CHARACTERIZATION OF STRUCTURAL PROTEINS OF DUCK-PLAGUE VIRUS

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THESIS

Submitted in partial fulfilment of the
requirement for the degree of

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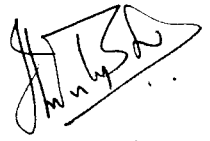
DECLARATION

I hereby declare that the thesis entitled "**CHARACTERIZATION OF STRUCTURAL PROTEINS OF DUCK PLAGUE VIRUS**" is a bonafide record of research work done by me during the course of research and that the 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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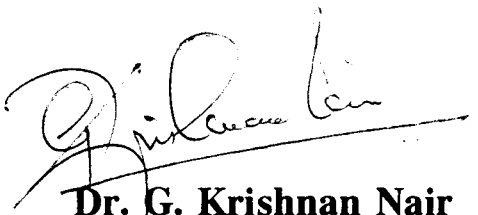
CERTIFICATE

Certified that the thesis, entitled "**CHARACTERIZATION OF STRUCTURAL PROTEINS OF DUCK PLAGUE VIRUS**" is a record of research work done independently by **Shri. J. Hudson Taylor**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.



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We, the undersigned members of the Advisory Committee of **Shri. J. Hudson Taylor,** a candidate for the degree of Master of Veterinary Science, agree that the thesis entitled "**CHARACTERIZATION OF STRUCTURAL PROTEINS OF DUCK PLAGUE VIRUS**" may be submitted by Shri. J. Hudson Taylor, in partial fulfilment of the requirement for the degree.

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To My dear pa, ma, thai
Davy & Dany

"This poor man cried, the LORD heard and delivered him for all his troubles"

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

BHV	-	Bovine herpes virus
CAM	-	Chorio allantoic membrane
CAS	-	Chorio allantoic sac
CEFC	-	Chicken embryo fibroblast culture
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
DVP-I	-	IVRI strain of DPV
DPV-V	-	Vaccine strain of DPV
EHV	-	Equine herpes virus
ELD_{50}	-	Embryo lethal dose 50
EM	-	Electron microscope
HSV	-	Herpes simplex virus
IBRV	-	Infectious bovine rhinotracheitis virus
IVRI	-	Indian Veterinary Research Institute
KD	-	Kilo dalton
PAGE	-	Poly acrylamide gel electrophoresis
Pi	-	Post inoculation
rpm	-	revolutions per minute
SDS	-	Sodium dodecyl sulphate
$TCID_{50}$	-	Tissue Culture Infective dose 50
TNE	-	Tris sodium chloride EDTA buffer
VBI	-	Veterinary biological institute

Introduction

INTRODUCTION

Duck population enjoys its domestication in the hands of poultry farmers in Asia as their homeland. Duck raising is widely popular in the coastal areas of Indian penninsula (Eastern and Southern states), where there are extensive availability of surface water and marshy land, unsuitable for other animals.

According to 1987 census, the duck population in India comes to around fifteen million and duck eggs to around five million (Sreenivasaiah, 1987). Eventhough the duck production is confined to a limited area of the country, it contributes about four crore rupees to the national income and provides employment to about two to three lakh rural families. Any effort to improve the duck industry will have a great impact on the lives of the farmers and will create ancillary job opportunities to many others (Sastry *et al.*, 1994).

With several advantages over poultry farming, duck contributes itself to be a reasonable source of protein for the human kind (Bulbule, 1982). They are relatively resistant to many diseases of poultry and have a very good foraging capacity. Ducks occupy second place to chicken in the production of table eggs in India.

One of the major threats to the flourishing duck industry is a fatal disease known as Duck Viral Enteritis (DVE), also known as Duck plague (DP). The disease confines itself to the anseriformes and is characterised by haemorrhagic enteritis, occurring in captive and free flying waterfowl (Leibovitz, 1971a). The disease is highly contagious, often fatal and results in heavy economic loss to commercial duck producers.

Several reports of the outbreak of this disease causing heavy mortality is received from different parts of India, since Mukerji *et al.* (1963a) had officially reported duck-plague for the first time in India in West Bengal. In Kerala, heavy mortality in ducks was reported from Alleppey district during 1976-1977 (Nair, 1978). Since then Kerala has become endemic for duck plague and regular reports are received from different waterlogged districts (Punnoose and Abdulla, 1976).

Thus it could be seen that duck-plague is continuing to be a great menace to duck farmers, affecting their economy. Effective control of the disease by periodical immunisation gained importance since 1964, which helped in meeting the raising demand for duck eggs and duck meat. However duck plague outbreaks have been reported in several parts of the country despite regular vaccination.

Though all the isolates of duck plague virus (DPV) are reported to be serologically identical, it has not been

subjected to detailed study at its molecular level, so far. With regard to study of the proteins of duck-plague virus, no published reports are available, though data on the protein profile of other herpes viruses are there. A knowledge of the viral proteins will help in developing a safe and potent vaccine against duck plague.

The studies in this line would also help to understand variations if any, among different isolates from different localities and the presently used vaccine strains. The development of a safe and potent sub-unit vaccine would be a boon to the duck farmers and the duck industry.

With the above objectives in view, the present study was undertaken for

(i) Identification of a suitable system to obtain a high titre of duck plague virus.

(ii) Characterisation of structural proteins of the duck plague virus isolates by Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Review of Literature

REVIEW OF LITERATURE

Duck plague, also known as duck viral enteritis (DVE), is an acute, contagious herpes virus infection of ducks, geese and swans, characterised by vascular damage, with tissue haemorrhages and free blood in body cavities, digestive mucosal eruptions, lesions of lymphoid organs and degenerative changes in parenchymatous organs (Leibovitz, 1991).

2.1 History

Baudet (1923) reported an outbreak of an acute haemorrhagic disease of domestic ducks in the Netherlands causing heavy mortality and suspected it to be fowl plague. DeZeeuw (1930) substantiated Baudet's findings and believed the agent to be a duck-adapted strain of fowl plague virus. Bos (1942) reexamined the above findings and concluded that the disease was not due to fowl plague virus, but was a new distinct viral disease of ducks which he termed duck plague. However only Jansen and Kunst (1949) had proposed it as the official name. Another outbreak was observed by Jansen *et al.* (1952) and found that ducks surviving this outbreak were immune to the earlier virus preserved by Bos. Several such outbreaks during 1923-1960 had been cited by Jansen (1961). Mukerji *et al.* (1963b) distinguished it from Newcastle

disease, fowl plague and duck hepatitis. The name Duck Viral Enteritis was introduced instead of duck plague by Leibovitz and Hwang (1968) in United States. Based on the pathological features and symptoms it was given the name Duck Viral Enteritis (Leibovitz, 1991).

2.2 Incidence and distribution

In addition to the Netherlands, DP had been reported from France (Lucam, 1949), Belgium (Devos *et al.*, 1964), USA (Leibovitz and Hwang, 1968), Britain (Hall and Simmons, 1972), Canada (Hanson and Willis, 1976), Thailand (Suwatviroj *et al*, 1977), Denmark (Prip *et al.*, 1983), Germany (Zielder *et al.*, 1984), China (Kunst, 1958; Wang *et al.*, 1984), Russia (Simonova *et al.*, 1984) and Austria (Pechan *et al.*, 1985).

Outbreak of DP in India was first officially reported from West Bengal in 1963 (Mukerji *et al.*, 1963a; Jansen and Kunst, 1964). Still reports of outbreaks are coming from West Bengal (Bhowmik and Chakrabarty, 1985; Bhowmik and Ray, 1987).

Duraiswamy *et al.* (1979) confirmed the presence of DP in Tamil Nadu. Outbreak of DP in Assam was reported by Chakrabarty *et al.* (1980) and virus isolation from such outbreak was reported by John *et al.* (1990). Duck plague was reported in Andhra Pradesh by Sreeramalu (1986).

In Kerala, during the period from April 1976 to January 1977, heavy mortality in ducks was reported in Alleppey district due to a disease which showed characteristic symptoms and lesions of duck plague (Nair, 1978). Regular reports of outbreaks are received from Alleppey, Kottayam, Pathanamthitta, Thrissur and other coastal areas. Duck plague outbreaks were also reported from vaccinated as well as unvaccinated flocks (Kulkarni *et al.*, 1995).

2.3 Aetiology

The causative agent of duck plague is a herpes virus in subfamily Alpha herpesvirinae (Mohanty and Dutta, 1981) and provisionally designated as Anatid herpes virus-I (AHV-I) by the herpes virus study group (Roizman, 1982).

2.3.1 Symptoms

Natural infection of duck plague was restricted only to anseriformes, not in other species of birds, mammals or man (Jansen, 1968). Egg production may drop 20 to 100 per cent (NewComb, 1968). An experimental infection caused respiratory or nervous signs primarily, leading on to spasms and paralysis, clinically similar to Newcastle disease (Kalinskii and Borisovich, 1969).

Deaths occurred within five days after the appearance of clinical signs, with an incubation period of three to seven days (Leibovitz, 1971a). Mortality may be severe as a result of dual infection of DP and latent bacterial infection (Dardiri, 1971). Deaths even within three days post infection (DPI) were reported by Roy *et al.* (1980).

Characteristic symptoms were high persistent flock mortality, drop in egg yield by 25 per cent, extreme thirst, droopiness, ataxia, ruffled feathers, nasal discharge, soiled vent, watery greenish diarrhoea and weakness with tremors (Leibovitz, 1971a; Rajan *et al.*, 1980). Symptoms with 78 to 100 per cent mortality among young ducks were also recorded (Suwatviroj *et al.*, 1977). Only 30 per cent mortality was recorded among one to three weeks old ducklings (Bhowmik and Chakrabarthy, 1985).

2.3.2 Lesions

The most striking lesions of this disease were the multiple petechiae throughout the body, particularly on serous membranes, heart and mucosa of the oesophagus. The mucosa of the intestines was also inflammed and showed petechiation (Jansen, 1964). Red coloured annular bands were seen at regularly spaced interval in the anterior and posterior jejunum (Leibovitz, 1969).

Leibovitz and Hwang (1970) made a detailed study about the above mentioned lesions of the disease in white pekin ducklings.

Closely packed petechiae gave a red "paint brush" appearance over the pericardium and the liver. Dark, mottled spleen appeared smaller than usual (Leibovitz, 1971b; Roy *et al.*, 1983; Bhowmik and Chakrabarty, 1985). Tiny haemorrhagic spots were found in the initial stages on the oesophageal mucosa and later yellowish or greyish diptheritic crusty plaques were seen (Dardiri, 1975).

Pinkish hepatic intranuclear inclusions could be demonstrated (Gailiunas and Dardiri, 1970; Montali *et al.*, 1976). Secondary infections were reported to be not essential for the development of typical lesions of duck plague (Proctor and Mathews, 1976).

2.3.3 **Virus morphology**

Hess and Dardiri (1968) reported that DPV suspensions could pass through membrane filters of 220 nm porosity but infectious virus was retained by 100 nm porosity membranes.

Electron microscopy of virus infected cell cultures 48 h post inoculation revealed virus particles in both the nucleus and cytoplasm of the cell (Breeze and Dardiri, 1968). Larger

particles of approximately 181 nm in diameter were observed in the cytoplasm.

In a negatively stained preparation, Proctor *et al.* (1976) described duck plague virus capsids as coated with extraneous substances, when free in suspension. Common forms found in the supernatent of cell culture were virions with diameter 120-160 nm. Usually a central herpes virus like capsid and blebs of the envelope were observed in damaged particles.

A morphogenesis study by Bergmann and Kinder (1982) revealed spherical nucleocapsids of 93 nm in diameter with a core about 61 nm in the cell nuclei. Simonova *et al.* (1984) observed spherical particles 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.

Tantaswasdi *et al.* (1988) described two types of virus particles in the cytoplasm of the hepatic cells, three to four days post infection. One was naked nucleocapsid measuring approximately 80 nm in diameter. Other consisted of an envelope having projections of 10 nm long, a nucleocapsid 80 nm in diameter and an electron-dense zone between the envelope and capsid.

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

2.4 Cultivation of duck plague virus

2.4.1 In chicken eggs, duck eggs and ducklings

The allantoic fluids containing the DP virus killed 10 to 60 per cent of the duck embryos by the sixth day post inoculation (Levine and Fabricant, 1950).

Primary isolation of the virus can best be obtained by propagation on the chorio-allantoic membrane (CAM) of nine to fourteen day old embryonating duck eggs, causing death after four days with extensive haemorrhage. Virulent virus passaged twelve times in duck eggs could be adapted in nine day old chicken embryos after three further passages. This caused death four to five days post inoculation with extensive haemorrhage of the embryos along with decrease in pathogenicity for ducks (Jansen, 1961). By 20th passage pathognesis for ducks had totally disappeared, resulting in perfect immunity against challenge. This avirulent virus was found to be effective for active immunisation (Jansen *et al.*, 1963).

Duck plague virus could be isolated in the chorio-allantoic membrane of nine to fourteen day-old embryonating duck eggs (Jansen, 1961; Jansen, 1964; Leibovitz, 1971a; John *et al.*, 1990). But Butterfield *et al.* (1969) and Dardiri (1975) preferred chorio-allantoic sac (CAS) route. CAS was later reported as the most ideal route of inoculation in mass DP vaccine production. 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). The DP virus was steadily propagated in CAS of developing duck and chicken embryos (Kalinskii and Borisovich, 1969). Reduction in mortality in lag phase, harvesting the embryos a day earlier than that of CAM route and higher yield of virus by CAM route was reported by Kalaimathi *et al.* (1985).

Mortality in duck embryos by virulent virus was maximum at 120 to 142 hours post inoculation with a lag phase of 96 hours, virus titres reaching $10^{6.3}$ ELD_{50} per ml (Butterfield and Dardiri, 1969). They stated that addition of embryo material to CAM and allanto-aminiotic fluid (AAF) would decrease the virus titre. Reproductivity of the disease in day old duckling was reported by Leibovitz (1971a) and Sarkar (1982).

Duck plague virus could not be isolated in chick embryos (Leibovitz, 1971a; Vetesi *et al.*, 1982). Bhowmik and

Chakrabarty (1985) also observed that chicks and chicken embryos were refractory to DP virus.

John *et al.* (1990) isolated a local virulent strain of duck plague virus by inoculation into the duck embryos by CAM route.

2.4.2 In cell cultures

Duck plague virus was first isolated in cell cultures by Kunst (1967) and noted the first cytopathic effect (CPE) within three to four days post infection. The development of virus in cell cultures was studied by electron microscopy of thin sections of infected cell cultures (Breeze and Dardiri, 1968). Dardiri (1969) also propagated the virulent DPV in duck embryo fibroblast cell culture.

The DPV infected cell cultures showed CPE at 24 to 60 h post infection which was characterised by rounding of cells, marked pyknosis thus resulting in very small grape like clusters (Kenwolf *et al.*, 1974; Nair, 1978). Gradually cells fused to form syncytia and giant cells (Leibovitz, 1971b). Solisch *et al.* (1983) demonstrated the importance of cytoplasmic inclusions in duck embryo cell cultures after examining 200 cell culture slides.

The virus was titrated in chicken and duck embryo cell cultures and the presence of new cell associated virus was detected at four hours pi with maximum titre at 48 hours (Leibovitz, 1971b).

Muscovy and Wood duck cell cultures gave good results with regard to virus yield, plaque quality and sensitivity. The virus in muscovy cells exhibited a latent period of six hours with maximum titre at 36 h (Kocam, 1976).

The virus could grow equally well on CCL-141 duck cell line originated from Pekin duck fibroblast cell lines were preferred due to availability, uniformity, known health history and more easily discerned plaques (Wolf *et al.*, 1976).

Burgess and Yuill (1981) concluded that less virulent DPV isolates could be isolated on muscovy duck embryo fibroblast cell culture using higher incubation temperature i.e., 39.5°C to 41.5°C instead of 37°C. Vetesi *et al.* (1982) demonstrated CPE in duck and goose embryo fibroblasts and kidney cells.

Kurochka *et al.* (1983) propagated chick embryo-adapted attenuated DPV in primary chicken monolayer cultures. Simonova *et al.* (1984) were also successful in propagation of attenuated Jansen strain of virus in chick embryo monolayer cell culture.

A virulent strain of duck plague virus was adapted to chick embryo fibroblast culture. Reduction in virulence with increasing passages in chick embryo fibroblast was recorded by Kalaimathi and Janakiram (1989).

Kalaimathi and Janakiram (1990) recorded the propagation of duck plague virus in chicken embryo fibroblast cell culture and found that as the number of passages increased the time taken to produce CPE decreased from 120 h initially to 60 to 72 h later.

2.5 Strain classification

Although DP strains have been noted with differences in virulence, all appear to be immunologically identical (Hess and Dardiri, 1968; Jansen, 1968; Speiker, 1977).

The DP virus is immunologically distinct from other avian viruses including fowl plague, Newcastle disease, duck hepatitis viruses (Bos, 1942; Jansen and Kunst, 1949; Levine and Fabricant, 1950; Dardiri and Hess, 1968) and herpes viruses (Roizman *et al.*, 1981).

Jansen (1961) demonstrated that all isolates during initial outbreaks in Netherlands were serologically homologous. There was no antigenic variation between Dutch strains and Indian strains (Jansen, 1964).

Plaque inhibition assay was used to compare Holland strain and American strain, indicating that there was no antigenic difference (Dardiri and Hess, 1968).

Sheridan-83, a non-pathogenic strain was isolated by Lin *et al.* (1984a) which was serologically indistinguishable from Holland and Long Island strains (Lin *et al.*, 1984b).

Six strains of DPV were detected employing reverse passive haemagglutination test and immunofluorescence test (Deng *et al.*, 1984). Lin *et al.* (1984b) studied that various isolates of different virulence were all serologically related biotypes of duck plague virus.

2.6 Immunogenicity and vaccination

Jansen and Kunst (1949) observed that the DP virus was not virulent for ducks on adaptation to chicken embryos after 20 passages, but was able to immunise ducks by intramuscular or oral route. Jansen (1964) was also successful in developing a chick embryo adapted DP virus, avirulent for the domestic ducks.

Twenty fifth passage in chicken embryos was completely safe for the adult ducks and the antigenicity of the duck embryo adapted DP virus was tested in healthy ducks which

withstood challenge 14 months post immunisation (Mukerji *et al.*, 1963b).

Jansen and Kunst (1967) declared the impossibility of reactivation of attenuated strain by means of 10 serial passages in duck eggs and hence confirmed it as a safe vaccine. Butterfield and Dardiri (1969) recommended the use of 0.05 per cent N-acetylazinidine (N-AEI) instead of 0.4 per cent Beta propiolactone (BPL) for the inactivation of chick embryo adapted DP virus.

However, Toth (1970) proved that the immunogenicity of the vaccine was unsatisfactory after inactivation either by BPL (or) N-AEI.

An apthogenic and immunogenic strain of the DP virus in fowls, tentatively designated as Sheridan-83 isolated in California, when inoculated into ducks offered protection against challenge with virulent Lake Andes (LA) strain (Lin *et al.*, 1984a). This strain used as vaccine virus, enabled the ducks to resist challenge with virulent DP virus (Lin *et al.*, 1984b).

Kalaimathi and Janakiram (1989 and 1991) adapted a virulent strain of DPV to chick embryo fibroblast and studied its immunogenicity. Their findings showed that it required a few more passages to reach an optimum attenuation and

immunogenicity to serve as an effective cell culture DP vaccine.

2.7 Protein profile of the herpes-viruses

Virus proteins can be separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to produce protein banding patterns or fingerprints which are unique for different viruses (Walpitta *et al.*, 1989). SDS-PAGE has been widely used to characterise viral proteins and to classify viruses (Waddell, 1979).

Herpes viral proteins are, among other viruses, the most carefully analysed and characterised (Honess and Roizman, 1973). SDS-PAGE has been utilized extensively in identifying and characterising herpes viral proteins (Powell and Courtney, 1975).

Eventhough there are reports on the protein profile of other herpes viruses, there are no published records on the protein profile of duck plague virus.

2.7.1 Herpes Simplex virus

Olshevsky and Becker (1970) determined the protein composition of HSV capsids, unenveloped and enveloped virions and identified nine proteins (designated I to IX) with

molecular weights ranging from 24 KD to 110 KD. They also demonstrated the presence of two proteins (II and III) in the empty viral capsids.

At least eight polypeptide components were obtained by Robinson and Watson (1971) while electrophoresisng purified HSV in seven per cent SDS polyacrylamide gel.

Cassai *et al.* (1975) compared the structural proteins specified by HSV types 1 and 2 and were able to reveal a wide range of 24 different polypeptide bands. They detected minor differences in their electrophoretic profiles on SDS-PAGE.

Comparison of the proteins of five herpes viruses viz., HSV-1, HSV-2, Bovine mammilitis virus (BMV), Equine abortion virus (EAV) and pseudorabies virus (PRV) revealed 30 electrophoretically distinct proteins in HSV-1 and HSV-2, 15 in BMV and 20 each in EAV and PRV. Their molecular weights ranged from 29 KD to 200 KD (Killington *et al.*, 1977).

Norrild and Vestargaard (1977) analysed extensively the purified HSV-1 proteins by SDS-PAGE and reported 20 different proteins ranging from 32.7 KD to 258 KD.

2.7.2 Cytomegalovirus (CMV)

Sarov and Abady (1975) obtained atleast 23 polypeptides by SDS urea PAGE of CMV with molecular weights ranging from

24.5 KD to 171 KD. In another study Fiala *et al.* (1976) detected 20 polypeptides ranging from 22 KD to 230 KD molecular weight in purified virus preparations of CMV.

Thirty three different viral structural polypeptides (VPs) were demonstrated by Kim *et al.* (1976) in 5 to 20 per cent polyacrylamide gels and their molecular weights ranged from 11 KD to 290 KD.

Analysis of proteins of purified human cytomegalovirus by SDS-PAGE, revealed 32 polypeptides with molecular weights ranging from 13.5 KD to 235 KD. It was also found that purified preparations of four strains of CMV showed a marked similarity in polypeptide composition (Gupta *et al.*, 1977).

Stinski (1977) showed that purified virus and dense bodies of CMV as analysed by SDS-PAGE revealed atleast 35 polypeptides detectable by Coomassie brilliant blue staining. The viral polypeptides ranged from 12 KD to 200 KD.

Twenty nine viral proteins were obtained by Chantler and Hudson (1978), from purified murine cytomegalovirus by using 10 per cent SDS-PAGE. These viral proteins included major and minor proteins.

Gibson (1981) showed atleast 22 different protein constituents of CMV (Colburn strain) by SDS-PAGE whose molecular weights ranged from 23 KD to 205 KD.

Purified human CMV were shown by SDS-PAGE to contain five glycoproteins on the envelope with molecular weights 52, 67, 95, 130 and 250 KD respectively (Farrar and Oram, 1984).

Proteins of extracellular virions, intracellular nucleocapsids (C-capsids and N-capsids) and the virion derived C-capsids of rat cytomegalovirus were analysed by SDS-PAGE. N-capsids were composed of three major proteins (142, 40 and 32 KD) and four minor proteins (74, 57, 38 and 15 KD) and seven new minor proteins (116, 87, 71, 67, 50, 35 and 34 KD) (Meijer *et al.*, 1984).

Fourteen polypeptide bands of nucleo capsid proteins of CMV strain AD-169 were detected by Sedarati and Rosenthal (1988) with their molecular weights ranging between 30 KD and 260 KD by SDS-PAGE.

2.7.3 Equine herpes virus (EHV)

Abodeely *et al.* (1971) identified 20 polypeptides in EHV-1 ranging in molecular weight from 13 KD to 115 KD. They also revealed that de-enveloped virus and envelope material contained atleast 14 and three polypeptides respectively.

Twenty eight structural proteins of EHV-1 were reported by Perdue *et al.* (1979) with their average molecular weights from 16 KD to 270 KD. They also showed that purified nucleocapsids contained five major structural proteins with molecular weights ranging from 18 KD to 148 KD.

Allen and Randall (1979) studied the structural polypeptides of the purified EHV-1 virions analysed by electrophoresis in SDS-polyacrylamide slab gels. Thirty four polypeptides ranging from 14 KD to 220 KD in molecular weight were observed.

Eleven glycoproteins on the envelope were identified by Turtinen and Allen (1982) of EHV-1 by electrophoresis whose molecular weight ranged from 24 KD to 260 KD.

2.7.4 Bovine herpes virus (BHV)

Sklyanskaya *et al.* (1977) found that mature virions of infectious bovine rhinotracheitis virus (IBR) contained 18 structural proteins with the molecular weights from 29 KD to 250 KD by PAGE.

Twenty one different polypeptides (VP_1 to VP_{21}) were found to be present in the purified extracellular enveloped IBR virus. Their molecular weights ranged from 31 KD to 275 in 10 per cent SDS-polyacrylamide gels (Pastoret *et al.*, 1980).

Mishra *et al.* (1981) described 25 structural polypeptides of IBR virus on 10 per cent gel with molecular weights from 14 KD to 330 KD. They also identified 11 of these polypeptides as glycoproteins.

Analysis of the viral polypeptides of IBR virus by five to 15 per cent linear gradient SDS-PAGE revealed that 33 virion polypeptides (VP_1 to VP_{33}) ranging in molecular weight from 13 KD to 275 KD were present in the complete virus particle (Bolton *et al.*, 1983).

Metzler *et al.* (1985) analysed European isolates of BHV-1 by SDS-PAGE and revealed 36 polypeptides with molecular weights ranging from 17 KD to 200 KD.

Analysis of proteins of BHV-1 isolated from calves with neurological disease by SDS-PAGE revealed atleast 31 distinct proteins (Metzler *et al.*, 1986).

Trepanier *et al.* (1986) demonstrated the proteins of partially purified BHV-1 by PAGE and showed 19 proteins with molecular weights from 14 KD to 145 KD.

Viral proteins of BHV-1 were analysed by five to 12 per cent linear gradient SDS-PAGE under reducing conditions, revealing 30 polypeptides whose relative mobilities ranged from 20 KD to 177 KD (Badia and Querol, 1988).

Twenty nine BHV-4 structural proteins had been identified by SDS-PAGE of purified labelled virus of which 10 of them were glycosylated (Dubuisson *et al.*, 1989).

Castro *et al.* (1992) compared the electrophoretic analysis of proteins of four virulent and four vaccine strains of IBR virus. The study revealed the presence or absence of proteins of 79 KD, 74 KD, 110 KD and 115 KD.

2.8.5 Pseudo rabies virus (PRV)

Ben Porat *et al.* (1970) revealed a difference of two proteins when analysing the proteins present in the infectious nuclear and cytoplasmic viral particles of PRV.

Analysis of the purified and enveloped nucleocapsids of PRV by Stevely (1975) showed that the enveloped particles contained atleast 20 proteins, whose molecular weights were in the range of 20 KD to 230 KD. He also demonstrated that the naked necleocapsids contained one major and seven minor proteins in the molecular weight range of 20 KD to 155 KD.

Hampl *et al.* (1984) identified 27 different viral proteins on analysing the envelope proteins of PRV, detected by PAGE. Their molecular weights ranged from 32 KD to 142 KD on 8 per cent polyacrylamide gels.

Comparison of proteins of attenuated strain MK-25 and A_2 strain of PRV revealed 18 polypeptides ranging from 20 KD to 270 KD in molecular weight (Khristova, 1986).

Hahn and Hahn (1987) resolved 34 protein bands of PRV by SDS-PAGE.

Todd *et al.* (1987a) consistently observed over 20 protein bands when purified PRV proteins were analysed by SDS-PAGE whose molecular weights were from 20 KD to 200 KD. In another study, over 200 proteins with molecular weights from 26 KD to 200 KD were observed when purified PRV preparation was analysed by PAGE (Todd *et al.*, 1987b).

2.8.6 Marek's Disease Virus (MDV)

Analysis of viral proteins by PAGE revealed that atleast eight proteins (designated VPI to VP VIII) were present in MDV (Chen *et al.*, 1972).

A comparison between the poypeptides of nucleocapsids of MDV strains with different biological properties and of antigenically related Herpes virus of Turkey strains by one and two dimensional gel electrophoresis revealed small differences in migration behaviour of a number of corresponding nucleocapsid polypeptides. Based on this the virus strains were differentiated into three groups (Westerbrink *et al.*, 1985).

Materials and Methods

MATERIALS AND METHODS

3.1 Materials

3.1.1 Ducklings

Ducklings from two sources were used to revive the virulent stock virus of DP (DPV-I).

(i) Three to four week old unvaccinated, healthy ducklings procured from the Duck Unit, University Poultry Farm, Mannuthy.

(ii) Two week old unvaccinated, healthy ducklings purchased from a private hatchery.

3.1.2 Eggs

(i) Duck eggs

Fertile eggs purchased from the University Poultry Farm, Mannuthy and private farms were used for the revival and titration of virulent viruses and preparation of duck embryo fibroblast (DEF) cell cultures.

(ii) Chicken eggs

Nine to eleven day old embryonated chicken eggs obtained from University Poultry Farm, Mannuthy were used for the

revival and titration of vaccine virus and preparation of chicken embryo fibroblast (CEF) cell cultures.

3.1.3 Biological products

(i) Vaccine virus

Vaccine strain of duck plague received from Veterinary Biological Institute (VBI), Palode, Kerala was used for propagation in chicken embryos and CEF cultures.

(ii) Virulent virus

a. The reference duck-plague virulent virus strain (Repository number - (Vir) AD/87-1) was obtained from Division of Avian Diseases, IVRI, Izatnagar. The virus was in the form of lyophilised liver suspension.

b. A field isolate of duck plague obtained from an outbreak in the Alleppey district was also used for the study.

3.1.4 Cell culture media

(i) Glasswares

Corning brand, neutral glassware were prepared as per the standard procedure (Cunningham, 1966).

(ii) Triple glass distilled water

Tap water distilled thrice in glass distillation apparatus was used to prepare different media/reagents.

(iii) Calcium magnesium free phosphate buffer saline (CMF-PBS)

Procured from Himedia laboratories as readymade dehydrated powder in vials.

(iv) 7.5 per cent sodium bicarbonate solution

Sodium bicarbonate - 7.5 g
Triple distilled water - 100 ml
Sterilized by filteration

(v) Trypsin

A stock solution of five per cent trypsin (1:250 SRL) was prepared in CMF-PBS, sterilised by filtration and stored at -20°C. When needed the working solution was prepared by diluting the stock solution with CMF-PBS to give a final concentration of 0.25 per cent.

(vi) Calf serum

Blood collected from cross-bred bull calves of about six months to one year was allowed to clot and refrigerated

overnight. The serum thus separated was collected in a sterile flask, inactivated at 56°C for 30 min, filtered and stored at -20°C until used.

(vii) Antibiotic solution

Benzyl penicillin	100000 I.U.
Streptomycin	1 g
Sterile distilled water	40 ml

The concentration of these antibiotics were decided in such a way that when one ml of this mixture was added to 100 ml, a final concentration of 250 I.U. of penicillin and 250 micrograms of streptomycin per ml was obtained. Two times the above concentration was used for egg inoculation and specimen inoculation.

(viii) Stock solutions

Dehydrated tissue culture media supplied by Himedia Laboratories were used to prepare the following stock solutions.

a. Hank's Balanced Salt Solution (HBSS) 10x

b. Minimum Essential Medium (MEM) with Earle's salt 10x

(ix) Working solutions

Working solutions were prepared from the above stock solutions.

a. Growth medium (GM) for Chicken Embryo Fibroblast (CEF) cell cultures

HBSS 10x	-	10 ml
Lactalbumin hydrolysate (LAH)	-	0.5 g
Yeast extract	-	0.2 g
Triple glass distilled water to make upto	-	100 ml

b. GM for Duck Embryo Fibroblast (DEF) cell cultures

MEM with Earle's salt 10x	-	10 ml
Triple glass distilled water to make upto	-	100 ml

After addition of 5 per cent of calf serum and antibiotics to the GM, the pH was adjusted to 7.3 to 7.4 using 7.5 per cent sodium bicarbonate just before use.

c. Maintenance Medium (MM)

The working solutions of HBSS/MEM (Earle's salt) with one per cent calf serum was used as MM.

3.1.5 Virus purification and concentration

Molecular biology grade/AR grade chemicals from SISCO Research Laboratories (SRL) were used.

(i) **TNE buffer**

Tris hydrochloride	-	7.9 g
Sodium chloride	-	1.95 g
EDTA	-	0.25 g
Distilled water	-	480 ml

pH was adjusted to 8.0 with 10N NaOH and the final volume was made upto 500 ml.

3.1.6 Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used for characterization of protein fractions of different isolates, following the procedure described by Laemmli (1970).

Reagents

Molecular biology grade/extrapure AR grade chemicals from SRL were used.

(i) Solution A

Acrylamide	-	30 g
N,N, Methylene bis acrylamide	-	0.8 g
Distilled water	-	100 ml

The solution was filtered through Whatman No.1 filter paper and stored at 4°C in an amber coloured bottle.

(ii) Solution B (pH 8.8)

Tris base	-	12.1 g

pH was adjusted to 8.8 with 10N HCl and was made upto 100 ml with double glass distilled water, filtered and stored at 4°C.

(iii) Solution C (pH 6.8)

Tris base	-	6.06 g
Distilled water	-	80 ml

pH was adjusted to 6.8 with 10N HCl. Final volume was made upto 100 ml, filtered and stored at 4°C.

(iv) SDS (one per cent)

SDS	-	1 g
Distilled water	-	100 ml

Filtered and stored at room temperature.

(v) Ammonium per sulphate

Ammonium per sulphate	-	50 mg
Distilled water	-	1 ml

Prepared freshly before use.

(vi) Electrode buffer (Tris glycine)

Tris base	-	3.03 g
Glycine	-	14.4 g
SDS	-	1 g
Distilled water	-	800 ml

pH was adjusted to 8.3 with 10N Hcl and the final volume was made upto one litre with double glass distilled water, filtered and stored at room temperature.

(vii) Resolving gel (10 per cent)

Solution A	-	6.7 ml
Solution B	-	6.0 ml
SDS	-	2.0 ml
Distilled water	-	5.2 ml
TEMED	-	10 ul
Ammonium per sulphate	-	0.1 ml
Total	-	20 ml

(viii) Stacking gel (five per cent)

Solution A	-	1.65 ml
Solution C	-	2.5 ml
SDS	-	1 ml
Distilled water	-	4.75 ml
TEMED	-	5 μl
Ammonium per sulphate	-	50 μl
Total	-	10 ml

(ix) Sample buffer

Solution C	-	8.5 ml
SDS	-	0.2 ml
Glycerol	-	1 ml
2-Mercapto ethanol	-	0.5 ml

Pinch of bromophenol blue.

(x) Staining solution

Coomassie brilliant blue R-250	-	200 mg
Methanol	-	50 ml
Acetic acid	-	10 ml
Distilled water	-	40 ml

The stain was prepared and filtered through Whatman No.1 filter paper and stored at room temperature.

(xi) Destain I

Methanol	-	50 ml
Acetic acid	-	10 ml
Distilled water	-	40 ml

(xii) Destain II

Methanol	-	25 ml
Acetic acid	-	50 ml
Distilled water	-	425 ml

3.2 Methods

3.2.1 Revival and passage of viruses

3.2.1.1 Virulent IVRI DP virus (DPV-I)

Four, 3 to 4 week old healthy ducklings were inoculated with 0.5 ml each of the virus reconstituted with two ml of sterile PBS, by intramuscular route. Liver and spleen were collected with utmost aseptic precautions from the birds, which died of DP infection and were preserved at -45°C. A 20 per cent W/V suspension of liver and spleen was made in PBS and centrifuged at 3000 rpm for 15 min. Antibiotics were

added to the supernatent at the rate of 500 I.U. of penicillin and 500 ug of streptomycin per ml and stored at -45°C.

The above supernatent was inoculated into twelve day old developing duck embryos (DDE) by CAM route as per the procedure described by Betts (1967), incubated at 37°C, candled daily and embryopathy was noticed. The dead embryos were chilled at 4°C for 24 h soon after candling. The clear allantoic fluid was collected centrifuged at 1000 rpm for 15 min and the supernatent was preserved at -45°C for use as antigen for inoculation in DEF monolayers.

3.2.1.2 Vaccine DP virus (DPV-V)

Eleven day old developing chick embryos (DCE) were inoculated with the vaccine virus by CAM route and harvested as per the above procedure. While harvesting, the whole embryos, CAM and AAF were collected. The embryo and CAM were chopped into pieces and stored at -45°C along with AAF. The tissues were homogenised and centrifuged at 5000 rpm for 15 min. The supernatent was collected, added antibiotics as in the case of DPV-I and preserved at -45°C for use as antigen for passage in DCE/CEF monolayers.

3.2.2 Isolation of virulent field virus (DPV-A)

Ailing and freshly dead ducks received from an outbreak in Alleppey district were utilised for virus isolation. Ailing birds sacrificed by exsanguination were subjected to detailed post-mortem examination. Liver and spleen were collected from the ailing and freshy dead birds and preserved at -45°C after adding three to four ml of tryptose phosphate broth. Using the preserved tissues, 20 per cent w/v suspension was made in PBS (pH 7.2) centrifuged at 3000 rpm for 15 min and the supernatent was treated with heavy dose of antibiotics (500 I.U. Penicillin; 500 ug Streptomycin per ml) and incubated at 37°C for one hour.

This formed the inoculum for embryonated duck eggs.

The above supernatent was inoculated into 12 day old DDE via CAM route and the AAF and CAM were harvested as described for the revival of DPV-I.

3.2.3 Propagation of virus in cell cultures

3.2.3.1 Preparation of primary DEF and CEF cultures

Ten actively moving, 12 day old embryonated duck eggs/10 day old embryonated chicken eggs were used for the preparation of DEF/CEF cultures. Standard procedure for DEF and CEF

cultures (Rovozza and Burke, 1973) was followed using 0.25 per cent trypsin solution by rapid trypsinization procedure. Viable cell count was made using trypan blue dye exclusion technique and the concentration was adjusted at 10^6 cells per ml throughout for seeding.

3.2.3.2 Infection of DEF and CEF monolayers

The tubes and prescription bottles showing confluent monolayers at 24 h were infected with the inoculum at the rate of 0.2 ml per tube and one ml per bottle. The DPV-I and DPV-A were inoculated into DEF cultures, while the DPV-V was inoculated into CEF cultures. Two bottles and two tubes were kept as uninfected controls. The cultures were examined under inverted microscope every 12 h post infection for the appearance of CPE. The bottles with maximum CPE were harvested by freezing and thawing thrice, centrifuging the contents at 5000 rpm for 15 min at 4°C and the supernatent was preserved at -45°C for purification and concentration of the virus.

3.2.4 Titration of the virus

The titration of all the three viruses (DPV-I, DPV-A and DPV-V) was done as per the method described by Villegas and Purchase (1980).

3.2.4.1 In embryonated eggs

The embryo lethal dose (ELD_{50}) of DPV-I and DPV-A was titrated in DDE while that of DPV-V was done in DCE.

Ten fold serial dilutions of the supernatents starting from 1 to 6 log 10 in chilled PBS (added with penicillin 500 I.U., Streptomycin 500 ug) were made using separate sterile pipette for each dilution. From each dilution, 0.1 ml dose per embryo was inoculated into five embryos by CAM route. After recording the deaths resulting between 36 h and six days, the 50 per cent end point of embryo lethal dose (ELD_{50}) was calculated as per the method illustrated by Reed and Muench (1938).

3.2.4.2 In cell cultures

The tissue culture infective dose ($TCID_{50}$) of DPV-I and DPV-A was titrated in DEF cultures while that of DPV-V was done in CEF cultures.

Ten fold dilutions of the supernatents were made as described above. From each dilution, 0.1 ml dose per tube having confluent monolayer was inoculated into five such tubes. The tubes were incubated at 37°C and observed every 12 h for CPE. Those tubes showing CPE were noted and the 50

per cent end point of tissue culture infective dose ($TCID_{50}$) was calculated as illustrated above.

3.2.5 Virus concentration and purification

The two virulent DP viruses (DPV-I and DPV-A) and the vaccine DP virus (DPV-V) were propagated in DEF and CEF cell cultures respectively. The supernatent obtained after the centrifugation of the infected cell lysate from the preliminary material for the concentration and purification of the virus.

The cell culture supernatent was centrifuged at 5,000 rpm for five minutes to sediment any coarse contaminants if any. This supernatent was centrifuged at 40,000 rpm for 4.5 h in a Servo Combi Plus No. 80 Rotar at 4°C.

The clear button like pellet was diluted with a minimum quantity of TNE buffer. The diluted pellet was again centrifuged in microfuge at 5000 rpm for 10 min. The clear supernatent was transferred to Ependrof tubes which contained the partially pure form of virus. This was later used for SDS-PAGE.

The same procedure was adopted for all the three strains of virus viz. DPV-I, DPV-A and DPV-V.

3.2.6 Direct electron microscopical examination of DPV

The DPV infected duck embryo/cell culture material was clarified by low speed centrifugation at 5000 rpm for 5 minutes. This supernatent was ultracentrifuged at 40,000 rpm for 4 hrs at 4°C in a Servo Combi Plus No.80 Rotor. The pellet obtained was resuspended in 0.3 ml of distilled water, centrifuged at 3000 rpm for 15 minutes and this supernatent was examined for the presence of the virus.

One drop of this suspension was placed on a formvar coated grid. The excess fluid was absorbed by a Whatman No.1 filter paper after 45 seconds. This charged grid was then stained with potassium phospho tungstate (PTA one per cent aqueous solution, pH 6.5) for 35 seconds. The excess PTA was blotted out and the grid was dried at room temperature for 10 minutes and examined in a Hitachi 600 A electron microscope at 75 KV.

3.2.7 SDS-PAGE

3.2.7.1 Sample preparation

Three strains (DPV-I, DPV-A and DPV-V) of DPV were used for protein characterization. Equal volumes of the purified sample and the sample buffer were mixed and heated in a water bath at 90°C for one minute, cooled and stored at 4°C.

3.2.7.2 Gel electrophoresis

The discontinuous system of polyacrylamide gel electrophoresis was carried out according to Laemmli (1970).

The gels were prepared in 16x20 cm glass plates supplied with the vertical electrophoresis apparatus with one mm thick spacer.

Ten per cent resolving gel was prepared (according to the composition given earlier) and poured in between the glass plates. Over this three ml of distilled water was added to get uniform surface and allowed to polymerise for 20 min.

After polymerisation, water was removed and five per cent stacking gel was added and allowed to polymerise for 20 min after inserting the comb. The comb was removed after complete polymerisation and each well was loaded with 20 µl quantities of different samples. Bovine Serum Albumin (BSA) and Lactalbumin were also loaded in the same way, as markers.

Electrophoresis was initially done at 100 V till the dye reached the surface of resolving gel and then the voltage was changed to 150 V and was continued till the dye reached the bottom of the gel.

The gel was removed and stained overnight with Coomassie blue and then destained with decolourizer-I for three hours,

followed by decolourizer-II, with frequent changes to complete destaining. After destaining the gel was kept on a transilluminator and photographed.

The position of the bands were traced on a transparency sheet. The molecular weights of marker proteins were plotted on a graph in relation to their migration. From this, molecular weights of viral proteins were calculated as per the method of Shapiro *et al.* (1967).

Results

RESULTS

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

4.1 Revival and passage of the viruses

4.1.1 IVRI strain (DPV-I)

Four ducklings were in inoculated with DPV-I for the revival of the virus. The ducklings started evincing symptoms from the 7th day PI like dullness, increased thirst, sticky eyes, anorexia, instability, nasal discharge initially watery becoming thick and mucoid at the later stages, swelling of the face and greenish vents due to greenish watery diarrhoea (Plate 1). Fifty per cent of the birds in the group died. A few hours before death, ducks preferred to sit with beak rested on some object or on the back in the wings. Sick birds when forced to move, had tremors of head, neck and body. The affected birds died.

On necropsy of the dead ducks, varying degrees of haemorrhage on the serous membranes, muscles and visceral organs were noticed. Petechial or ecchymotic haemorrhages on heart and abdominal walls were seen. Necrotic foci and

Plate 1. Clinical signs of duck plague in a duckling

Plate 2. Gizzard muscle necrosis in a duckling infected with DPV-A

petechiae on the liver, enlargement and congestion of liver and spleen were noted. Pathognomonic lesions were seen in the cases viz., yellowish, crusty diptheritic plaques in the oesophagus, catarrhal inflammation of the proventriculus, whitish necrotic areas in the gizzard musculature (Plate 2). Red paint brush appearance of the pericardium was also seen. Liver and spleen of these birds were processed and used as virus source for inoculation of DDE.

Twelve day-old duck embryos inoculated with 20 per cent suspension of liver and spleen from the above ducklings, succumbed between fourth and sixth day PI showing edema, haemorrhage all over the body and generalized congestion of CAM (Plate 3). Embryo liver and spleen were enlarged and showed petechiae and necrotic foci (Table 1).

4.1.2 Vaccine strain (DPV-V)

The reconstituted DPV-V, inoculated into DCE by CAM route, died within 72 to 120 hrs PI, with characteristic, extensive haemmorhages on the CAM and the whole embryo. Maximum mortality was noted within 120 hrs Pi (Table 1).

The whole embryo along with the AAF and CAM were collected and used for further passages and inoculation in cell cultures.

Plate 3. Congestion of embryo and CAM (inoculated with Alleppey strain of DPV)

4.2 Isolation of virulent field virus (DPV-A)

Ailing ducks were procured from a disease outbreak in Alleppey district. The symptoms by these birds were very similar to those observed on experimental infection of DPV-I. The birds were seen squatting in the corners with mucoid nasal discharge and marked respiratory distress. Pasty eyelids were also seen. All the birds obtained from the field outbreak died with two days of procurement.

Gross lesions observed during the post-mortem examination were similar to that of DPV-I. Most of the birds had red annular bands at regular intervals on the mucosa of the small intestine.

Twelve day old DDE were inoculated with the processed material suspected to contain DPV-A, at the rate of 0.2 ml per egg by CAM route. Majority of the embryos died within three to six days. Generalized congestion of the embryo and CAM were seen (Plate 3). The extremities of the embryo, the abdomen and occipital region were severely congested. The embryonic liver showed necrotic foci (Table 1). The harvested material was serially passaged thrice to increase the virulence which was evident from the reduction in time taken for death of the embryos from six days to three to four days.

4.3 Propagation of virus in DEF/CEF cell cultures

4.3.1 IVRI strain (DPV-I)

A satisfactory uniform monolayer of DEF was formed within 18 to 24 hours of seeding (Plate 4). Elongated, spindle shaped fibroblast could be noted. DPV-I was inoculated at the one ml per bottle and 0.2 ml per tube. DPV-I produced marked CPE from the first passage in cell cultures. The infected cell cultures showed characteristic rounding and clumping of cells within 36-48 hours PI. The foci of infection enlarged slightly during the next 24 hrs thus resulting in very small, grape like clusters.

Gradually the infected DEF cells fused to form syncytia (Plate 5). By 72 hrs PI a large portion of cells was detached from the glass surface. Vacuolation of cytoplasm and eosinophilic intranuclear inclusion bodies were noticed on microscopic examination of coverslip cultures stained by May-Grunwald Giemsa staining (Plate 6 and Table 1). Passaging thrice serially in cell cultures reduced the time taken for production of CPE to 18 to 24 hours PI.

4.3.2 Alleppy strain - DPV-A

Complete monolayer of DEF cell cultures were formed by 24 to 36 hrs. The DEFC monolayer was inoculated with the

Plate 4. Normal monolayer of duck embryo fibroblast. May-Grunwald Giemsa stain x 400

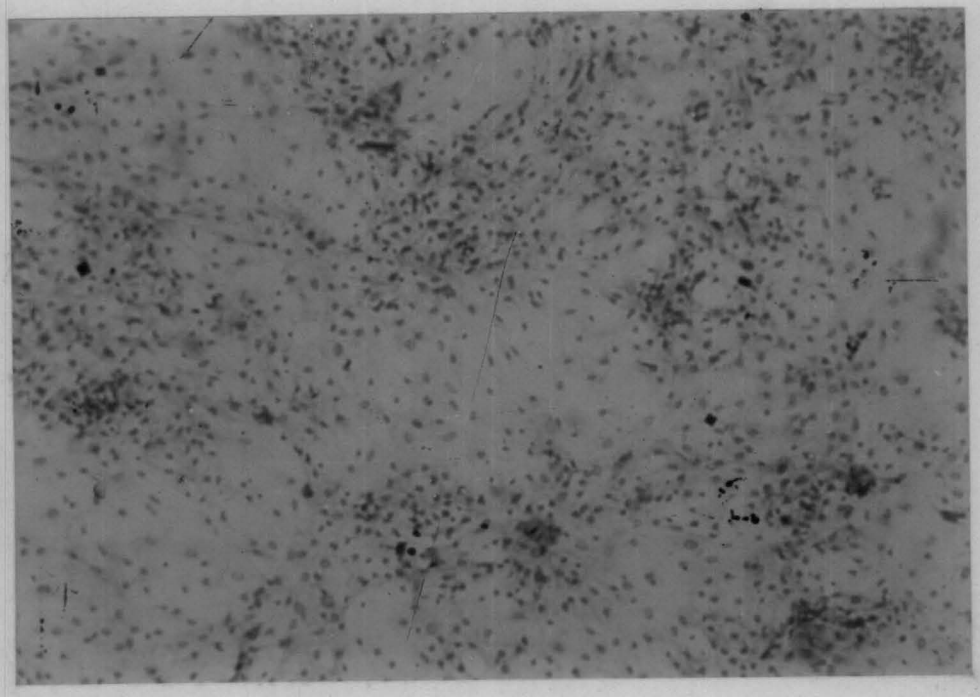
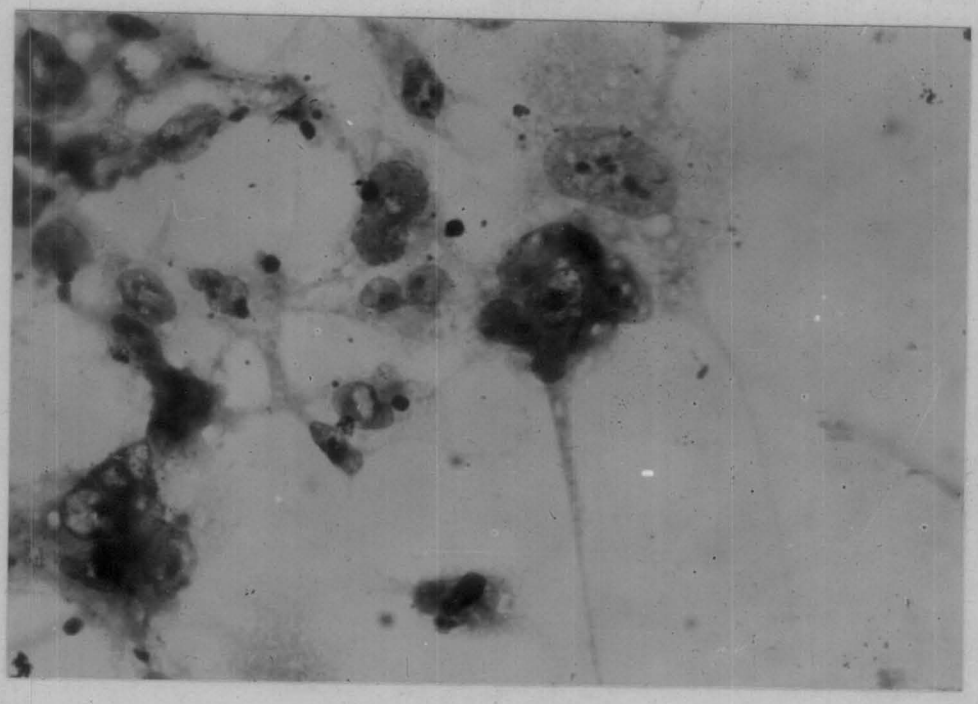


Plate 5. Syncytium formation in duck embryo fibroblast cell culture 48 hours P.I. with DPV-A. May Grunwald Giemsa stain x 1200

Plate 6. Intranuclear inclusion bodies and vacuolation observed in duck embryo fibroblast. 48 hours P.I. with DPV-A. May Grunwald Giemsa stain x 1200



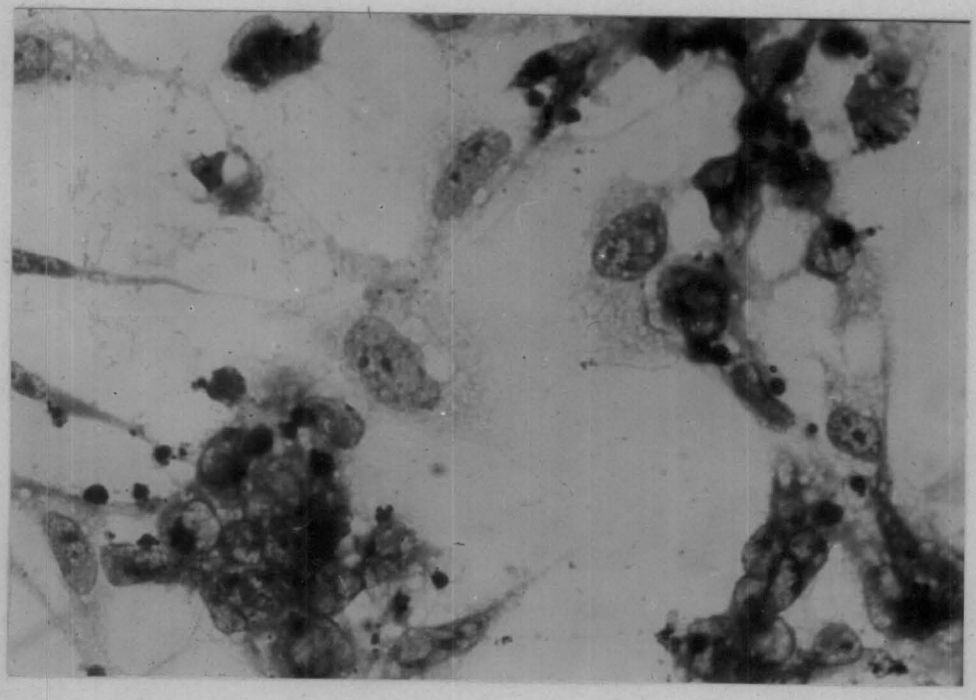


Table 1. Embryonic lesions and cytopathic changes of DPV-I, DPV-A and DPV-V

Sl. No.	Isolate	Lesions		Cytopathic changes	
		DDE	DCE	DEFC	CEFC
1.	IVRI DPV-I	Death of embryo between 4-6 days P.I. Edema, haemorrhage all over the body. Liver and spleen showed necrotic foci and petechae	Not significant	CPE within 36-48 hr. Rounding and clumping of cells. Vacuolation and eosinophilic intranuclear inclusion bodies seen	-
2.	DPV-A Alleppey	Death of embryo between 3-6 days P.I. Generalized congestion of embryo and CAM. Severe congestion on the extremities	Not significant	CPE within 48-72 hr PI. Granular changes of cytoplasm. Syncytium formation and intranuclear inclusion bodies seen	-
3.	DPV-V Vaccine	-	Death of embryo between 72-120 hr PI. Charateristic extensive haemorrhage on CAM and embryo	-	CPE within 48-60 hr. Rounding and clumping of cells syncytrum formation and intranuclear inclusion bodies seen

DDE - Developing duck embryo
DCE - Developing chick embryo
DEFC - Duck embryo fibroblast culture
CEFC - Chick embryo fibroblast culture

processed embryo material. The cell culture did not show marked CPE during the initial two passages but on subsequent passage rounding and clumping of cell were seen from third day Pi. Maximum CPE was observed as the infected cells were detached from the glass surface at 96 hrs (Table 1).

Microscopic examination of stained cover slip cultures of 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 (Plate 6). The time of occurrence of maximum CPE and complete degeneration of cells decreased to 36 hrs as the number of passages increased.

4.3.3 Vaccine strain (DPV-V)

After the formation of a complete CEF monolayer DPV-V produced CPE at 120 hrs Pi in the first two passages. The time of onset of CPE was found to decrease with increasing passages. Accordingly the time taken for producing CPE decreased from 120 hrs to 60 hrs (Table 1).

Characteristic changes observed in the DPV-V infected CEF cell cultures were same as that was produced by other two strains in DEFC.

4.4 Titration of the viruses

4.4.1 IVRI strain

4.4.1.1 In duck embryos

Ten fold dilutions of DPV-I prepared in chilled PBS was inoculated by CAM route at the rate of 0.2 ml per egg for titration of the virus. The embryo lethal dose (ELD_{50}) of this strain was found to be $10^{5.27}$ per ml of the inoculum (Table 2).

4.4.1.2 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 of DPV-I in maintenance medium. $TCID_{50}$ was calculated to be $10^{5.75}$ per ml of the inoculum (Table 2).

4.4.2 Alleppey strain DPV-A

4.4.2.1 In duck embryos

DPV-A titrated in 12-day old DDE had an ELD_{50} of $10^{4.86}$ per ml of inoculum (Table 2).

4.4.2.2 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 2).

Table 2. Titre values of DPV-I, DPV-A and DPV-V

Sl. No.	Isolate	Titre of DPV	
		ELD_{50}	$TCID_{50}$
1.	IVRI DPV-I	$10^{5.27}$	$10^{5.75}$
2.	Alleppey DPV-A	$10^{4.86}$	$10^{5.25}$
3.	Vaccine DPV-V	10^{4}	$10^{4.5}$

ELD_{50} - Embryo lethal $dose_{50}$

$TCID_{50}$ - Tissue culture infective $dose_{50}$

4.4.3 Vaccine strain DPV-V

4.4.3.1 In chick embryo

Vaccine strain had an ELD_{50} of $10^{4.0}$ per ml when titrated in 10 day old DCE (Table 2).

4.4.3.2 In chick embryo fibroblast cultures

DPV-V had a $TCID_{50}$ of $10^{4.5}$ per ml in CEF cell cultures.

4.5 Direct electron microscopic examination of the virus

The processed DDE and DEF culture samples, on examination in electron microscope revealed enveloped viral particles, with the morphological features of herpes virus (Fig.7). The size of these particles ranged between 150-190 nm with a core size of 70-90 nm (Plate 7).

4.6 Protein profile of the viruses

Partially purified DP virus when subjected to protein analysis by SDS-PAGE, the various polypeptides of the virus got separated into different bands. These bands were visible when stained with Coomassie brilliant blue dye. The electrophoretic pattern of the viral proteins of IVRI strain, Alleppey strain and vaccine strain of duck plague virus are presented in Plate 8 and Fig.1.

Plate 7. Electron microscopy of DPV-A x 80,000

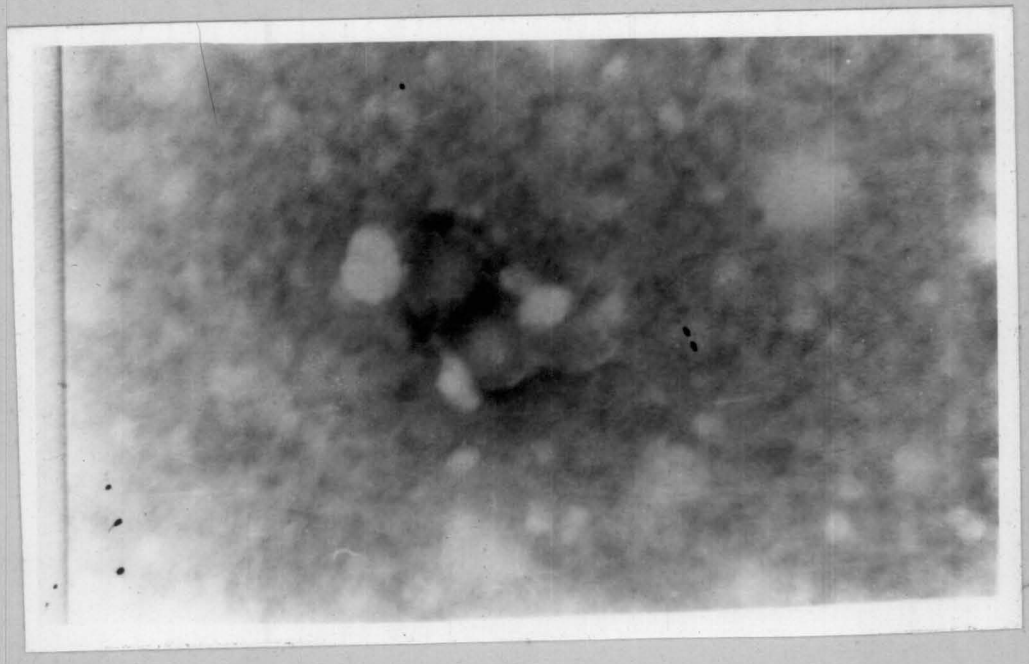


Plate 8. SDS-PAGE pattern of DPV-isolates

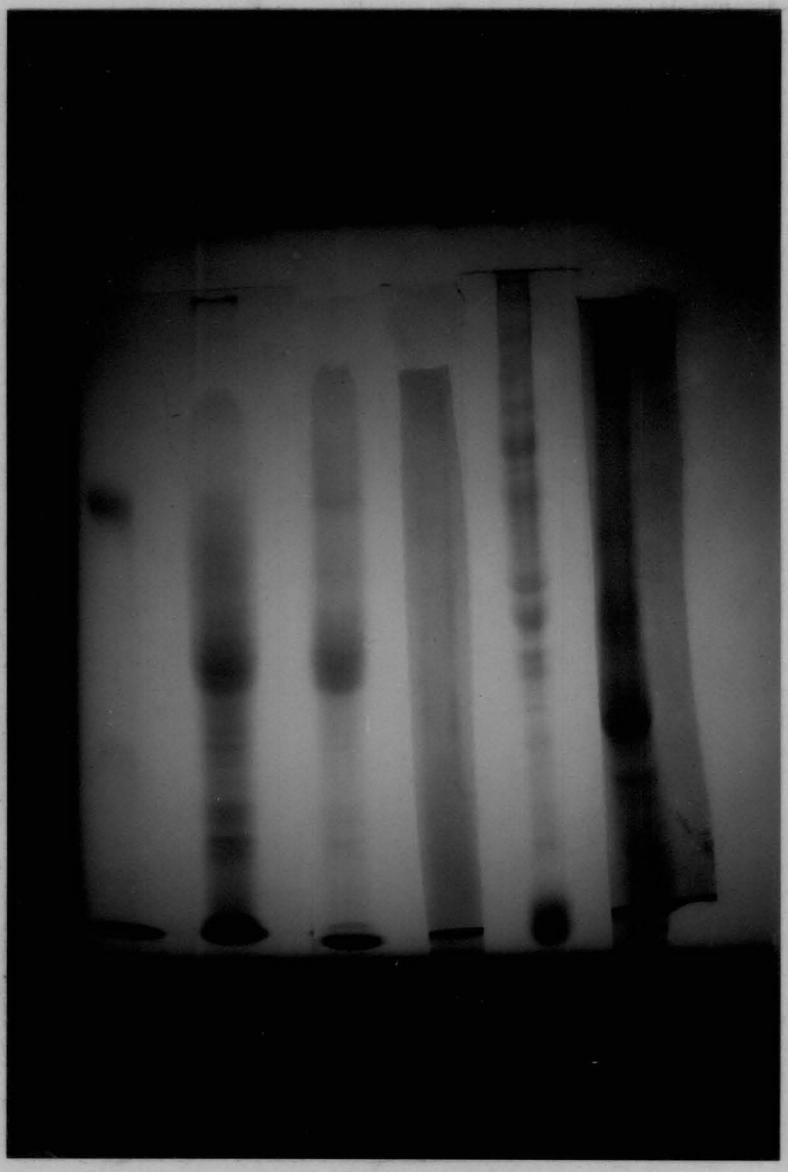


Fig.1 Diagrammatic representation of SDS-PAGE patterns of DPV

I - Protein markers

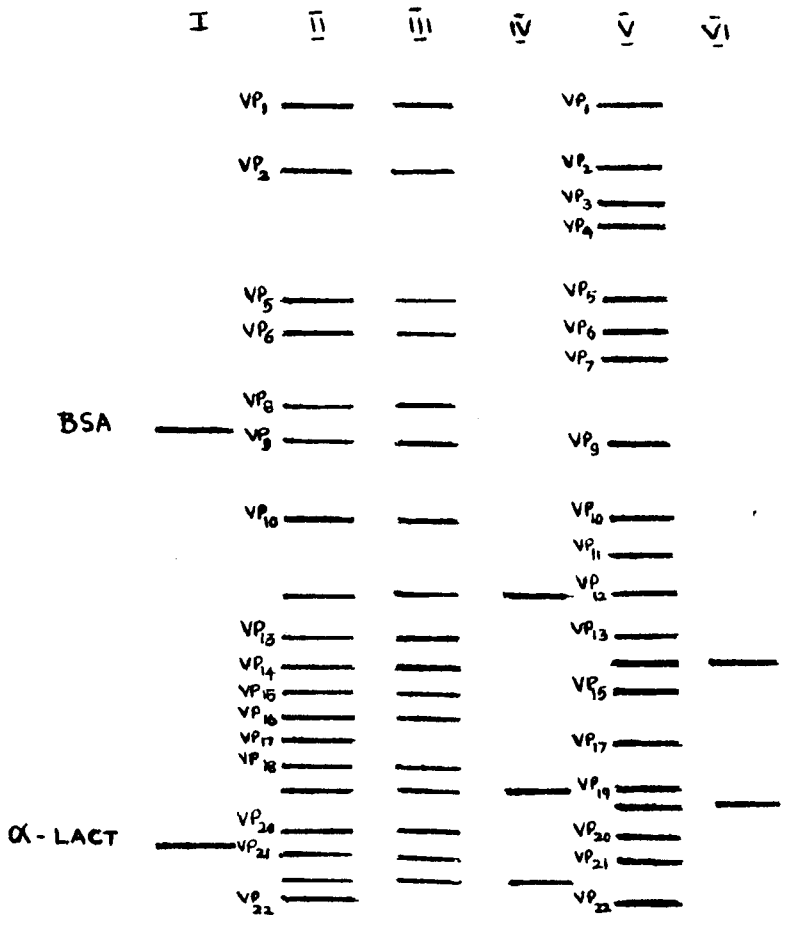
II - DPV (IVRI-strain)

III - DPV (Alleppey strain)

IV - Control duck (Control)

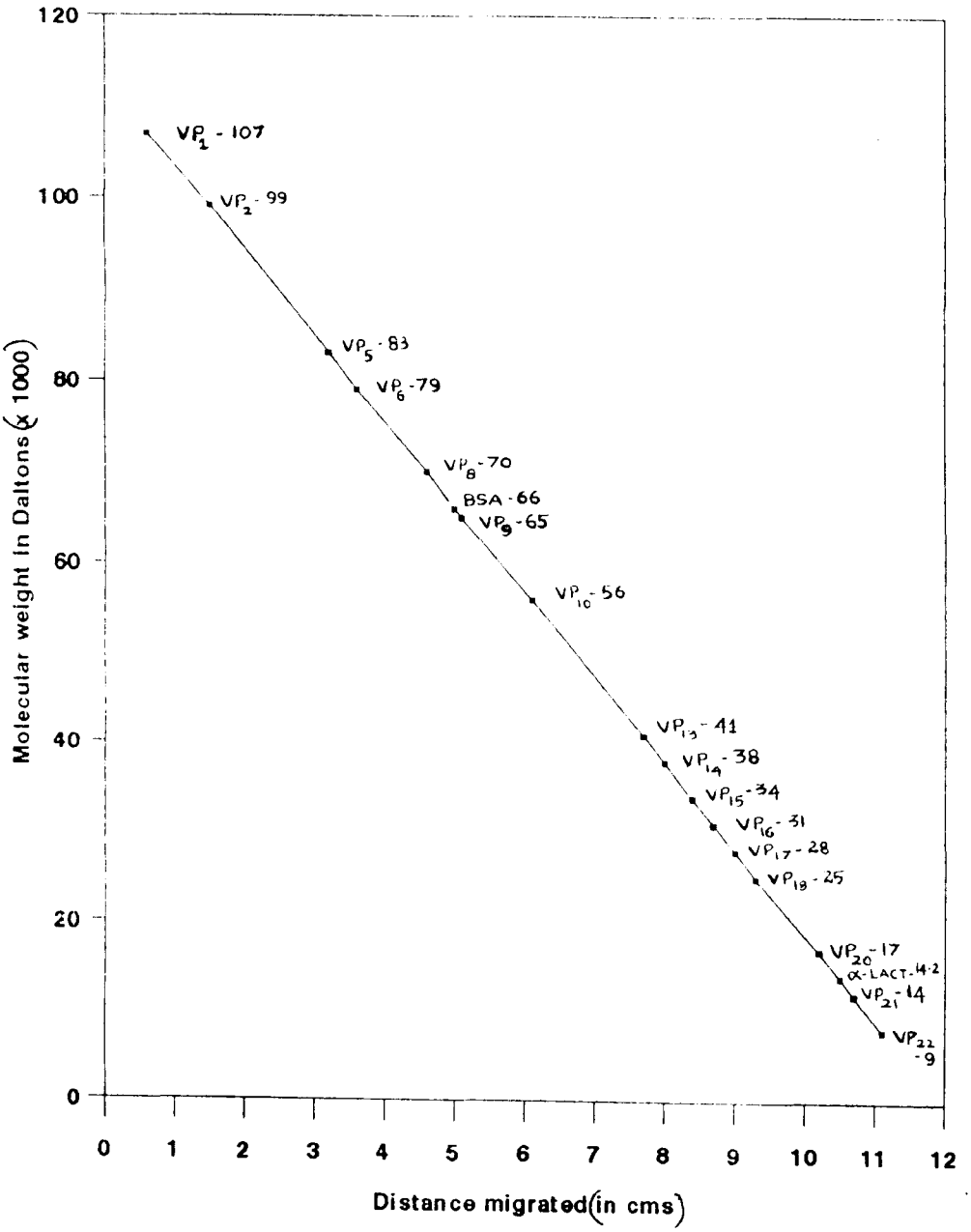
V - DPV (Vaccine strain)

VI - Control (Chicken)


I
II
III
IV
V
VI
VP1
VP2
VP3
VP4
VP5
VP6
VP7
VP8
VP9
VP10
VP11
VP12
VP13
VP14
VP15
VP16
VP17
VP18
VP19
VP20
VP21
VP22
BSA
α - LACT

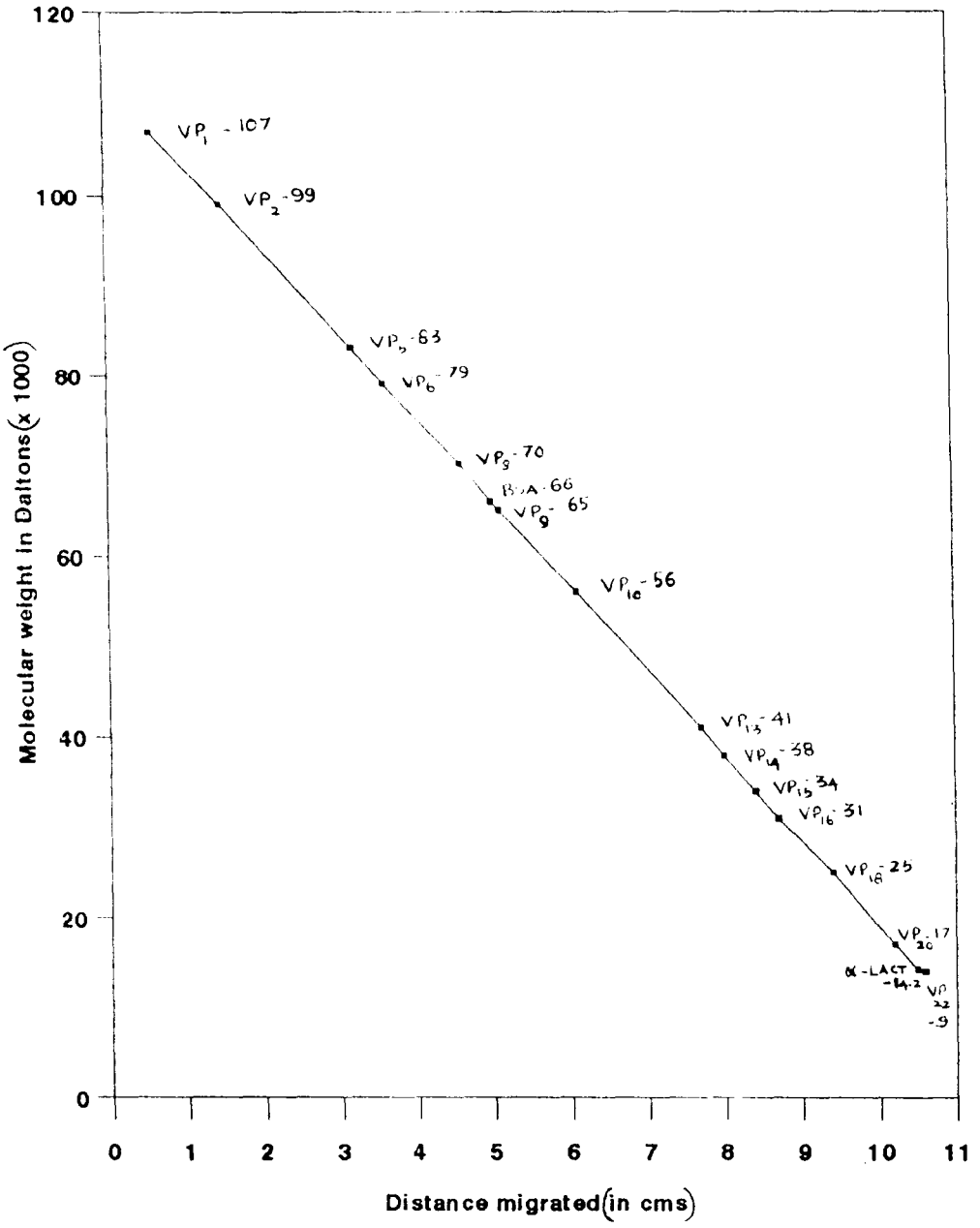
MOLECULAR WEIGHT DETERMINATION OF PROTEINS OF DPV (IVRI STRAIN)

Fig.2



MOLECULAR WEIGHT DETERMINATION OF PROTEINS OF DPV (ALLEPPY STRAIN)

Fig.3



MOLECULAR WEIGHT DETERMINATION OF PROTEINS OF DPV (VACCINE STRAIN)

Fig.4

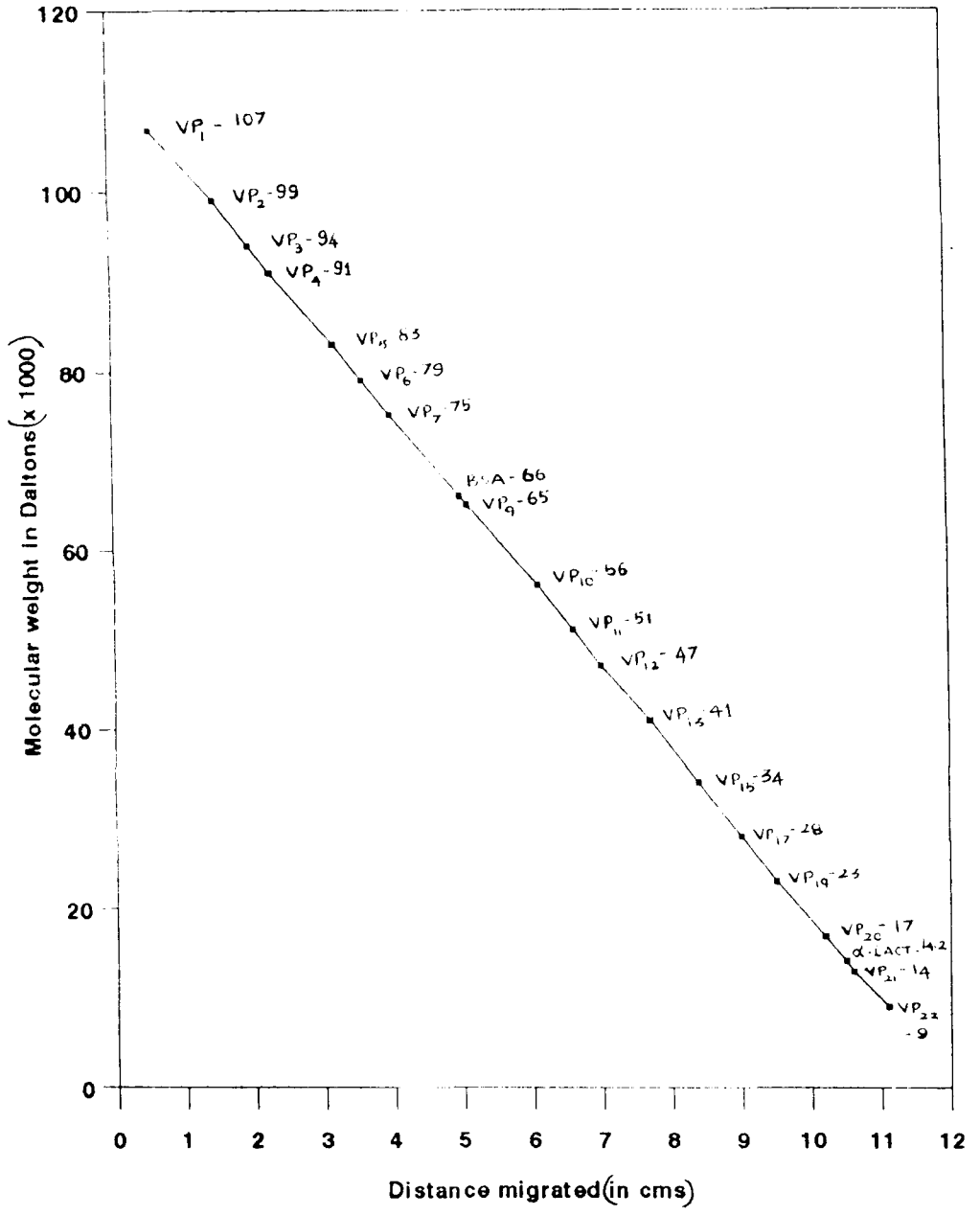


Table 3. Molecular weights of different protein fractions of duck plague virus

Protein fractions	IVRI		Alleppey		Vaccine	
	Distance migrated cm	Mol.Wt KD	Distance migrated cm	Mol.Wt KD	Distance migrated cm	Mol.Wt KD
VP_1	0.6	107	0.6	107	0.6	107
VP_2	1.5	99	1.5	99	1.5	99
VP_3					2.0	94
VP_4					2.3	91
VP_5	3.2	83	3.2	83	3.2	83
VP_6	3.6	79	3.6	79	3.6	79
VP_7					4.0	75
VP_8	4.6	70	4.6	70	-	-
VP_9	5.1	65	5.1	65	5.1	65
VP_{10}	6.1	56	6.1	56	6.1	56
VP_{11}					6.6	51
VP_{12}					7.0	47
VP_{13}	7.7	41	7.7	41	7.7	41
VP_{14}	8.0	38	8.0	38	-	-
VP_{15}	8.4	34	8.4	34	8.4	34
VP_{16}	8.7	31	8.7	31	-	-
VP_{17}	9.0	28	-	-	9.0	28
VP_{18}	9.4	25	9.4	25	-	-
VP_{19}					9.6	23
VP_{20}	10.2	17	10.2	17	10.2	17
VP_{21}	10.6	14	10.6	14	10.6	14
VP_{22}	11.1	9	-	-	11.1	9
	16 Proteins		14 Proteins		18 Proteins	

The molecular weights of these proteins were calculated by comparing the distance migrated by known molecular weight protein markers namely α -lactalbumin and Bovine Serum albumin.

The IVRI strain of DP virus resolved sixteen proteins while the Alleppey strain resolved only fourteen proteins. Alleppey strain showed a similar band pattern as that of the IVRI strain except that it lacked 28 KD (VP_{17}) and 9 KD (VP_{22}) proteins.

The vaccine strain showed a different pattern of protein bands from the virulent strains. Eighteen polypeptides could be demonstrated in the vaccine strain.

The molecular weights of the sixteen proteins resolved in the IVRI strain were VP_1 (107KD), VP_2 (99KD), VP_5 (83KD), VP_6 (79KD), VP_8 (70KD), VP_9 (65KD), VP_{10} (56KD), VP_{13} (41KD), VP_{14} (38KD), VP_{15} (34KD), VP_{16} (31KD), VP_{17} (28KD), VP_{18} (25KD), VP_{20} (17KD), VP_{21} (14KD) and VP_{22} (9KD) (Table 3 and Fig.2).

The fourteen proteins resolved in the Alleppey strain had molecular weights of VP_1 (107KD), VP_2 (99KD), VP_5 (83KD), VP_6 (79KD), VP_8 (70KD), VP_9 (65KD), VP_{10} (56KD), VP_{13} (41KD), VP_{14} (38KD), VP_{15} (34KD), VP_{16} (31KD), VP_{18} (25KD), VP_{20} (17KD) and VP_{21} (14KD) (Table 3 and Fig.3).

Vaccine strain showed eighteen proteins whose molecular weights were 107KD (VP_1), 99KD (VP_2), 94KD (VP_3), 91KD (VP_4), 83KD (VP_5), 79KD (VP_6), 75KD (VP_7), 65KD (VP_9), 56KD (VP_{10}), 51KD (VP_{11}), 47KD (VP_{12}), 41KD (VP_{13}), 34KD (VP_{15}), 28KD (VP_{17}), 31KD (VP_{16}), 23KD (VP_{18}), 17KD (VP_{20}) 14KD (VP_{21}) and 9KD (VP_{22}) (Fig.4). The vaccine strain revealed the presence of six new proteins which were not present in the virulent strains. The new proteins were VP_3 (94KD), VP_4 (91KD), VP_7 (75KD), VP_{11} (51KD), VP_{12} (47KD) and VP_{19} (23KD). Of the remaining twelve proteins ten proteins were similar to those present in both the virulent strains while VP_{17} (28KD) and VP_{122} (9KD) were present only in IVRI strain and not in Alleppey strain (Table 3).

Discussion

DISCUSSION

The present study was conducted to understand the differences in the protein profile of two virulent strains of DPV: viz., DPV-I (IVRI) and DPV-A (Alleppey) and a vaccine strain (DPV-V) which is used extensively throughout Kerala.

Difference in symptomatology, lesions in ducks in natural and experimental infections, embryopathy and cytopathic effects in cell cultures produced by the three strains of DPV were investigated. DPV strains, cultured in DEF/CEF monolayers were used as virus source for concentration and partial purification of the virus. This concentrated and partially purified virus was used for protein analysis by PAGE analysis.

5.1 Clinical manifestations

On experimental inoculation of DPV-I into four week old ducklings, the incubation period for the development of the disease was 6 to 8 days. Leibovitz (1971a) recorded an incubation period of three to seven days while Roy *et al.* (1980) had reported death even within three days.

The chief clinical signs observed in the infection with both the virulent strains were anorexia, purulent nasal discharge, ataxia, photophobia, lacrymation and squatting

posture. The symptoms were agreeable with the findings of Leibovitz (1971a) and Rajan *et al.* (1980). Fifty per cent of the ducklings experimentally inoculated with DPV-I succumbed to the disease. The remaining birds recovered after two weeks during which period, the symptoms of DP were evident. Contrastingly, Alleppey isolate DPV-A produced high mortality among the infected ducklings. 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 which were experimentally infected with DPV-V did not show any evidence of the disease, being the vaccine strain.

5.2 Lesions

5.2.1 In ducks

The most striking lesions which were observed on post-mortem of ducks in natural and experimental infection with DPV-A and DPV-I were congestion and ecchymotic haemorrhages on the serous membranes, heart and other visceral organs. They were strikingly similar to those observed and described by earlier workers (Jansen, 1961, 1964 and 1968; Leibovitz, 1971a; Proctor, 1976; Duraisamy *et al.*, 1979 and Roy *et al.*, 1983). Necrotic lesions on the mucosa and musculature of the gizzard and haemorrhagic bands at regular intervals in the

small intestine were prominent in infection with DPV-A than DPV-I. Rajan *et al.* (1980) had reported similar findings from an outbreak of DP. No significant lesions were noted in the ducklings inoculated with the vaccine strain of DPV.

5.2.2 In embryonated eggs

The duck embryos inoculated with the virulent strains of DPV died within 4 to 6 days PI with characteristic edema, congestion of CAM and haemorrhage all over the body. Similar lesions were also observed by Jansen (1961) and Kalinskii and Borisovich (1969). On inoculation with DPV-I strain in duck eggs, the embryos showed severe generalized congestion from the first passage itself. DPV-A produced severe congestion at the extremities when inoculated in DDE. Initially DPV-A caused only mild lesions which increased gradually on subsequent passages. This may be because the DPV-I might have undergone several passages before its supply, unlike DPV-A which is a fresh virulent field isolate. The increase in the severity of the lesion and the reduction in time for the production of embryopathy with every passage of DPV-A in DDE may be an indication that the virus got adapted to DDE. This is in accordance with the findings of Bhowmik and Chakraborthy (1985) and Kulkarni (1993).

DPV-V produced severe congestive lesions on CAM and the whole embryo, following experimental inoculation in DCE.

Maximum mortality of the embryos were noticed between 72 and 120 hrs PI. This is in agreement with the findings of Dardiri (1975). The vaccine strain of DPV produced lesions only in DCE because this was adapted for growth in embryonating chicken eggs, following 12 passages in DDE and three blind passages in DCE. Serial passage of DPV in DCE reduced the virulence and hence it served as a safe and potent vaccine (Jansen, 1964).

5.3 Cytopathy in cell cultures

The virulent strains viz., DPV-I and DPV-A were propagated in DEF and the vaccine strain DPV-V in CEF monolayers.

Rounding and clumping of cells was observed by 36 hours PI on first passage itself in DEF culture. A similar pattern of cytopathic effect was reported by Kunst (1967) and Dardiri (1969). Gradually there was fusion of fibroblastic cells to form a syncytium and vacuolation of the cytoplasm by 36 hrs PI of DPV-I. Formation of syncytium and vacuolation in DEF culture 36 hrs PI of DPV-I seen in this study was in agreement with the observation of Kenwolf *et al.*, 1974; Nair, 1978 and Panisup *et al.*, 1990).

Eosinophilic intranuclear bodies were noticed on microscopic examination of coverslip cultures. This coincides

with the findings of Panisup *et al.* (1990) who had reported only the presence of intranuclear inclusions in infected cells of DEF cultures. But Breeze and Dardiri (1968) and Bergmann and Kinder (1982) had reported the presence of both intranuclear and intracytoplasmic inclusion bodies. The presence of only eosinophilic intranuclear inclusion bodies in infected cells on light microscopy was also reported by Leibowitz (1971a) and Rajan *et al.* (1980).

DPV-A did not produce marked CPE on the first two passages. But on subsequent passages, the CPE appeared marked, which was similar to that of the one produced by DPV-I. As the number of passages increased, the time taken for the production of CPE decreased from 72 hrs on third passage to 36 hrs in the fifth. This is in accordance with the finding of Kalaimathi and Janakiram (1990) who reported a reduction in time taken for production of CPE from 120 hrs on the first passage to 60 to 72 hrs in the twelfth passage.

The vaccine strain produced similar CPE in CEF culture initially at 120 hrs Pi. The time taken for producing CPE gradually decreased to 60 hrs on subsequent passages. This concurs with the finding of Kalaimathi and Janakiram (1990) who observed a reduction in time taken for the production of CPE with increase in the number of passages.

5.4 Titration of DPV strains

The titre of all three strains were found to be higher in cell cultures, ($TCID_{50}$) than in embryonated eggs (ECD_{50}).

5.4.1 Embryo Lethal Dose 50 (ELD_{50})

The ELD_{50} of DPV-I, A and V were $10^{5.27}$ per ml, $10^{4.26}$ 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). Butterfield *et al.* (1969) have reported a much higher titre (ELD_{50}) of $10^{6.3}$ per ml for virulent strain of DPV.

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

The three strains of DPV (I, A and V) had titres of $10^{5.75}$ per ml, $10^{5.75}$ per ml and 10^{5} per ml respectively in DEF/CEF cultures. 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.

It could be concluded that tissue culture system is better than the embryonating eggs since the $TCID_{50}$ of all the three strains were higher than the ELD_{50}.

5.5 EM examination of DPV

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

5.6 Protein profile of the viruses

Two virulent strains of DP virus namely DPV-I and DPV-A and a vaccine strain of DP virus namely DPV-V were concentrated and partially purified to study the protein profile of this virus.

Eventhough there are reports on the protein profile of other herpes viruses, there are no published records on the protein profile of duck plague virus.

The virulent strains of DP virus namely DPV-I and DPV-A were found to have sixteen proteins and fourteen proteins respectively. The 28KD (VP_{17}) and 9KD (VP_{122}) protein bands found in the IVRI strain were absent in the Alleppey strain. The molecular weights of the sixteen proteins present in the IVRI strain ranged from 9KD to 107KD. The Alleppey strain had

resolved fourteen proteins whose molecular weights ranged from 14KD to 107KD.

Killington *et al.* (1977) compared to proteins of EHV and PRV and showed that both contained twenty proteins ranging from 29KD to 200KD. The vaccine strain DPV-V shows around eighteen proteins which ranged from 9KD to 107KD.

Twenty polypeptides could be identified in EHV-1 ranging from 13KD to 115 KD (Abodeely *et al.*, 1971). The range of the molecular weight of the proteins resolved in this study also is closer to the above observation ie., 9KD to 107KD.

Sklyanskaya *et al.* (1977) found that mature virions of infectious bovine rhinotracheitis virus (IBR) contained 18 structural proteins. This is in proximity to our detection that the three DPV strains viz. DPV-I, DPV-A and DPV-V contained 16, 14 and 18 proteins respectively. Nineteen proteins were shown by Trepanier *et al.* (1986) in BHV-1 with molecular weights ranging from 14KD to 145KD.

Comparing the protein profile of two virulent DP viruses namely DPV-I and DPV-A with that of a vaccine strain DPV-V, the vaccine strain was found to posess six additional proteins namely VP_3, VP_4, VP_7, VP_{11}, VP_{12} and VP_{19}. At the same time it lacked four proteins namely VP_8, VP_{14}, VP_{16} and VP_{18} which were present in both the virulent strains. Castro *et al.* (1992)

had also compared the proteins of four virulent and four vaccine strains of IBR virus and revealed that they differ in presence or absence of proteins viz. 79KD, 74KD, 110KD and 115KD.

Ben Porat *et al.* (1970) showed a difference of two proteins in PRV when comparing two different strains. Stevely (1975) demonstrated at least 20 protein in PRV while Khristova (1986) revealed 19 proteins in the attenuated strain of PRV.

Chen *et al.* (1972) revealed eight proteins on analysing the protein profile of Marek's disease. Westerbrink *et al.* (1985) showed that there are migration differences in the proteins while comparing the antigenically related strains of Herpes virus of Turkey.

The presence or absence of protein bands between strains may help in their identification. It is seen here that both the virulent strains possess identical proteins except that strain '1' had two additional proteins (28 KDa and 9KDa). Eleven proteins are common for all the three strains. Four proteins (70, 38, 31 and 25 KD) were seen only in the virulent strains. To use these proteins as a marker for virulent strains needs protein analysis of more number of virulent strains. Absence of these four proteins in the vaccine strain could be due to deletion mutation during the process of attenuation. Difference between the virulent and vaccine

strain was also evident as the latter resolved six (94, 91, 75, 51, 47 and 23 KDa) unique proteins. These variations between the virulent and vaccine strains also indicate the possibility of an antigenic distinctiveness of the parent strain of DPV from which the vaccine strain was evolved. However there variations cannot considered as a reason for vaccination breakdown unless the role of these proteins in the protective immunity has been worked out. It should also be seen that the number of proteins that can be resolved by PAGE analysis depends on various factors.

Deng *et al.* (1984) had detected six strains of DPV employing reverse passive haemaglutination test and immunofluorescence. At the same time Lin *et al.* (1984b) showed that various isolates of different virulence were all serologically related to biotypes of duck plague virus. The difference in the protein patterns of the DP viruses, in the present study indicates the possibility of strain variation. Only the structural proteins of two virulent and one vaccine strain of DP virus had been analysed. To confirm the possibility of strain variation a detailed analysis of the envelope and nucleocapsid proteins of the virus has to be made which has to be supported by a detailed DNA profile study.

Summary

SUMMARY

Two virulent strains of duck plague virus namely DPV-I (procured from IVRI, Izatnagar) and DPV-A (isolated from Alleppey district) and one vaccine strain DPV-V (procured from VBI, Palode) were used in this study for the analysis of structural proteins. The symptoms and lesions produced by each of the strains in the naturally and experimentally infected birds, pathological alterations and changes in embryonated eggs, cytopathic changes in cell cultures and protein profile were used for comparison of strains.

Revival of the IVRI strain was done by inoculation into ducklings. The inoculated ducklings succumbed to the disease showing characteristic symptoms and lesions of DP. Liver and spleen of the dead ducks were processed and utilised for the inoculation into DDE. The inoculated embryos died within 3-6 days Pi, showing congestive lesions on CAM and body of embryo. The allantoic fluid, CAM and embryo liver were processed and inoculated into DEF monolayers. On infection with DPV-I, monolayers showed rounding and clumping of fibroblast cells, syncytium formation, extensive vacuolation in the cytoplasm and production of intranuclear inclusion bodies which were characteristic of the CPE produced by herpes viruses. By 48-72 hrs Pi the monolayer started getting detached from the

glass surface. No lesions were produced on DCE inoculated with DPV-I.

Alleppey strain also produced similar symptoms and lesions of DP as observed in DPV-I infection. DPV-A infection produced a higher mortality among the ducks. Pathognomic lesions viz., gizzard muscle necrosis and annular haemorrhagic bands in the small intestine were characteristic and pronounced on infection with this strain of DPV. DPV-A on inoculation into DDE produced same lesions as that of DPV-I but showed severe congestion in the extremities. On infecting the monolayers with DPV-A, the time taken for production of CPE was longer and the CPE was more similar to DPV-I infection.

Vaccine strain was inoculated into ducklings and DCE. No symptoms and lesions were noticed in inoculated ducks while congestion of CAM and whole body was seen in DCE inoculated with DPV-V. Congested whole embryo and CAM were processed and used for infection in CEF monolayer. CPE in CEF monolayer by DPV-V was similar to that produced by DPV-I and DPV-A in DEF monolayers.

The ELD_{50} and $TCID_{50}$ of DPV-I in DPE and DEF cultures were $10^{5.27}$ and $10^{5.75}$ per ml respectively. DPV-A had a ELD_{50} of $10^{4.86}$ per ml in DDE and $TCID_{50}$ of $10^{5.25}$ per ml in DEF cultures. The ELD_{50} (in DCE) and $TCID_{50}$ (in CEF culture) of DPV-V were 10^{4} and

$10^{4.5}$ per ml respectively. It could be concluded that the tissue culture system is better than the embryonating eggs since the $TCID_{50}$ values are greater than ELD_{50} in all the three strains of DPV.

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 DEF culture and CEF culture were used as source for the partial purification of the virus. The concentrated virus pellet obtained was a partially purified one.

The partially purified virus pellet was subjected to discontinuous system of polyacrylamide gel electrophoresis on a ten per cent gel. The protein fragments were made visible after staining the gel with Coomassie brilliant blue. The molecular weights of the protein fragments were estimated by comparison of the distance migrated by them with that of standard molecular weight markers. Bovine Serum Albumin and Lactalbumin were used as protein markers in this study.

The three strains of DPV viz. DPV-I, DPV-A and DPV-V resolved into 16, 14 and 18 bands respectively. DPV-A showed a similar band pattern as that of the DPV-I except that it lacked 28 KD (VP_{17}) and 9KD (VP_{22}). The vaccine strain showed

a different pattern of protein bands from the virulent strains. Eighteen polypeptides could be demonstrated in the vaccine strain.

The molecular weights of the sixteen proteins present in the IVRI strain and Alleppey strain ranged from 9KD to 107KD and 14KD to 107KD respectively. The vaccine strain also resolved proteins which ranged from 9KD to 107KD.

The vaccine strain was found to possess six additional proteins namely VP_3, VP_4, VP_7, VP_{11}, VP_{12} and VP_{19}. At the same time it lacked four proteins namely VP_8, VP_{14}, VP_{16} and VP_{18} which were present in both the virulent strains.

The possibility of the occurrence of strain variation as indicated by the difference in the protein patterns of the DP viruses under study is discussed.

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CHARACTERIZATION OF STRUCTURAL PROTEINS OF DUCK-PLAGUE VIRUS

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ABSTRACT OF A THESIS

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ABSTRACT

Two virulent strains of duck plague virus - DPV-I (IVRI) and DPV-A (Alleppey isolate) and a vaccine strain - DPV-V (VBI Palode) were investigated for the differences in clinical manifestations in naturally and experimentally infected ducks, morphological changes in developing duck/chicken embryo (DDE/DCE) and cytopathic effects in duck embryo fibroblast/ chicken embryo fibroblast culture (DEFC/CEFC) and chicken embryo fibroblast culture (CEFC).

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

Embryonated duck eggs were used for passaging DPV-I and isolating DPV-A, while embryonated chicken eggs were used for propagating DPV-V. All the three strains produced mortality of embryos with congestion on CAM and body of the embryo. DPV-A produced more congestion on the extremities of the embryos.

Duck embryo fibroblast cultures were used for culturing the virulent strains while chicken embryo fibroblast cultures were used for culturing the vaccine strain. All the three strains produced characteristic CPE, with rounding and clumping of cells, syncytium formation, vacuolation of cytoplasm and formation of intranuclear inclusion bodies. The time for the production of CPE decreased on successive passage.

Titration of the three strains of DPV was done in embryonated eggs (ELD_{50}) and cell cultures ($TCID_{50}$). DPV-I, DPV-A and DPV-V had an ELD_{50} of $10^{5.27}$, $10^{4.86}$ and 10^{4} per ml respectively. $TCID_{50}$ of DPV-I and A in DEF culture were $10^{5.75}$ per ml and $10^{5.25}$ per ml respectively and that of DPV-V in CEFC was $10^{4.5}$ per ml. The tissue culture system gives the best titre than the embryonating eggs for all the three DPV strains.

On electron microscopy, the field isolate of DPV showed particles ranging from 170-190 nm in diameter.

Protein analysis of the virulent strains viz. DPV-I and DPV-A by SDS-PAGE revealed sixteen and fourteen proteins respectively. Mild difference of two proteins (VP_{17} and VP_{22}) was noticed between the two strains. DPV-A lacked the 28KD and 9KD protein bands.

The vaccine strain DPV-V on electrophoresis showed a different pattern of protein bands from the virulent strains. Eighteen proteins could be resolved in the vaccine strain.

The molecular weight of sixteen proteins of DPV-I ranged from 9KD to 107KD while the fourteen proteins of DPV-A ranged from 14KD to 107KD. The proteins of the vaccine strain also ranged from 9 KD to 107 KD.

The possibility of the occurrence of strain variation as indicated by the difference in the protein patterns of the DP viruses under study is discussed.