INTRASPECIES TYPING OF ORTHOPOXVIRUSES

by

MADALENE RICHARDSON

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He that dwelleth in the secret place of the Most High,

shall abide under the shadow of the Almighty.

I will say of the Lord, He is my refuge and my fortress,

my God, in Him will I trust.

Psalm 91: 1, 2.

ABSTRACT

Characterization of viruses within the Orthopox genus has, in the past, been done by evaluating a spectrum of biological properties which gave different reactions in the different species. More recently analysis of genomic DNA by restriction endonucleases has proved a valuable additional way of identifying species and revealing intra-species variations among the orthopox viruses. This thesis reports the results of investigations of poxvirus isolates in which both biological characterization and DNA analysis have been used.

Specimens of scabs from sick buffaloes in 18 outbreaks of buffalopox in India were extracted and virus was isolated from 13 of them by inoculation on chick embryo chorioallantoic membranes. The lesions resembled those of vaccinia and the isolates were confirmed as orthopox viruses by their susceptibility to neutralization by anti-vaccinial serum. Purified preparations of each isolate were made and the virion DNA extracted from them, was analysed by digestion with endonuclease Hind III, followed by electrophoresis to separate the resulting DNA fragments. The profiles of fragment sizes in the Hind III digest were indistinguishable from those of vaccinia isolates, but differed from other representative orthopoxviruses. This was the first examination of any DNA from the buffalopox virus.

Six isolates were characterised in more detail. In biological tests, one of the six showed the ceiling temperature, rabbit virulence, cytopathic effect in cell monolayers and sensitivity to anti-vaccinial serum which is characteristic of vaccinia, and

is regarded as an isolate of vaccinia. The other five had a lower ceiling temperature, were avirulent when inoculated in rabbits and were 10 - 50 fold less sensitive than vaccinia to neutralization with anti-vaccinia serum. In these properties they differed from vaccinia and resembled the buffalopox virus described by Baxby and Hill in 1971.

Examination of virion DNA, using other restriction endonucleases showed that all five isolates could be grouped together and distinguished from a group of five vaccinia strains in digests with the endonuclease Sac I, Xho I and Eco RI. The sixth isolate which was biologically like vaccinia was also in the vaccinia group in these DNA analyses. Digestion of DNA obtained from all 12 of the non-vaccinia buffalo isolates showed the same profile and this profile differed from that of any of the vaccinia strains and also from the buffalopox isolate made 15 years previously in North India and described by Baxby and Hill.

In another series of experiments the viral DNA from a number of isolates of monkeypox was analysed. One of these isolates was the first to be made from a wild caught animal (a squirrel) and the question was whether this isolate could be identified with isolates made from humans who had become infected in the same area of Zaire. All the isolates had previoulsy been characterized biologically as being monkeypox virus. DNA was prepared from the squirrel isolate and from two human isolates from about the same time and district. No difference could be detected in the profiles of fragment sizes generated by digestion with the endonucleases Hind III or Pst I. To substantiate this

apparent identity, DNA was prepared from another 9 monkeypox isolates from human infections with monkeypox in Liberia, Sierra Leone, Nigeria and Zaire as well as from five outbreaks in captive monkeys in Europe or America. The isolates covered the period from 1958 to 1986. The fragment size profiles established by electrophoresis of Pst I digests of all these DNA preparations were compared and found to fall into one of three distinct patterns. All the animal isolates from outside Africa had the same pattern as the isolates from Liberia and Sierra Leone. A second pattern was given by the two isolates from Nigeria and all the seven isolates from Zaire showed a third pattern. These observations supported the idea that the squirrel had been infected with the same variant of monkeypox virus that was causing the sporadic human infections in that country. The significance of the difference between the three patterns was investigated by constructing maps showing the location of Pst I cleavage sites on the genome of one isolate from each of the three main areas. A map had been published for Hind III cleavage sites on the genome of the Denmark strain of monkeypox virus. DNA from the Denmark strain was digested with Hind III, separated by electrophoresis in agarose gels and the fragments transferred by blotting onto nylon membranes. Fragments of DNA excised from gels after electrophoresis of Pst I digests were labelled by nick translation with ^{32}P-nucleotide and hybridized to the Hind III blots in order to determine the part of the genome from which each fragment came. By repetitions of this technique it was possible to construct Pst I maps of the genomes of representative strains of each of the three geographical variants of the virus. The sites which were different in the various isolates were found to be located in the centre and

towards the left hand end of the genome maps.

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CHAPTER 1

TYPING OF ORTHOPOXVIRUSES

Viruses are divided into 3 major classes, namely animal viruses, plant viruses and bacterial viruses. They consist of either a DNA or RNA genome, which is surrounded by a protective shell, and can also be enclosed within an envelope that contains both protein and lipid (Joklik 1980).

Animal viruses are classified with regard to 4 terms, namely 1: the type of the nucleic acid (RNA or DNA) and the strandedness (single or double stranded) of it, 2: the molecular weight of the nucleic acid or the percentage of nucleic acid of the virion, 3: the outline of the virion/nucleocapsid and 4: the kinds of host infected or the kinds of vector (Fenner et al. 1974).

Host range cannot be a criterion for the classification of animal viruses because each animal species is subject to infection by a wide variety of viral agents and a virus species can infect several animal species. The pathogenic pattern of the disease caused by the virus can also be varied. The system of classification is thus based on the physical and chemical properties of the virus particles themselves. Morphologic similarity corresponds closely to similarity in virion components (Joklik 1980).

The genus Orthopoxvirus belongs to the family Poxvirdae.

Poxviruses are brick-shaped or oval , complex particles (with the exception of Parapoxviruses), with a whorled surface filament pattern. The family contains a double stranded linear DNA genome that is cross-linked covalently at or within 50 base pairs of its end. It has the largest genome amongst the animal viruses (Joklik 1980).

A feature that seperates Poxviridae from most other DNA viruses is the fact that they multiply in the cytoplasm of infected cells (Joklik 1980). The only other DNA viruses (apart from poxviruses) which replicate in the cytoplasm are the iridoviruses. The poxvirus genome codes for a number of enzymes that are used in the virus's own replication.

The genera (subgroups) of animal Poxviridae are the Orthopoxvirus, Leporipoxvirus, Avipoxvirus, Capripoxvirus, Suipoxvirus, Parapoxvirus and some ungrouped viruses. See table 1 for the classification of poxvirus. The Orthopox genus (or Variola-Vaccinia Subgroup) contains variola virus, vaccinia, cowpox, ectromelia (mousepox), monkeypox, taterapox, raccoonpox and horsepox (Kaminjolo et al. 1974).

Members of the subgroups have been shown, by complement fixation or fluorescent antibody, to share a common group "NP" antigen. The "NP" antigen was prepared by alkaline extraction of dried virus particles and contains all its DNA. It is suggested that this antigen is associated with the internal protein which has been shown to be associated with the viral DNA (Woodroofe & Fenner 1962).

Table 1: The classification of the Poxviruses (Moss 1985).

Family: POXVIRIDAE

Subfamilies	Genera	Members
Chordopoxviridae (poxviruses of vertebrates)	Orthopoxvirus	camelpox, ectromelia, monkeypox, vaccinia, variola
	Parapoxvirus	bovine pustular stomatitis chamois contagious ecthyma milkers node, orf
	Avipoxvirus	canarypox, fowlpox, juncopox, pigeonpox, quailpox, sparrowpox
	Capripoxvirus	goatpox, lumpy skin disease, sheeppox
	Leporipox	myxoma, rabbit fibroma, squirrel fibroma
	Suipox	swinepox virus
	Unclassified	Molluscum contagiosum, tanapox, Yaba monkey tumour pox
Entomopoxviridae (insect poxviruses)	A	Melontha melolontha virus
	B	Amsacta moori virus
	C	Chironimus luridus

Several viral antigens are revealed by complement fixation, precipitation, virus neutralization and heamagglutinin-inhibition tests. Some of the viruses, though distinct in biological behaviour, are very close antigenically. Hybrids of 2 closely related poxviruses growing in the same cells, have characteristics derived from both. The activity of heat inactivated poxvirus will also be restored within cells in which another poxvirus is multiplying (Joklik et al. 1960). Members of the variola-vaccinia subgroup show relatedness in base sequence homology which corresponds well to immunological findings.

Infectious poxvirus particles contain a transcriptional system which can synthesize functional mRNA that is polyadenylated, capped and methylated. A large number of enzymes for performing these functions are packaged in the virus particle and they are coded for by the large genome (Moss 1985).

The genome of poxviruses consists of a single linear double stranded DNA molecule with covalent cross-links at the termini of the molecule. Identical sequences occur at the termini of the genome and these "inverted terminal repeats" have been described for various species within the orthopox group (Wittek et al. 1978; Mackett & Archard 1979). Repetitive elements, called tandem repeats, are present within the inverted terminal repeat. The function of these repetitive sequences is not known (Wittek & Moss 1980; Wittek 1982)

In this study two viruses within the Orthopoxvirus genus were more closely investigated. Biological and genomic

characteristics of the 2 species were studied and observations were made regarding the variations which exist within a species. These intraspecies variations were further investigated.

The first study (Section II) reports on the isolation and biotypical characterization of viruses from a number of specimens taken from buffalo, in India, which suffered from a pox disease. The characterization of the DNA extracted from these viruses is also reported.

In the second study (Section III) DNA studies were used to distinguish between a number of monkeypox strains. The biological characteristics of the viruses have previously been well documented, and the investigation of genomic variations presents a tool for intraspecies typing of these strains.

Chapters in Section I describe the methods which were used in these studies.

SECTION I

METHODS FOLLOWED

CHAPTER 2

INTRODUCTION TO VIRUS AND DNA CHARACTERIZATION OF ORTHOPOXVIRUSES

Within the Orthopoxvirus genus, the historically most important viruses are the ones causing smallpox (variola major, alastrim) and the viruses that have been studied most are the strains of vaccinia. Identification within the subgroup has been made on biological characteristics and more recently on genomic characterization.

2.1: BIOLOGICAL STUDIES

Assignment of a virus to the genus Orthopoxvirus can be made by the close serological relationship between all the members (Baxby 1975).

This distinguishes Orthopoxvirus from all the other poxvirus subgroups, but the antigenic relationship within the group does not form a sufficient basis to classify it into species. This was done on the basis of the spectrum of biological characters each member exhibits.

Biological tests that were used in classification included the pock character on Chick Chorioallantoic Membrane (CAM), the ceiling temperature, the lesions produced in rabbit skin, the cytopathic effect (CPE) in tissue culture (TC) systems and heamagglutinin (HA) production.

The biological properties of viruses within the orthopox group were reviewed by Baxby (1975).

All the orthopoxviruses will produce pocks on chick chorioallantoic membranes (CAM) in 48 - 72 hours and members of the orthopox group will grow in a large range of cell types. Smallpox produces small, white dome shaped pocks on CAM, which are very similar to pocks produced by monkeypox virus when eggs are incubated at 36 - 37°C. At 35 - 36°C, monkeypox virus produces small pocks with a definite haemorrhagic centre (Von Magnus et al. 1959; Gispen et al. 1967).

Differentiation between monkeypox and smallpox pocks on CAM can be made by their different ceiling temperatures. Monkeypox will produce pocks at 39°C, while smallpox virus fail to do so above 38,5°C (Bedson & Dumbell 1961).

Camelpox virus, which has a very limited host range, produces small opaque white pocks on CAM, which can be confused with those of smallpox and monkeypox viruses. Smallpox and camelpox can be differentiated by the CPE they form in some TC systems. It is easier to distinguish camelpox from variola major than from other varieties of smallpox that have been isolated.

Vaccinia strains were divided into more and less virulent forms, called neuro- and dermovaccinia respectively. Dermovaccinia produced opaque white pocks on the CAM, while neurovaccinia produced flat ulcerated pocks with a tendency to haemorrhage.

Differences in pock character for different species of vaccinia virus were described, as well as in their HA production, heat resistance, virulence in mice and rabbits and lesions produced on rabbit skin. Vaccinia virus grows in a wide range of mammalian cells and was the first virus to be cultivated in tissue culture. Vaccinia plaques in TC are often larger than those produced by other members of the subgroup. CPE is usually characterized by the production of "strand cells". Cowpox, buffalopox and monkeypox usually produce similar CPE.

Vaccinia strains also produce pocks above 40 - 41°C (Bedson & Dumbell 1961) and do not produce A-type inclusions. Differentiation between different vaccinia strains is made difficult by the variation in the biological characters of the different viruses within the species.

Some viruses isolated from outbreaks of pox in buffaloes have a pock character on CAM similar to that of vaccinia. A virus, named buffalopox virus, was found to be different from vaccinia (Baxby & Hill 1971). It had a lower ceiling temperature than vaccinia, closer to that of variola major, and could be differentiated by cross agar gel diffusion, immunoelectrophoresis, serum neutralization and complement fixation tests (Kataria & Singh 1970, Lal & Singh 1973). Mathew (1977a; 1977b) compared buffalopox, vaccinia and cowpox viruses with regard to plaque formation in TC systems with a description of the macro- and microscopic changes. Chandra et al. (1984) studied the CPE of buffalopox in 2 further TC systems. Physico-chemical studies have also shown that the virus differs

from vaccinia in the appearence of it's internal core and subsurface layers (Hill et al. 1972).

Cowpox was differentiated from vaccinia, using serological and histological techniques. The pocks produced by cowpox on the CAM are characterized by an intense haemorrhagic reaction. Vaccinia pocks, although sometimes haemorrhagic, will be larger than those produced by cowpox and cowpox will not produce pocks above 40°C. Cowpox forms lesions in rabbit skin which are large and indurated with a purple-black centre. Cowpox also induces large eosinophilic inclusions in the cytoplasm of infected cells (A-type inclusions), which are only produced by one other member of the subgroup, namely ectromelia virus. Biologically different strains of cowpox virus have been illustrated by comparison of biological properties of various strains. Cowpox virus also produces mutants without the haemorrhagic pock quality with a frequency of about 1/100. These mutants vary slightly in virulence and show the loss of a particular antigen.

Ectromelia (mousepox) virus produces small, slow growing white irregularly shaped pocks which do not grow above 39°C. Tissues infected with this virus show large eosinophilic inclusions, but not to the same extent for all strains. Variations in mouse pathogenicity and plaque character have been described for different strains. (Baxby 1975).

See table 2.1.1 and 2.1.2 for some of the biological properties of viruses of the Orthopox group of Poxviruses.

Table 2.1.1: Biological properties of smallpox-like Orthopoxviruses (Baxby 1975).

VIRUS	PROPERTIES			
	Pock character on CAM	Ceiling Temperature ^{o}C	Rabbit skin Lesion	HA production
Variola major	small, opaque white	38.5	transient nodule, no serial passage	high
variola minor (alastrim)	small, opaque white	37.5	transient nodule, no serial passage	low
monkeypox	small, opaque haemorrhagic	39.0	indurated lesion purple centre	high
camelpox	small, opaque white	38.5	transient nodule, no serial passage	high
ectromelia	irregular, small opaque white	39.0	transient nodule, no serial passage	

Table 2.1.2: Biological properties of vaccinia-like Orthopoxviruses (Baxby 1975).

VIRUS	PROPERTIES			
	Pock character on CAM	Ceiling Temperature ^{o}C	Rabbit skin Lesion	CPE in TC cells* (days)
vaccinia	pale, haemorrhagic ulcerated/large opaque white	41.0	indurated with purple centre, strain variations	1.5 - 2
cowpox	haemorrhagic	40.0	indurated with purple centre	2
buffalo-pox	pale, haemorrhagic ulcerated/large opaque white	38.5	erythematous red nodule	2- 3

* RK 13 and Vero cells

2.2: SEROLOGICAL TYPING

The members of the variola-vaccinia subgroup of poxviruses are closely related antigenically and their classification on a serological basis was attempted.

Baxby (1977) reported that heat-inactivated vaccinia does not induce neutralizing antibody to all orthopoxviruses and that heat-labile surface antigens were involved. Cross neutralization tests with antisera which had been separately absorbed with viruses of the orthopox group, gave evidence for the involvement of 4 neutralizing agents and their distribution between 13 virus strains was determined. A 5th antigen was also discovered for some viruses. No antigen was found to be specific to a virus, but they differed in their distribution. Viruses differing in their biological character, shared the same antigens however. Thus this technique does not present a clear ground for subdivision (Baxby 1982).

Differentiation could be made between variola, monkeypox and vaccinia infections, using absorption of sera with heterologous antigens (Gispen et al. 1976; Esposito et al. 1977).

Studies on the antigens of buffalopoxvirus and the serological reaction in infected buffaloes have been reported by Grover et al. (1980a; 1980b).

Walls et al. (1981) describe the differentiation of sera from patients who were naturally infected with human monkeypox and variola, and individuals who were immunized with vaccinia virus, with an adsorption radioimmunoassay (RIA). (A RIA for differentiation of orthopoxviruses was described by Hutchinson et al. in 1977.) The study confirmed that there is a common antigen

group on variola and vaccinia virus, not shared with monkeypox virus, and that vaccinia and monkeypox viruses have antigens in common that variola virus does not have. This method provides a way of identifying, serologically, the poxvirus responsible for infection long after the acute phase of illness.

2.3: FURTHER METHODS OF CHARACTERIZATION

Additional means for the differentiation and identification of orthopoxviruses have been investigated.

Poxvirus induces activity of enzymes concerned with nucleic acid metabolism. DNA polymerase and thymidine kinase are virus coded and differences in their properties from that in uninfected cells may provide useful markers for characterization of the virus concerned. Thymidine kinase preparations from several variola isolates were found to be sensitive to inhibition by thymidine triphosphate, while preparations from other orthopoxviruses (vaccinia, cowpox and monkeypox) were all resistant to inhibition. This could provide a valuable additional marker for variola and variola-related viruses (Bedson 1982).

Structural proteins from variola, monkeypox and vaccinia viruses were studied, using SDS-polyacrylamide gel electrophoreses. The viruses could be differentiated by their unique pattern in a certain molecular weight region (Esposito et al. 1977). Sera were made species specific by heterologous absorption with a glycoprotein and a phosphoprotein of similar size in each of the

virus species. Turner and Baxby (1979) studied the structural polypepties of orthopoxviruses, using the same technique and could divide the viruses in 5 groups. The differentiating polypeptides were located on the surface and subsurface layers of the virion. They reported minor differences in the polypeptides of monkeypox virus strains from human and monkey outbreaks.

Monoclonal antibodies were raised which reacted specifically with monkeypox virus (although it did not neutralize the virus) but not with cowpox, variola and vaccinia virus isolates. The antigenic determinant which was recognized by the monoclonal antibodies was located on a 15.5 kilodalton monkeypox virus polypeptide which was unaltered and present in all 8 of the monkeypox isolates that were used, but not in the other orthopoxviruses that formed part of the study (Roumillat et al. 1984)

Computer classification methods were introduced for comparing the genomic maps of othopoxviruses, drawing dendograms from qualitive binary information. This method is useful for summarizing the relationships of aligned nucleotide sequences (Gibbs & Fenner 1984).

2.4: GENOMIC ANALYSIS

Studies on the genome structure and make-up of poxviruses has provided additional methods in the typing, inter- and intraspecies, of the orthopox group.

The pattern of fragment sizes obtained by digests of genomic DNA with various restriction endonucleases could be used in distinguishing between different orthopox species as well as variations within these species. Gangemi and Sharp (1976) described the use of the restriction enzyme from Heamophilus influenzae (Hind III). This enzyme cleaved the DNA of the vaccinia virus into 14 fragments. Restriction also revealed a small size difference in the fragment size in one of the resulting fragments (fragment B) when 2 strains were compared.

Muller et al. (1977) could clearly differentiate between restriction patterns of viruses from the Orthopoxvirus genus and a virus belonging to the Avipoxvirus genus, which indicates a low degree of genetic relatednes.

Esposito et al. (1978) reported the use of Hind III, Sal I and Bam HI restriction enzymes for restriction analysis of 12 closely related orthopox viruses. Hind III cleavage profiles were used to group the viruses into 4 species, namely cowpox, vaccinia, monkeypox and variola. Isolates within species could be differentiated by comparing gel electrophoresis patterns produced by Sal I.

Mackett and Archard (1979) constructed maps showing the location of cleavage sites for Hind III and Xho I in the genus of 14 orthopox viruses. They found the distibution of Hind III sites

within the internal region of orthopoxvirus genomes highly conserved, however, this conservation was less marked with Xho I. Species specific differences resulted largely from extensive near terminal variation in length and sequences of the DNA.

Restriction endonuclease analysis of poxvirus genomes has proved to be the most precise and reliable method of differentiation. Physical maps, showing the order of restriction fragments allow a more detailed comparison of closely related viruses. Species specific differences in genome structure consist mainly of extensive near terminal variations in sequence and in length (with some of these sequences species unique). All orthopox DNAs show a high degree of sequence conservation in the central region of the genome. Heterogeneity in the lengths of the terminals of vaccinia virus (McCarron et al. 1978), as well as for temperature sensitive mutants of vaccinia virus, has been described. (Wittek 1982).

Esposito and Knight (1985) analysed restriction patterns and constructed restriction maps of 38 orthopoxviruses. Hind III maps correlated to a high degree, but variations occured in the middle and especially terminal cleavage sites, which provided a basis for discerning species, strains and variants. Two types of monkeypox virus DNA, each with 2 variants, were differentiated and the differences correlated with geographical origin of the isolates.

Pilaski et al. (1986) differentiated cowpox-like viruses that have been isolated from outbreaks of pox in zoo-kept animals between 1960 and 1984 by restriction analysis. 6 Elephant isolates, 1 rhinoceros isolate and 2 okapi isolates were investigated and could be differentiated.

The use of hetero-duplex analysis for the detection of differences in the genomes of orthopox viruses were described by Kinchington et al. (1984). Sequence divergence between corresponding fragments was indicated and small regions of heterogeneity between the genomes of variola and monkeypox viruses were located.

CHAPTER 3

METHODS USED IN VIRUS GROWTH AND DNA PREPARATION

3.1: OBTAINING VIRUS FROM SCAB SPECIMENS

Virus sometimes had to be recovered from scab specimens that were sent via the World Health Orginization from areas where outbreaks of poxdisease occured.

Scabs taken from infected animals were sent in 50% glycerol saline in cooled packs. 1 or 2 scabs were removed and put into an all glass Tenbroeck grinder together with 1 ml Mc Ilvains Buffer (4 mM, pH 7.4). The material was carefully ground until it was all fine and then the liquid containing the fine material was taken out and centrifuged for a few seconds (Eppendorf microfuge). The supernatant fluid (SNF) was taken off and used as an inoculum onto CAM as a 1/5 dilution in 10% PBS with double (2 ml per 100 ml) the normal antibiotics concentration (Appendix). 3 Eggs were inoculated per sample and the virus obtained after 3 days incubation was used as stock virus or cloned for a pure stock of the virus. If no pocks were obtained the procedure was repeated with the remaining scabs.

3.2: PURIFICATION OF POX VIRUS

Orthopoxvirus strains (vaccinia, buffalopox, monkeypox, camelpox and others) were grown and virus was purified following a basic

method.

Virus stocks were kept at -20°C in 50% Glycerol (final concentration). Virus stocks were prepared by vigorously shaking 3 confluently infected chorioallantoic membranes (CAM) of a specific virus strain in a Universal bottle, 20% filled with sterile glass beads (BDH, 5 mm) for 1 minute with 1 ml of 70% Glycerol in TE Buffer (10 mM Tris, 1 mM EDTA, pH 9) per membrane. The bottles were left on ice for 30 - 60 minutes and then centrifuged at 110 g for 6 minutes. The clarified supernatant fluid (SNF) was the virus stock to be stored.

For the production of a purified preparation of poxvirus, a dilution of the stock virus was made. The dilution must have a virus titre, measured in pockforming units per ml (pfu/ml), that would give a confluent spread of pocks on the CAM. This concentration was established by titration of the stock virus on CAM and using the appropriate dilution.

The virus dilution was inoculated onto the CAMs of 11 - 13 day old (optimally 12 day old) embryonated hens eggs. Each egg was candled to see that it was embryonated, alive and the right age. A pencil was used to mark the shell on the centre of the air sac and an area on the lateral side of the egg which is not directly neighbouring a bloodvessel. A spring-loaded punch (McCarthy & Dumbell 1961) was used to perforate the shell at these 2 points. A melted mixture of pertoleum jelly and parafin wax was spread over the opening of the lateral side of the egg (the egg was placed with this punch hole in a dorsal position). The tip of an

old-fashioned fountain pen was put through the opening of the air sac and when the wax mixture had set, the pen was used to scrape away the wax in the immediate area of the dorsal punch hole. A drop of saline was put on this area.

The pen was held in one hand and a rubber suction teat in the other. The nib of the pen was carefully put in through the dorsal punch hole and with a slight downward pressure pulled back so that a tear was made in the shell membrane, but not through the CAM. The saline flowed into the created air space and the space was increased by sucking air from the air-sac with the suction bulb. The eggs were candled to check that the CAMs had dropped correctly and the eggs were left with the dropped CAMs facing upwards at 36^{o}C for 2 hours after which inoculation took place.

A dilution of virus from the 50% glycerol stock was made in 1\10 dilution of Phosphate Buffered Saline (PBS) to which antibiotics (Appendix) were added at 1 ml per 100 ml of diluted PBS. The virus titre in pfu/ml was chosen according to the purpose of virus growth, either to obtain confluent growth or to have pocks that were well separated to allow for counting. Of this viral suspension 0.1 ml was inoculated onto each CAM through the dorsal punch hole, using a 1 ml syringe and 26G needle. After inoculation, the punch hole was sealed with the wax mix and each inoculum was carefully spread on the membrane by moving the egg with a rotational movement. The eggs were then incubated at 36^{o}C for 3 days.

The membranes were harvested by cutting open each egg along the lateral middle line, emptying the contents into disinfectant and preserving the upper half of the shell to which the infected membrane was attached. The shell and the CAM were trimmed to the borders of the infected area and this part of the membrane was removed from the shell with a pair of forceps and washed in saline by swirling. Pocks could best be observed if the membrane was spread in a petri dish backed by a dark background. Pocks were evaluated and counted or the membranes were processed further as in the case of virus purification.

About 6 of the confluent membranes were put in a Universal bottle with sterile glass beads (as described before) and 1.5 ml of Mc Ilvains Buffer (4 mM, pH 7.4) (Appendix) was added per membrane. During the course of procedures the solutions used were cold and the membranes were kept at $4^{o}C$ to inhibit lytic action.

The bottles containing the harvested membranes were balanced for centrifugation, firmly closed, wrapped in paper toweling (to preserve low temperature and make leaks detectable) and vigorously shaken for 1 minute. They were then centrifuged at 110 g for 6 minutes and the SNF taken off and kept. A further volume of Mc Ilvains Buffer was added to the membranes and they were shaken and centrifuged as before. The SNFs were pooled and left to stand on ice for 45 - 60 minutes, after which it was centrifuged at 1200 g for 15 minutes.

The clarified SNFs were spun at 15000 g for 60 minutes with a

small cushion of 36% (1M) Sucrose in each tube. After spinning, the SNF was discarded and the yellowish virus pellet carefully resuspended in a small volume of the TE Buffer pH 9. The virus suspension was layered on 10% Dextran T10 (Pharmacia Fine Chemicals) over a 36% (1 M) sucrose volume which was half of the volume of the Dextran solution; usually 10 ml and 5 ml. The virus was pelleted through this gradient at 15000 g for 60 minutes and again resuspended in a small volume of TE Buffer (pH 9). The colour of the virus pellet should be milky white. This final purification step was repeated if the pellet was very yellow. It was found that a further purification step through a sucrose density gradient (20 - 60%) was not necessary. The resuspended virus was stored at $4^{o}C$ for up to a few weeks.

Low speed centrifugation steps were done in a Sigma 301 K Refrigerated Centrifuge, using sealed buckets into which the Universal bottles fitted. The high speed centrifugations were done an a IEC B20-A Centrifuge, using a swing-out rotor head.

3.3: PREPARING VIRUS STOCK FROM A SINGLE POCK

When the characteristics of a specific virus were investigated it was necessary to ensure that the virus used as stock virus originated from a single virus of the kind and that the virus stock did not contain a mixture of viruses.

A 10^{2} dilution of the original viral stock was made in 10% PBS, filtered through a 0.65 um filter (Millipore) and immediately

diluted further and inoculated onto prepared CAMs. 0.1 ml of the filtered virus was inoculated onto the membrane and 3 eggs were used per virus. The eggs were incubated at 36°C for 3 days after which the membranes were harvested. Harvesting was done from above the membrane after the shell was carefully broken away with a pair of forceps. A single pock was chosen and lifted up with a pair of sterile forceps and cut free. This single pock was put in a Universal bottle with glass beads and 1 ml of 4 mM Mc Ilvains Buffer (pH 7.4) was added. The bottle was shaken vigorously for 1 minute and then centrifuged at 110 g for 6 minutes. The SNF was removed and inoculated neat or at a low dilution (1/10) onto three CAMs (0.1 ml per egg). The eggs were incubated for 3 days at 36°C after which virus was harvested to become the new virus stock.

By filtering the virus suspension, theoretically only single virus particles were let through the filter and the pocks produced on the membrane originated from a single virus particle.

3.4: EXTRACTION OF DNA FROM PURIFIED POX VIRUS

Viral DNA was extracted from purified viral suspensions of a specific viral strain that was prepared from about 12 confluent CAMs.

The volume of the virus suspension in TE Buffer was measured and an equal volume of Lysis Buffer was added (2% Na n-lauryl sarcosinate or 2% SDS; 54% Sucrose; 200 mM B-mercapto ethanol in

100 mM Tris pH 7.8). (The Na n-lauryl sarcosinate has the advantage that it can be stored at 4°C.) This was left until lysis of the virus particles was complete (suspension became clear); usually about 15 minutes at room temperature. Proteinase K (Boeringer Mannheim) (Appendix) to a concentration of 100 ug/ml was added and the mixture incubated at 56°C (waterbath) for 90 minutes. The protein debris was removed by adding an equal volume of phenol (see Appendix) and inverting the tube until the phases were well mixed. It was then spun; 3 minutes at 1600 g or 15 seconds in a microfuge (Eppendorf) until the 2 phases had separated. The phenol layer was pipetted off and discarded. The phenol extraction was repeated and phenol appeared clearer than after the first spin. Residual phenol was removed by two chloroform extractions with an equal volume of Chloroform:Isoamyl alcohol :: 24:1 (v/v). After mixing and spinning as in the phenol step, the aqueous phase contained the DNA. The chloroform removed remaining protein as well as traces of phenol.

The volume of the DNA containing aqueous phase was measured and brought to a concentration of 0.3 M Sodium-acetate by addition of 1 M stock and mixing. DNA was precipitated by adding 2.5 x the volume of cold absolute ethanol. Without inverting the tube, the viral genomic precipitate and was spooled out, using a thin glass rod. The DNA was shaken off into a volume of RNAse Buffer (0.01M Tris pH8; 0.1M NaCl) and allowed to dissolve completely. Ribonuclease A (Sigma) (Appendix) was added to a concentration of 25 ug/ml and incubated for 1 hour at 37°C.

The enzyme was removed with phenol and chloroform as described

before and DNA was precipitated with Na-acetate and absolute ethanol. Again the DNA was spooled out into either distilled water or a TE Buffer (10 mM Tris, 0.1mM EDTA; pH 8). The DNA was left to dissolve at 4°C and was used for restriction endonuclease analysis.

A method is described by Esposito et al. (1981) for the extraction of intact orthopox DNA from virus infected cell cultures without the necessity of purifying virion particles first.

CHAPTER 4

METHODS USED IN BIOLOGICAL COMPARISONS

4.1: POX LESIONS ON CHICK CHORIOALLANTOIC MEMBRANES (CAM)

The appearance of pocks formed by orthopox viruses on CAMs was investigted. Pock character of viruses of this subgroup have been studied extensively and is an important aspect in characterizing the virus.

The CAMs of 11 - 13 day old embryonated chicken eggs were dropped as described in Chapter 3. A virus dilution from the 50% Glycerol stock was made in 1/10 PBS to a dilution that produced a suitable number of easily distinguishable pocks on the membrane. Of this viral suspension, 0.1 ml was inoculated onto the CAM and the eggs were incubated at 36^{o}C for 3 days. 2 - 3 Membranes were used per virus.

The membranes were harvested (Chapter 3) and washed in saline that contained a small volume (1 - 5%) of 10% Formalin. This gave a certain degree of firmness to the membrane and caused it to lie flat on the surface of the petri dish in which it was spread. Pocks were observed, using a 10 x magnifying eyepiece with engraved measuring units, which allowed measuring of pock size in mm. The petri dish was turned upside down and the lens held to the bottom of the dish (membranes usually remained in position). The pock appearance was noted, the diameter of about 5

of the pocks was measured and the mean diameter calculated.

4.2: GROWTH ON CAM AT AN ELEVATED TEMPERATURE

Temperature affects the growth of individual poxviruses and the ceiling temperature (highest temperature at which pock formation on the CAM occurs) of a virus species is a characteristic property of such a virus and can be used to distinguish between viruses within the subgroup.

The efficiency of pock formation on CAM seems to be impaired as the ceiling temperature of a specific species is approached. Individual pocks become smaller and the yield of infective virus from pocks grown near the ceiling temperature was less. Above the ceiling temperature pock formation is prevented and multiplication of the virus does not occur (Bedson & Dumbell 1961).

No attempt was made to determine the ceiling temperatures of viruses that were investigated because of a lack of suitable equipment (temperature stable incubators, waterbaths). However, the effect on pock formation when infected CAMs were incubated at 40^{o}C, was determined.

The CAMs of 12 day old embryonated hen's eggs were dropped and the eggs were inoculated with dilutions of the different viruses at an infectious concentration that would produce 50 - 100

lesions per membrane. 2 Eggs per dilution were inoculated and incubated for 3 days at 40°C in a cabinet incubator (Hearson) of which the temperature was maintained by means of a water jacket. The temperature inside the incubator was monitored with a thermometer in water and although small variations (±0.5°C) occured during the 3 days, the mean temperature was maintained at 40°C. A group of control eggs was also inoculated with comparative dilutions and incubated for 3 days at 36°C. CAMs were harversted, washed and spread on petri dishes for the observation of pock appearance.

4.3: VIRUS GROWTH IN TISSUE CULTURE

Vaccinia virus was the first virus to be cultivated in tissue culture. It will grow in a wide variety of cells and readily form plaques in a continuous cell sheet. The first changes occur 1 - 4 hours after infection, when the infected cells lose their characteristic shape and begin to round off, thereby breaking the continuity of the cell sheet. Cytopathic effects include the formation of giant cells and basoph lic and eosinoph lic inclusions. Infected cells are killed, possibly by a virus specified protein (Bernkopf et al. 1959; Bablanian 1968)

The cytopathic effect of some orthopoxviruses on tissue culture cells was investigated. Observations were made with regard to plaque size and appearance. The tissue culture cells that were used to illustrate plaque formation of the poxviruses, were CV1 cells, a monkey kidney cell line, RK 13 cells, a rabbit kidney cell line and Human Embryo Fibroblasts, primary cells.

The cells were grown in tissue culture flasks or tubes (Falcon) on Eagles Minimum Essential Medium (Flow Laboratories) with 10% Foetal Calf Serum (FCS) (State Vaccine Institute, Pinelands),3 mM Bicarbonate and Antibiotics (Appendix) (1 ml stock per 500 ml medium) at 37°C and maintained in MEM + 4% FCS at 33°C.

Cell monolayers were infected as soon as they become confluent. A suitable dilution of the virus stock (50% Glycerol) was made in MEM (without serum). The medium was aspirated off the cell sheet and a volume of the virus dilution (just enough to cover the whole cell sheet) was inoculated onto the cells. The flask was gently rocked to spread the inoculum and the cells were incubated at 36°C for 1 hour, allowing for adsorbtion of the virus particles to the cells. The inoculum was removed and the cell sheet washed once with MEM, after which the cells were incubated at 36°C with MEM + 5% FCS until plaque formation was sufficient; 2 - 3 days.

The SNF was aspirated from the infected cell sheet and the cells were fixed with freshly filtered Strong Carbol Fuchcin by covering the cell sheet with the fixative and then washing it after a suitable interval (about 30 seconds) by flooding with water. Pouring off of fixative will leave a residue on the cell sheet. After rinsing off excess fixative, the TC flask was left to dry. The cell sheets stained a dark purple-red colour and the plaques (poxviral CPE) were visible as clear areas in the cell sheet. This method was also used for virus titrations (mostly in CV1 cells) as it provided an easily countable display of virus

infection, which was calculated in plaque forming units per ml (pfu/ml). The sizes of plaques were measured using a calibrated 10 x magnifying eyepiece, on the 3rd day of infection and the mean plaque diameter was calculated.

4.4: CYTOPLASMIC INCLUSION BODIES

When cells are infected with poxviruses, 2 types of cytoplasmic inclusions are formed which differ morphologically and tinctorially.

The B-type inclusions are granular and well defined. They stain Feulgen positive for DNA and usually contain virus particles (Malherbe & Strickland-Cholmley 1980). They correspond to the DNA factories (Cairns 1960).

The inclusions are often described as being eosinophylic (acidophylic) but also show other tinctorial properties. These properties are not absolute and depend on the method of fixation and staining agents used. The inclusions of variola and vaccinia viruses are sometimes called Guarnieri bodies (basophil hematoxylinophil).

Vaccinia virus grows in a wide range of cells with the production of cytoplasmic inclusions (basophylic in Vero cells). Monkeypox can be cultivated in a wide range of cell types and produces loculated basophylic cytoplasmic inclusions (Malherbe &

Strickland-Cholmley 1980). Cowpox and ectromelia viruses cause the formation of large eosinophylic inclusions (A-type) which are not produced by any other members of the subgroup (Baxby 1975). Some cowpox strains occlude virus within the A-type inclusion; in others the inclusions are empty. An "occlusion factor" has been recognized which determines whether the A-type inclusion will contain virus (Ichihashi & Dales 1973).

The ability of the buffalopox viruses to produce A-type cytoplasmic inclusion bodies when grown on CAMs, was investigated. Cowpox (Whipsnade strain) was used as a positive control.

A virus dilution that would give a spread of separate, well developed pocks on CAM (1×10^3 pfu/ml) was inoculated into eggs as described before and grown at $36^{\circ}C$ for 3 days. 2 - 3 Eggs per virus were used and the membranes were harvested in a way that caused little damage to the membrane itself and that did not cause much haemorrhage. This was done by supporting the egg, the infected membrane facing upwards. The shell above the membrane was carefully broken away and the infected membrane was lifted up and an area of it was cut loose and spread in a petri dish.

Rectangular pieces (0.75 x 1 cm) of firm paper (white card) were prepared and each membrane was stretched over one of these with well formed pocks spaced over the surface of the paper. The edges of the membrane were trimmed to the edges of the paper and both were submerged and left in Helly's Fixative (Appendix) for one hour.

The membranes were marked by naming them on the backs of the different pieces of paper.

After fixation, the pieces of paper were lifted out of the fixative and the membranes taken off. Each membrane was trimmed with straight edges to a trapesium shape, wrapped in gause material and enclosed in a plastic cassette. The cassettes were marked on their botom bases, washed overnight in running water and processed. Processing was routinely done by the Department of Histology (Medical School, UCT). The membranes were embedded into paraffin wax blocks, the base of the cassette serving as a base for the block. Each membrane was positioned in the wax block so that the cutting edge corresponded to the long side of the membrane. Slides were prepared from thin sections of the membrane and these were stained.

Helly's Fixative contains mercuric chloride which is a powerful protein precipitant that penetrates and hardens tissue fairly quickly. It fixes both nuclei and cytoplasm well and doesn't distort the tissues. In conjunction with other fixing agents, it produces a brown/black granular "mercury pigment" precipitate, which can be removed from the section by alcoholic iodine treatment.

Paraffin wax was removed from the slide by xylene treatment and then the slide were taken through absolute, 96% and 70% ethanol. The section was then placed in 0.5% Iodine in 70% ethanol (Gram's Iodine) for 3 minutes, rinsed in tap water and put into 5% Sodium Thiosulphate ("hypo") until the brown colour

disappeared (30 seconds). It was then washed in running tap water for 1 - 2 minutes after which staining proceeded.

Haematoxylin and Eosin (Appendix) were used to stain the slides. After bringing the slide to water, it was dipped into haematoxylin twice and then immediately rinsed in water. Slides were blued in Scott's tap water (Appendix) for 30 seconds and washed well in running water. Counterstaining was done in Eosin for 30 seconds and then slides were differentiated through 70%, 96% and absolute ethanol (2 changes of each) to xylene. After 2 changes in xylene, the slides were mounted with coverslips and Depex mounting fluid.

The time for staining in haematoxylin was considerably shorter than prescribed in the methods. Where staining with haematoxylin was allowed for 1 - 2 minutes as is standard, the cytoplasms of membrane cells stained so darkly, that observations were difficult.

The staining agents used, were as prepared in the Diagnostic Virology Laboratory (Medical School, UCT). Methods were followed, with slight variations, as suggested by Culling (1963) and Drury and Wallington (1967).

Slides were studied under an Olympus light microscope and magnifications of 10 x 10, 10 x 40, and 10 x 100 (oil immersion) were used; 10 x 40 being the most suitable.

4.5: NEUTRALIZATION OF VIRUSES

Poxviruses are antigenically highly related. Surface antigens as well as internal antigens are shared by the different species of othopoxviruses.

The ability of a serum raised againt vaccinia antigens to neutralize buffalopox virus was determined. Freeze-dried, pooled anti-vaccinia rabbit serum was obtained from Dr C. Rondle. The serum dilution giving a 50% reduction in plaque number was determined by inoculating aliquots of a suitable virus concentration with dilutions of the antiserum. After incubating the virus-serum mixture for 1 hour at 37^{o}C (as described by Boulter 1957), the mixture was inoculated onto CV1 monolayers (not CAM as described by Boulter). The virus inoculum titre was calculated to produce the near maximal countable plaque number in the controls.

CV1 cells were seeded into 24-well plates (Costar, Falcon, Nunc). 1 Plate was prepared for each virus investigated and the cells were infected when they had just reached confluency.

The freeze-dried anti-vaccinia serum was reconstituted in 1 ml of distilled water. Dilutions of serum were made in MEM (without FCS) to 2 x the dilutions that were eventually desired. A dilution of a virus (from 50% Glycerol stock) was made in MEM to 2 x the titre that would produce a suitable number of plaques when inoculated onto the cells.

A volume of the virus dilution made for each virus investigated was mixed with an equal volume of each serum dilution, which resulted in mixes with 1 x the serum and 1 x the virus dilution. The mixes were incubated at 36°C for 1 hour and used to inoculate onto the cells.

The SNF were aspirated from each well of the 24-well plate and 0.2 ml of the appropriate inoculum (virus and serum dilution after incubation) were inoculated per well. 4 Wells were used per dilution and 5 serum dilutions as well as a control of virus without serum were used as inocula. The inoculum was spread by gently rocking the plate and all plates were incubated at 36°C for an hour to allow for adsorbtion of the virus to the cells.

After incubation, the inoculum was aspirated off and replaced with 1 ml of MEM + 5% FCS per well. Plates were incubated (in 5% CO_2 atmosphere) at 36°C for 2 days. (Plaques were usually clearly visible and countable after 2 days.) The cell sheets were stained with Strong Carbol Fuchcin, washed and plaques were counted when the plates had dried. An agar overlay was not used as plaques could be counted before secondary plaques had appeared.

For each virus tested, it was estimated which serum dilution caused a reduction of 50% in the number of plaques per well. It was noted whether there was a difference between this value for the vaccinia strains tested and for the buffalo isolates that were used.

4.6: VIRUS INFECTIVITY IN RABBITS

The ability of buffalopox isolates to produce lesions in rabbit skin and the characters of the resulting lesions were compared to the lesions produced by equivalent doses of vaccinia virus.

A dilution of each virus containing 10^5 pfu was inoculated intradermally into the shaved skin of the back and flanks of albino rabbits. 0.1 ml was inoculated per dilution (using a 1 ml syringe and a 26G needle) and as many as 10 different viruses and/or dilutions could be put in the skin of one rabbit, marking the areas of inoculation.

The rabbits were observed daily and any lesion development was noted and the size of lesions was measured.

CHAPTER 5

METHODS USED IN DNA COMPARISONS

5.1: DIGESTION OF ORTHOPOX DNA BY RESTRICTION ENZYMES

The Orthopox DNA (Chapter 3), dissolved in a Tris-buffer or water and typically at a concentration of 0.5 - 1.5 mg/ml, was subjected to digestion with restriction enzymes.

Restriction enzymes produce fragmentation of the double stranded DNA. A specific enzyme would recognises a specific sequence of base pairs in the DNA chain and catalyses the breaking of the strands at or in the vicinity of that point. The resulting fragments can be separated by electrophoresis in an agarose gel.

A digestion reaction mixture with a volume of 10 ul consisted of 1 - 3 ul of DNA (depending on the concentration), 1 ul of a 10 x concentrated restriction enzyme buffer (which is recomended for use with a specific enzyme), 1 ul of restriction enzyme at 10 units per ul (diluted in 1 x buffer) and distilled water to make up the volume. The water was added to the DNA and the enzyme was added last. The reaction was set up in an Eppendorf tube and incubated in a waterbath at 37^{o}C for 3 hours. Most restriction reactions would be complete after 1 hour, but the time was increased to ensure complete digestion of the large Orthopox genome.

The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. The EDTA chelates divalent ions and was incorporated in a 10 x Stop Buffer (Appendix), which was used if the digested fragments were to be electrophoresed. 1 ul of 10 x Stop Buffer was added to the reaction mixture and the tube was spun in a microfuge for a few seconds to bring all condensed liquid to the bottom of the tube.

The restriction enzyme was removed if the generated restriction fragments were required for further reactions (cloning, nick translation). This was done by treatment with phenol and chloroform as described before. The volume of the digest was increased with distilled water to about 200 ul for a more comfortable working volume. After precipitation with absolute ethanol, the tube was left in dry ice ($-70^{o}C$) for 5 - 10 minutes or at $-20^{o}C$ for a few hours. The precipitated DNA was spun down at 12000 g for 5 minutes and the SNF carefully poured off. The DNA pellet was dried under vacuum in a Speed Vac Concentrator (Savant) and a volume of 70% ethanol was added to dissolve all remaining salt that precipitated with the DNA. The pellet was spun down again and dried as before, after which the DNA was dissolved in water.

The restriction endonucleases that were used as well as the 10 x restriction buffers were supplied by Amersham International. The enzymes Eco RI, Hind III, Pst I, Sac I and Xho I were used. See Table 5.1.1 and 5.1.2 for High- , Medium-, and Low-Salt Buffers (Maniatis et al. 1982) that can be used instead of commercially supplied buffers.

It was found that Eco RI produced star activity, which is a loss of sequence specificity in enzyme action, leading to a loss of the restriction pattern. This can occur because of conditions such as high endonuclease concentration, the substitution of magnesium with manganese, low ionic strength reaction conditions, a high pH value and the presence of organic solvents such as glycerol (New England Biolabs Catalog 1986/87; Oliver & Ward, 1985). Star activity was eliminated by shortening of the incubation time and using the High Salt restriction buffer. The number of enzyme units per digestion could also have been reduced.

Table 5.1.1: Restriction Enzymes and 10 x Buffers

Enzymes and 10 x Buffers as supplied by Amersham		Recognition sequence for 5'-> 3' strand	Alternate 10 x Buffer
Eco RI	E8	G"AATTC	High Salt
Hind III	E8	A"AGCTT	Medium Salt
Pst I	E5	CTGCA"G	Medium Salt
Sac I	E14	GAGCT"C	Low Salt
Xho I	E4	C"TCGAG	High Salt

Table 5.1.2: Make-up of Non-commercial Buffers (10 x) for Restriction Endonuclease Digestions

Buffer	NaCl	Tris-Cl pH 7.5	MgCl2	Dithiothreitol
Low	0	100 mM	100 mM	10 mM
Medium	500 mM	100 mM	100 mM	10 mM
High	1 M	500 mM	100 mM	10 mM

As suggested in Maniatis et al. (1982).

5.2: GEL ELECTROPHORESIS OF DNA

The restricted DNA fragments were separated according to size by electrophoresis in agarose gels. The fragments move under electrical force , but because double stranded DNA fragments are negatively charged under neutral pH conditions, electrophoretic mobility is determined by fragment sizes (almost independent of basepair composition or sequence) (Rickwood & Hames 1982). The resulting spectrum of fragments can be specific for the virus species from which the DNA was prepared and could be used to distinguish between different species and even between strains within a species. The fragments are named alphabetically according to size. The biggest fragment (which will migrate the slowest) is the A-fragment for that particular restriction digest. With decrease in fragment size, the fragments are named

with subsequent letters of the alphabet.

Electrophoresis was done in horizontal gel apparatus with gel dimensions of 12.5 cm x 22 cm and cathode and anode reservoirs that are in contact. The electrophoresis buffer was a TAE Buffer (0.04M Tris-acetate, 0.001M EDTA ; See Appendix for 50 x working solution) which was used fresh for every run. Agarose (Seravac, LSL) was weighed out (usually 0.8% w/v) and dissolved in TAE buffer using a microwave oven. It was allowed to cool to at least 56^{o}C before gels were poured. Glass or perspex slabs were used as bases for the gels and masking or insulation (electrical) tape was used to dam off the edges of the plate, forming walls of about 1 cm high, so that the melted agarose could be contained in it. Combs with tooth size and number of teeth that would suit the purpose of the gel and number of samples to be electrophoresed were supported about 2 cm inside and parallel to the one short edge of the plate. The teeth of the comb were about 1 mm from the surface of the plate so that wells formed when the warm agarose was poured onto the plate. The gels had a thickness of about 3 mm and were left to set at room temperature for 20 - 30 minutes.

When the agarose had set, the comb was taken out carefully and the tape removed. The gel, with the glass plate, was put into the electrophoresis tank and the tank filled with buffer until the gel was covered to a depth of 1 - 2mm. The samples were then loaded.

Samples, mixed with Stop Buffer (Appendix), were loaded into the

wells with a micro-pipette (Gilson); 10 - 15 ul per well. The presence of glycerol in the loading buffer makes the sample heavy and it replaces the TAE buffer in the well. After loading, the electrodes were connected to a power pack, with the cathode at the end where the samples were loaded and the anode side at the other end of the gel.

Where good resolution of fragments were desired, gels were run about 1.4 Volt/cm for 16 hours (overnight). Agarose gels were also used to check the presence, concentration or state of DNA. In these cases a minigel was run where the agarose was poured into the gel mould and carrier tray that accompanied a Minnie Submarine Agarose Unit (Hoefer Scientific Instruments, Model HE 33),using an appropriate comb and samples were electrophoresed at approxamitely 10 Volts/cm for about 1 hour. The dyes that were included in the Stop- or Loading buffer give an indication of the distance the fragments have moved in the gel.

DNA was stained with Ethidium Bromide which was added to a volume of the TAE buffer to a concentration of 1 ug/ml. The Ethidium Bromide was mixed well with the buffer. The gel, usually still on its base plate, was immersed in this and left to stain for 20 - 30 minutes. When minigels are run, Ethidium Bromide was incorporated into the melted agarose at about 2 drops of a 100 ug/ml solution per 50 ml agarose. This saved time, but was only done when making a checking gel since Ethidium Bromide can interfere with the migration of fragments as it forms a complex with DNA.

This complex has a fluorescence excitation spectrum and the fluoresence of DNA bound Ethidium Bromide is at least 10 x more than that of free Ethidium Bromide. The best fluoresence, with the lowest degree of photonicking, for this complex is at 300 - 302 nm, but lamps with this limit emit light at the red end of the visible spectrum, which had to be removed by a filter. (Rickwood & Hames 1982)

A Fotodyne Incorporated DNA transilluminator was used which contain 300 nm light bulbs, as well as an ultraviolet glass filter, which is used to transmit the radiation needed to excite fluoresence and absorb other radiation (254 shortwave and longwave UV) that is emitted from the light source. (Fotodyne Incorporated , Transilluminator Manual)

After staining, the gel was placed onto the transilluminator glass filter plate, the illuminator switched on and the DNA fragments in the gel observed. When the gel background was overstained, the contrast with the fluorescing DNA fragments was too small, and the gel needed to be destained. This was done by leaving the gel in TAE buffer for a period (about 1 hour) or further electrophoresing the gel if it was at the same time necessary to separate the fragments further.

At all times when Ethidium Bromide was present, handling was done wearing gloves. A protective UV-safe face screen was worn when using the transilluminator and care was taken not to scratch the surface of the glass filter on the apparatus and to keep it clean and dry.

5.3: PHOTOGRAPHY OF ELECTROPHORESIS GELS

Gels were photographed on polaroid and/or negative black and white film, when the fragments were stained and fluorescing.

The camera that was used, was a Mamiya RB 67 Pro-S which was fixed above the transillumionator on a permanent stand and which could be equiped with a Mamiya Polaroid hand pack film holder. A red filter was installed within the lense assembly and the photographs were taken onto Ilford FP4 120 mm, Medium Speed black and white film and/or Polaroid Type 667 Coaterless black and white Land film.

Polaroid film was exposed for 1 second and the black and white film for 30 - 45 seconds. Acutol developer, 0.2% Acetic Acid Stop Bath and Amfix Fixer with S-type Hardener (Maybaker) were used according to prescribed methods for the developing of negatives. A Paterson Developing tank was used to develop, stop fix and wash the film in and Photoflo (Kodak) was added to the wash. Films were hung to dry and negatives were stored in a Paterson Negative file.

5.4: DETERMINING OF CROSS-LINKED END FRAGMENTS

The covalently cross-linked end fragments of some monkeypox viruses was established. This was done by denaturing all the fragments that were generated by a restriction reaction. The end fragments of the genome easily renature, because of the

covalently closed "hair-pin" nature of it (De Filippes 1976), and it co-migrates with the corresponding fragments in a non-denatured digest. The internal fragments do not renature easily and do not electrophorese as well distinguishable bands.

Two equal volumes of a restriction digest were set up for the viruses of interest and the reactions were stopped by addition of EDTA to a concentration of 50 mM. Phenol and chloroform extractions were done and the DNA precipitated. One of each digest was redissolved in 4 volumes 95% formamide (Merck) in 50 mM Tris-Cl pH 7.5, heated to 65^{o}C on a heating block for 10 minutes to denature the DNA fragments and kept on ice. The other digests of each were redissolved in 10 ul distilled water and left on ice.

Both digests were mixed with 1 ul of Loading buffer (Appendix) and the denatured and normal digests were electrophoresed next to each other. The samples were electrophoresed at 1.4 Volts/cm for 16 hours and the DNA stained with Ethidium Bromide (Archard & Mackett 1979; Mackett 1981).

The fast renaturing cross linked end fragments migrated with the corresponding non-denatured fragments as a clearly distinguishable band. See figures 12.2.3 and 12.2.4.

5.5: SOUTHERN BLOTTING

DNA fragments were immobilized onto a nylon membrane by using a

procedure that was originally developed for use with nitro-cellulose membranes. (Southern 1975).

The fragments were denatured and single stranded fragments carried over onto the membrane. This is called a Southern blot and was used in hybridization reactions. The membrane that was used, Hybond-N Membrane (Amersham International), is a nylon membrane with a high binding capacity for DNA, RNA and protein.

DNA was digested with a specific restriction enzyme (digestion volume of 100 ul) and the fragments were separated through electrophoresis in a 0.8% agarose gel. The geltank apparatus used was specifically made to correspond to an apparatus that allows multiple hybridization reactions per blot, the Deca-probe apparatus. The gel had dimentions of 184 x 170 mm and a ten tooth comb was used. 10 ul of the digestion mix was loaded per well. Electrophoresis was done at 1.4 Volts/cm for 16 hours after which the DNA was stained with Ethidium Bromide and visualized by 300 nm transilluminated light. See Figure 5.5.1. The orientation of the gel was marked by cutting off one corner.

The DNA fragments were denatured by gently rocking the gel in a Denaturing Solution (1.5 M NaCl; 0.5 M NaOH). The gel was covered by the solution for 15 - 30 minutes and this was repeated twice. The denaturing solution was replaced by the Neutralizing Solution (1.5 M NaCl; 0.5 M Tris•HCl pH 7.2; 0.001 M Na_2EDTA) and the gel was gently rocked in this for 30 minutes. This was repeated twice and the single stranded DNA was ready to be

transfered to the Hybond-N membrane.

A large piece of plastic wrap was spread on a flat surface and a piece of 3MM blotting paper (Whatman), slightly bigger than the size of the gel, which had been well pre-wetted in 20 x SSC (Appendix), was centered on this. The gel was placed upside down onto the blotting paper and Hybond-N membrane which had been cut the same size as the gel was carefully placed onto the gel. Care was taken that no air bubbles were trapped under the membrane and the positions of the wells were marked on the membrane with a pencil. The membrane was also cut at the corresponding corner the gel was cut.

A piece of blotting paper, the same size as the gel was placed on top of the membrane and was followed by many layers of paper toweling cut to the size of the gel. The paper was weighed down by 2 - 3 bricks and a glass plate could be placed beneath the bricks to spread weight evenly. The borders of the plastic wrap were folded over to the edges of the bottom blotting paper to prevent liquid loss through evaporation. As the 20 x SSC moved through the gel to the blotting paper, the DNA which it carried along was trapped onto the nylon membrane. At all times gloves were worn. Method guidelines were taken from Amersham Membrane Transfer and Detection Methods (1985).

The wet paper towels were replaced with dry ones and the blotting was left to proceed overnight. When no more liquid was absorbed by the paper toweling, the layers were removed and the nylon

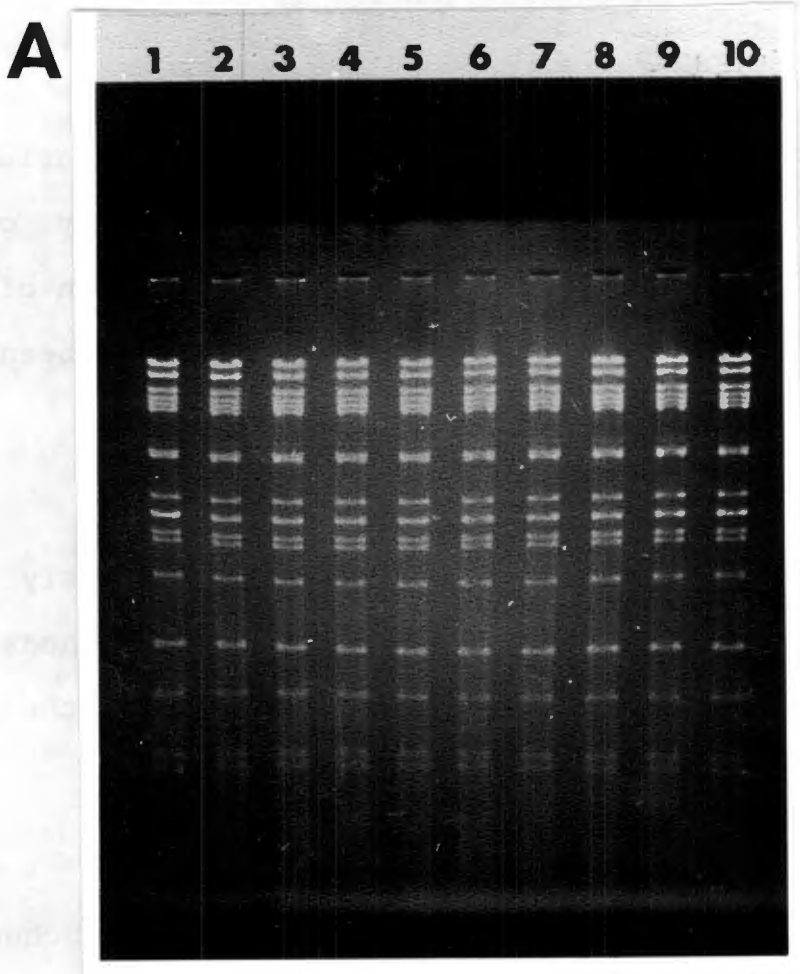
membrane (blot) rinsed in 2 x SSC to wash off pieces of adhering agarose. It was left to drip dry and placed, DNA side down, on a piece of plastic wrap onto the transilluminator and exposed to UV-light (300 nm) for 2 - 4 minutes. The blot was wrapped in plastic wrap to prevent it from drying out and was stored until needed. Blots prepared like this were sealed additionally in plastic filing envelopes, named, dated and kept at 4°C.

5.6: PREPARATION OF HYBRIDIZATION PROBES

The DNA that was used as probes for hybridization to the immobilized DNA fragments could be obtained by growing up plasmids into which specific DNA fragments have been cloned. It could also be recovered from fragments that have been separated in agarose gels.

Fragments recovered from agarose gels were mostly used and recovering could be done in several ways. Methods included electro-elution onto DEAE paper or recovering the DNA from excised gel strips that contained the fragment.

The prepared N45 DEAE Membranes (Schleicher & Schuell) were inserted into a slit in the agarose gel, just ahead of the desired fragment. The fragment was electrophoresed onto the paper, eluted off and precipitated. This procedure gave satisfactory recovery of small fragments, but not of large fragments (more than 3 kb).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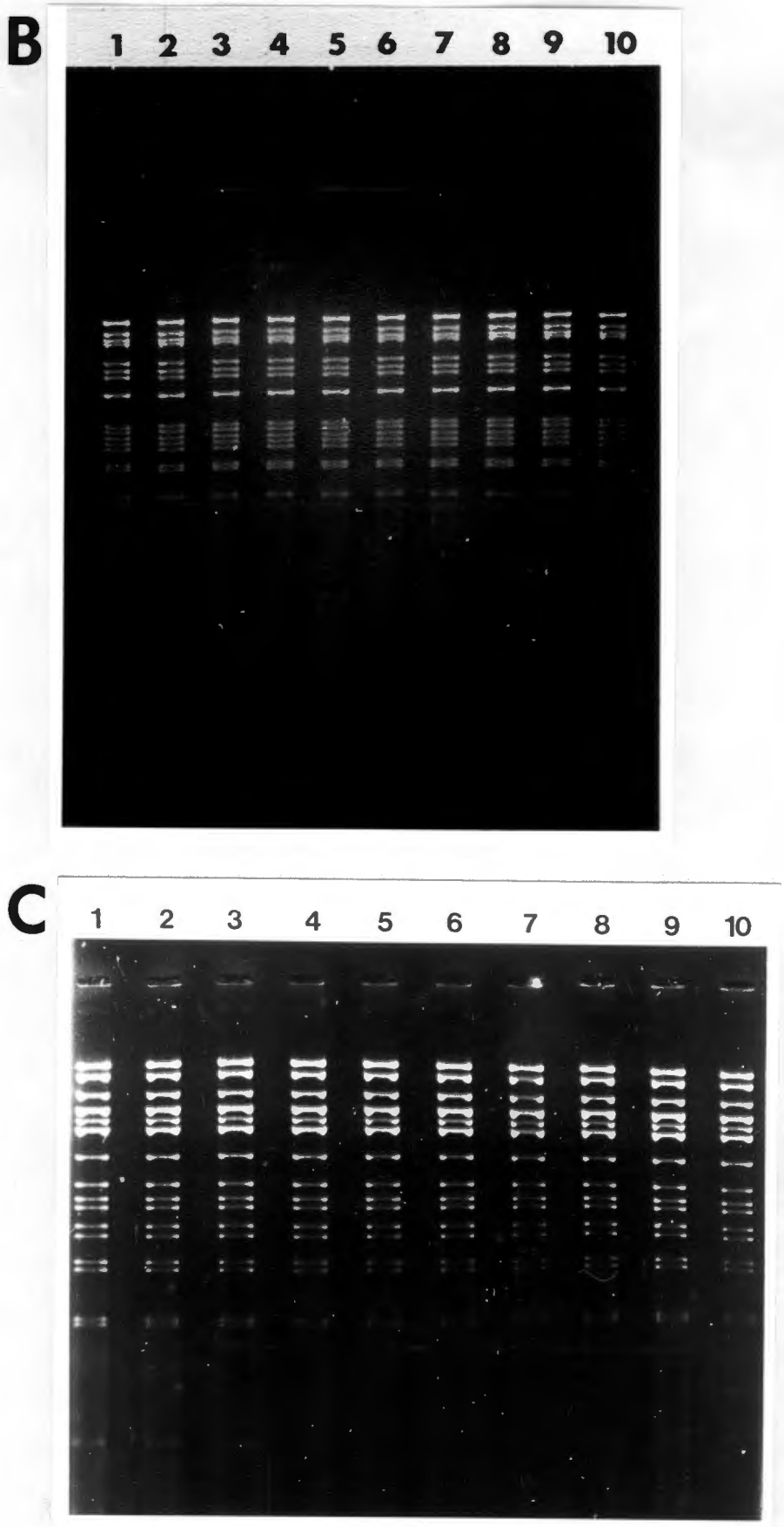


Fig 5.5.1: 0.8% Agarose gel (1.4 Volt/cm for 16 hours) of Monkeypox Denmark, restricted with Hind III (A), Pst I (B) and Sq 249 restricted with Pst I (C) for blotting. Gel tracks correspond to chambers in Deca-probe apparatus.

When using the Bio-trap Apparatus (Schleicher & Schuell) the fragment containing agarose was excised from the gel and chopped up finely. It was put in the apparatus with TAE buffer and electrophoresed under high voltage to trap the fragment in the space formed by the 2 membranes at the anode end of the apparatus. DNA moved through the first membrane, but the second membrane does not allow passage of DNA. The recovery of fragments in this way was good, but the process was too time consuming, considering the number of fragments that needed to be isolated.

Excised gel fragments were also chopped up, each put in an Eppendorf tube and an equal volume of phenol was added. The tube was vortexed, frozen and then centrifuged in a microfuge to separate the 2 phases. The aqueous phase contained the DNA, while the agarose remained in the phenol phase. The recovery was not satisfactory with this method.

The method that produced the best results was the Freeze-Squeeze method. This method was followed, with some alteration, as described by Rickwood and Hames (1982). Viral DNA was digested with a specific restriction enzyme with 10 x the concentration of DNA and digestion volume that would be used for 1 geltrack. A 0.8% agarose gel was prepared, using a comb that produces a wide (18 mm) slot. The sample was loaded and electrophoresed overnight or longer at approximately 1.4 Volt/cm to obtain maximum resolution of the fragments and separation over the maximum length of the gel.

It was sometimes necessary to electrophorese some fragments for longer periods to separate them sufficiently. A PC 750 Pulse Controller (Hoefer) was used to create an electrophertic field with alternating directions at set time intervals. This was done to separate fragments that migrated very close together, so that they could be exised from the gel without interfering with neighbouring fragments. The conditions chosen depended on the size of the fragments concerned. For larger fragments, the pulse intervals were increased and for small fragments it was decreased.

The bands were visualized and excised from the gel with a scalpel blade and individual fragments were identified in alphabetical order according to the distance they had migrated (which depended on the size of the fragment). The use of transilluminated UV-light was restricted as much as possible during excision of bands to prevent photo-nicking of DNA.

At first the excised gel strips were wrapped (similar to a sweet) in plastic wrap and the ends of the wrap were secured with insulation or masking tape, but later dialysis tubing (Spectrapor, 10 mm) was used.(See Appendix for preparation of dialysis tubing.) The gel strip was put inside the tubing with a pair of forceps and the ends of the tubing closed off by a matching plastic clip (Spectrum Medical Instruments.Inc.). The strips were put at -20^{o}C overnight or until frozen. All handling was done wearing gloves.

When a gel strip was frozen, the dialysis tubing containing it

was rolled between the fingers to break up the agarose and when it had thawed, the tube was punctured with a sharp object (scalpel blade). The buffer, which contained the DNA specific to that fragment oozed out of the small hole and was collected into an Eppendorf tube. As much of the fluid as possible was collected, but pieces of agarose avoided. Up to 300 ul was collected in this way.

The yield of various fragments was checked by running an aliquot (10 - 15 ul) of each in a minigel at 10 Volt/cm for 2 hours. See Figures 5.6.1 and 5.6.2.

Sometimes DNA was concentrated (phenol, chloroform, precipitation) in a small volume (30 ul) of water, which cleaned the fragments from TAE buffer and any agarose. Centrifuging (microfuge) during these steps was done at 12000 g for an increased time of 15 minutes at $4^{o}C$ for a better yield of recovered fragments. This procedure produced large enough quantities of specific DNA fragments of suitable quality for hybridization procedures. In most cases though, the fragments were used directly in nick translation procedures.

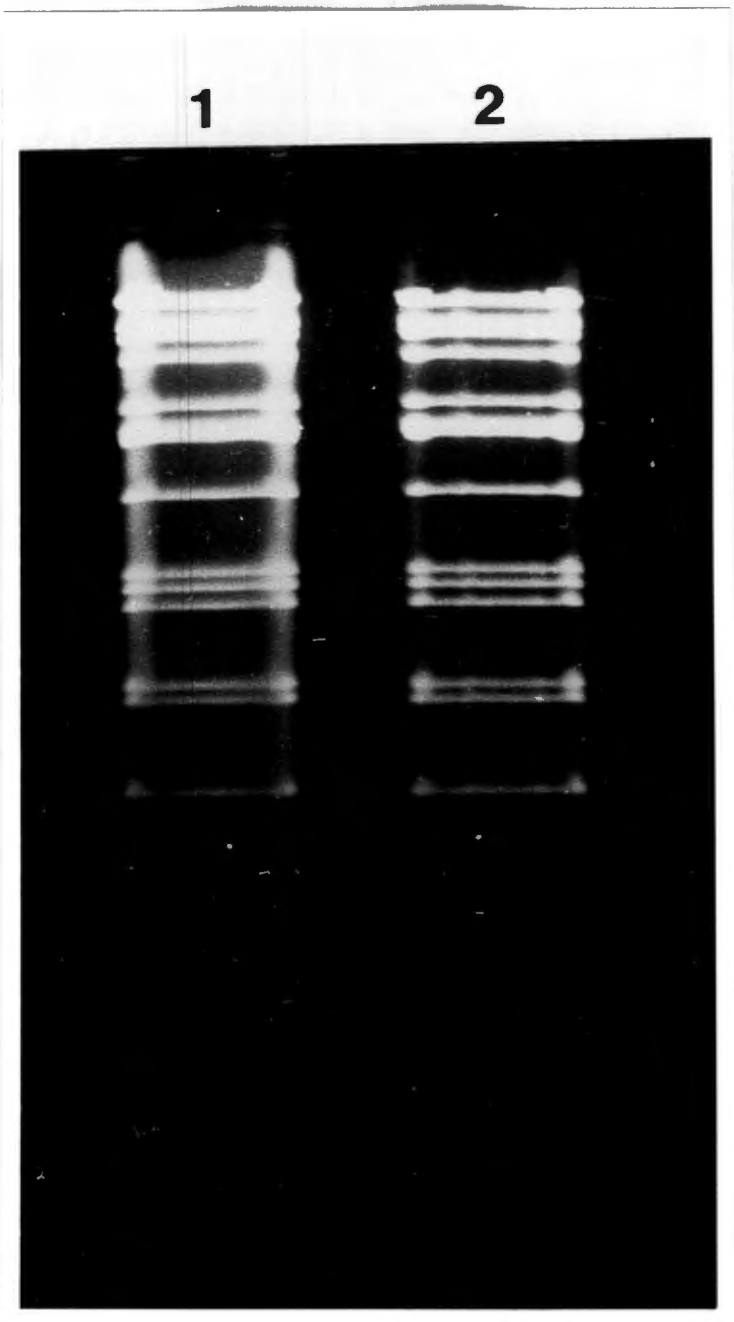


Fig 5.6.1: Mp Benin restricted with Pst I. 100 ul of digestion mix was divided and electrophoresed in 2 wide slots in 0.8% agarose gel at 1.4 Volts/cm for 20 hours.

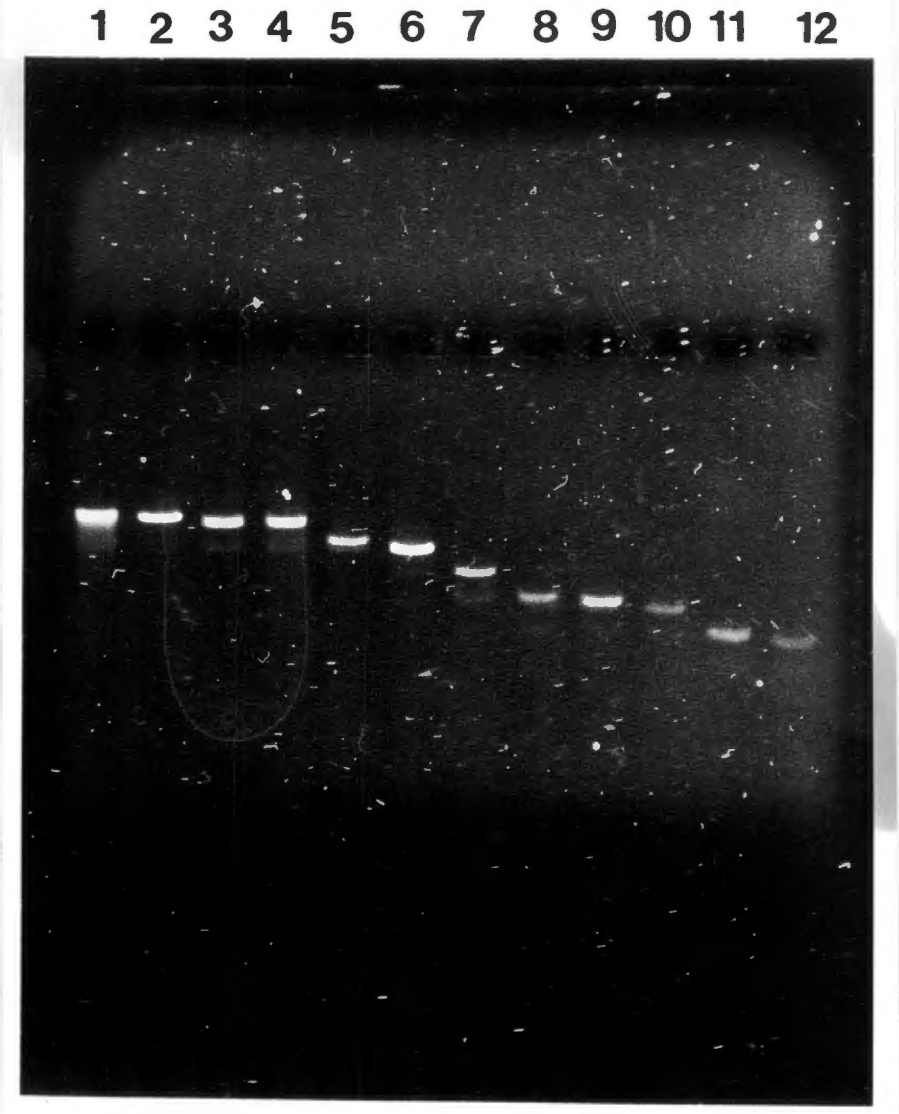


Fig 5.6.2: Fragments A - L of Mp Benin after Freeze-Squeeze elution from a preparative gel. 15 ul of each eluted volume of 100 - 300 ul was loaded.

5.7: NICK TRANSLATION AND HYBRIDIZATION

Radioactive nucleotide residues were incorporated into double stranded DNA by nick translation reactions.

One strand of the molecule was nicked by DNase I and DNA polymerase I (purified from Echerichia coli) catalyzed the addition of nucleotide residues to the 3'-hydroxyl terminus left by the nick and also the removal of nucleotide residues from the 5'-side of the nick. This resulted in the movement of the nick along the DNA and by providing the 4 nucleotides, of which one (usually dCTP) was radioactively labelled with ^{32}P, ^{32}P-labelled DNA was prepared. The reaction was incubated at 15°C, a temperature at which the maximum extent of the reaction was theoretically limited to one complete replacement of the existing nucleotide sequence. The radiolabelled double stranded DNA fragments were denatured and used as hybridization probes to the Southern blots that were prepared.

The blot was put in a plastic filing envelope and the bag sealed with a bag sealer (Tew Impulse Sealer). A small opening was left through which pre-hybridization mixture (about 30 ml) was pipetted into the bag. All air bubbles were squeezed from the bag and the opening was sealed. The blot with the pre-hybridization mix was left at 42°C in a shaking water bath for at least 2 hours.

The pre-hybridization mix was made up as follows (for 100 ml):

30 ml 20 x SSC and 70 ml distilled water into which 0,25 g of Blotto Powder (Johnson et al. 1984) was dissolved. Blotto Powder is dried milk powder (Carnation Low Fat) which was used instead of Denharts Solution and salmon sperm DNA (Maniatis et al. 1982) to prevent non-specific binding of probes.

The nick translation reagents were obtained from Amersham International in the form of Nick Translation Kits. The radioactive nucleotide that was mostly used was [a-^{32}P]dCTP, but [a-^{32}P]dATP was also used. The rest of the nucleotides (dATP, dTTP, dGTP) were obtained as a ready mix in the nucleotide buffer solution provided with the kit. Single nucleotides could be obtained and mixed to suit the reaction conditions (when using ^{32}P-ATP). Radionucleotides were obtained from Amersham International.

The nick translation reaction was done as prescribed in the Amersham Nick Translation Booklet, but half of the prescribed volumes were used.

10 ul of a specific eluted DNA fragment was mixed with 20 ul distilled water and 10 ul of the nucleotide buffer solution (Solution 1) in an Eppendorf tube. 5ul of [a-^{32}P]dCTP with a specific activity of 400 Ci/mmol was added. When radionucleotide with a specific activity of 3000 Ci/mmol was used, 3 ul and 2 ul of distilled water was added. 5ul of enzyme (Solution 2) was added and after mixing, the tube was spun briefly in a microfuge to bring all of the contents to the bottom of the tube. The reaction was incubated in a water bath at 15^{o}C for 90 minutes.

At all times radioactive reagents were handled with gloves and work was done behind a perspex screen that shielded the body.

During the reaction incubation time, a spin column was prepared for each probe. The plunger of a 1 ml Tuberculin syringe (B-D) was taken out and the rubber stopper at the end of the plunger was taken off and cut in half. The section of the stopper open on both sides was kept. Siliconized glass wool (Appendix) was put into the syringe and pushed down with the plunger to form a plug in the bottom of the syringe. The piece of stopper was pushed down onto the glass wool plug and the syringe was ready to be stacked.

Sephadex G50 (Pharmacia Fine Chemicals) sepharose which had been equilibrated (Appendix) in nick translation buffer (150 mM NaCl; 10 mM EDTA; 1.0% Sodium Dodecyl Sulphate/SDS; 5 mM Tris-HCl pH 7.5) was allowed to settle. It was sucked up with a pasteur pipette and put into the syringe, avoiding the formation of air bubbles. The syringe was completely filled and centrifuged at 1600 g for 4 minutes to pack the sephadex. The syringe was put into a tube before spinning to collect the nick translation buffer that is spun through. The packed column should fill about 3/4 of the syringe.

On completion of the nick translation reaction, 4 ul of Stop buffer was added to the reaction and the contents of the tube loaded onto the sephadex column, followed by 200 ul of Nick translation buffer. A decapped Ependorf tube was placed in the bottom of the tube containing the column, into which the effluent

was collected. The columns were centrifuged at 1600 g for 4 minutes. This procedure removed unincorporated nucleotides from the reaction mix. The effluent contained the double stranded DNA probe into which radioactively labelled dCTP (or dATP) had been incorporated. The probe was denatured for 5 minutes at 95°C and used for hybridization.

Hybridization reactions were done using the PR 200 Deca-probe Apparatus (Hoefer). This apparatus consists of 2 perspex slabs, one with 10 slots across the length of it. Southern blots were prepared from agarose gels with corresponding geltracks. A blot was placed between the 2 slabs and lined up with the slots. Rubber o-rings sealed off the tracks, creating hybridization units and making it possible to hybridize a specific probe only to one of the DNA tracks immobilized on the blot.

This apparatus allowed the use of up to 10 probes per blot. If it had not been available, the blot would have had to be cut up into strips and each strip would have had to be sealed with it's hybridization mix. For autoradiation the strips would have needed to be carefully lined up.

The blot was taken from the water bath and out of the filing envelope and set up on the deca-probe apparatus. It was necessary to trim the blot to allow proper sealing of the apparatus and also to make sure that the blot was quite wet.

The hybridization probes were loaded into the different hybridization chambers according to the way the specific experiment was planned and the chambers filled up with hybridization mix (for 100 ul: 50 ml of pre-hybridization mix and 50 ml of formamide). The openings into the hybridization chambers were closed with a piece of tape and the apparatus was moved to mix and spread the reagents in the chamber. Pre-hybridization mixture was put into the chambers that were not used. Careful note was made of which probe was loaded into which chamber and the sealed apparatus was put in a flat plastic container (Tupperware) with a lid and left shaking overnight in a waterbath at 42^{o}C.

The next day, the probes were pipetted out of the chambers and kept. When all the chambers were empty, the screws were loosened and the blot taken off. It was washed free of unbound or non-specifically bound probe. The first wash had a low stringency (2 x SSC; 0.1% SDS; and 0.25% Blotto powder) and the blot was put in a container, covered with the wash and gently shaken for 30 minutes. This was repeated twice and then the first wash was replaced by a second more stringent wash (0.1% SSC; 0.1% SDS). This was also repeated twice for 30 minutes. The washes were discarded into a radioactive discard bin.

The presence and quantity of radioactivity was monitored with a Geiger Counter (Mini Instruments). The difference in registered reading on the counter between the spin column after spinning through the probe and the cleaned probe itself, was an indication of how effective the incorporation of the radioactive nucleotides

were. The counter gave an indication of how strong the probe had been and it was also used to detect in which areas of a blot more radioactivety was present.

5.8: AUTORADIOGRAPHY

After completion of the washes, the blot was allowed to dry somewhat but it was kept damp. It was wrapped in plastic wrap and put into an autoradiographic cassette (Kodak; Curex) with a sheet of X-ray film (Kodak; Amersham; Cronex) which was placed between the blot and the intensifying screen of the cassette. The film was loaded in a dark room under a safelight and a corner of it was cut to orientate it with regard to the blot.

The cassette was wrapped in aluminium foil and put at -70°C. Depending on the strength of the probe, bands that hybridized might be visible on the X-ray film within an hour. The film was left on the blot long enough for bands to be clearly visible (few hours up to a few days). The film was removed from the cassette in a darkroom under a safelight and developed (DPX or Ilford Developer, 3 minutes; 2% Acetic Acid Stop Bath, 1 minute; Maybaker Amfix Fixer with S-type Hardener, 2 minutes; wash in running water, 15 minutes; hang to dry). Hybridization was indicated by the activation of silver particles on the X-ray film by the radioactive emission in the areas where probes have bound to the blot, which showed up as black lines.

5.9: RE-USE OF BLOTS

Blots could be used more than once if they were kept damp, since the DNA probes then binds reversably to the membrane. The blot was boiled for 30 - 60 minutes in a 0.1% SDS solution and was allowed to cool down to room temperature. An X-ray film was exposed to the blot to determine if all radioactivity was removed. The blot was ready for re-use if no bands appeared. Blots have been used up to 3 times, but the smaller fragments might not be present in sufficient quantities if used more often.

5.10: ESTIMATION OF FRAGMENT SIZES

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

The DNA from bacteriophage lambda cl 857 Sam 7 (Amersham International), restricted with Hind III, was used as a molecular marker. The sizes of the fragments generated were known and the distance the fragments had moved in the gel was plotted (x-axis) against the $\log_{10}$ of their molecular size in kb (y-axis) to form a standard curve. The distance the fragments of unknown size had moved in the same gel at the same time was measured and the sizes of these fragments were determined from the standard curve. For fragments larger than 20 kb estimations are difficult because of the very slow migration of these big fragments, and size

estimations cannot be regarded as absolute. When Orthopox viruses were restricted, fragments with molecular size of more than 20 kb were often generated and estimations of genome size and fragment size were considered to be approximations.

SECTION II

INTRASPECIES TYPING OF BUFFALOPOX VIRUS

CHAPTER 6

INTRODUCTION TO BUFFALOPOX

6.1: HISTORY OF THE DISEASE

References to pox disease which occured mostly in buffaloes in India, were made in the 1930 s (Wariyar 1937). Reliable laboratory methods for the identification of pox viruses were not yet available at that time and outbreaks of the disease were often said to be associated with smallpox epidemics and vaccination programmes.

Speculations were that the causative virus could have been vaccinia, since buffalo were used for the production of smallpox vaccines in India. A different virus could also have been responsible as the lesions produced by different viruses in one animal species can be similar. Three virus isolates from outbreaks of pox in buffaloes, investigated by Baxby and Hill, appeared to be strains of vaccinia virus, while a fourth isolate could be differentiated from the three by neutralization tests, plaque production in RK 13 cells and in ceiling temperature tests. Although this fourth isolate has the same ceiling temperature as variola, it differs from it in other characteristics. This virus was therefore given a separate identity and was called the Buffalopox Virus. (Baxby & Hill 1971)

Serological studies have shown that buffalopox virus is closely related to vaccinia and cowpox viruses, but not identical. It

should be placed in the vaccinia-variola subgroup of poxviruses and no serological relationship was established between buffalopox and sheeppox or goatpox viruses, although it shares a common group antigen with some of these viruses. (Lal and Singh, 1972; 1973; 1977).

Information about the manifestation and behaviour of the disease was collected during an extensive outbreak of pox disease in buffalo in the Dhule district of the Maharastra state, India, during December 1975 to April 1976. (Reported by Mathew et al. "1976").

Dhule has a very big cattle market where cattle from various areas is sold, and then dispenced throughout the state of Maharastra. An outbreak of disease in the Dhule district usually spreads immediately along the cattle traffic routes. This pattern was also established for the buffalopox outbreaks with it's origins in Raven (Dhule district) and in Dhule itself.

The spread of the disease was aided by the fact that the milking buffaloes of a village were brought together twice daily in a central milking place. Any necessary veterinary care was given on these occasions. Virus thus easily spread from one animal to another through the infected hands of the milkmen, handlers and through the equipment. Two or three neighbouring villages also share a common pond and outbreaks were reported to be affecting villages that make use of the same pond. Although buffalo were the main species infected, other farm species such as white

cattle, goats and even horses have shown pox lesions. Cases have also been reported in human beings, chiefly among milkers and attenders of the infected buffaloes.

Out of 29 outbreaks that were reported from various villages during this time, about 700 buffaloes and 120 white cattle were affected. In Raven village, about 400 buffaloes were affected with about 20 deaths in the buffalo calves The morbidity rate among buffaloes was about 70% with no deaths in adult animals.

This monitored outbreak terminated when all the cattle markets, including the main market at Dhule, were closed down. Mass media were used to communicate hygienic practices and a mass vaccination of humans, buffaloes and cattle with smallpox vaccine was undertaken. A "good take" of the vaccine was observed with no adverse reactions. The disease subsided in this area after the mass vaccination program.

6.2: CLINICAL PRESENTATION

The clinical presentation of the disease was summarized from accounts by Mathew et al. ("1976"), Lal & Singh (1977) and Mallick & Dwivedi (1982).

The disease shows the typical stages of pox disease. Papules appear on the teats and udder which are followed by a vesicular stage. The vesicles form into pustules which contain cheesy pus with centralized necrosis. Scab formation happens within 2 weeks

and the scab usually falls off within 5 - 7 days after formation, leaving a pink scar at the site. Pox lesions were mostly seen on the teats, udder, medial aspect of both thighs and (in a few cases) on the quater, lips and around the nostrils. Thickening of the teats and stenosis of the milk duct were observed, leading to mastitis. A generalized form of the disease was seen in a few young animals with lesions on the vulva and perineal region and on the prepuce of male buffalo calves. The disease could also spread to the eyes and many reports mention lesions on the inner and outer earflap, spreading down to the parotid region. Calves whose mother's teats were infected showed 1 or 2 typical vesicles on the tongue. These animals would protrude their tongues because of pain and the high mortality amongst such calves was attributed to starvation. Disease in adult animals leads to a decrease in milk production.

Complete recovery of the buffaloes was noticed within 20 - 30 days and in cows (white cattle), it was within 7 - 15 days. It was noted that the disease did not spread easily from buffalo to cattle or other species of animals. Lesions that were experimentally produced in cattle were milder, and in sheep and goats far milder, than lesions produced in buffaloes (Ramakrishnan & Ananthapadmanabhan 1957).

The infection in human beings occured mainly in the milkers who developed isolated pox lesions on various parts of their bodies, but infection did not spread from one person to another. The incubation time was 7 - 8 days and the lesions were about 2 cm in diameter. Typically, 6 - 20 lesions would be seen on fingers and

hands (including the palms), and sometimes lesions would appear on the face. The lesions were always localized and no generalized lesions were noticed in human beings. Pox scabs would take about 15 days to fall off and the infected person would complain of pain at the lesion site with a slight fever and a tingling and numbness in the hands (Mathew et al." 1976"). Baxby and Hill (1971) reported that one of them became infected, (local lesion on the thumb), while investigating buffalopox virus and that virus was isolated from the small papule that formed, which was identified as having the properties of buffalopox virus.

6.3: STATUS OF THE DISEASE

There are known endemic areas in India where buffalopox appears every year. Dhule, Jalgaon, Akola, Nasik and Pune often report outbreaks. During 1984 - 1985 the buffalopox incidence in Pune district was so severe that the pox nodules on the face and hands of the milkers could be mistaken for smallpox. These outbreaks would have previously been controlled by use of smallpox vaccine, but this is no longer available. (Extract from Status Report 1985)

Information of smallpox vaccination programes and the use of vaccine to contain the outbreaks of buffalopox is difficult to obtain. Correspondence with health personel in India tells that vaccination of the general population in the districts Pune, Jalgaon, Nasik, Dhule and the adjacent areas, was stopped on 3

April 1979, and that no vaccination has been performed thereafter. The vaccine which was used in that area was a smallpox vaccine produced in the USSR. (Correspondence to K R Dumbell 1986). This vaccine was obtained together with 2 other vaccine strains stated to be widely used in India (Correspondence 1986).

Buffalopox has been listed as an important zoonosis by a joint FAO/WHO Expert Committee on zoonosis (WHO Techincal Report Series 1967). It is of economical importance especially because of the occurence of mastitis with the disease. Attenuation of the virus has been attempted by serial passage in cell culture with the aim of developing a live attenuated vaccine strain (Dogra & Sharma 1981), but smallpox vaccine has also been used to induce protective immunity in buffaloes (Ramakrishnan & Ananthapadmanabhan 1957).

6.4: SOURCES OF STRAINS

Specimens from infected buffaloes in Maharastra, India, during December 1985 to February 1987 were received for investigation via The World Health Orginization (Geneva).

Specimens received were scabs from infected animals sent in 50% Glycerol saline. Virus was isolated from 13 of the 18 specimens and 2 more viruses were obtained from Dr Baxby, namely a buffalopox virus isolated in Pune during 1986 and the type culture of buffalopox originating from Hissar in 1967.

Table 6.4.1 gives a summary of the origin and extent of outbreaks from which virus was isolated. Vaccinia viruses used originated from the USSR, the King Institute, Madras and the Patwadangar Institute, Nanital, and were made available through WHO. Vaccinia strains Dairen (DIE), Wyeth and Lister, as well as other viruses used, were available in the collection of Prof K. R. Dumbell.

An investigation was made into the biological characteristics of the buffalopox viruses that were isolated and the genomic structure was also examined. Comparisons were made with other poxviruses and between different isolates of buffalopox virus.

Table 6.4.1: Buffalopox Virus Outbreaks from which virus was isolated.

ISOLATE		DATE	VILLAGE/DISTRICT	HUMAN INVOLVEMENT
0-BP	B1	1971	Hissar	?
V78/85	B2	Dec.1985	Ratnagiri	Yes
V79/85	B3	Dec.1985	Beed	?
V81/85	B4	Dec.1985	Ratnagiri	Yes
V31/86	B5	Feb.1986	Pune	Yes
V115/86	B6	May 1986	Solapur	Yes
3903	B7	Dec 1986	Dhulia/e	Yes
3904	B8	Dec 1986	Dhulia	Yes
3905	B9	1986/87	Dhulia	No
3906	B10	1986/87	Dhulia	?
3907	B11	1986/87	Dhulia	?
3908	B12	Dec 1986	Dhulia	?
2-BP	B13	1986	Pune	?
339	B14	Feb.1987	Dhulia	No
340	B15	Feb.1987	Dhulia	No

CHAPTER 7

RESULTS OF INVESTIGATION OF BIOLOGICAL CHARACTERISTICS

The biological characteristics of the viruses isolated from outbreaks of poxdisease in buffalo in India were investigated. Methods as described in Chapter 4 were used and the results are set out in this chapter.

7.1: POCK PRODUCTION ON CAMS

The pock characters on CAM of viruses from outbreaks of buffalopox and of some vaccinia strains were investigated.

The vaccinia strains as well as the buffalo isolates produced lesions on CAM. There was some variation in the appearance and the size of the lesions, but these cannot be regarded as significant. The heterogeneous character of pocks as described by Baxby and Hill (1971) and Seghal et al. (1976) were observed. Major differences in the pock characters of vaccinia strains and buffalo isolates were not greater in extent than those which occur between different vaccinia strains. The pocks were different in appearance and larger than the ones produced by monkeypox viruses and did not show the haemorrhagic quality of cowpox virus.

See fig 7.1.1 - 7.1.7 for pocks on CAM as produced by isolates from different areas within Maharastra state, India, and pocks of

the USSR vaccinia strain. Table 7.1.1 gives an outline of pock character and the measured diameter of the pocks produced on CAM by buffalo isolates and some vaccinia strains.

Fig 7.1.1: Pocks produced on CAM by buffalo isolate V78/85 after 3 days at 36°C.

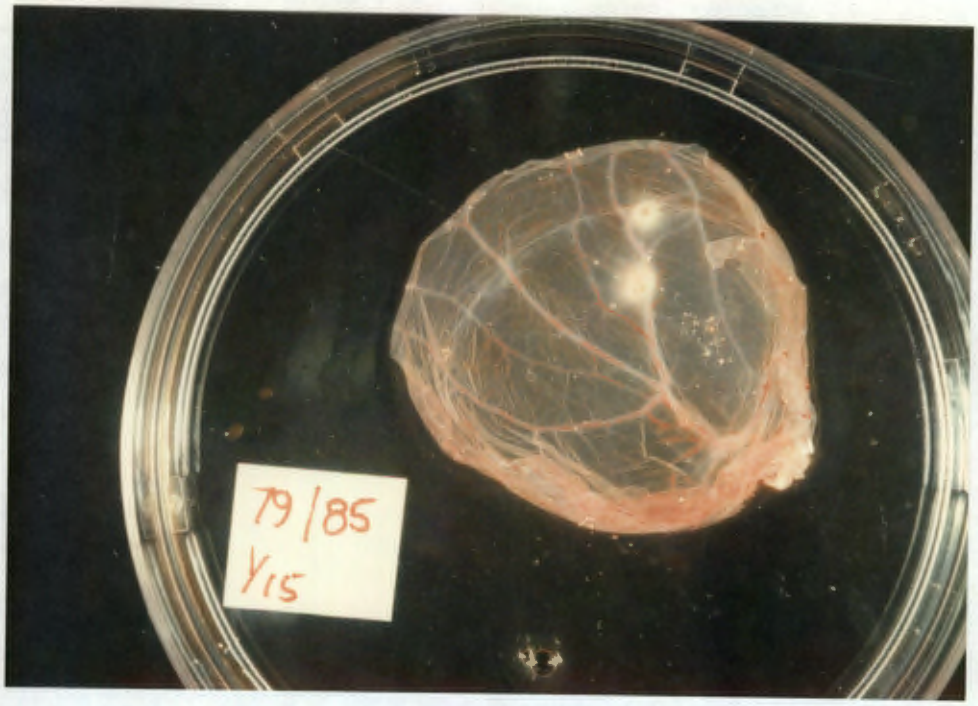


Fig 7.1.2: Pocks produced on CAM by buffalo isolate V79/85 after 3 days at 36°C.

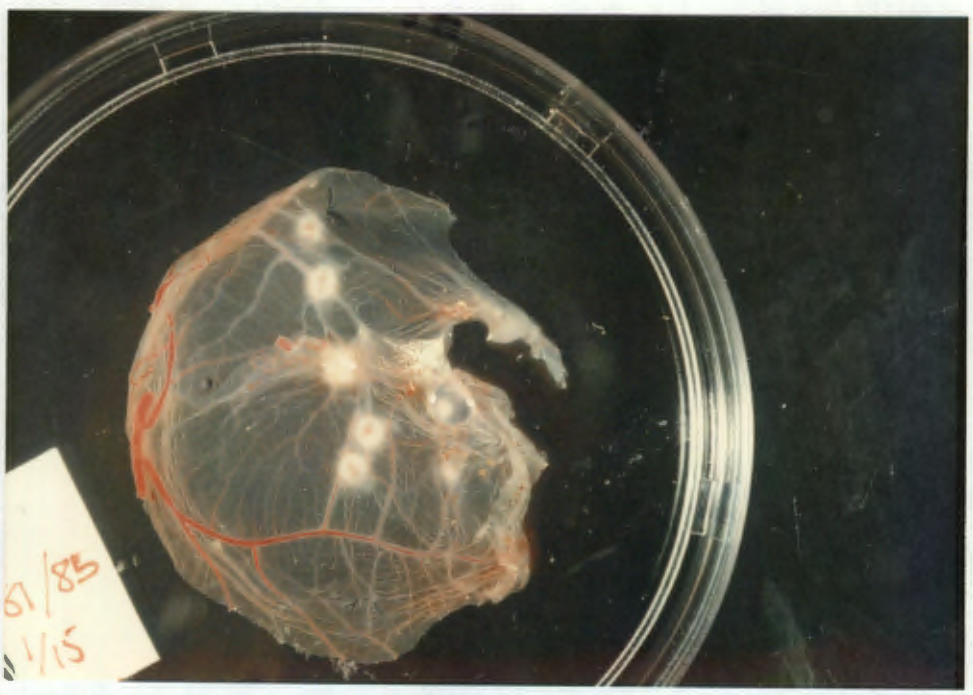


Fig 7.1.3: Pocks produced on CAM by buffalo isolate V81/85 after 3 days at 36oC.

Fig 7.1.4: Pocks produced on CAM by buffalo isolate V31/86 after 3 days at 36°C.

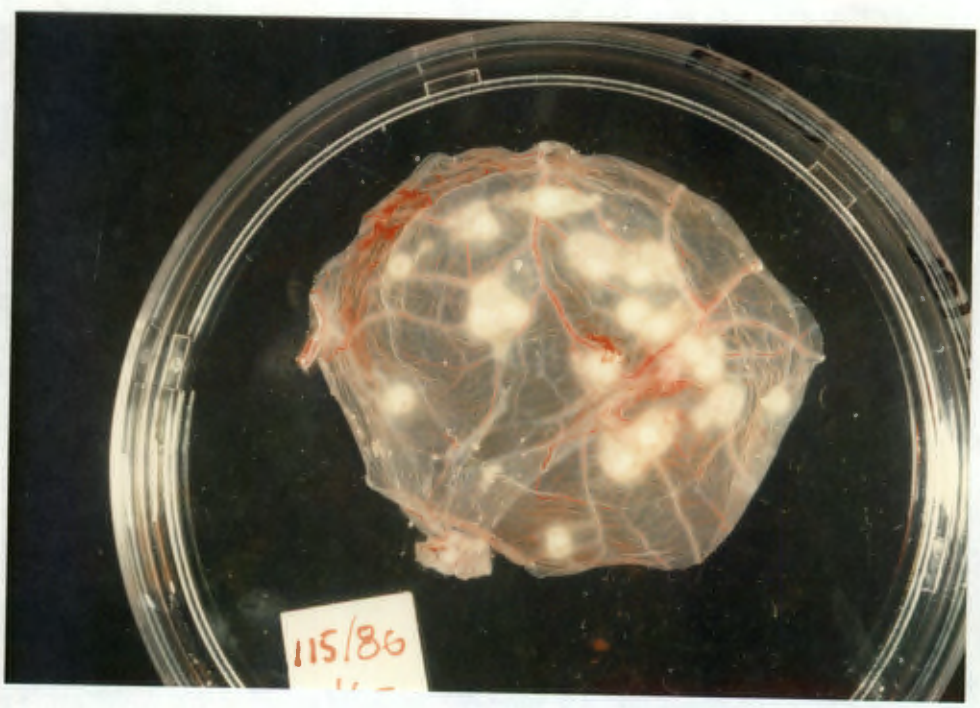


Fig 7.1.5: Pocks produced on CAM by buffalo isolate V115/86 after 3 days at 36°C.

Fig 7.1.6: Pocks produced by buffalo isolate 3903 after 3 days at 36°C.

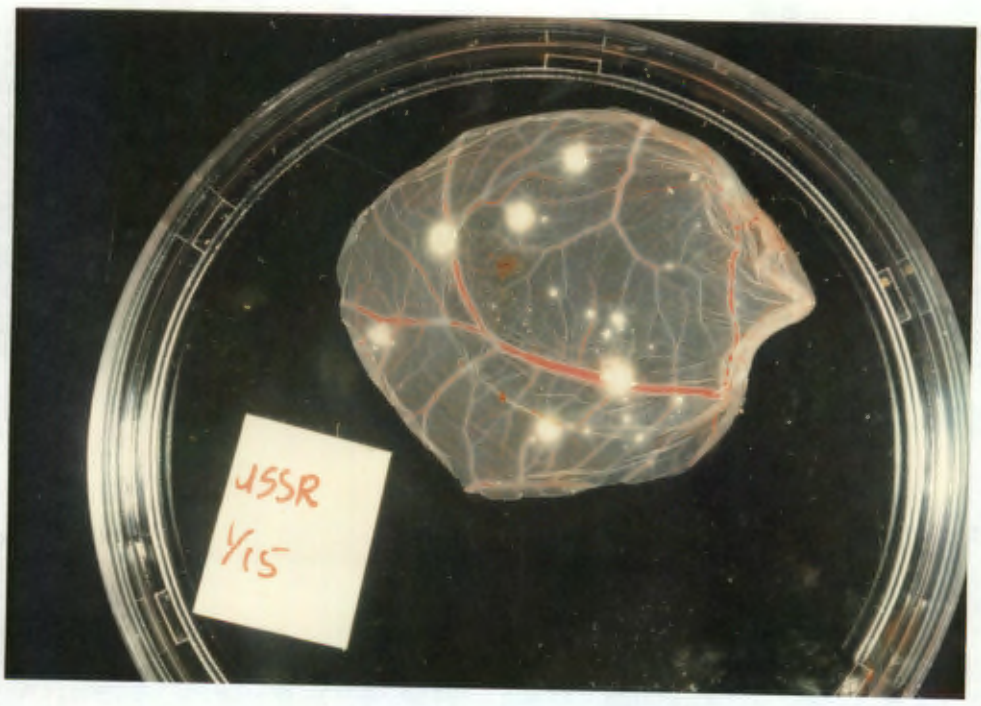


Fig 7.1.7: Pocks produced by the USSR vaccinia strain after 3 days at 36°C.

Table 7.1.1: Appearance of pocks produced by buffalo isolates and vaccinia viruses on CAM after incubation at 36°C for 3 days.

VIRUS		APPEARANCE OF POCKS	DIAMETER, mm (mean of 5 measurements)
0-BP	B1	Grey, slightly ulcerated, slight capillary haemorrhage	2.6
V78/85	B2	Grey-white, ulcerated, hollow or dense centre	2.0
V79/85	B3	Grey, some ulcerated, slight capillary haemorrhage	2.2
V81/85	B4	Opaque, more dense around boarder, capillary haemorrhage	1.4
V31/86	B5	Grey, ulcerated, slight capillary haemorrhage	1.9
V115/86	B6	Opaque, capillary haemorrhage	2.5
3903	B7	Grey, ulcerated, slight capillary haemorrhage	2.3
3904	B8	Grey, ulcerated, slight capillary haemorrhage	2.4
3905	B9	Grey, central capillary haemorrhage	2.4
3906	B10	Opaque grey, capillary haemorrhage	2.2
3907	B11	Grey-white,dense, ulcerated, slight capillary haemorrhage	3.0
3908	B12	Grey-white, slight central capillary haemorrhage	2.5
2-BP	B13	Opaque,some ulcerated, capillary haemorrhage	1.7
339	B14	Opaque-grey, capillary haemorrhage	2.3
340	B15	Grey-white, dense, dome shaped, slight capillary haemorrhage	2.5
DIE		Grey, dense, ulcerated, slight capillary haemorrhage	2.0
USSR Vacc.		Grey,some ulcerated	2.0
King Inst.		Grey ulcerated, some opaque with capillary haemorrhage	2.3
Patwadangar		Grey, ulcerated,	2.7

7.2: GROWTH AT 40°C

The effect on pock formation on CAM of some of the buffalo isolates when the eggs were incubated at 40°C was varied and differed from the effect shown by vaccinia virus.

The buffalo isolates what were tested were V78/85, V79/85, V81/85, V31/86 and V115/86. The vaccinia viruses tested, were grown from the USSR vaccine, King Institute vaccine and Patwadangar vaccine. Vaccinia DIE, Radebe and Lenny (from the collection of Prof K. R. Dumbell) were also used.

Incubation of the eggs at 40°C did not affect the growth on CAM of the vaccinia strains. Pocks produced by these viruses at this temperature were still large, dense and like the ones produced at 36°C. There was a slight decrease in the number of pocks when incubated at the higher temperature. This corresponds with the fact that the ceiling temperature for vaccinia strains was found to be 41°C (Bedson & Dumbell 1961). The ability to produce at 40°C characteristic pocks like those at 36°C, was impaired in the instance of all but one of the buffalo isolates tested.

V79/85 did not produce any pocks at 40°C, while V81/85, V31/86 and V115/86 produced a few small, thin pocks. The pocks produced by V78/85 at 40°C looked the same as those at 36°C.

It appeared that the ceiling temperatures of the 4 buffalo isolates were significantly less than those of the vaccinias,

while isolate V78/85 had a ceiling temperature equal to that of vaccinia virus. This virus might not be a true buffalopox virus and could be considered to be more closely related to the vaccinia strains.

See Table 7.2.1 for a summary of the results obtained. Problems were experienced with the maintenance of temperature at 40°C and the influence of this elevated temperature on the pock formation of the other buffalo isolates was not investigated.

Table 7.2.1: The effect of growth at 40°C on pock formation of buffalo isolates and of Vaccinia strains.

Virus Inoculated	Appearance of pocks	Effect on pock numbers
V78/85 (B2)	Large, vaccinia like	Almost unaffected
V79/85 (B3)	No pocks	
V81/85 (B4)	Pocks very small	Much reduced
V31/86 (B5)	Pocks wide spread, but thin	Reduced
V115/86 (B6)	Pocks smaller and less dense	Reduced
USSR Vaccinia	Large pocks	Almost unaffected
King Inst Vacc	Large pocks	Almost unaffected
Patwadangar	Large pocks	Almost unaffected

7.3: CYTOPATHIC EFFECT IN TISSUE CULTURE

All of the viruses obtained from outbreaks of buffalopox produced a cytopathic effect (CPE) in tissue culture. All formed plaques in CV1 cells which were clearly visible within the 2nd day after inoculation. The cells rounded up and a clear area formed which was visible when the cell sheets were fixed and stained. All the isolates that were tested in HEF cells produced CPE. They were V78/85, V79/85, V81/85, V31/86 and V115/86. The cells formed strands over the plaques and cell rounding occured. The plaques formed by V78/85 were bigger than those formed by the other tested isolates. Plaques were also produced in RK 13 cells.

The sizes of plaques in CV1 and RK 13 cells were measured after 2 and 3 days and also compared with the plaques produced by vaccinia strains in the same cell lines. Table 7.3.1. shows the mean plaque diameter for the 6 buffalo isolates that were grown on CV1 and RK 13 monolayers.

Table 7.3.1: Plaque diameter produced in CV1 cells and RK 13 cells by buffalo isolates and vaccinia strains after 3 days incubation at 36°C

VIRUS	PLAQUE DIAMETER IN TC CELLS AFTER 3 DAYS (mean value of 10 measurements, mm)	
	CV 1 cells	RK 13 cells
V79/85	0.8	1.0
V81/85	0.7	1.1
V31/86	0.7	1,1
V115/86	0.8	1.1
3903	0.8	1.1
V78/85	0.9	1.1
USSR vaccinia	1.1	1.3
DIE vaccinia	1.2	1.0
King Inst.	1.2	1.4
Patwadangar	1.1	1.1

7.4: FORMATION OF A-TYPE CYTOPLASMIC INCLUSION BODIES

Thin sections of CAMs infected with viruses from outbreaks of pox in buffalo were stained with Heamatoxylin and Eosin (H+E) and investigated for the presence of A-type cytoplasmic inclusion bodies. The buffalo isolate viruses used, were V78/85, V79/85, V81/85 and V31/86. The Cowpox Whipsnade strain (from the collection of Prof K. R. Dumbell) was used as a positive control.

No A-type eosinophylic inclusion bodies were seen in the sections of the buffalo isolates. In the membranes infected with cowpox, eosinophylic inclusions were seen. These were oval shaped, homogneous with a slight halo and were typical of A-type inclusions.

In V81/85 vacuolar spaces were seen in the cytoplasm of cells in the infected areas of the membrane, but no inclusion bodies. V79/85 and V31/86 showed some eosinophilic inclusion bodies, but these were irregular in shape and the contents appeared granular. V78/85 did not appear to have any cytoplasmic inclusions and large vacuolar spaces were observed in areas with a high degree of infection. Similar findings to these were described by Mathew (1977), where B-type inclusion bodies were produced by buffalopox viruses in different tissue culture systems.

7.5: NEUTRALIZATION OF VIRUSES

Five of the buffalo isolates were incubated with different dilutions of an anti-vaccinia serum and the ability of the serum to neutralize the virus was compared with the neutralizing effect the serum had on vaccinia strains.

The dilutions of serum that were used varied from 1/10 to 1/1280. Only one dilution of a virus was used which produced a countable number of pocks per TC well. 4 Wells per serum dilution were used and 4 control wells (without neutralizing serum) were put up for every virus tested. The cells were incubated for 2 days after inoculation before fixing and counting

of the plaques.

A 50% decrease in number of plaques formed were not obtained for all the buffalo viruses with the chosen serum dilutions, except in the case of 2. The point of 50% reduction of plaque count for the USSR and DIE vaccine strains were reached with a much higher dilution of serum (1/1000) than for any of the buffalo isolates so that the anti-vaccinia serum did not have the same neutralizing effect on the buffalo isolates that it had on the vaccinia viruses. The serum had neutralizing powers on 2 buffalopox strains for which the 50% end points were established (V115/86 and 3903 at about serum dilution 1/80)and probably also for the ones where the end points didn't fall within the range of the experiments. From these results, it could be said that the buffalopox viruses are not the same in antigenic make-up as the vaccinia virus strains, although some antigens may be shared by the two virus species. V78/85 showed the 50% end point with much higher serum dilutions than the other buffalo isolates (similar to the vaccinia strains, DIE and USSR), that were tested at the same time. The end point for the other buffalo isolates was at a serum dilution at least 10 x less.

7.6: LESIONS PRODUCED IN RABBIT SKIN

5 Buffalopox viruses were inoculated into shaved rabbit skin as described before. Stock dilutions of the virus were diluted in 10% PBS to titres of 10^5 and 10^3 pfu/0.1ml and the lesions they produced were compared to the lesions formed by 3 strains of

vaccinia virus and the buffalo isolate V78/85.

The buffalopox viruses were V79/85, V81/85, V31/86, V115/86 and 3903. The vaccinia strains used were the USSR, King Institute and Patwadangar strains. The lesions produced by the vaccinia strains were big papular lesions (18 mm) with necrosis while the buffalopox viruses produced much smaller lesions (9 - 12 mm), which didn't become necrotic. The lesions produced by V78/85 were larger than the ones produced by the buffalopox viruses and closer in appearance to those produced by the vaccinia strains. No generalization of pocks occured and the lesions started resolving after day 7. Measurements were done on day 5. These findings correspond to those described by Baxby and Hill (1971) for the buffalopox virus type strain.

CHAPTER 8

DNA COMPARISONS OF BUFFALOPOX ISOLATES

8.1: INTRODUCTION

The distribution of Hind III restriction cleavage sites in the genomes of orthopox viruses, shows that the map positions of an internal region of about 60 megadaltons (half of the genome) are highly conserved between the different species (Mackett & Archard 1979; Esposito & Knight 1985).

Profiles of fragment sizes are thus characteristic of the different species and show only minor variation in separate isolates of the same species (Mackett & Archard 1979). Consequently the Hind III profiles have proved useful as a means of species identification in the Orthopoxvirus genus. Some variations are seen in the conserved region , but these are characteristic of particular species.

Patterns of fragment size in digestions with other endonucleases are not so constant within isolates of the same species and are therefore useful in the investigation of intraspecies variation.

In this chapter it is shown that buffalopox isolates share the same Hind III pattern as vaccinia virus. In digests with Pst I, Sac I, Xho I and Eco RI, the buffalo isolates, except isolate

V78/85, which has been shown to be vaccinia (Chapter 7), show close similarity among themselves and their patterns are clearly different from those of the vaccinia strains.

8.2: METHODS

Virus was grown on CAM from a suitable dilution of the virus stock and the DNA was extracted from purified viral preparations as described in Chapter 3. Endonuclease restriction digests of DNA from the buffalo isolates and other viruses from the orthopox group were set up and the fragments that were generated were separated by gel electrophoresis. The fragments were stained with Ethidium Bromide and visualized by transilluminated light (300 nm). DNA from Bacteriophage lambda cl 875 Sam 7 (Amersham International) was used as a molecular marker. See Chapters 3 and 5.

8.3: RESULTS

Restriction of various orthopox viruses with Hind III clearly differentiated between the different species (Esposito et al. 1978; Mackett & Archard 1979). Monkeypox, cowpox and vaccinia DNAs were easily distinguished from one another (fig 8.3.1). The vaccinia strains and the buffalo isolates, however, gave almost the same fragment pattern. A slight variation in the distance the B fragments of DIE, USSR, 0-BP and the rest of the buffalo isolates had migrated, was noticed. See fig 8.3.1.

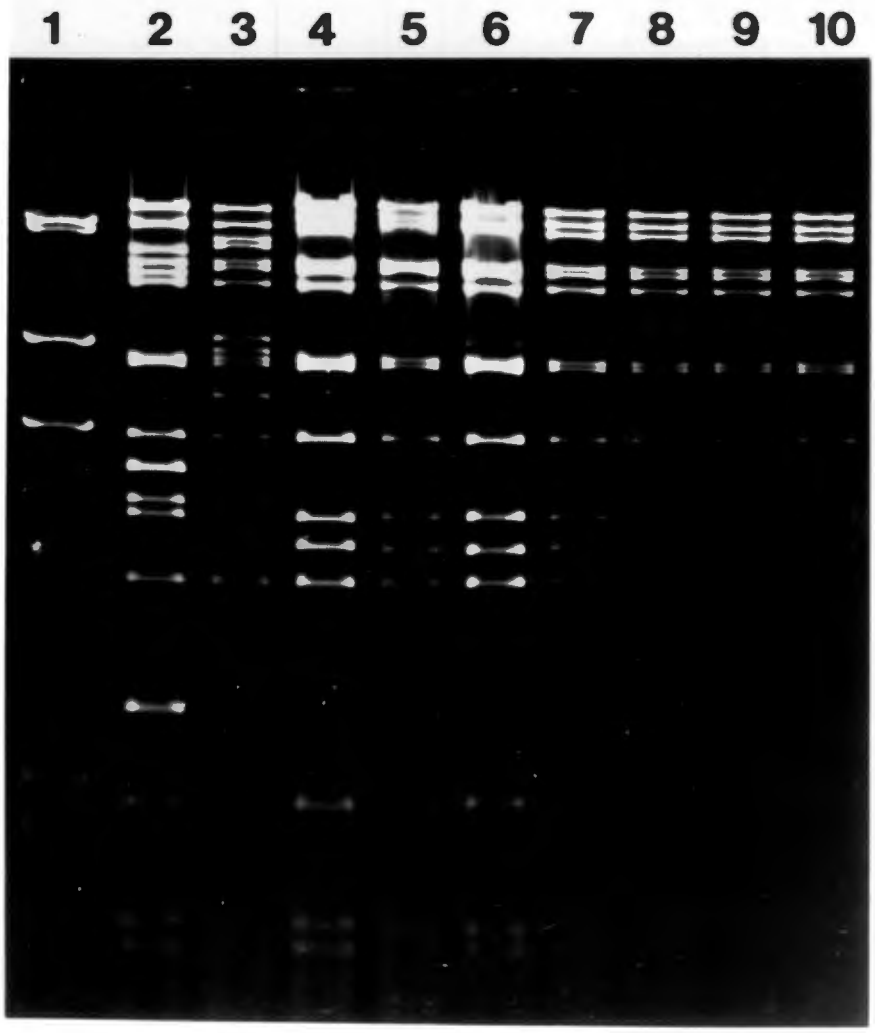


Fig 8.3.1: Hind III restriction fragments of orthopox virus species, buffalo isolates and lambda DNA. Lanes 1 - 10. 1: lambda DNA; 2: Mp Denmark; 3: Cowpox LB; 4: vaccinia DIE; 5: vaccinia USSR; 6: 0-BP; 7: 3903; 8: 3904; 9: 3905; 10: 3906. Electrophoresis was done in a 0.8% agarose gel at 1.4 Volt/cm for 16 hours.

With the restriction enzyme Pst I, clearly distinguishable variations were observed between the vaccinia strains and the buffalo isolates, but also between the different vaccinia strains and between the different buffalo isolates. Vaccinia King Institute and Patwadangar corresponded with regard to the Pst I restriction pattern and they resembled the vaccinia USSR Pst I pattern more closely than that of vaccinia DIE. Variation in Pst I patterns for the buffalo isolates occured. V79/85 and V81/85 showed the same fragment pattern, which differed from that of 0-BP. The pattern of V78/85, which has the biological character of vaccinia, also differed substantially from those of the buffalo isolates. See fig 8.3.2.

DNA from a further 6 buffalo isolates were restricted with Pst I. In comparison with V79/85 and V81/85, the patterns observed were identical for all 8 isolates. See fig 8.3.3. DNA from the remainder of the buffalo isolates as well as from virus 2-BP (received from Dr Baxby) were restricted with Pst I. The same constant pattern shown by the buffalo isolates, (illustrated in fig 8.3.3) was obtained (not shown). It appeared that Pst I could be used to distinguish between vaccinia strains and buffalo isolates and also that the different buffalo isolates from Maharastra, V78/85 excluded, shared a constant pattern.

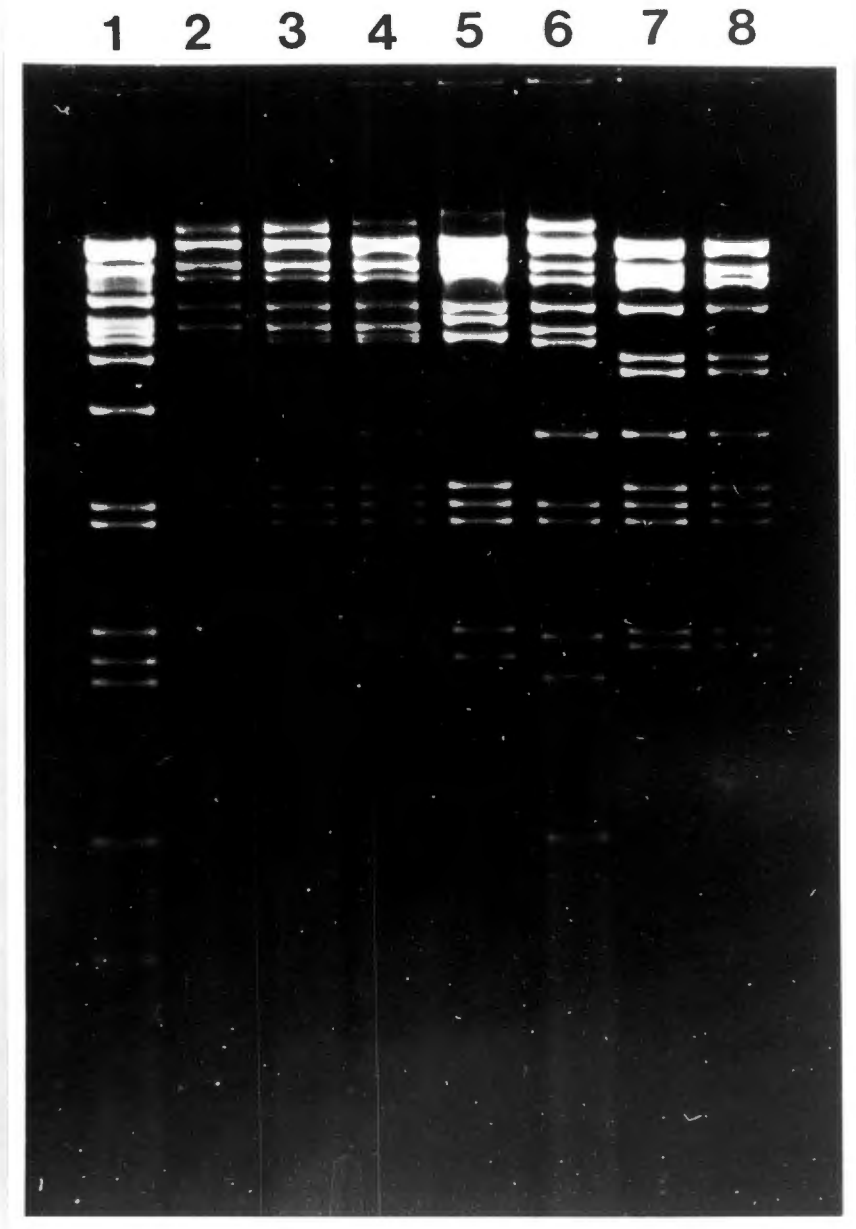


Fig 8.3.2: Pst I restriction patterns of vaccinia species and four buffalo isolates. Lanes 1 - 8. 1: vaccinia DIE; 2: vaccinia King Institute; 3: vaccinia Patwadangar; 4: vaccinia USSR; 5: 0-BP; 6: V78/85; 7: V79/85; 8: V81/85. Electrophoresis was done in a 0.8% agarose gel at 1.4 Volt/cm for 16 hours.

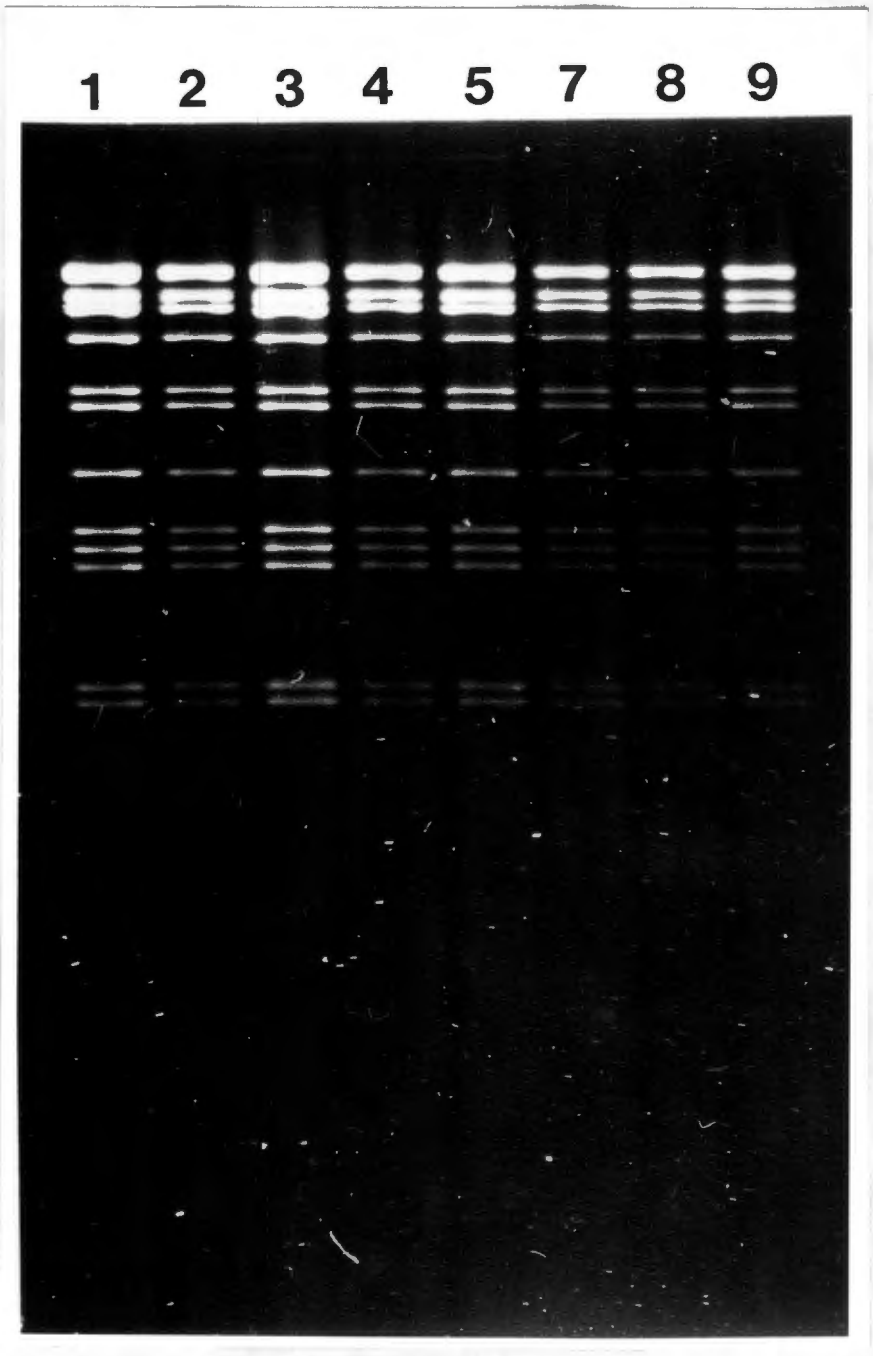


Fig 8.3.3: Pst I restriction patterns of buffalo isolates. Lanes 1 - 8. 1: V79/85; 2: V81/85; 3: V31/86; 4: V115/86; 5: 3903; 7: 3904; 8: 3905; 9: 3906. Electrophoresis was done in a 0.8% agarose gel at 1.4 Volt/cm for 16 hours.

5 Isolates, representing 5 areas of isolation (Beed, Ratnagiri, Pune, Solapur and Dhulia) within Maharastra, India were further investigated. Restriction patterns from these were compared with those obtained from 3 vaccine strains as well as with the vaccinia-like buffalo isolate V78/85 (Ratnagiri) (See Chapter 7).

With the restriction enzyme Xho I, the fragment patterns for the 5 buffalo isolates corresponded to a high degree and could be differentiated from that of the vaccinia strains and V78/85. The pattern for V78/85 was closely related to that of King Institute and USSR vaccinia strains. See fig 8.3.4.

Sac I restriction of the 5 buffalo isolates differentiated them from vaccinia strains and V78/85, with the constant appearance of bands in the 15 kb range, which did not appear in V78/85 and the vaccinia strains. All the buffalo digests had a band in the 6 - 7 kb region, the same size as one in vaccinia. Unlike vaccinia, the buffalopox viruses had a second band in this size region, which varied slightly in size between the isolates. In V81/85, V31/86 and 3903 the two fragments are evident, but in V79/85 and V115/86, the two bands are close enough to coalesce into a single band, evident by its greater intensity after staining with Ethidium Bromide. See fig 8.3.5.

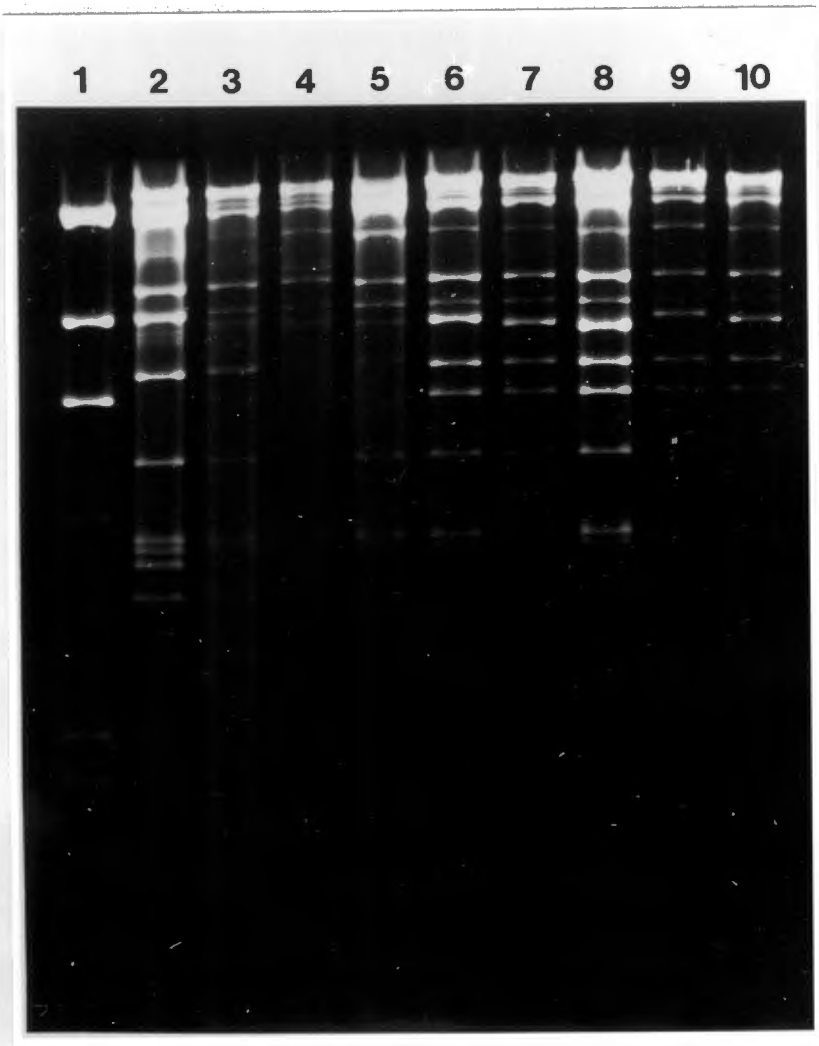


Fig 8.3.4 Xho I restriction patterns for vaccinia strains and buffalo isolates. A molecular marker was digested with Hind III. Lanes 1 - 10. 1: lambda DNA (Hind III); 2: vaccinia DIE; 3: vaccinia USSR; 4: vaccinia King Institute; 5: V78/85; 6: V79/85; 7: V81/85; 8: V31/86; 9: V115/86; 10: 3903. Electrophoresis was done in a 0.8% agarose gel at 1.4 Volts/cm for 16 hours.

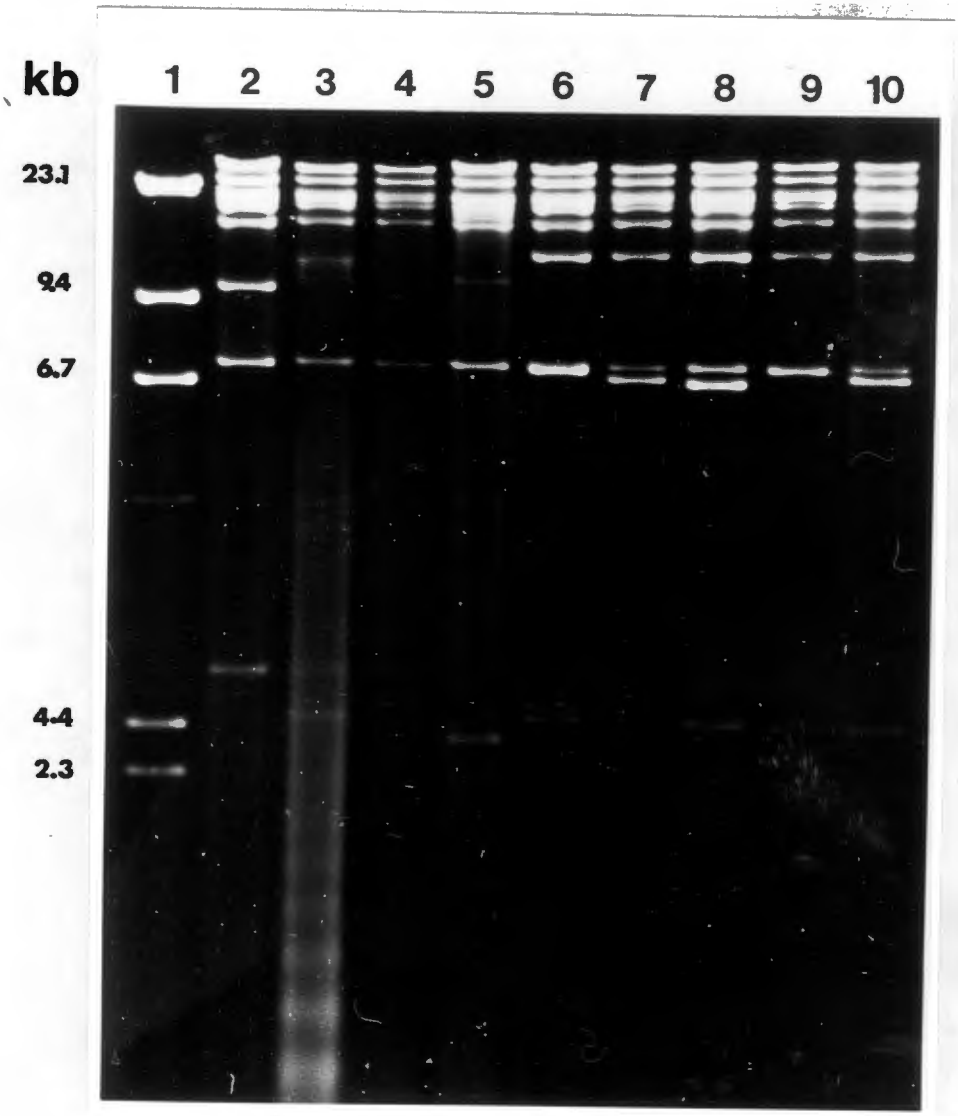


Fig 8.3.5: Sac I restriction patterns for vaccinia strains and buffalo isolates. A molecular marker was digested with Hind III. Lanes 1 - 10. 1: lambda DNA (Hind III); 2: vaccinia DIE; 3: vaccinia USSR; 4: vaccinia King Institute; 5: V78/85; 6: V79/85; 7: V81/85; 8: V31/86; 9: V115/86; 10: 3903. Electrophoresis was done in a 0.8% agarose gel at 1.4 Volt/cm for 16 hours.

With Eco RI digestion, a much larger number of fragments was produced and the more complex patterns did not so easily show the difference between the group of vaccinias (and V78/85) and the group of buffalopox viruses. The buffalopox group however showed a pattern which was constant except for one band in the 5 - 9 kb region which varied in size between isolates, being larger in V79/85 and V115/86 and smallest in V31/86. See fig 8.3.6.

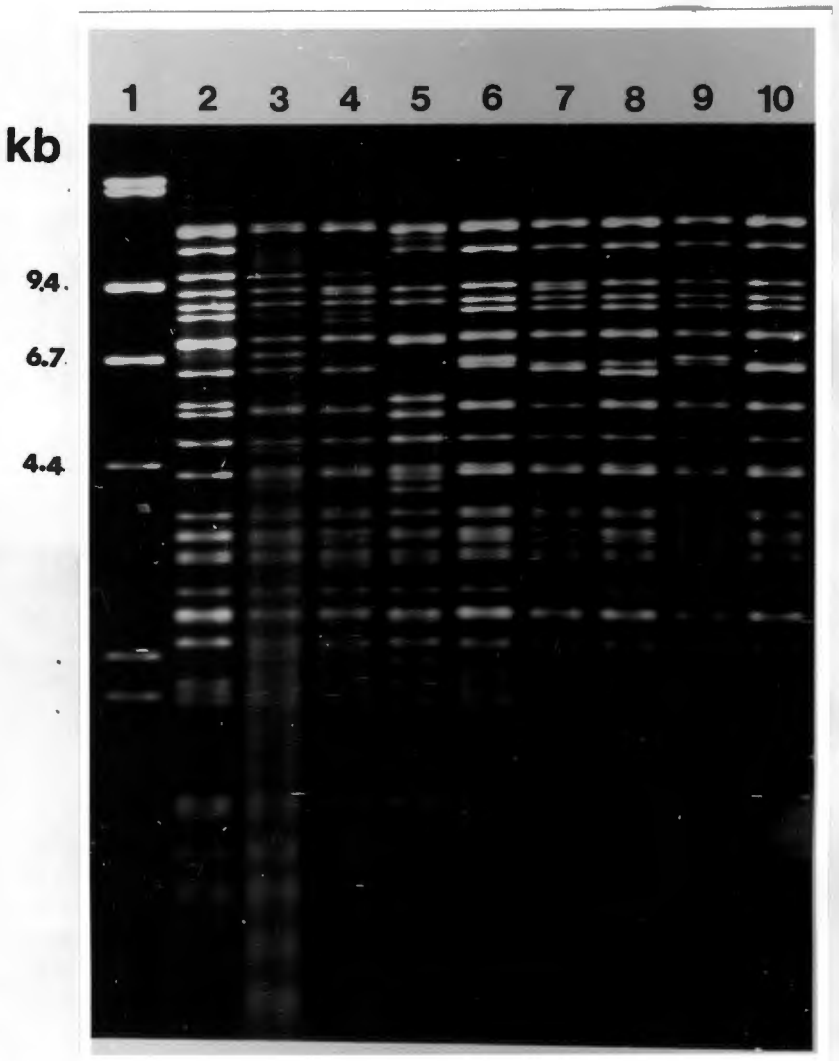


Fig 8.3.6: Eco RI restriction patterns for vaccinia strains and buffalo isolates. A molecular marker was digested with Hind III. Lane 1 - 10. 1: lambda DNA (Hind III); 2: vaccinia DIE; 3: vaccinia USSR; 4: vaccinia King Institute; 5: V78/85; 6: V79/85; 7: V81/85; 8: V31/86; 9: V115/86; 10: 3903. Electrophoresis was done in a 0.8% agarose gel at 1.4 Volt/cm for 16 hours.

CHAPTER 9

DISCUSSION OF INTRASPECIES TYPING OF BUFFALOPOX VIRUS

Virus was isolated from scab specimens that were taken from buffaloes that suffered from pox disease in Maharastra, India from 1985 - 1987. 18 Specimens were received and virus was recovered from 13 of these. A further 2 viruses were received from Dr Baxby and were included in the study. 0-BP is the original buffalopox strain type (isolated in North India) that was investigated by Baxby and Hill (1971), and 2-BP was isolated from a buffalopox outbreak in Pune, Maharastra during the same time the outbreaks occured from which the other specimens were received.

9.1: EVALUATION OF BIOLOGICAL CHARACTERISTICS

Virus was amplified (from a 50% Glycerol stock of the virus) on CAMs and DNA was extracted from the purified virus particles to be used in restriction analysis studies. The biological character of the buffalo isolates was compared mostly with those of vaccinia strains. It was confirmed that the buffalopox virus belongs to the genus Orthopoxvirus by the fact that anti-vaccinia rabbit serum was able to neutralize the infectivity of the virus in cell culture. A 50% decrease in plaque number was obtained for the vaccinia species DIE and USSR at a serum dilution of 1/1000 and buffalo isolate V78/85 was also neutralized by serum at this dilution. Two buffalo isolates , V115/86 and 3903 showed

a 50% decrease in plaque number at a serum dilution of 1/80, a more than ten fold increase in serum concentration. The serum dilutions that were used did not span a wide enough range to estimate the 50% end points for the other buffalo isolates (V79/85, V81/85, V31/86) that were included in the experiment, but it was clear that all three were more than ten times less sensitive than the vaccinia strains.

The absence of A-type inclusion bodies confirmed that the buffalo isolates were not variants of cowpox virus. This was also illustrated by the pock character on CAM. The pocks produced by the buffalo isolates at 36°C could be described as vaccinia-like. Most of the isolates produced pocks with a slight haemorrhagic centre, but to a much lesser degree than the haemorrhage which is shown by cowpox virus. The slight haemorrhage was not so noticable with the vaccinia strains in the same experiment. V78/85 also did not show haemorrhage and produced some dense white pocks. The heterogeneous pock character that was reported in earlier studies of buffalopox virus was not obvious but it was noted that slight variations in the pock character of each of the buffalo isolates occured in different eggs or in different experiments.

The incubation of infected CAMs at 40°C severely affected the ability of buffalopox virus to produce pocks on CAM. V79/85 produced no pocks at this temperature, while the pocks of V81/85, V31/86 and V115/86 were similar and thinner than at 36°C. Pock numbers were also much reduced at the higher temperature. Isolate V78/85 and the vaccinia strains USSR, King Institute and

Patwadangar did not show alteration in pock character and a small reduction was observed in the number of pocks. This corresponds with the establishment of the ceiling temperature for vaccinia virus at 40 - 41°C (Bedson & Dumbell 1961).

V78/85 seems to be more closely related to the vaccinia strains than the other buffalopox isolates and can be regarded as a vaccinia virus. It is noted that Baxby and Hill (1971) characterized 4 buffalo isolates and found 3 of them indistinguishable from vaccinia virus. The buffalo isolates did not show the same degree of virulence in rabbit skin as the vaccinia strains (and V78/85). The vaccinias (and V78/85) produced larger plaques in tissue culture monolayers and produced CPE more rapidly than the buffalopox viruses.

Evaluation of the biological characters of the buffalo isolates, leads to the conclusion that all the buffalo isolates are orthopox viruses and that all (except V78/85) are distinguishable from vaccinia virus (by ceiling temperature, neutralization test, rabbit virulence and CPE in tissue culture). They differ from cowpox (no A-type inclusion bodies) and from monkeypox (pock character - Von Magnus et al. 1959; Gispen et al. 1967). Viruses recovered from the buffalopox outbreaks were regarded as being equilvalent to the buffalopox virus first described by Baxby and Hill (1971). V78/85 exhibited a biological character close to that of the vaccinia strains used in the study and was considered to be a vaccinia strain in the further investigations.

9.2: EVALUATION OF RESTRICTION ANALYSES

The restriction patterns that were observed after electrophoretic separation of fragments produced by restriction digests of the buffalopox DNAs, made further typing of the virus possible .

The restriction endonuclease Hind III clearly distinguished monkeypox, cowpox and vaccinia viruses from each other, but with this enzyme, the buffalo isolates presented the same fragment pattern as the vaccinia strains and would be classified as vaccinia viruses. The restriction enzyme Pst I, though, showed that a distinct differentiation can be made between the genomes of the vaccinias and the buffalo isolates. With Pst I digests of further buffalo isolates it was seen that all of them except V78/85 and 0-BP had an identical Pst I fragment pattern. V78/85 was shown not to be a buffalopox virus and the variation in the pattern for 0-BP, is probably explained by the fact that it was isolated many years before and in Hissar, a different part of India.

Five representative isolates were further characterized by restriction analysis with Sac I, Xho I and Eco RI. With all 3 enzymes, the buffalopox viruses presented a characteristic restriction pattern, which was distinguishable from that of V78/85 and the vaccinia strains which were included in the study.

The use of restriction enzymes made it possible to distinguish between buffalopox virus and vaccinia, although it was also shown (Hind III) that the virus is genomically closely related to

vaccinia. Two distinct fragment patterns were observed for buffalopox viruses with Pst I. Although all the Maharastra isolates (V78/85 excluded) had the same Pst I profile, this pattern was significantly different from that of the Hissar buffalopox isolate. Variations in the genomes of buffalopox viruses (including the 5 viruses which were further investigated) made it possible to distinguish variations within the buffalopox strains. These studies on the DNA of buffalopox virus were the first studies to be done on the genome of this virus.

SECTION III

INTRASPECIES TYPING OF MONKEYPOX VIRUS

CHAPTER 10

INTRODUCTION TO MONKEYPOX

10.1: REPORTED OUTBREAKS

Monkeypox was discovered in 1958 in an outbreak of non-fatal pox-like disease in a captive monkey colony in Copenhagen, Denmark (Von Magnus et al. 1959). The virus isolated from the affected animals was shown to be an orthopoxvirus and could be differentiated from those previously recognized.

In 1959 an outbreak of monkeypox occured in animal quarters in Philadelphia, USA and in 1962, monkeypox was recognized in the primate colony of a research institute in Washington D.C. (Cho & Wenner 1973).

In 1964 an outbreak of monkeypox was reported in the Rotterdam Zoo (Netherlands), where gaint anteaters, orangutans, chimpanzees, gorillas, guenons, squirrel monkeys, maqaques, gibbons and marmosets were affected. Monkeypox virus was isolated from a number of animals of various species. (Cho & Wenner 1973). Viruses isolated from these animals were studied by Gispen et al. (1967). The pock lesions formed on CAM by the isolated monkeypox virus resembled those formed by variola, but could be distinguished from variola by infectivity in rabbit skin and by the ability of monkeypox to produce pocks on CAM at 39^{o}C.

During 1964 - 1965, monkeypox virus was isolated from a silent monkeypox infection in captive Asian monkeys (Review Arita & Henderson 1968; Cho & Wenner 1973) and in 1966 from a langur in the USA (personal communication from C. Espana to Arita et al. 1972). A chimpanzee that arrived at an animal colony in Paris from Sierra Leone in November 1968, suffered from monkeypox soon after arrival and the causative agent was identified as being monkeypox virus (Milhaud et al. 1968).

Serological studies with sera from almost 2500 monkeys originating from South-East Asia and the African continent, gave negative results for monkeypox antibodies, but it was found that neutralizing antibody persisted at a high titre in animals that had been infected in captivity (Arita et al. 1972).

In September 1970, a 9 month old boy with suspected smallpox was admitted to a hospital in the Equatorial Province of the Republic of Zaire. This became the first recognized case of a human infection caused by monkeypox virus (Lanyj et al. 1972). The monkeypox virus isolated, was differentiated from variola by it's pock character at 35^{o}C, it's ceiling temperature and it's rabbit virulence (Marennikova et al. 1972).

Between October 1970 and May 1971, six cases of suspected smallpox were reported in Liberia, Nigeria and Sierra Leone. All occured in unvaccinated individuals with no spread to close household contacts (Forster et al. 1972). Monkeypox was identified as the causative virus in each case.

From 1970 to December 1975, 20 cases of monkeypox were recorded in West and Central Africa. Twelve of the 20 cases occured in the Republic of Zaire and other countries involved were Liberia, Sierra Leone, The Ivory Coast and Nigeria (Arita & Henderson 1976)

Surveillance of human monkeypox has continued in West and Central Africa and special surveillance and research activities were developed in Zaire, where 92% of all the cases have been discovered up to 1985.

Outbreaks of human monkeypox have continued sporadically in Zaire. They are associated with the rainforests, of which the larger percentage is situated in that country. People affected usually lived in small villages in the tropical rainforest and most of them within 100 m of the edge of the forest. Zaire contains 56% of the tropical rainforest area of Western and Central Africa and the surveillance of monkeypox there has been more intensive than elsewhere. In 1982 and 1983, the seasonal peak incidence of human monkeypox corresponded to the period of highest rainfall in the area, namely July - August (Arita et al. 1985). However, Breman et al. (1980) reported that, based on occurence from 1970 - 1979, monkeypox appeared more frequently in the dry season, similar to the seasonal spread of smallpox.

The last reported case of smallpox in West and Central Africa occured in June 1970 (Arita & Henderson 1976). Routine smallpox vaccination officialy ceased in Zaire in 1980, with a few sporadic vaccinations in 1981. Vaccination against smallpox

would protect against monkeypox as both are orthopox viruses. Children under 10 years were at the highest risk, accounting for 84% of 143 cases. Presumed person to person transmission did occur within households and a few cases occured in hospitalized children who had been in contact there with human cases (Arita et al. 1985).

In its clinical aspects, monkeypox is similar to smallpox, except for the frequent occurence of lymphadenopathy before the rash appears in the case of monkeypox. It gives rise to an extensive rash with significant associated mortality, particularly in children (Memoranda: World Health Orinization 1984), with a lesser degree of severity in people with visible vaccination scars (Jezek et al. 1987).

In screening sera from human outbreaks in Kole Zone, East Kassai, Zaire, monkeypox-specific antibodies were found in sera from 27 individuals (0.8% of people tested). The prevalence rate was four times higher in the 5 - 9 year age group than in children of the 0 - 4 years group, but was the highest in the 15 - 19 year age group (Jezek et al. 1987).

The secondary attack rate (number of secondary cases divided by the number of contacts) is a reasonable measure of the contagiousness or transmissibility of human monkeypox. It was found to be considerably lower than that of variola virus. The longest chain of transmission so far recorded for the disease consisted of 4 serial cases (Jezek, Arita et al. 1986), while the

majority of primary cases failed to give rise to even one infection among young contacts (Jezek, Marennikova et al. 1986).

The similarity between the clinical manifestation of human infections with monkeypox and variola viruses made human monkeypox the most important orthopox infection in man for surveillance and research (Arita et al. 1985) and one of the priorities is to identify the natural reservoir of this virus (Memoranda: Bulletin of the World Health Orginization 1984).

10.2: ANIMAL RESERVOIRS

Surveys were conducted to determine the animal reservoir of monkeypox. Serum surveys of animals showed orthopoxvirus antibodies in a small percentage of several animal species, particularly in monkeys, squirrels and terrestrial rodents (Arita et al. 1985; Khodakevich et al. 1986).

In July 1985 an epidemiological team collected animals within 40 km of Yamvuku village, Bumba Zone, Zaire (where a human case occured in 1981). One of the 38 animals sampled, was a squirrel with skin erruptions and monkeypox virus was isolated from the skin, lungs, spleen and kidneys (Khodakevich et al. 1986). This sick squirrel (Funisciurus anerythrus) was found on 21 July 1985 by a resident of Bodjoki village about 50 m away from the village. It was killed with a stick and brought to Yambuku. The body was examined and dissected the same evening. Round and oval flat superficial lesions of 2 - 3 mm in diameter were seen on the

abdomen and more distincly in the upper and lower inguinal areas (Khodakevich et al. 1987a).

This was the first time that monkeypox virus had been isolated from an animal infected in the wild. Another squirrel that was captured near a village where human cases were reported in 1981, 1982 and 1984 had specific antibodies to monkeypox virus. No monkeypox specific antibodies were found in terrestrial rodents. Samples from 5 of 51 Funisciurus anerythrus squirrels that were collected in 1979 had recently been shown to have monkeypox specific antibodies and F anerythrus seems to be a priority species to consider as a possible reservoir host of monkeypox virus (Khodakevich et al. 1986).

In 1985 a study was conducted in Northern Zaire in the human environments and among the animals that are suspected of maintaining virus transmission. Data suggested that most human cases have been infected in the area immediately (20 m - 3 km) surrounding the villages or in the agricultural area (3 - 4 km away from the villages). These areas are inhabitated by terrestrial and arboreal rodents and bats, while the larger animals have abandoned it (Khodakevich et al. 1987b).

In a further study (January - February 1986), no antibodies were found in 233 rodents tested, but a 24.7% incidence of monkeypox specific antibodies were found in 320 F. anerythrus species, suggesting that these animals sustain virus transmission in the areas surrounding human settlements. A consistently high level of antibodies were also found among Heliosciurus rufobrachium

squirrels, suggesting that this species is also involved in the transmission (Khodakevich et al. 1987b).

Primates tested during the survey represented animals (3 positives) of the arboreal level of the primary forest; species that occupy the upper stratum of the forest. Squirrels occupy several strata of the primary tropical forest, some sharing the upper stratum with the primates, but the importance of these primates in sustaining the virus in nature is difficult to judge.

Information on the population density of squirrels in the upper stratum of tropical forest is lacking, but these animals are highly populous in the lower stratum of decidious forests. The nuts of the oil-palm trees (Elaeis guineensis), which are in abundance in the agricultural areas, are a source of food for these squirrels and maintain a high population density in that area (Khodakevich et al. 1987b).

The question arises whether the virus isolated from the sick squirrel that was found in 1985, is the same as the virus that was recovered from humans. If so, it would mean that the squirrel is part of the reservoir from which humans are infected.

The virus isolated from the squirrel is biologically typical of monkeypox virus. An analysis of the genome of this isolate was undertaken and compared with that of other isolates of monkeypox virus, especially those of human isolates, to find what

intraspecies distinctions are possible.

10.3: MONKEYPOX VIRUSES USED

The various monkeypox viruses that were used in these studies are listed in Table 10.3.1. The African isolates of monkeypox were isolated at and obtained from either the Centre of Disease Control, Atlanta, USA (CDC) or the Research Institute for Viral Preparations, Moscow, USSR (IVP). Dr S. S. Marennikova of IVP provided strains Z 241, Z 369 and MP 1324. The others were provided by Dr J. H. Nakano of CDC. Mp Denmark was a gift from Dr P. von Magnus and Mp Rotterdam from Dr R Gispen. Other strains were made available through the smallpox eradication unit of the World Health Organization. These contributions are greatly appreciated by the author.

Table 10.3.1: Monkeypox isolates from which DNA was prepared for use in this study.

Strain (Reference)	Date of original isolate	Animal species from which virus was recovered	Place
Mp Denmark (1)	1958	Macacus cynomolgus	Copenhagen
Mp Prier (2)	1959	captive monkeys	Philadelphia,
Mp McConnel (3)	1962	captive monkeys	Washington DC USA
Mp Rotterdam (4)	1964-1965	anteaters, orangutan monkeys	Rotterdam Zoo Netherlands
Mp Espana (2)	1966	langur	California, USA
Mp Paris (5)	1968	chimpanzee	Paris, France
Mp Liberia I (6)	1970	human	Liberia
Mp Liberia II (6)	1970	human	Liberia
Mp 266	1970	human	Sierra Leone
Mp 82	1971	human	Nigeria
Mp Z 241	April 1972	human	Elonga village, Katako-Kombe, Zaire
Mp Z 369	May 1973	human	Lisala Zone, Zaire

Strain	Date of original isolate	Animal species from which virus was recovered	Place
Mp Benin (7)	1978	human	Nigeria
Mp 1324	21 September 1979	human	Bokonzo village Gemena, Zaire
Sq 249	21 July 1985	squirrel	Bodjoki village, Bumba, Zaire
V85-1-240	15 August 1985	human	Bodjoki village, Bumba, Zaire
V86-1-21	10 December 1985	human	Bonbanza village Bumba, Zaire
V86-1-112	28 January 1986	human	Bopoma village, Bongadanza, Zaire

The numbers in parentheses refer to the references in which the outbreaks were described. Ref 1: Von Magnus et al. (1959); Ref 2: Arita et al. (1972); Ref 3: Cho & Wenner (1973); Ref 4: Gispen et al. (1967); Ref 5: Milhaud et al. (1969); Ref 6: Forster et al. (1972); Ref 7: Arita & Gromyko (1982).

CHAPTER 11

DNA COMPARISONS OF MONKEYPOX VIRUS

11.1: INTRODUCTION

Restriction endonucleases which recognize a 6 base pair sequence, cut orthopox DNA 5 - 50 times. Polymorphism in fragment sizes and the location of cutting sites in a linear map of a genome offer a way of making detailed fine comparisons between isolates of the same species of poxvirus.

Restriction maps have been constructed for monkeypox (Mp Denmark) for the enzymes Bgl I, Sma I, Hind III, Kpn I, Sal I, Xho I and Sac I (Esposito & Knight 1985) and the Hind III maps provided a basis for discerning species, strains and variants of different orthopox viruses. Mackett and Archard (1979) compared the Hind III patterns of 3 monkeypox isolates for restriction enzymes Hind III and Xho I, while Esposito and Knight (1985) compared the Hind III restriction patterns of 12 monkeypox isolates. Esposito and Knight comment that the isolates show slight variations in size of some of the Hind III fragments.

These techniques were applied to determine whether the monkeypox virus that was isolated from a sick squirrel in Zaire in 1985 is sufficiently closely related to the isolates from human monkeypox cases in the same area to be regarded as coming from the same natural maintenance cycle. DNA from the squirrel isolate was

therefore compared with human monkeypox isolates from Zaire and also with human isolates from other regions in Africa and with the isolates from captive animals in Europe and America. Comparison of generated restriction fragments from monkeypox viruses isolated from these different sources will give an indication of the degree of conservation in the genome from one isolate to another. The restriction enzymes Hind III, Pst I and Eco RI were used in this study.

11.2: METHODS

DNA was extracted from purified virus (after amplification on CAM). The DNA was restricted and the fragments generated were separated by gel electrophoresis, stained with Ethidium Bromide and visualized by transilluminated light (300 nm). (Chapter 3 and 5).

11.3 RESULTS

With Hind III, all the monkeypox isolates had the same pattern of fragment size distribution and the terminal fragment (J), was the only one to vary significantly in size, see fig 11.3.1. This confirms the findings of Esposito and Knight (1985).

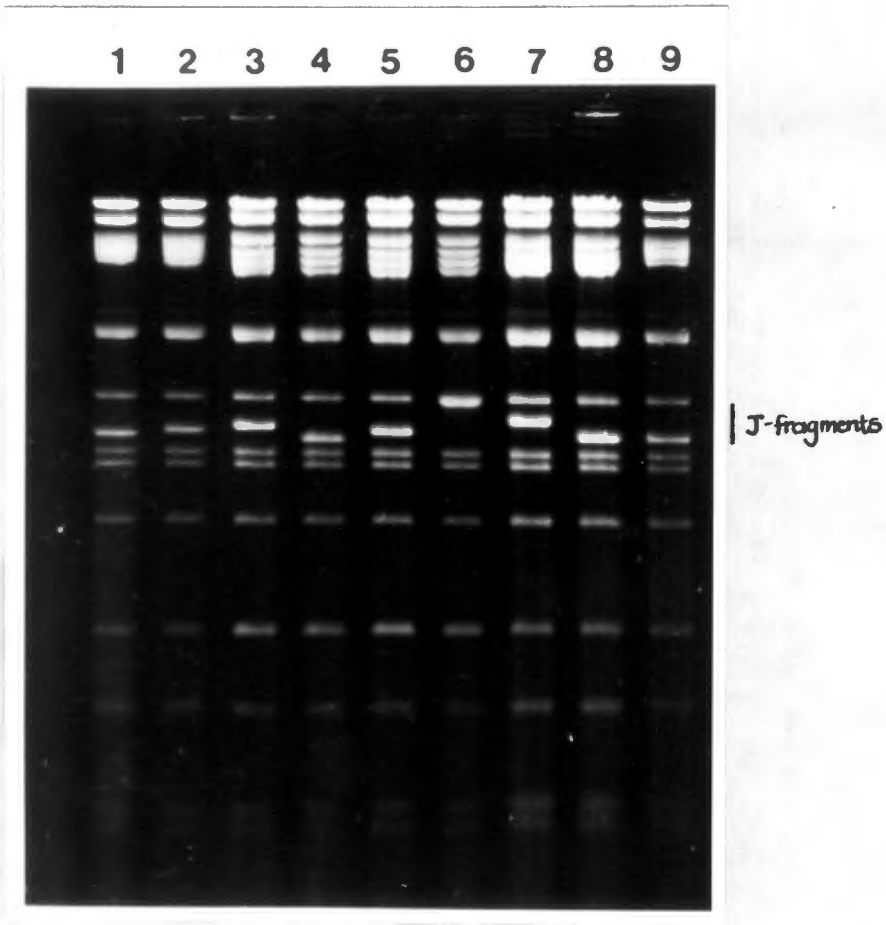


Fig 11.3.1: Restriction patterns of monkeypox isolates restricted with Hind III. Lanes 1 - 9. 1: Sq 249; 2: V86-112; 3: Mp Denmark; 4: Mp 266; 5: Mp Espana; 6: Mp Liberia I; 7: Mp 82; 8: Mp Benin, 9: Sq 249. Electrophoresis was done in a 0.8% agarose gel at 1.4 Volt/cm for 16 hours.

With the restriction enzyme Pst I, the squirrel isolate showed the same pattern as isolates from humans infected in the same area and the pattern could be distinguished from isolates originating in other areas. Isolates from Nigeria gave a distinct pattern and the isolates from the West Coast (Sierra Leone and Liberia) another clearly distinguishable one. See fig 11.3.2 and 11.3.3.

Patterns were thus characteristic of the endemic origin of the virus and, within Nigeria and Zaire, the respective patterns appeared to have remained constant over the several years that separated different isolates. All isolates obtained from captive animals gave the pattern that was typical of the West Coast isolates, which suggests that this region was the origin of the monkeypox virus which appeared in Europe and the USA. The routes of transfer of virus in outbreaks in captive animals are unknown, except in the case of a chimpanzee that became sick with monkeypox shortly after it arrived in Paris from Sierra Leone (Milhaud et al. 1969). The decrease in outbreaks of monkeypox in captive animals after 1966 was probably due to the decrease in export of animals and the stricter control on contact and handling of animals while they are in transit.

In the Pst I restriction patterns of the Zaire isolates, variation occured in the lengths of fragments D and H, which are shown in Chapter 12 (fig 12.2.3) to be the terminal fragments of the genome which include the hair pin end loops. The basic Pst I pattern, however, was constant for all the Zaire isolates examined (figs 11.3.2 and 11.3.3)

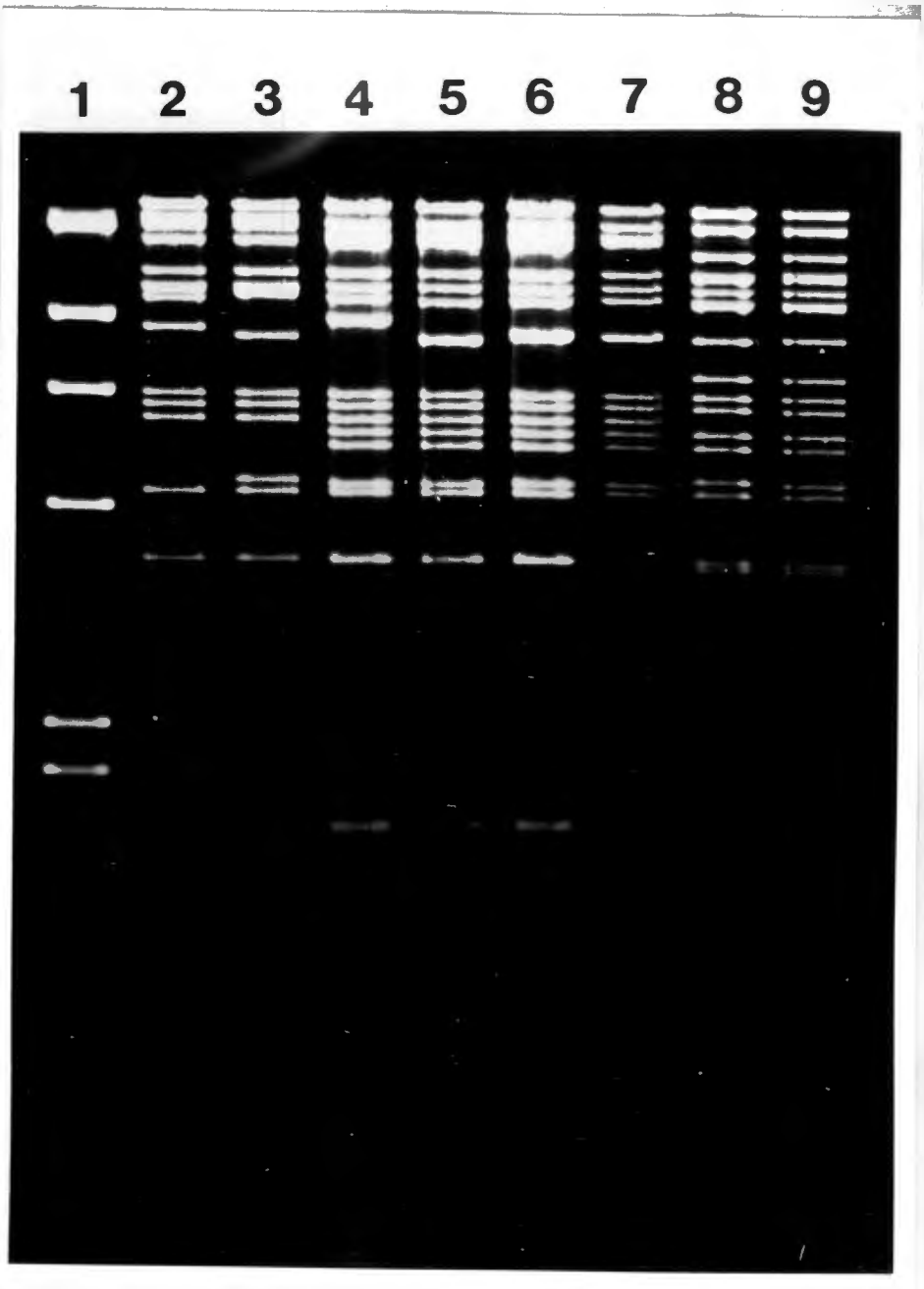


Fig 11.3.2: Pst I restriction fragments of monkeypox isolates. A molecular marker lambda was digested with Hind III. Lanes 1 - 9. 1: lambda DNA (Hind III); 2: Mp 82; 3: Mp Benin; 4: Mp Liberia I; 5: Mp 266; 6: Mp Denmark; 7: Mp Espana; 8: Z 369; 9: V86-112. Electrophoresis was done in a 0.8% agarose gel at 1.4 Volt/cm for 16 hours.

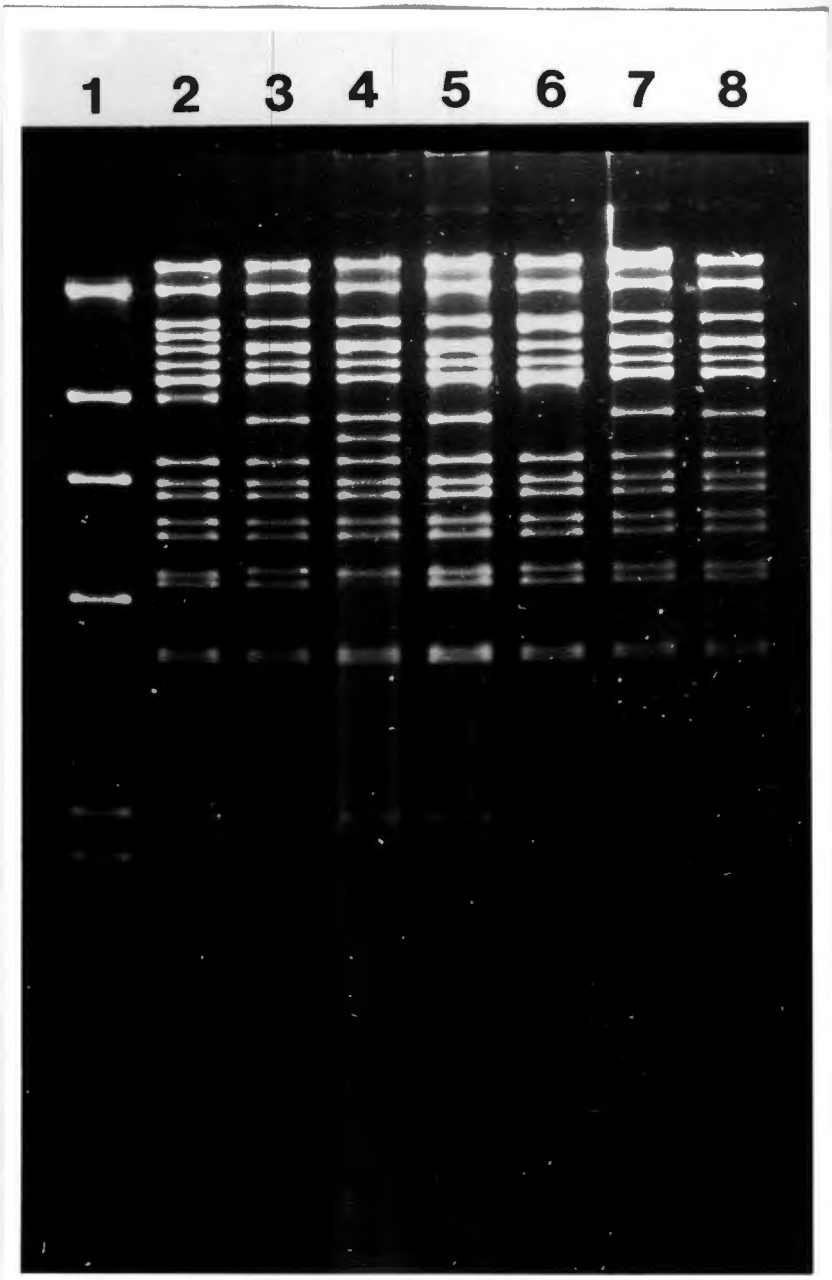


Fig 11.3.3: Pst I restriction fragments of the Zaire-isolates of monkeypox. Lambda DNA restricted with Hind III was used as a molecular marker. Lanes 1 - 8: 1: lambda DNA (Hind III); 2: Z 241; 3: Z 369; 4: Mp 1324; 5: V85-240; 6: V86-21; 7: V86-112; 8: Sq 249. Electrophoresis was done in 0.8% agarose at 1.4 Volt/cm for 16 hours.

A limited study with Eco RI showed similarity in the patterns of the Zaire isolates (including the squirrel isolate), which could be differentiated from the pattern produced by Mp Denmark with the same restriction enzyme.

Thus, the monkeypox virus in 3 different areas had developed to give 3 distinct patterns in restriction digests of their DNA genomes. The close similarity of the squirrel isolate and Zaire isolates strongly suggests that the isolates came from the same epidemiological maintenance cycle.

The differences in Pst I patterns were constant between the different endemic areas and it was decided to locate the regions of difference on the genome. This was done by determining the location of Pst I cleavage sites on genomes of one representative virus from each area (as detailed in Chapter 12).

CHAPTER 12

CONSTRUCTION OF PST I RESTRICTION MAPS FOR THREE MONKEYPOX STRAINS

Pst I restriction sites were established for Mp Denmark, Sq 249 and Mp Benin, each of which were considered to be representative of the West Coast, Zaire and Nigerian isolates of the monkeypox virus (Chapter 11).

In constructing a restriction map de novo, restriction enzymes are used that will not produce more than 20 fragments when the genome to be mapped is restricted with it. With 2 of these enzymes (A and B), single and double digests are done and the sizes of the fragments produced are determined. Fragments that remain the same size after restriction with A and with A+B are contained completely within a B fragment. Fragments from an A digest that disappear after the double digest will be replaced by 2 or more smaller fragments of which the size will add up to the same size as the A fragment that disappeared. The same applies to fragments created by B. When an A fragment wholly contains a B fragment and the neighbouring B fragment stretches into the next A fragment, the internal terminal pieces of the 2 A fragments will add up to the size of the B fragment bridging the 2 A fragments when a double (A+B) digest is done.

Inspections of generated fragments and sizes are done and more enzyme combinations are included. In this way, a rough restriction map is constructed for 2 or more enzymes.

In linear DNA with cross-linked termini, the ends can be identified by their ability to renature quickly when DNA fragments (after restriction digest) are allowed to re-anneal after denaturing of all the fragments.

Confirmation of the 2 or more tentative restriction maps is done by hybridization reactions, where the mapping results are confirmed if A and B (and others) fragments from the same area on the genome cross hybridize to each other. Once a map for any genome has been confirmed, it becomes easy to construct maps for other nucleases and also for non-identical, but closely related genomes of the same group. These principles and others were followed by De Fillipes (1982) to map vaccinia virus DNA.

In this study, the already constructed and confirmed Hind III restriction map for Mp Denmark (Mackett & Archard 1979) was used to compile Pst I maps for Mp Denmark and two other monkeypox strains. Methods used included the determining of sizes produced by Pst I restriction, determining the end fragments and doing hybridization reactions with radiolabelled fragments excised from gels. The methods used are detailed in Chapter 5.

12.1: METHODS

The sizes of Mp Denmark Hind III, Mp Denmark Pst I, Sq 249 and Mp Benin Pst I were estimated. An agarose gel with Hind III digests of lambda DNA and Hind III and Pst I digests of the appropriate monkeypox isolates were run at 1 Volt/cm for 20 hours (see fig 12.1.1). The digests were all heated at 65^{o}C for 5 minutes just

prior to loading the gel to break all non-covalent binding of fragments. If this was not done, a fraction of the lambda Hind III D fragment was non-covalently bound to some of the lambda Hind III A fragment and it would migrate as a fragment of a higher molecular weight.

The migration distance of the lambda Hind III fragments was measured and plotted against the $\log_{10}$ of the known sizes (Appendix) to form a standard curve. The migration distances of monkeypox fragments was measured and the $\log_{10}$ of sizes read from the standard curve. From these values the estimated sizes (in kb) of the various monkeypox fragments were calculated.

The covalently cross-linked end fragments of the 3 monkeypox types were established by denaturing all the fragments of a Pst I digest of the virus in the presence of formamide. This was also done for some of the Zaire isolates and it could be seen which fragments are positioned at the ends of the genome. See figures 12.2.3 and 12.2.4.

Hybridizations were done with Mp Denmark Hind III fragments, cloned into plasmids, to blots of Pst I digests of viruses representing the 3 described endemic areas. Later Mp Denmark Hind III, Mp Pst I or Sq 249 Pst I fragments that were immobilized onto nylon membranes were hybridized to [a-^{32}P]dCTP labelled (sometimes [a-^{32}P]dATP) probes that were prepared from fragments (Mp Denmark Pst I, Sq 249 Pst I, or Mp Benin Pst I) that were separated in and recovered from an agarose gel. After

hybridization and washing, autoradiographs which had been exposed to the hybridized blot, gave visual representations of which fragments hybridized where.

The deca-probe apparatus was mostly used in these reactions and a nick translated Pst I digest of the whole genome of the virus that was being mapped was usually loaded in the first hybridization chamber to mark the position of all the bands in the other chambers. Blots were boiled in 0.1% SDS for re-use.

Pst I Blot, Hind III probes: One strain of each of the three monkeypox Pst I types was digested with Pst I, electrophoresed and blotted. 3 Lanes of each were run next to each other and the tenth lane consisted of one of the Zaire isolates digested with Pst I (Mp Z 369). The first lane of every group was probed with ^{32}P- labelled Hind III N fragment of Mp Denmark, cloned into the plasmid pBR 329 and the third lane of each with the labelled Hind III K fragment of Mp Denmark, cloned into pBr 329. The middle lanes of each set of three were probed with a labelled Mp Denmark Pst I digest as a positive control and ^{32}P-labelled pBR 329 was used as a negative control in the tenth hybridization chamber.

The Mp Denmark Hind III N fragment is situated at both ends of the genome, while the Hind III K fragment of the same virus is found only at the right hand end of the genome, but may share some sequence homology with the left hand end of the Hind III F fragment as part of the right hand end of the inverted terminal repeat of the genome. A fragment which hybridizes to Hind III N

could be situated at either or both ends of the genome, while the K fragment will hybridize to fragments situated inside the right hand end of the genome and fragments that overlap the left hand end of Hind III F. See fig 12.2.14.

Pst I probes: For establishing the positions of Pst I sites in the Mp Denmark genome, Pst I fragments of the virus were radioactively labelled and hybridized to a Mp Denmark Hind III blot. When the position of Sq 249 Pst I fragments were established, the Mp Denmark Pst I digest (for deca-probe apparatus) was also immobilized on a nylon membrane and the probes were obtained from Sq 249 Pst I digests and fragments from these digests. Mp Denmark Hind III , Mp Denmark Pst I and Sq 249 Pst I blots were used for hybridization of radioactively labelled Mp Benin fragments.

12.2: RESULTS

All the appropriate monkeypox strains restricted with Hind III/Pst I and the molecular marker restricted with Hind III, were separated in a 0.8% agarose gel (fig 12.2.1). The migration distance in mm was plotted against the $\log_{10}$ of the known molecular size (in kb). See fig 12.2.2 for the resulting standard curve. The migration distances of fragments of known size were used to determine (calculating from the reading on the standard curve) what size (kb) the fragments were. Table 12.2.2 gives the fragments and their estimated lengths.

The published known sizes for Mp Denmark Hind III fragments could

also be used in size determinations. This was not done, because of small discrepancies that exist between values published by different authors. See table 12.2.1 for comparison between estimated and published sizes. The difficulty in making accurate size deteriminations for big (slow migrating) fragments is a reason for these differences.

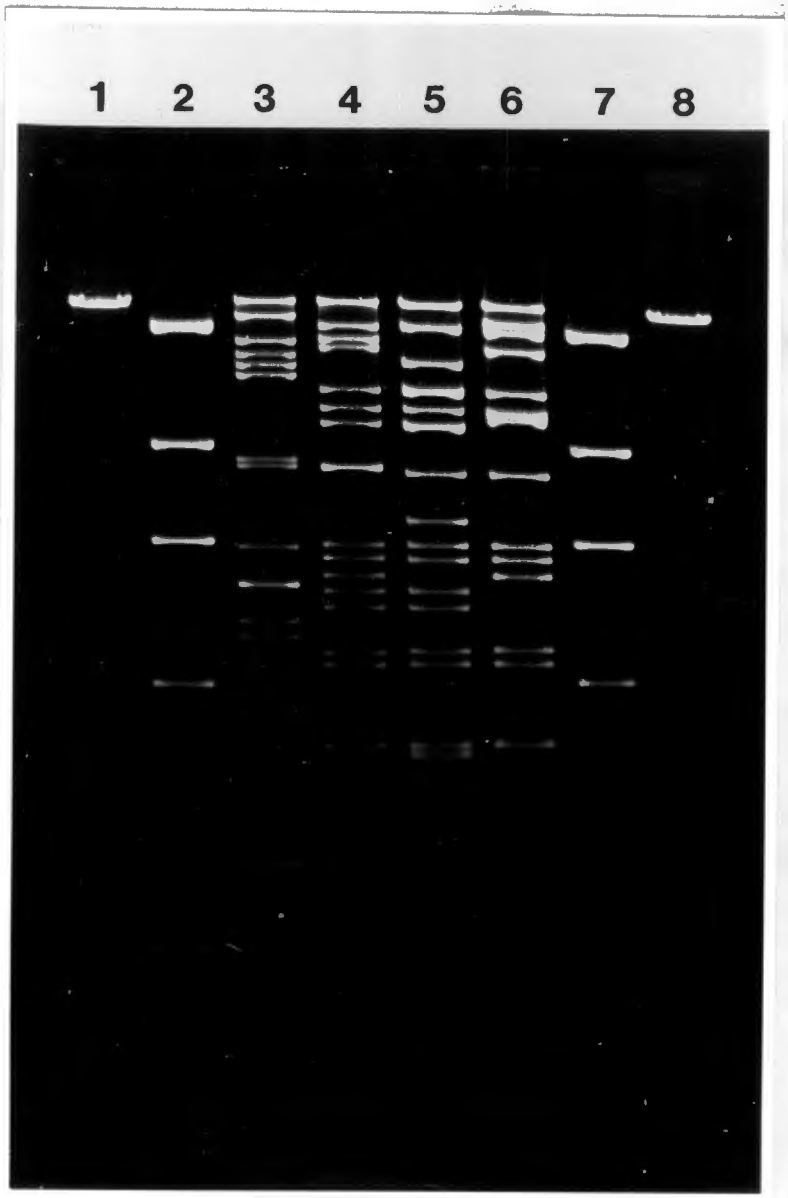


Fig 12.2.1: 0.8% Agarose gel with restriction digests of molecular marker and 3 different virus strains. Lanes 1 - 8. 1: Bacteriophage lambda, unrestricted; 2: Lambda Hind III; 3: Mp Denmark Hind III; 4: Mp Denmark Pst I; 5: Sq 249 Pst I; 6: Mp Benin Pst I; 7: Lambda Hind III; 8: Lambda unrestricted. All samples were heated at 65^{o}C for 5 minutes just prior to loading and electropohoresis was done at 1 Volt/cm for 20 hours.

Table 12 2 1: Fragment sizes of Mp Denmark Hind III restriction digest fragments as published and as determined by the author.

FRAGMENTS	MOLECULAR SIZE (kb) MP DENMARK HIND III FRAGMENTS				
	Author	Ref 1	Ref 2	Ref 3	Ref 4
A	48.0	45.0	45.5	46.9	36.5
B	28.8	25.5	25.8	26.2	29.0
C	20.0	17.6	17.7	18.2	19.6
D	17.8	15.5	15.6	15.8	17.0
E	16.2	14.3	14.4	15.1	16.0
F	15.1	13.4	13.5	13.7	14.4
G	8.9	8.9	9.0	8.9	9.0
H	8.7	8.4	8.5	8.5	8.7
I	6.6	6.5	6.5	6.2	6.3
J, J	5.8	5.8	5.8	5.5	5.7
K	5.2	5.1	5.2	5.1	5.0
L	4.7	4.8	4.9	4.9	4.7
M	4.0	3.9	3.9	4.0	3.8
N, N	2.8	2.7	2.7	2.3	2.8
O	2.1	2.1	2.2	1.7	2.1
P	1.5	1.6	1.6	1.5	1.6
Q	1.4	1.4	1.5	1.4	1.5
TOTAL LENGTH:	206.2	191.0	192.8	193.7	192.2

Ref 1: Mackett & Archard (1979); Ref 2: Dumbell & Archard (1980)
Ref 3: Esposito et al. (1981); Ref 4: Esposito & Knight (1985).

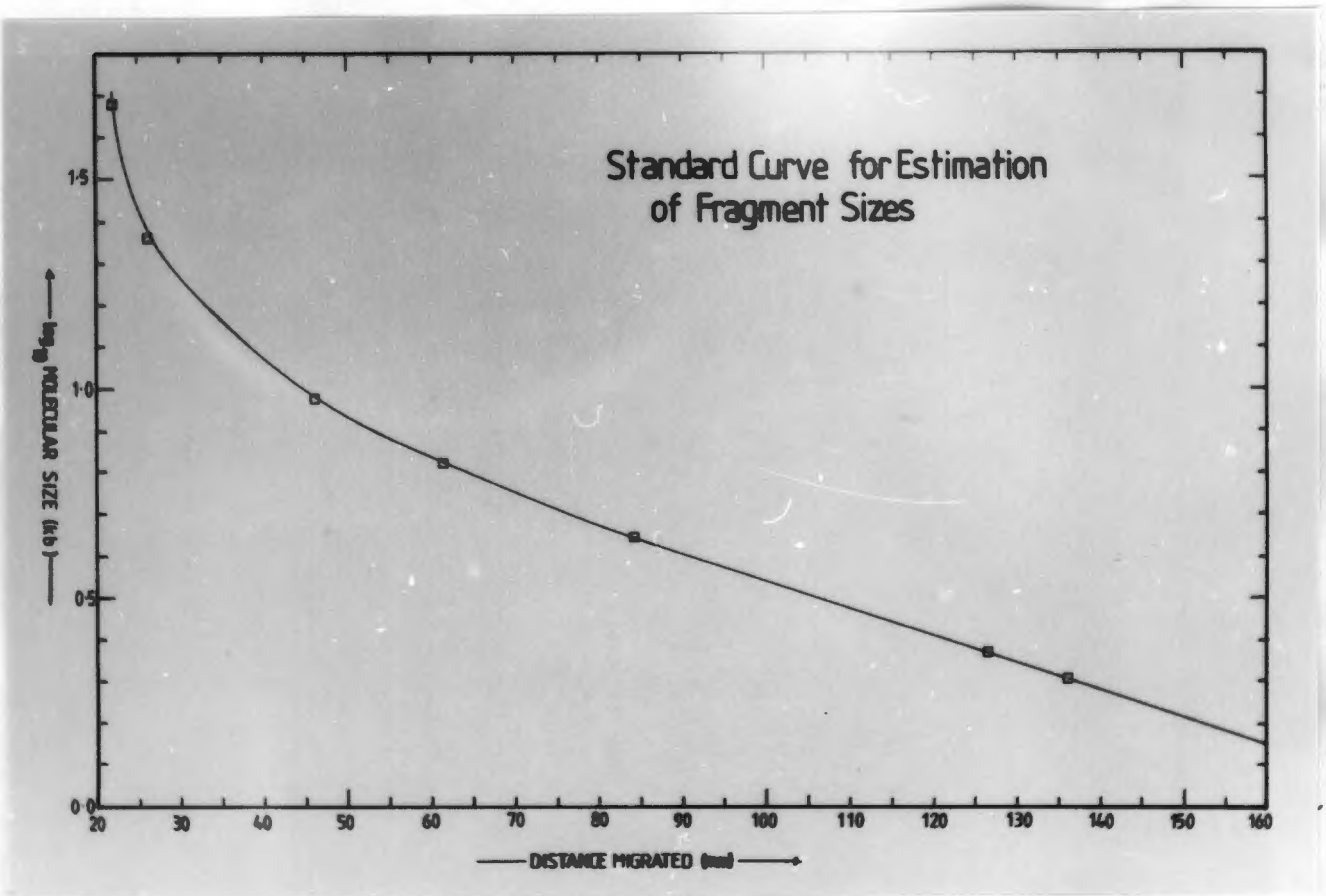


Fig 12.2.2: Standard curve for estimation of fragments sizes. The distance a fragment migrated (x-axis) was measured in mm from the inner edge of the loading well. The $\log_{10}$ of molecular size (in kb) was plotted on the y-axis. Molecular sizes of the molecular marker, Bacteriophage lambda cl 857 Sam 7, were taken from Oliver and Ward (1985).

Table 12.2.2: The estimated sizes of fragments generated (kb) by Hind III/Pst I restriction digests of 3 different monkeypox strains.

Molecular size	Fragments of Viruses Investigated			
	Mp Denmark		Sq 249	Mp Benin
kb	Hind III	Pst I	Pst I	Pst I
48.0	A	-	-	-
39.8	-	A	A	A
28.8	B	-	-	-
25.1	-	-	-	B
24.0	-	B	B	-
21.9	-	-	-	C
21.4	-	C	-	-
20.0	C	-	-	-
19.4	-	D	-	-
18.3	-	-	-	D
17.8	D	-	-	-
16.6	-	-	C	-
16.2	E	-	-	-
15.1	F	-	-	-
13.8	-	E	-	-
13.5	-	-	D D	E
12.4	-	F	-	-
12.0	-	-	E	-
11.8	-	-	-	F F
11.1	-	-	-	G
11.0	-	G	-	-
10.7	-	-	F F	-

Molecular size	Fragments of Viruses Investigated			
	Mp Denmark		Sq 249	Mp Benin
kb	Hind III	Pst I	Pst I	Pst I
8.9	G	-	-	H
8.7	H	H H	-	-
8.4	-	-	G	-
7.1	-	-	H	-
6.6	I	I	I	I
6.3	-	J	-	J
6.2	-	-	J	-
6.0	-	K	-	K
5.8	J J	-	-	-
5.7	-	L	K	-
5.4	-	M	L	-
5.2	K	-	-	-
4.8	-	N	M	L
4.7	L	-	-	-
4.6	-	O	N	M
4.0	M	-	-	-
3.8	-	P	O	N
3.7	-	-	P	-
2.8	N N	-	-	-
2.3	-	-	Q	-
2.1	O	-	-	-
1.7	-	Q	R	O
1.5	P	-	-	-
1.4	Q	-	-	-

Molecular size	Fragments of Viruses Investigated			
	Mp Denmark		Sq 249	Mp Benin
kb	Hind III	Pst I	Pst I	Pst I
Total length:	206.2	204.1	201.1	196.0

The covalently closed hairpin ends of several monkeypox viruses were identified. Figure 12.2.3 and 12.2.4 illustrate how the length of the end fragments vary from one isolate to another. Although the same basic Pst I restriction pattern was preserved in all the monkeypox isolates from Zaire, the end fragments vary in length. Table 12.2.3 gives a summary of Pst I digest fragments situated at the termini of the genome as it was established for the various monkeypoxes that were investigated.

Table 12.2.3: Monkeypox isolates and the Pst I restriction fragments that were identified as quickly renaturing covalently closed "hairpin" ends of the specific isolate.

Virus Isolate	Pst I Fragments
Mp Denmark	H, H
Z 241	D, H
Z 369	D, G
Mp Benin	B, H
Mp 1324	D, G
Sq 249	D, G
V85-240	D, G
V86-21	D, G
V86-112	D, H

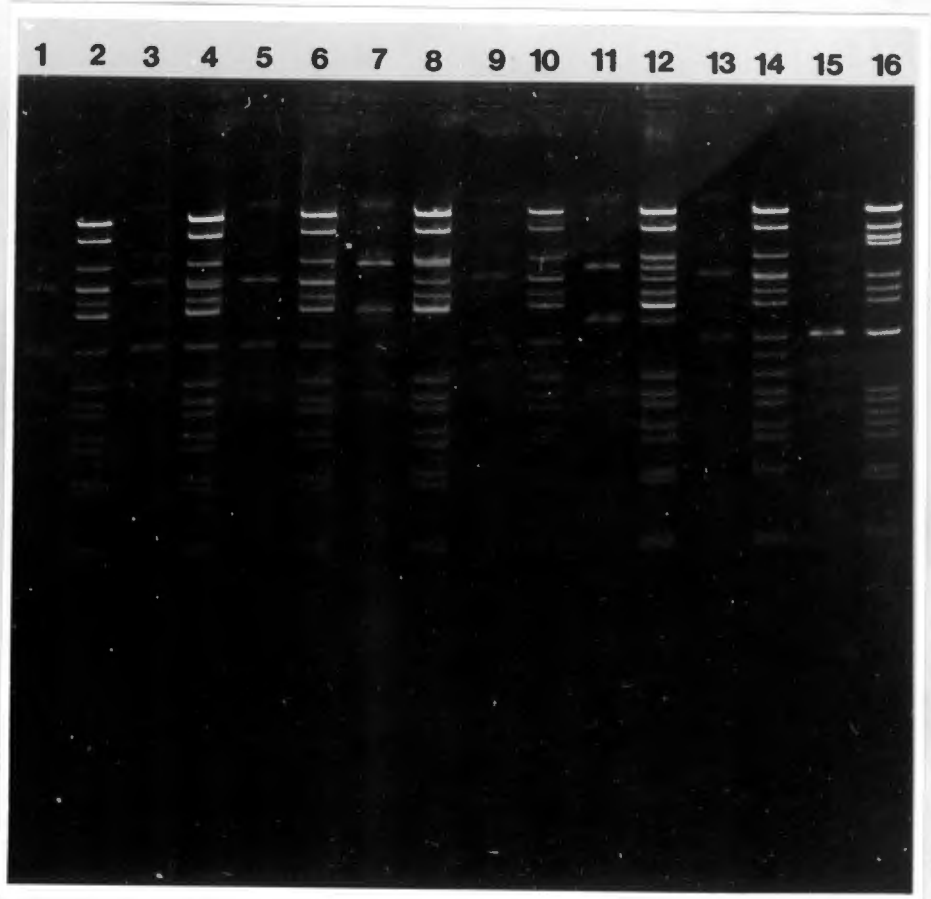


Fig 12.2.3: Denatured (95% formamide, 65°C) monkeypox Pst I digests were electrophoresed in 0.8% agarose gel at 1.4 Volt/cm for 16 hours, together with non-denatured Pst I digests of the same virus. Lanes 1 - 16. 1: Sq 249, denatured; 2: Sq 249; 3: V112-86, denatured; 4: V112-86; 5: V85-240, denatured; 6: V85-240; 7: V86-21, denatured: 8: V86-21; 9: Z 369, denatured; 10: Z 369; 11: Z 241, denatured; 12: Z 241; 13: Mp 1324, denatured; 14: Mp 1324; 15: Mp Denmark, denatured; 16: Mp Denmark.

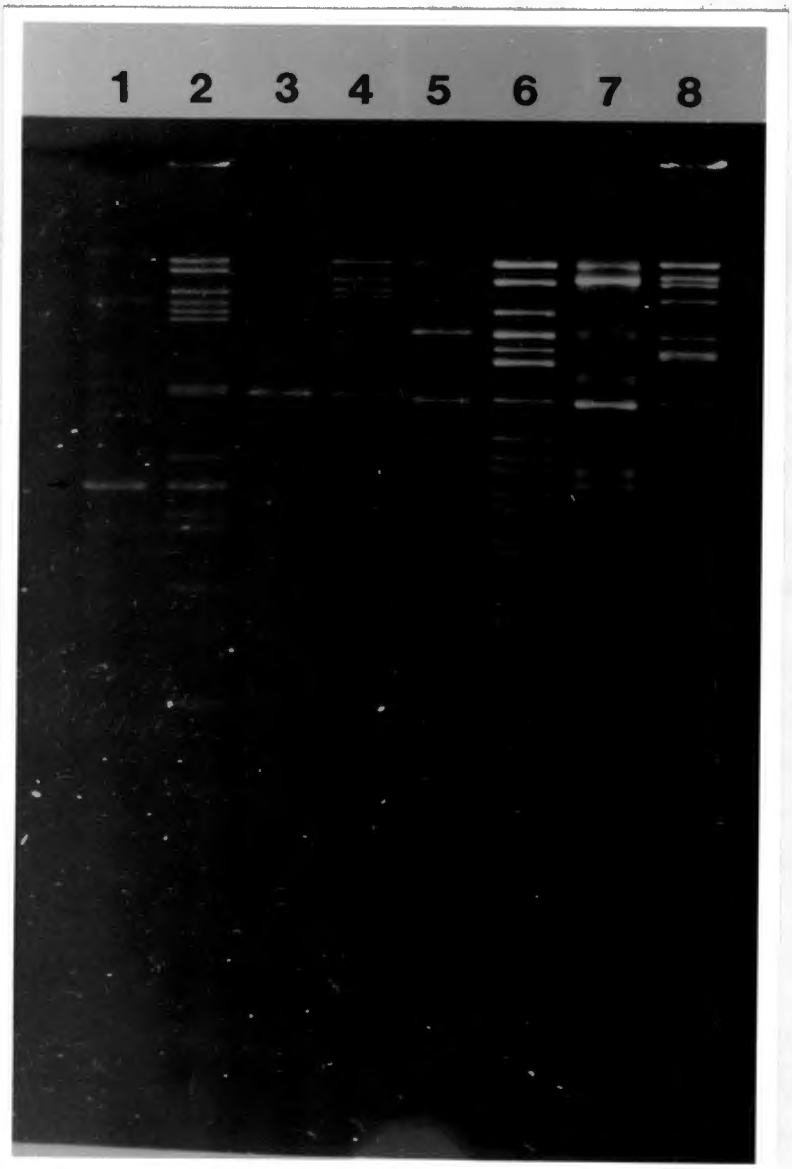


Fig 12.2.4: Denatured (95% formamide, 65°C) monkeypox restriction (Hind III/Pst I) digests were electrophoresed in a 0.8% agarose gel at 1.4 Volt/cm for 16 hours together with non-denatured digests of the same viruses. Lanes 1 - 8. 1: Mp Denmark Hind III, denatured; 2: Mp Denmark Hind III; 3: Mp Denmark Pst I, denatured; 4: Mp Denmark Pst I; 5: Sq 249 Pst I, denatured; 6: Sq 249 Pst I; 7: Mp Benin Pst I, denatured; 8: Mp Benin Pst I.

After the hybridization reaction, the obtained autoradiographs were used to compile a Pst I restriction map for Mp Denmark, Sq 249 and Mp Benin.

When inspecting results of the hybridization between cloned Mp Denmark Hind III clones N and K and Pst I digests of Sq 249, Mp 266 and Mp 82, Hind III N hybridized to Sq 249 Pst I G and D, while Hind III K hybridized to Sq 249 Pst I M/N and R. Hind III N hybridized to Mp 266 Pst I H (double fragment), while K hybridized to N/O and Q and slightly to H. Hind III N hybridized to Mp 82 Pst I B, H and L, while Hind III K hybridized to Mp 82 Pst I L and N and slightly to B. When Mp 82 and Mp Benin Pst I restriction patterns were compared, it was seen that Mp 82 Pst I N very probably corresponds to Mp Benin Pst I O and MP 82 Pst I L to Mp Benin Pst I M. It was deduced that Mp Denmark Hind III would hybridize to Mp Benin Pst I O and L. The labelled plasmid control did not hybridize to any of the viral sequences, so that none of the hybridization signals can be attributed to the plasmid vector. See fig 12.2.5.

After further hybridization reactions, fragments that hybridized to each other were read off. Where Mp Denmark Pst I fragments were used as radioprobes to a Mp Denmark Hind III blot, the fragments that hybridized to each other are summarized in Table 12.2.3 and figures 12.2.6 - 12.2.8 show some of the autoradiographs that conveyed the information.

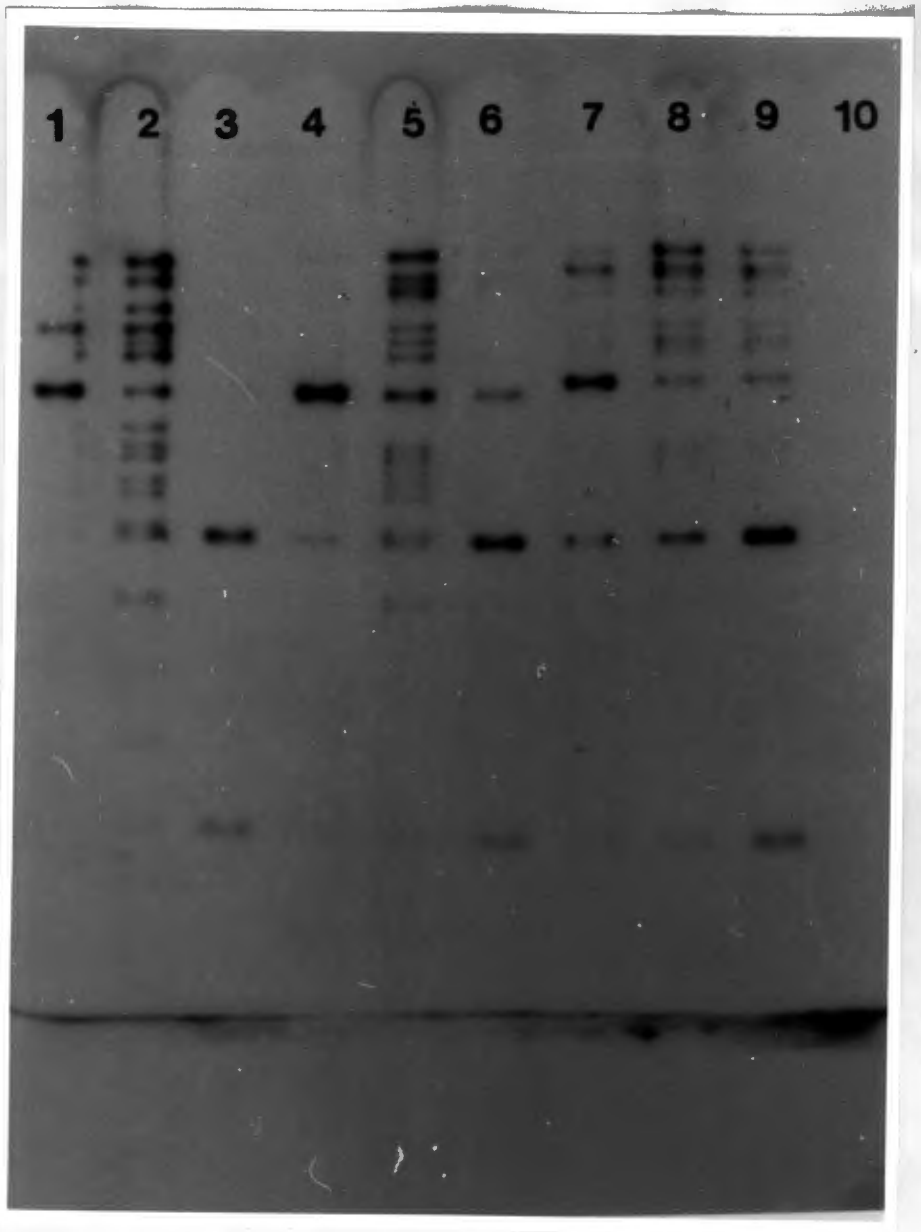


Fig 12.2.5: Autoradiograph of 3 monkeypox strains (Southern blot), hybridized to Mp Denmark Hind III probes. Lanes 1 - 3: Sq 249 Pst I; Lanes 4 - 6: Mp 266 Pst I; Lanes 7 - 9: Mp 82 Pst I; Lane 10: Mp Z 369 Pst I. Lanes 1, 4 and 7 were probed with ^{32}P-labelled Mp Denmark Hind III N cloned into pBR 329. Lanes 2, 5 and 8 were probed with ^{32}P-labelled Mp Denmark. Lanes 3. 6 and 9 were probed with ^{32}P-labelled Mp Denmark Hind III K cloned into pBR 329. Lane 10 was probed with ^{32}P-labelled pBR 329. The hybridizations were done in the deca-probe apparatus and the X-ray film exposed for 8 hours.

Table 12.2.3: Mp Denmark Pst I fragments, labelled with [a-^{32}P]dCTP and the Mp Denmark Hind III fragments that it hybridized to.

Mp Pst I ^{32}P-labelled fragments	Mp Denmark Hind III fragments
A	A, B
B	C, E, I, O
C	O, F
D	A, D
E	G, I
F	H, L
G	B
H	J, N
I	K/L, M
J	C
K	C
L	D, H
M	D/E
N	B, K
O	K
P	A
Q	K

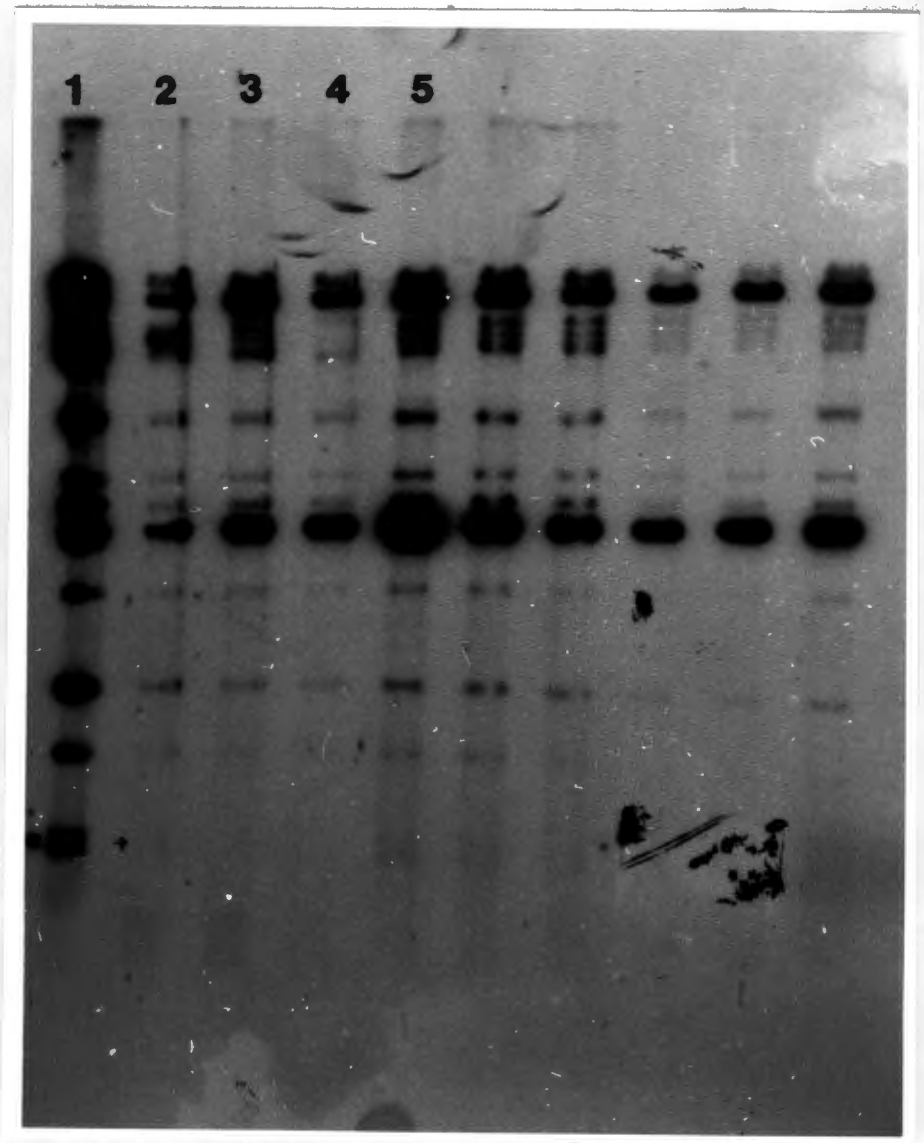


Fig 12.2.6: Autoradiograph of ^{32}P-labelled Mp Denmark Pst I fragments hybridized to a Mp Denmark Hind III blot (deca-probe apparatus). Lanes 1 - 5. 1: Mp Denmark Pst I whole genome; 2: Pst I M; 3: Pst I N; 4: Pst I P; 5: Pst I Q. The probes leaked between hybridization chambers, lighting up in lanes that were not in use. Signals of high intensity were considered to be real. The X-ray film was exposed for 8 hours.

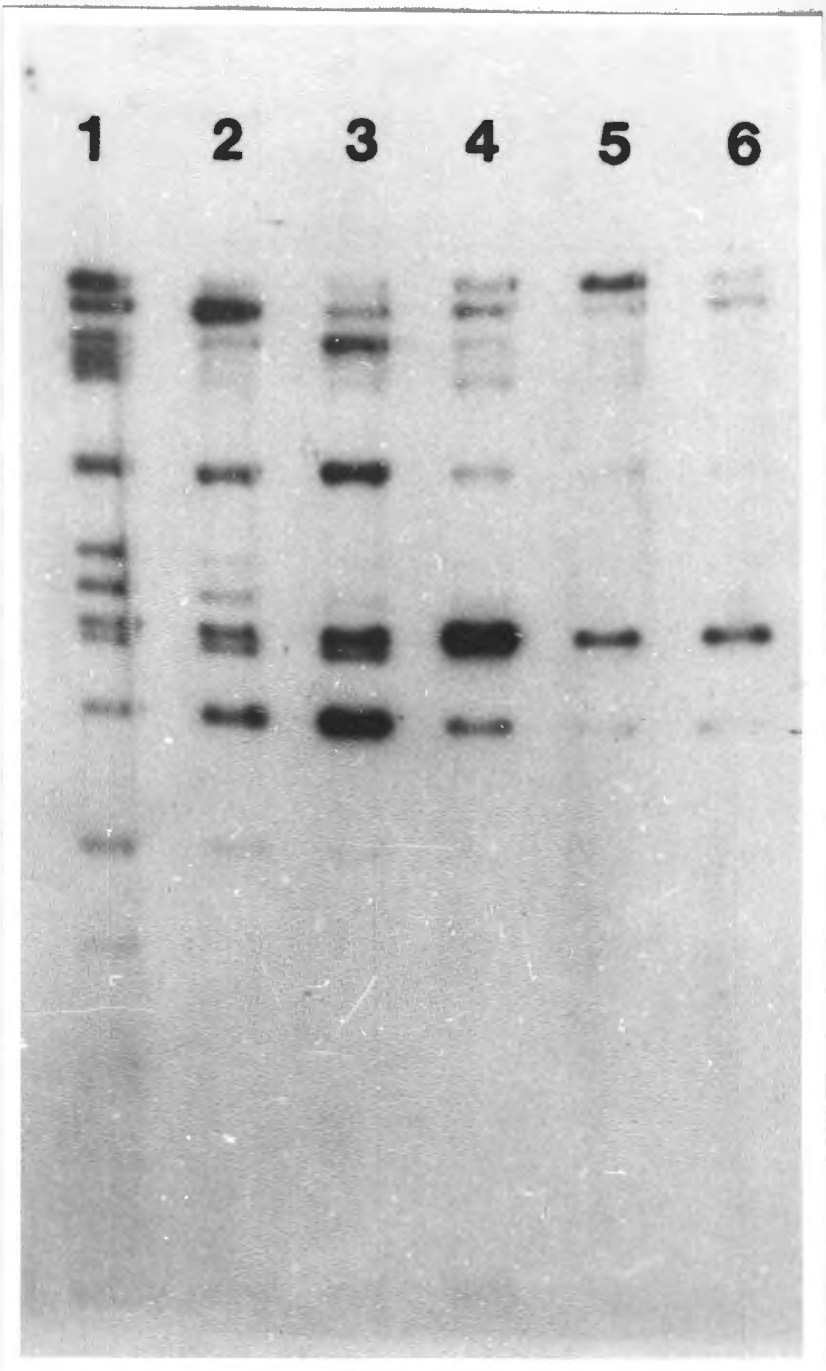


Fig 12.2.7: Autoradiograph of ^{32}P-labelled Mp Denmark Pst I fragments hybridized to a Mp Denmark Hind III blot (deca-probe apparatus). Lanes 1 - 6: 1: Mp Denmark Pst I whole genome; 2: Pst I G; 3: Pst I I; 4: Pst I O; 5: Pst I P; 6: Pst I Q. Before use the blot was boiled in 0.1% SDS to wash off previous hybridized probes (fig 12.2.6), but traces have remained which were disregarded in reading the results.

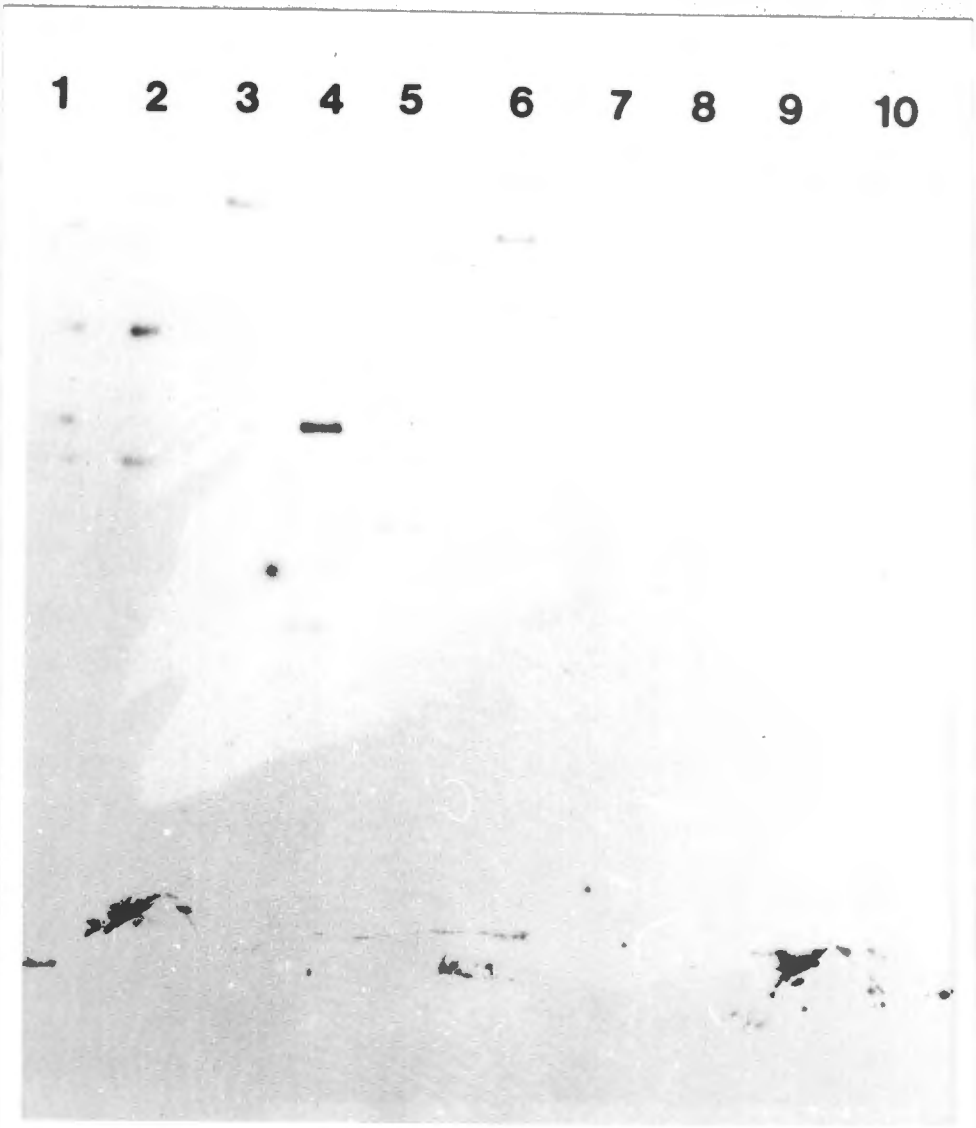


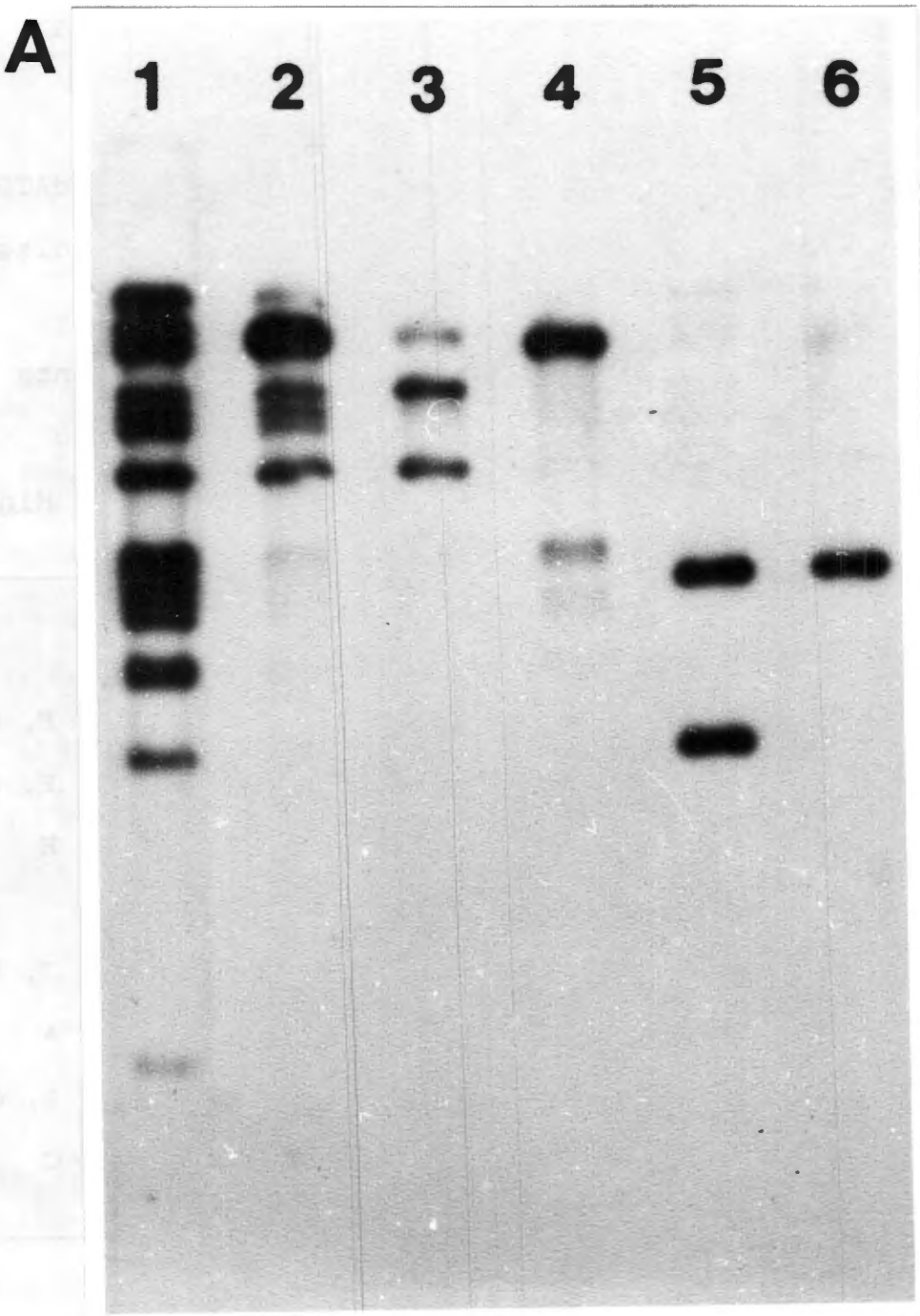
Fig 12.2.8: Autoradiograph of ^{32}P-labelled Mp Denmark Pst I fragments hybridized to a Mp Denmark Hind III blot (deca-probe apparatus). Lanes 1 - 10. 1: Empty; 2: Pst I F; 3: Pst I G; 4: Pst I H; 5: Pst I I; 6: Pst I J; 7: Pst I K; 8: Pst I L; 9: Pst I N; 10: Pst I P. The blot was used for the 3rd time after boiling in 1.0% SDS. The X-ray film was exposed for 5 days.

Sq 249 Pst I fragments were hybridized to Mp Denmark Hind III and Mp Denmark Pst I blots. Table 12.2. 4 summarizes the results obtained from the autoradiographs. These hybridizations confirmed the results from the Mp Denmark Pst I hybridizations to Mp Denmark Hind III blot. See figures 12.2.9 - 12.2 11.

Table 12.2.4: Sq 249 fragments labelled with [a-^{32}P]dATP and the Mp Denmark Pst I and Hind III fragments that it hybridized to.

Sq 249 Pst I ^{32}P-labelled fragments	Mp Denmark Fragments it hybridized to	
	Pst I	Hind III
B	B	
C	C	F, O, P
D, D	C, E, H	F, G, I, J
E	F	H
F	D, G	
G	H	J, N
H	D	A
O+P, P	K, P	A, C
Q	K	C

Fragments A, I, J, K, L, M, N and R were not used as probes as it was assumed that they would hybridize to the Pst I fragments of Mp Denmark that they co-migrate with. It was also assumed that Pst I P of Mp Denmark corresponds to Pst I O of Sq 249, so that Sq 249 Pst I P was responsible for hybridization of Mp Denmark Pst I K and Mp Denmark Hind III C.


A
1
2
3
4
5
6

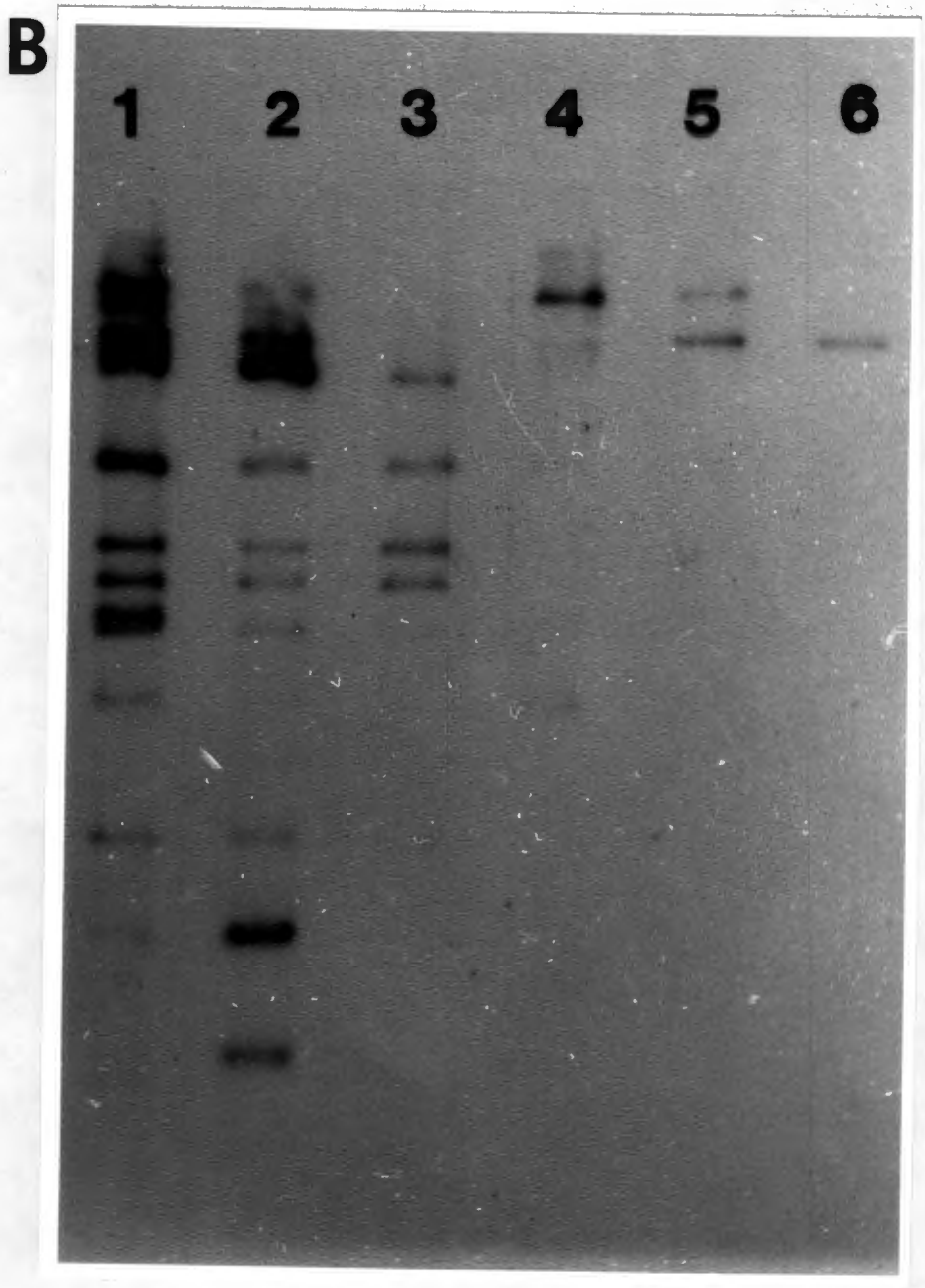


Fig 12.2.9 (A & B): Autoradiograph of Sq 249 Pst I ^{32}P-labelled fragments hybridized to a Mp Denamark Pst I blot (A) and a Mp Denmark Hind III blot (B). Lanes 1 - 6. 1: Sq 249 Pst I whole genome; 2: Pst I C; 3: Pst I D; 4: Pst I H; 5: Pst I O+P; 6: Pst I Q. X-ray film exposed overnight.

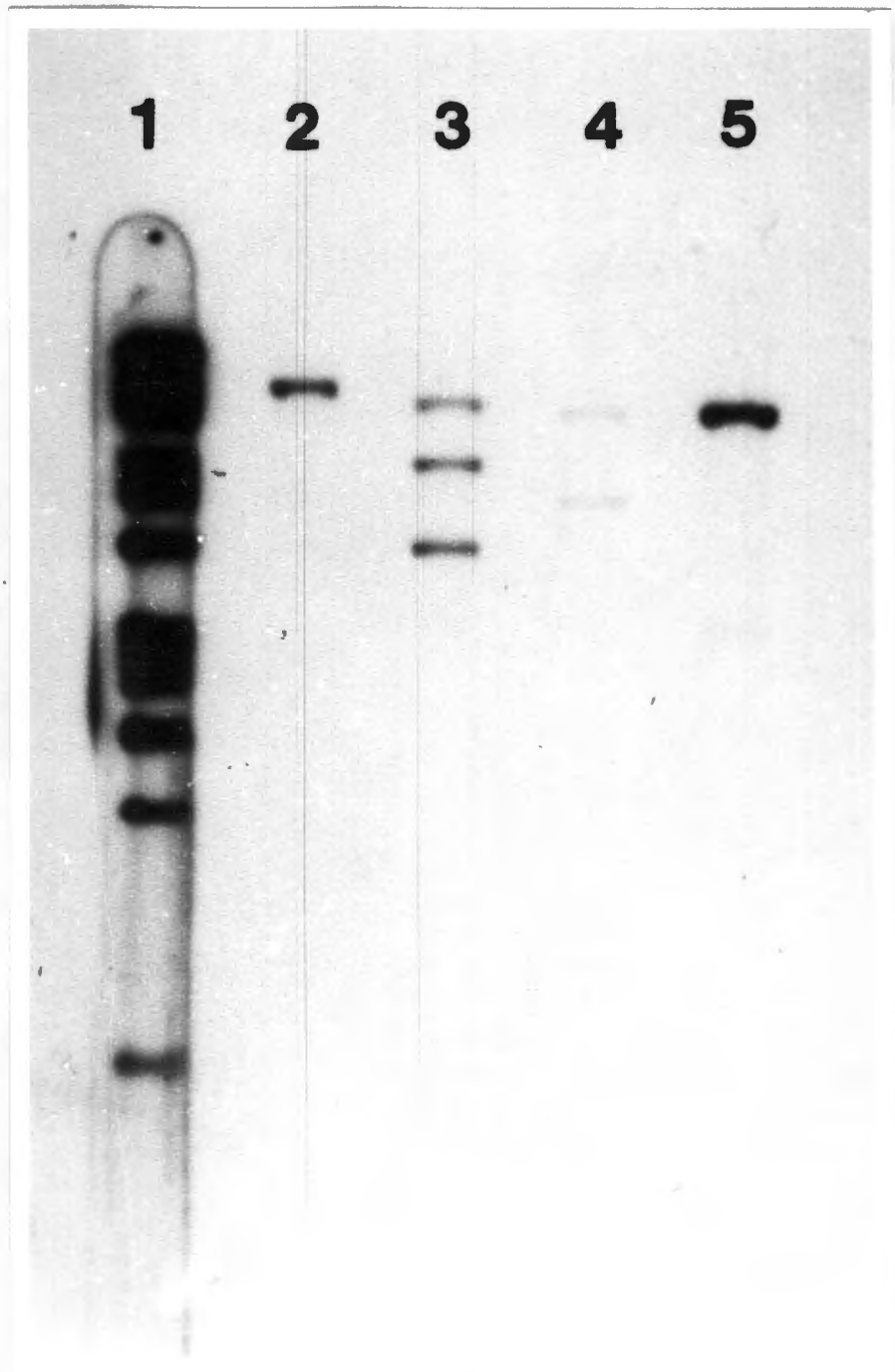


Fig 12.2.10: Autoradiograph of Sq 249 Pst I ^{32}P-labelled fragments hybridized to a Mp Denmark Pst I blot (deca-probe apparatus). Lanes 1 - 5: 1: Sq 249 Pst I whole genome; 2: Pst I B; 3: Pst I D; 4: Pst I F; 5: Pst I H. X-ray film exposed overnight.

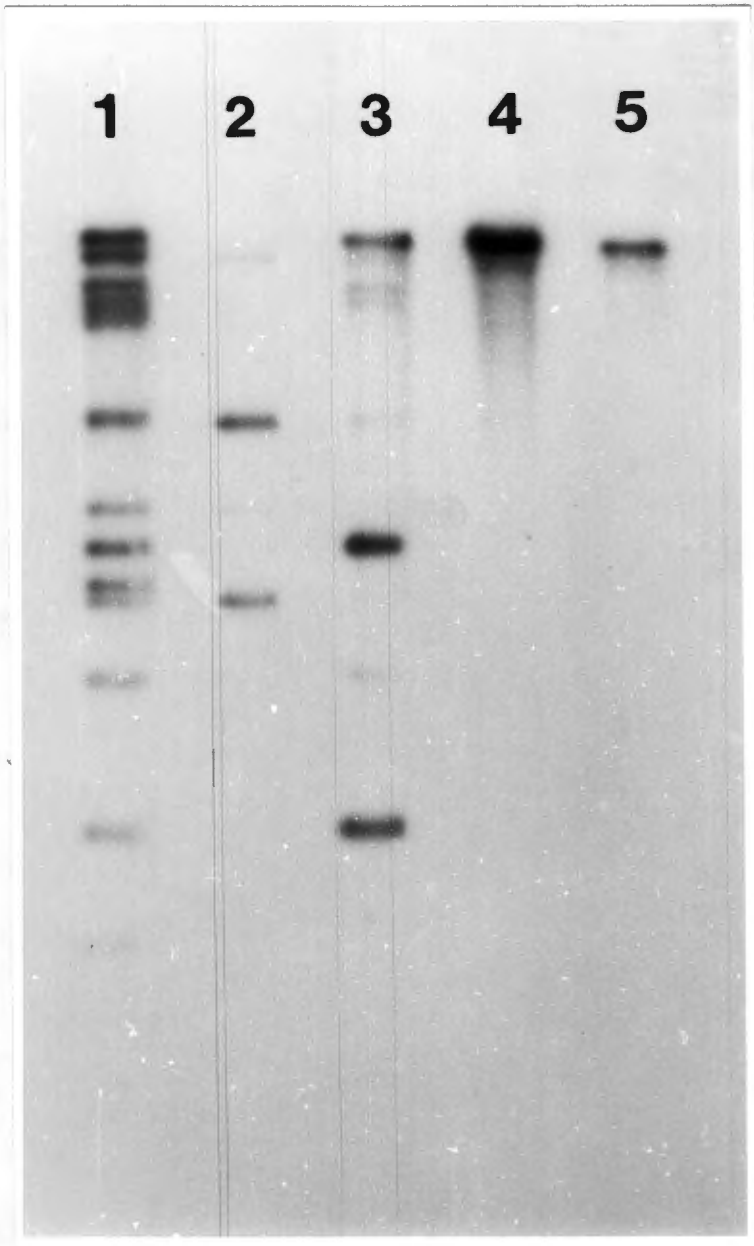
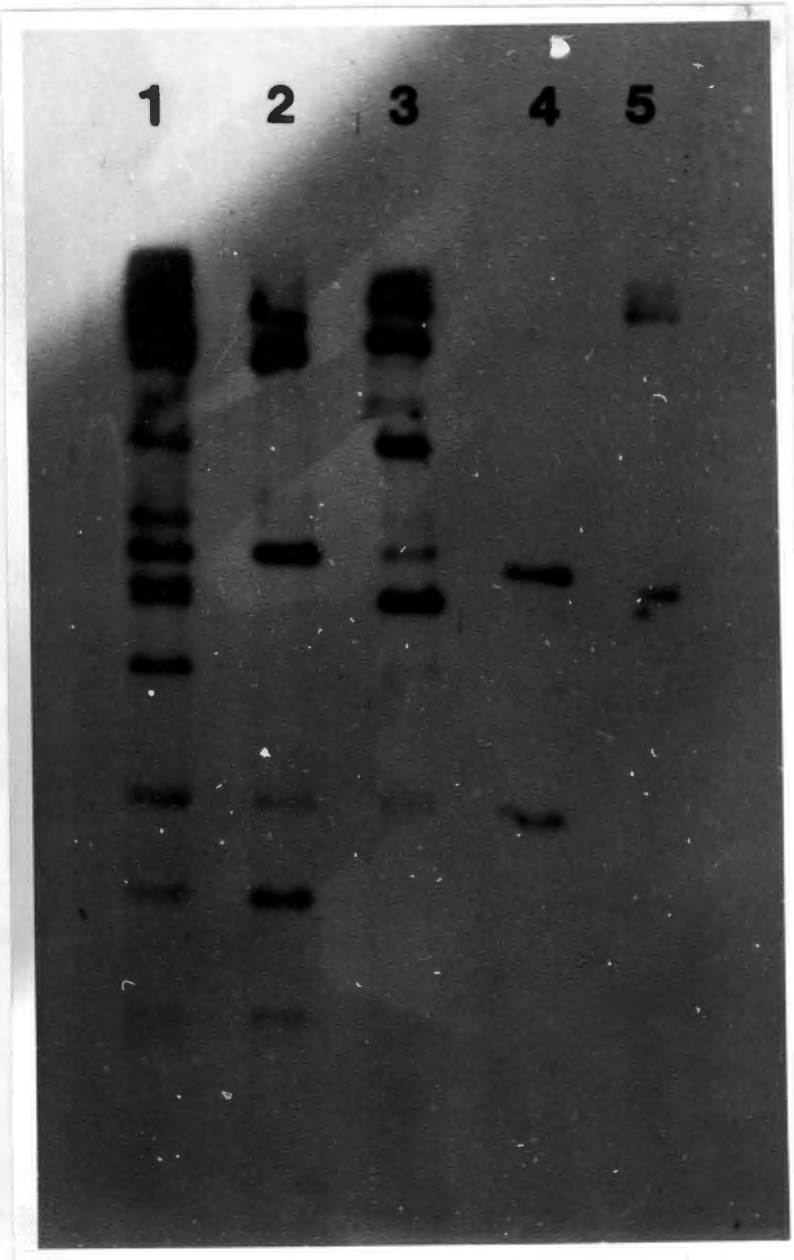


Fig 12.2.11: Autoradiograph of Sq 249 Pst I ^{32}P-labelled fragments hybridized to Mp Denmark Hind III blot (deca-probe apparatus). Lanes 1 - 5: 1: Sq 249 Pst I whole genome; 2: Pst I E; 3: Pst I G; 4: pUC 19 - Sq 249 Pst I H; 5: Mp Denmark Pst I P. (Note Sq 249 Pst I H has been cloned into plasmid pUC 19.)

A



The Benin Pst I fragments were hybridized to Mp Denmark Hind III and Pst I blots as well as to a Sq 249 Pst I blot, which also confirmed results of previous hybridizations. Table 12.2.6 summarizes the results. Figures 12.2.12 and 12.2.13 show some of the autoradiographs obtained.

Table 12.2.6: Mp Benin Pst I fragments, labelled with [a-^{32}P]dCTP and the fragments that it hybridized to.

Mp Benin ^{32}P-labelled fragments	Fragments it hybridized to: Mp Denmark Hind III	Mp Denmark Pst I	Sq 249 Pst I
B	J, F, O, P, N	C, H	
C/D	D, E	B, D	B, F, H
D/C	D, E	B, D	B, F, H
F+G	B, D, G/H, L	F, G, L, M	
H	J, N	H	D, G
K			P, Q
L	K, B	N	
M	K	O/N	N
N			O
O	K, B	O/N	R

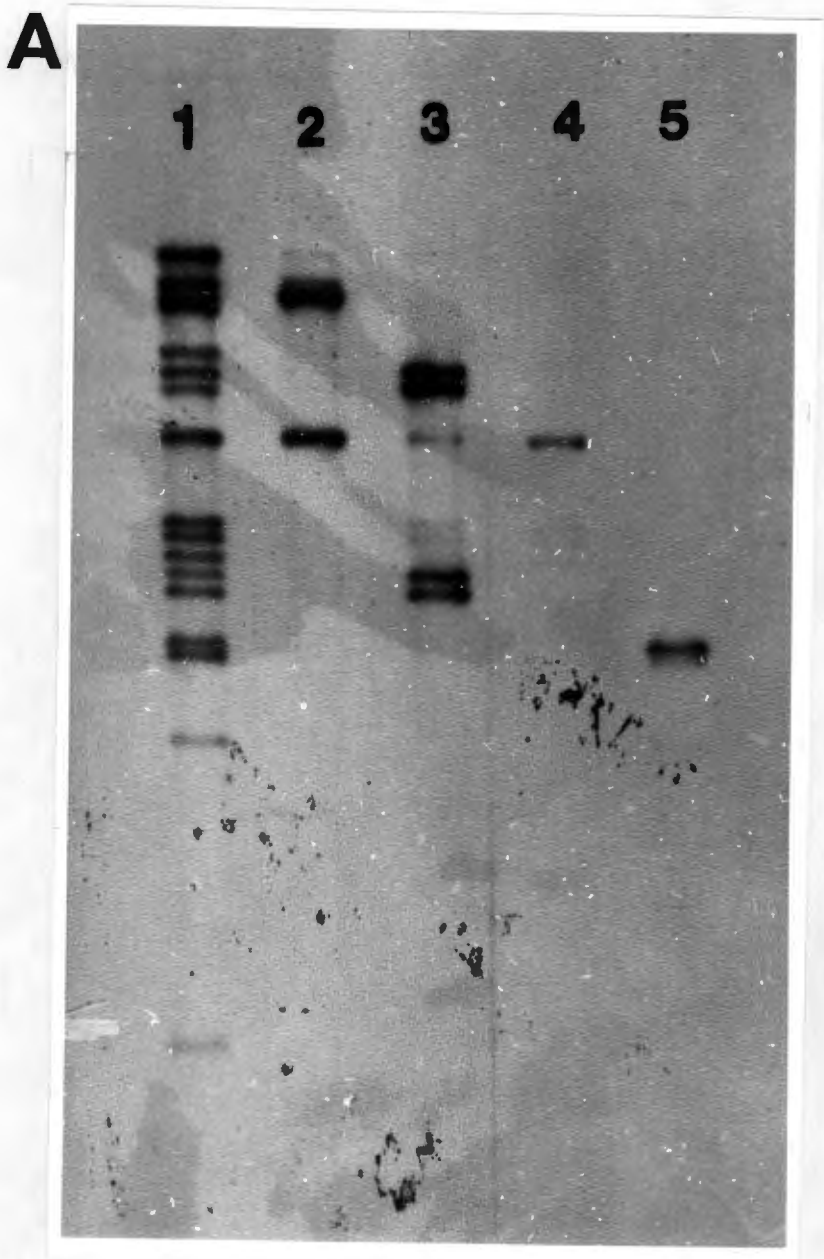


Fig 12.2.12: Autoradiographs of Mp Denmark Hind III (A) and Pst I (B) blots that were probed with ^{32}P-labelled Mp Benin Pst I fragments. Lanes 1 - 5. 1: Mp Benin Pst I whole genome; 2: Pst I B; 3: Pst I F+G; 4: Pst I H; 5: Pst I L. The same probes were used for both blots. X-ray film exposed overnight.

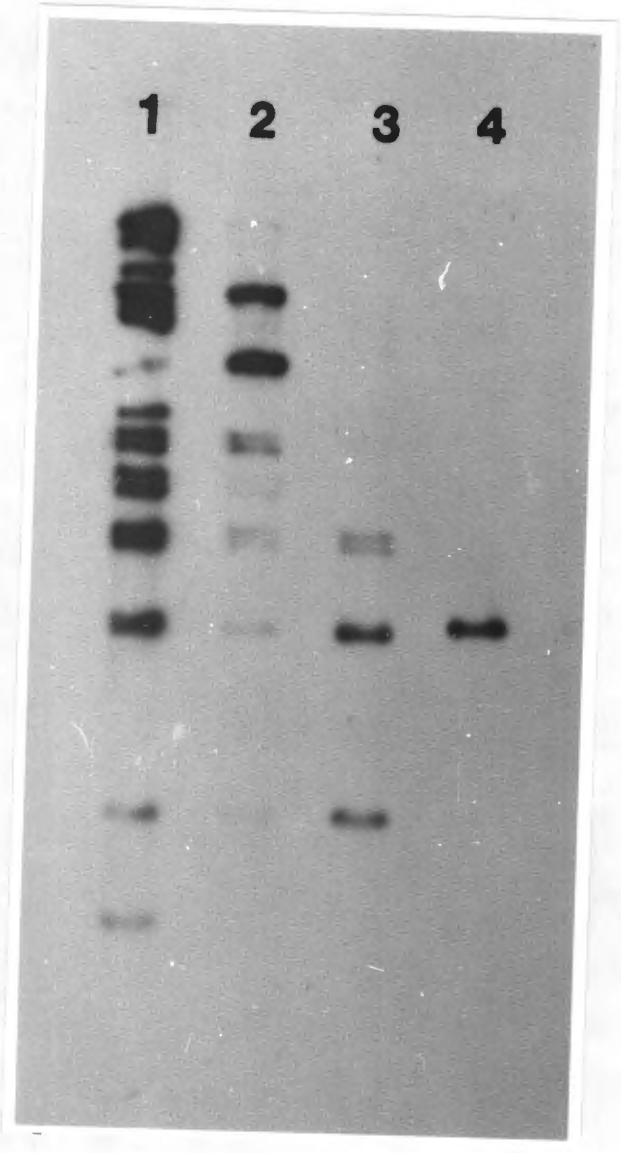


Fig 12.2.13: Autoradiograph of Sq 249 Pst I blot (deca-probe apparatus) probed with ^{32}P-labelled Mp Benin Pst I fragments. Lanes 1 - 4. 1: Mp Benin whole genome; 2: Pst I H; 3: Pst I K; 4: Pst I N. X-ray film exposed overnight.

Considering the results obtained from the hybridization reactions, the determination of end fragments and the estimated fragment sizes, the restriction sites for Pst I in the genomes of the 3 strains of monkeypox were established to be in the positions illustrated by figure 12.2.14.

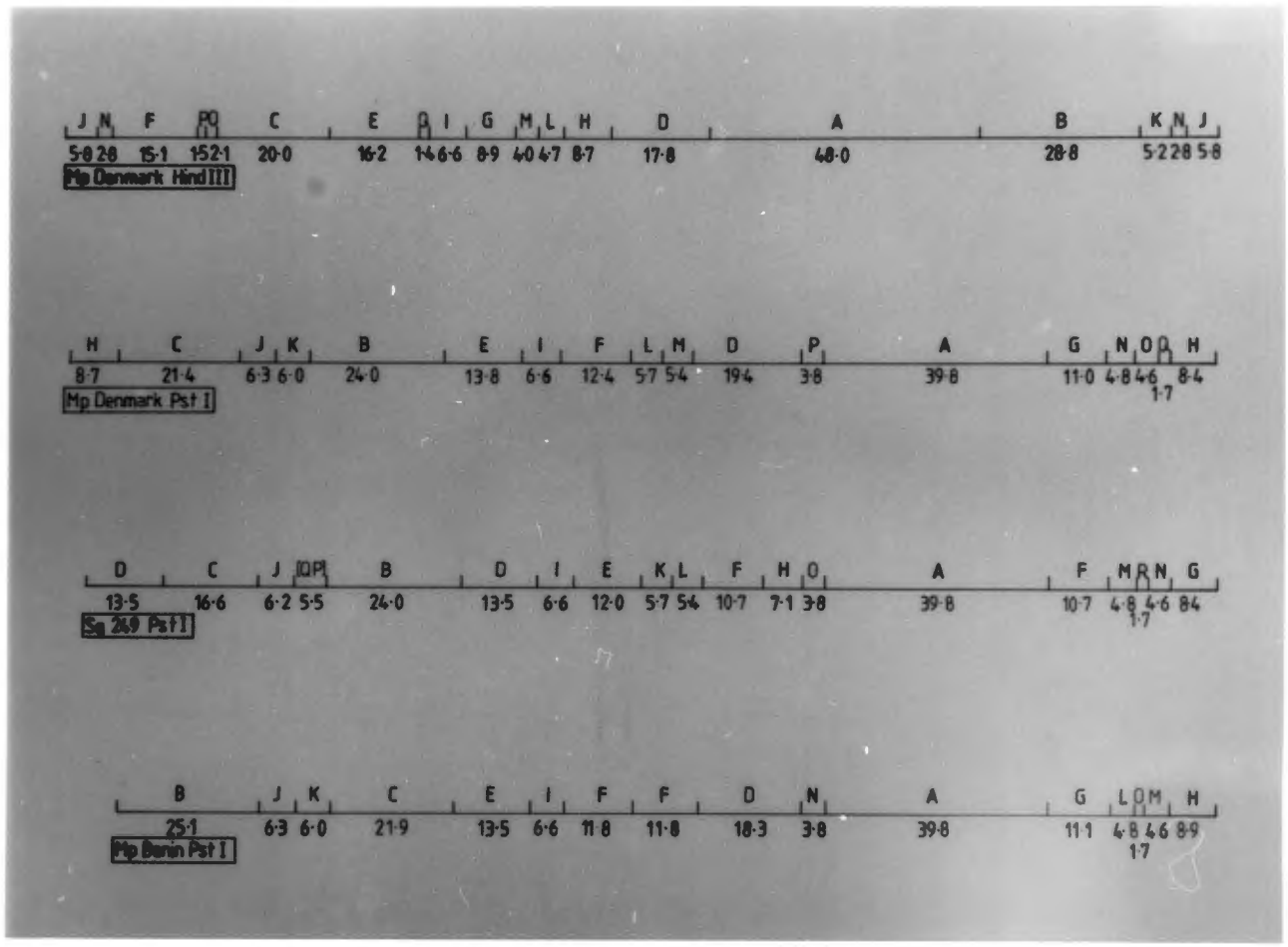


Fig 12.2.14: Hind III restriction map of Mp Denmark, with restriction sites as published before (Mackett & Archard 1979; Dumbell & Archard 1980; Esposito et al. 1981; Esposito & Knight 1985). Pst I restriction maps for Mp Denmark, Sq 249 and Mp Benin as it were constructed from results of hybridization reactions.

CHAPTER 13

DISCUSSION OF INTRASPECIES TYPING OF MONKEYPOX VIRUS

13.1: EVALUATION OF RESTRICTION ANALYSES

DNA was prepared from monkeypox virus that was obtained from various outbreaks of monkeypox since 1958. Viruses originated from disease in captive animals and more recently, humans. The sporadic occurrence of human monkeypox in Central and Western African rainforest areas (especially in Zaire), raised the question of which animal was acting as a reservoir host for the monkeypox agent. Virus was isolated from a sick squirrel, which was the first recorded case of monkeypox in an animal caught in the wild. The restriction analysis of DNA from this squirrel monkeypox virus and from human isolates from the same area was done to establish to what degree the viruses are related.

DNA from viruses that were isolated from human monkeypox cases in Zaire, was shown to be similar to that of the squirrel isolate with the restriction enzyme Pst I. Variations in fragment length noted were those in fragments that correspond to the termini of the genome. The same basic restriction pattern was observed for virus that was isolated in 1972 and the ones isolated in 1985. Eco RI restriction digests of the same set of viruses confirmed these results. It can therefore be concluded that the squirrel monkeypox and the virus from human isolates are the same, and form part of the same chain of transmission.

The restriction patterns of isolates that originated outside Zaire, were investigated and compared with those of the Zaire-isolates. These included 5 strains from captive animals in Europe and the USA, as well as 2 from Liberia and Sierra Leone. A further 2 originated from Nigeria.

Hind III did not distinguish between viruses from the different areas. Slight variations in the length of one of the fragments were observed between strains. This fragment represents the terminal fragments in a Hind III digest of Mp Denmark and probably also is the terminal Hind III fragments for the other monkeypox viruses.

Three distinct Pst I patterns were distinguished which were linked to the origin of the isolates. European, American, Liberian and the isolates from Sierra Leone all presented one pattern. The isolates from Nigeria exhibited a second pattern, while the isolates from Zaire presented a third pattern.

13.2: CONSTRUCTION OF PST I RESTRICTION MAPS

Mp Denmark was chosen as a representative of the European and West African types as a Hind III map of this genome was already available (Mackett & Archard 1979). Mp Benin represented the Nigerian group and Sq 249 the Zaire isolates. Pst I restriction sites on the genomes of these three viruses were established by cross-hybridization reaction. The Hind III restriction map for Mp Denmark, as established by Mackett and Archard (1979) and

confirmed by Esposito and Knight (1985), was used as a basis of these determinations.

The freeze-squeeze method for elution of fragments from agarose gels proved to be very effective, with a high recovery of DNA, which could be used directly in nick translation reactions.

The linkage order of the Pst I fragments of Mp Denmark was first established by cross-hybridizations to the separated Hind III fragments blotted onto nylon membrane. Cross-hybridizations were then done onto blots of Pst I digests of Mp Denmark to establish Pst I cleavage sites in the squirrel and Nigerian isolates and it confirmed the results that were obtained previously. Fragments of very high molecular weight (> 20kb) were produced with both Hind III and Pst I digestions of the DNA of the 3 virus strains and estimations of the sizes of these may only be approximate. The calculated genome size, by the sum of individual fragment sizes, differed only by about 7% from the genome sizes that have been published before.

On inspection of the 3 Pst I maps constructed from the obtained data, variations between the 3 genomes were found in the centre region as well as in the left hand end of the genome. A site appeared within the D fragments of Mp Denmark and Mp Benin to create the F and H fragments of Sq 249. A cutting site disappeared in the Mp Benin F fragments which consisted to two fragments in the corresponding area of Mp Denmark and Sq 249. The K fragments of Mp Denmark possess an extra site in Sq 249.

The left hand end of the genome was shortened for both Sq 249 and Mp Benin and in the case of the latter, only consisted of one fragment (B), while it consisted of 2 fragments in Mp Denmark and Sq 249. The variations in Pst I restriction maps for these viruses were thus found to be less dramatic than the marked difference in patterns in the electrophoresed gels.

Restriction analysis of the monkeypox viruses made distinctions between different strains possible and the investigation of these differences gave some information which will be useful in determining the origin and animal host of the monkeypox virus.

APPENDIX

ANTIBIOTIC STOCK SOLUTION (used at 1ml per 100 ml)

2×10^6 units Penicillin

2 g Streptomycin

2 g Neomycin

100 ml Physiological Saline (0.85% NaCl w:v)

Sterile filter.

EOSIN

70% of a 2% Eosin Solution in water and 30% of a 2% Phloxine in water.

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 Haematoxylin in absolute ethanol. Dissolve Alum in water (heat if necessary). Mix the 2 solutions together and heat to boiling point. Cool slightly and add Mercuric oxide. Cool rapidly. Add Glacial acetic acid before use. Filter.

SCOTT'S TAP WATER

$NaHCO_3$	3.5 g
$MgSO_4$	20 g
Tap water	1 liter

Dissolve salts separately, then mix. Thymol is added to prevent moulds.

HELLY'S FIXATIVE

Mercuric chloride	5.0 g
Potassium dicromate	2.5 g
Sodium Sulphate	1.0 g
Distilled water	100 ml
Formalin	5 ml, added immediately before use.

PHOSPHATE BUFFERED SALINE

NaCl	8.0 g
KCl	0.2 g
KH_2PO_4 (anhydrous)	0.91 g
or	
$Na_2HPO_4.2H_2O$	1.14 g
or	
$Na_2HPO_4.12H_2O$	2.28 g
Distilled water	1 liter

pH 7.5

Mc ILVAIN'S 4 mM BUFFER, pH 7.4

Stock Solution A: Citric acid 0.1 M

Stock Solution B: $Na_2HPO_4.12H_2O$ 0.2 M

For pH 7.4: Mix 1.38 ml of Solution A and 18.7 ml of Solution B. Add 800 ml of distilled water and pH with Solution A or B. Make up to 1 liter.

50 X TAE (Maniatis et al. 1982)

Tris base	242.0 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8)	100 ml

Dissolve and make up to 1 liter with distilled water.

PREPARATION OF PHENOL (Maniatis et al. 1982)

Commercial phenol (Merck) stored at -20°C (in small aliquots (200 ml)) after it was liquified.

Allow to warm to room temperature.

Melt at 68°C

Add Hydroxyquinoline to a final concentration of 0.1%.

Extract several times with an equal volume of 10 M Tris (pH 8), until the pH of the aqueous phase is higher than pH 7.6.

Store at 4°C under an equal volume of 0.1 M Tris (pH 8), containing 2% B-mercaptoethanol.

PREPARATION OF PROTEINASE K (Maniatis et al. 1982)

Powdered Proteinase K (Boehringer Mannheim) was dissolved in sterile distilled water at 20 mg/ml. It was aliquoted into 50 or 100 ul volumes and stored at -20°C.

PREPARATION OF RNAse (Maniatis et al. 1982)

Pancreatic Ribonuclease A (Sigma) was dissolved in 10 mM Tris (pH 7.5) and 15 mM NaCl - 10 mg/ml. It was heated at 100°C for 15 minutes and allowed to cool slowly to room temperature. It was dispensed in aliquots (50 ul) and stored at -20°C.

10 X STOP BUFFER

Glycerol v/v	50%
Na_2EDTA pH 8	100 mM
SDS w/v	1.0%
Bromophenol Blue	0.1%
Cyanol	0.1%

The EDTA was ommited from the buffer for use as a 10 x Loading Buffer.

20 X SSC (Maniatis et al. 1982)

NaCl	175.3 g
Sodium citrate	88.2 g

Dissolve in 800 ml distilled water. Adjust to pH 7 with 10 N NaOH. Adjust volume to 1 liter.

PREPARATION OF DIALYSIS TUBING (Maniatis et al. 1982)

Dialysis tubing (Spectrapor, 10 mm) is cut into 80 mm lengths. Boil for 10 minutes in a large volume of 2% Sodium Bicarbonate and 1 mM EDTA. Rinse thoroughly in distilled water. Boil for 10 minutes in 1 mM EDTA and allow to cool. Store submerged at 4°C. Before use, wash with distilled water. Wear gloves when handling.

PREPARATION OF SEPHADEX G50 (Maniatis et al. 1982)

30 g of Sephadex G50 (Pharmacia Fine Chemicals), fine or medium grade, is added to 250 ml TE pH 8 buffer (10 mM Tris, 1mM EDTA) in a 500 ml bottle. The powder must be dispersed well. Leave at room temperature overnight or heat at 65°C for 1 - 2 hours or autoclave for 15 minutes at 15 lb/in^2. Allow to cool to room temperature. Decant SNF and raplace with an equal volume of TE pH 8 buffer.

SILICONIZING GLASS WOOL

Work in a chemical hood.
Place glass wool in a glass beaker.
Add enough dichlorodimethylsilane (BDH) to wet all glass wool.
Swirl around and pour off.
Allow glass wool to dry.
Rinse in water several times.
Dry in oven at 180°C for 2 hours.

RESTRICTION FRAGMENT SIZES (IN KB) OF BACTERIOPHAGE LAMBDA CL 857 SAM 7, WHEN RESTRICTED WITH HIND III. (Oliver & Ward 1985)

Fragment	Length of Fragment (kb)
A	23.130
B	9.416
C	6.682
D	4.361
E	2.322
F	2.027
G	0.564
H	0.125

ABBREVIATIONS

A	Adenosine
C	Cytidine
CAM	Chick Chorioallantoic Membrane
CPE	Cytopathic Effect
Ci	Curie
cm	Centimeter
dATP	Deoxy-adenosine triphosphate
dCTP	Deoxy-cytidine triphosphate
DNA	Deoxyribo-nucleic acid
EDTA	Ethylenediaminetetra-acetic-acid
FCS	Foetal Calf Serum
G	Guanosine
HA	Haemagglutinin
H+E	Haematoxylin and Eosin
kb	Kilobase pairs
ml	Millilitre
mg	Milligram
mm	Millimeter
mM	Millimolar
MEM	Eagles Minimum Essential Medium
M	Molar
N	Normal
pfu/ml	Plaque or Pock forming units per millilitre
PBS	Phosphate Buffered Saline
RIA	Radiolinked Immunoassay
SDS	Sodium Dodecyl Sulphate
SNF	Supernatant Fluid

T	Thymidine
TC	Tissue Culture
Tris	Tris(hydroxymethyl)amino methane
ug	microgram
ul	microlitre
UV	Ultra Violet

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