RESTRICTION ENDONUCLEASE ANALYSIS OF DUCK PLAGUE VIRAL DNA

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled **"RESTRICTION ENDONUCLEASE ANALYSIS OF DUCK PLAGUE VIRAL DNA"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled **"RESTRICTION ENDONUCLEASE ANALYSIS OF DUCK PLAGUE VIRAL DNA"** is a record of research work done independently by **Mrs. Sangeetha Vijaysri**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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LIST OF ABBREVIATIONS

BHV	-	bovine herpes virus
CAM	-	chorioallantoic membrane
CAS	-	chorioallantoic sac
CEFC	-	chicken embryo fibroblast culture
CHV	-	caprine herpes virus
CMFPBS	-	calcium magnesium free phosphate buffered saline
CPE	-	cytopathic effects
DCE	-	developing chicken embryo
DDE	-	developing duck embryo
DEF	-	duck embryo fibroblast
DEFC	-	duck embryo fibroblast culture
DP	-	duck plague
DPV	-	duck plague virus
DPV-A	-	Alleppey strain of DPV
DPV-I	-	IVRI strain of DPV
DPV-V	-	vaccine strain of DPV
EBSS	-	Earle's balanced salt solution
EDTA	-	Ethylene diamine tetra acetic acid
EHV	-	equine herpes virus
ELD_{50}	-	embryo lethal dose 50
EM	-	Electron microscope
FHV	-	feline herpes virus
HBSS	-	Hank's balanced salt solution
HSV	-	herpes simplex virus
IBRV	-	infectious bovine rhinotracheitis virus
ILT	-	infectious laryngotracheitis
ILTV	-	infectious laryngotracheitis virus
IPVV	-	infectious pustular vulvovaginitis virus

IVPM	-	Institute of veterinary preventive medicine
IVRI	-	Indian veterinary research institute
Kbp	-	kilobase pair
KV	-	Kilovolt
M	-	Molarity
MDV	-	Marek's disease virus
N	-	Normality
NDV	-	Newcastle disease virus
NP40	-	Nonidet P40
nm	-	nanometer
OD	-	optical density
PAGE	-	poly acrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PCR	-	polymerase chain reaction
PHA	-	Passive haemagglutination
pi	-	post inoculation
PRV	-	pseudo rabies virus
PTA	-	phospho tungstic acid
RE	-	restriction endonuclease
REA	-	restriction endonuclease analysis
RFLP	-	restriction fragment length polymorphism
RHV	-	Reindeer herpes virus
rpm	-	revolutions per minute
S	-	Svedberg unit
SDS	-	sodium dodecyl sulphate
TBE	-	Tris boric acid EDTA buffer
$TCID_{50}$	-	tissue culture infective dose 50
TE	-	Tris EDTA buffer
TNE	-	Tris sodium chloride EDTA buffer
Tris HCl	-	Tris hydrochloride
VBI	-	Veterinary biological institute

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Introduction

INTRODUCTION

Duck farming is a popular occupation in the eastern and southern states of India. In Kerala, the climatic conditions, topography and presence of extensive waterlogged areas provide an excellent habitat for these birds. Duck rearing has contributed much to augment the income of marginal farmers and agricultural labourers. Compared to poultry, these hardy birds can withstand better, the variations in ecological and geoclimatic conditions and are found to be less susceptible to the wide range of avian diseases. Their good foraging capacity and their ability to act as agents for biological control of certain invertebrate vectors are added advantages. Therefore duck husbandry is fast becoming a valuable addition to the overconcentrated poultry industry, especially in the waterlogged areas of Kerala State.

The managemental and rearing practices followed by the majority of duck farmers, like free range system and movement of flocks during harvesting seasons and summer months to paddy fields and available water sources, subject these birds to great stress and expose them to various avian pathogens. Among the diseases that afflict ducks, duck plague (DP) caused by an alpha herpes virus, is an important one, causing heavy morbidity and mortality. Since a wide range of water birds, especially mallard ducks, are also affected by this disease,

it is suspected that wild birds may act as reservoirs of this viral pathogen (Burgess *et al.*, 1979).

Mukerji *et al.* (1963a) were the first to report the incidence of DP in India, from West Bengal. Now this disease has been found to be endemic in this country. DP was first recorded in Kerala by Punnoose and Abdulla in 1976. Since then, outbreaks of this disease have occurred almost every year in various parts of the state. Considering the widespread nature and severity of DP, it has now become a great threat to Kerala's duck population of nearly 8.5 lakhs.

Diagnosis of field outbreaks of DP has been based mainly on clinical findings and post mortem lesions. Although vaccination has been practised as a prophylactic measure in India since 1963, this disease often appears in vaccinated flocks also. Routine tests such as plaque reduction, virus neutralization and immunofluorescence have revealed that there are no antigenic differences between field isolates and vaccine strains. Various studies in India and abroad have shown that duck plague virus (DPV) isolates from different parts of the world are serologically homogenous (Jansen, 1961, 1964; Dardiri and Hess, 1968; Spieker, 1977, Lin *et al.*, 1984a, 1984b). This calls for molecular characterisation of the various isolates of DPV, to unearth differences, if any, between the serologically homologous strains.

Restriction endonucleases (RE) are the molecular scissors that bind specifically to and cleave double-stranded DNA at specific sites within or adjacent to a particular sequence of bases called recognition sequences. Therefore restriction endonuclease analysis (REA) can be used to distinguish isolates with different nucleotide sequences in their genome. This technique has been employed by various workers to identify different strains of the same virus and to study their relationship with other viruses.

There are many reports on the molecular characterisation of different herpes viruses with the aid of REs. Qiao (1992) has recorded that vaccine and virulent strains of DPV varied in their restriction pattern. Gardner *et al.* (1993) used molecular characterisation of anatid herpes virus to study its relatedness to other avian herpes viruses, its latency and the epidemiology of infections. No attempt has been made till date to compare the field isolates of DPV from Kerala with the vaccine strain commonly used here. Such a quest on the pathogenicity and molecular characterisation would give valuable insights into the characteristics of the viral strains, besides bringing to light, factors associated with the epidemiology of the disease and the possible causes of vaccination failures.

Therefore, the present investigation was undertaken to

1. Study the clinical and pathomorphological manifestations of the virulent and vaccine strains of DPV in ducklings and embryos and cytopathic effects in cell cultures.

2. Titration of various virus strains in cell cultures/ embryonated eggs.

3. Restriction enzyme analysis of the virulent and vaccine strains of DPV to detect molecular differences if any, between them.

Review of Literature

REVIEW OF LITERATURE

Duck plague (DP), also named as duck viral enteritis is an acute disease affecting ducks, geese and swan, of all ages and is usually associated with high morbidity and mortality. This contagious herpes virus infection is characterised by vascular damage with petechial haemorrhages in mucous membranes and tissues, especially of the gastrointestinal tract, free blood in the body cavities and lesions in lymphoid and parenchymatous organs (Leibovitz, 1991).

2.1 History

Although Baudet (1923) had reported an outbreak of an acute haemorrhagic disease in ducks in Netherlands, it was Jansen and Kunst (1949) who distinguished this disease from other viral diseases like duckling hepatitis, fowl plague and Newcastle disease. They proposed the name DP, which was later approved by the XIV International Veterinary Congress. Leibovitz and Hwang (1968) reported this disease from the United States for the first time and renamed it duck viral enteritis, based on the clinical signs.

2.2 Incidence and distribution

Duck plague had been recognised as a major disease of ducks in Europe, Asia (Jansen, 1963, 1968 and Jansen and

Kunst, 1964) and in the American continent (Leibovitz and Hwang, 1968). The first official report of the disease in India was from West Bengal in 1963 (Mukerji *et al.*, 1963a and b). Since then, the disease was reported from Kerala (Punnoose and Abdulla, 1976; Nair, 1978), Tamil Nadu (Duraiswamy *et al.*, 1979), Assam (Chakraborty *et al.*, 1980, John *et al.*, 1990) West Bengal (Bhowmik and Chakraborty, 1985) and Andhra Pradesh (Sreeramalu, 1986), which showed the endemic nature of this disease in India. In Kerala, heavy losses were incurred regularly by the duck farmers in waterlogged areas of Allepey, Kottayam, Pathanamthitta and Thrissur districts. The disease incidence was found to occur mostly during March to May (Kulkarni, 1993). Kulkarni isolated DPV in 1991 from vaccinated and unvaccinated ducks from six outbreaks in Kerala (Kulkarni *et al.*, 1995).

2.3 Aetiological agent

Breeze and Dardiri (1968) reported that DP was caused by a DNA virus. This belonged to alpha herpes virinae subfamily of the family Herpes viridae (Mohanty and Dutta, 1981).

2.3.1 Physiochemical and biological properties

DPV was found to be sensitive to ether and chloroform (Hess and Dardiri, 1968). They reported that it was inactivated by exposure for 18 hours at 37°C to trypsin,

chymotrypsin and pancreatic lipase but was resistant to the action of papain, lysozyme, cellulase and R Nase. Infectivity of the virus was retained for 30 days at 22°C but was destroyed by heating to 56°C in 10 minutes. This virus was found sensitive to the usual disinfectants and was inactivated by pH below 3 and above 10 (Leibovitz, 1991).

DPV was found to be a non-haemagglutinating (Jansen, 1961) and non-haemadsorbing virus which formed plaques and inclusion bodies in infected cell cultures (Dardiri and Hess, 1968). In the presence of complement, antibodies to DPV lysed infected duck embryo fibroblasts (Lam, 1984). Newcastle disease virus (NDV) effectively blocked multiplication of DPV but DPV did not interfere with NDV multiplication (Breeze and Dardiri, 1968).

2.3.2 Antigenic properties

DPV was found to be immunologically distinct not only from other avian viruses like NDV, fowl plague virus and duck hepatitis virus (Jansen and Kunst, 1949, Levine and Fabricant, 1950 and Dardiri and Hess, 1968) but also from other herpes viruses (Roizman *et al.*, 1981).

Jansen (1964) demonstrated that there was no antigenic variation between the Dutch and Indian strains. Dardiri and Hess (1968) also arrived at a similar conclusion on comparing

the American Long Island strain with the Holland strain, by plaque inhibition assay. Spiecker (1977) confirmed the serologic homogeneity of various DPV isolates.

Lin *et al.* (1984a) isolated a nonpathogenic strain (Sheridan 83) and found that the virulent strains like Holland strain and Long Island strain were serologically indistinguishable from nonpathogenic strains.

Deng *et al.* (1984) opined that reverse passive haemagglutination test (PHA) and immunoflourescence test were superior to the conventional plaque assay in the detection of DPV. Kulkarni (1993) stated that the simplicity and specificity of PHA was particularly advantageous when a large battery of serum samples was being screened.

2.4 Epizootiology

Anatids, ranging from domestic ducks, geese and swan to wild water fowl were reported to be the main hosts of DPV. Natural infection occurred mainly by direct and indirect contact. Leibovitz (1991) suggested that infected waterbodies played a significant role in the transmission of infection. According to him, all ages of ducks above one week were susceptible to DPV and opined that mechanical transmission by blood sucking arthropods could not be ruled out. In Kerala, all DP infected flocks during 1990-93 had a migratory history

and there were no reports of the disease in organised duck farms, which had no access to external water source (Kulkarni, 1993). A carrier state was suspected in wild ducks (Burgess *et al.*, 1979) and Leibovitz (1991) considered the mallard ducks as possible natural reservoirs of DPV.

Jansen (1964) recovered the viral agent from eggs removed from cloaca of infected domestic duck. Leibovitz (1971) and Prip *et al.* (1983) opined that transovarian transmission was not of great significance. Ziedler *et al.* (1984) also could not isolate the virus from eggs laid during natural outbreaks. There were no reports of excretion of the virus in experimentally infected birds, which had earlier been vaccinated (Kulkarni, 1993). Effect of the virus on fertility and hatchability varied depending on strain of virus and species of duck affected, muscovy ducks being more susceptible than white pekin.

2.5 Symptoms and lesions

Leibovitz (1971) found that the incubation period ranged from 3-7 days in domestic ducks. Characteristic signs of DP included drop in egg production, droopiness, ataxia, extreme thirst, lachrymation, photophobia and gluing of eyelids, nasal discharge, ruffled feathers, soiled vent with greenish watery diarrhoea, weakness along with tremors and high flock

mortality. Ailing birds stayed away from water, assuming a squatting posture with drooping wings. Hoarseness of voice and laboured breathing were noticed and mortality was found to be more severe in the presence of secondary bacterial infections (Dardiri, 1971). In subacute cases diphtheroid inflammation of cloaca was noticed.

On necropsy of infected birds, catarrhal proventriculitis, gizzard muscle necrosis, erythematous lesions of digestive tract, haemorrhages and necrosis of visceral organs and gonads, serosal haemorrhages and collection of free blood within body cavities and hollow organs were found (Leibovitz, 1969). Dardiri (1975) and Das *et al.* (1990) noted longitudinal rows of haemorrhage in the oesophagus and cloaca, which later became yellow or gray with haemorrhagic diphtheritic pseudomembranes and considered these lesions as pathogenomonic for DP. Haemorrhages were also seen in the bursa of Fabricius, ovary, conjunctiva, syrinx, lymphoid organs, liver, spleen and heart. There was red paint brush appearance of pericardium and mottled appearance of liver and spleen (Rajan *et al.*, 1980; Roy *et al.*, 1983; Bhowmik and Chakraborthy 1985). Chennakesavalu *et al.* (1987) demonstrated intranuclear inclusion bodies and also Cowdry type A inclusion bodies in the liver and epithelium of oesophagus and cloaca. Barr *et al.* (1992) reported the presence of intranuclear inclusion bodies in epithelial cells

of bursa of Fabricius, stratified squamous epithelium of oesophagus, intestine, splenic periarteriolar reticular cells and harderian glands.

2.6 Diagnosis

Diagnosis of DP was usually based on symptoms and lesions, supported by histopathological studies. Isolation and identification of the virus, in 12-14 day old embryonated duck eggs or ducklings or duck embryo fibroblast cell cultures provided confirmation, even in the absence of diagnostic morphological alterations. Virus neutralisation test, using anti DP serum could be effectively done in duck embryos, chick embryos or cell cultures (Dardiri and Hess, 1967). They also developed a plaque assay which could be inhibited by specific anti DP serum for identification of the virus. Agar gel diffusion test (Cottral 1978) immunofluorescence test (Erickson *et al.*, 1974) reverse passive haemagglutination test (Deng *et al.*, 1984), counter immunoelectrophoresis (John *et al.*, 1989) electron microscopic examination of the virus (Tantaswasdi *et al.*, 1988) and immunoperoxidase staining (Islam *et al.*, 1993) were found to be of value in the diagnosis of DP.

2.6.1 Isolation of DPV

2.6.1.1 Ducklings

The most sensitive method for isolation of DPV was found to be by infecting day old ducklings, preferably specific pathogen free with 0.5 ml of tissue suspension, by intramuscular route. If virus was present in the inoculated material, ducklings died with typical symptoms and lesions of DP (Leibovitz, 1971). Sarkar (1982) also reported the reproducibility of the disease in day old ducklings.

2.6.1.2 Embryonated eggs

Primary isolation of the virus could be done on chorioallantoic membrane (CAM) of 12-14 day old embryonated duck eggs, causing death in 4-6 days with extensive haemorrhages (Jansen, 1961). Mortality by virulent virus in duck embryo was maximum at 120-192 hours post inoculation (*pi*) with the virus titres reaching $10^{6.3}$ ELD_{50} per ml (Butterfield and Dardiri, 1969).

Chicken and developing chicken embryos (DCE) were found to be refractory to DPV (Bhowmik and Chakraborthy, 1985; Kulkarni, 1993). However DPV could be adapted to grow in DCE, following 12 passages in duck embryos and three blind passages in chicken embryo (Jansen, 1964). According to Dardiri (1975)

mortality of infected chicken embryo depended on virulence and concentration of DPV in the inoculum. They opined that maximum number of deaths on inoculation of DCE adapted DPV usually occurred between 72-192 hours *pi*. The virus concentration was highest in CAM and amnioallantoic fluid, while it was low in yolk and embryo.

By the twentieth passage in DCE, DPV totally lost its pathogenecity for ducks (Jansen, 1961) and this attenuated virus could be effectively used for active immunisation against DP (Jansen *et al.*, 1963). Jansen (1961, 1964) indicated that CAM was the route of choice for DPV but Butterfield *et al.* (1969) and Dardiri (1975) opined that chorioallantoic sac (CAS) route was superior to CAM. CAS route yielded higher titre of virus and the reason was attributed to simultaneous exposure of more number of cells to the virus through the medium of allantoic fluid (Kenneth and Lauffer, 1953).

Reduction in number of embryo mortality in the lag phase and earlier mortality in embryos inoculated by CAS route absolved laborious processing of older embryos, along with an increase in quantum of final harvest (Kalaimathi *et al.*, 1985).

2.6.1.3 Cell cultures

DPV was first isolated in duck embryo fibroblast cell cultures (DEFC) by Kunst (1967). He recorded the appearance of cytopathic effects (CPE) within 3-4 days *pi*.

Breeze and Dardiri (1968) studied the development of the virus in cell cultures by election microscopic (EM) examination of thin sections of infected cell cultures. They demonstrated the presence of both intranuclear and intracytoplasmic inclusions in infected cells. These inclusions produced by the virus were found to be either eosinophilic or basophilic.

Muscovy and wood duck cell cultures gave best results with regard to sensitivity, yield and plaque quality of DPV. In muscovy cell cultures, the virus exhibited a latent period of 6 hours with development of maximum titre of virus 36 hours after inoculation (Kocan, 1976).

Leibovitz (1971) titrated the virus in both chicken embryo and duck embryo fibro blast cultures (CEFC and DEFC). He detected the presence of cell associated virus as early as four hours *pi* with maximum virus titre occurring 48 hours *pi*. He also reported that the virus was present extracellularly 6-8 hours pi and reached maximum titre after 60 hours.

Burgess and Yuill (1981) recommended an elevated incubation temperature of 39.5° to 41.5°C for isolation of strains of low virulence, which lacked the ability to produce CPE and plaques in DEFC incubated at 37°C and at 5 per cent carbon dioxide level. This technique was faster and less expensive than duckling inoculation or immunofluorescence.

A duck embryo fibroblast cell line known as CCL 141 was found to be suitable for direct isolation and identification of DPV as well as for virus titration (Wolf *et al.*, 1974). This pekin duck fibroblast cell line was preferred, inspite of lesser virus yield than primary cell cultures, due to its easy availability, uniformity, known health history and easily discerned plaques.

Although CEFC were found refractory to DPV, virus attenuated by serial passage in duck and chicken embryos could be propagated in it (Dardiri and Hess, 1968 and Dardiri, 1969). They developed a plaque assay for titration of DPV and also a plaque inhibition assay using anti DP serum for virus identification.

A progressive drop in mortality rate and a decrease in the severity of reactions were observed in ducklings and duck embryos inoculated with a virulent strain of DPV, adapted to CEFC (Kalaimathi and Janakiram, 1989). Successive passages in CEFC increased the titre from $10^{4.8}$ at the first passage to

$10^{6.83}$ at the twelfth passage and the time taken for development of CPE decreased from 120 hours initially to 60-72 hours in the last passage (Kalaimathi and Janakiram, 1990).

Kurochka *et al.* (1983) propagated DPV, already adapted to chicken embryos, in CEFC and also in suspensions of one million cells per ml, in a muscle protein hydrolysate medium containing 10 per cent serum, inoculated with $10^4 TCID_{50}$ of virus per ml. Simonova *et al.* (1984) successfully propagated an attenuated strain of DPV, called Jansen's strain, in CEFC.

Strains of DPV, like Utrecht strain, attenuated in chicken embryos and CEFC was recommended for active immunisation against DP (Intervet, 1993).

2.6.2 Morphology and structure of the virus

Breeze and Dardiri (1968) observed typical herpes virus particles in the nucleus and cytoplasm of infected cells by EM. Bergmann and Kinder (1982) also used EM to study morphology and maturation of DPV. They encountered two types of virus particles, spherical nucleocapsids 93 nm in diameter and nucleoides (cores) 61 nm in diameter in the infected cell nucleus. Cowdry type A intranuclear inclusion bodies were observed in advanced phase of nucleocapsid production. Their studies revealed that encasement of nucleocapsids by interior

nuclear membrane was followed by occurrence of particles 126-129 nm in diameter, in perinuclear spaces and cytoplasm.

Barr *et al.* (1992) stated that in some cases a single viral envelop surrounding 2-3 nucleocapsids could be seen. Simonova *et al.* (1984) observed a sperical particle measuring upto 394 nm (average 150-250 nm) with a nucleoid 20-25 nm, icosahedral internal capsid 50-60 nm and an external capsid 90-100 nm.

Mature virions differed structurally from other members of herpes family, because they acquired an electron dense material in their cytoplasmic matrix, to form a tegument, which was found surrounded by an envelope obtained by passing through membrane investing tubules, vesicles and vacuoles (Tantaswasdi *et al.*, 1988). They stated that virus particles varying in size from 150-384 nm (mean 200 nm) were embedded in an osmiophilic matrix and that large cytoplasmic inclusions were formed by accumulation of these in cytoplasmic spaces of infected cell.

Panisup *et al.* (1990) reported the production of pinkish intranuclear inclusion bodies, not only in infected tissues, but also cell cultures, inoculated with DPV.

2.6.3 Molecular biology

Breeze and Dardiri (1968) reported that this virion contained DNA and that exposure to DNase led to removal of central core of the virion, without affecting the envelope, but RNase had no such effect on the ultrastructural morphology of the virus. Fluorescence of intranuclear inclusion bodies in infected cell cultures, stained with acridine orange was also consistent with presence of DNA in this virion (Leibovitz, 1971).

Based on a comparison with T4 DNA, the DNA of DPV was found to have a sedimentation coefficient of 59.7 S and a molecular size of 180 Kbp (Gardner *et al.*, 1993). They stated that the viral DNA had a mean molecular mass of 1.18×10^8 daltons and a buoyant density of 1.723 g/cm^3 in cesium chloride, corresponding to a G+C content of 64.3% which was the highest reported for any avian herpes virus, in alpha herpes virinae subgroup. They suggested that the viral duplex DNA was linear with no covalently closed termini or significant base modifications but with single strand nicks or gaps.

Fenner *et al.* (1993) stated that alpha herpes viruses have a lenear double stranded genome ranging from 120-240 Kbp in size with a G+C ratio ranging from 32 per cent to 74 per cent.

2.7 Restriction enzyme analysis of herpes viruses

Restriction endonuclease analysis (REA) of the DNA of different herpes viruses was done by many workers to identify different isolates, to study their relatedness and to investigate the epidemiology and latency of infections. This technique was found to be of great help to understand the genomic peculiarities of isolates collected from different sites in the body, varying geographic locations and races anthropologically heterogeneous.

2.7.1 Herpes simplex virus

Cortini and Wilkie (1978) investigated the order of RE fragments of HSV-1 and HSV-2 DNA from physical maps using molecular hybridization technique and further cleavage of these RE fragments with other RE. They found that the molecular weight of various regions, which constituted HSV-2 genome, was very similar to the corresponding molecular weight of fragments on HSV-1 genome.

Chancy *et al.* (1983) compared the distribution of RE sites of 84 isolates of HSV-1 collected from U.K., U.S.A., Canada and Japan. They stated that variability of RE sites, in contiguous genomic segments, which included both non coding and coding sequences, showed a marked heterogeneity, indicating that some viral gene sequences were more variable

than others, especially in virus isolates from distinct anatomical sites and different geographical locations. Sakaoka *et al.* (1987) studied 172 epidemiologically unrelated HSV strains isolated from Japan and Sweden and found that HSV strains from geographically separate countries and anthropologically different races had distinct distribution in RE recognition sites.

Umene *et al.* (1984) determined RFLP of HSV 1 using southern hybridization with specific ^{32}P labelled cloned HSV DNA fragments. Umene (1987) also investigated the variation of RE cleavage pattern between HSV 1 strains, using REs recognising 4 bp DNA sequences (eg. Hae III, Hha I) instead of enzymes recognising 6 base pair DNA sequences (Bam HI, Sal I). Umene and Yoshida (1993) studied the genomic profiles of 66 strains of HSV type 1, isolated in Japan with regard to RFLP and length variation of fragments containing reiterations. They were the first to identify a RFLP marker (VR 23) peculiar to the predominant genotype of HSV 1.

Polyacrylamide gel electrophoresis (PAGE) of RE digests of HSV-2 DNA enabled the use of enzymes that cleaved DNA into more number of fragments thus improving sensitivity of the analysis (Kinnunen *et al.*, 1987).

Kintner and Brandt (1994) developed a method of rapid isolation of HSV, DNA analogous to miniprep method of

bacterial plasmid isolation. In this method, they lysed the infected cell cultures by freeze thaw cycles to release the virus and removed the cell nucleus by centrifugation. Viral DNA was extracted, following digestion with proteinase K, by phenol-chloroform and precipitated with ethanol. It was claimed that this method required only a small amount of infected cells as source of viral DNA, no radio labelling and that the DNA separated was sufficiently pure to be used for restriction fragment length polymorphism (RFLP) analysis.

2.7.2 Bovine herpes virus

Numerous workers all over the world had attempted to analyse the relation between the RE pattern of various isolates of bovine herpes virus (BHV) 1 and the diverse clinical manifestations of this virus infection. Mishra *et al.* (1983) were able to categorise isolates from Canada, United States and Europe on the basis of restriction pattern into three strains and nine substrains but were unable to associate strains with specific clinical signs. Seal *et al.* (1985) also found that isolates from respiratory infections and other clinical syndromes showed 95 per cent genetic homology by REA and nucleic acid hybridisation studies of viral DNA.

Kataria *et al.* (1992) purified a respiratory isolate of BHV-1 by potassium tartrate density gradient centrifugation.

They reported that the Hind III pattern of the Indian isolate was similar to that of European and American isolates.

Metzler *et al.* (1985, 1986) compared various field isolates of BHV-1 from Europe and Argentina, with reference strains, by SDS PAGE, RE analysis and by discriminating their reactivity with a panel of monoclonal antibodies.

Strains differentiated by REA experimental infection and reciprocal cross protection displayed different levels of virulence, inspite of having a basic similarity in the restriction pattern (Castrucci *et al.*, 1988, Naeem *et al.*, 1991). Castrucci *et al.* (1988) stated that the different strains were mutually protective in that calves were generally found to be refractory to challenge inoculation with either homologous or heterologous viruses. However, both strains failed to produce neutralising antibodies in experimental calves.

Hamelin *et al.* (1990) differentiated one reference, two vaccine and three wild type strains of BHV-1 with Bgl I, Pst I, PVu I and PVu II. They reported that this simplified DNA fingerprinting method was useful, not only in controlling genetic stability of BHV-1 vaccine during production but also to differentiate vaccine strain from clinical isolates.

Viral DNA from 10 herpes viruses, isolated from six sheep and four goats were examined by REA with respect to their relatedness to one another, to bovine herpes viruses type 6 (caprine herpes virus) and two strains of BHV-1: infectious bovine rhinotracheitis virus (IBRV) and infectious pustular vulvovaginitis virus (IPVV). Most of the isolates were found to differ from each other by one or more bands, although basically similar in RE pattern to IBRV. They were different from BHV-6 and IPVV (BHV-1). In fact some of the viruses yielded RE patterns, indistinguishable from those previously reported for three modified live IBRV vaccine strains. However, on immunoprecipitation most of the isolates reacted with IBRV sera. Some isolates also reacted with anti BHV-6 serum, indicating the antigenic relationship of the former to the latter (Whetstone and Evermann, 1988).

Miller *et al.* (1988) compared an isolate of BHV-1, from an aborted foetus (FI) with other BHV isolates like Colorado-1, Iowa and K 22 by REA of viral DNA and classified their isolate as type-2 IPVV virus, subtype a. Further, they assessed the abortifacient properties of three subtypes of BHV-1: Cooper, FI and K 22, distinguishable by REA, by intravenous inoculation into pregnant heifers. They found that heifers given Cooper and FI strains aborted, while those administered K 22 strain delivered full term calves (Miller *et al.*, 1991).

Santen and Chang (1992) cleaved BHV-4 DNA into various fragments with REs Eco RI, Hind III, Pst I and went on to clone and map the genome of this virus.

A genomic comparison of BHV-1, caprine herpes virus (CHV-2) and reindeer herpes virus (RHV-1) was done by REA and southern blot hybridization (Rimstad *et al.*, 1992 and Vanderplasschen, 1993). It was found that RHV-1 was a distinct species within the family herpes viridae, although it showed a higher degree of cross reactivity, than BHV-1 and CHV-2, with other herpes viruses. Restriction maps of cervid herpes viruses 1 and 2, which were antigenically related to BHV-1, was distinguished by southern blot hybridization with Hind III restriction.

Thiry *et al.* (1992) compiled the genomic variations of BHV type 4, to build a dendrogram, showing three levels of divergence between various isolates. They classified this virus under subfamily gamma herpes virinae, because of its similarity in molecular biology to Epstein Barr virus and herpes virus saimiri.

D'offay *et al.* (1993) isolated and characterised some BHV-1 isolates from cases of encephalitis in North America. They found that the RE pattern of the field isolates resembled that of reference strains like respiratory BHV-1, Cooper

strain and neuropathogenic strains - Australian N 569 and Argentine A 663.

Clark *et al.* (1993) opined that RE pattern using Pst I, of BHV-1, isolated from big horn sheep in California was found to be similar to the Cooper strain of IBRV. They stated that all isolates of BHV from big horn sheep were typical of either field strains or vaccine strains of BHV-1 common in cattle in U.S.A.

Celedon *et al.* (1994) concluded that BHV-1 isolates obtained from diverse lesions and geographic locations in Chile were genomically different from each other and from the other standard reference strains.

Phenotypic variants of 2 strains of BHV-1, Azelle and 111/3, were compared with their parent strains by digestion with Hind III, Pst I, Bam HI and Eco RI. Electrophoretic pattern of the DNA of phenotypic variants was found to be similar to that of the parent strain (Rola and Zmudzinski, 1995).

Gupta *et al.* (1995) cloned the Hind III digested fragments of an Indian isolate of BHV-1 into PBR 322 vector, and compared the restriction pattern of these recombinant clones with previously reported maps to identify the viral inserts.

DNA of BHV type 1, from fatal cases of encephalitis, was compared with that of vaccine strain 758-43 using the REs, Pst I and Hind III. This study showed that the encephalitis outbreak had occurred due to administration of a mixed live vaccine of BHV-1, bovine viral diarrhoea virus and parainfluenza 3 virus, by intranasal route, instead of the prescribed intramuscular route (Horiuchi *et al.*, 1995).

2.7.3 Equine herpes virus

DNA of equine herpes virus 1 (EHV 1) was purified using rate zonal centrifugation in 10-15 per cent sucrose in Tris sodium chloride EDTA (TNE) buffer pH 8 by Whalley *et al.* (1981). They studied the physical map of genome of Australian isolates of EHV-1 using REs Eco RI, Bgl II, Bam HI. In another study the electrophoretic patterns of ^{32}P tagged viral DNA, digested with the same three REs, clearly discriminated nonabortificient and abortificient strains of EHV-1 (Sabine *et al.*, 1981).

Turtinen *et al.* (1981) used REA, PAGE and plaque reduction test, to distinguish field strains of EHV-1 from laboratory strains like Army 183, Ky A-ha and KyA-M. REA revealed strain related variation in the location of several Bam HI restriction enzyme cleavage sites, with alteration in the number and mobility of restriction fragments.

Studdert (1983) isolated EHV from cases of respiratory disease and aborted equine foetuses, and compared them by RE DNA fingerprinting. This data, together with an analysis of the epidemiological pattern, strongly supported the view that foetal and respiratory strains were separate species: EHV-1 and 4 respectively (Studdert *et al.*, 1984). However Uppal *et al.* (1993) found no significant difference, in REA, between respiratory isolates of EHV and those from abortion cases in India using enzymes Bam HI and Pst I.

Six isolates of EHV 3 produced a mixture of small and large plaque variants in equine foetal kidney cells, under methyl cellulose (Kamada and Studdert, 1983). The cleavage sites, generated by Bam HI, producing fragments of large plaque variants, were found to be distinct from that of small plaque variants.

Allen *et al.* (1983) conducted molecular epizootiological studies of EHV-1 infections by REA of viral DNA.

Homology and variation in restriction pattern of reference strain and field strains of EHV were studied by autoradiography of RE patterns with a ^{35}S labelled DNA probe, derived from reference strain of EHV (Chowdhury *et al.*, 1986).

Browning *et al.* (1988) used REA to compare the genome of equine and asinine herpes viruses.

Crandell *et al.* (1988) isolated and compared the REA fingerprint of EHV-1 isolates from cattle with EHV-1 Subtypes Army 183 and RQ strain.

Kirisawa *et al.* (1993) studied the genomic diversity among EHV-1 strains, isolated in Japan, using the RE: Bam HI. To identify the variable fragments, the genomic DNA of EHV-1 cleaved with Bam HI were cloned in plasmid PVC-18 and physical maps of the viral DNA were constructed by combination of southern blot analysis and double enzyme digestion of the cloned fragments. By using these cloned fragments as probes in southern blot analysis, the area of heterogeneity observed among EHV-1 field isolates was located in both terminals of UL, centre of UL, IR, US and TR region of the genome.

Kirisawa *et al.* (1994) compared virulent field strains with vaccine strains of EHV, attenuated by serial passage in bovine kidney cell lines. They located the regions of variability using similar procedure, by blotting and DNA probes.

Inter and intra strain genomic variation of EHV-1 isolates was compared by Bonass *et al.* (1994). They stated that both types of variation could occur due to mutation of DNA, causing loss of Bam HI sites.

2.7.4 Pseudorabies virus (PRV) (Aujeszky's Disease Virus)

REA was used to compare the genome of various strains of PRV from disease outbreaks from different countries (Todd and McFerran, 1985; Christensen *et al.*, 1987; Huang *et al.*, 1988; Kim *et al.*, 1988; Shibata *et al.*, 1992; Banks, 1993; Echeverria *et al.*, 1994 and Pancic, 1995). This technique was also employed to compare the field isolates with vaccine strains (Paul and Mengeling, 1982; Pritchett *et al.*, 1984; Arno *et al.*, 1985 and Gielkins *et al.*, 1985. Kukidi *et al.* (1990) used REA to identify probable routes of virus introduction, in cases of fresh outbreaks of Aujeszky's disease. Yamada *et al.* (1992) opined that the ability to differentiate strains of PRV, from different geographic areas on the basis of genome fragmentation might be useful in studying the epidemiology of outbreaks of the disease.

Mengeling *et al.* (1983) examined the DNA of PRV by REA following treatments like plaque purification of stock virus, serial passage in swine and serial passage of virus in cell cultures of high and low multiplicity of infection, to see if such treatments would select or induce virus populations with different predomination number or location of enzyme cleavage sites. Their studies revealed that plaque purification and *in vivo* passage produced alteration in RE pattern but not much alteration was noticed on serial passage in cell cultures.

Wathen and Pirtle (1984) also reported minor variation in electrophoretic mobility of certain restriction enzyme fragments, on serial passage in swine.

Jestin *et al.* (1990) used silver staining technique of DNA restriction fragments for ultra rapid identification of the different strains of PRV.

Banks (1993) used RFLP and polymerase chain reaction (PCR) to discriminate between the strains of PRV and relating these results to pig movement records, he was able to trace the spread of the virus in new outbreaks.

Lipowski *et al.* (1994) labelled purified DNA of PRV to obtain a biotinylated DNA that was highly sensitive and specific for hybridisation with Aujeszky viral DNA. This probe also enhanced the detection limits of restriction fragments compared to restriction pattern analysis in ethidium bromide stained agarose gel. Their results suggested that this probe could be used for characterization of PRV genome, even if only minute amounts of viral DNA were available.

2.7.5 Macropodid herpes virus

Johnson *et al.* (1985) differentiated various isolates of macropodid herpes viruses into two distinct strains, macropodid herpes 1 and 2 based on REA, DNA composition, dot

blot hybridisation and analytical ultracentrifugation in cesium chloride. Further, the DNA of macropodid herpes virus 2 was analysed using RE and molecular cloning techniques (Johnson and Whalley, 1987).

2.7.6 Feline herpes virus

Rota *et al.* (1986a and b) characterised the genome of Feline herpes virus 1 (FHV-1) and compared the DNA and polypeptide pattern of FHV with herpes isolates from dog. They concluded that canine and FHV were nearly identical except that canine isolates showed a small extension of the largest Bam HI fragment. Purified FHV DNA was analysed by RE and r 5' exonuclease digestion, blot hybridisation and EM. The genomic heterogeneity isolated from cats suspected to have feline viral rhinotracheitis was analysed by examining the digestion pattern with REA (Maeda *et al.*, 1995). They classified the field isolates into three major genotypes and successfully identified the heterogeneous regions in the viral genome.

2.7.7 Rodent herpes virus

The DNA of rodent herpes virus was characterised by REA, dot blot hybridisation and dried gel hybridisation to differentiate mouse cytomegalovirus, rat cytomegalovirus and mouse herpes virus strain 76 (Hamelin and Lussier, 1992).

2.7.8 Avian herpes viruses

Restriction enzyme studies of infectious laryngotracheitis virus (ILTV), Mareks disease virus (MDV) and DPV have been invaluable to distinguish between strains, study disease epidemiology and determine passage history.

2.7.8.1 Infectious laryngotrachietis virus

Leeb *et al.* (1987) investigated the genomic isomerism of ILTV and herpes virus Saimiri 1 using REA and scanning densitometry.

Guy *et al.* (1989) compared vaccine, reference and field strains of ILTV by REA. He opined that vaccine and reference strain had a similar restriction pattern while the field strain differed from the vaccine strain on REA.

Keller *et al.* (1992) compared the RE fingerprints of ILTV DNA from 13 Pennsylvania field isolates with vaccine and reference strains to evaluate the possible contribution of mutation of ILTV vaccine strain to outbreaks of ILT.

The effect of serial *in vivo* passage on expression of virulence and DNA stability of ILTV of low virulence was studied by REA of viral DNA by Kotiw *et al.* (1995). Based on their report that serial passage did not cause genomic alteration, it was postulated that live ILTV vaccines could

cause outbreaks of clinical disease in the event of inadequate or incomplete vaccination procedures.

2.7.8.2 Marek's disease virus

Studies done by Silva and Barnett (1991) as well as earlier reports suggested that the RE pattern of MDV provided a simple and accurate method to differentiate between the three MDV serotypes, and virus strains within each serotype and determine whether the viruses had been passaged extensively in cell cultures.

2.7.8.3 Herpes viruses of wild birds

Herpes viruses isolated from falcons, pigeons and psittacines were compared by REA of their DNA by Aini *et al.* (1993). They found the RE pattern of falcon and pigeon herpes viruses was strikingly similar to each other and different from that of herpes viruses isolated from psittacines.

2.7.8.4 Duck plague virus

Molecular characterisation of DPV was done by Qiao (1992) and Gardner *et al.* (1993). Qiao (1992) compared the cleavage pattern of virulent and vaccine strains of DPV using seven restriction enzymes. Hinf I, Eco RI, Hind III, Pst I, Xho I, Bam HI and Sal I. He stated that Hinf I had no site of action on DPV DNA and Eco RI RE patterns of both strains were similar

while RE studies using Hind III, Pst I, Xho I, Bam HI and Sal I could be used to distinguish the 2 strains. Hind III cut the DNA of two strains into 9 and 12 fragments, Pst I into 11 and 15 fragments and Xho I into 12 and 15 fragments respectively. Bam HI cut both DNA into 10 fragments but there were obvious differences in size of the fragments.

Gardner *et al.* (1993) studied the various molecular characteristics of Holland Strain of DPV DNA, along with its restriction pattern. They observed 15 to 22 bands on agarose electrophoresis of DPV DNA cut by Bam HI, Eco RI, Pst I and Bgl II. Hind III and Xho I digested DPV into more than 30 fragments.

Materials and Methods

MATERIALS AND METHODS

3.1 Glassware and containers

Borosil brand of glassware and Laxbro plastic ampoules (for specimen collection) were used. They were prepared for laboratory work employing standard procedures (Hoskins, 1967).

3.2 Reagents

Analytical grade/molecular grade chemicals and reagents were used. The various solutions required for this study were prepared as per manufacturers recommendations or as discribed in Sambrook *et al.* (1989).

3.2.1 Phosphate buffered saline (PBS)

Solution A

Sodium chloride	-	8.0 g
Potassium chloride	-	0.2 g
Disodium hydrogen phosphate ($Na_2H\ PO_4\ 7H_2O$)	-	1.608 g
Potassium dihydrogen phosphate ($KH_2\ PO_4$)	-	0.2 g
Distilled water	-	800 ml

Solution B

Calcium chloride ($CaCl_2$)	-	0.1 g
Distilled water	-	100 ml

Solution C

Magnesium chloride ($MgCl_2$ $6H_2O$)	-	0.1 g
Distilled water	-	100 ml

Solution A, B and C were autoclaved at 15 lb at 121°C for 15 minutes and stored separately at 4°C and mixed just before use under sterile conditions.

3.2.2 Calcium magnesium free phosphate buffered saline (CMFPBS)

Sodium chloride	-	8.0 g
Potassium chloride	-	0.2 g
Disodium hydrogen phosphate (Na_2H PO_4 $7H_2O$)	-	1.608 g
Potassium dihydrogen phosphate (KH_2 PO_4)	-	0.2 g

This was made up to 1000 ml with distilled water and sterilized at 121°C at 15 lb pressure for 15 minutes.

3.2.3 Phosphate glycerol saline

Glycerol	-	1 part (V/V)
PBS	-	1 part

3.2.4 Trypsin solution

Trypsin 1:250 - (SRL)	-	5 g
CMF PBS	-	100 ml

This solution was filtered by sterilisation. This was diluted 20 times with CMF PBS to get a 0.25 per cent working solution.

3.2.5 Lactalbumin hydrolysate (DIFCO)

0.5 per cent solution of this was made in tissue culture medium before sterilisation.

3.2.6 Yeast extract (DIFCO)

0.2 per cent solution of this was prepared in tissue culture medium before sterilisation.

3.2.7 Sodium bicarbonate solution (7.5 per cent)

Sodium bicarbonate	-	7.5 g
Distilled water	-	100 ml

This was sterilised by filtration.

3.2.8 Earle's balanced salt solution (EBSS)

10 x stock solution

Solution I

Calcium chloride	-	2.0 g
Distilled water	-	200 ml

Solution II

Sodium chloride (NaCl)	-	68 g
Potassium chloride (KCl)	-	4.0 g
Magnesium sulphate ($MgSO_4$ $7H_2O$)	-	2.0 g
Disodium hydrogen phosphate (Na_2H PO_4 H_2O)	-	1.25 g
Glucose	-	10.0 g
Phenol red	-	0.2 g
Distilled water	-	800 ml

After mixing solution one and two, added two ml of chloroform and stored in tightly stoppered bottle at 4°C Diluted one part of the 10 x stock solution with nine parts of distilled water, sterilised by autoclaving at 10 lbs pressure for 45 minutes and was stored at 4°C. pH of this was adjusted to 7.4 with sodium bicarbonate before use. EBSS was also prepared by reconstituting 9.6 g dehydrated EBSS (Himedia) powder in one litre distilled water. This was sterilised by filtration and pH was adjusted to 7.4 before use.

3.2.9 Hanks balanced salt solution (HBSS)

Solution I

Sodium chloride (NaCl)	-	160 g
Potassium chloride (KCl)	-	8 g
Magnesium sulphate ($MgSO_4$ $7H_2O$)	-	4 g
Distilled water	-	800 ml

Calcium chloride ($CaCl_2$)	-	2.8 g
Distilled water	-	150 ml

When the salts became completely dissolved, the solution was made upto 1000 ml. This was mixed well and two ml chloroform was added and stored in tightly stoppered bottle at 4°C.

Solution 2

Disodium hydrogen phosphate ($Na_2H\ PO_4\ H_2O$)	-	2.27 g
Potassium dihydrogen phosphate ($KH_2\ PO_4$)	-	1.2 g
Glucose	-	20.0 g
Distilled water	-	800 ml

When salts were completely dissolved 0.4 per cent phenol red was added and made upto 1000 ml with distilled water. Two ml chloroform was added and this was stored at 4°C in a tightly stoppered bottle.

Hanks balanced salt solution was prepared by adding one volume of solution one and one volume of solution two to 18 volumes of distilled water. This was autoclaved at 10 lb pressure for 45 minutes or was sterilized by filtration. pH of the solution was adjusted to 7.4 with 7.5 per cent sodium bicarbonate solution.

Hanks balanced salt solution was also prepared by dissolving 9.76 g of Dehydrated HBSS (Hi-Media) powder in one litre distilled water. This was sterilised by filtration. The pH of this solution was adjusted to 7.4 with sodium bicarbonate solution.

3.2.10 Calf serum

Blood was collected from calves, below two months maintained at the University Livestock Farm, Mannuthy and the serum was separated. This serum was clarified, sterilised by filtration, inactivated at 56°C for 30 minutes, distributed into small aliquots and was stored at -20°C. Sterile calf serum was used at five per cent level in growth medium and two per cent level in maintenance medium.

3.2.11 Antibiotic solution

Streptomycin	-	1 g
Penicillin	-	1,000,000 units
Sterile distilled water/HBSS	-	40 ml

One ml of this solution was added to 100 ml of tissue culture medium to give a final concentration of 250 IU penicillin and 250 ug streptomycin per ml.

3.2.12 Sodium dodecyl sulphate (SDS) Ten per cent

SDS	-	10 g
Distilled water	-	80 ml

The volume was made upto 100 ml with distilled water.

3.2.13 Nonidet P-40 (Sigma)

3.2.14 Proteinase K (Sigma) 5 mg/ml

Proteinase K	-	5 mg
Distilled water	-	1 ml

This was stored at -50°C.

3.2.15 RNase (Boehringer) 10 mg/ml

Ten mg of RNase was dissolved in one ml of 100 mM. Tris HCl and this was boiled for 15 minutes to destroy any DNase present. This was stored at -50°C.

3.2.16 Ethanol

Commercially available rectified spirit was distilled twice with acid and alkali and stored in glass stoppered bottle.

3.2.17 Phenol

Commercially available crystalline phenol was distilled. This was equilibrated with Tris HCl to prepare tris equilibrated phenol.

3.2.18 **Chloroform** (SRL) was used

3.2.19 **Isoamyl alcohol** (SRL) was used

3.2.20 **Sodium acetate (3 M)**

Sodium acetate	-	12.305 g
Distilled water	-	40 ml

The pH was adjusted to 5.2 with glacial acetic acid and the final volume was made upto 50 ml with distilled water.

3.2.21 **Ethidium bromide** (Stock solution 10 mg/ml)

One gram of ethidium bromide was dissolved in 100 ml distilled water with constant stirring with a magnetic stirrer for one to two hours and the solution was then transferred to an amber coloured bottle, wrapped in aluminium foil and stored at room temperature.

3.2.22 **Tris hydrochloride (Tris HCl) (1M)** (Stock solution)

Tris HCl	-	15.76 g
Distilled water	-	80 ml

The pH was adjusted to 8.0 with 10 N HCl and the final volume was made upto 100 ml with distilled water.

3.2.23 **Ethylene diamine tetraacetic acid (EDTA) (0.5 M)** (Stock solution)

EDTA	-	18.612 g
Distilled water	-	80 ml

The pH was adjusted to 8.0 with 10 N NaOH and the final volume was made upto 100 ml with distilled water.

3.2.24 **Sodium chloride (1 M)** (Stock solution)

Sodium chloride	-	10.8 g
Distilled water	-	80 ml

The volume was made upto 100 ml with distilled water.

3.2.25 **Tris borate EDTA buffer (TBE) 5 x**

Tris base	-	54.0 g
Boric acid	-	27.5 g
0.5 M EDTA (pH8)	-	20 ml
Distilled water	-	900 ml

The pH was adjusted to 8 and the volume was made upto 1000 ml with distilled water. One part of this was diluted with 9 parts distilled water and used.

3.2.26 Tris sodium chloride EDTA buffer (TNE)

1 M sodium chloride	-	10 ml
1 M Tris HCl pH 8	-	1 ml
0.5 M EDTA pH 8	-	0.2 ml
Distilled water	-	88.8 ml

3.2.27 Tris EDTA buffer (TE)

1 M Tris HCl pH 8	-	1 ml
0.5 M EDTA pH 8	-	0.2 ml
Distilled water	-	98.8 ml

3.2.28 Sample buffer (6 x)

Bromophenol blue	-	0.25 g
Xylene cyanol FF	-	0.25 g
Sucrose	-	40 g
Distilled water	-	100 ml

This solution was made up to 100 ml in distilled water.

3.2.29 Restriction enzymes (obtained from 'Genei', Bangalore)

Eco RI	-	10 IU/ul
Bam HI	-	10 IU/ul

Pst I	-	10 IU/ul
Xho I	-	10 IU/ul
Hind III	-	10 IU/ul
Lambda Hind III digest Molecular weight marker	-	500 ug/ml

This Hind III digested lambda DNA had eight fragments of 23.130, 9.416, 6.557, 4.361, 2.322, 2.027, 0.564 and 0.125 Kbp size.

3.3 Ducklings and embryonated eggs

Four week old white pekin ducklings were employed for experimental studies. Embryonated duck and chicken eggs were procured from the University Poultry Farm at Mannuthy or purchased locally.

3.4 Collection of materials

Morbid and dead ducks with clinical symptoms of DP were procured from Alleppey district during a disease outbreak. Ailing birds were kept under observation and materials were collected on death of the birds.

3.5 Virus strains

3.5.1 Virulent strains

A virulent strain, DPV-I was procured in freeze dried form, from the Division of Avian Diseases, Indian Veterinary Research Institute (IVRI), Izatnagar, Uttar Pradesh. The field strain DPV-A was isolated from birds in an outbreak of DP in Kuttanad area of Alleppey district.

3.5.2 Vaccine strain (DPV-V)

Lyophilized chick embryo adapted DPV-V was obtained from Veterinary Biological Institute (VBI) Palode, Kerala.

3.6 Virus revival and isolation

3.6.1 Virulent strains

3.6.1.1 Duck plague virus received from IVRI (DPV-I) was reconstituted in two ml sterile PBS and inoculated into four one-month old ducklings by intramuscular route at a dose rate of 0.5 ml per bird. Liver and spleen of the infected birds which succumbed were used as virus source for inoculation into DDE.

3.6.1.2 Liver and spleen collected from birds which died during the disease outbreak after showing classical symptoms

of DP, were used as virus source for field strain of DPV (DPV-A).

The liver and spleen of ducks which died during the disease outbreak and also experimentally infected birds were collected in tryptose phosphate broth/glycerol phosphate saline and stored at -20°C. These were homogenised in Tenbroeck's tissue grinder with silica gel and frozen and thawed thrice. The supernatent was collected after clarifying at 5000xg for 30 minutes. Antibiotics (penicillin 500 IU, streptomycin sulphate 500 ug, gentamicin 50 units per ml) were added to this supernatent and this was used as virus source for duck embryo inoculation.

3.6.2 Vaccine strain (DPV-V)

Lyophilised vaccine was reconstituted in two ml of sterile PBS and used for chicken embryo inoculation by CAM route.

3.6.3 In embryonated eggs

Twelve-day old duck embryos, showing brisk movement on candling, were selected for isolation of virulent strain and field strain of DPV. The vaccine strain was passaged in 10-day old healthy chicken embryo. The processed material containing antibiotics was inoculated at the rate of 0.2 ml

per embryo by CAM route and the eggs were sealed and incubated at 37°C. The control eggs were inoculated with 0.2 ml sterile PBS. Embryos were candled twice daily and those that died within 24 hours of inoculation were discarded. Following death of the embryo it was chilled at 4°C overnight before harvesting. Live embryos were refrigerated on the sixth day, chilled at 4°C overnight and then harvested. The harvested embryos were examined for lesions of DP. The amnioallantoic fluid, CAM, liver and spleen of the embryos were collected and stored in sterile vials at -50°C. Serial passage of each of the virus strains was done thrice in embryonated eggs, in order to increase the titre of the virus. This virus stock was then preserved, processed and used for inoculation in cell cultures.

3.6.4 Chicken and duck embryo fibroblast cell cultures

Cell cultures of chicken and duck embryo fibroblasts were prepared from 12-day old embryonated eggs in tissue culture bottles, Roux flasks and test tubes using standard procedures (Kunst, 1967). When confluent monolayers were formed (18-24 hours) they were washed with PBS and infected with virus materials harvested from infected embryos. One ml of the virus suspension was inoculated into Roux flask, 0.5 ml into tissue culture bottle and 0.1 ml into test tube. The infected monolayer was incubated at 37°C for one hour for virus

adsorption and then the inoculam was poured off, washed gently in tissue culture medium and fed with maintenance medium containing 2 per cent foetal calf serum. This was incubated at 37°C.

Duck embryo fibroblast cell cultures (DEFC) were used for propagation of the virulent strains of DPV (DPV-A and DPV-I). The vaccine strain was grown on CEFC . The monolayer was examined under inverted microscope at 12 hour intervals for development of cytopathic effects (CPE). On development of CPE the supernatent and the cells were used as virus source for DNA extraction.

3.7 Titration of virus

3.7.1 In embryonated eggs (ELD_{50})

All the three strains of DPV were titrated in embryonated eggs. Ten fold dilutions upto 10^{-6} of the virus were prepared in HBSS. Four embryonated eggs were inoculated with 0.2 ml of each dilution of the virus by CAM route. The virulent strains of DPV were inoculated into 12-day old DDE. Ten-day old DCE was used for inoculation of the vaccine strain.

The inoculated eggs were candled twice daily. Embryos that died within 24 hours of inoculation were discarded. All the embryos were chilled overnight at 4°C, on observation of

death on candling upto six days *pi* and harvested the next morning.

The CAM, embryo and liver and spleen of the embryo were examined for specific lesions of DP. The titre of the three strains of virus (ELD_{50}) was calculated as described by Reed and Muench method (1938).

3.7.2 In cell cultures (TCID50)

Cell cultures of chicken and duck embryo fibroblasts were prepared in test tubes. Serial ten fold dilutions upto 10^{-6} of the virulent strains - A and I, and the vaccine strain were prepared in maintenance medium. Three test tube cultures were inoculated with each dilution of the virus. The cell cultures were incubated at 37°C and observed every 12 hours for CPE. The titre ($TCID_{50}$) of virus was calculated as described by Reed and Muench method (1938).

3.8 Electron microscopic examination of the virus

The DPV infected material from duck embryo/cell culture was clarified twice by low speed centrifugation at 3000 rpm for 30 minutes. This supernatent was subjected to ultracentrifugation at 33,000 rpm in a Servo Combi Plus No.80 Rotor for three hours at 4°C. The virus pellet was resuspended in 0.3 ml of distilled water, centrifuged at 3000 rpm for 15

minutes and this supernatent was used for EM examination. One drop of this virus suspension was placed on formavar coated grid. After 45 seconds, the excess fluid was absorbed in a Whatman No.1 filter paper. This charged grid was then stained with potassium phospho tungstate (PTA one per cent aqueous solution, pH 6.5) for 35 seconds. After blotting the excess PTA, the grid was dried at room temperature for 10 minutes and examined in a Hetachi 600A electron microscope at 75 KV.

3.9 DNA analysis

3.9.1 DNA extraction

DNA extraction was done by the procedure described by Sambrook *et al.* (1989). The virulent strain from IVRI (DPV-I), the field strain isolated from Alleppey (DPV-A) and the vaccine strain procured from VBI Palode (DPV-V), were used for nucleic acid extraction.

Both DPV-I and DPV-A were grown in DEFC and DPV-V in CEFC. The nucleic acid was extracted from both intracellular and extracellular virus.

The infected cells were harvested when 80-100 per cent cells showed CPE. These cells were pelleted by centrifugation at 5000 rpm for 10 minutes. Both the supernatent and cell pellet were collected separately. The supernatent was

subjected to ultracentrifugation at 33,000 rpm in Servo Combi Plus No.80 Rotor for three hours at 4°C in order to pellet the extracellular virus. The virus pellet was suspended in 0.1 M NaCl, 0.01 M Tris HCl, 0.001 M EDTA. The cellular fraction was washed twice in PBS and suspended in 0.1 M NaCl, 0.01 M Tris Hcl pH 7.5, 0.001 M EDTA containing 0.5 per cent Nonidet P 40 (NP 40). Following a 10 minute period in an ice bath interrupted by three bursts (30 seconds) of vortexing, the nuclear pellet was removed by centrifugation at 5000 xg for 10 minutes.

The supernatent containing the virus, obtained from the lysed cells and the virus pelleted by ultracentrifugation of cell culture fluid were incubated with ribonuclease A at the rate of 20 ug per ml for 30 minutes at 37°C. This was brought to 0.5 per cent in SDS and the mixture was incubated for three hours at 37°C, with proteinase K (Sigma) at a final concentration of 200 ug per ml.

The DNA released from the virions was extracted twice with an equal volume of tris phenol, the aqueous phase was again extracted, first with phenol chloroform isoamyl alcohol (25:24:1) and then with chloroform isoamyl alcohol (24:1). Nucleic acid was precipitated from the aqueous phase by addition of two volumes of freshly distilled cold absolute ethanol, following adjustment of the solution to a final

concentration of 0.3 M sodium acetate pH 5.2. This was kept at -20°C for two to five hours.

The precipitated nucleic and was pelleted by centrifugation at 14,000 rpm for 20 minutes at 4°C. The resultant pellet was vacuum dried and dissolved in 30 ul tris EDTA buffer (TE buffer pH 8).

3.9.2 Assessment of concentration and purity of DNA

The concentration and purity of DNA were assessed by spectrophotometry. Ten ul of the DNA sample was diluted to one ml with distilled water. The spectrophotometer (SL 159 UV Vis Spectrophotometer) was set to zero with distilled water at 260 and 280 nm wavelength. The DNA samples from each of the three strains were taken in turn in the cuvette and OD was measured at 260 nm and 280 nm. One OD at 260 nm was taken as 50 ug per ml of double stranded DNA. Ratio of OD 260/OD 280 indicated the purity of the DNA samples. DNA samples having ratio of less than 1.8 were again subjected to DNA extraction procedure (Sambrook *et al.*, 1989) to obtain sufficiently pure DNA.

3.9.3 Restriction enzyme digestion

Restriction endonucleases cleave the DNA at specific sequences of bases (recognition sequences) within the DNA

molecule. The five enzymes used in the study were Eco RI, Bam HI, Hind III, Pst I and Xho I.

Sequence specificities and source of restriction enzymes (Murray *et al.*, 1990)

RE	Bacterial source	Sequence cleaved
Eco RI	Escherichia coli RY 13	5' G A A T T C 3' C T T A A G
Bam HI	Bacillus amyloliquefaciens H	5' G G A T C C 3' C C T A G G
Xho I	Xanthomonas holcicola	5' C T C G A G 3' G A G C T C
Pst I	Providencia stuartii 164	5' C T G C A G 3' G A C G T C
Hind III	Haemophilus influenzae Rd	5' A A G C T T 3' T T C G A A

All five REs produce sticky ends on cleaving the DNA strand (Murray *et al.*, 1990).

Two ug of viral DNA of each of the three strains, two ul of 10x RE assay buffer and two ul of RE were taken in microfuge tubes containing distilled water so that the total reaction volume in each tube was 20 ul. This was incubated at 37°C overnight in a waterbath. The RE were then inactivated by heating to 56°C for 10 minutes.

The samples were prepared for agarose electrophoresis by mixing 20 ul of the RE digest with 4 ul of sample buffer.

3.9.4 Agarose gel electrophoresis

Two hundred and forty milligram of agarose was added to 30 ml of 0.5 x tris borate EDTA buffer (TBE). The slurry was kept in a boiling water bath to melt the agarose. This was then cooled to 60°C. Ethidium bromide was added to a final concentration of 0.5 ug per ml.

Agarose was poured onto a tray 15 cm x 7 cm size with comb fitted for formation of wells at one end and allowed to solidify. After solidification the combs were removed and the gel was transferred into a submarine gel electrophoresis tank containing TBE buffer, with the wells at the cathode end. The DNA digests of the three strains mixed with sample buffer were loaded into 3 wells. Lambda DNA Hind III digest, which was used as the molecular weight marker was loaded into the 4th well. Electrophoresis was done at two volts per cm for six to eight hours from cathode to anode. The electrophoresis was stopped when the sample dye had migrated to the anode end of the gel.

The agarose gel was placed on a ultraviolet transilluminator for visualisation of the DNA fragments. The

fluorescent bands observed for each strain were counted and photographed.

3.9.5 Estimation of total molecular size and molecular weight of DNA

The molecular sizes of restriction enzyme digests of DNA were estimated by comparison of the distance migrated by restriction fragments to that of standard molecular weight marker. Lambda DNA fragments obtained by digestion with Hind III was used as standard. By plotting the values of distance migrated by lambda Hind III digested DNA fragments (X-axis) versus the $\log_{10}$ Kbp values of their molecular sizes (Y-axis) on graph paper a linear curve was obtained. The molecular size of restriction fragments of DPV with each RE was determined by interpolation of the curve from the values of distance migrated (cm) by them. The $\log_{10}$ Kbp values so obtained on Y-axis was calculated to antilog so as to obtain the molecular size in Kbp of the restriction fragment.

The molecular weight (Daltons) of the restriction fragments were computed based on the relation 1.575 Kbp=10^6 Daltons (Sambrook *et al.*, 1989).

Results

RESULTS

Three strains of DPV, namely DPV-I procured from IVRI, DPV-A isolated from Alleppey and DPV-V, the vaccine strain from VBI Palode were utilised for this study.

4.1 IVRI strain

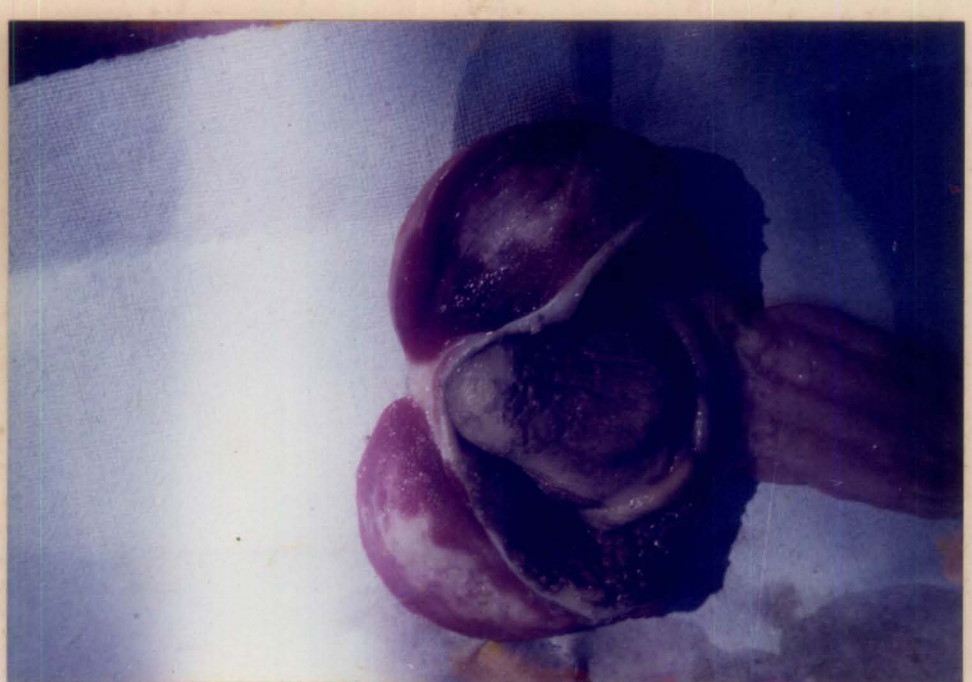
4.1.1 Virus revival

Virulent strain of DPV from IVRI was inoculated into four one-month old ducklings for revival of virus. Clinical manifestations of DP appeared 5-7 days *pi*. Anorexia, lethargy, lachrymation and photophobia, nasal discharge and greenish watery diarrhoea were the main clinical signs (Fig.1). When the affected birds were confined to cages, they adopted a squatting posture in the dark corners of the cage, with the beak resting either on the floor or on its back. Few of the inoculated birds succumbed to the virus infection on the 8th day *pi*.

Post mortem of dead ducks, revealed varying degrees of haemorrhage on the serous membranes, muscles and visceral organs. Congestion, haemorrhages and necrotic foci were observed on the liver and spleen. Most of the birds had crusty diphtheritic plaques in the oesophagus, catarrhal proventriculitis and whitish necrotic areas in the gizzard

Fig.1 Duckling showing clinical signs of duck plague

Fig.2 Necrosis of gizzard muscle in duckling infected with DPV-A



musculature (Fig.2). Haemorrhagic areas (paint brush appearance) were seen on the myocardium. Liver and spleen of these birds were processed and used as virus source for inoculation of DDE.

4.1.2 Passage of virus in DDE

Generalised congestion of CAM and body of embryo was observed on inoculation of DPV-I (Fig.3). Most embryos succumbed within three to five days *pi*. Embryo liver had whitish necrotic foci.

4.1.3 Passage of virus in DEFC

A uniform monolayer of DEF was formed within 18 to 24 hours of seeding (Fig.4). DPV-I produced marked CPE from the first passage in cell cultures. Rounding and clumping of cells were seen 36-48 hours *pi* (Fig.5). By 64 hours *pi* a large portion of the monolayer was detached from the glass surface. Syncytium formation was observed due to fusion of infected DEF (Fig.6). Vacuolation of cytoplasm and eosinophilic intranuclear inclusion bodies were noticed on microscopic examination of 'coverslip cultures' stained by May Grunwald giemsa technique (Fig.7). Serial passage in cell cultures reduced the time taken for production of CPE to 18 to 24 hours *pi*.

ig.3 Embryo and CAM. Congestion seen in I (inoculated with IVRI strain) and A (inoculated with Alleppey strain) C - control

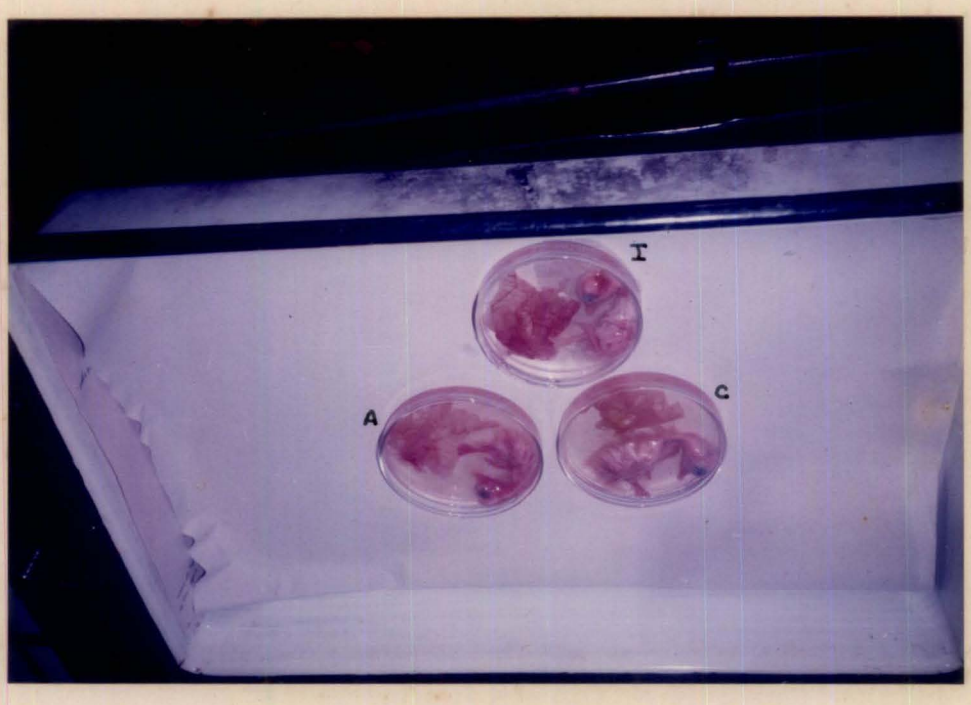
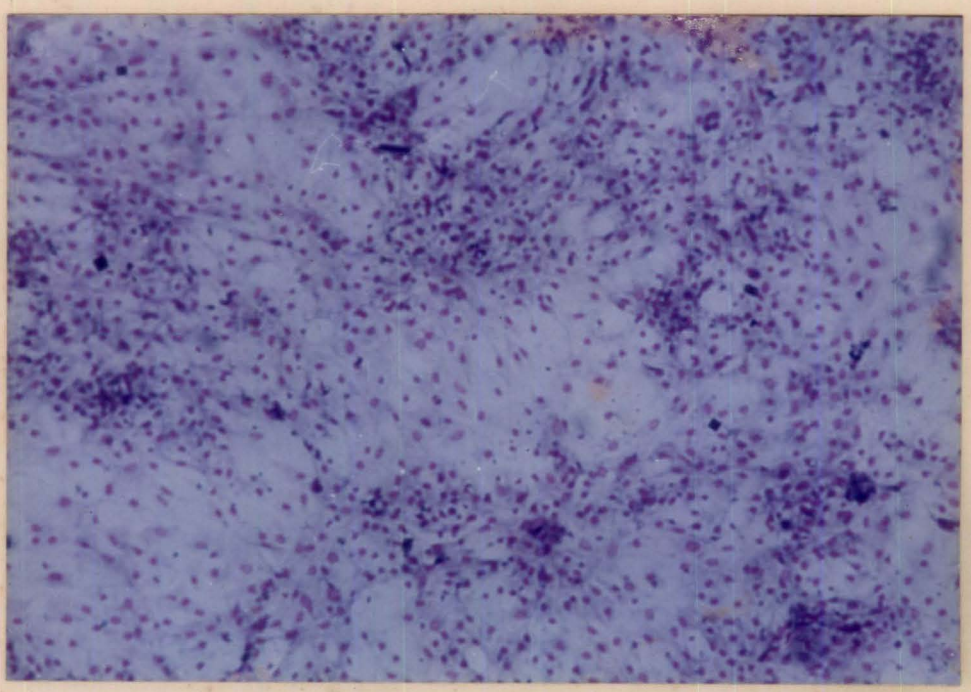

I
A
C

Fig.4 Normal monolayer of duck embryo fibroblast. May Grunwald Giemsa st ain x 400

Fig.5 Duck embryo fibroblast monolayer showing rounding and clumping of cells. 36 hours *pi* with DPV-I. Grunwald Giemsa st ain x 400



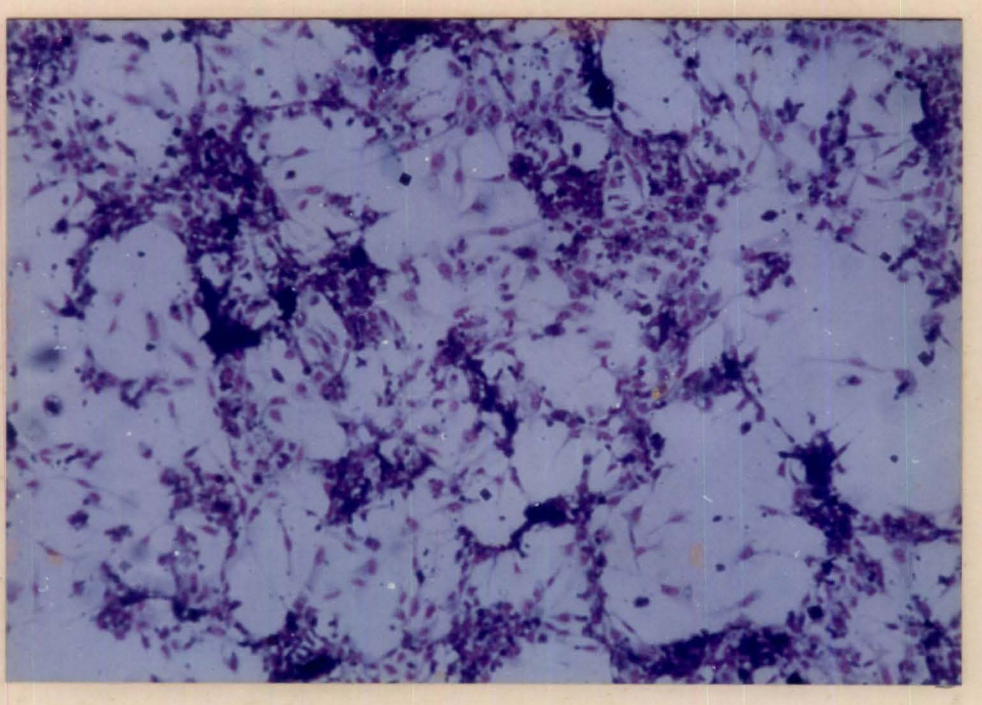
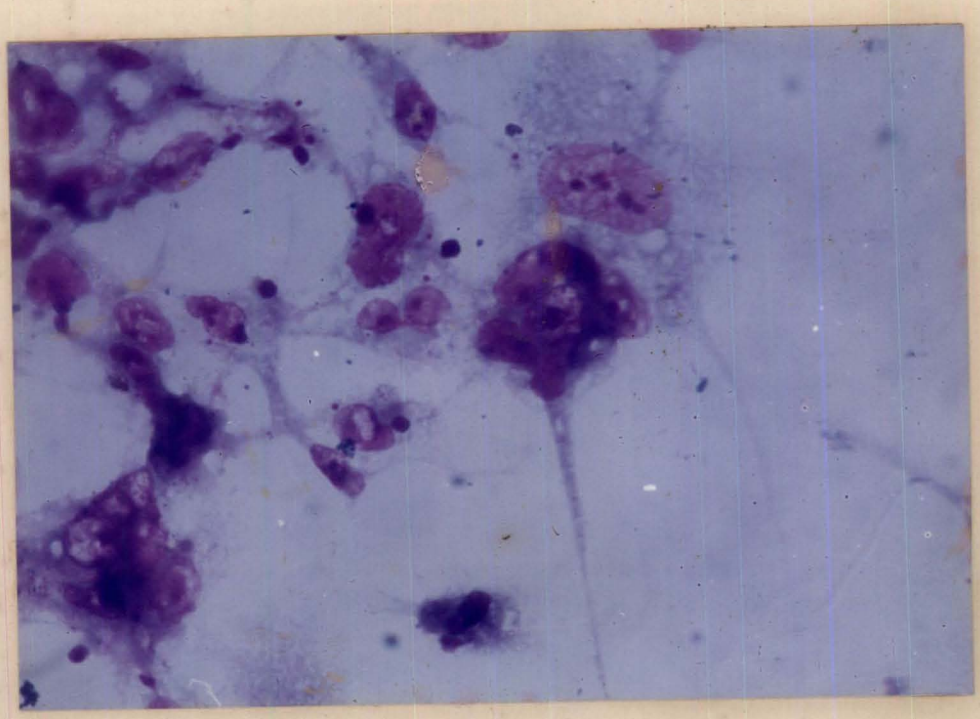
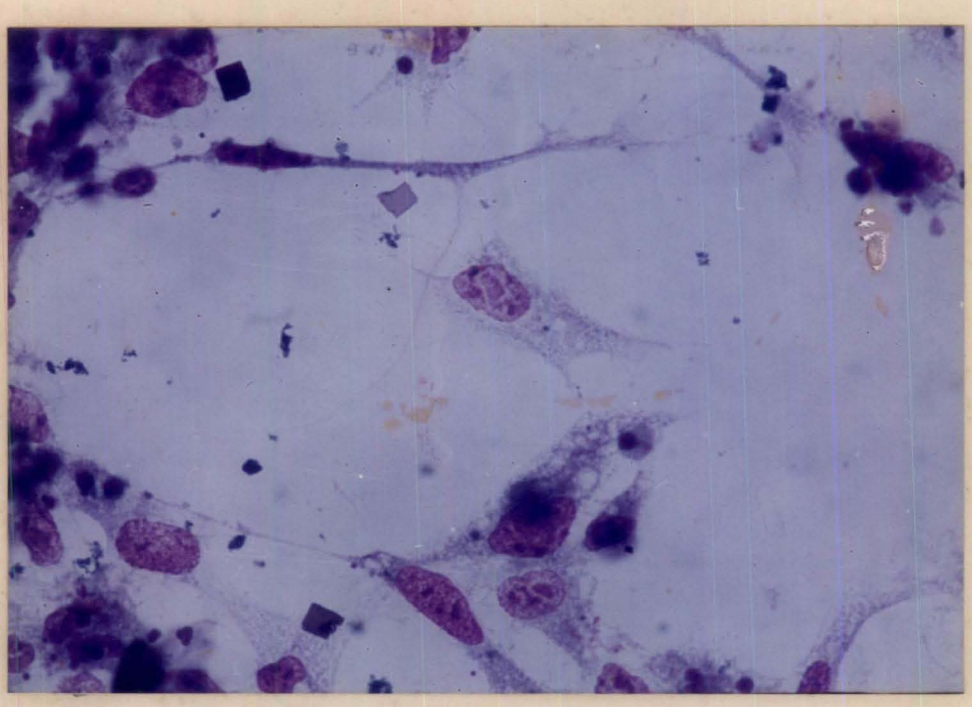


Fig.6 Syncytium formation in duck embryo fibroblast cell culture. 48 hours *pi* with DPV-I. Grunwald Giemsa st ain x 1200

Fig.7 Intranuclear inclusion bodies observed in duck embryo fibroblast cell culture. 48 hours *pi* with DPV-I. Grunwald Giemsa st ain x 1200





4.1.4 Titration of virus

4.1.4.1 In duck embryo

Ten fold dilutions of DPV-I prepared in maintenance medium was inoculated by CAM route at the rate of 0.2 ml per egg for titration of virus. The embryo lethal dose (ELD_{50}) of this strain was $10^{5.27}$ per ml of inoculam (Table 1).

4.1.4.2 In duck embryo fibroblast culture

The tissue culture infective dose ($TCID_{50}$) of DPV-I was assessed by examination of coverslip cultures inoculated with ten fold dilutions (upto 10^6) of DPV-I in maintenance medium. $TCID_{50}$ was calculated to be $10^{5.75}$ per ml of inoculum (Table 2).

4.2 Alleppey strain

Ducks showing symptoms of DP were procured from a disease outbreak in Alleppey district. All the classical symptoms of DP, as observed on experimental infection of DPV-I, were seen. During later stages of the disease nasal discharge and respiratory distress were marked, with the birds squatting in dark corners of the cage. Gluing of eyelids was observed. All the birds obtained from the field outbreak succumbed within two days of procurement.

Table 1. Titration (ELD_{50}) of DPV-I in duck embryo

Titration	No. of eggs per dilution of DPV-I	No. of		Cumulative		+ve ratio	Percentage infected
		Infected	Non-infected	+ve	-ve		
10^{0}	4	4	0	21	0	21/21	100
10^{-1}	4	4	0	17	0	17/17	100
10^{-2}	4	4	0	13	0	13/13	100
10^{-3}	4	4	0	9	0	9/9	100
10^{-4}	4	3	1	5	1	5/6	83.3
10^{-5}	4	2	2	2	3	2/5	40.0
10^{-6}	4	0	4	0	7	0/7	0

$$\text{Proportionate distance} = \frac{83.3-50}{83.3-40} = \frac{33.3}{43.3} = 0.7690$$

$$\text{Titre } (ELD_{50}) = 10^{4.77}/0.2 \text{ ml of inoculum}$$

$$= 10^{5.27} \text{ per ml}$$

Table 2. Titration ($TCID_{50}$) of DPV-I in duck embryo fibroblast culture

Titration	No. of tubes per dilution of DPV-I	No. of CPE +ve	No. of CPE -ve	Cumulative +ve	Cumulative -ve	+ve ratio	Percentage infected
10^{0}	3	3	0	14	0	14/14	100
10^{-1}	3	3	0	11	0	11/11	100
10^{-2}	3	3	0	8	0	8/0	100
10^{-3}	3	2	1	5	1	5/6	83.3
10^{-4}	3	2	1	3	2	3/5	60.0
10^{-5}	3	1	2	1	4	1/5	20
10^{-6}	3	0	3	0	7	0/7	0

$$\text{Proportionate distance} = \frac{60.0-50}{60.0-20} = 0.2500$$

$$\text{Titre } (TCID_{50}) = 10^{5} \times 10^{0.25}$$

$$= 10^{5.25}/0.2 \text{ ml of inoculum}$$

$$= 10^{5.75} \text{ per ml}$$

Pathomorphological changes observed on postmortem, were similar to that produced by DPV-I. In addition to those lesions described for DPV-I, most birds had red annular bands, at regular intervals on the walls of the small intestine.

4.2.1 Virus isolation

4.2.1.1 In duck embryo

Processed material suspected to contain DPV-A, inoculated into 12 day old DDE at the rate of 0.2 ml per egg by CAM route, caused death of majority of the embryos in four to six days. Generalised congestion of embryo and CAM was seen. The extremities of the embryo were severely congested and embryonic liver had necrotic patches. Serial passage in embryonated eggs caused reduction in time taken for death of embryo from six days to three to four days (Table 7).

4.2.1.2 In duck embryo fibroblast culture

The DEFC monolayer, inoculated with processed embryo material, did not show marked CPE during the first two passages but subsequently rounding and clumping of cells were seen from third day *pi*. Microscopic examination of stained coverslip culturesof DEFC, infected with DPV revealed the cytoplasm to be highly granular, with large vacuoles. Syncytium formation and intranuclear inclusion bodies were observed in the infected DEF. Depending on the stage of

development, the inclusion bodies were either eosinophilic or basophilic.

4.2.2 Titration of DPV-A

4.2.2.1 In duck embryo

DPV-A titrated in 12 day old DDE had an ELD_{50} of $10^{4.86}$ per ml of inoculum (Table 3).

4.2.2.2 In duck embryo fibroblast culture

The $TCID_{50}$ of DPV-A, assessed by inoculating three coverslip cultures per dilution of virus, upto 10^{-6}, was found to be $10^{5.25}$ per ml of inoculum (Table 4).

4.3 Vaccine strain

4.3.1 DCE inoculation

The reconstituted DPV-V, inoculated in DCE by CAM route caused embryo mortality in four to five days *pi*. Both embryo and CAM were severely congested.

4.3.2 CEFC inoculation

DPV-V passaged in CEFC produced CPE after three days on first passage. On serial passage, the time taken for development of CPE was reduced to 36-48 hours. All the

Table 3. Titration (ELD_{50}) of DPV-A in duck embryo

Titration	No. of eggs per dilution of DPV-A	No. of		Cumulative		+ve ratio	Percentage infected
		Infected	Non-infected	+ve	-ve		
10^{0}	4	4	0	19	0	19/19	100
10^{-1}	4	4	0	15	0	15/15	100
10^{-2}	4	4	0	11	0	11/11	100
10^{-3}	4	3	1	7	1	7/8	87.5
10^{-4}	4	3	1	4	2	4/6	66.6
10^{-5}	4	1	3	1	5	1/5	20
10^{-6}	4	0	4	0	9	0/9	0

$$\text{Proportionate distance} = \frac{66.6-50}{66.6-20} = \frac{16.6}{46.6} = 0.3562$$

$$= 10^{4.3562}/0.2 \text{ ml}$$

$$\text{Titre } (ELD_{50}) = 10^{4.36}/0.2 \text{ ml of inoculum}$$

$$= 10^{4.86} \text{ per ml}$$

Table 4. Titration ($TCID_{50}$) of DPV-A in duck embryo fibroblast culture

Titration	No. of tubes per dilution of DPV-A	No. of CPE +ve	No. of CPE -ve	Cumulative +ve	Cumulative -ve	+ve ratio	Percentage infected
10^{0}	3	3	0	16	0	16/16	100
10^{-1}	3	3	0	13	0	13/13	100
10^{-2}	3	3	0	10	0	10/10	100
10^{-3}	3	3	0	7	0	7/7	100
10^{-4}	3	3	0	4	0	4/4	100
10^{-5}	3	1	2	1	2	1/3	33.3
10^{-6}	3	0	3	0	5	0/5	0

Proportionate distance $= \frac{100-50}{100-33} = 0.7496 = 0.75$

Titre ($TCID_{50}$) $= 10^{4} \times 10^{0.75}$

$= 10^{4.75}$/0.2 ml of inoculum

$= 10^{5.25}$ per ml

characteristic changes, as produced by the other two strains in DEFC, were also seen in this case.

4.3.3 Titration of DPV-V

4.3.3.1 In chicken embryo

Vaccine strain had an ELD_{50} of $10^{4.0}$ per ml, when titrated in 10 day old DCE (Table 5).

4.3.3.2 In chicken embryo fibroblast culture

DPV-V had a $TCID_{50}$ of $10^{4.5}$ per ml in CEFC (Table 6).

The major characteristics of all the three strains are presented in Table 7.

4.4 Electron microscopic examination of the virus

The processed DDE and DEFC samples revealed enveloped viral particles, with morphological features of herpes virus (Fig.8). The size of these particles ranged between 150-190 nm with a core size of 70-90 nm.

4.5 Concentration and purity of extracted DNA

The concentration and purity of DNA of the three strains of DPV were assessed by spectrophotometry. The virus strains were concentrated by differential centrifugation and processed

Table 5. Titration (ELD_{50}) of DPV-V in chicken embryo

Titration	No. of eggs per dilution of DPV-V	No. of		Cumulative		+ve ratio	Percentage infected
		Infected	Non-infected	+ve	-ve		
10^{0}	4	4	0	16	0	16/16	100
10^{-1}	4	4	0	12	0	12/12	100
10^{-2}	4	4	0	8	0	8/8	100
10^{-3}	4	3	1	4	1	4/5	80
10^{-4}	4	1	3	1	4	1/5	20
10^{-5}	4	0	4	0	8	0/8	0
10^{-6}	0	0	0	0	0	0	0

$$\text{Proportionate distance} = \frac{80-50}{80-20} = \frac{30}{60} = 0.5$$

Titre (ELD_{50}) = $10^{3.5}$/0.2 ml of inoculum

= 10^{4} per ml

Table 6. Titration ($TCID_{50}$) of DPV-V in chicken embryo fibroblast culture

Titration	No. of tubes per dilution of DPV-V	No. of CPE +ve	No. of CPE -ve	Cumulative +ve	Cumulative -ve	+ve ratio	Percentage infected
10^{0}	3	3	0	15	0	15/15	100
10^{-1}	3	3	0	12	0	12/12	100
10^{-2}	3	3	0	9	0	9/9	100
10^{-3}	3	3	0	6	0	6/6	100
10^{-4}	3	2	1	3	1	3/4	75
10^{-5}	3	1	2	1	3	1/4	25
10^{-6}	3	0	3	0	6	0/7	0

$$\text{Proportionate distance} = \frac{75-50}{75-25} = 0.5$$

$$\text{Titre } (TCID_{50}) = 10^{4} \times 10^{0.5}$$

$$= 10^{4.5} \text{ per 0.2 ml of inoculum}$$

$$= 10^{5} \text{ per ml}$$

Fig.8 Electron microscopy of DPV-A x 80000

Table 7. Certain biological characters of DPV-I, DPV-A and DPV-V

Sl. No.	Isolate	Lesions		Cytopathic changes		Titre of DPV	
		DDE	DCE	DEFC	CEFC	ELD_{50} (per ml of inoculum)	$TCID_{50}$
1.	IVRI DPV-I	Death of embryo in 3-5 days *pi* severe congestion of embryo and CAM. White necrotic spots on liver of embryo	No significant gross lesions	CPE in 36-48 hours on the first passage Rounding and clumping of cells Syncytium formation intranuclear inclusion bodies	--	$10^{5.27}$	$10^{5.75}$
2.	Alleppey DPV-A	Death of the embryo in 4-6 days *pi* Severe congestion of CAM and embryo especially at the extremities. Necrosis of embryo liver	No significant gross lesions	CPE in 48-72 hours from third passage. Rounding and clumping of cells. Granular changes of cytoplasm, vacuolation syncytium formation and intranuclear inclusion bodies	--	$10^{4.86}$	$10^{5.25}$
3.	Vaccine DPV-V	--	Death of embryo in 4-5 days *pi* with generalised congestion of embryo and CAM	--	CPE in 36-48 hours Rounding and clumping of cells. Syncytium formation and intranuclear inclusion bodies from the second passage	10^{4}	10^{5}

DDE developing duck embryo
DCE developing chicken embryo
DEFC duck embryo fibroblast culture
CEFC chicken embryo fibroblast culture
CPE cytopathic effects
ELD_{50} Embryo lethal $dose_{50}$
$TCID_{50}$ Tissue culture infective $dose_{50}$

by phenol chloroform extraction, to obtain the viral DNA. The optical density (OD) at 260 nm for the three strains DPV-I, DPV-A and DPV-V were 0.366, 0.319 and 0.294 and that at 280 nm was 0.199, 0.169 and 0.162 respectively (Table 8).

The concentration of DNA in each sample was calculated by the formula OD260 x 50 (a constant for double stranded DNA) x 100 (dilution factor). DNA concentration of the three strains was DPV-I = 1830 ug per ml, DPV-A = 1595 ug per ml and DPV-V = 1470 ug per ml.

The purity of the DNA was measured by calculating the ratio of OD 260/280. The ratio was 1.84, 1.90 and 1.80 for DPV-I, DPV-A and DPV-V respectively.

4.6 Restriction endonuclease analysis

DNA of the three strains was digested with Eco RI, Bam HI, Pst I, Xho I and Hind III. The enzyme digested was electrophoresed in 0.8 per cent agarose gels containing ethidoum bromide 0.5 ug per ml. Hind III digested Lambda phage DNA was used as molecular weight marker. The Hind III digested lambda DNA had eight fragments of 23.130, 9.416, 6.557, 4.361, 2.322, 2.027, 0.564 and 0.125 Kbp size. The molecular size and weight of the various restriction fragments were estimated by comparison of the distance migrated by them

Table 8. Concentration and purity of DNA of DPV strains

	DPV-I	DPV-A	DPV-V
OD 260	0.366	0.319	0.294
OD 280	0.199	0.169	0.162
Purity (ratio)	1.84	1.9	1.8
Concentration (ug/ml)	1830	1595	1470

OD = Optical density

$$\text{Purity} = \text{OD}\ \frac{260}{280}$$

Concentration = OD 260 x 50 (a constant for double stranded DNA) x 100 (dilution factor)

Fig.9 RELATIONSHIP BETWEEN DISTANCE MIGRATED (cm) BY RESTRICTION FRAGMENTS OF DNA AND THE LOG $_{10}$ VALUE OF THEIR MOLECULAR SIZE (Kbp)

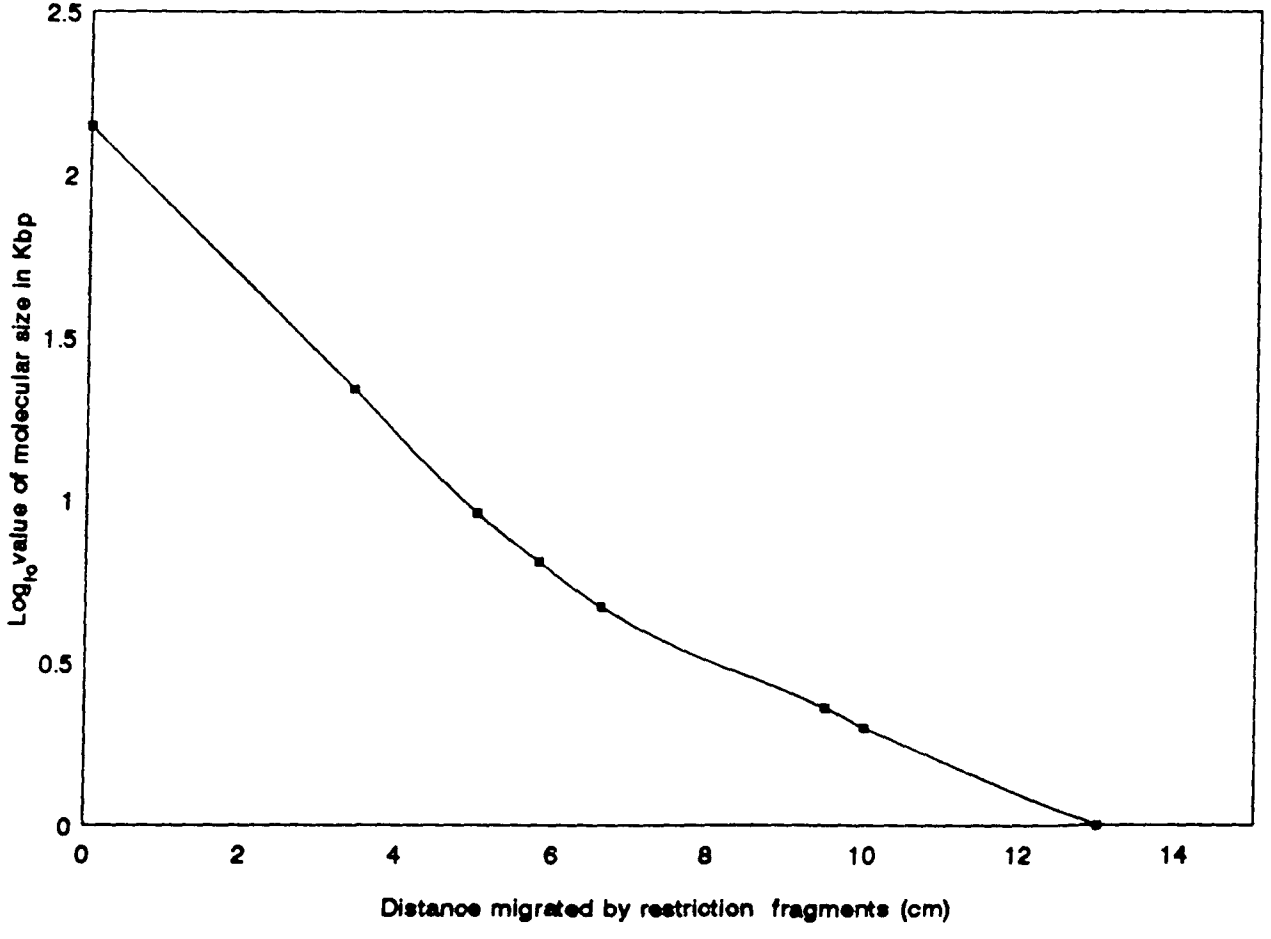
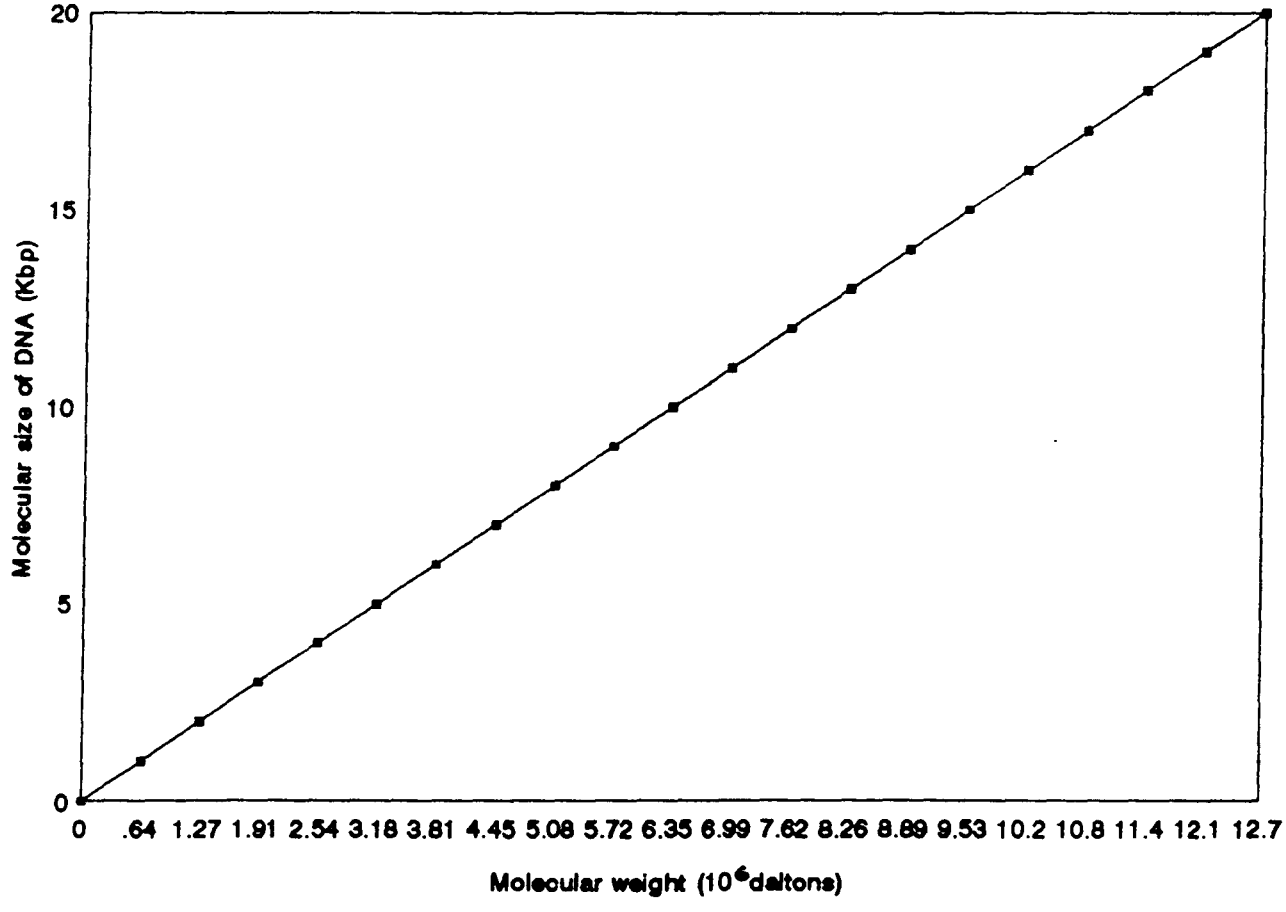


FIG.10 RELATIONSHIP BETWEEN MOLECULAR SIZE OF DNA (Kbp) AND ITS MOLECULAR WEIGHT (daltons)



10^6daltons = 1.575 Kbp (1bp of the sodium salt of DNA weighs 635 daltons.)

in agarose gel with that of the molecular weight marker fragments (Fig.9 and 10).

4.6.1 Eco RI

The results of Eco RI digestion of DPV-I, DPV-V and DPV-A, DNA, are presented in Figure 11 and 12 and Table 9. The DNA, of DPV-I, V and A were digested into 17 fragments each by this RE. Restriction fragments of DPV-I ranged from 2.188 to 43.650 Kbp with a total molecular size of 186.581 Kbp and molecular weight of 118.463×10^6 Daltons.

Restriction enzyme digestion of the genome of DPV-V yielded DNA fragments, with the smallest fragment being 2.188 Kbp and the largest 47.860 Kbp with a total molecular size of 186.403 Kbp and molecular weight of 118.351×10^6 Daltons.

The DNA of DPV-A digested with Eco RI yielded fragments ranging from 2.188 to 47.86 Kbp with a total estimated molecular size and molecular weight of 186.113 Kbp and 118.167×10^6 Daltons respectively.

4.6.2 Bam HI

The DNA of DPV-I digested with Bam HI yielded 14 fragments ranging in size from 2.399 to 43.65 Kbp having a total molecular size of 182.009 Kbp and molecular weight of 115.561×10^6 Daltons.

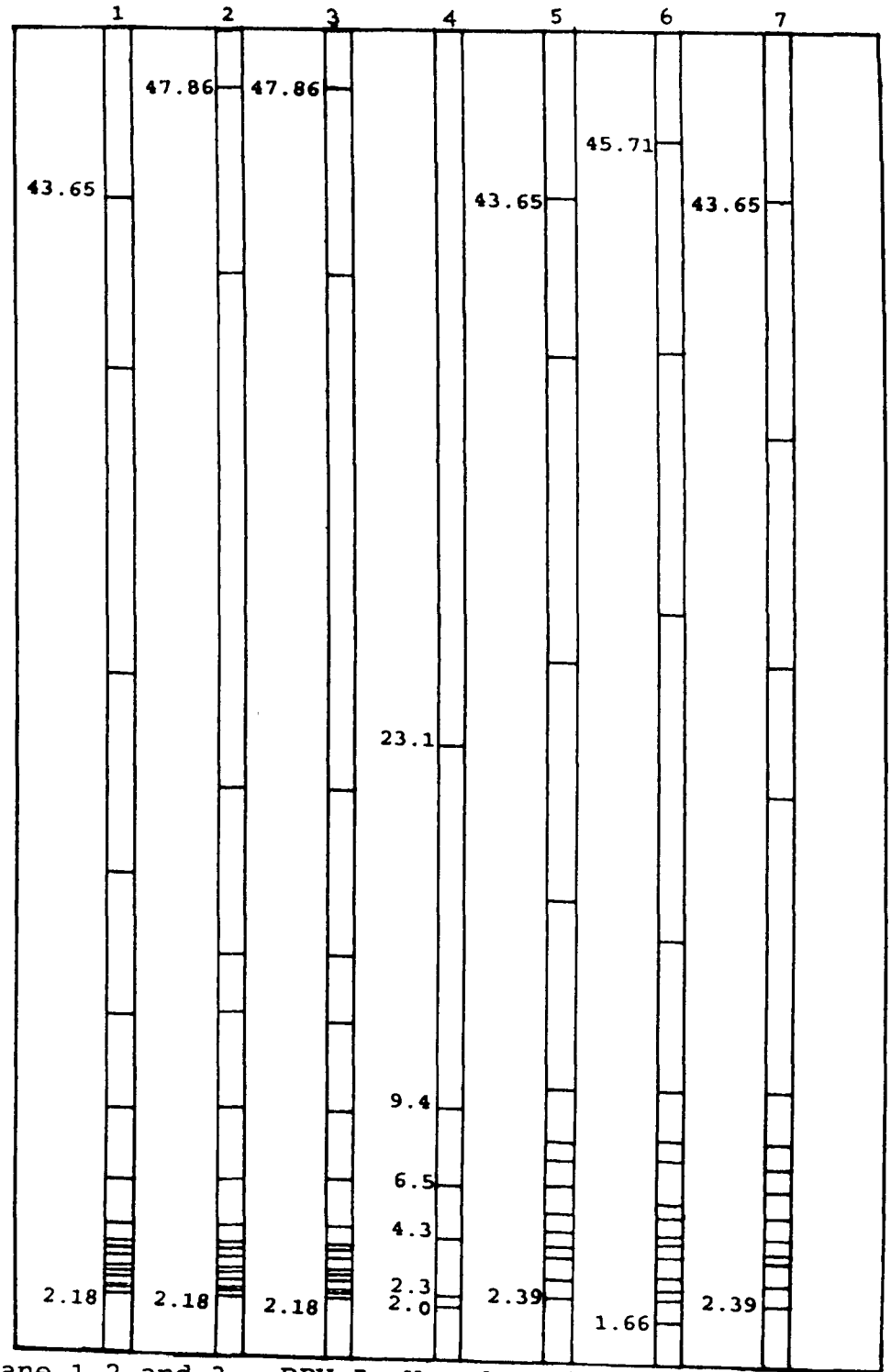
Fig.11 Electrophoresis in agarose gel. Restriction pattern of DPV-I, V and A strains on digestion with Eco RI and Bam HI

Lane 1,2 and 3 - DPV-I, V and A digested with Eco RI

Lane 4 - Lambda Hind III digest - molecular weight marker

Lane 5,6 and 7 - DPV-I, V and A digested with Bam HI

Fig.12 Pictorial representation of restriction pattern of DPV-I, V and A strains on digestion with Eco RI and Bam HI



Lane 1,2 and 3 - DPV-I, V and A digested with Eco RI

Lane 4 - Lambda Hind III digest - molecular weight marker

Lane 5,6 and 7 - DPV-I, V and A digested with Bam HI

Table 9. Molecular size of restriction fragments (Kbp) of the three strains of DPV DNA digested with Eco RI

Sl. No.	Eco RI		
	I	V	A
1.	43.650	47.860	47.860
2.	37.750	40.740	40.740
3.	25.700	21.380	21.350
4.	18.200	15.140	14.140
5.	12.880	12.880	12.590
6.	9.120	9.120	9.120
7.	6.607	6.607	6.607
8.	4.786	4.786	4.786
9.	4.169	4.169	4.169
10.	3.981	3.981	3.981
11.	3.631	3.631	3.631
12.	3.236	3.236	3.236
13.	3.020	3.020	3.020
14.	2.754	2.754	2.754
15.	2.512	2.512	2.512
16.	2.399	2.399	2.399
17.	2.188	2.188	2.188
Total molecular size (Kbp)	186.581	186.403	186.113
Molecular weight (10^6 Daltons)	118.463	118.351	118.167
Fragment number	17	17	17

Table 10. Molecular size of restriction fragments (Kbp) of the three strains of DPV DNA digested with Bam HI

Sl. No.	Bam HI		
	I	V	A
1.	43.650	45.710	43.650
2.	37.750	37.750	34.670
3.	26.300	28.180	26.300
4.	17.380	15.850	21.380
5.	10.230	10.230	10.230
6.	8.318	8.318	8.318
7.	7.586	7.586	7.586
8.	6.607	6.026	6.607
9.	5.495	5.495	5.495
10.	4.786	4.786	4.786
11.	4.365	4.365	4.365
12.	3.981	3.981	3.981
13.	3.162	3.162	3.162
14.	2.399	2.512	2.399
15.		2.399	
16.		1.660	
17.			
Total molecular size (Kbp)	182.009	188.010	182.929
Molecular weight (10^6 Daltons)	115.561	119.371	116.145
Fragment number	14	16	14

The DNA of DPV-V yielded 16 fragments on digestion with this enzyme. The fragment size ranged from 1.660 to 45.710 Kbp with a total size of 188.010 Kbp and molecular weight of 119.371×10^6 Daltons (Fig.11&12, Table 10).

The DNA of DPV-A was digested into 14 fragments ranging from 2.399 to 43.65 Kbp with a total size of 182.929 Kbp and molecular weight of 116.145×10^6 Daltons.

4.6.3 Xho I

The DNA of DPV-I was digested by this RE into 21 restriction fragments ranging from 1.313 to 47.86 Kbp with a total molecular size of 174.554 Kbp and molecular weight of 110.828×10^6 Daltons.

The DNA of DPV-V also yielded 21 fragments with a similar size range of 1.313 to 47.86 Kbp but the total molecular size of the virus genome was 176.028 Kbp with a molecular weight of 111.76×10^6 Daltons.

Genome of DPV-A was cleaved into 23 restriction fragments by Xho I, ranging from 1.259 to 47.86 Kbp with the estimated total molecular size being 176.794 Kbp and molecular weight 112.25×10^6 Daltons respectively (Figure 13&14, Table 11).

4.6.4 Pst I

This is illustrated in Figure 13 and 14 and Table 12. The genome of DPV-I was digested into 21 fragments with a total molecular size of 178.737 Kbp and molecular weight of 113.484×10^6 Daltons. The smallest fragment was 1.738 Kbp and the largest 25.12 Kbp in size.

The DNA of DPV-V was divided into 22 fragments ranging from 1.585 to 30.9 Kbp with a total molecular size and weight of 185.041 Kbp and 117.486×10^6 Daltons respectively.

The DNA DPV-A was digested into 21 fragments with a total molecular size of 181.508 Kbp and molecular weight of 115.243×10^6 Daltons. The restriction fragments ranged from 1.514 to 27.54 Kbp.

4.6.5 Hind III

The DNA of DPV-I was divided into 23 fragments ranging from 1.514 to 38.02 Kbp with a total molecular size of 184.267 Kbp and molecular weight of 116.995×10^6 daltons DPV-V yielded 22 restriction fragments, the smallest being 1.66 Kbp and the largest 38.02 Kbp. The total molecular weight was 117.845×10^6 daltons.

The DNA of DPV-A yielded 21 DNA fragments ranging from 1.778 to 36.31 Kbp with a total molecular size of 180.567 Kbp

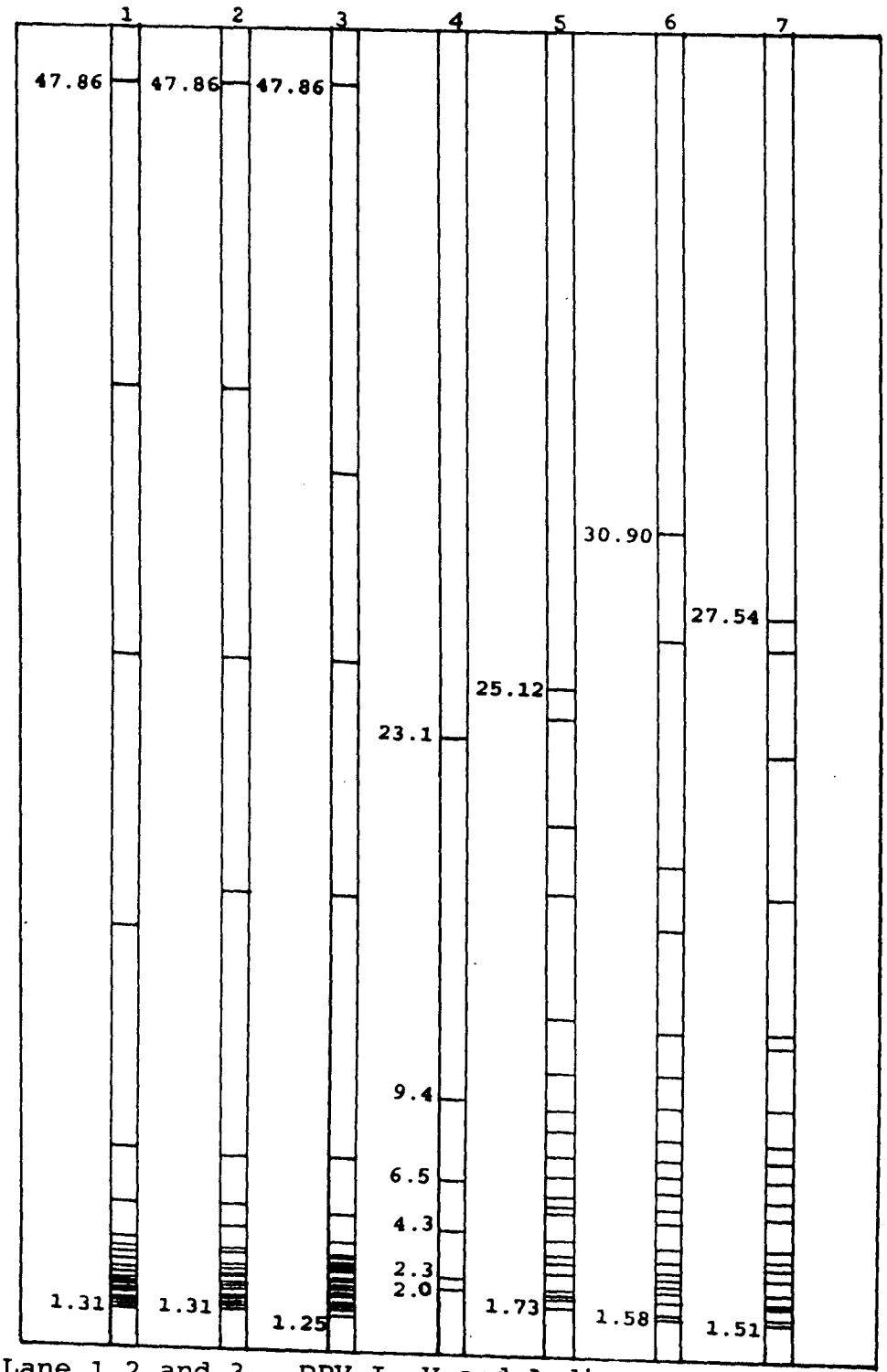
Fig.13 Electrophoresis in agarose gel. Restriction pattern of DPV-I, V and A strains on digestion with Xho I and Pst I

Lane 1,2 and 3 - DPV-I, V and A digested with Xho I

Lane 4 - Lambda Hind III digest - molecular weight marker

Lane 5,6 and 7 - DPV-I, V and A digested with Pst I

Fig.14 Pictorial representation of restriction pattern of DPV-I, V and A strains on digestion with Xho I and Pst I



Lane 1,2 and 3 - DPV-I, V and A digested with Xho I

Lane 4 - Lambda Hind III digest - molecular weight marker

Lane 5,6 and 7 - DPV-I, V and A digested with Pst I

Table 11. Molecular size of restriction fragments (Kbp) of the three strains of DPV DNA digested with Xho I

Sl. No.	Xho I		
	I	V	A
1.	47.860	47.860	47.860
2.	36.310	36.310	33.110
3.	26.300	26.300	26.300
4.	15.850	17.380	17.380
5.	7.586	7.244	7.244
6.	5.495	5.495	5.129
7.	4.169	4.677	4.169
8.	3.802	3.802	3.631
9.	3.548	3.631	3.469
10.	3.162	2.951	3.162
11.	2.630	2.630	3.020
12.	2.399	2.399	2.818
13.	2.188	2.239	2.512
14.	2.089	2.089	2.291
15.	1.905	1.905	2.188
16.	1.738	1.738	1.905
17.	1.698	1.622	1.820
18.	1.585	1.585	1.698
19.	1.514	1.445	1.622
20.	1.413	1.413	1.445
21.	1.313	1.313	1.413
22.			1.349
23.			1.259
Total molecular size (Kbp)	174.554	176.028	176.794
Molecular weight (10^6 Daltons)	110.828	111.763	112.250
Fragment number	21	21	23

Table 12. Molecular size of restriction fragments (Kbp) of the three strains of DPV DNA digested with Pst I

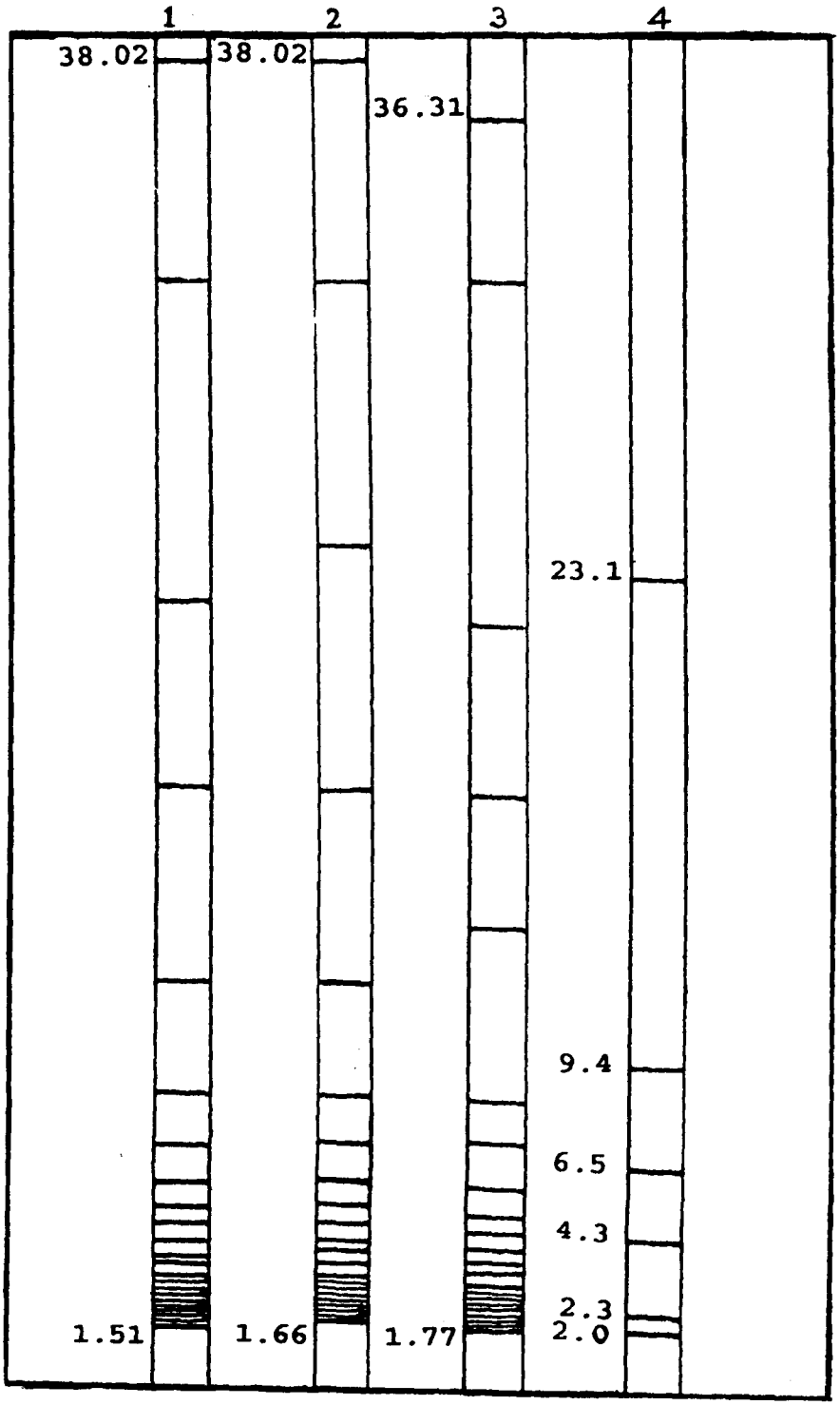
Sl. No.	Pst I		
	I	V	A
1.	25.120	30.900	27.540
2.	23.990	26.920	26.300
3.	19.950	18.200	22.390
4.	17.380	15.850	17.380
5.	12.590	12.020	12.020
6.	10.230	10.230	11.480
7.	8.913	9.120	9.120
8.	8.318	7.943	7.586
9.	7.244	7.244	7.244
10.	6.457	6.607	6.457
11.	5.888	6.166	5.888
12.	5.495	5.495	5.129
13.	5.129	4.898	3.981
14.	4.169	3.981	3.631
15.	3.631	3.548	3.311
16.	3.311	3.162	2.951
17.	2.951	2.818	2.239
18.	2.289	2.512	1.905
19.	2.009	2.239	1.820
20.	1.905	1.905	1.622
21.	1.738	1.698	1.514
22.		1.585	
23.			
Total molecular size (Kbp)	178.737	185.041	181.508
Molecular weight (10^6 Daltons)	113.484	117.486	115.243
Fragment number	21	22	21

Fig.15 Electrophoresis in agarose gel. Restriction pattern of DPV-I, V and A strains on digestion with Hind III

Lane 1,2 and 3 - DPV-I, V and A digested with Hind III

Lane 4 - Lambda Hind III digest - molecular weight marker

Fig.16 Pictorial representation of restriction pattern of DPV-I, V and A strains on digestion with Hind III



Lane 1, 2 and 3 - DPV-I, V and A digested with Hind III

Lane 4 - Lambda Hind III digest - molecular weight marker

Table 13. Molecular size of restriction fragments (Kbp) of the three strains of DPV DNA digested with Hind III

Sl. No.	Hind III		
	I	V	A
1.	38.020	38.020	36.310
2.	31.620	31.620	31.620
3.	22.390	25.120	21.880
4.	16.980	16.980	16.980
5.	11.480	11.480	13.178
6.	8.318	8.318	8.318
7.	6.918	6.918	6.918
8.	5.754	5.754	5.754
9.	5.012	5.012	5.012
10.	4.571	4.571	4.571
11.	3.981	3.981	3.981
12.	3.631	3.631	3.631
13.	3.311	3.311	3.311
14.	2.951	2.951	2.951
15.	2.818	2.818	2.818
16.	2.630	2.630	2.630
17.	2.455	2.455	2.455
18.	2.291	2.291	2.291
19.	2.138	2.138	2.138
20.	2.042	2.042	2.042
21.	1.820	1.905	1.778
22.	1.622	1.660	
23.	1.514		
Total molecular size (Kbp)	184.267	185.606	180.567
Molecular weight (10^6 Daltons)	116.995	117.845	114.646
Fragment number	23	22	21

Table 14. Restricton fragments number, estimated total molecular size and molecular weight of DPV-I V and A on digestion with restriction enzymes

Sl. No.	Restriction enzyme	No. of fragments			Molecular size (Kbp)			Molecular weight in 10^6 Daltons		
		I	V	A	I	V	A	I	V	A
1.	Eco RI	17	17	17	186.581	186.403	186.113	118.463	118.351	118.167
2.	Bam HI	14	16	14	182.009	188.010	182.929	115.561	119.371	116.145
3.	Xho I	21	21	23	174.554	176.028	176.794	110.828	111.763	112.250
4.	Pst I	21	22	21	178.737	185.041	181.508	113.484	117.486	115.243
5.	Hind III	23	22	21	184.267	185.506	180.567	116.995	117.845	114.646
	Total				181.230	184.218	181.582	115.067	116.964	115.290

and molecular weight of $114.646x10^6$ daltons. This is seen in Figure 15 and 16 and Table 13.

The genome of the three strains of DPV-I, V and A had mean molecular sizes of 181.230; 184.218 Kbp and 181.582 Kbp and the molecular weights were $115.067x10^6$, $116.964x10^6$ and $115.290x10^6$ Daltons respectively for I, V and A.

Discussion

DISCUSSION

This study was conducted to assess the molecular differences in the DNA of virulent strains of DPV: DPV-I (IVRI) and DPV-A (Alleppey) and vaccine strain (DPV-V) that is being used presently in Kerala state.

Differences in clinical manifestations, lesions in ducks in natural and experimental infections, embryo pathology and CPE in cell cultures, produced by the three strains of DPV were investigated. DPV strains, cultured in DEFC and CEFC were used as virus source for DNA extraction. The concentration and purity of the DNA were assessed prior to digestion with five REs to compare the restriction profile of the three strains.

5.1 Clinical manifestations

An incubation period of 6-8 days was required for development of the disease, on experimental inoculation of DPV-I into four week old ducklings. Das *et al.* (1990) have reported a similar incubation period for DP.

The chief clinical signs observed in infection with both the virulent strains were lachrymation, photophobia, nasal discharge, ataxia, anorexia, and squatting posture. These

symptoms were in agreement with the findings of Leibovitz (1991). However on experimental infection with DPV-I, only 50 per cent of the ducklings succumbed to the disease. The remaining birds, recovered after two weeks of the disease, during which period, the symptoms of DP were evident. Alleppey strain produced high mortality in infected flocks. The comparatively low virulence of DPV-I could be due to several passages in embryo or cell cultures, that this strain might have undergone, prior to supply. Ducks experimentally infected with DPV-V did not show any evidence of the disease.

5.2 Pathomorphology

5.2.1 In ducks

Congestion, haemorrhage and necrosis of visceral organs, digestive tract lesions and serosal haemorrhages were observed on postmortem of ducks, in natural and experimental infection with DPV-A and DPV-I. This is in confirmation with the observations of the lesions of DP recorded by Leibovitz (1971), Duraiswamy *et al.* (1979) and Roy *et al.* (1983). Necrotic lesions on the mucosa and musculature of the gizzard and haemorrhagic bands seen at regular intervals in the small intestine were more prominent in infection with DPV-A than DPV-I. Rajan *et al.* (1980) had reported similar findings from an outbreak of DP during 1976 in Alleppey district. No

lesions were observed in ducklings inoculated with the vaccine strain of DPV.

5.2.2 In embryonated eggs

Both the virulent strains of DPV produced congestion of the CAM and on the body of DDE. Generalised congestion on the body of embryo was marked from the first passage itself on DPV-I inoculation. Congestion was more severe at the extremities in DDE inoculated with DPV-A. On first passage of DPV-A in DDE, the lesions were mild, increasing in severity with each passage. This was probably because DPV-I might have under gone several passages in DDE prior to supply, unlike DPV-A. The increase in severity of lesions and reduction in the time taken for its production, with each passage of DPV-A in embryonated eggs may be an indication of adaptation of the virus to DDE. Both the virulent strains of DPV failed to produce lesions in DCE. This confirms the findings of Bhowmik and Chakraborthy (1985) and Kulkarni (1993).

The vaccine strain produced congestive lesions on CAM and on the body of DCE, following experimental inoculation. Maximum number of deaths of DCE inoculated with DPV-V occurred between 72 and 120 hours *pi*. This agrees with the findings of Dardiri (1975). Only the vaccine strain of DPV produced lesions in DCE because this was adapted for growth in embryonated chicken eggs, following 12 passages in DDE and

three blind passages in chicken embryo. Serial passage of DPV in DCE reduces the virulence of the virus making it safe for use as vaccine (Jansen, 1964).

5.2.3 In cell cultures

The virulent strains of DPV were grown in DEFC and vaccine strain in CEFC.

Rounding and clumping of cells was observed by 36 hours *pi* on first passage itself in DEFC. Similar CPE was reported by Kunst (1967). There was fusion of fibroblastic cells to form a syncytium and vacuolation of the cytoplasm at 36 hours *pi* of DPV-I. Syncytium formation and vacuolation of cytoplasm observed in DEFC 36 hours *pi* of DPV-I seen in the present study were in agreement with the observations made by Panisup *et al.* (1990) and Fenner *et al.* (1993). Although Breeze and Dardiri (1968) Bergman and Kinder (1982) and Barr *et al.* (1992) have reported the presence of both intranuclear and intracytoplasmic inclusion bodies on EM of infected cells, only intranuclear inclusion bodies were observed on light microscopy in this study. This agrees with the findings of Panisup *et al.* (1990) who has reported only the presence of intranuclear inclusions in infected cells of DEFC. Leibovitz (1971), Rajan *et al.* (1980), Chennakesavalu *et al.* (1987) and Richter and Horzinek (1993) also reported the presence of only

eosinophi lic intranuclear inclusion bodies in infected cells on light microscopy.

Alleppey strain did not produced marked CPE on the first two passages. From the third passage, similar CPE as produced by DPV-I was observed. As the number of passages increased the time taken for production of CPE decreased from 72 hours on the third passage to 36 hours on the fifth passage. Kalaimathi and Janakiram (1990) have reported a reduction in time taken for production of CPE from 120 hours on the first passage to 60 to 72 hours in the twelfth passage which is in agreement with the present finding.

The vaccine strain produced similar CPE in CEFC in 48 to 72 hours *pi* on first passage and the time taken for production of CPE decreased with serial passage in CEFC. This concurs with the finding of Kalaimathi and Janakiram (1990) who observed reduction in time taken for production of CPE with each passage in cell cultures.

5.3 Titration of DPV strains

Titre of all three strains of DPV was higher in cell cultures ($TCID_{50}$) than in embryonated eggs (ELD_{50}).

5.3.1 Embryo lethal dose$_{50}$ (ELD_{50})

The ELD_{50} of DPV-I, A and V were $10^{5.27}$ per ml. $10^{4.86}$ per ml and 10^{4} per ml respectively. This is in general agreement with the ELD_{50} ($10^{5.2}$/ml) reported by Kalaimathi and Janakiram (1990) although Butterfied and Dardiri (1969) have reported a much higher titre (ELD_{50}) of $10^{6.3}$ per ml for virulent strain of DPV.

5.3.2 Tissue culture infective dose$_{50}$ ($TCID_{50}$)

The three strains of DPV (I, A and V) had titres of $10^{5.75}$ per ml, $10^{5.25}$ per ml and 10^{5} per ml respectively in DEFC/CEFC at third passage level. Kalaimathi and Janakiram (1990) reported an increase in titre of the virus from $10^{4.8}$ on the first passage to $10^{6.83}$ on the twelfth passage in cell cultures.

5.4 EM examination of DPV

The size of the virus particles of DPV-A observed under EM ranged between 170-190 nm. This falls within the size range of 150-250 nm observed by Simonova *et al.* (1984). The core size of DPV-A was found to be 70-90 nm. Bergmann and Kender (1982) recorded a core size of 61 nm for DPV, and Panisup *et al.* (1990), opined that the core size of DPV ranged from 70 to 100 nm. This indicated that the virus observed is DPV, a member of herpes viridae.

5.5 Concentration of DNA

The concentration of DNA was highest for DPV-I (1830 ug per ml) and lowest for DPV-V (1470 ug per ml) with that of DPV-A (1595 ug per ml) falling in between.

5.6 Restriction endonuclease analysis

In recent years REA has been used with increasing frequency to study the genome of viruses, for identification and differentiation of strains and to assess the extent of genetic heterogeneity and relationship between viruses. This method of typing the organisms, avoids the confusing antigenic relationships, yielding fewer ambiguities in positive identification. REA has proved to be a powerful tool in epidemiological studies, to elucidate the cause of infections, to identify the subtype of viruses responsible for it and to differentiate field isolates from vaccine strains.

In this study three antigenically homologous strains of DPV were compared by REA with five REs in order to detect differences if any, at the molecular level.

5.6.1 Eco RI

This enzyme digested both the virulent strains (DPV-I and A) and the vaccine strain into 17 fragments. The fragment size for I strain ranged from 2.188 to 43.65 kbp while for V

and A strains it ranged from 2.188 to 47.86 kbp. The size of the restriction fragments of DPV-I differed from those of the other two strains, which were nearly identical. The main difference was in the first 4 fragments of DPV-I which were lighter than those of DPV-V and DPV-A. The RE pattern of the three strains of DPV clearly differed from the reports of Qiao (1992) who recorded the presence of only 7 fragments which were similar for both strains. But Qiao (1992) also noted a difference in size of first fragment between the strains. Gardner *et al.* (1993) reported the production of 17 restriction fragments on digestion of Holland strain of DPV with Eco RI. Although this agrees with our finding in fragment number, the size of the restriction fragments recorded by them (1.9 to 31 kbp) differed markedly from those detected in this study. It appears that this enzyme is not effective to distinguish the Alleppey isolate of DPV from the vaccine strain. Total molecular size of the various fragments of DNA of the three strains was almost similar when digested with this enzyme.

5.6.2 Bam HI

This RE digested both the virulent strains (DPV-I and A) into 14 fragments ranging from 2.399 to 43.650 kbp and the vaccine strain into 16 fragments ranging from 1.66 to 45.71 Kbp. Both the virulent strains had a restriction pattern nearly similar to each other except for the size of the second

and fourth fragments. The vaccine strain differed from DPV-I and DPV-A in the size of six and seven fragments respectively apart from having two additional fragments. Therefore, this enzyme could clearly distinguish the virulent strains from the vaccine strain. Qiao (1992) has reported that although this enzyme cleaved both the virulent and vaccine strains into 10 fragments, the strains could be differentiated by differance in molecular size of 7 of the restriction fragments. Gardner *et al.* (1993) recorded the presence of 15 fragments ranging from 1.8 to 24.5 kbp with this enzyme. The results of the present investigation agree with the work of Gardner *et al.* (1993) as far as the number of fragments are concerned. Bam HI is found to be more effective in differentiating the various strains as seen from our result. Total molecular size of the genome of both the virulent strains was almost equal, while vaccine strain had a larger molecular size.

5.6.3 Xho I

This enzyme cleaved DPV-I and V into 21 fragments ranging in size from 1.313 to 47.86 kbp, and DPV-A into 23 fragments ranging from 1.259 to 47.86 kbp. In spite of cleaving the I and V strains into the same number of fragments, there was difference in the molecular size of 8 fragments between these two strains. The field isolate from Alleppey showed a marked difference in the size of 12 fragments when compared to vaccine strain and 16 fragments when compared to DPV-I apart

from having two additional fragments. Therefore DPV-A can be clearly distinguished from the other 2 strains based on both number and size of restriction fragments by using this RE. The estimated total molecular size of genome of DPV-I, V and A were 174.554, 176.028 and 176.794 kbp respectively, which is lower than the total genomic size recorded with any other RE. This may be because some of the low molecular weight fragments might have been lost on electrophoresis. Qiao (1992) has reported the presence of 13 fragments in the virulent and 14 fragments in the vaccine strain of DPV with differences in their molecular sizes. Gardner *et al.* (1993) has reported the presence of more than 30 restriction fragments on Xho I digestion of Holland strain of DPV. The findings of this study is notably different from those of both Qiao (1992) and Gardner *et al.* (1993).

5.6.4 Pst I

The enzyme cleaved both the virulent strains of DPV (I and A) into 21 fragments, the I strain ranging from 1.738 to 25.12 Kbp and A strain from 1.514 to 27.54 Kbp. The vaccine strain was cleaved into 22 fragments ranging from 1.585 to 30.9 Kbp. Majority of the fragments were less than 10 Kbp in size. The vaccine strain differed from DPV-I in the molecular size of 17 fragments and DPV-A in the size of 15 fragments apart from the fact that DPV-V had one additional terminal fragment. Both the virulent strains differed in the size of

11 of the their fragments. The molecular size of the genome of DPV-I, V and A on digestion with Pst I were 178.737, 185.041 and 181.508 respectively. Qiao (1992) has recorded the presence of 11 fragments in the virulent strain and 14 fragments in the vaccine strain of DPV both having the same range but differing in the size of 9 of the fragments. Gardner *et al.* (1993) reported that virulent DPV was cleaved into 22 fragments with Pst I with majority of the fragments being less than 10 Kbp in size. This shows some similarity with this study, although there is much difference from the findings of Qiao (1992).

5.6.5 Hind III

This RE cleaved DPV-I into 23, DPV-V into 22 and DPV-A into 21 fragments. The heaviest fragment produced by cleavage with Hind III was 38.02 kbp for both I and V strain and 36.310 kbp for A strain. The size of the lightest fragment recorded on DPV-I, V and A DNA digestion with Hind III were 1.514, 1.660 and 1.788 kbp respectively. Both DPV-I and A differed from DPV-V in the size of 4 of their fragments. The virulent strains also differed from each other not only in the number of fragments but also in the molecular size of some of the fragments. The total molecular size of the genome of DPV-I, V and A were 184.267, 185.606 and 180.567 Kbp respectively. Qiao (1992) has recorded the presence of 9 fragments in the virulent and 12 fragments in the vaccine strain of DPV with

the virulent strain differing from the vaccine strain in the size of 5 of its fragments. Gardner *et al.* (1993) has observed more than 30 restriction fragments on Hind III digestion of Holland strain of DPV. The results of this REA do not agree with the findings of either Qiao (1992) or Gardner *et al.* (1993). All the three strains of DPV when digested with Hind III produced different number of fragments with difference in the molecular size of some of the fragments showing the possiblity of delineation of the three strains of DPV based on REA with this enzyme.

The average molecular size of DPV-I, V and A as per this study were 181.23 Kbp, 184.218 Kbp and 181.582 Kbp, their molecular weights being 115.067 x 10^6, 116.964 x 10^6 and 115.29 x 10^6 respectively. The molecular size of the genome of DPV-V was larger than that of the other two strains on digestion with most of the REs used in this study. However the genomic size of all three strains falls well within the range of 120-240 Kbp as stated for herpes viruses (Fenner *et al.*, 1993). Qiao (1992) has recorded a molecular weight of 1.43-22 x 10^6 Daltons for DPV. Gardner *et al.* (1993) estimated the molecular mass of the genome of DPV to be 119 x 10^6 Daltons based on comparison of the sedimentation coefficient of DPV DNA with T_4 DNA in linear neutral sucrose gradient. They stated that this agreed with the average molecular mass of DPV genome (117.6x10^6 Daltons) computed by REA with 4 REs. The molecular size and mass of DPV genome

recorded in the present study differed from the observations of Qiao (1992) but concurred with the findings of Gardner *et al.* (1993).

5.7 Conclusion

Variation in the number of restriction fragments and size of homologous restriction fragments between the different strains of a virus is apparently the result of stepwise deletion or addition of discrete units of DNA during intra cellular replication of viral genome (Lonsdale *et al.*, 1980). This might possibly explain the differences in the restriction profile of the three strain observed in this study. Differences in the restriction pattern of Alleppey and the vaccine strains raises the question of whether the vaccine strain is able to induce sufficient protection against the virulent field strain of DPV, in vaccinated birds. Since DPV produces a number of restriction fragments this may be helpful in cloning of desired fragments to create recombinants.

Although four of the five REs (Bam HI, Xho I, Pst I and Hind III) used in this investigation, produced differences in the number and molecular size of fragments, in the restriction profile of the three strains of DPV, it was felt that this method alone is not sufficient for differentiation of DPV strains. It would be beneficial to further evaluate the use

of the above four REs with more number of DPV strains, obtained from different geographic locations.

Further investigation into the possibility of carrier birds, virus latency and reactivation of latent virus based on genetic studies, will be necessary to explain satisfactorily, the enigmatic aspect of epizootiology of this viral disease. Since various factors involved in the culturing of virus strains and agarose electrophoresis of their DNA can influence the restriction profile, sequencing and identification of the various genes would be needed to draw specific conclusions about the genomic differences between the various strains of DPV.

Summary

SUMMARY

Three strains of DPV: DPV-I (procured from IVRI, Bareilly, Uttar Pradesh), DPV-A (isolated from Alleppey district) and DPV-V (vaccine strain from VBI, Palode) were used in this investigation. The symptoms and lesions produced by each of the strains in infected ducks, pathomorphological alterations changes in embryonated eggs, cytopathic changes in cell cultures and REA were used for comparison of strains.

The IVRI strain was revived by inoculation into ducklings. Inoculated ducks succumbed to the disease showing typical symptoms and lesions of DP. Liver and spleen of dead ducks, were processed and used for inoculation of DDE. DPV-I caused death of inoculated embryos in 3-6 days *pi*, with congestive lesions on CAM and body of embryo. Allantoic fluid, CAM and embryo liver were processed and inoculated into DEFC. No lesions were produced on DCE inoculated with DPV-I. Typical CPE, characteristic of herpes viruses, with rounding and clumping of fibroblast cells, syncytium formation, extensive vacuolation in the cytoplasm and production of eosinophilic intranuclear inclusion bodies were seen on DPV-I infection. By 48-72 hours *pi* the monolayer started getting detached from the glass surface.

The ELD_{50} and $TCID_{50}$ of DPV-I in DDE and DEFC were $10^{5.27}$ and $10^{5.75}$ per ml respectively.

Alleppey strain produced similar symptoms and lesions of DP as observed in DPV-I infection. Higher mortality was seen on DPV-A infection. Gizzard muscle necrosis and annular haemorrhagic bands in the small intestine were more pronounced on infection with this strain of DPV. The lesions produced in DDE by DPV-A were similar to that of DPV-I infection but the extremities showed more severe congestion. This strain did not produce any lesions in DCE. The CPE produced by DPV-A in DEFC was similar to that produced by DPV-I, but the time taken for development of CPE was longer. The ELD_{50} (in DDE) and $TCID_{50}$ (in DEFC) of DPV-A were $10^{4.86}$ and $10^{5.25}$ per ml respectively.

Vaccine strain was inoculated into ducklings and DCE. No symptoms and lesions were observed in inoculated ducks, while congestion of CAM and body of embryo was seen in DCE inoculated with DPV-V. Materials collected from DCE were processed and inoculated with CEFC. CPE seen in CEFC were similar to that produced by DPV-I and A in DEFC. DPV-V had a ELD_{50} of 10^{4} per ml in DEC and $TCID_{50}$ of 10^{5} per ml in CEFC.

Electron microscopy of the field isolate of DPV revealed particles ranging in size from 170-190 nm with a core size of 70-90 nm.

Virus strains grown in DEFC and CEFC were used as source of virus for DNA extraction. The DNA concentration of DPV-I, V and A were 1830 ug per ml 1470 ug per ml and 1595 ug per ml, respectively.

Extracted DNA of the three strains was digested by Eco RI, Bam HI, Xho I, Pst I and Hind III. Electrophoresis was done in 0.8 per cent agarose gel containing ethidium bromide (0.5 ug per ml) to compare the restriction profile of each strain. The DNA fragments were observed in a U-V transilluminator and photographed. The molecular sizes of the restriction fragments were estimated by comparison of the distance migrated by them with that of standard molecular weight marker. Hind III digest of Lambda DNA was used as molecular weight marker in this study.

All the three strains of DPV were cleaved into 17 fragments with slight differences in fragment size on digestion with Eco RI. DPV-I differed in the size of some of the heavy fragments while the size of restriction fragments of DPV-V and A were very similar.

Both the virulent strains (I and A) were cleaved into 14 fragments and the vaccine strain into 16 fragments on digestion with Bam HI. Most of the fragments of DPV-I and A were similar in size. The vaccine strain differed from DPV-I and A in the size of five and seven fragments respectively.

Digestion of the DNA of DPV-I and V with Xho I yielded 21 fragments with difference in the size of eight of the fragments. Alleppey strain was digested into 23 fragments. This strain differed from DPV-I in the size of 13 fragments and DPV-V in the size of 14 fragments.

Restriction enzyme Pst I cleaved both the virulent strain into 21 fragments and vaccine strain into 22 fragments. Although both DPV-I and A were digested into the same number of fragments they differed in the size of 11 of their fragments. DPV-V differed from DPV-I in the size of 16 fragments and DPV-A in the size of 15 fragments, apart from having two additional fragments.

Hind III digested DPV-I, V and A into 23, 22 and 21 fragments. Both the virulent strains (I and A) differed from DPV-V and also from each other in the size of 4 of their fragments.

The estimated molecular weight of the genome of DPV-I, V and A were 115.067 Megadalton 116.964 Megadalton and 115.290 Megadalton.

Though all the five REs produced some differences in restriction profile of the three strains of DPV, Bam HI, Xho I, Pst I and Hind III were found to be more useful in distinguishing the three strains used in this investigation.

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* Originals not seen

RESTRICTION ENDONUCLEASE ANALYSIS OF DUCK PLAGUE VIRAL DNA

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ABSTRACT

Differences in the clinical manifestations of infected ducks, pathomorphology in developing duck embryo (DDE), and developing chicken embryo (DCE) and cytopathic effects in duck embryo fibroblast culture (DEFC) and chicken embryo fibroblast culture (CEFC) caused by two virulent strains of duck plague virus - DPV-I (procured from I.V.R.I., Izatnager) and DPV-A (isolated from Alleppey) and a vaccine strain - DPV-V (obtained from V.B.I., Palode) were investigated in this study. Restriction endonuclease analysis (REA) was done to assess molecular differences between the strains.

Both the virulent strains produced typical symptoms and lesions of duck plague (DP). However, the level of mortality and severity of certain lesions like gizzard muscle necrosis and haemorrhagic bands in the small intestine were more pronounced in DPV-A infection. DPV-V did not produce any symptoms or lesions on experimental inoculation into ducklings.

Embryonated duck eggs were used for passage of DPV-I and isolation of DPV-A, and DCE was used for propagation of DPV-V. Mortality of embryos with congestive lesions on the CAM and body of the embryo were observed for all the three strains. DPV-A produced more severe congestion on the extremities of

inoculated embryos. Both the virulent strains (I and A) failed to produce lesions in DCE.

Both the virulent strains were cultured in DEFC and DPV-V in CEFC. All three strains produced CPE, characteristic of herpes viruses, with rounding and clumping of cells, syncytium formation, vacuolation of cytoplasm and formation of eosinophilic intranuclear inclusion bodies. DPV-A and DPV-V took more time for production of CPE in the first passage with the time taken for CPE production decreasing with successive passages.

The three strains of DPV were titrated in embryonated eggs (ELD_{50}) and cell cultures ($TCID_{50}$). DPV-I and A had an ELD_{50} of $10^{5.27}$ per ml and $10^{4.86}$ per ml respectively in DDE. The ELD_{50} of DPV-V was 10^{4} per ml in DCE. $TCID_{50}$ of DPV-I and A in DEFC were $10^{5.75}$ per ml and $10^{5.25}$ per ml respectively and that of DPV-V in CEFC was 10^{5} per ml.

Virus particles ranging in size from 170-190 nm with a core size of 70-90 nm were observed on electron microscopic examination of processed and concentrated DPV-A material collected from infected DDE.

Strains of DPV cultured in DEFC/CEFC were used as virus source for DNA extraction. DPV-I, V and A had DNA concentrations of 1830 ug per ml, 1470 ug per ml and 1595 ug per ml respectively.

Restriction enzyme analysis was done using Eco RI, Bam HI, Xho I, Pst I and Hind III. Eco RI cleaved all the three strains of DPV into 17 fragments. The restriction profile of DPV-V and A were nearly identical to each other with slight variation from DPV-I in fragment size.

The RE Bam HI digested both the virulent strains of DPV (I and A) into 14 fragments which were similar to each other except for the size of two fragments. DPV-V was cleaved into 16 fragments with differences in the size of six to seven fragments on comparison with DPV-I and A.

The RE Xho I cleaved DPV-I and V into 21 fragments and DPV-A into 23 fragments. All three strains differed from each other in the size of several fragments.

Both the virulent strains (DPV-I and A) yielded 21 fragments and DPV-V 22 fragments on digestion with Pst I. Majority of the fragments were below 10 Kbp in size and there was variation in the size of 11-17 fragments between the strains.

Restriction enzyme Hind III cleaved DPV-I, V and A into 23, 22 and 21 fragments respectively. Apart from the difference in fragment number, all the three strains differed from each other in the size of 4 of their fragments.

The average molecular size of the genome of DPV-I, V and A estimated by REA with five REs were 181.23 Kbp (Molecular weight 115.067 Megadalton), 184.218 Kbp (116.964 Megadalton) and 181.582 Kbp (115.290 Megadalton) respectively.

Of the five REs used in this investigation Bam HI, Xho I, Pst I and Hind III were found to be more useful in differentiation of the three strains of DPV.