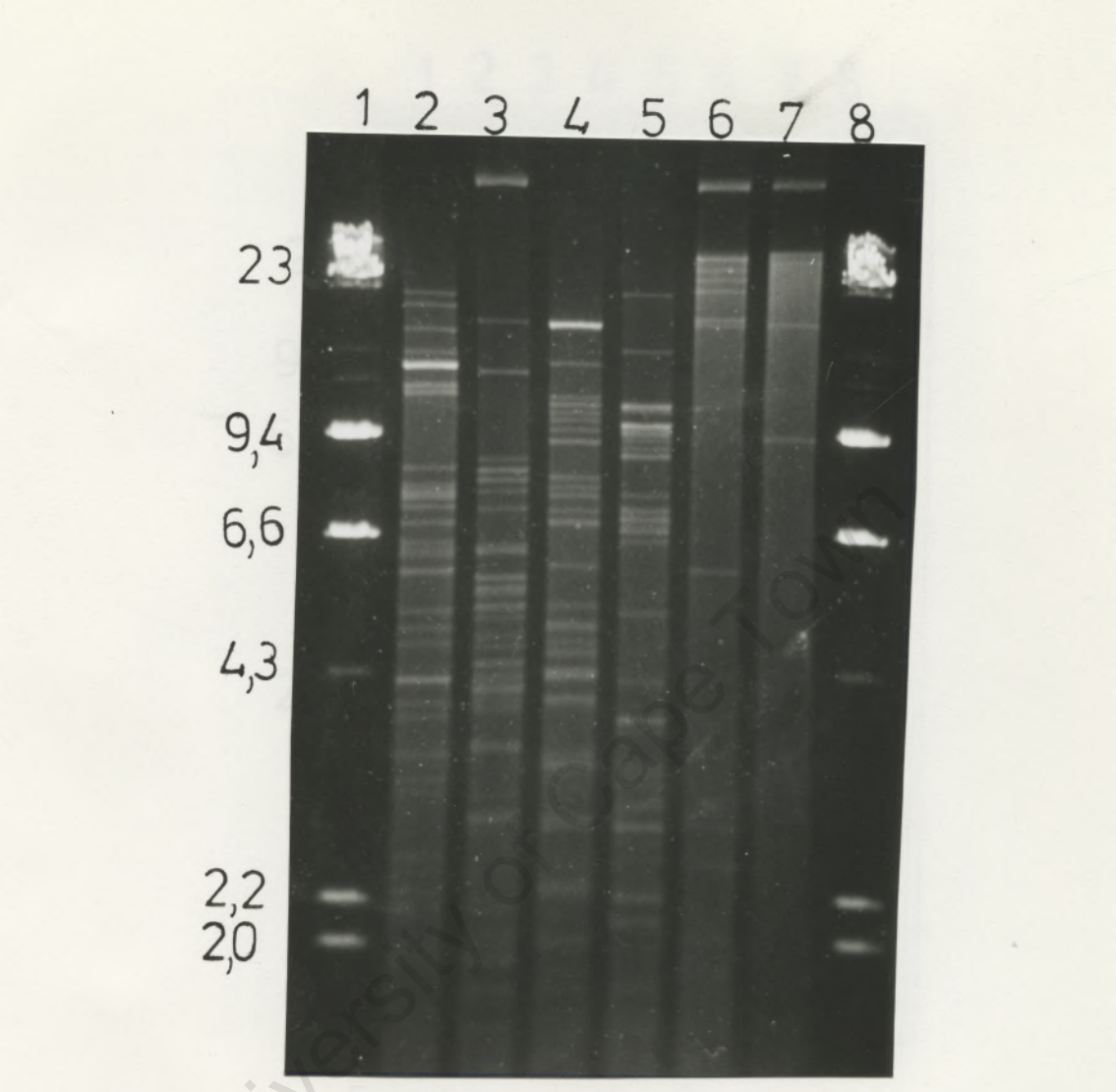

Figure 12.4.1.(A): The restriction profiles of the parrotpox isolate digestion with various enzymes and separated on a 0,8% agarose gel electrophoresed at 2 V/cm for 18 hours.

Parrotpox isolate digested with:

Lane 2: BamH I; lane 3: Bgl II; lane 4: Eco RI; lane 5: Hind III; lane 6: Pst I and lane 7: Sma I.

Lanes 1 and 8 contain the lambda Hind III size markers.

Figure 12.4.1.(B). : The autoradiograph of the parrotpox isolate digested with various enzymes and hybridised to 32P labelled oligonucleotide. Lanes 1 and 8: Lambda Hind III size markers; lanes 2 to 7 Parrotpox isolate viral DNA digested with: lane 2: BamH I; lane 3: Bgl II; lane 4: EcoR I; lane 5: Hind III; lane 6: Pst I and lane 7: Sma I. An arrow indicates the positive signal.

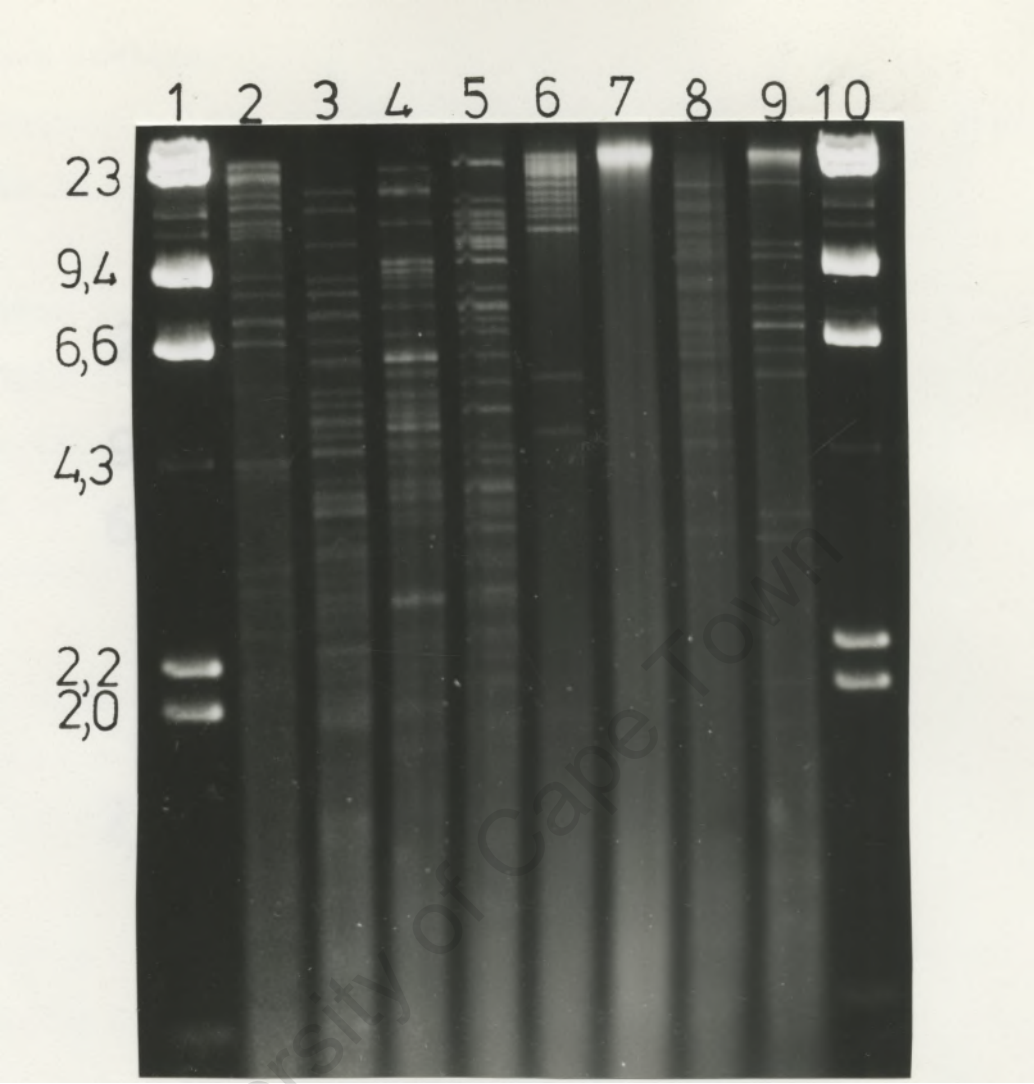

Figure 12.4.2.(A).: The restriction profiles of the fowlpox virus digested with various enzymes. The fragments were separated on a 0,8% agarose gel at 2 V/cm for 18 hours.

The fowlpox viruses was digested with

Lane 2: BamH I; lane 3: Bql II; lane 4: Eco RI; lane 5: Hind III; lane 6: Pst I; lane 7: Sma I; lane 8: Xba I and lane 9: Xho I.

Lanes 1 and 10 are the Lambda Hind III size markers.

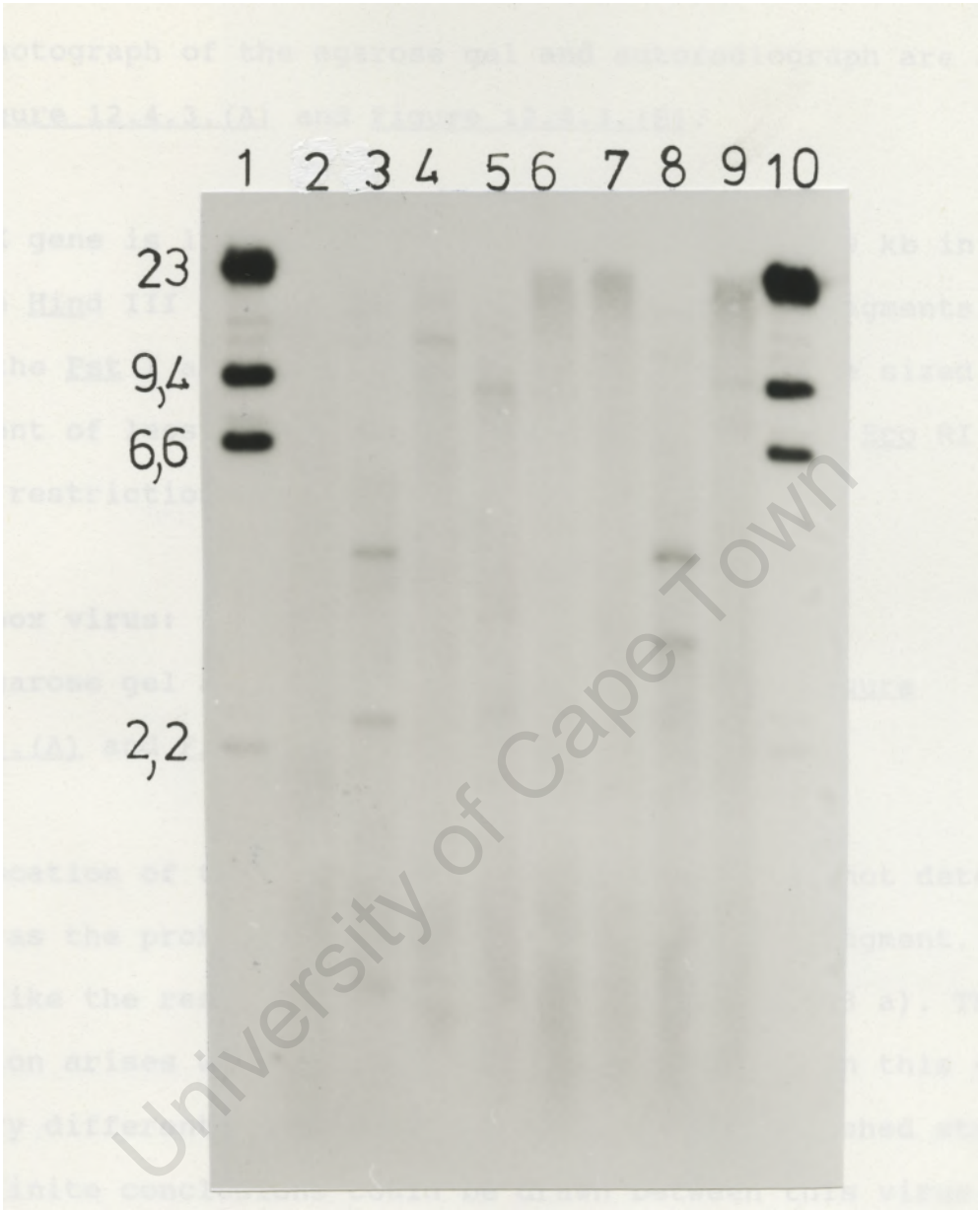

Figure 12.4.2.(B).: The autoradiograph of the fowlpox virus digested with various restriction enzymes and hybridised to the ^{32}P labelled oligonucleotide. The lanes are as noted in Figure 12.4.2.(A).

Penguinpox isolate:

The photograph of the agarose gel and autoradiograph are shown as Figure 12.4.3.(A) and Figure 12.4.3.(B).

The TK gene is located on a band of approximately 9 kb in size of the Hind III digest, and on very large sized fragments of both the Pst I and SMA I digests. A more manageable sized fragment of less than 4 kb is found in the Bgl II, Eco RI and Xba I restriction profiles.

Quailpox virus:

The agarose gel and autoradiograph are shown as Figure 12.4.4.(A) and FiCTUre 12.4.4.(B).

The location of the TK gene of quailpox virus was not determined as the probe did not bind to a single DNA fragment. This is unlike the results of Schnitzlein, et al., (1988 a). The question arises whether the strain of virus used in this study is very different from the virus used in the published study. No definite conclusions could be drawn between this virus and the results published by Schnitzlein, et al., (1988 a). A further observation was made that in this study the probe appeared to bind to two fragments in each digest with equal intensity.

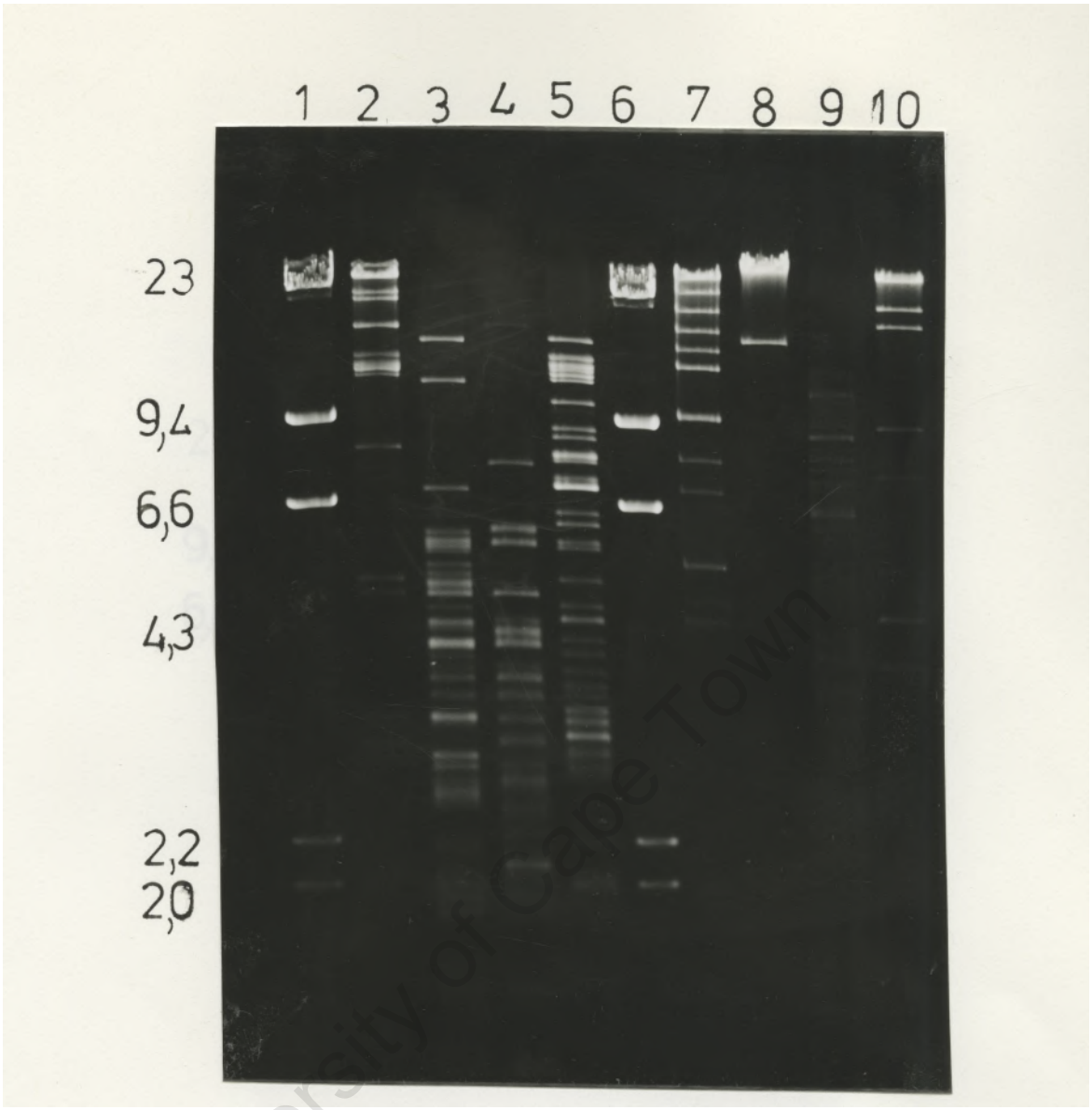

Figure 12.4.3.(A).: The restriction profiles of the penguinpox isolate digested with various restriction enzymes. The fragments were separated on a 0,8% agarose gel at 2 V/cm for 18 hours.

The penguinpox isolate was digested with:

lane 2: BamH I; lane 3: Bgl **II**; lane 4: Eco RI; lane 5: Hind III; lane 7: Pst I; lane 8: Sma I; lane 9: Xba I and lane 10: Xho I. Lanes 1 and 6 and the Lambda Hind III size markers.

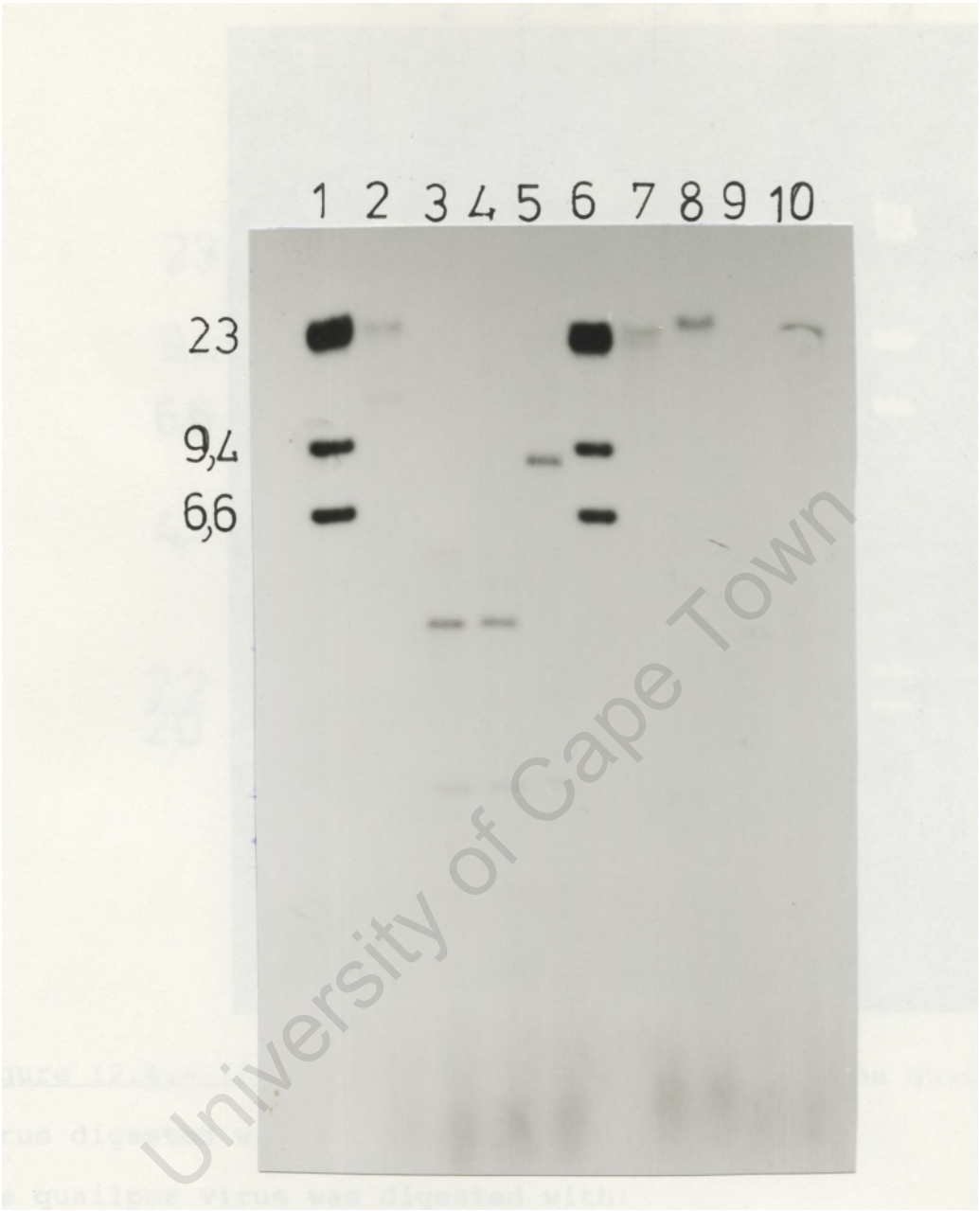

Figure 12.4.3.(B).: The autoradiograph of the penguinpox isolate digested with various restriction enzymes and hybridised to ^{32}P-labelled oligonucleotide. The lanes are as marked in Figure 12.4.3.(A).

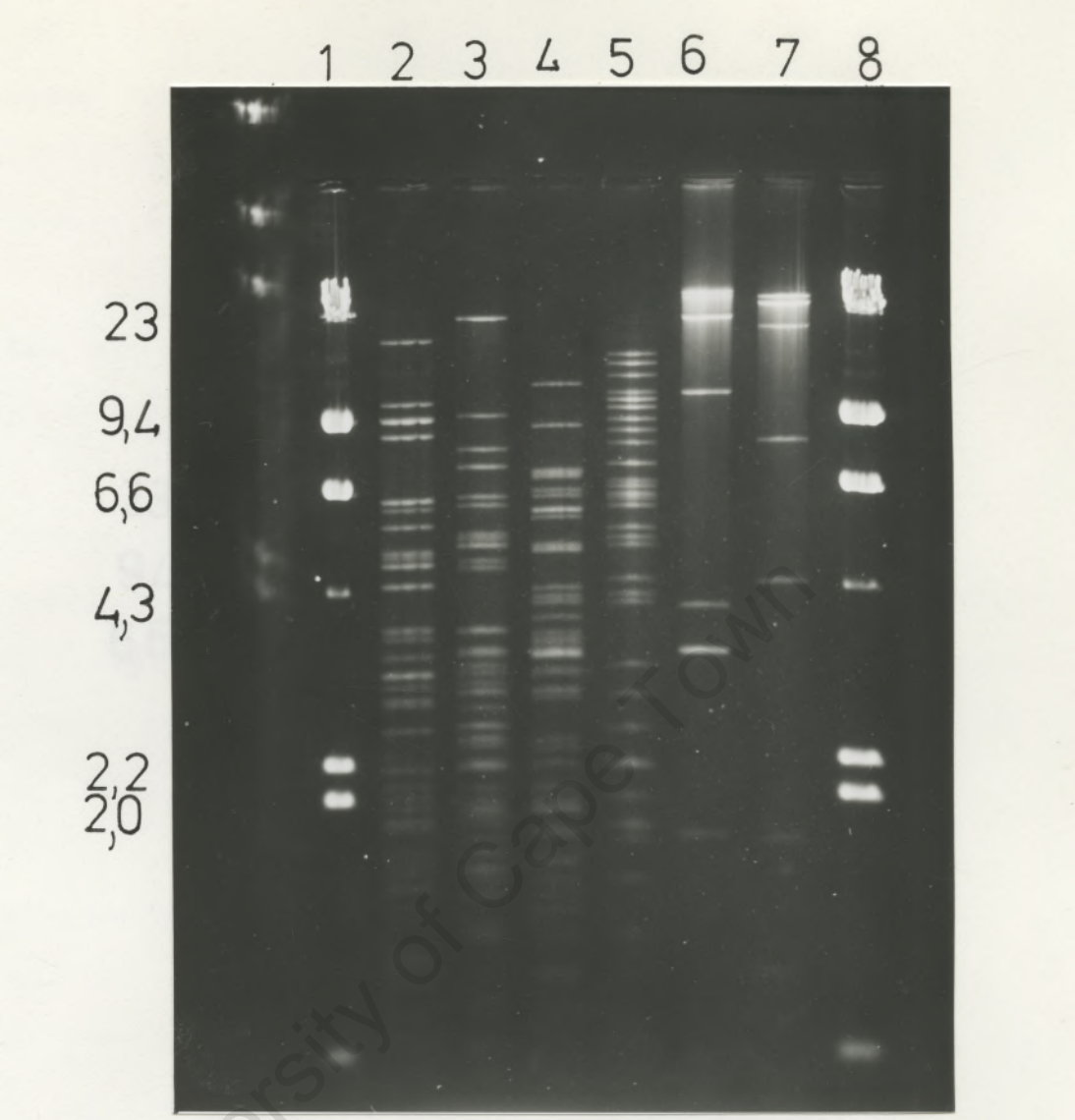

Figure 12.4.4.(A).: The restriction profiles of the quailpox virus digested with various restriction enzymes.

the quailpox virus was digested with:

Lane 2: BamH I; lane 3: Bgl II; lane 4: Eco RI; lane 5 Hind III; lane 6: Pst I and lane 7: Sma I restriction enzymes.

Lanes 1 and 8 are the Lambda Hind III size markers.

The fragments were separated on a 0,8% agarose gel at 2 V/cm for 18 hours.

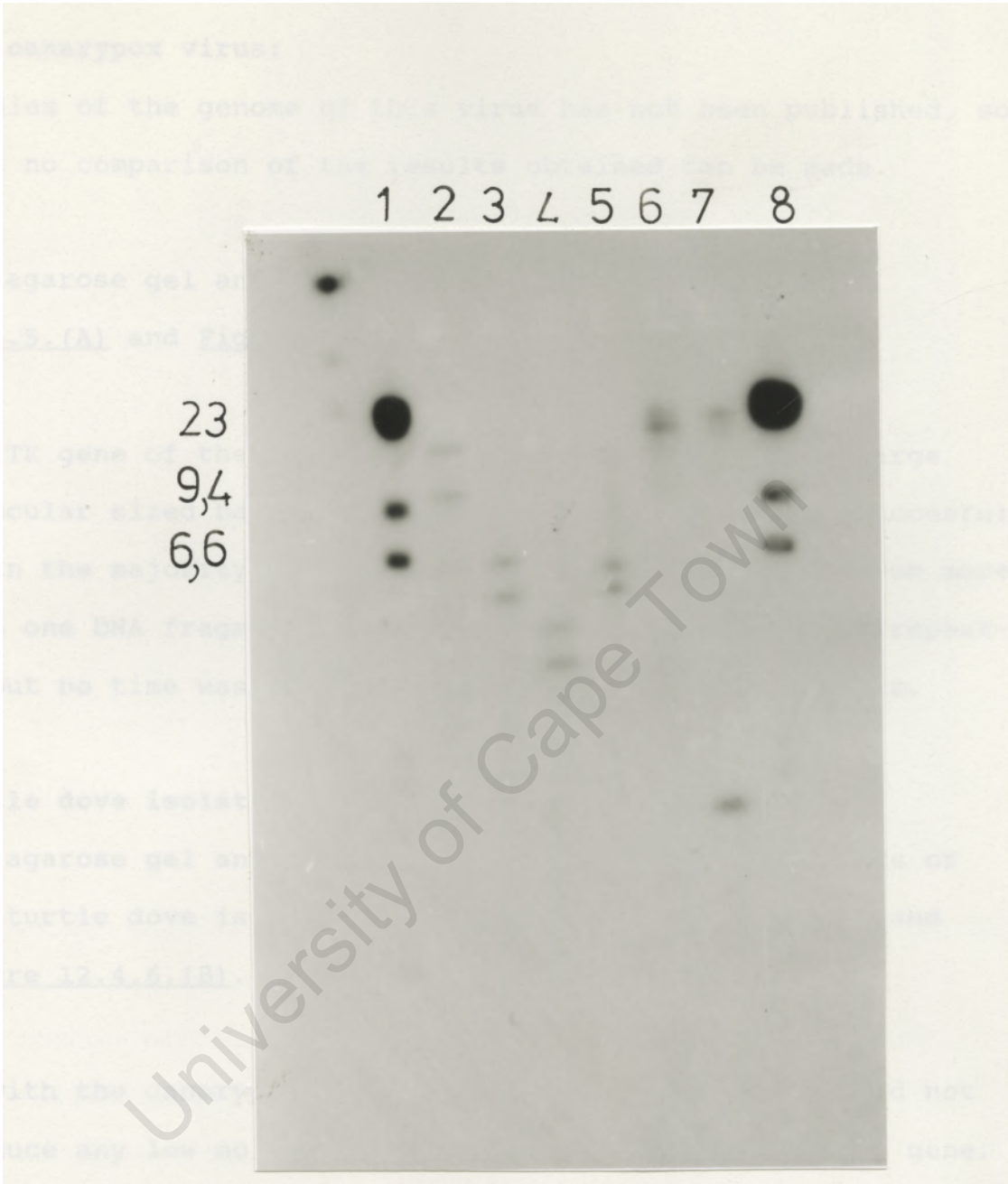

Figure 12.4.4.(B).: The autoradiograph of the quailpox virus digested with various enzymes and hybridised to the 32P labelled oligonucleotide. The lanes are as labelled in Figure 12.4.4.(A).

The canarypox virus:

Studies of the genome of this virus has not been published, so that no comparison of the results obtained can be made.

The agarose gel and autoradiograph are shown as figure 12.4.5.(A) and Figure 12.4.5.(B).

The TK gene of the canarypox virus is located on the large molecular sized bands, however this experiment was unsuccesful as in the majority of the digests there was a signal from more than one DNA fragment. These hybridisations need to be repeated but no time was available for the further experiments.

Turtle dove isolate:

The agarose gel and the autoradiograph of the digestions of the turtle dove isolate are shown in Figure 12.4.6.(A) and Figure 12.4.6.(B).

As with the canarypox virus, the turtle dove isolate did not produce any low molecular sized bands containing the TK gene. The smallest fragment was located in the Eco RI digest where the band was 9,4 kb in size. These restriction profiles, like the canarypox virus, also hybridised more then once to the oligonucleotide probe.

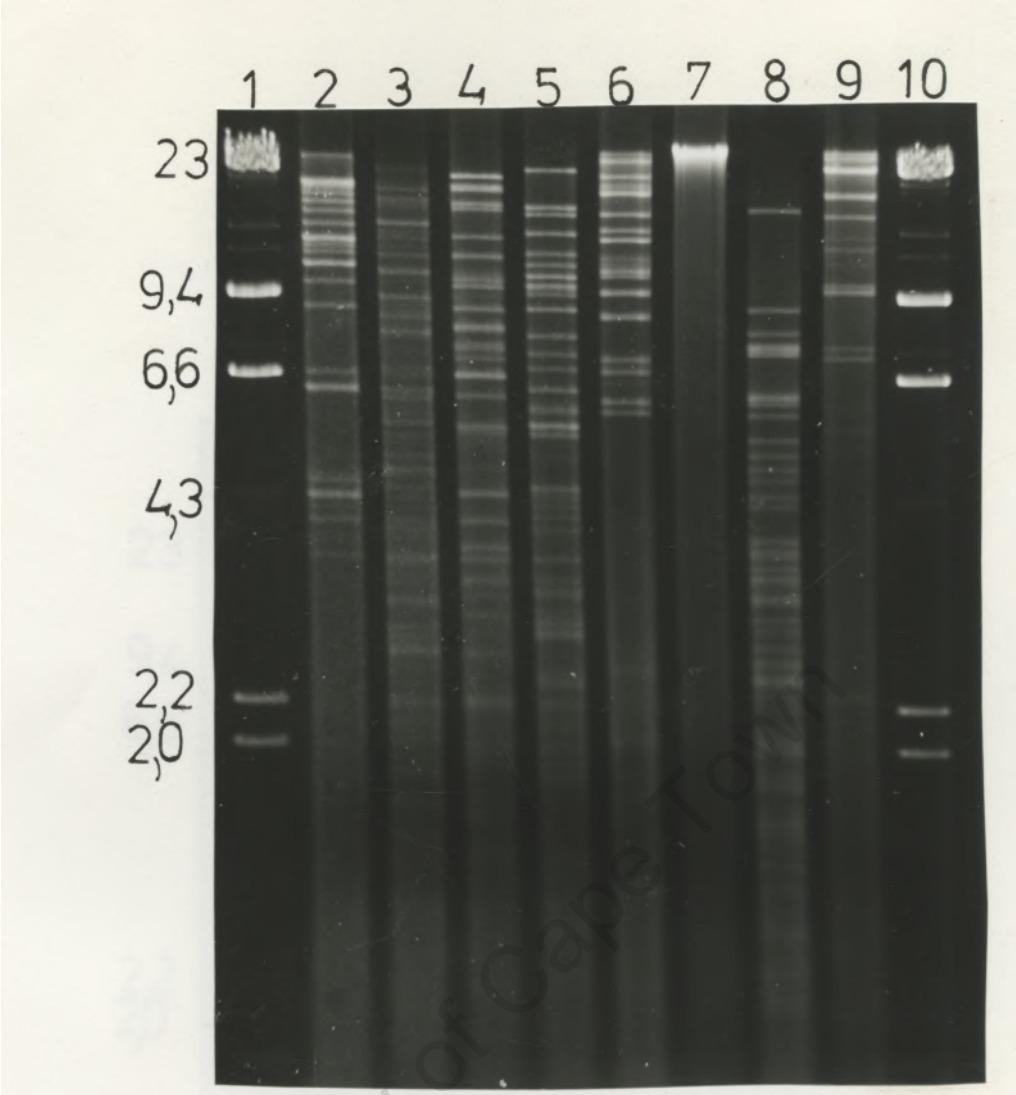

Figure 12.4.5.(A).: The restriction fragments of the canarypox virus was separated on a 0,8% agarose gel was electrophoresised for 18 hours at 2 V/cm and stained with ethidium bromide.

The canarypox virus was digested with:

Lane 2: BamH I; lane 3: Bgl II; lane 4: Eco RI; lane 5: Hind III; lane 6 Pst I; lane 7: Sma I; lane 8: Xba I and lane 9: Xho I. Lanes 1 and 10 contain the molecular sized marker of Lambda Hind **III**

Figure 12.4.5.(B).: The autoradiograph of the canarypox virus digested with various enzymes and hybridised to ^{32}P labelled oligonucleotide. The lanes are as marked in Figure 12.4.5.(A).

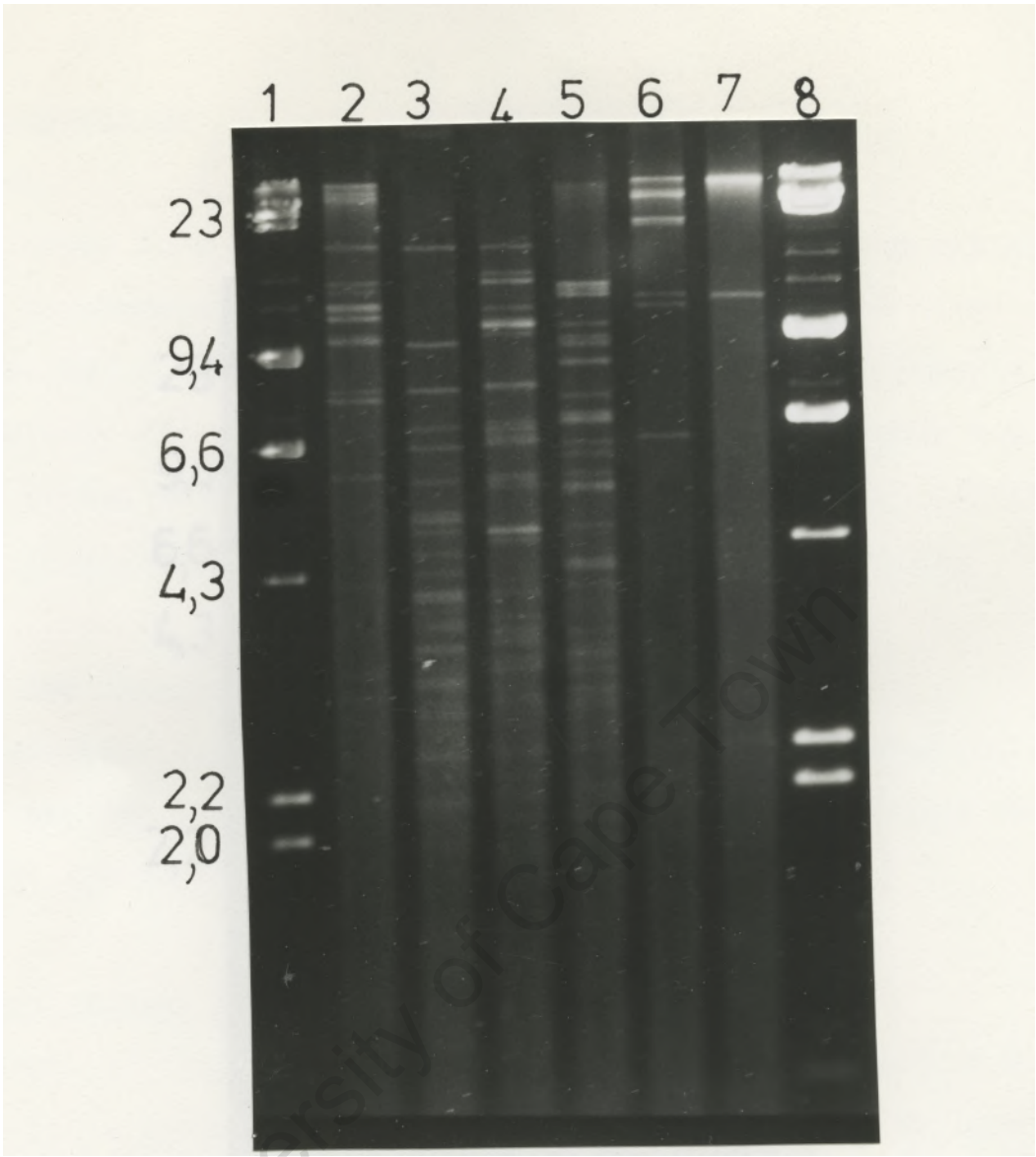

Figure 12.4.6.(A).: The restriction profiles of the turtle dove isolate digested with various restriction enzymes. The fragments were separated on a 0,8% agarose gel at 2 V/cm for 18 hours. The Lambda Hind III molecular size makers were electrophoresed in lanes 1 and 8.

The turtle dove isolate was digested with:

Lane 2: BamH I; lane 3: Bgl II; lane 4: Eco RI; lane 5: Hind III; lane 6: Pst I and lane 7: Sma I.

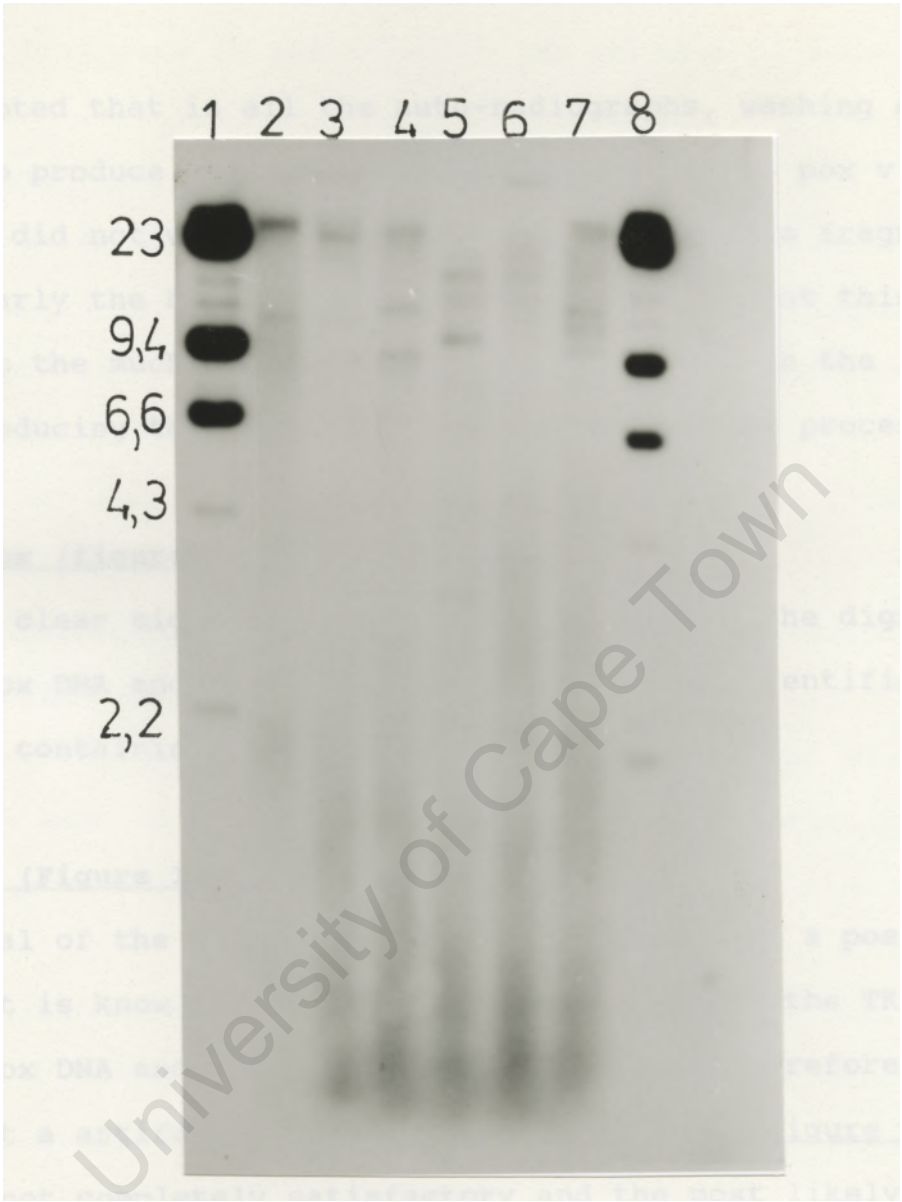

Figure 12.4.6.(B).: The autoradiograph of the turtle dove isolate digested with various restriction enzymes and hybridised with the ^{32}P labelled oligonucleotide. The lanes as marked for Figure 12.4.6.(A).

12.5: DISCUSSION

Comment:

It was noted that in all the auto-radiographs, washing extensively to produce a single positive signal in the pox viral digests, did not wash the signal out of the lambda fragments, particularly the higher bands. It is suggested that this may be due to the much greater concentration of DNA in the lambda bands, reducing the efficiency of the washing out process.

Parrot pox (figures 12.4.1. (a) and (B))

A single clear signal was obtained from each of the digests of parrot pox DNA and it can be assumed that this identifies the fragment containing the TK gene.

Fowl pox (Figure 12.4.2. (A) and (B))

IN several of the digests more than one band gave a positive signal it is know that there is a single copy of the TK gene in fowlpox DNA and the duplicate signals must therefore represent a artifact. The original gel shown in Figure 12.4.2. (A) was not completely satisfactory and the most likely for the duplicated signal is that the DNA was only partially digested.

Penguin pox (Figure 12.4.3. (A) and (B)).

In these digests a signal strong in all except the Pst I

digest this may be due to incomplete separation of the two largest fragments in one of which the TK gene is seen to lie.

Quail pox (Figures 12.4.4. (A) and (B)).

Each of these digests yield two signals in the auto-radiograph. The original seems to show well-defined fragments and at the moment no explanation can be offered in the results in this auto-radiograph. It is possible that there might be a complete or partial second copy of the TK gene in this virus.

Canary pox (Figures 12.4.5. (A) and (B) and Turtle dove isolate (Figures 12.4.6. (A) and (B).

These two experiments were unsatisfactory as clear signals were not seen in the auto-radiograph. These experiments need to be repeated but time was not available to do this.

The comparison of the restriction profiles of the parrotpox isolate tb the other viruses confirms the earlier conclusion that the avipox virus genera consist of viruses producing different restriction profiles classified in the same genera.

SECTION V

CHAPTER THIRTEEN

GROWTH OF AVIPDXVIRUS IN MAMMALIAN CELLS IS BLOCKED AFTER VIRAL DNA REPLICATION

13.1: GROWTH OF AVIPDX VIRUSES IN TISSUE CULTURE.

Avipox viruses are normally grown on CAMs of chick and duck embryos or in avian cell cultures. In cell cultures some of the avipox viruses produce CPE, which is characterized by refractive, rounded cells and degeneration after 3 to 6 days, while other viruses may not produce any CPE (Tripathy, et al., 1981).

Avipoxviruses cannot be passaged in mammalian cell cultures and do not produce infective virus particles in those cells (Randall, et al., 1964 and Burnett and Frothingham, 1968). The part of the virus growth cycle which is blocked has never been investigated.

However Taylor and Paoletti (1988) and Taylor, et al., (1988 a and b) showed that a recombinant fowlpox virus, expressing rabies glycoprotein in avian cells and hosts, was capable of inducing rabies immunity when injected into dogs, cats and rabbits. This indicates that the block to viral growth must occur only after some degree of viral expression has occurred.

Tests were therefore conducted to determine whether cytoplasmic sites of viral DNA replication can be identified in various mammalian or non-mammalian cell lines inoculated with different avipox viruses.

13.2: METHODS

The cells and culture media have been previously described in Chapter 3.

Poxvirus DNA replication occurs in the cytoplasm and the presence of cytoplasmic DNA was determined using an *in* situ detection test developed for the detection of mycoplasma contamination in cell cultures by the fluorescent Hoechst 33258 stain (Chen, 1977). All the solutions and buffers are listed in Appendix A under DNA fluorescent stain reagents.

13.2.1. DETECTION OF DNA USING HOECHST 33258

The cells were grown on coverslips placed in tissue culture tubes at 37°C. The growth medium was removed and the cells were infected with 1 pfu/cell of purified virus. Maintenance medium consisting of 5 % FCS in MEM or DMEM was added to the tubes. The coverslips were harvested at the following intervals: 0, 6, 18, 24, 30 and 48 hours by replacing the medium with 3 - 4 ml of cold fixative. This was left for 5 minutes at ambient temperature, the fixative removed and the process

repeated leaving the fixative for 10 minutes. The coverslip was allowed to dry. As there were various time intervals at which coverslips were harvested, they were stored at -20°C until all were then collected. This was necessary as the working stain dilution degraded because the fluorescent stain is unstable.

The coverslips were removed from -20°C and dried completely. The Hoesht working solution was stirred for 30 minutes and used to stain coverslips at room temperature in a humidified container for 30 minutes. They were washed 3 times with distilled water and mounted while wet with citric acid - disodium phosphate mounting buffer. They were examined the same day for fluorescence under a fluorescence microscope. It was important for the coverslips to be examined immediately as the stain is sensitive to both light and heat and degrades after 24 hours even if kept in the dark at 4°C.

13.3: RESULTS

A positive result was indicated by the appearance of cytoplasmic fluorescence in the cell. A negative control of HF cells is shown in Figure 13.3.1. The nuclei were seen as large bright bodies within the cell. The cytoplasm of the negative cells was clear and fluorescence-free. Certain cell cultures stained positive for mycoplasma. These were seen as faint

background fluorescence (Figure 13.3.4.).

In contrast, the cells infected with avipox viruses contained small, discrete bright fluorescent bodies in the cytoplasm (Figure 13.3.2 to Figure 13.3.6). These viral replication bodies were seen with all avipox viruses used in the study, and in all the cell cultures used. The results are tabulated in Table 13.1.

Table 13.1.: The time, post-inoculation, in hours at which foci of DNA replication bodies of viruses could be seen using the DNA fluorescent stain.

Cell culture used	Avipox virus used						
	FPV	PaPV	PePV	PiPV	QPV	CPV	TPV
CV1	24	24	24	30	24	24	24
HF	18	18	24	30	18	18	24
HeLa	18	18	24	30	18	18	18
MDBK	24	30	24	30	24	24	24
RK13	18	24	18	24	18	18	24
MDCK	24	24	24	24	24	24	24
CEF	18	18	18	18	18	18	18

The abbreviation of the viruses are FPV - fowlpox virus; PaPv - parrotpox virus; PePV - penguinpox virus; PiPV - pigeonpox virus; QPV - quailpox virus; CPV - canarypox virus; TPV - turkeypox virus.

Vaccinia virus is not included in this table as for all the cell cultures vaccinia DNA replication bodies could be seen at

18 hours post- inoculation. This time has been reported to be as low as 8 hours (Moss, 1991), however this period was not investigated in this study.

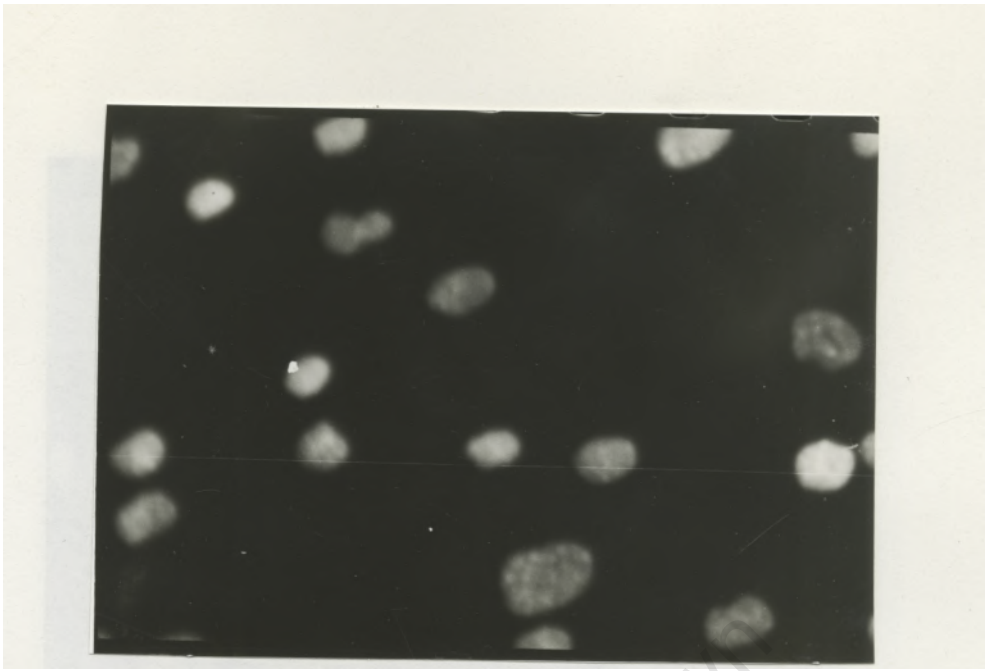

Figure 13.3.1.: A photograph showing uninfected HF cells with only the nucleus staining.

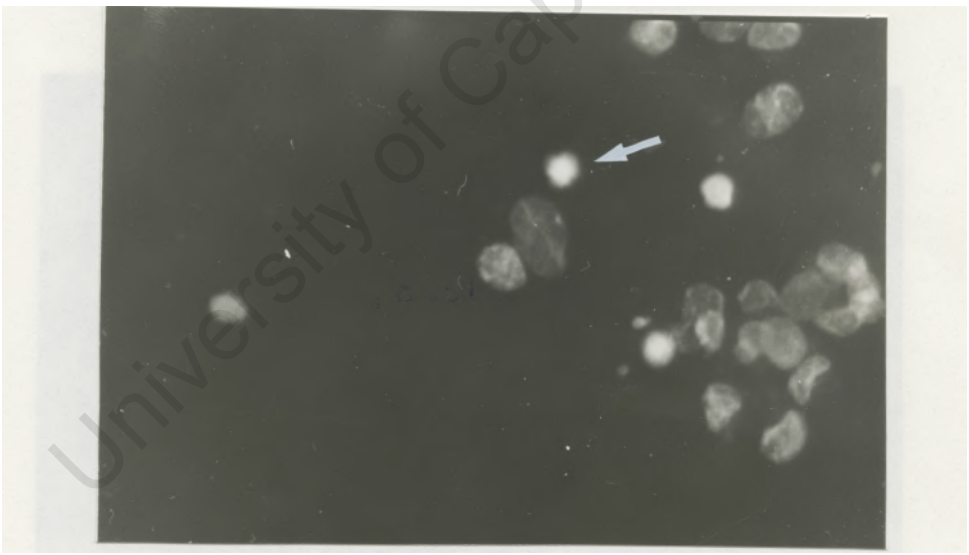

Figure 13.3.2.: A photograph showing vaccinia virus infected cells after 18 hours. The replication bodies are shown as small, discrete, bright fluorescent bodies (indicated by the arrow).

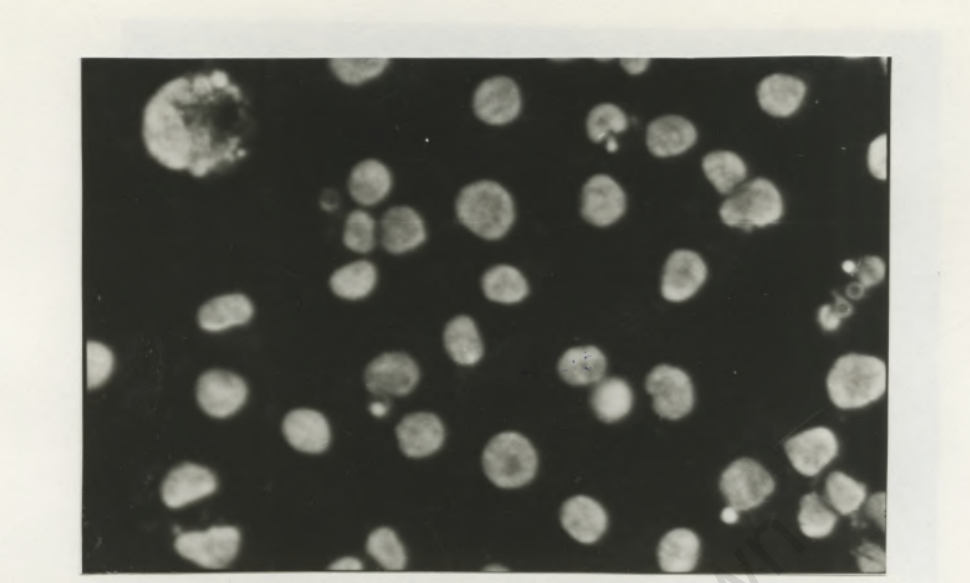

Figure 13.3.3.: A photograph of fowlpox infected HF cells after 24 hours showing the replication bodies as small, discrete and brightly fluorescing foci.

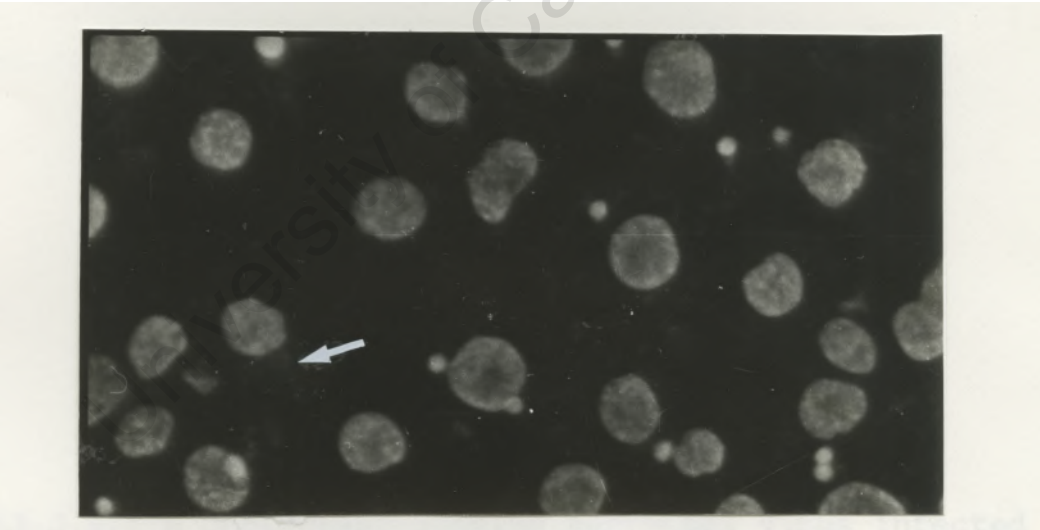

Figure 13.3.4.: A photograph of fowlpox virus infected CV1 cells showing foci of viral DNA fluorescing strongly. These cells are contaminated with mycoplasma which causes the faint fluorescence seen in the cytoplasm (indicated by arrow).

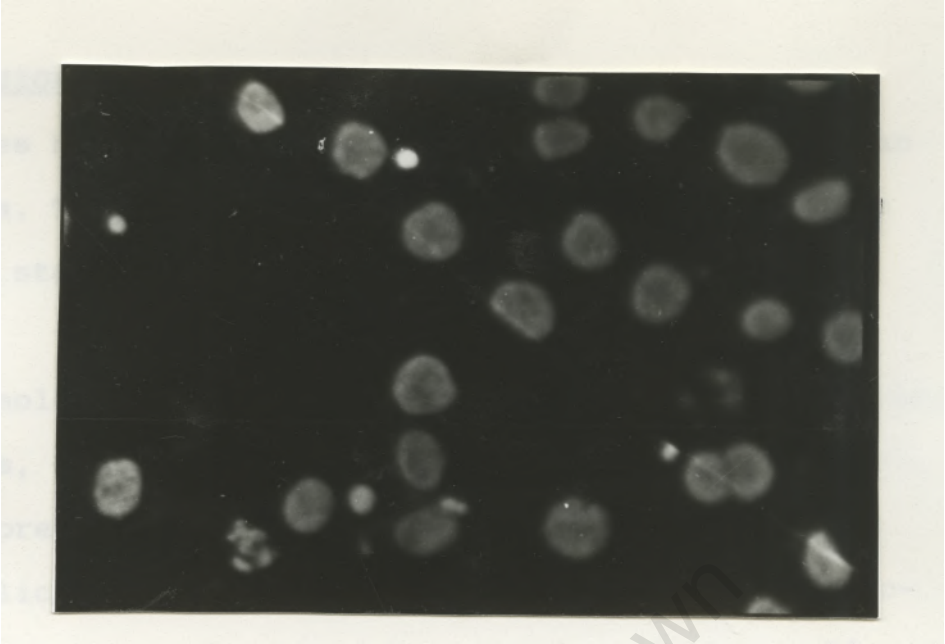

Figure 13.3.5.: A photograph of penguinpox virus infected CV1 cells after 24 hours. Viral DNA is seen as bright fluorescent bodies in the cytoplasm.

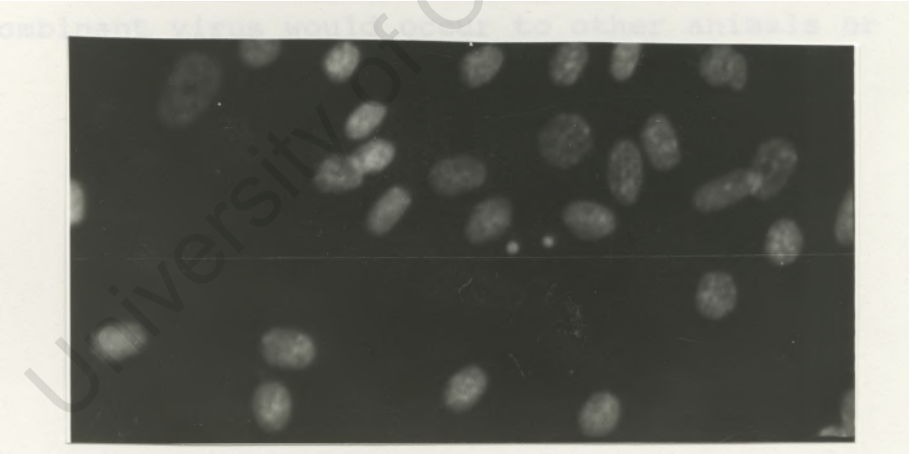

Figure 13.3.6.: A photograph showing parrotpox virus infected CV1 cells, 24 hours post-infection. The replication factories, which are bright and discrete, are seen against a faint background of mycoplasma in the cytoplasm.

13.4; DISCUSSION AND CONCLUSION

Avipox viruses have been shown to replicate DNA in mammalian cell cultures. The growth cycle of the virus appeared to be blocked at a stage later in the replication cycle.

Taylor and Paoletti (1988) have already shown that recombinant fowlpox virus, expressing rabies glycoprotein in avian cells, will also express the protein in mammalian cells and hosts. This has implications for the development of veterinary vaccines. A recombinant avipox vaccine used in animals would elicit an immune response to antigens introduced into host cells. There would be a further advantage in that the avipox virus could not complete a replication cycle and so no spread of the recombinant virus would occur to other animals or humans.

SECTION VI

CHAPTER FOURTEEN

CONCLUSIONS

14.1: ISOLATION OF A NOVEL AVIPDX VIRUS - PENGUINFOX VIRUS.

A virus was isolated from scrapings taken from a diseased penguin at SANCCOB, Milnerton. This virus was classified as an avipox virus using classical biological characterisations of the virus and identified as a novel virus using molecular biology techniques.

This is the first recorded instance of the isolation of an avipox virus from Jackass penguins. The first virus isolate was from a single bird admitted to SANCCOB. Two further isolations were made a year later, from two birds admitted to SANCCOB on different days.

The three penguinpox isolates were found to be morphologically similar. On the basis of restriction endonuclease digestions, the genomes were found to be similar, differing only in the terminal fragments. Differences within the terminal fragments of orthopox viruses have been used as a basis of strain differentiation (Baroudy, et al., 1982). Differences found the terminal repeats of the three penguin pox isolates suggested that these three viruses are strains of the same virus. The three isolates appeared to be strains of the same virus.

Avipox virus infection is apparently a latent infection within the Jackass penguin population found around the Cape Peninsula, and not introduced when the birds were admitted to SANCCOB. The virus infection could occur when the birds are stressed such as when found coated with oil or on admission to SANCCOB.

The isolation of a pox virus from a fowl and a turtle dove confirmed the fact the presence of other avipox viruses in the Cape Peninsula. Comparison of the restriction endonuclease digestion profiles of the local fowl, turtle dove and penguinpox isolates showed them to be three different viruses. The isolation of three avipox viruses in the Cape Peninsula suggested that there are probably many other avipox viruses in the wild bird population. This fact was emphasized by Tripathy, et al., (1973), when he reported that over 60 types of wild birds are infected with avipox viruses.

14.2: IDENTIFICATION AND PURIFICATION OF THE PENGUINPDX VIRUS

The first penguinpox virus was identified on the basis on the following studies:

(i) electron microscopy;

(ii) morphology;

(iii) histology studies and;

(iv) restriction endonuclease digestion profiles.

(i) The electron micrograph studies: These were conducted on purified penguinpox virus showed a structure Of a typical pox virus with a bi-lipid layer and surface tubules. The size of the virus particle is within the range for pox viruses. A fatty coat surrounding the virus can be seen (Figure 6.3.1.). This feature has not been seen with orthopox viruses but it confirms the evidence of Randall, et al., 1964, where it is stated that fowlpox virus has a fatty coat and over 40% of the virus contains lipids.

This fatty coat presented problems during purification. Standard purification methods developed for orthopox viruses could not utilised to purify the avipox viruses. On titration, extracts of avipox viruses a titre 5 logs lower was obtained when compare to an orthopox virus. It was assumed that the fatty coat was responsible for aggregating the avipox virus particles and that they were being lost during the centrifugation steps. Joklik (1962), used an organic solvent, Gentron, to purify poxvirus. A similar fluorocarbon, Arklone-X, was used in this study and the solvent resulted in an increase of 5 logs of virus titre. The use of Arklone at the initial stage of extraction increase the titre. This confirms the suggestion of Gafford, et al, 1967, that the use of an organic solvent could purify the avipox viruses.

(ii) Morphology studies: The pock morphology on CAMs could not be used as a definitive means of identifying different strains of avipox virus. The pocks of the penguin isolate were fine, small and discrete. These differed from those of the reference viruses which were large and in most cases haemorrhagic in nature. The reference viruses have, however been passaged over several generations on CAMs and in cell environments. This could have resulted in morphological changes taking place.

(iii) The histological studies: This work showed that the penguinpox isolate is an avipox virus, based on the presence of A-type inclusions similar to these described for fowlpox virus (Tripathy, et al., 1981).

(iv) Restriction endonuclease digestion studies: DNA restriction profiles were obtained by digestion of viral DNA of the penguin pox virus and of three other avipox viruses, (viz. fowlpox, canarypox and quailpox), with Bam HI, Hind III, Pst I, Sal I, Xba I and Xho I. None of these profiles showed any significant similarities. The novel penguin pox isolate appeared to be a distinct virus, quite dissimilar from the other viruses tested.

After Sam HI and Hind III digestion, electrophoretic profiles displayed by the quailpox and fowlpox viruses were found to be

similar to those profiles recently published by Schnitzlein, et al., (1988 b). In many of the digests, fragments larger than 25 kb were visualised. These fragments were showed minimal separation by gel electrophoresis and it was difficult to calculate their size with accuracy. Estimations of the entire genome size were concluded from profiles showing well separated fragments. With respect to the genome size, the penguinpox virus was comparable to the fowlpox and canarypox viruses.

A novel finding was that quailpox virus appeared to have a smaller genome than the other avipox viruses.

A conclusion drawn from the digestion profiles shows that the genome, but not the size, of the penguinpox virus differed significantly from the quailpox, fowlpox, canarypox and parrotpox viruses.

These comparisons, done on the avipox viral genomes, (the first such study to be undertaken), show the viruses to be different in all respects. No homology studies were undertaken but examination of these digests with that of the orthopox viruses (Muller, et al., 1977) reveals that the avipox viruses are very dissimilar and that maybe they should be classified into different sub-genera on the based on their

diverse morphological characteristics and the fact that of these avipox viruses do not offer cross-protection within the entire avipox group (Winterfield and Reed, 1985).

14.3: ADDITIONAL PROJECTS

Two additional projects emanated from this study:
They were

(i) the location of the TK gene in avipox viruses; and

(ii) a study into the replication of avipox viruses in mammalian cells.

(i) The TA gene study: This study revealed that the TK gene was located on different sizes of fragments. This is not surprising as the genomes of the viruses gave different restriction profiles. However to determine the position of the gene on the genome, further homology studies has to be conducted. Unsuccessful attempts were made to clone a 2,2 kb fragment of the parrotpox virus containing the TK gene from parrotpox DNA into bacterial vectors as part of this project.

(ii) The replication of the avipox virus in mammalian cells: It was reported that fowlpox recombinants were able to induce an immune response in mammalian hosts (Taylor, et al., 1988 b). This was unexpected as it is known that avipox viruses cannot replicate in mammalian cells (chapter 6). During this

study, mammalian cells infected with avipox virus were examined using a fluorescent stain to detect DNA replication. The presence of discrete pockets of fluorescence in the cytoplasm of the infected cells revealed that viral DNA replication did occur. Viral replication must have been interrupted after DNA replication as no infectious were isolated from tissue fluids.

14.4: CONCLUSION

The successful outcome of this study represents the first isolation of an avipox virus from Jackass penguins. The isolation of two similar viruses a year later confirmed the presence of a pox virus which is latent in the penguin population. The isolation of avipox viruses from other birds in the Cape Peninsula suggests that there are other avipox viruses in the avian population.

APPENDIX A.

1% ACID ALCOHOL

Concentratrated hydrochloridic acid	1 ml
96% ethanol	99 ml

Mix well.

ACTIVATED TRYPSIN VERSENE (ATV)

5% stock trypsin	1 ml
PSN stock solution	0,5 ml
Trypsin Base	10 ml

Mix the above in 90 ml of sterile water under aseptic conditions.

CARBOL FDCHSIN STAIN

Basic fuchsin	5 g
phenol crystralline)	25 g
alcohol 95%	50 ml
water	500 ml

Mix the fuchsin and phenol and heat for 5 minutes. Add the alcohol and filter through a Whatman number 1 filter before use.

THE HOECHST STAINS - DNA FLUORESCENT STAINS

THE STOCK SOLUTION

Bisbenzimid H3325B	5 mg
Sterile PBS	100 ml

Dissolve the powder in the PBS by stirring for 30 minutes at room temperature. Wrap the bottle in aluminium foil and store in the dark at 4°C.

THE WORKING SOLUTION

Stock solution	0,5 ml
Sterile PBS	45 ml

Mix the stock solution with PBS, stirring for 30 minutes at room temperature in a bottle wrapped in aluminium foil.

THE FIXATIVE

Glacial acetic acid	25 ml
Methanol	75 ml

DMix the fluids together and store at 4°C.

THE MOUNTING SOLUTION

O,1 M citirc acid	22,2 ml
0,2 M Na2HPO4.2H2O	27,8 ml
Glycerol	50 ml

Mix the above solutions at room temperature and adjusted the pH to 5,5. Store at 4°C wrapped in aluminium foil.

EOSIN

Eosin solution 2 % (w/v made in distilled water) 70m1

Phloxine solution 2 % (w/v made in distilled water) 30m1

Mix the solution together before use.

FORMALIN

Aqueous formaldehyde	37 ml
Methanol	7 ml
1120	56 ml

Mix together and store at room temperature.

GLYCEROL – 80%

Glycerol	80 ml
H,0	20 ml

Mix the two fluids together, autoclave and store at room temperature.

HAEMATOXYLIN

Haematoxylin	2,5 g
Absolute ethanol	50 ml
Ammonium alum	50 g
Distilled water	500 ml
Mercuric oxide	1,5 g
Glacial acetic acid	20 ml

Dissolve the haematoxylin in absolute ethanol while dissolving

the ammonium alum in water, heating if neccessary. Mix the two solutions and heat to boiling point. Cool the solution slightly and add the mercuric oxide. Cool the solution before adding the glacial acetic acid. Filter the solution before use.

1 M HEPES BUFFER

Hepes	23.8 g

Dissolve the powder in 80 ml of 0.3 M NaOH. Adjuxt the pH to 7.2 and make the volume to 100 ml with distilled water. Filter sterilise the solution through a 0,22 gm filter.

IODINE ALCOHOL

Grams triple strength iodine is diluted 1:3 in 96% ethanol, to give an iodine alcohol solution.

KINASE BUFFER – 10 X CONCENTRATED

1 M TRIS-Cl (pH 7,6)	50 ml
1 M MgC12	1O ml
1 M Spermidine	0,1 ml
1 M dithiothreitol	5 ml
0,5 M EDTA	0,2 ml

Mix the above and make the volume to 100 with distilled water.

LOADING BUFFER

glycerol	50 ml
SDS 10% (w/v)	10 ml
bromophenol blue	0,1 g
cyanol	0,1 g

Mix the above with water at room temperature and make the volume to 100 ml. Dispense the solution in 10 ml amounts and keep at room temperature.

LURIA AGAR

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	15 g
Distilled water	1000 ml

Dissolve the agar in 500 ml distilled water. Dissolve the tryptone, yeast extract and NaCl in 300 ml distilled water. Mix the solutions and adjust the volume to 1000 ml. Sterilise by autoclaving.

LYSIS BUFFER

Na n-lauryl sarcosinate (36%)	17,5 ml
sucrose	54 g
TRIS pH 7.8 buffer	100 ml

Mix the compounds together, sterilise by autoclaving and

dispense in 2 ml amounts. Before use add 200 mM 8-mercaptoethanol.

McILVAINS1 PH 7.4 BUFFER

Solution A : 0.1 M Citric acid

Solution B : 0.2 M Na2HPO4.12H2O

Mix 1.83 ml of solution A and 18.7 ml of solution B. Add 800 ml of distilled H2O. Adjust the pH of the solution with either solution A or B. Make the solution up to 1 000 ml. Sterilize by autoclaving.

EAGLE'S MINIMAL ESSENTIAL MEDIUM (MEM) AND DULBECCO'S MODIFIED EAGLE'S ESSENTIAL MEDIUM (DMEM)

Make the MEM or DMEM to the manufacturer's specifications and sterilise through a 0,22pm filter. During filtering dispense the medium in 500 ml amounts and store at 4°C. Add antibiotics (PSN), sodium bicarbonate and foetal calf serum to the medium immediately proir to use.

MEM DILENT

Make the MEM dilent by adding 1 ml of a 1M Hepes solution to 100 ml MEM. Adjust the pH of the solution to 7.4 by the addition of HC1 or NaOH. Filter sterilize the solution through a 0,22 pm filter.

PHENOL

Liquidify commercial phenol at 56°C. Add hydroxyquinoline at a final concentration of 0,1 %. Extracct the phenol several times with an equal volume of 1 M TRIS pH 8 buffer until the pH of the aqueous phase is higher than pH 7,8. Store the phenol at 4°C under an equal volume of 1 M TRIS pH 8 buffer containing 2% B-mercaptoethanol.

PHOSPHATE BUFFERED SALINE (PBS)

NaC1	8,0 g
KCl	0,2 g
KH,PO4	0,12 g
Na2HPO4 (anhydrous)	0,91 g OR
Na2HPO4.2H2O	1,14 g OR
Na2HPO4.12H2O	2,28 g

Dissolve the salts in 900 ml distilled water. Adjust the pH to 7,5 and make the volume to 1000 ml. Dispense into 100 ml volumes and autoclave for 15 minutes.

PBS DILUENT

PBS	10 ml
water	90 ml

Mix the PBS and water and autoclave. Add 1 ml of the PSN solution and store at 4°C.

THE PSN SOLUTION.

PSN solution is the antibiotic solution which contains penicillin, streptomycin and neomycin.

Penicillin	2 x 106 Units
Streptomycin	2 g
Neomycin	2 g

Reconsitute the above antibiotics in a total volume of 100 ml physiological saline. Mix the solution well, filter sterilize through a 0,22gm filter and stoer dispensed in 2,5 ml at -20°C.

RNAse BUFFER

TRIS pH 8	1 ml
NaC1	O,58 g

Mix the salt in the TRIS buffer and make the solution up to 100 ml. Sterilize the solution by autoclaving.

SCOTT'S WATER SUBSTRATE.

NaHCO4	3.5 g
MgSO4	2O g

Dissolve the two compounds separately then mix together carefully to make a volume of 100 ml with distilled water.

SEPHADEX G50

Sephadex G50, fine grade	30 g
TE buffer pH 8	500 ml

Suspend the sephadex G50 in 250 ml TE pH 8 buffer in a 500 ml bottle. Disperse the powder and leave overnight at room temperature. Change the TE buffer pH8 carefully and autoclave the suspension. Allow to cool completelyand change the buffer again. Store the suspension at 4°C.

SSC BUFFER – 20 X CONCENTRATED

NaC1	175,3 g
Na-citrate	88,2 g

Dissolve the salts in 800 ml distilled water. Adjust the Ph to 7,0. Make the volume to 1000 ml and sterilize by autoclaving and store at room temperature.

STOP BUFFER

Glycerol	50 ml
SDS	0.1 g
0,5 M EDTA (pH8)	0,1 ml
bromophenol blue	0,1 g
cyanol	0,1 g

Dissolved the above in 30 ml distilled water and make the volume to 100 ml. Dispense the solution in 10 ml and store at room temperature.

TRYPAN BLUE SOLUTION

Trypan blue powder	5 g
physiological saline	100 ml

Dissolve the powder in the saline, filtere through Whatman number 1 paper after which filtere sterilized through a 0,22gm filter. Dispense the dye in 2 ml amounts and store at 4°C.

TRYPSIN

Trypsin powder	5 g
Trypsin base	10 ml
H_2O	90 ml

Add the trypsin base to the water and acidify to a bright yellow by adding concentrated HCl. Sprinkle the trypsin powder onto the surface of the trypsin base. Leave the trypsin to dissolve slowly without stirring or agitating. Filter sterilize through a 0.2 gm filter.

TRYPSIN BASE.

NaC1	30 g	
KC1	2 g	
KH2PO4	1.2 g	
Na2HPO4	9.1 g	OR
Na2HPO4.2H2O	11.2 g	OR
Na2HPO4.12H2O	22.8 g	
Phenol (0.4% solution)	25 ml	
glucose	5 g	

Dissolve the above chemicals in 700 ml of distilled water. Dissolve 2 g of EDTA in about 100 ml of distilled H2O. Add the EDTA solution to the above salt solution. Adjust the pH to 7.8 with 1 N NaOH. Make the solution to 1000 ml and fliter sterilize through a 0.2 gm filter. Dispense the solution in 20 ml amounts. For use add 10 ml of the base to 90 ml of steril-ized distilled water.

TRYPTONE BROTH

Tryptone	10 g
NaCl	5 g
Distilled water	1 000 ml

Dissolve the compounds in 800 ml distilled water and make the volume to 1 000m1. Sterilize by autoclaving.

ZENKER BASE.

MgCl	5g
Potassium dichromate	2.5 g
Na-sulphate	1 g
Distilled water	100 ml

Disslove the compounds in water to make a stock solution.

A working solution: Add 5 ml of formalin to the stock solution.

APPENDIX B

GENERAL CHEMICALS

The following are supplied by Merck, Darmstadt, Germany: phenol, chloroform, isoamylalcohol, NaCl, THIS salt, glycerol, concentrated HCl, xylol, absolute ethanol, methnol, acetic acid, glacial acetic acid, formaldehyde, ethidium bromide and KCl.

The following were supplied by BDH, Poole, England: surcose, EDTA, NaHCO2, Na-acetate, glycerol, DPX mounting fluid, KH2PO4, NaH2PO4, MgSO4, B-mercaptoethanol, Na n-lauryl sarcosinate, citric acid and HC1.

Sigma, St Louis, United States of America supplied: SDS, cyanol, bromophenol blue and trypan blue.

The agarose, Sephadex G50, Dextran T10 was supplied by Pharmacia, Fine Chemicals, Uppsala, Sweden.

The black and white negative film, developer and autoradiograph film were obtained from Ilford, Cheshire, England.

The developer, fixer, hardener as well as a different

autoradiograph film were supplied from May Baker, Port Elizabeth, South Africa.

APPENDIX C

One- and three-letter codes used for the amino acids.

Amino acid	Codes	
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

APPENDIX D

The Hind III restriction fragment sizes in base pairs of the bacteriophage Lambda (Oliver and Ward, 1985)

A	23 130
B	9 416
C	6 682
D	4 361
E	2 322
F	2 027
G	564
H	125

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