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PRODUCTION AND APPLICATION OF MONOCLONAL ANTIBODIES AGAINST DUCK PLAGUE VIRUS

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

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1999

DECLARATION

I. here by declare that this thesis entitled **Production and application of monoclonal antibodies against duck plague virus** is a bonafide record of the research work done by me during the course of research and the thesis has not previously fotmed 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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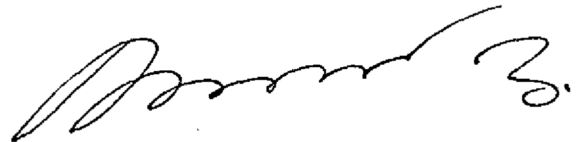

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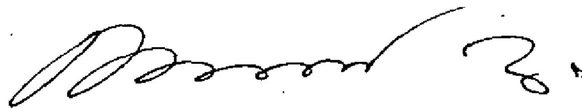

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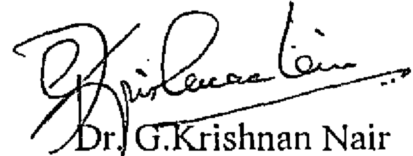

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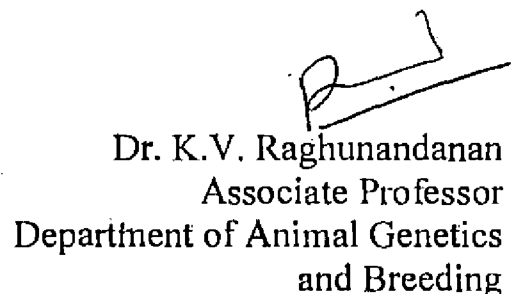

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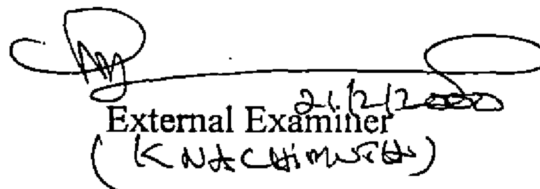

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TO

RESPECTED DADA, SOU. AAI
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LIST OF ABBREVIATIONS

AAF	Allanto amniotic fluid
AB	Avidine Biotin
ADV	Aujeszky"s disease virus
AEI	Actylziridine
AGPT	Agar gel precipitation test
BHV	Bovine herpes virus
BMV	Bovine mammilitis virus
CAM	Chorio allantoic membrane
CAS	Chorio allantoic sac
CCL 141	Certified cell line 141
CMF-PBS	Calcium-Magnesium free Phosphate buffer saline
CPE	Cytopathic effect
CEF	Chicken embryo fibroblast
DCE	Developing chicken embryo
DDE	Developing duck embryo
DEF	Duck embryo fibroblast
DP	Duck plague
DPV	Duck plague virus
DVE	Duck viral enteritis
DPV-A	Alleppey strain of DPV
DPV-I	IVRI strain of DPV
DPV-V	Vaccine strain of DPV
ELD_{50}	Embryo lethal dose 50
EAV	Equine herpes virus
ELISA	Enzyme linked immunosorbent assay
HVT	Herpes virus of turkeys
HAT	Hypoxanthin AminopterinThyamidine
HT	Hypoxanthin Thymidine
HBSS	Hank's balanced salt solution
HSV	Herpes simplex virus
FAT/IFT/IF	Fluorescent Antibody test
FCS	Fetal calf serum
IM/IEM	Immuno electron microscopy
IBR/IBRV	Infectious bovine rhinotracheitis/virus
IVRI	Indian Veterinary Research Institute
IVPM	Institute of veterinary preventive medicine
PI	Post inoculation/ Post infection
PAGE	Poly acralamide gel electrophoresis
Rpm	Revolutions per minute
gp	Glycoprotein
ILT/ILTV	Infectious laryngotracheitis Virus
IP/IPT	Immunoperoxidase/test
Mab/Mabs	Monoclonal antibodies
MDV/VVMDV	Marek's disease virus/ very virulent
MATSA	Marek's associated tumor specific antigens

PRV	Pseudorabies virus
PEG	Poly ethylene glycol
PHA	Passive haemagglutination
RBCs	Red blood cells
SNT	Serum neutralization test
$TCID_{50}$	Tissue culture infective dose 50
VBI	Veterinary biological institute
VNI	Virus neutralization index
VNT	Virus neutralization test

INTRODUCTION

INTRODUCTION

Duck rearing is a popular enterprise among farmers and laborers in water logged areas of the coastal states of India, particularly West Bengal, Andhra Pradesh, Orissa, Kerala and Assam. Availability of marshy land unsuitable for cultivation facilitates duck farming. Moreover, because of low cost of production, duck meat and eggs are cheaper and hence preferred. One of the major threats to the flourishing duck industry is a fatal disease known as Duck Plague (DP) or Duck viral enteritis. The disease is caused by a herpes virus and the birds of Anatidae family are highly susceptible to the infection. The disease is characterized by hemorrhagic enteritis and is seen in captive as well as free flying waterfowls. It is highly contagious, often fatal and results in heavy economic loss to the duck farmers.

Since the first official report of DP by Mukerji et al. (1963) from West Bengal, several outbreaks of DP causing heavy mortality have been reported from different parts of India.

Heavy mortality in ducks was reported during 1976-77 from Alleppy district of Kerala (Nair, 1978). Since then Kerala has become endemic for duck

DP and regular reports are received from different water logged districts of the state (Punnoose and Abdulla, 1976 ; Kulkarni, et al.,1995).

Thus DP continues to be a great threat to the farmers, thereby affecting their economic condition. Effective control of the disease by periodical vaccination gained importance since 1964. This has helped to control the disease to some extent. However, DP outbreaks have been reported from several parts of the country despite regular vaccination. There may be many reasons for the failure of the vaccine to confer protective immunity to the birds. One of the reasons always stated is the conventional chicken adapted vaccine which is reported to have low titers and poor immunogenicity (Kulkarni, 1993 , Bordolai, et al.,1994). Another factor which may also be responsible for the failure of the vaccination could be the local strain which differ in the virulence/antigenicity.

Although all the isolates of Duck Plague Virus (DPV) are reported to be serologically identical, it has not been subjected to a detailed investigation at molecular level so far. There are few reports on protein profile of different isolates (Sangeetha,1996; Gardner, et.al. 1993 and Taylor, 1997). Taylor (1997) tested three different isolates for its protein profile by Poly Acrylamide Gel Electrophoresis (PAGE) and it was observed that the three isolates viz. Vaccine, Alleppy and IVRI were having few proteins which were specific to each isolate. He opined that the protein which are not common may be responsible for

conferring the virulence of a very high degree to a particular isolate and in absence of it making it completely avirulent for the ducks. As it was not clear whether the isolates were really differing at molecular level, a further probe in this regards was warranted, which might throw some light on vaccinal breakdown and help in future in development of a better vaccine against DP.

Since the initial description of the Monoclonal Antibodies (Mabs) by Köhler and Milstein (1975), these have made a profound impression on all areas of biological research and biotechnology. The basic property of a Monoclonal antibody, that of combining specifically with one epitope can be used to supply wealth of data. This specificity of reaction provides the basis for the detection of the subtle differences between the related viruses and thus differentiation of one virus from another. The use of antibody of uniform specificity thus permits a very fine analysis of epitope and epitope antibody interaction and therefore of structural alteration in the target molecule. Indeed this approach greatly exceeds conventional serology in specificity, and has led to the recognition of differences between viruses previously thought identical (Carter and Meulen, 1984).

Information on the histological and intracellular location of antigen is obtained from techniques such as Immunofluorescence (IF), Immunoperoxidase (IP) and Immuno electron microscopy (IEM). Detection of tissue antigen by

using polyclonal serum has drawbacks such as false positive detection of the antigen and inability to differentiate closely related viruses or strains of the viruses.

Moreover the background staining results in wrongly diagnosing the antigen (Guy, et al. 1992). On the other hand, Mabs are highly specific in detecting the tissue antigens and bind to only particular antigen against which these are produced. Guy et al. (1992) and Malmarugan (1997) observed that the staining with hyperimmune serum resulted in nonspecific background staining due to antibodies produced in response to contaminants in the immunization preparations.

Thus Mabs are the reagents of very high specificity and therefore have many advantages over the polyclonal serum which is used conventionally in the diagnosis. With this view, the present research was conducted with following objectives,

a) To produce Monoclonal antibodies against Duck Plague Virus.

b) To study the efficacy of Mabs in diagnosis and in detecting the tissue antigens in comparison with polyclonal serum.

c) To evaluate the strain variation in duck plague virus , if any.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 Duck Plague (DP)

Outbreak of an acute haemorrhagic disease in ducks was reported in the Netherlands by Baudet (1923). There was high mortality, petechial haemorrhages throughout the body and enteritis and the disease was suspected to be fowl plague. De Zeeuw (1930) observed identical outbreak and opined that the disease was caused by a duck adapted strain of fowl plague virus.

Bos (1942) reported the third outbreak with similar findings but contradicted with the earlier reports and indicated that the disease was not the same as fowl plague as hens and pigeons could not be infected with the material collected from the infected birds and suggested that the infectious agent could be an independent virus.

At the XIV th International Veterinary Congress Jansen and Kunst (1949) proposed the name Duck Plague because of the viremic characteristic of the disease.

Jansen *et al.* (1952) reported another outbreak with heavy mortality , however, the ducks surviving this outbreak were demonstrated to be immune to the virus of an earlier outbreak. An elaborate review of duck plague outbreak till 1961 in the Netherlands was presented by Jansen (1961).

In addition to the Netherlands the disease has been reported from almost all duck rearing countries (Leibovitz 1991).

Leibovitz and Hwang (1968) proposed that the disease may be named as Duck Viral Enteritis (DVE) on the basis of clinical symptoms and pathognomonic pathological lesions presented by the diseased birds.

Mukerji *et al.* (1963) were the first to report the occurrence of the DP from West Bengal in India. A viremic infection mainly in adult ducks characterized by high infectiousness, rapid course and high mortality was observed in a Government duck farm. The symptoms recorded were nasal discharge and wet eyes, trembling and drooping of wings .There was profuse greenish white watery diarrhoea and death. The disease was subsequently reported by Jansen (1964), Bhowmik and Chakraborty (1985) and Bhowmik and Ray (1987) from West Bengal.

The disease was reported from Kerala by Punnoose and Abdulla (1976). Heavy mortality in ducks was reported during April 1976 to January 1977 in Alleppy district of Kerala state. Characteristic symptoms were noticed in ailing birds and postmortem findings were suggestive of duck plague (Nair 1978). Regular outbreaks of DP had been reported from Alleppy, Kottayam, Pathanamthitta, Thrissur and other coastal areas of the state (Anonymous,1976).

Kulkarni *et al.* (1995) reported outbreak of DP in vaccinated as well as in unvaccinated flocks and isolated duck plague virus from the ailing birds.

Duraiswamy *et al.* (1979) reported the disease from Tamil Nadu, Chakraborty *et al.* (1980) from Assam and Sreeramulu (1986) from Andhra Pradesh.

2.2 Duck plague Virus (DPV)

The causative agent of DP is a herpesvirus in the sub family Alpha herpesvirinae (Mohanty and Dutta,1981) and provisionally designated as Anatid herpes virus - I (AHV - I) by herpes virus study group.

DPV is a non hemagglutinating and non haemadsorbing virus (Jansen, 1961). The virus is sensitive to ether and chloroform. Infectivity

of the virus could be destroyed by heating at 56°C for 30 min . The virus was shown to get inactivated by exposure to pH below 3 and above 11 for 45 min and by the treatment with trypsin, Chymotrypsin and pancreatic lipase for 18 hours at 37°C (Hess and Dardiri,1968).

DPV produces intranuclear inclusions in infected cells and forms plaques in cell cultures (Dardiri and Hess,1968).

Electron microscopy of virus infected cell cultures revealed virus particles in both nucleus and cytoplasm of the cell after 48 hours post inoculation. Larger particles of approximately 181 nm diameter were observed in the cytoplasm (Breeze and Dardiri,1968).

In a negatively stained preparation, Procter et al. (1976) observed that the virus replicated in hepatic macrophages, hepatocytes and bile duct epithelium . In ultra thin sections herpes virus-like nucleocapsid and virions were found in nucleus and cytoplasm of the infected cells.

Bergman and Kinder (1982) studied morphology and maturation of DP virus in thin sections obtained from organs of experimentally infected day old chicks . Spherical nucleocapsids of 93 nm diameter were recorded in the cell nuclei. Encasement of the nucleocapsid by the interior nuclear membrane

resulted in virus particles of 126 nm or 129 nm diameter in perinuclear space and in cytoplasm.

Tantaswasdi *et al.* (1988) described two types of virus particles in the cytoplasm of the hepatic cells three or four days post infection. One had naked nucleocapsid morphologically similar to those seen in the nucleus. The other type was mature virion measuring about 200 nm in diameter. It consisted of an envelope 80 nm in diameter and an electron dense zone between the envelope and the capsid.

Panisup *et al.* (1990) cultivated a field isolate in duck embryo fibroblast (DEF) cell culture. Characteristic plaques and intranuclear eosinophilic inclusion bodies were seen four days post infection. The infected cells showed extensive vacuolisation in the cytoplasm and irregular nuclear bleb formation.

Taylor (1997) observed enveloped virus particles with morphological features of herpes virus in DEF cell culture. The size of these particles ranged between 150 to 190 nm with a core size of 70 to 90 nm.

2.3 Cultivation of Duck plague virus

2.3.1 Ducklings

Ducklings had been used by many workers for propagation of DPV (Sarkar, 1982 ; John *et al.*, 1990 a ; Senthil kumar,1997). John *et al* (1990 b) used six day old Khaki Campbell ducklings for virus isolation . The ducklings were inoculated intramuscularly with 0.5 ml of inoculum per bird. The inoculum was prepared from liver and spleen as 50 per cent (w/v) suspension collected from natural outbreak of DP. The inoculated ducklings showed clinical signs 3-4 days post inoculation. Petechial haemorrghage all over the body, haemorrhagic foci on liver and dark red spleen were noticed in PM findings.

2.3.2 Embryonated eggs

Isolation of DPV was done by inoculating tissue suspension onto the chorio allantioic membrane (CAM) of embryonated duck eggs. The embryo death was recorded 4 to 6 days post inoculation with extensive haemorrhges throughout the body (Jansen,1961; Kalaimathi and Janakiram, 1989; John *et al.*,1990 b)

Jansen (1961) reported that the virus after twelve passages in duck embryo and three passages in chicken embryo was able to cause the death of chicken embryo within four to five days post inoculation with extensive

haemorrhages. The pathogenicity for ducks decreased rapidly in chicken eggs and by 20th passage the virus was avirulent for ducks.

Butterfield and Dardiri. (1969 b) studied the distribution of DPV in embryonated chicken and duck eggs. It was observed that attenuated DPV when inoculated onto CAM , amnio allantoic fluid and CAM yielded high titer of virus. However, virulent virus infected duck embryos yielded high titer of virus when inoculated into chorio allantoic sac (CAS) or yolk sac.

Bhattacharya *et al.* (1977) revived vaccine strain of DPV in duck embryo. Twelve days old duck embryos were inoculated by CAM route at the rate of o.1 ml suspension of 1:50 diluted seed virus. The embryo death was recorded on fourth day. Back passaging in duck embryo resulted in the revival of the seed virus and it gained virulence.

Kalaimathi *et al.* (1985) used two different routes for inoculation of DPV viz. CAM and CAS. Nine days old embryonated chicken eggs were inoculated by CAM and CAS routes. The embryo death was recorded four to five days post inoculation. Highest yield of DPV was recorded in eggs inoculated by CAS route and therefore it was recommended for large scale vaccine production.

Glavits *et al.* (1990) inoculated a virulent strain of DPV into allantoic cavity of goose eggs and observed that the virus replicated in kidneys, myocardium, gizzard muscles and CAM of the embryo.

Taylor (1997) passaged virulent as well as vaccine strain of DPV in developing duck embryos and chicken embryos respectively. Embryo death was recorded 4 to 6 days post inoculation with oedema and haemorrhages all over the body and generalized congestion of the CAM.

Senthil kumar (1997) inoculated 11 days old developing chicken embryos with vaccine strain of DPV and observed embryo death within 4 to 6 days with necrotic areas all over the body and liver and congestion of spleen.

2.3.3 Cell culture

Kunst (1967) passaged DPV in duck embryo fibroblast cell culture and observed cytopathic effect (CPE) three to four days post inoculation. Inclusion bodies were observed in the nucleus as well as cytoplasm of the cell inoculated with DPV.

Cultivation of DPV in duck embryo fibroblast cell culture had been reported by many workers (Dardiri,1969; Kocam 1976; Burgess and Yuill, 1981). Similarly chicken embryo fibroblast (CEF) was also used as a system

for cultivation of DPV by Kurochka *et al.* 1983);Kalaimathi and Janakiram (1989); John (1988) and Senthil Kumar (1997).

Replication of DPV was studied in primary cell culture (DEF) and CCl 141 cell line. Both types of cells were accurate for quantifying the virus and new virus progeny was noticed 18h post inoculation. Though primary cell cultures yielded five to six times more virus compared to CCl 141, authors recommended that the cell line is more useful as plaques were easily detectable and it had known health history (Kenwolf and Quimby, 1976).

A virulent strain of DPV was adapted to CEF cell cultures. Twelve passages were made in CEF and its effect on virulence was studied at 5 th ,8 th and 12th passage level by inoculating ducklings and duck eggs. A reduction in the mortality and embryo death was recorded over passages. First passage virus killed 89.5 per cent inoculated embryos compared to 20 per cent by 12 th passage virus (Kenwolf and Quimby, 1976).

A reduction in time required for appearance of CPE was noticed when a virulent DPV was adapted to CEF cell cultures. CPE noticed at 60 th hour post inoculation in first three passages. As the passages increased, earlier onset of CPE was observed and at 12 th passage it was at 24 h post inoculation (Kalaimathi and Janakiram,1990) .

Senthilkumar (1997) passaged vaccine strain of DPV in CEF cell culture. Reduction in appearance of CPE was noticed over passages. At first passage the CPE was observed at 96-h , however at 10 th passage CPE was observed at 24-h post inoculation. Similarly the time taken for complete desquamation was also reduced from 120 h post inoculation in first passage to 80-h at 10th passage.

Anonymous (1998) attenuated virulent strain of DPV (DPV-A) by passaging the seed material in duck and chicken embryo and in chicken embryo fibroblast cell cultures. The seed material had a pathgenicity index (PI) 1.56 at 0 passage and a titre of $10^{4.5}$ ELD_{50} /ml. The virus was passaged 15 times in duck embryo and the titre and pathgenicity index was assessed at 5, 10, and 15th passage. The PI was reduced from 1.56 to 0.88 and a slight increase in titre was observed as the passages increased. It was further attenuated in chicken embryo for 15 passages and a reduction in PI from 0.88 to 0.17 was observed. This seed material was then passaged in CEF cell culture for 15 times. ELD_{50} of the virus was $10^{4.5}$ and pathgenicity index of 0.07 at 15 th CEF cell culture passage level.

2.4. Purification and concentration of herpes virus.

Concentration and purification procedures utilized for the preparation of viral antigens are essentially aimed at reducing the fluid and separate it from the host material (Williams and Chase , 1960).

Purified fractions of enveloped and deenveloped equine herpes virus were obtained by sucrose gradient centrifugation followed by dialysis and ultra centrifugation in a sartoris colloidal bag (Abodeely, *et al.*, 1971).

Chen,*et al.* (1972) propagated Marek's disease virus in roller bottle cultures of DEF, precipitated with 50 per cent saturated ammonium sulphate and layered onto 20 to 65 per cent sucrose gradient and centrifuged at 21,000 rpm for 90 min at 4°C. The purified virus band was noticed at the interface between 20 and 65 per cent gradient.

Perdue *et al.* (1974) obtained a purified fraction of EHV-I by precipitating with PEG 6000 at a final concentration of 70 and 23 percent and then layered onto 5 to 30 percent Dextran T10 gradients and centrifuged for one h at 52000 g.

Purified naked and enveloped nucleocapsides of pseudorabies virus were obtained by Stevely (1975). The cell lysate supernatant was

layered onto 36 ml gradient (12 to 32 percent w/v of Dextran T 10). The gradient was centrifuged for oneh at 20000 rpm. The virus zone was collected and centrifuged onto a discontinuous sucrose gradient (20 to 40 per cent).

Kallington *et al.*(1977) studied the protein profile of the five herpes viruses viz.HSV-1, HSV-2,Bovine mamilitis virus (BMV), Equine abortion virus (EAV) and Pseudorabies virus (PRV). Thirty distinct proteins were observed in HSV -1 and 2 , 15 in BMV and 20 each in EAV and PRV. Their molecular weight ranged from 29 to 200 kd.

Infectious Bovine Rhinotracheitis (IBR) virus grown in bovine embryo kidney cell culture was concentrated and purified in Ficoll density gradient. The polypeptide concentration of virus was studied by PAGE . The mature virus was found to contain 18 structural proteins with molecular weight ranging from 250000. to 29000 daltons (Sklyanskaya, *et al.*,1978).

BHV I was purified by pelleting thrice (First in minimum essential medium and then in phosphate buffered saline) at 120,000 g for 60 min each time and resuspended in 3 ml distilled water. This was centrifuged at 100,000 g for 30 min in caesium chloride gradient with a final density of 1.26 g/cm (Pastoret *et al.*, 1980).

IBR virus was purified by layering the viral suspension onto a 10 percent Potassium tartarate and one percent Tritan X-100. which was performed over linear 15 -45 Potassium tartrate gradient and centrifuged at 60,000 g for 60 min in an SW 25 rotor (Bolton *et al.*,1981).

Westerbrink *et al.* (1985) performed rate zonal centrifugation of Marek's Disease Virus (MDV) by layering the cell lysate onto a 36 ml, 15- 20 percent linear sucrose gradient in PBS.

Live attenuated chicken embryo adapted DPV cultivated in duck embryo was purified by treating infected amnio allantoic fluid with protamine sulphate and PEG . The purified virus was found to contain a titre of $10^{8.5}$/ 0.1 ml. The same virus was used for virological studies and vaccine production.(Rao, *et al.* ,1992).

Gardner, *et al.* (1993) purified the DPV by layering the cell lysate supernatant onto 12 to 62 per cent W/V linear sucrose gradient and centrifuged at 40,000 rpm for 4 h at 4°C.

2.5 Immunity

Jansen (1964) demonstrated that chicken adapted strain of DPV was completely avirulent for the ducks and could be used for active immunization.

In an experiment ducks vaccinated with chicken adapted vaccine showed neutralizing antibodies. The antibodies could be demonstrated in the majority but not in all vaccinated birds and this was due to interference phenomenon that provoked non antibody defense. This finding was of practical significance as the vaccination could be performed on farms where an outbreak of duck plague is already developing.(Jansen, 1964).

Butterfield and Dardiri (1969 a) Vaccinated mallard ducks with attenuated DPV and observed that there was no correlation between the mortality and level of antibodies produced by attenuated vaccine.

Butterfield and Dardiri (1969 b) inactivated chick embryo adapted DPV by 0.05 percent acetylaziridine (AEI) and 30 min by 0.4 percent propiolactone. The AEI inactivated or live attenuated virus produced comparable levels of antibodies to that of DEF propagated virus. Though the AEI inactivated vaccine produced low serological response it protected the ducks against the challenge whereas propiolactone preparation gave no protection.

Toth (1970) tested chicken embryo adapted duck virus enteritis modified live vaccine. The dose of 1 ml of vaccine did not cause disease or mortality among 260 ducks inoculated. Among 258 challenge tested

ducklings 0.77 per cent mortality was recorded whereas among 200 non vaccinated controls the mortality was 88.0 per cent. The full protection against challenge virus infection in vaccinated ducklings as early as 4th day post vaccination was attributed to interference phenomenon.

Toth (1971) inoculated white Pekin breeder ducks with modified live virus vaccine. One group was vaccinated once and the other twice, 19 weeks later. The vaccinated birds were tested for neutralizing antibody development and resistance against challenge inoculation with virulent virus. Single vaccination did not induce any antibody, whereas two times vaccination did induce a low level of antibody. High level of neutralizing antibody was detected in vaccinated birds challenged with virulent virus.

A lack of correlation between neutralizing antibody titres and resistance against challenge with virulent virus was attributed to the interference phenomenon. Absence of neutralizing antibodies in unvaccinated birds kept along with the breeders confirmed that vaccinated birds did not spread the vaccine virus.

Dardiri (1975) observed that ducks surviving the natural or experimental infection were solidly immune. A total of 2936 sera samples were examined. Hundred per cent of the sera samples from non infected flocks had

a virus neutralizing index in the range of 0.00 to 1.25 log 10 . On the other hand, recovered birds had VNI of 0.75 log 10 or less. Comparison of the VNI of sera obtained at 0, 21 and 42 days PI from ducks that survived experimental infection indicated that there was an increase of 1 to 2 log in the VNI. It was concluded that antibody level of log 1.75 or more signified infection of ducks with virulent DPV.

Birds infected with lethal and nonlethal strain of DPV were challenged between two months and four years. None of the ducks infected with non lethal virus died after challenge whereas all but five of 25 infected with lethal virus died. All the survivors from challenge developed neutralizing antibody titres though these were not detectable before challenge (Burgess and Yuill, 1982).

Lin *et al.* (1984 a) isolated a non pathogenic strain of DPV (Sheridan 83) and vaccinated white Pekin ducks with this strain. The ducks inoculated with this virus developed resistance to the challenge by Lake Andes strain of DPV. The hyperimmune sera from the birds, neutralized all the three strains of DPV *Viz.*; Sheridan 83, Holland and Lake Andes strain.

Lam and Lin (1986) observed that vaccination of ducks with apathogenic strain of DPV resulted in protection against challenge with

virulent virus. Antisera produced in vaccinated ducks were able to transfer resistance against the challenge to the recipient ducks and concluded that antibody mediated response was important in protecting the ducks from DEV infection.

Sergeev *et al.* (1990) vaccinated the ducks with live attenuated AKV vaccine and observed that the vaccine protected 60 percent of the ducks from challenge infection five months after vaccination.

Senthil kumar (1997) vaccinated the ducks with 5th and 10th passage virus by intramuscular route. The mean serum neutralization titre of the ducks vaccinated with 5th passage virus was 64, with VNI of 1.8 on 20th day post vaccination. However, the 10th passage vaccination group had a mean titre of 54 and serum neutralization index of 1.73 on 20th day post vaccination. Fifth as well as 10th passage virus protected the birds against challenge, while all the birds from control group (unvaccinated) showed typical clinical signs of DP and died 7 to 9 days post challenge.

2.6 Diagnosis

Diagnosis of DP is mainly based on the clinical findings, macroscopic and microscopic lesions and isolation and identification of the virus. Various serological tests have been used for the diagnosis of the disease.

Mukerji *et al.* (1965), Dardiri (1975) and Mukit *et al.* (1988) used serum neutralization test for the diagnosis of DPV, plaque assay by Dardiri and Hess (1968) and agar gel diffusion test by John (1988). Immunoflurorescence (Erickson, *et al.*, 1974), reverse passive haemagglutination (Kulkarni, 1993), counter immuno-electrophoresis (John, *et al.*, 1989), electron microscopy (Tantaswadi, *et al.*, 1988), immunoperoxidase test (Islam, et al., 1993) and enzyme linked immuno-sorbent assay (chandrika,1996) have been successfully employed in the diagnosis of DPV.

2.6.1 Enzyme Linked Immuno Sorbent Assay (ELISA)

Enzyme immuno assay introduced by Engvall and Perlman (1971) has been used in herpes viruses by many workers. The test was used in Equine herpes virus by (Dutta, *et al.*, 1983, Galosi, *et al.*, 1993) Banks (1985) and Afshar *et al.* (1987) in swine herpes virus, Collins *et al.* (1984) and Edwards *et al.* (1986) in bovine herpes virus ; Herring *et al.* (1980); Bolton *et al.* (1983), Suribabu *et al.* (1984) and Florent and Wiseman (1990) in Infectious bovine rhinotracheitis virus.

An indirect ELISA with modified solid phase and improved specificity was developed by Block (1988) to assay antibodies to adeno associated virus, infectious bursal disease, New Castle disease, infectious laryngotracheitis, Marek's disease, herpes virus of Turkeys, reovirus and infectious bronchitis virus. The test was highly specific, reproducible, rapid and simple to perform. In nearly all the cases it was more sensitive than agar gel precipitation test.

An ELISA for detection of antibodies to ILT virus in chicken was developed and compared with SNT (York, *et al.*, 1983). ELISA titres were found to be 16 to 32 fold higher than those obtained by SNT. Treatment of the serum or the plate and change in the composition of the diluent did not have any influence on the sensitivity of the ILT assay.

Adair *et al.* (1985) compared the sensitivity of the four serological tests *viz.*, ELISA, SNT, FAT and AGPT for detecting antibodies to ILT in chicken sera and reported ELISA to be slightly more sensitive than SNT and AGPT, but comparable with FAT.

Fuchs *et al.* (1985) compared different serological tests and observed that micro SNT was slightly more sensitive than FAT and ELISA but all the

three tests were equally sensitive in quantitative assay, while AGPT was unsuitable because of low sensitivity.

Srivastava *et al.* (1982) developed micro ELISA technique for detection of antibodies to MDV. This test was carried out on serum samples of fowls that had been vaccinated with HVT. It detected antibodies cross reacting with MDV in all sera samples whereas gel precipitation test detected the same only in 43 per cent cases.

A reproducible ELISA using MDV infected cells for detection of antibodies to MDV was described by You-Quan Cheng (1985). Two types of antigens, purified virus and MDV infected CEFs were tested. The latter produced highly specific reaction with very less non specific reaction. ELISA was reported to be 20 to 40 fold more sensitive than IFT.

Reports on application of ELISA in DPV are very scanty. Chandrika (1996) developed a field diagnostic kit for DPV and suggested that DOT-ELISA has potential to be used as an alternative to VNT because of its sensitivity, rapidity and simplicity and can be used in geographically remote areas.

Malmarugan (1997) used plate, DOT and strip ELISA and compared these with PHA in DPV. ELISA was found to be more specific and 20 to 40 fold higher titres were obtained than that of PHA. ELISA detected antibodies in low titred sera and author suggested its use in inapparent and typical conditions and also to determine the immune status of vaccinated birds.

2.6.2 Immunoperoxidase test

The immunoperoxidase technique has been widely used as a rapid method for the identification and localization of viral antigens in infected host cells and in various cell cultures.

The test has been successfully used for the diagnosis of several viral infections including herpes viruses. Matsuoka *et al.* (1987) demonstrated the Aujeszky's disease virus (ADV) antigens in formalin fixed and paraffin embedded brain tissue sections . The test was found to be effective in diagnosis of ADV along with routine histological procedure.

Guy *et al.* (1992) developed indirect IPT for detection of ILT virus antigen in frozen tissue sections. IPT was compared with IFT, histopathology and viral isolation for detecting the presence of ILT virus in trachea of experimentally infected chicken. The sensitivity and specificity of IPT was 72

and 93 per cent and that of IFA was 53 and 90 per cent respectively. Histopathological detection of ILT virus was highly specific (98 per cent) but sensitivity was poor (42 per cent).

Islam *et al.* (1993) adapted avidin biotin (AB) peroxidase method of IPT for the detection of DPV antigen in formalin fixed paraffin embedded tissue sections of liver and spleen of experimentally infected domestic ducks. The positive reactions were localized mostly in the nucleus and less frequently in the cytoplasm of few hepatocytes and kupffer cells of the liver and lymphocytes and reticular cells of the spleen.

Islam and Khan (1995) used AB method of IPT to study the primary replication and tissue distribution of DPV in domestic ducks following oral infection . The virus replicated primarily in the mucosa of digestive tract especially in the oesophagus as early as 24 h post infection , then spread to the bursa of Fabricius, thymus, spleen and liver. The epthelial cells and macrophages of these organs were the principal sites of virus replication. However, viral antigens were also detected in lymphocytes. In all the cases the antigen was localized both in the nucleus and cytoplasm of the cell.

Ogino *et al.* (1996) demonstrated IBR virus (BHV I) by avidin biotin complex immuno peroxidase method in the tissues of two aborted fetuses which had been stored for 25 years after fixing in formalin and embedding in

paraffin. IBR viral antigens were detected as necrotic foci in various organs of the fetuses.

Malmarugan (1997) used IPT for the detection of the DPV antigens in paraffin embedded tissue sections of liver and spleen of infected ducks. The reaction was found mostly in the nucleus and also in the cytoplasm. Some non specific reactions and dark background staining was observed and was attributed to the use of polyclonal antiserum. Eighty five per cent of liver and 80 per cent of spleen sections revealed positive reaction. The test was recommended for the diagnosis of DPV.

2.6.3 Virus Neutralization test (VNT)

Virus neutralization test (VNT) has been used to assess the immunogenicity of DPV vaccine or to study the virulence of different strains of DPV by many workers. Various systems have been used to perform VNT.

The test was carried out in 9 to 11 days old embryonated eggs (Mukerji, *et al.*, 1965; Dardiri and Hess, 1968; Mukit, *et al.*, 1988) as well as in cell cultures (Lin, *et al.*, 1984b; Kalaimathi and Janakiram, 1991; Bordoalai, *et al.*, 1994 and Senthilkumar, 1997).

Jansen (1961) observed that sera from ducks immunized against DPV neutralized the homologous virus and also virulent virus. The ducks inoculated with this serum virus mixture remained healthy . It was concluded that the neutralization was stable.

Mukerji *et al.* (1963) used serum from ducks recovered from a natural outbreak and mixed it with virulent virus. The serum virus mixture was inoculated into healthy adult ducks. The experimental ducks remained healthy, however, all the control ducks inoculated with virulent virus died. The experimental ducks challenged on 10th day succumbed to the challenge as these were unvaccinated.

Jansen and Wemmenhove (1966) observed that though the ducks did not show serum neutralization titres, were protected against the challenge and therefore suggested that animal test was more reliable.

The responses of White Pekin ducks to different vaccines were measured by VNT and challenge test with virulent virus. The acetylaziridine (AEI) inactivated vaccine produced higher levels of antibodies than orally administered DEF propagated virus. It also protected the ducks against the challenge, however, the propeolactone vaccine gave no protection (Butterfield and Dardiri 1969 b).

Mukit *et al.* (1988) collected sera samples from three different flocks of ducks and observed marked differences in virus neutralization titres. The average neutralization indices were 1.5, 2.3 and 3.8 in unvaccinated, vaccinated and recovered ducks respectively.

Kalaimathi and Janakiram (1991) passaged the virulent DPV in chicken embryo fibroblast cell culture and 5th and 12th passage virus was used as vaccine. The micro SNT was performed on the sera from 5th and 12th passage virus vaccinated birds and the titres observed were 40 and 80 respectively, however, there was sharp increase in SN titres post challenge.

The efficacy of two different vaccines was studied by Bordolai *et al.* (1994) whose SN indices were ranging from 0.24 to 1.78 over six weeks. The conventional vaccine group had VNI 1.12 and cell culture vaccinated birds had VNI 1.78. There was sharp increase in VNI post challenge.

SNT was performed with the serum from birds vaccinated with 5th and 10th passage DPV virus. The mean SN titre in 5th passage group was 64 with VNI of 1.8 on 20th day post vaccination. However, a mean titre of 54 with and VNI 1.73 was observed in 10th passage group. Low titres in 10th passage group was reasoned out to be because of loss of immunogenicity after 5th passage (Senthilkumar, 1997).

2.7 Monoclonal Antibodies (Mabs)

Since the initial description of hybridoma cell lines secreting monoclonal antibodies of predefined specificity by Kohler and Milstein (1975) the technique has been applied to many viruses and bacteria and has made profound impression on all areas of biological research and biotechnology.

Koprowski *et al.* (1977) pioneered the production of Mabs against viruses and viral antigens. Mabs have now been produced against representative members of most taxonomic virus groups.

There is no published evidence in the literature about the production and application of Mabs in duck plague virus. However Mabs have been reported and been used for characterization and diagnosis of other herpes viruses.

Ikuta *et al.* (1983 a) produced Mabs against Marek's disease virus (MDV) and Herpes virus of turkeys (HVT). Fifty and 14 hybridoma clones produced antibodies specific to MDV and HVT respectively. Of these 14 MDV and 5 HVT clones produced antibodies that recognized antigenic sites common to both the viruses.

Lee *et al.* (1983 a) developed 50 antibody secreting hybridomas specific for three different serotypes of Marek's disease virus. Eleven hybridomas were used for serotyping a panel of 15 pathogenic and non pathogenic strains of MDV and HVT, representing three serotypes. One Mab was type common, 8 reacted only with pathogenic and attenuated strain of MDV, one reacted with non pathogenic strain of MDV and one with HVT strain only.

Van-Duren-Little, *et al.* (1984) produced Mabs against BHV type 1. The antibodies reacted with six glycoproteins of the 11 glycoproteins identified for BHV 1 and were useful in demonstrating inter relationship between some of the glycoproteins of BHV1.

Collins *et al.* (1984) produced Mabs against BHV 1 and used these to study the neutralizing determinants of BHV 1 polypeptides.

Shimizu *et al.* (1989) and Sinclair *et al.*(1989) characterized neutralizing and non-neutralizing Mabs against Equid herpes viruses. Three Mabs neutralized subtype 1 but not subtype 2 virus. It was opined that neutralizing antibodies were useful in vaccine production and non neutralizing for identifying virus strain variation.

Stable hetrohybridomas secreting Mabs against cattle herpes virus 1 (BHV1) were developed. Three of these hybridomas were secreting neutralizing antibodies and were useful in immunological research ,diagnosis and therapy (Anonymous. 1989).

York *et al.* (1990) defined the antigens of infectious Laryngo trachaetis virus using Mabs. Group 2 Mabs which recognized nonglycosylated component of the 205 k d complex could be used to screen for the expression of the recombinant viral protein for development of a recombinant viral vector vaccine.

Natraj and Srikumaran (1994) developed T cell hybridoma specific for BHV 1 glycoproteins (gp). One hybridoma was specific for BHV 1 gp 91, 2 specific for gp IV and three for gp III.

Seven mouse hybridoma cell lines producing Mabs against an encephalitogenic strain of BHV 1.3 were established. Only one Mab neutralized the virus without complement and five with addition of complement but none of the mab neutralized respiratory strain of BHV 1 (Chang, *et al.,* 1994).

Winkler *et al.* (1995) studied neutralizing Mabs to BHV1 (Cooper strain) for viral specificity with the help of Immunoperoxidase test and immuno electron microscopy. The infected cells revealed peroxidase activity by red brown granular deposits in the nucleus and cytoplasm.

2.8 Procedure

There is vast variation in the literature regarding myeloma cell line, strain of mice, immunization protocol, antigen purification and its use for immunization and other aspects of Mab production. Various workers have tried modifications to the original procedures adopted by Kohler and Milstein (1975). These modifications have helped to simplify the procedure, retaining the basic concept of Mab production.

In their classical experiment Kohler and Milstein (1975) used BALB /c mice and immunized with a potent antigen Sheep RBCs. Two myeloma cell lines of BALB/c origin P 1 Bul and P3-X 63 Ag 8 were used and inactivated Sendai virus was used as the fusing agent.

The procedure of Mab production had been reviewed by Goding (1980), Groth and Scheidegger (1980), Galfre and Milstein (1981) and Zola and Brooks (1983).

Ikuta *et al.* (1983 b) produced Mabs against surface and secreted glycoproteins of MDV and HVT. Hybridomas were formed between mouse myeloma cells and spleen cells from the mice immunized with the virus. The authors opined that purified virus specific antigens were more effective than unpurified virus infected cell homogenate.

Lee *et al.* (1983a) inoculated MDV and HVT infected cell suspension with Freund's complete adjuvant into the foot pad and intra peritoneal in 5 female BALB /c mice. Second injection was given three weeks later with complete adjuvant. A final injection was given intravenous without adjuvant after an additional 10 to 14 days. Serum samples obtained from orbital bleeding were tested for antibodies and mice showing titres between 1:100 and 1:1000 were selected for further fusion.

Lee *et al.* (1983 b) developed antibody secreting hybridoma to MD tumor associated surface antigen (MATSA) by somatic cell hybridization between mouse myeloma cell line SP2/0-Ag/14 and spleen cells from MSB 1 immunized mice. Each of five female i.v. injection was given after an additional 10 to 14 days. Polyethylene glycol (PEG) 1000 , 35 per cent and 5 per cent dimethyl sufoxide (DMSO) in 1 ml was used as fusing agent.

Collins *et al.* (1984) immunized BALB /c mice with three injections of purified cattle herpes virus 1 (BHV 1). Spleen cells from immunized mice were fused with myeloma cells SP 2/0-Ag 14 to produce hybridoma. Mab secreting hybridomas were cloned by limiting dilution.

Van-Duren-Little *et al.* (1984) injected BALB/c mice with purified Bovine herpes virus type 1 in complete Freund's adjuvant. Second injection was given 2 weeks later with incomplete Freund's adjuvant, final i.v. injection in phosphate buffered saline was given three days prior to fusion. Positive clones were cloned by limiting dilution.

Metzler *et al.* (1985) immunized BALB /c mice with partially purified cattle herpes virus 1 BHV-1) in complete Freund's adjuvant. Three weeks later mice were given booster injection on three consecutive days. The spleen was removed next day and spleen cells were fused with X 63 Ag 8.654 nonsecreting myeloma cell line using PEG. Hybrids were cloned by limiting dilution method.

Chang *et al.* (1986) immunized BALB /c mice with 100 ug of purified Infectious Bovine Rhinotracheatis virus (IBRV) and a booster of 50 ug of virus per mouse at three weeks interval. The spleen cells were fused with

mouse myeloma cell line P 3 X 63 Ag 8 653. HAT media was used as selection system and the supernatant from hybridomas were tested by ELISA.

Live bovine herpes virus 1 (BHV-1) clarified by sedimentation through a 40 per cent sucrose cushion was mixed with complete Freund's adjuvant and injected i.p. into BALB/c mice. Neutralization positive mice were boosted with an intravenous injection of virus 3 days prior to fusion of spleen cells with P 3 X 63-Ag 8.653 myeloma cell line. Neutralizing antigenic areas on the glycoprotein of BHV 1 were identified by reciprocal competition radioimmuno assay using Mabs (Marshall, *et al.*, 1988).

Sinclair *et al.* (1989) produced Mabs to equid herpes virus type 1. Ten to twelve week old BALB /c mice were injected i.p. with EHV 1 in buffer. After 6 to 8 weeks mice were injected i.v. with antigen in phosphate buffered saline pH 7.4. Spleen cells were fused with mouse myeloma cell line NSO 3 days after the final injection and culture supernatants were tested with ELISA. Positive hybrids were subcloned twice by limiting dilution.

Shimuzu *et al.* (1989) developed Mabs with neutralizing activity to EHV-1 propagated in ESK cell culture. Partially purified virus emulsified in complete Freund's adjuvant was administered. Three weeks later a second injection was given with incomplete adjuvant, followed by last

intra peritoneal injection of virus suspension alone. After 3 more days spleen cells were fused with P3U1 myeloma cells. Hybridomas were selected in HAT medium and screened with the help of ELISA.

Nakajima *et al.* (1989) used BALB /c mice to produce Mabs against Marek's disease virus serotype 2. Sonicated MDV 2 strain infected cell suspensions emulsified in Freund's complete adjuvant was given on day one. After 4 to 8 weeks animals received booster injection of virus infected cells without adjuvant. Spleen cells from immunized animals were fused with P3 X63-Ag 8U1 myeloma cells. The positive hybrids were detected by immunofluorescence.

Stable heterohybridomas secreting Mabs against cattle herpes virus 1 (BHV1) were produced. A young cattle was immunized by direct injection into the lymphnodes with BHV-1 emulsified in Freund's adjuvant. Cells from these nodes were isolated and fused with SP 2/0 myeloma cells in presence of PEG.Sensitive hybrids were again fused with fresh lymph node cells from the same animal. Cells from this fusion were selected and fused again with fresh lymph node cells to produce heterohybridomas which secreted Mabs reacting strongly with BHV 1 (Anon, 1989).

York *et al.* (1990) produced Mabs against infectious laryngotracheatis virus (ILTV) by immunizing adult BALB /c mice with partially purified ILT virus, SA 2 strain (Vaccine strain). The mice received 2 s/c injections (100 ug) one month apart in Freund's complete and incomplete adjuvant respectively. An intra peritoneal injection was given four days before fusion. Mice were immunized similarly with a virulent strain. Two hundred million splenocytes were fused with 40 million NS 1 myeloma cells using 50 per cent PEG and positive hybridomas were cloned by limiting dilution.

The tetradomas were produced by a triple fusion sequence. Lymph node cells from a calf hyper immunized with BHV 1 were fused with SP 2/0 cells and Mab producing cattle mouse heterohybridoms were selected. These were then refused with primed lymph nodes similar to first fusion and cattle-cattle X mouse triomas were selected. These were then fused with primed lymph nodes and cattle X mouse tetradomas were obtained (Anon, 1992).

2.9 Applications of Mabs.

Applications of Mabs are very vast and this biotechnology has been used in all aspects of biomedical research. Mabs have been used in diagnostic virology, taxonomy and epidemiology and has simplified the

molecular biological and biochemical investigations. Mabs have helped to study the interaction of the virus and the host. The major application of mab has been in the field of identification of viral strains and variants. This has been reviewed by Mc Cullough (1986) and Raybould (1989).

Apart from the applications of Mabs in virology and viral diseases, this biotechnology has been used in monitoring ovulation and detecting the pregnancies in cattle (Booman, *et al.*, 1984). Rapid diagnostic assays for early detection of the infectious diseases, direct use for passive immunization, modification of the physiological functions to increase production and immuno therapy in cancer are some of the applications highlighted by Raybould (1989). It has also been used in identification of parasites (Gamble and Graham, 1983), Detection of toxins and drug residues in animal feed (Candish, *et al.*, 1985) and management of human tumors (Abrams and Oldham, 1985). It has also been used in fish farming and plant science (Sander and Dietzgen, 1984 and Furytya, *et al.*; 1987).

2.10 Monoclonal antibodies in the diagnosis

Rapid and accurate diagnosis of viral diseases is of obvious importance to the clinician and to public health. Mabs are most reliable reagents with very high specificity and constant quality and helped in accurate and quick diagnosis of the infection.

Carter and Meulen (1984) used Mab based immunofluorescence test for early diagnosis of the cytomegalo virus. The method was found to be simple and easier than time consuming viral isolation.

Crouch and Acreas (1984) reported that Mab based diagnostic systems were useful in detecting subclinical viral infection and helped in tracing the epidemiology of neonatal diarrhea of calves.

ELISA has been the test of choice for screening hybridoma supernatant and testing the immune status of the immunized mice used for fusion.

Chang *et al.* (1986) used Mab based ELISA to test the hybridoma supernatant and further used high titred Mabs to study the virus neutralization.

Sinclair *et al.* (1989) and Shimuzu *et al.* (1989) produced Mabs to EHV-1. The culture supernatants were tested for specific antibodies against EHV-1 with the help of ELISA.

Nakajima *et al.* (1989) compared Immunofloresceence (IF) with ELISA for screening hybridoma to very virulent MDV (VVMDV). Out of 58 hybridoma clones that were screened, 36 gave positive reaction in IF and

22 gave positive reaction in ELISA. None of the Mabs gave positive reaction in both IF and ELISA.

Karacu and Nagio (1993) utilized a Mab based blocking ELISA (B-ELISA) and compared it with conventional indirect ELISA and VN test. It was observed that B -ELISA which offered both convenience of the conventional indirect ELISA and serotype specificity of the VN test was useful for field application in IBV diagnosis and evaluation of response to IBV vaccination.

Moer *et al.* (1993) used indirect ELISA for testing EHV1 specific Mabs and compared it with IFA and IPT test. The blocking ELISA was able to differentiate EHV-1 and EHV-4 serum samples.

Immunoperoxidase test has been used for the detection of the viral antigens in the tissue sections and has many advantages over IFT and RIA.

Guy *et al.* (1992) observed that staining with hyper-immune anti sera resulted in non specific background staining due to antibodies produced in response to contaminants in the immunizing preparations. Mab based IPT staining resulted in high quality and high sensitivity in ILT virus detection.

Moer *et al.* (1993) used a type specific Mab and tested the different EHV strains with indirect ELISA, IFT and IPT. The Mab did not give reaction with other EHV in all the tests.

Chang et al. (1994) produced Mabs against encephalitogenic BHV type 1.3 and respiratory BHV type 1.1. Two of the Mabs L6G and O7E were conjugated with peroxidase and used for the detection of BHV 1.3 antigens in formalin fixed brain tissue of experimentally infected calves.

Winkler *et al.* (1995) studied three neutralizing Mabs specific for BHV-1 by IPT and IEM. Red brown granular deposits were observed in the nucleus and the cytoplasm. IP activity was not observed in the negative controls.

Malmarugan (1997) performed IPT in DPV with hyper-immune serum raised in ducks. Dark brown background staining was observed along with brown deposits in the nucleus as well as cytoplasm.It was opined that , Mabs may be helpful in reducing background staining and would be more accurate in detecting the viral antigens in tissue sections.

Virus neutralization test (VNT) has been used by Lee *et al.* (1983 a) to study the neutralization activity of Mab ascitis fluid against cell

free MDV and HVT. High titred Mabs reacted with individual specific serotype and confirmed the classification of MDV .It was opined that a panel of Mab was essential to study intratypic strain variability of MDV.

Abdel-Magid *et al.* (1992) produced a panel of Mabs against BHV-1. Three Mabs were neutralizing and reacted with 130/75, 150 and 77 k da glycoprotein. A fourth Mab was non neutralizing and recognized the 97 k da glycoprotein.

Karaku and Nagio (1993) used VNT to detect specific antibodies to IBV using Mabs. A good correlation was observed between results of blocking ELISA (B-ELISA) and VNT. Serotype specific antibodies were detected in the chicken sera.

Chang *et al.* (1994) used Mab based VNT to distinguish BHV type 1.3 and respiratory BHV type 1.1. One Mab neutralized the virulent virus without the complement, however five Mabs required complement for neutralization.

Winkler *et al.* (1995) studied three neutralizing Mabs specific for BHV type 1. Virus specificity was studied with the help of IPT and IEM.

MATERIALS AND METHODS

MATERIALS AND METHODS

3..1 Materials

3.1.1 Eggs

3.1.1.1 Duck eggs

Fertile duck eggs purchased from the University Poultry Mannuthy farm were used for revival of the virulent virus strain of virus and preparation of duck embryo fibroblast cell culture.

3.1.1.2. Chicken eggs

Nine to 11 day old embryonated chicken eggs obtained from University poultry farm, Mannuthy, were used for preparation of chicken embryo fibroblast cell culture.

3.2 Ducklings

Three to four weeks old unvaccinated, healthy ducklings were procured from duck unit, University poultry farm, Mannuthy and used for revival of virulent DP stock virus.

3.3 Biologicals

3.3.1 Vaccine virus

Vaccine strain of DPV was received from Institute of Animal Health and Veterinary Biologicals, Palode, Kerala and IVPM, Ranipet, Tamil Nadu and used as seed virus for bulk Production in tissue culture.

3.3.2 Virulent virus

The reference duck plague virulent virus strain [repository Number - (vir) AD/87-1] was obtained from Division of Avian Diseases, IVRI, Izatnager, UP. The virus was lyophilised liver suspension.

3.3.3 Alleppey strain

A field isolate of DPV collected from an outbreak in Alleppey district was used in the study. The virus was adapted to CEF and preserved for further use.

3.4 Glass-ware

Borsil brand glass-ware and Tarson make plastic wares were used for tissue culture work.

3.5 Solutions

3.5.1 Triple glass distilled water (TDW)

Tap water distilled thrice in glass distillation apparatus was used to prepare differents media and reagents throughout the study.

3.5.2 Calcium-Magnesium free Phosphate buffered saline (CMF-PBS)

CMF-PBS was prepared from ready made dehydrated powder obtained from M/S Himedia laboratories, Mumbai. The solution was prepared as per the guidelines of the manufacturer.

CMF-PBS........ One vial

TDW............1,000 ml

The PH was adjusted to 7.4 and sterilized by autoclaving at 15 lb pressure for 15 min.

3.5.3 Sodium bicarbonate solution (7.5 per cent)

Sodium bicarbonate.......7.5 g

TDW.........................100 ml

The solution was sterilized by filtration using membrane filter of 0.45 um pore size and stored at 4 °C until use.

3.5.4 Trypsin solution (5 per cent)

Trypsin (1:250).........5 g

CMF PBS................100 ml

The solution was sterilized by filtration using membrane filter of pore size 0.45 um and stored at –20° C till use. The working solution of trypsin (0.25 per cent) was prepared by diluting in CMF-PBS prior to use.

3.5.5 Antibiotic solution

Streptomycin........... 1 g

Benzyl Penicillin.......10,00000 IU

Sterile TDW..............100 ml

One ml of solution was added to 100 ml media to obtain final concentration of 250 ug of streptomycin and 250 IU of benzyl penicillin per ml of media.

Gentamycin 2 ml (80 mg)

Sterile saline 40 ml

100 X stock solution was prepared and stored at 4 ° C till use . At the time of use 1 ml stock solution was added to 100 ml of medium to get final concentration of 20 ug per ml.

3.6 Calf serum

Blood collected from healthy male calves below one year age was used as source of serum. Clear serum was collected, clarified by centrifugation and inactivated at 56 ° C for 30 min. The serum was sterilized by filtration using 0.22 um membrane filter and stored in small aliquotes at –20° C until use.

3.7 Media

3.7.1 Cell culture media

Dehydrated cell culture media supplied by M/S Himedia laboratories Mumbai were used for cell culture throughout the study.

3.7.2 Hanks Balanced Salt Solution (HBSS)

HBSS dehydrated powder	1 vial
TDW	1000 ml
Yeast extract	2 g
Lactalbumin hydrolysate	5 g

The medium was sterilized by filtration using 0.22 um membrane filter and stored at 4 ° C for further use.

3.7.3 Growth medium

HBSS	94 ml
Calf serum	5 ml
Antibiotics	1 ml

The pH was adjusted 7.2 to 7.4 with sterile sodium bicarbonate prior to use.

3.7.4 Maintenance medium

HBSS	96 ml
Calf serum	3 ml
Antibiotics	1 ml

The pH was adjusted as above.

3.8 Staining of cover slip cultures

3.8.1 May Grunwald Giemsa stain

May Grunwald stain	2.5 g
Absolute methanol	1000 ml

The stain was dissolved in absolute methanol and allowed to age for one month.

Giemsa stain	1 g
Glycerol	66

Giemsa powder was dissolved in glycerol at 55° C, kept overnight. Sixty ml of absolute methanol was added and kept at room temperature until use.

3.9 Virus concentration and purification

Molecular biology grade chemicals were used for concentration and purification of the virus.

3.9.1 Tris sodium EDTA buffer (TNE buffer)

Tris hydrochloride	7.9 g
Sodium chloride	.1.95 g
EDTA	0.25 g
TDW	480 ml

The pH was adjusted to 8.0 and final volume was made up to 500 ml.

3.10 Materials and biologicals for plate ELISA

3.10.1 Antigen for coating

DPV vaccine, IVRI and Alleppy strains concentrated, purified and stored in small aliquotes were used in 1:100 dilution as coating antigen for plate ELISA. The dilution of the antigen was arrived by checker board titration.

3.10.2 ELISA plates

Tarson brand plastic plates with 96 U bottom wells were used throughout the study.

3.10.3 CMF-PBS

The working solution of CMF-PBS was prepared as indicated in 3.5.2.

3.10.4 Phosphate buffered saline Tween 20 (PBST) pH 7.2

CMF-PBS	1000 ml
Tween 20	500 ul

The pH was adjusted to 7.2

3.10.5 Bovine serum albumin (BSA) 2 per cent

BSA	2 g
PBST	100 ml

3.10.6 Carbonate bicarbonate buffer pH 9.6

Sodium carbonate (Na_2CO_3)	1.59 g
Sodium bicarbonate($NaHCO_3$)	2.93 g
TDW	1000 ml

The pH was adjusted to 9.6

3.10.7 Sodium citrate buffer 0.05 M , pH 4.2

Sodium citrate	14.71 g
TDW	1000 ml

The pH was adjusted to 4.2

3.10.8 Substrate solution

ABTS (2'-2-azino-di-ethyl	
Benzthiazolin-6-sulfonic acid)	11 mg
Sodium citrate buffer	50 ml
Hydrogen peroxide	25 ul

Prepared just prior to use and immediately dispensed in the wells.

3.10.9 Goat anti mouse peroxidase conjugate

This was procured from M/S Bangalore Genei, Bangalore and Sigma chemicals, USA as 1 ml solution. Ten ul of conjugate was diluted in 20 ml of PBST at the time of use (1:2000). Dilution of the conjugate was ascertained by checker board titration.

3.11 Indirect immuno peroxidase test (IPT)

3.11.1 CMF-PBS pH 7.4

This was prepared as per the method described earlier (3.5.2)

3.11.2 Tris HCL pH 7.6

0.2 M Tris (2.42 g/100 ml) - Solution-A

0.2 N HCL -Solution-B

50 ml of A + 38.4 ml of B to make up to 200 ml with TDW.

3.11.3 Trypsin

Trypsin (1:250) - 0.25 g

TDW - 100 ml

3.11.4 Substrate solution

0.05 per cent 3,3' diaminobenzidine tetrahydrochloride (DAB) was prepared by dissolving 5 mg DAB in 10 ml Tris-HCl buffer and 30 ml of 33 per cent H_2O_2 (0.01 % H_2O_2) was added just prior to use.

3.12 Materials for hybridoma production

3.12.1 Glassware and plastic-ware

Borosil brand glassware and Tarson and Nunc make plastic plates and flasks were used in the study. The glassware were sterilized as per the standard procedure.

3.12.2 Chemicals and reagents

Freund's complete adjuvant, Freund's incomplete adjuvant, RPMI 1640 media, L glutamine, HAT medium supplement (Hypoxanthine, Thymidine, Aminopterine), Fetal calf serum and polyethylene glycol of Sigma USA make was used for hybridoma and monoclonal antibody production.

3.12.2.1 RPMI 1640

RPMI 1640 available as premixed powder was dissolved in 1000 ml sterile TDW. To this 2 g of Sodium bicarbonate, 300 mg glutamine, 4 ml antibiotic solution (Gentamycin -20 mg / ml) and 4 ml of 2-mercaptoethanol (5x105 M) was added. The pH was adjusted to 7.2 to 7.4 with 1N NaOH or 1N HCl and filtered through 0.22 um membrane filter and stored at 4° C until use.

3.12.2.2 PEG solution

PEG 5 g

Sterile RPMI medium 5 ml

PEG sterilized by autoclaving at 15 lb pressure for 15 min and when hot 5 ml of sterile RPMI medium was added.

3.12.3 Fetal Calf Serum (FCS)

FCS stored in small aliquotes was added at the rate of 20 per cent to the RPMI 1640 medium to make growth medium myeloma cell line as well as for hybridoma.

3.12.4 Biologicals

3.12.4.1 Antigens for Immunization

Vaccine strain of DPV (DPV- V) cultivated in CEF cell culture and partially purified was used for immunization of BALB/c mice.

3.12.4.2 Experimental Animals

BALB/c mice received from National Institute of Nutrition, Hyderabad and maintained in the Department of Microbiology, served as source for hybridoma work and splenocyte donor.

3.12.4.3 Myeloma Cell line

SP 2/0-Ag-14, a BALB/c origin, non secreting plasmacytoma cell line obtained from National Facility for Animal tissue culture, Pune and Department of Animal Biotechnology, Madras Veterinary College, Chennai, was used for fusion with splenocytes from donor mice.

3.13 Methods

3.13.1 Revival of DPV strains

3.13.1.1 Vaccine strain

The lyophilised vaccine received from VBI Palode/Ranipet was reconstituted in 5 ml of CMF-PBS and used as seed virus for inoculation in DCE and large scale production in CEF cell culture.

3.13.1.2 Alleppey strain

Virulent Alleppey strain of DPV, adapted to grow in CEF in the Department of Microbiology, was used as seed virus for bulk production.

3.13.1.3 IVRI strain

IVRI strain of DPV received as lyophilized liver suspension was reconstituted in CMF-PBS and inoculated into five ducklings.The liver and spleen from the dead birds were collected, processed and used as seed virus for inoculation into 11 day old duck embryo by CAM route at the rate of 0.1 ml suspension per embryo . The inoculated eggs were incubated at 37 ° C and embryo mortality was monitored at 24 h intervals. The dead embryos were collected and chilled at 4° C overnight.

The embryos were harvested and examined for DP lesions. The AAF and CAM were collected. The CAM was homogenized in AAF and centrifuged at 5000 rpm for 5 min. The supernatant was collected and preserved at –20 ° C after addition of antibiotics at the rate of 500 IU Benzyl penicillin and 500 ug of streptomycin. This was used for further passages in DEF cell culture.

3.14 Propagation of DPV in cell culture

3.14.1 Preparation of primary CEF cell culture

Eleven day old embryonated chicken eggs were used for preparation of CEF cell culture. The standard procedure of Freshney (1994) was used for this purpose.

The embryos were collected aseptically in a Petri dish containing CMF-PBS. The embryos were decapitated , evisceratedand cut into 1 to 2 mm pieces. The tissue fragments were transferred to a beaker and minced in to a homogeneous

suspension. The tissue suspension was then trypsinised thrice with 0.25 per cent trypsin solution for 15 min each. The trypsinised tissue suspension was pooled, filtered through muslin cloth and centrifuged at 800 rpm for 5 min to pellet the cells.The cells were washed thrice with CMF-PBS and resuspended in growth medium. The cell concentration was adjusted to 1 x 10^{8} cells per ml and seeded in Roux flasks and test tubes with cover slip at the rate of 75 ml and 1 ml respectively. The bottles and tubes were incubated at 38.5° C.

3.14.2 Cultivation of DPV vaccine strain in CEF

The flasks and tubes showing confluent monolayer at 24 h of incubation were inoculated with DPV (Vaccine strain) at the rate of ten ml per Roux flask and 0.1 ml per test tube. The monolayers were then incubated at 38.5° C for 45 min for virus adsorption. The monolayers were washed with maintenance medium and refed with the same and incubated at 38.5° C. Suitable controls were incorporated in the procedure. The monolayers were examined at 12 hourly interval under inverted microscope for appearance of CPE .

The bottles and tubes showing complete CPE were frozen at –20° C and thawed at room temperature three times to release the virus from the cells. The contents from all the bottles were pooled and centrifuged at 3000 rpm for 30 min in a refrigerated centrifuge at 4° C. Clear supernatnt was collected and stored at -20° C until further use .

3.14.3 Cultivation of DPV Alleppey Strain in CEF

The same procedure adapted for cultivation of vaccine strain was also used for cultivation of Alleppey strain of DPV.

3.14.4 Cultivation of DPV IVRI strain in DEF

The same procedure for preparation of primary cell culture and cultivation was adapted for IVRI strain of DPV except that the primary cell culture system used was DEF, prepared from 11 day old duck embryos.

3.15 Virus titration and assessment of $TCID_{50}$

$TCID_{50}$ for vaccine, Alleppey strain and IVRI strain was assessed in CEF and DEF cell culture respectively. The test tube monolayer cultures were inoculated with DP virus. Serial ten fold dilutions of the virus were made in maintenance medium and each dilution was inoculated into four tubes at the rate of 0.1 ml of virus suspension per tube. The tubes were incubated at 38.5 °C and observed at 12 hourly interval for appearance of CPE. The test tubes showing CPE were noted down and 50 per cent end point was calculated at the end of 96 hours of incubation as per the method of Reed and Muench (1938).

3.16 Virus concentration and purification

The method adapted by Taylor (1997) for concentration and purification of DPV was followed with slight modifications. The tissue culture supernatent preserved at –20°C was thawed and centrifuged for 5 min for clearing the coarse

particles if any. Clear supernatant was then centrifuged at 1,00,000 g for 4.5 hours in Beckman ultra centrifuge at 4°C.

The small button like pellet was diluted with minimum quantity of TNE buffer. The diluted pellet was again centrifuged in microfuge at 5000 rpm for 5 min and clear supernatant was transferred to the Eppendorf tubes. This partially purified virus was used for priming the BALB /c mice for hybridoma production, raising the polyclonal hyperimmune serum and as antigen for coating the ELISA plates.

The same procedure was adapted for all the three strains of DPV *Viz.*, Vaccine, Alleppey and IVRI.

3.17 Raising of polyclonal serum

The method suggested by Anonymous (1992) was followed for raising polyclonal serum in mice against three strains of DPV with slight modification. Protein concentration of the partially purified virus was ascertained as per the Biuret method and was adjusted to 100 ug of protein per 50 ul of solution. Fifty ul of virus suspension emulsified in FCA was inoculated intra peritoneally on day one into five BALB /c female mice of 6 to 8 weeks age.

A second injection of same quantity was given with FIA after 15 days of first inoculation and a booster injection was given without adjuvant after another

15days (45th day). The animals were test bled 7 days after the last injection and serum was collected. Five mice each were used for raising the polyclonal serum against the three strains of DPV. The polyclonal serum was pooled and preserved for future use at -20°C. The same procedure was adapted for raising polyclonal serum against all the strains under study.

3.18 Plate ELISA

The ELISA was performed as described by Voller et al. (1976) with minor modifications.

Optimum dilution of coating antigen and goat antimouse peroxidase conjugate was determined by checker board titration and same was used throughout the study.

All the wells of the 96 well ELISA plate were coated with 100 ul of 1 in 100 diluted antigen in ELISA coating buffer, pH 9.6 wrapped in aluminium foil and stored at 4°C overnight. The plates were washed next day 3 to 4 times by emptying and refilling with 200 ul of PBST in each well. The plates were mopped and dried by tapping on a plain cloth. The uncoated sites were blocked with 2 per cent BSA and incubated at 37°C for 60 min.

The plates were washed as before and sera samples (Hyper immune serum) were added starting from 1:100 to 1:12800 dilution in duplicate rows at

the rate of 100 ul per well and incubated at 37° C for one h. The wells were washed dried and charged with goat anti mouse peroxidase conjugate and incubated at 37°C for one h. After incubation the wells were washed thoroughly 4 to 5 times with PBST, and freshly prepared 100 ul of substrate was added and incubated at room temperature for 30 min.

The plate was observed every 10 min for development of colour reaction. After maximum reaction, the plates were read in a multiscan ELISA reader at 405 nm . Suitable controls viz. substrate control, HRP control, Known + ve control, Known -ve control were incorporated . The substrate controls were blanked to read the sample.

3.19 . Immunoperoxidase test

Tissue collected from birds infected with IVRI and Alleppey strain of DP were fixed in 10 per cent buffered formalin and subjected to paraffin embedding. The sections of 4 to 6 um thickness were cut and mounted onto the slide. Prior to the test , the sections were deparafinised and subjected to 0.25 per cent trypsin treatment for 1 h. The sections were treated with hyper-immune serum (1 in 10 dilution) and incubated at 37°C for 45 min in a humid chamber.

The slides were washed in PBS thrice for 5 min and kept in methanol bath containing 0.5 per cent H_2O_2 at room temperature for 30 min for removing endogenous peroxidase. These sections were washed in PBS and treated with

goat anti mouse peroxidase conjugate (1:1000) for 45 min at 37°C. Excess conjugate was removed by thorough washing with PBS for 3 to 4 times. Freshly prepared substrate solution (DAB) in tris HCl buffer containing 0.01 per cent H_2O_2 was added and allowed to react for 2 to 5 min at room temperature.

The slides were washed in PBS and dehydrated through graded series of ethanol followed by xylol and mounted in DPX mountant / 50 per cent glycerol phosphate buffered saline. The slides were examined under light microscope and the peroxidase activity was observed as dark brown precipitate.

3.20 Virus Neutralization Test (VNT)

Serial two fold dilutions of serum from 1:2 to 1:128 were made in tissue culture maintenance medium pH 7.2. Equal quantity of 100 $TCID_{50}$ of DPV viruses (Vaccine/ Alleppey/ IVRI) was added and incubated at 37°C for 60 minutes for neutralization of virus. Three or four tubes with confluent monolayers were inoculated for each dilution with 0.1 ml of serum virus mixture and incubated at 38.5°C.

The inoculated tubes were examined at 24 h interval until the virus control tubes showed CPE. The tubes in each dilution showing CPE or no CPE were noted down. The naturalization titre and virus neutralization index (VNI) was calculated as per the method of Reed and Muench (1938). Uninoculated control and antigen control were incorporated in the test.

3.21 Methods for Hybridoma Production

3.21.1 Immunization of mice

Method described for raising polyclonal serum (3.17) was adapted for priming mice for hybridoma production with slight modification.

Five BALB/c mice were immunized as per the same dose and procedure described for polyclonal serum up to three injections. One more booster was given 15 days after the third injection with 100 ul of antigen without adjuvant (60th day). Final injection was given with 100 ul of antigen, three or four days prior to fusion.

3.21.2 Maintenance of Myeloma cell line

The myeloma cell line (SP2/0-Ag14) was maintained in the Department of Microbiology in RPMI 1640 medium. The cells were allowed to grow and after complete monolayer formation the cells were subcultured. The monolayers were maintained in complete RPMI medium with 10 per cent FCS. The cells subcultured two or three days prior cell fusion so as to have the logarithmic growth phase. The cells in logarithmic phase and having over 90 per cent viability were only used for fusion.

3.21.3 Feeder cell Preparation

The splenocytes from a healthy normal BALB/c mouse were used as feeder cells and prepared one day prior to fusion. The mouse was sacrificed following

aseptic precautions and spleen was collected and placed in a Petridish containing 10 ml RPMI medium with double strength antibiotics. The capsule and fat adhering to the spleen was removed and spleen was transferred to another Petridish containing 10 ml RPMI medium. The splenocytes were collected by repeatedly inoculating the medium into the spleen using 20 G needle and 10 ml syringe. After complete removal of cells from the spleen the medium containing splenocytes was carefully collected in a centrifuge tube. The splenocytes were centrifuged at 1200 rpm for 5 min . The supernatant was removed and the cell pallet was resuspended in HAT medium. The viable cell count was made and the concentration was adjusted 2 to 3 X 10^6 cells per ml and splenocytes were distributed into the 96 well tissue culture plate at the rate of 100 ul per well and incubated at 37°C with 5 per cent CO_2 tension. The same plates were used next day to distribute the fused cells.

3.21.4 Preparation of myeloma cells for fusion

On the day of fusion, the SP 2/0 cells in logarithmic phase of growth were harvested from the bottles and washed in serum free medium once by centrifuging at 1200 rpm for 5 min. The viable cell count was taken and cells with more than 90 per cent viability were used for fusion.

3.21.5 Preparation of splenoctytes for fusion

One BALB /c mouse immunized with DPV vaccine strain and showing good immune response (high ELISA titre) was selected as the donor of splenocyte The procedure adapted for feeder cell preparation was used for collection of

splenocytes. The splenocytes were resuspended in serum free medium after washing and viable cell count was taken. The splenocytes with more than 95 per cent viability were only used for fusion.

3.21.6 Cell count

Hundred ul of cell suspension was taken from known volume and mixed with equal volume of stain. The mixture was carefully applied between the cover slip and haemocytometer, over the counting chamber. The viable cells which excluded the dye were counted against the dead cells which were stained. The cells from four large squares were counted and total count was calculated as

Total no. of cells per ml = Mean cells per square X 2 X 10

During feeder cell preparation and for fusion, viable cell count was taken for splenocytes as well as myeloma cells. Dye exclusion method using the trypan blue solution was used and the required cell concentration was adjusted to (1:10, Splenocytes : Myeloma cells).

3.21.7 Cell Fusion

Method described by and Galfre and Milstein (1981) and Zola and Brooks (1989) was used with slight modification. After taking the viable count the volume of splenocyte and myeloma cell was adjusted so as to have the cell ratio 1:10 respectively. The cells were suspended in plain RPMI medium in a 50 ml conical bottom plastic centrifuge tube and the mixture was centrifuged at 1200 rpm for 5

min. The supernatant was removed and the cell button was loosened with little residual medium by gentle tapping so as break the large clumps.

One ml of PEG 4000 (Sigma) was added to the cell mixture over a period of 30 seconds with continuous gentle tapping of the tube. The tube was kept undisturbed for 90 seconds. The PEG cellmixture was further diluted with RPMI medium as one ml RPMI medium over a period of one minute thrice, followed by 1.5 ml medium over a period of one min thrice, 2.5 ml of medium over a period of one min twice and 5 ml medium over a period of one min four times. The tubes were gently agitated while adding the medium so as to achieve uniform dilution of the PEG.

After completion of fusion process the contents of the tubes were gently mixed and centrifuged at 800 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in HAT medium with 20 per cent FCS. This fused cell suspension was then plated out in to the 96 well cluster plates at the rate of 100 ul per well which were already having feeder cells. The plates were incubated at 37°C with 5 per cent carbon dioxide tension.

3.21.8 Maintenance of fused cells

The fused cells in 96 well plate were checked every day for development of the hybrids and medium was changed after every three days. The hybrids were maintained in HAT medium till three to four changes and then two to three changes

with HT medium were made. After a total of 5 to 6 changes in HAT and HT medium the hybrids were maintained in plain RPMI medium with 15 per cent FCS.

3.21.9 Screening of hybrids

When the clones attained 80 percent confluency and the culture supernatent was collected and screened for the presence of antibody against DPV by indirect ELISA as described earlier, except for the replacement of hyperimmune polyclonal serum by hybridoma supernatant and wells were put for each supernatant.

The hybridoma supernatant showing double the OD value than that of negative control was taken as positive.

3.21.10 Cloning of the hybrids

Cloning under limiting dilution technique described by Goding (1980) and Galfre and Milstein (1981) was followed. The positive clones were expanded by transferring into a 24 well cluster plate containing feeder cells. The cells were cloned when there was confluent growth.

3.21.11 Cloning by limiting dilution

When positive hybrids attained confluent growth, recloning was done to establish monoclonality. The cells were harvested and a cell count was made and

the suspension was serially diluted in such away that 100 ul of suspension contained 1000, 100, 10 and 1 cell each. The dilutions were made in RPMI medium and the cells were distributed at the rate of 100 ul / well . Two rows of 96 well plate were each used for all the dilutions. However, four rows were used for the last dilution i.e.1 cell per 100 ul of suspension. The plates were incubated at 37°C with 5 per cent CO_2 tension.

The wells were observed for the presence of clones daily and the wells containing clones developed from single cell (last dilution 4 wells) were identified and the growth and development was monitored . When the cells attained 80 per cent confluency , the supernatant was tested for the presence of antibodies by indirect ELISA and positive clones were further expanded into 24 well cluster plate and tissue culture bottles containing feeder cells for bulk production of the desired antibodies.

3.21.12 Bulk production of Mabs

The hybridoma clones secreting Mabs against DPV vaccine strain were grown in tissue culture bottle containing 10 per cent FCS. The supernatant was collected and stored at –20°C for futher use.

3.22 Applications of Monoclonal antibodies

Mabs produced were tested against the three strains of DPV Viz., Vaccine , Alleppey and IVRI. ELISA was performed to study the cross reactivity

of Mabs on the same lines and procedure used for testing the polyclonal serum except for, the serum was replaced by the hybridoma supernatant, .rest of the procedure remained the same.

Efficacy of the Mabs in localization of the tissue antigens was ascertained by IPT in the liver and spleen tissue sections and in the CEF monolayers in case of vaccine strain .The procedure described earlier for IPT was followed except the serum was replaced by Mab. The Mab was diluted 1:2 for the test.

Neutralizing ability of Mab against the three strains of DPV was tested by performing VNT on the same lines as described earlier except the mabs replaced the polyclonal serum.

3.23 Cryopreservation

3.23.1 Materials

3.23.1.1 Cryovials

Tarson brand cryovials were used for cryopreservation of myeloma cell line and the hybrids.

3.23.1.2 Medium

RPMI 1640 medium was used for cryopreservation of the cells and the hybrids.

3.23.1.3 Dimethyl sulfoxide (DMSO)

Merek brand DMSO was used as cryoprotectant during freezing of the cells.

3.23.1.4 Freezing medium

Fetal calf serum	0.8 ml
DMSO	0.1 ml
RPMI 1640	0.1 ml

3.24 Method

Method described by Zola and Brooks (1989) and Anonymous (1992) was followed with modifications as under.

The myeloma cells in the logarithmic phase of growth were collected by repeated flushing. The cells along with the medium were collected in a conical centrifuge tube and washed two times with RPMI 1640 medium. The supernatant was carefully removed and cell pellet was loosened. The freezing medium was added dropwise while constantly shaking the tube. The medium along with the cells was collected in a cryovial, sealed and labeled. The vial was transferred to a thermocol box and kept at 4°C for 4 h. Subsequently the vials were shifted to -20°C for 3 h. After a total of 7 h the vials were kept in a liquid Nitrogen (LN_2) freezing chamber and slowly the vapors were released. The cells were allowed to freeze in to LN_2 vapours for 30 min and the vials were immediately shifted to LN_2 container.

3.25 Revival of the cells

For checking the viability of the cells, the vials kept in LN container were taken out and immediatply dropped in a beaker containing water at 40° C. As soon as the cell suspension thawed the vials were wiped with 70 per cent alcohol and contents were diluted in 9 ml medium and centrifuged at 1200 rpm for 5 min. The cell pellet was resuspended in growth medium and transferred to culture bottle. The cells were incubated at 37°C with 5 per cent CO_2 tension.

3.26 Survivability and longevity trials

A trial to ascertain the survivability and longevity of the hybrids was conducted to determine the appropriate time of collection of the supernatant and maximum antibody production. The trial was conducted as follows.

The positive hybrids were allowed to grow in a tissue culture bottle and at the confluent growth the cells were collected by repeated flushing. The cells were counted and the number was adjusted to 1000 cells per ml of the medium. The cells were then dispensed in to 24 well cluster plate. The growth and antibody production was monitored every 24 h. The supernatant was collected and tested for antibody production by indirect ELISA. The parameters of growth such as total number of cells, coverage of the wells and change in colour of the medium i.e. pH change were noted down every 24 h up to a period of 120 h. ELISA was performed as per the procedure described earlier.

RESULTS

RESULTS

Three strains of DPV, namely the vaccine strain, DPV-V, IVRI strain DPV-I and a local strain isolated from Alleppey district of Kerala state, DPV-A were utilised in the present study.

4.1 Revival and passage of the virus

4.1.1 Vaccine strain (DPV-V)

4.1.1.1 Chicken embryo

Five, 11 day old embryonated chicken eggs were inoculated with reconstituted vaccine virus via CAM route. The embryo death was recorded 80 to 120 h post inoculation (PI). The dead embryos showed extensive hemorrhages all over the body, congestion of spleen and liver and highly congested CAM (Plate 1). The embryo along with AAF and CAM was collected and further processed for passages in CEF cell culture.

4.1.1.2 Chicken embryo fibroblast cell culture

After 24 h of seeding of the cells in the tissue culture bottles, a confluent monolayer of CEF cell was observed. The monolayer showed long, elongated, spindle shaped fibroblast cells. Coverslip cultures of normal monolayer unstained and stained with May Grunwald Giemsa showed cells

Plate 1. Congestion of CAM inoculated with
vaccine strain of DPV
1-Normal 2-Infected

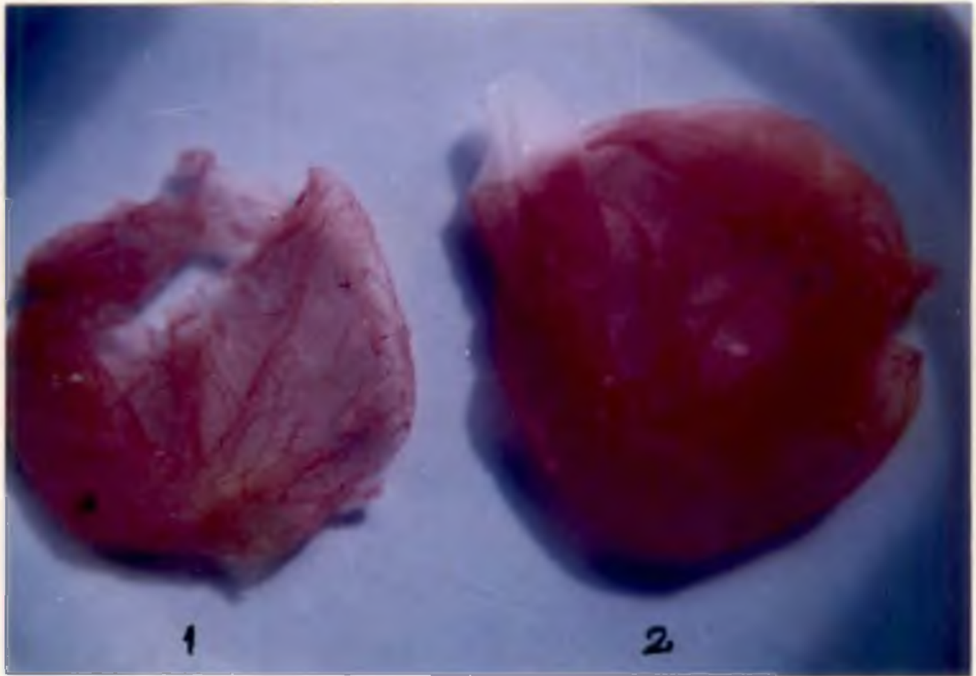

Plate 1

containing acidophilic cytoplasm and oval shaped centrally located nuclei (Plate 2 and 3).

4.1.1.3 Cytopathic effect in CEF cell cultures

The CEF cell cultures infected with DPV vaccine strain showed CPE 48 to 96 h PI. The CPE was characterised by rounding of cells, clumping and vacuolation, elongation of cytoplasm, bridge formation and syncytium formation. Complete disquamation of the cells and monolayer was noticed at 120 h PI (Plate 4 and 5).

The infected monolayer stained with May Grunwald Giemsa stain showed eosinophlic intranuclear inclusion bodies (Plate 6).The culture supernatant containing virus was pooled and stored at -20° C until further processing.

4.1.2 Alleppy strain (DPV-A)

The local strain of DPV isolated from Alleppy district of Kerala state and adapted to grow in CEF cell cultures was used for further passaging. The tissue culture fluid of earlier passages maintained in the department was used as seed virus and confluent monolayer of CEF were infected with the same. The CPE observed in the CEF monolayer were similar to those observed with the vaccine strain.

Plate 2. Normal monolayer of chicken embryo fibroblast (Unstained X100)

Plate 3. Normal monolayer of CEF (May Grunwald Giemsa stain X 400)

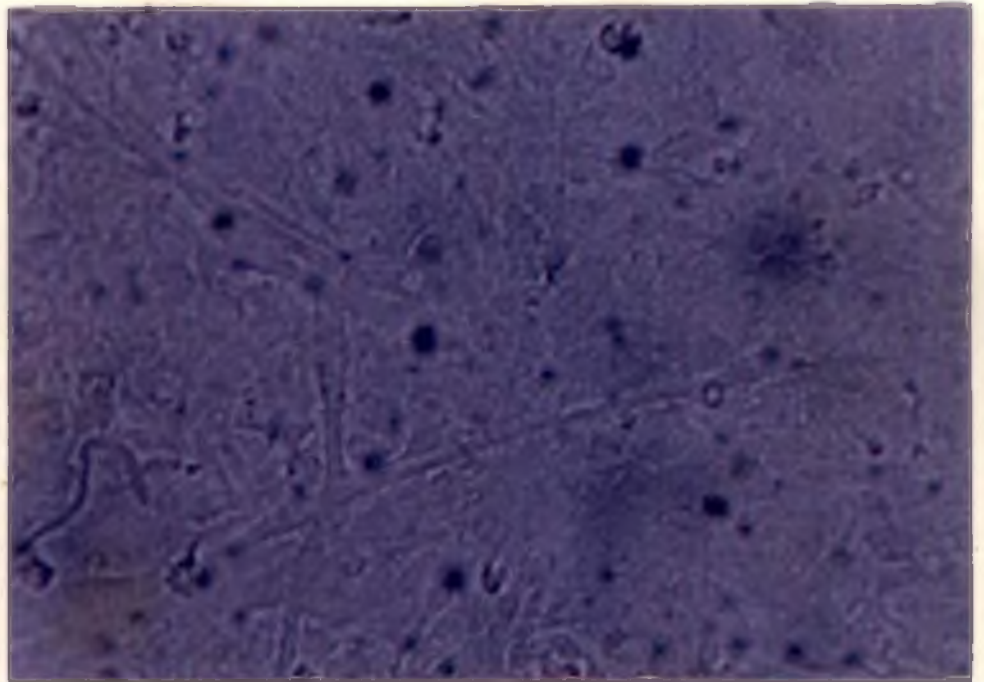

Plate 2

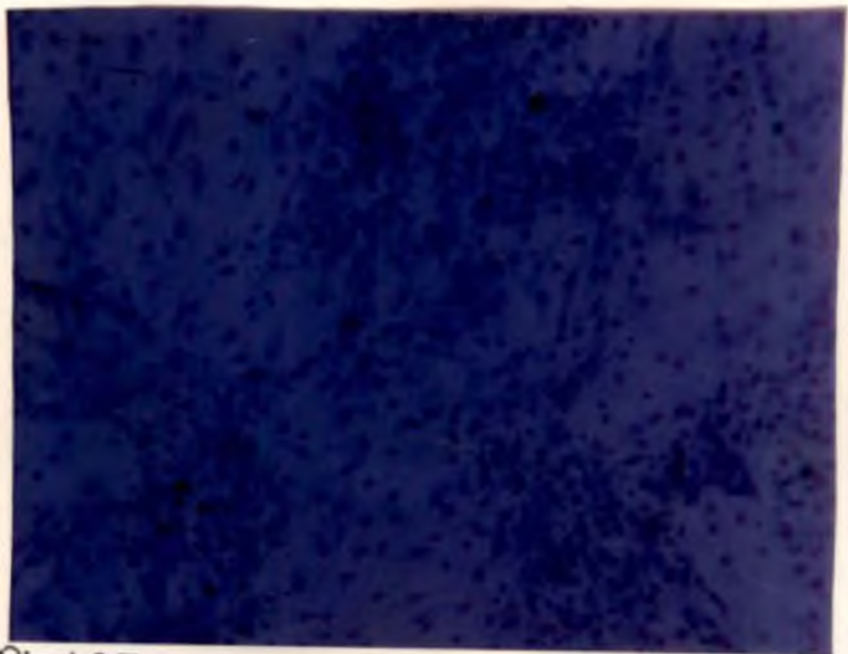

Plate 3

Plate 4. CPE in CEF infected with
DPV vaccine strain
(Unstained X100)

Plate 5. CPE in CEF infected with DPV vaccine strain
(stained May Grunwald Giemsa stain X 100)

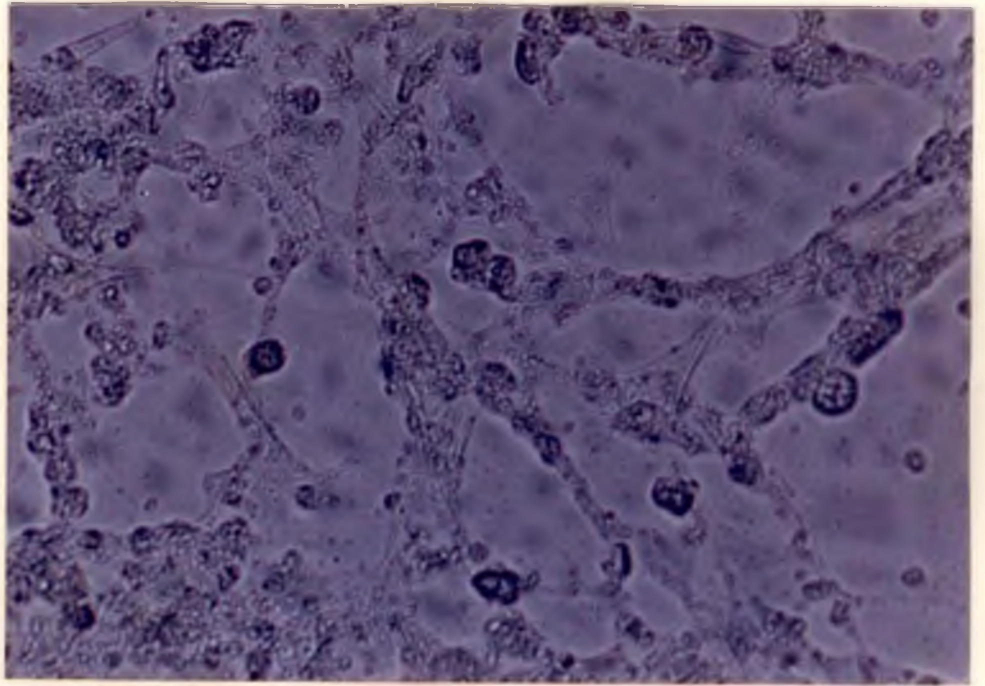

Plate 4

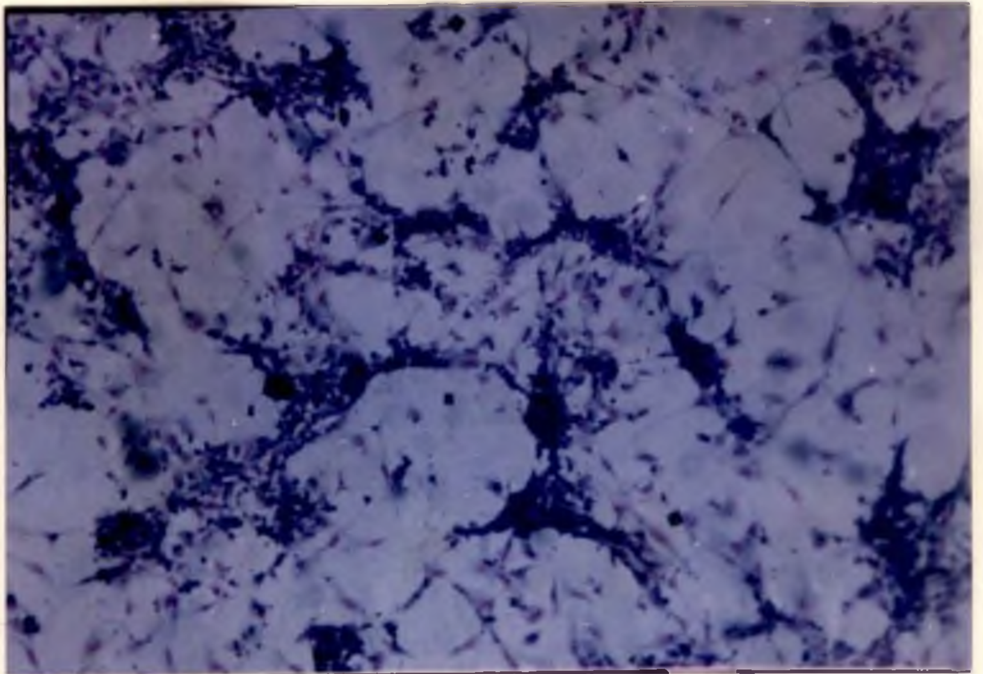

Plate 5

Plate 6. CPE in CEF infected with DPV vaccine strain
Intra nuclear inclusions (May Grunwald stainX1000)

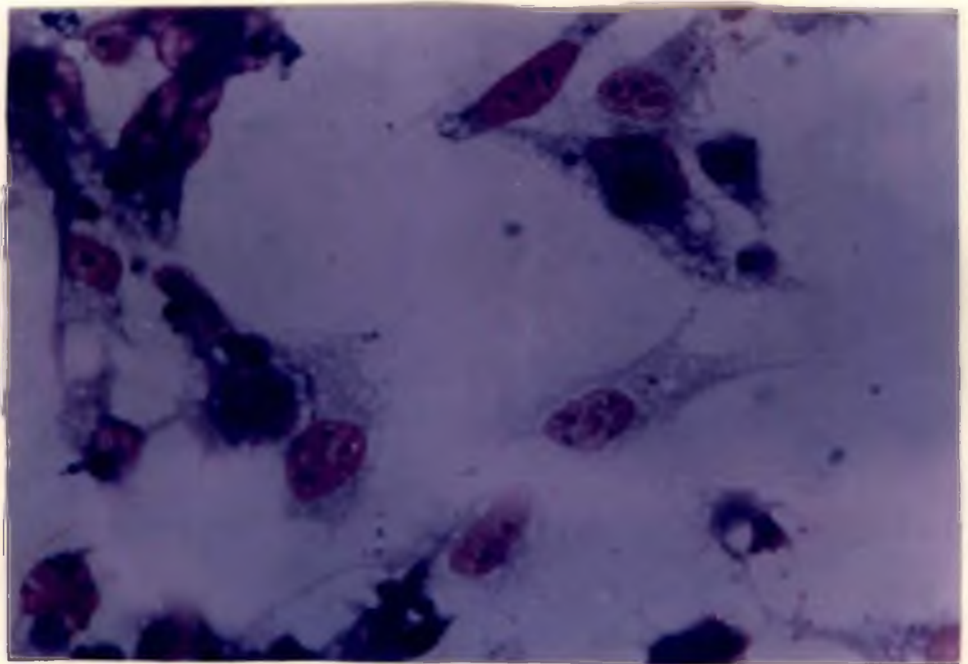

Plate 6

4.1.3 IVRI strain (DPV-I)

4.1.3.1 Ducklings

Five ducklings were inoculated with the seed virus (DPV-I) received from Indian Veterinary Research Institute (IVRI), Izatnagar, UP, India. The ducklings started showing the symptoms from 5th day PI. The symptoms observed were dullness, increased thirst, sticky eyes, nasal discharge, swelling of the face and head and soiled vent due to greenish watery diarrhea. Seventy per cent birds inoculated with the virus died on 7th day PI. The birds preferred to sit with breast resting on the floor and beak turned on the back prior to death. When the ailing birds were forced to move, showed the tremors of the head neck and body. All the ailing birds died 7th day PI. On necropsy of the dead birds, varying degree of hemorrhages were noticed on serous membranes, muscles and visceral organs. Necrotic foci and petechial hemorrhages on liver, enlargement and congestion of liver and spleen were pathgomonic. Crusty diptheric plaques in the esophagus, catarrhal inflammation of the proventriculus, white necrotic areas in the gizzard muscles were typical DPV lesions observed. Liver and spleen of these birds were processed and used as seed virus for inoculation in developing duck embryos.

4.1.3.2 Duck embryo (DDE)

A 20 per cent suspension of liver and spleen , collected from dead birds was inoculated in 11 day old DDE. Embryo death was noticed between 4 th to 6 th day PI. Oedema and congestion all over the body and congested CAM were noticed. Enlarged liver and spleen with petechial hemorrhages and necrotic foci were evident. AAF and CAM were further processed for passaging in DEF cell cultures.

4.1.3.3 Duck embryo fibroblast cell culture

DPV-I produced marked CPE at 48 h PI and was characterised by rounding and clumping of cells, bridge formation and syncytium formation.The complete sloughing of the monolayer was observed 120 h PI. Coverslip cultures stained with May-Grunwald Giemsa stain showed vacuolation in cytoplasm and eosinophlic intranuclear inclusion bodies.

4.2 Titration of the Virus

4.2.1 Vaccine strain (DPV-V)

Pooled virus suspension was subjected to titration in CEF cell cultures to assess the tissue culture infective dose ($TCID_{50}$). The $TCID_{50}$ was calculated after the control tubes showed the CPE. The $TCID_{50}$ for vaccine strain of DPV was $4.7X10^5$ /ml of the inoculum .

4.2.2 Alleppy strain (DPV-A)

The DPV -A strain cultivated in the CEF cell cultures was pooled and titrated in the same system to asses the $TCID_{50}$ of the virus.The $TCID_{50}$ for the Alleppy strain of DPV was $3.2X10^4$ /ml of the inoculum.

4.2.3 IVRI strain (DPV-I)

The DPV-I cultivated in DEF cell cultures was pooled over passages and was subjected to titration. The $TCID_{50}$ of the virus suspension was ascertained in DEF cell culture. It was observed that the $TCID_{50}$ for DPV-I strain was 10^5 /ml of the inoculum.

4.5 Raising of polyclonal serum

The mice inoculated with all the three strains of DPV *Viz.*,DPV-V, DPV-A and DPV-I showed high antibody titers by ELISA (Table 1). Four mice inoculated with DPV-V showed ELISA titers above 1:12800 (Plate 7). All the mice inoculated with DPV-A were having ELISA titers above 1:12800 (Plate 8). However in case of mice inoculated with DPV-I the titers were 1:6400. The serum samples of the mice from respective groups were pooled and used for further testing.

4.6 Cross reactivity of polyclonal serum

4.6.1 ELISA

Cross reactivity of the polyclonal serum raised against DPV-V, DPV-A and DPV-I was studied by employing plate ELISA test. The results are presented in Table 2.

It was observed that , polyclonal serum raised against vaccine strain (DPV-V) reacted with the homologus virus and had a titre of 1:12800. It also reacted with heterologous virus strains *Viz.*,DPV-A and DPV-I with the same specificity and similar titers were observed with these strains (Plate. 9). Poly clonal serum raised against DPV-A showed a titre of 1:12800 with homologus

Plate 7 ELISA titres of mice inoculated with DPV-V
A to H 1:100 to 1:12800,
3 and 4-Mice no.1
5 and 6- Mice No.2
7 and 8 –Mice No.3
9 and 10- Mice No.4
11and 12- Mice No.5

Plate 8 ELISA titres of mice inoculated with DPV-A
A to H 1:100 to 1:12800,
3 and 4-Mice no.1
5 and 6- Mice No.2
7 and 8 –Mice No.3
9 and 10- Mice No.4
11and 12- Mice No.5

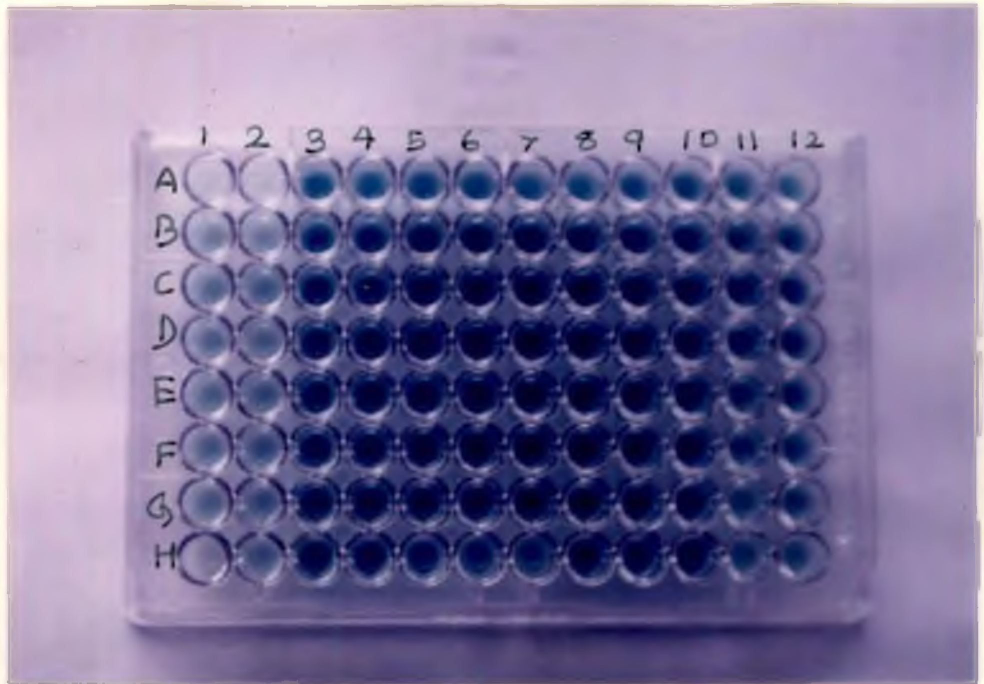

Plate-7

Plate 8

Table 1. ELISA titres of mice inoculated with three strains of DPV

ELISA titres		
Vaccine	Alleppey	IVRI
1:12800	1:12800	1:6400
1:12800	1:12800	1:6400
1:12800	1:12800	1:6400
1:12800	1:12800	1:6400
1:12800	1:12800	1:6400

Table 2. Cross reactivity of polyclonal serum with ELISA

Strain	Polyclonal ELISA Titres		
	Vaccine	Alleppey	IVRI
Vaccine	1:12800	1:12800	1:12800
Alleppey	1:12800	1:12800	1:12800
IVRI	1:6400	1:6400	1:6400

strain and with other two strains of the DPV under study. However, DPV-I showed a titer of 1:6400 with homologus virus strain and heterologous strains (Plate 10 and 11).

4.6.2 Immunoperoxidase test

Detection of viral antigens in the tissue sections by using polyclonal serum was done by employing immunoperoxidase test. The results of IPT with all the three polyclonal serum and its cross detection of the tissue antigens are presented in Table 6. Polyclonal serum raised against DPV-V detected homologous tissue antigens in CEF monolayer and it also detected DPV-A and DPV-I in the liver and spleen tissue sections collected from the birds after experimental inoculation. Polyclonal serum raised against DPV-A and DPV-I also detected homologues virus and heterologous virus strains in tissue sections or CEF monolayers. The tissue sections stained with IPT showed dark brown deposits at the site of antigen localisation, which were easily identified by light microscopy. Though the antigen detection was clearly marked by dark brown reaction, background tissue also stained brown to faint yellow (Plate 12). Similar staining reaction was observed with polyclonal serum raised against other two strains of DPV under study.

Plate 9 . Cross reactivity of DPV-V polyclonal serum against three strains of DPV

A to H , 1:100 to 1:12800

A-1 and 2 substrate control

B-1 and 2 HRP control

Column 4 and 5- DPV-V

Column 7 and 8-DPV-I

Column 10 and 11- DPV-A

Plate 10. Cross reactivty of DPV-A polyclonal serum against three strains of DPV

A to H , 1:100 to 1:12800

A-1 and 2 substrate control

B-1 and 2 HRP control

Column 4 and 5- DPV-V

Column 7 and 8-DPV-I

Column 10 and 11- DPV-A

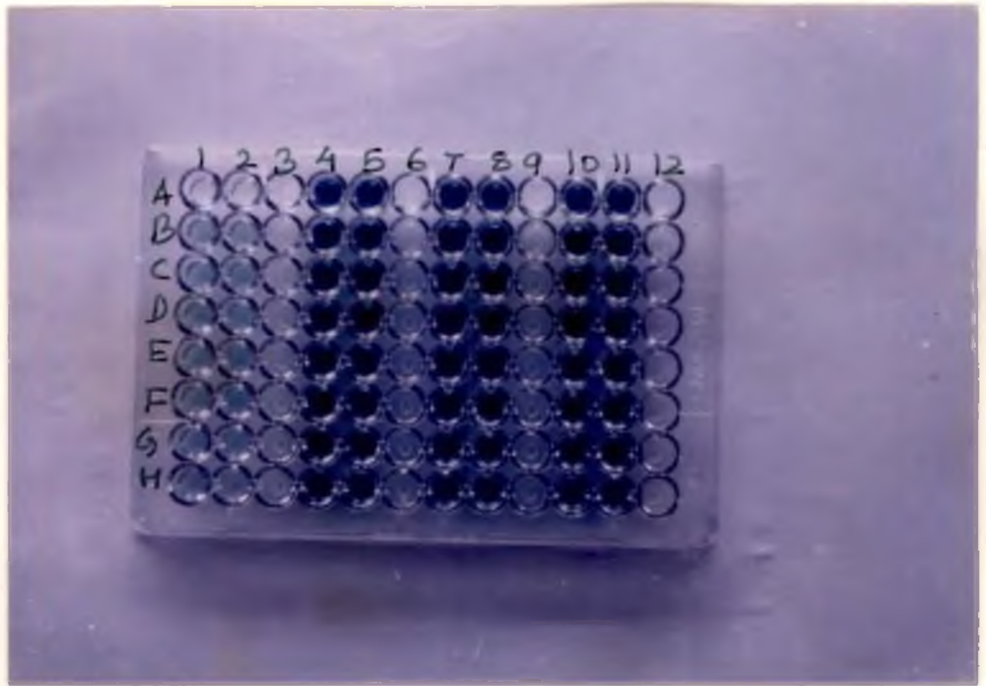

Plate-9

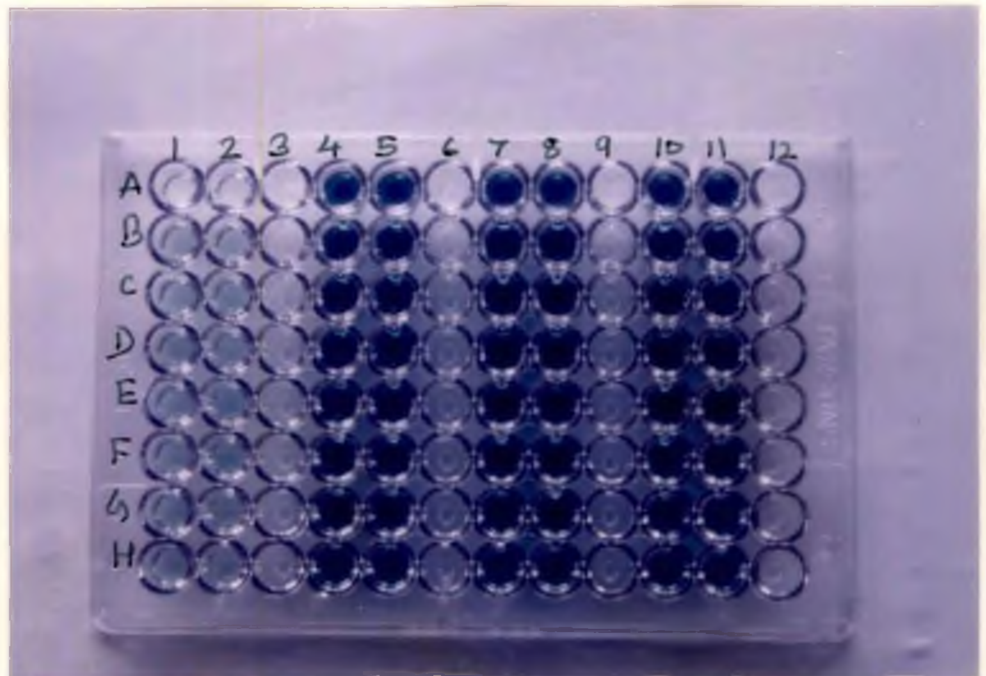

Plate.10.

Plate 11 Cross reactivty of DPV-I polyclonal serum against three strains of DPV

A to H , 1:100 to 1:12800
A-1 and 2 substrate control
B-1 and 2 HRP control
Column 4 and 5- DPV-V
Column 7 and 8-DPV-I
Column 10 and 11- DPV-A

Plate 12. Immuno peroxidase test with poly clonal serum (X 400)

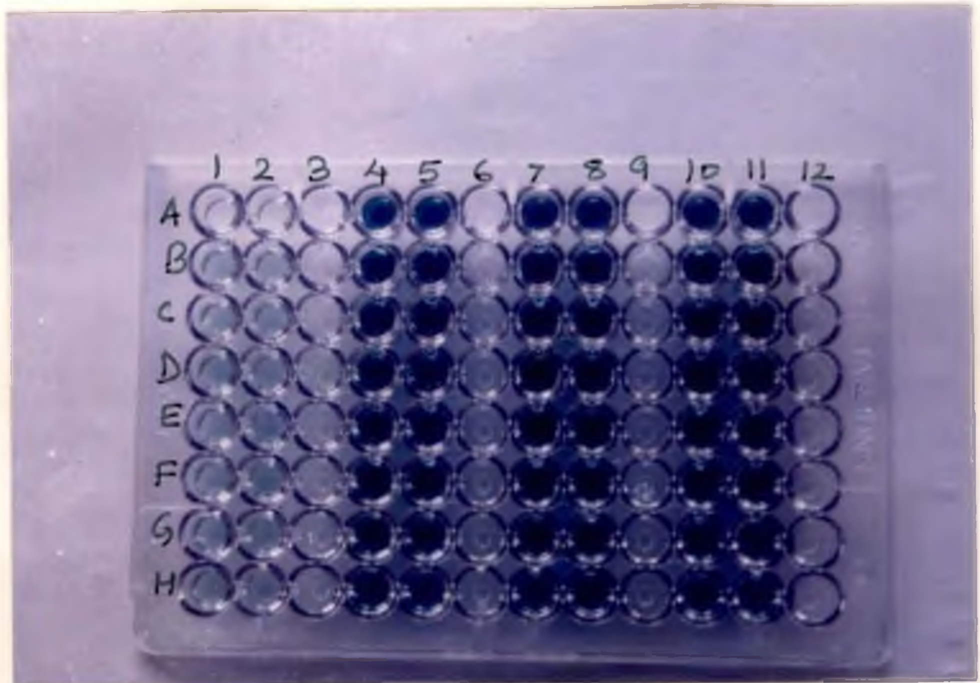

Plate. 11

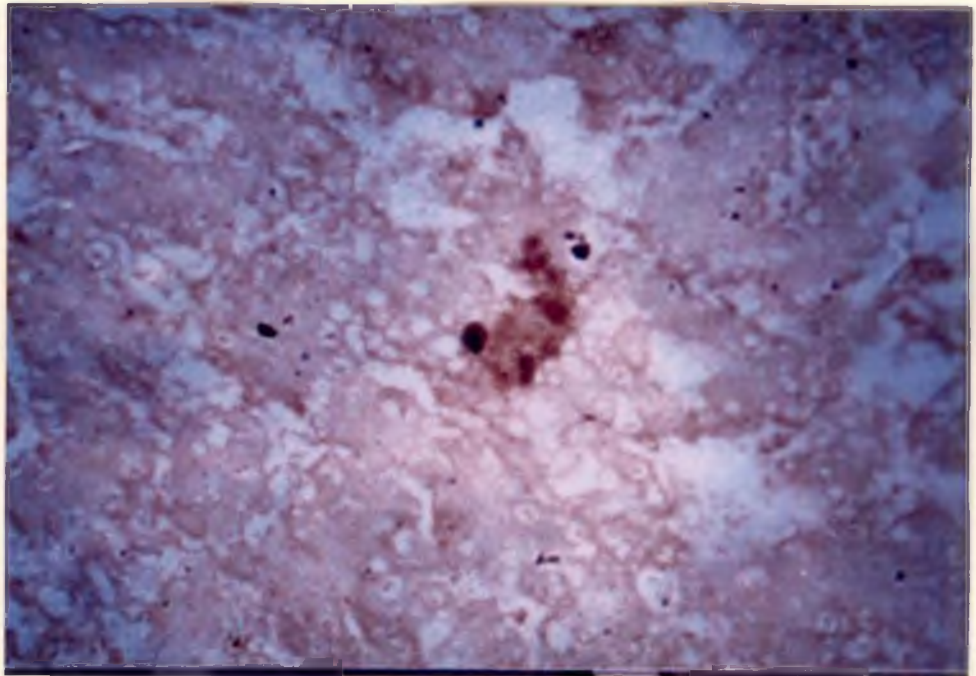
Plate - 12

Table 3. Cross reactivity of polyclonal serum with IPT

Strain	Polyclonal ELISA Titres		
	Vaccine	Alleppey	IVRI
Vaccine	+ Ve	+ Ve	+ Ve
Alleppey	+ Ve	+ Ve	+ Ve
IVRI	+ Ve	+ Ve	+ Ve

+ Ve indicates detection of viral antigens

4.6.3 Virus neutralization test

The virus neutralization test was performed by utilizing polyclonal serum against the three strains of DPV. The virus neutralization titers (VNT) and virus neutralization index (VNI) of the polyclonal serum against all the three strains of DPV under study have been presented in Table 4.

Polyclonal serum raised against DPV-V showed a VNT of 64 and VNI 1.8 against homologous virus. However the VNT against DPV-A and DPV -I was 48 with VNI of 1.65 for both the heterologous strains. Polyclonal serum raised against DPV-A neutralized the homologous virus at a titre of 32 with a VNI of 1.5 , It also neutralized the vaccine strain with same VNT and VNI. However it neutralized DPV-I with a titre of 24 and VNI 1.35. Polyclonal serum raised against DPV-I neutralized homologous strain, and had a titre of 48 with a VNI of 1.65. The same polyclonal serum neutralized other two strains i.e.DPV-V and DPV-A with a VNT of 32 and VNI of 1.5

Table 4. VN titres and VNI of polyclonal serum with homologous and heterologous strains

Polyclonal serum	Vaccine		Alleppey		IVRI	
	VNT	VNI	VNT	VNI	VNT	VNI
Vaccine	1:64	1.8	1:48	1.65	1:48	1.65
Alleppey	1:32	1.5	1:32	1.5	1:24	1.35
IVRI	1:32	1.5	1:32	1.5	1:48	1.65

4.7 Monoclonal Antibody production

4.7.1 Priming of mice and antibody titers

The mice inoculated with DPV-V were utilized as splenocyte donor in Mab production. The mice used for raising polyclonal serum were further primed with DPV-V and used as splenocyte donor in fusion procedure. The antibody titers were tested with the help of indirect ELISA. The test was performed in ELISA plate and the results were recorded within 30 min after the final step i.e. addition of the substrate. The test was read at 405 nm and the OD values which were double than the negative were considered as positive.

The results of ELISA titers of the mice inoculated with DPV-V and utilized as splenocyte donor are presented in Table 4 and Plate 6.

4.7.2 Maintenance of Myeloma cell line

Myeloma cell line SP 2/0 was maintained in RPMI 1640 tissue culture medium. A confluent growth was observed within 72 h of seeding.The cells formed a complete sheet of monolayer. At this stage the bottle was split in to 2 and further maintained in the same medium. Three days prior to fusion the cell line was seeded into two milk dilution bottles. A confluent monolayer with complete covering of the surface of the bottle was observed 72 h post seeding.

The cells were in log phase and actively dividing and were used for fusion (Plate 13).

4.7.3 Feeder cell preparation

One day prior to the fusion the plates were seeded with the feeder cells .The cells were collected from a normal healthy mice and the cell count was made as per the standard procedure. It was observed that by repeated flushing of the medium the cells get released from the spleen and a good recovery of the cells was seen. The cell concentration of the suspension was made and found to be 1 x 10^7 to 10^8 during all the fusions attempted.

4.7.4 Viable cell count

Viable cell count was made by the dye exclusion method for feeder cells as well as for splenocytes on the day of fusion. The cells which took the trypan blue stain were considered as non viable and remaining cells were only counted as viable. It was observed that the viability of the feeder cells and that of splenocytes was above 92 per cent and 95 per cent respectively. The splenocytes with viability of more than 90 percent were only used for fusion in all the fusions undertaken.

Plate 13. Normal myeloma cell line (X 400)

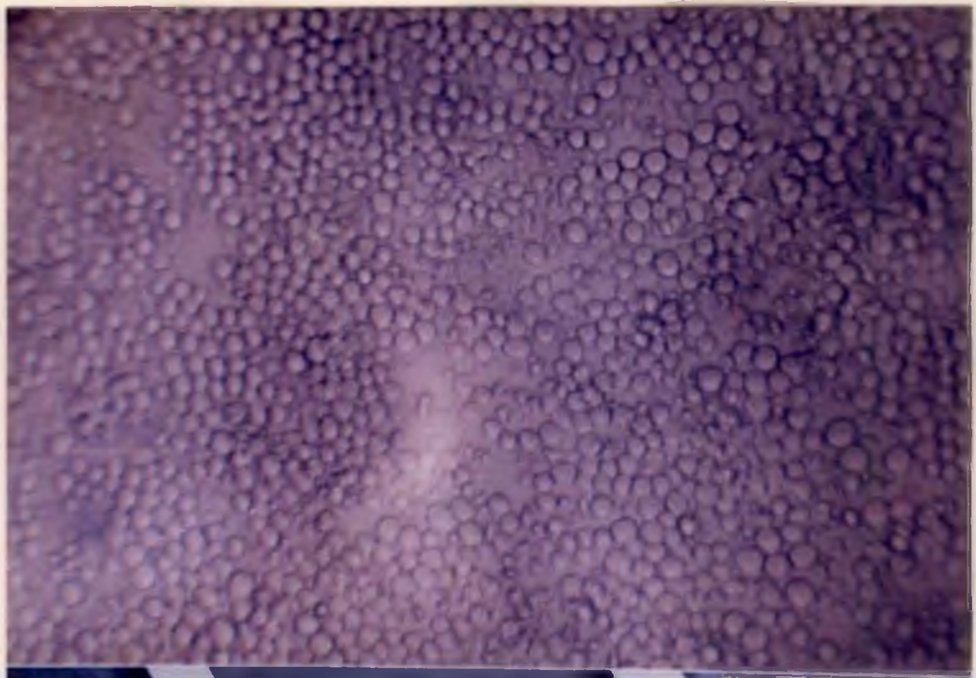

Plate-13

4.8 Fusion

The fusion of the myeloma cells and splenocytes from the donor mice was performed as per the standard procedure. The splenocytes were recovered from the mice primed with DPV -V and the cell count was taken . It was observed that the splenocyte recovery was optimum during all the fusion attempted.

After mixing of the myeloma cells and splenocytes in presence of poly ethylene glycol 4000 (PEG 4000) the cell mixture diluted and centrifuged . The clump formed was firm and was detached by slow mixing.The fused cells were then seeded in the tissue culture plates which were having feeder cells. Clumps of the splenocytes and the myeloma cells were observed on the day of fusion in the wells after seeding. These fused cells were maintained in HAT medium. The plates were monitored daily for the growth of colonies. It was observed that, small colonies of the hybrids were visible from 10th day post fusion. colonies were seen more distinctly on 12th to 15 day onwards (Plate 14 and 15).

4.9 Maintenance of the hybrids

Once the hybrids were spotted in the wells, the growth was monitored daily and at the stage when the well was covered 80 per cent the hybrids were shifted to the 24 well cluster plate. Initially the growth of the cells was slow but

Plate 14. Hybrid clones in 96 well cluster plate on 12th day (X 100)

Plate 15. Hybrid clones in 96 well cluster plate on 12th day (X 400)

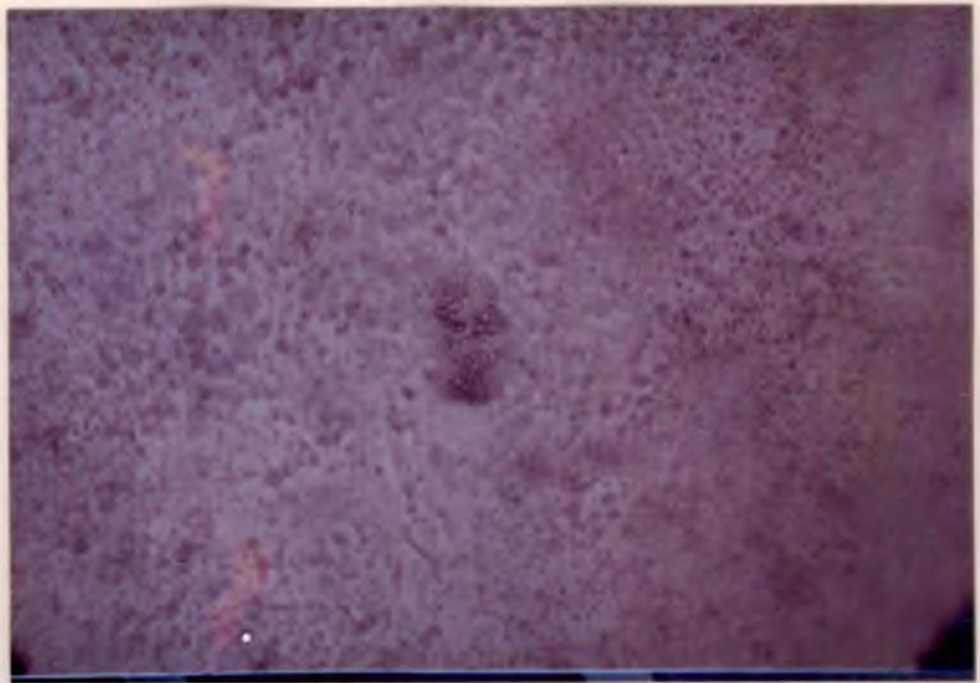
Plate-14

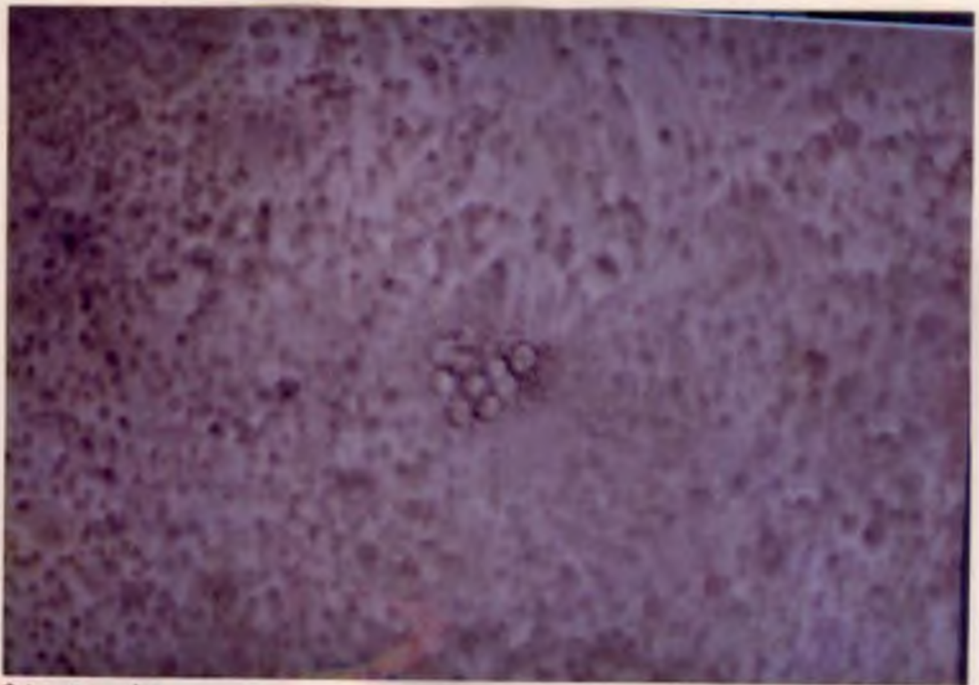
Plate-15

as soon as the cells were shifted to 24 well cluster plate and maintained in plain RPMI medium the hybrids showed a satisfactory growth (Plate 16).

4.10 Testing of Hybridoma supernatant

The hybridoma supernatant, from the wells showing the hybrid colonies was tested with indirect ELISA. Paired wells were put for each dilution. The positive hybrids were selected based on the colour reaction in ELISA and were further propagated (Plate 17 and 18).

4.11 Cloning of the hybrids

Cloning of the hybrids was done by the limiting dilution method. The positive were collected from the 24 well cluster plate and the cells were diluted to achieve 1, 5, 10 and 100 and 1000 cells per ml. It was observed that, out of 96 wells of the plate 2 to 4 wells containing 1 cell each showed development of colonies from single cell (Plate 19, 20, 21,22 and 23).

The supernatant from these wells was again tested for antibody which was confirmed by the development of the color reaction in ELISA. The hybrids positive for the desired antibodies against DPV-V were only processed further.

Plate 16. Hybrid clones expanded in 24 well cluster plate

Plate 17. Clones developing from single cell after limiting dilution

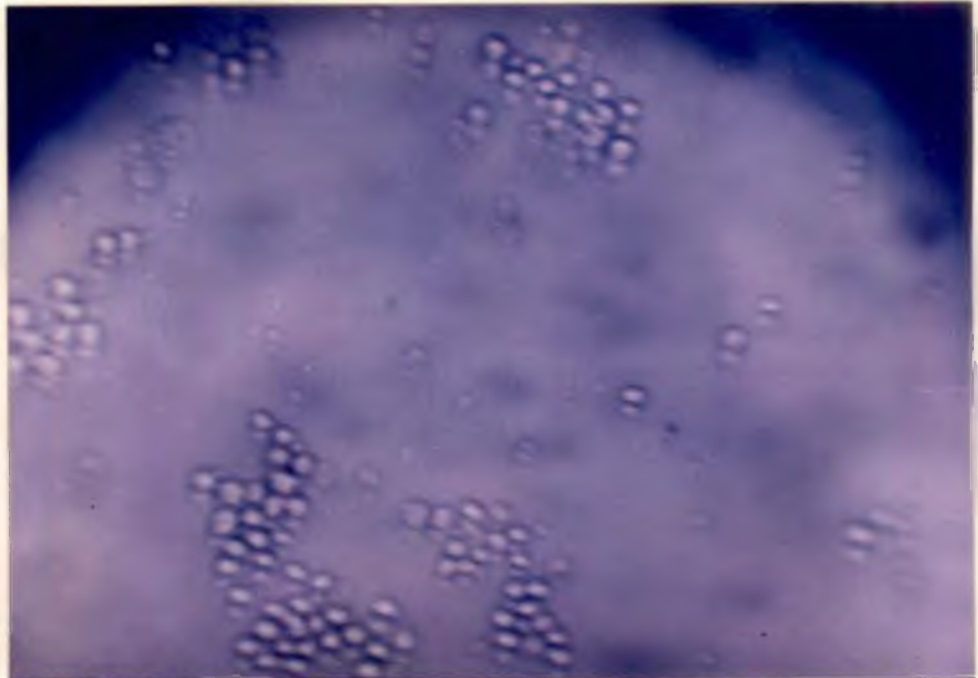

Plate.16

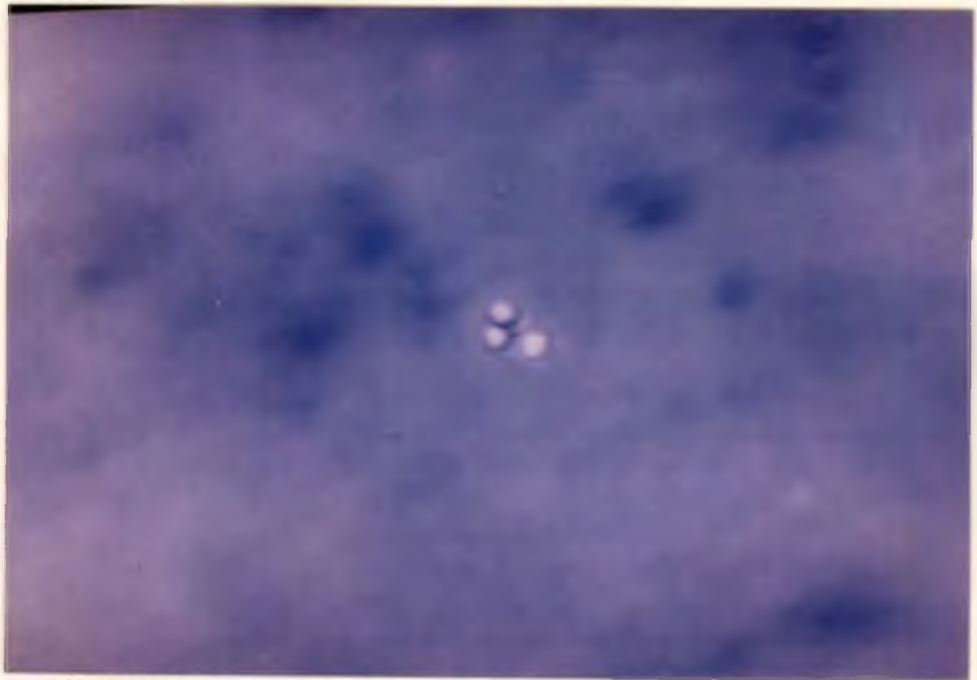

Plate.17

Plate 18. Clones developing from single cell after limiting dilution

Plate 19. Colony developing from single cell after limiting dilution

Plate-18

Plate-19

Plate 20. Colony developing from single cell after limiting dilution

Plate 21. Colony developing from single cell after limiting dilution

Plate-20

Plate-21

Plate 22. Testing of hybridoma supernatant by ELISA

A-1, Substrate control
B-1, HRP control
C-1, + Ve control
E-1,F-1, -Ve control
D-4,5 , F-5,6 and 9,10
+ Ve hybridoma

Plate 23. Testing of hybridoma supernatant by ELISA

A-1, Substrate control
B-1, HRP control
C-1, + Ve control
E-1, F-1, -Ve control
B-5, 6 , E-8,9
+ Ve hybridoma

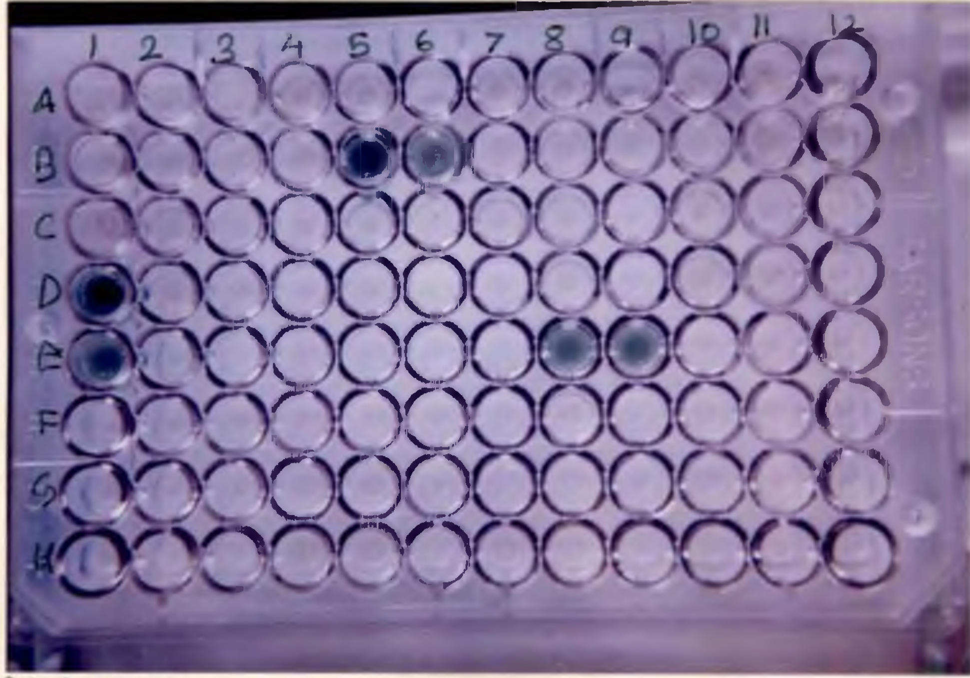

Plate - 23

Plate-22

4.12 Monoclonal antibodies

A total of seven Mabs producing clones were further propagated and Mabs were collected and stored for further testing.

4.13 Cross reactivity by ELISA

All the seven Mabs were tested for cross reactivity against homologous as well as heterologous strains using indirect ELISA. The results are presented in the Table 5.

Mab 1 reacted with homologous virus and the titre was observed to be 1:32. It also reacted with DPV-A and DPV-I with the same titre. No differences were observed and Mab reacted with all the three strains with same titre. The OD values observed up to 1:32 titre were double the negative and were considered as positive (Plate 24).

Plate 24. Cross reactivity of Mab against three strains of DPV

4 and5 DPV-V

7 and 8 DPV-I

10 and11 DPV-A

A to H 1:2 to 1:256

Plate 25. Cross reactivity of Mab against three strains of DPV

4 and5 DPV-V

7 and 8 DPV-I

10 and11 DPV-A

A to H 1:2 to 1:256

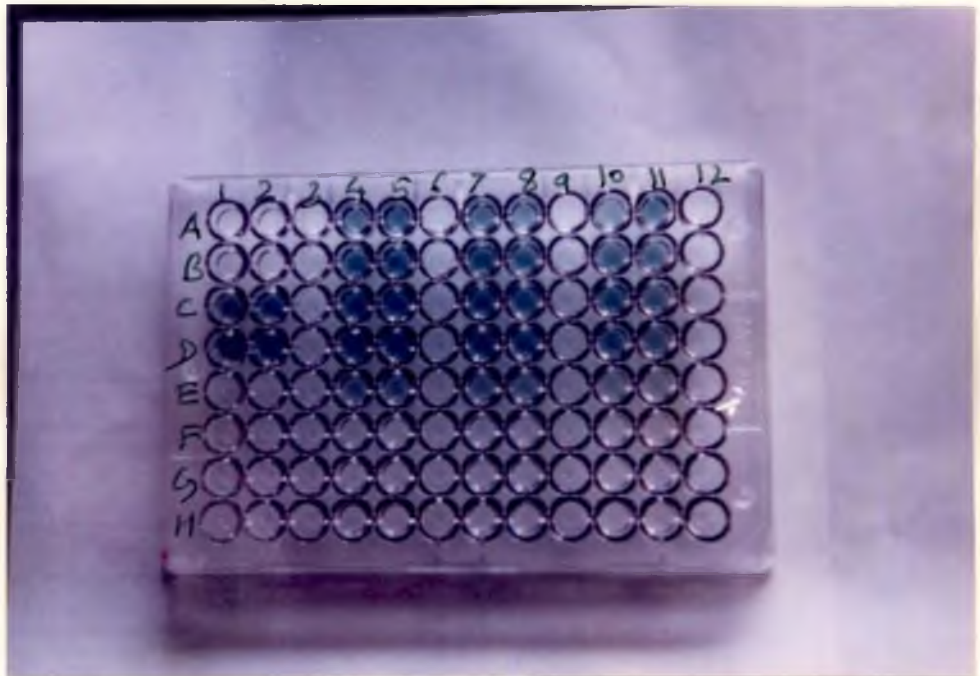

Plate-24

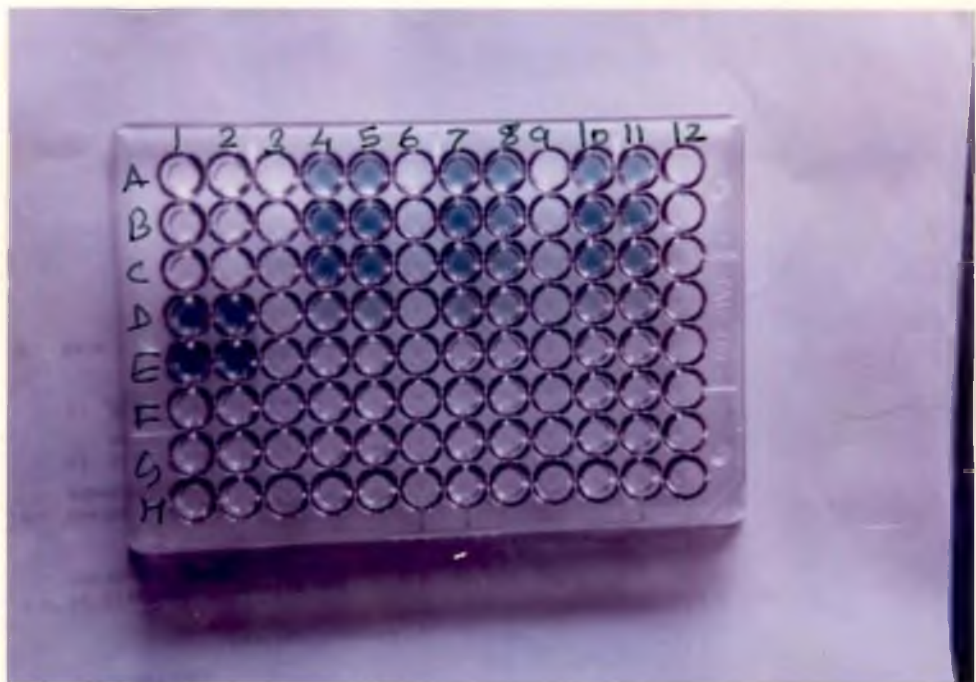

Plate-25

Table 5. Cross reactivity of Mabs against three strains of DPV by ELISA

Monoclonal antibody	ELISA titres		
	Vaccine	Alleppey	IVRI
Mab1	1:32	1:32	1:32
Mab2	1:8	1:8	1:8
Mab3	1:16	1:16	1:16
Mab4	1:16	1:16	1:16
Mab5	1:8	1:8	1:8
Mab6	1:16	1:16	1:16
Mab7	1:8	1:8	1:8

Mab 3 ,4 and 6 showed a titre of 1:16 with homologous and other two strains of DPV in indirect ELISA (Plate 25).

However MAb 2, 5 and 7 showed a titre of 1:8 with homologous as well as heterologous strain of DPV (Plate26).

4.14 Detection of the tissue antigens

The panel of seven Mabs was used to study the efficacy in detecting the tissue antigens by IPT. The test was performed on the coverslip cultures of DPV-V and on liver and spleen sections for DPV-A and DPV-I. All the seven Mabs detected the tissue antigens in the CEF cell cultures as well as liver and spleen tissue sections. The reaction was clear and dark brown spots were observed at the antigen deposit site. The background staining was negligible and thus the detection was easy and accurate. No differences were observed in tissue antigen detection with all the seven MAbs, However, when compared to polyclonal serum, background staining was reduced considerably (Table 6 and Plate 27 and 28).

Plate 26. Cross reactivity of Mab against three strains of DPV

4 and5 DPV-V

7 and 8 DPV-I

10 and11 DPV-A

A to H 1:2 to 1:256

Plate 27. Immuno peroxidase detection of DPV by Mab

Plate-26

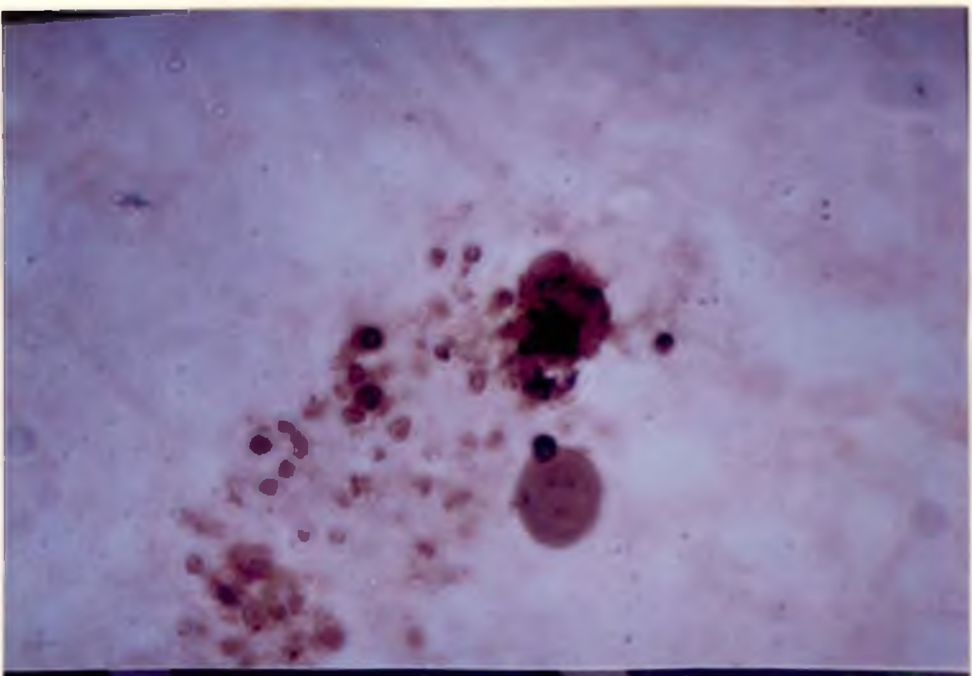

Plate-27

Plate 28. Immuno peroxidase detection of DPV by Mab

Plate 29. Longevity and of hybrids ELISA titres
3 and 4 24 h supernatant
5 and 6 48 h supernatant
7 and 8 72 h supernatant
9 and 10 96 h supernatant
11 and 12 120 h supernatant

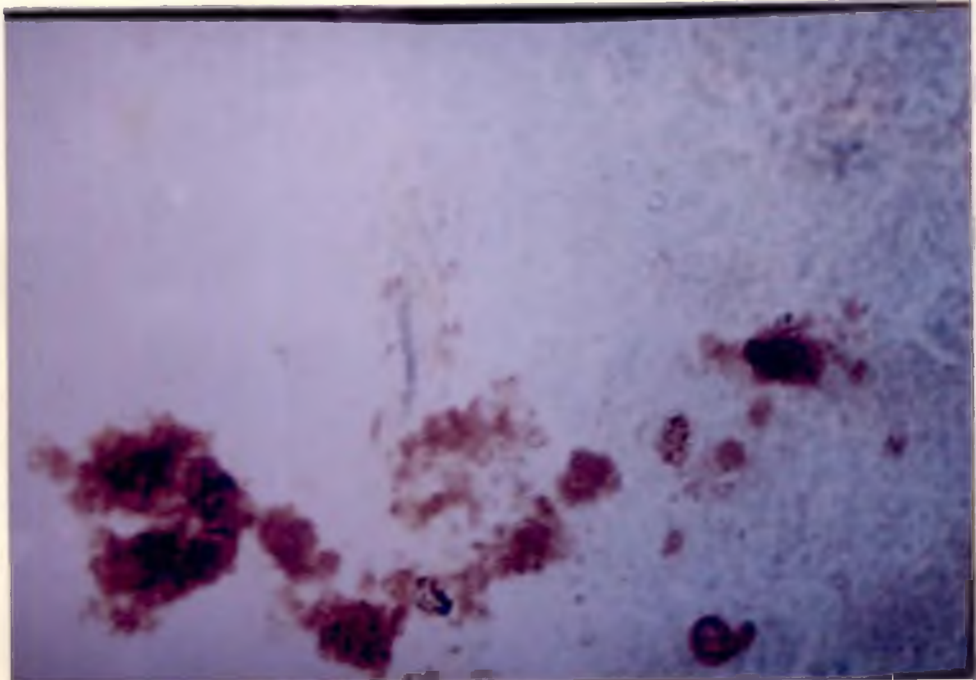

Plate-28

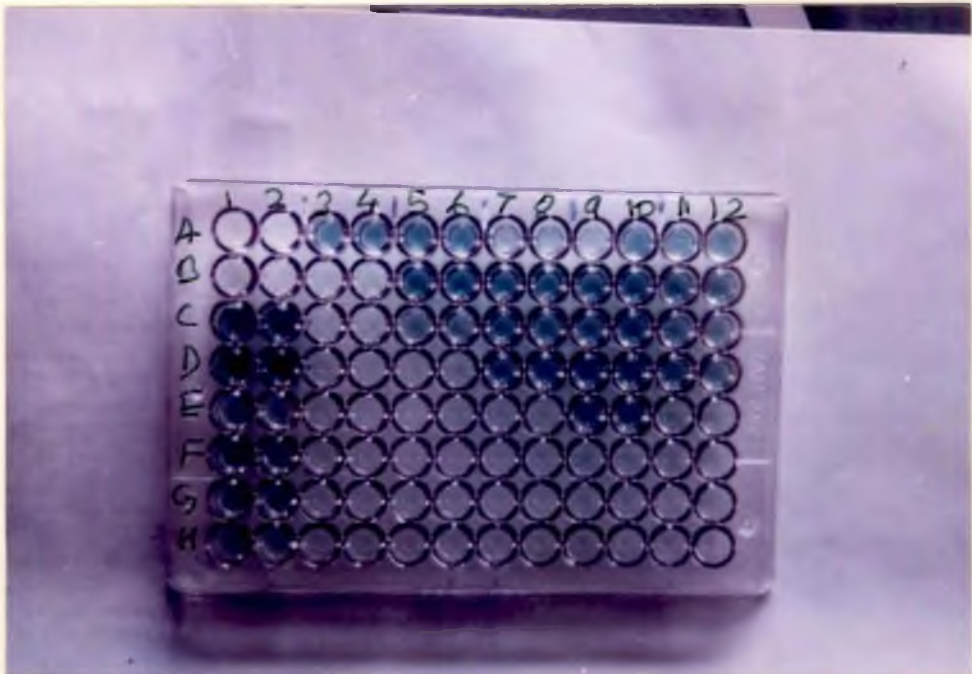

Plate-29

Table 6. Cross reactivity of Mabs against three strains of DPV by IPT

Monoclonal antibody	Vaccine	Alleppey	IVRI
Mab1	+Ve	+Ve	+Ve
Mab2	+Ve	+Ve	+Ve
Mab3	+Ve	+Ve	+Ve
Mab4	+Ve	+Ve	+Ve
Mab5	+Ve	+Ve	+Ve
Mab6	+Ve	+Ve	+Ve
Mab7	+Ve	+Ve	+Ve

4.15 Virus Neutralization

All the seven Mabs were tested for virus neutralization in CEF cell culture for DPV-V and DPV-A, however DEF was used for DPV-I. The results are presented in Table 7.

All the Mabs neutralized the homologous as well as heterologous virus with same specificity and intensity. Mab 5 and 7 however did not neutralize the virus at all and may be nonneutralizing.

Mab 2, 4 and 6 neutralized the homologous virus with a VNT of 6, the VNI for the same was 0.79. These also neutralized DPV-A and DPV-I with a VNT of 4 and VNI of 0.75. It was observed that the titers for all the three strains were not differing and were within same dilution.

Mab 1 showed a slight higher titre as compared to other but VNT with homologous as well as heterologous strains was similar.

Table 7. VN titres and VNI of Mabs with homologous and heterologous strains.

Polyclonal serum	Vaccine		Alleppey		IVRI	
	VNT	VNI	VNT	VNI	VNT	VNI
Mab 1	1:8	0.9	1:8	0.9	1:8	0.9
Mab 2	1:6	0.79	1:4	0.75	1:4	0.75
Mab 3	1:2	0.39	1:2	0.35	1:2	0.35
Mab 4	1:6	0.79	1:4	0.75	1:4	0.75
Mab 5	Non neutralizing					
Mab 6	1:6	0.79	1:4	0.75	1:4	0.75
Mab 7	Non neutralizing					

4.16 Cryo preservation

Myeloma cell line as well as positive hybrids were cryopreserved. The cells in log phase were harvested from the tissue culture bottle counted and frozen as per the standard procedure. After revival it was observed that, the viability of the cells was very low and most of the cells had cold shock which was evident from shrunken appearance of the cells . However after 2 to 3 passages the growth was satisfactory.

4.17 Survivability and longevity trial

The trial was conducted to ascertain the appropriate time of collection of the hybridoma supernatant. The cells after seeding into the wells were observed daily for a period of 120 h. The standard parameters like change in pH and 80 per cent coverage were also noted down. The supernatant was tested for antibody titre by ELISA. The results are presented in (Plate 29). The cell growth was constant up to 48 h and between 48 to 72 h fast growth with change in pH was observed. Simultaneous titers were also noted and were highest between 72 to 96 h. The cell coverage in the well was about 70 to 80 percent at 72 h Thereafter as the cell number and the coverage increased, sloughing of the layer as well as cell death was evident. It was maximum at 120 h and simultaneous titers were also lower as compared to 72 and 96 h.

DISCUSSION

DISCUSSION

Since 1976 Kerala is endemic for Duck plague and regular outbreaks of DP have been reported in vaccinated as well as unvaccinated flocks from the duck rearing areas of the state. One of the reasons for the DP in vaccinated flocks always attributed is the possible strain variation. Though antigenic variations have not been reported from the outbreaks in the state by conventional serological tests (Kulkarni, 1993) and even at molecular level (Sangeetha, 1996 and Taylor 1997) , a detailed probe in to the reasons for the breakdown of immunity is warranted.

Conventional serology employes use of the polyclonal serum as standard diagnostic reagent. Apart from having many advantages like convenience, easy availability, use in common tests like agglutination and precipitation , it serves as a poor reagent when minor differences in the antigen are required for molecular characterization.

Hence, the present investigation was conducted to produce the Monoclonal antibodies against the vaccine strain of DPV and compare it with the polyclonal serum raised against the three strains of DPV employed in the study viz.,Vaccine, Alleppy and IVRI. Further the cross reactivity of the

polyclonal serum in diagnosis of DP and detection of tissue antigens was compared in relation to the Mabs.

The strain variation in DPV , if any could also be investigated at epitope level with the help of Mabs.

5.1 Revival of the DPV strains

5.1.1 Vaccine strain

Vaccine strain of DPV was revived in 11 day old chicken embryo by inoculating via CAM route.The embryo death was recorded 4 to 5 days PI with hemorrhages all over the body, congestion of spleen and CAM and necrotic foci on Liver. Similar lesions were recorded by Jansen (1961), Mukerji *et al.* (1965) and Senthilkumar (1997).

5.1.2 Cytopathic effect in CEF cell culture

Chicken embryo fibroblast infected with vaccine strain of DPV revealed rounding and clumping of cells at 48 h PI. Kalaimathi and Janakiram (1990); Sangeetha (1996) and Senthiikumar (1997) also observed similar changes in CEF cell culture infected with DPV vaccine strain. Syncytium formation and vacuolation observed at 96 h PI is in agreement with the observations of Kalaimathi and Janakiram (1990); Panisup *et al.* (1990) and Senthilkumar (1997). Desqammation of the cells

and monolayer was noticed at 120 h PI (John 1988; Kaliamathi and Janakiram, 1990 and Senthilkumar, 1997).

Coverslip cultures of DPV infected CEF revealed eosinophlic intranuclear inclusion bodies and this is in agreement with the observations of Panisup *et al.* (1990) ; Sangeetha (1996) and Senthilkumar (1997). Intranuclear inclusion bodies were also demonstrated in the tissue sections from the infected birds stained with hematoxylin and eosin under light microscope. (Leibovitz, 1971 and Rajan, *et al.*, 1980). However Breese and Dardiri (1968); Bergman and Kinder (1982) and Barr *et al.* (1992) observed intranuclear and intracytoplasmic inclusion bodies under electron microscope.

5.1.3 Alleppy strain

Alleppy strain of DPV (DPV-A) adapted to grow in CEF cell culture was further passaged in the same system. The CPE observed in case of DPV-A was similar to that of DPV-V in the same cell culture. The time required for initiation of CPE and detachment of the monolayer was also similar.The time required for initiation of CPE was on par with DPV-V and suggested complete adaptation of the DPV-A to CEF cell culture. In the present case DPV-A was serially passaged in CEF and the same was used which may be the reason for similar observation. In adaptation of DPV to CEF cell culture, Kaliamathi and Janakiram (1990); Bordolai *et al.*(1994)

and Senthilkumar (1997) observed a reduction in time taken for the appearance of CPE. Our findings correlate with the above.

Taylor (1997) also observed a decrease in time taken for appearance of the CPE in DEF infected with DPV-A and DPV-I. After complete adaptation the time required was constant between 36 to 48 h PI. This report confirms our finding regarding adaptation and CPE in DPV-A strain .

5.1.4 IVRI strain (DPV-I)

5.1.4.1 In ducklings

The lesions observed in ducklings experimentally infected with DPV-I were congestion and hemorrhages on serous membranes, heart and other visceral organs. The death was recorded in all the ailing birds within 5 to 7 days PI. Similar findings were observed by Jansen (1961,1964,1968); Leibovitz (1971); Duraiswamy *et al.* (1979) and Taylor (1997).

5.1.4.2 In embryonated eggs

DPV-I produced similar lesions in duck embryo (DDE) as those seen in DCE when DPV-V was cultivated. However the severity of the lesions was very high and marked with severe congestion all over the body and CAM. Jansen (1961) and Taylor (1997) also observed similar lesions in DDE infected with virulent virus. The probable explanation for the severity

of the lesions in DDE with a virulent strain may be that the system serves as the natural host for the virus. Increased severity of the lesions and reduction in the time taken for the production of embryopathy is also in accordance with the findings of Bhowmik and Chakraborty (1985) and Kulkarni (1993).

5.1.4.3 In cell culture

The cell culture system used for the revival of DPV-I was DEF. The cytopathy produced by DPV-I in DEF was similar to one that was seen in CEF infected with DPV-V and DPV-A. No differences were observed in the time taken for appearance of CPE and sloughing of the cells and may be because of the adaptation of the virus to the cell culture system.Similar pattern of CPE was observed by Kunst (1967); Dardiri (1969); Kenwolf and Quimby (1976) , Nair (1978); Panisup *et al.* (1990); and Taylor (1997).

5.2 Titration of DPV strains

The tissue culture infective dose of all the three strains under study was evaluated in CEF cell culture for DPV-V and DPV-A and in DEF for DPV-I.

The DPV-V had a $TCID_{50}$ of $4.7X10^{5}$ per ml of the inoculum. Kalaimathi and Janakiram (1990) also observed a titre of $10^{4.8}$ in first passage which increased up to $10^{6.8}$ in 12 th passage. Taylor (1997) and

Sangeetha (1996) reported a titre of 10^{5} for DPV -V in CEF cell culture. Senthilkumar (1997) also observed a titre of $10^{5.67}$ in 5 th passage virus and $10^{6.77}$ in 10 th passage virus cultivated and titrated in CEF cell culture. Findings in the present study are in agreement with the above. The titre observed in the present study may also be because of the fact that the supernatant over passages was pooled.

DPV-A had a titre of $TCID_{50}$ of $3.2X10^{4}$ per ml of the inoculum which is also in agreement with Taylor (1997). He had used DEF for cultivation of the DPV-A and for subsequent titration and recorded a titre of $10^{5.75}$. The system used for cultivation is different which may be the reason of slightly lower titres in the present study.

$TCID_{50}$ for IVRI strain of DPV in present study was 10^{5}. Taylor (1997) also observed similar titres and recorded a $TCID_{50}$ of $10^{5.75}$.The results are in complete agreement with the report and may be because of the similar system and conditions used in these studies.

5.3 Concentration and purification of DPV

All the three strains used in the present study were partially purified by centrifuging the culture supernatant at 100000g for 4.5 h in ultra centifuge. Metzler *et al.* (1985) partially purified the cattle herpes virus (BHV-1)and used it for inoculation into mice for priming the mice for Mab production. The procedure adapted for partial purification in the present study has resulted in good recovery eliminating nonspecific contaminants in the preparation and was successfully used for raising polyclonal serum as well as priming the mice for Mab production.

Reports on purification of DPV are very scanty, Rao *et al.* (1992) purified live attenuated chick embryo adapted DPV by treating amnioallantoic fluid with Protamine sulphate and PEG. The authors opined that thus purified virus could be used for virological studies and vaccine production.

Gardner *et al.*(1993) also purified DPV by layering the cell lysate supernatant onto 12 to 62 per cent W/V linear sucrose gradient and centrifuged at 40000 rpm for 4 h at 4° C.

The procedure adapted in the present study was similar to that above except for the gradient centrifugation. However the results in the present

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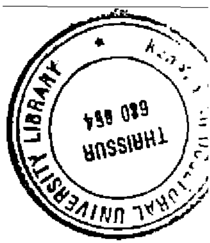

study of antibody tires by ELISA and SNT confirm that this procedure can be used particularly in situations where complete purification cannot be achieved. For virological and molecular studies partial purification seems to be appropriate.

5.4 Antibody response and cross reactivity

5.4.1 Enzyme Linked Immuno Sorbent Assay (ELISA)

ELISA is the test of choice for detection of antibodies particularly in the mice primed for Mab production. This test was used in the present study for evluating the antibody titres in primed mice as well as to ascetain the titres in mice used for raising the polyclonal serum. There is an isolated report on use of plate ELISA in the diagnosis of DPV.

The mice inoculated with the three strains of DPV *Viz*. DPV-V, A and I showed an antibody titre of 1:12800, 1:12800 and1:6400 repectively. As the mice were given fortnightly inoculations with adjvant a strong immune response was elicited. The higher titres can also be attributed to partially purified virus antigens used for ELISA. Bolton *et al*. (1983) and York *et al*. (1983) also reported similar finding with IBR and ILT viruses respectively.

Cross reactivity of the polyclonal serum was tested with plate ELISA. Two rows of the ELISA plate were coated with antigen (DPV-V). Similarly other antigens in the study were also used for coating two rows each in the same plate. Polyclonal serum raised against one strain was used in a single plate which was coated with the different antigens. The polyclonal serum raised against DPV-V reacted with same specificity with all the three strains of DPV. The titres recorded with DPV-V polyclonal serum were 1:12800 1:12800 and 1:6400 for homologous , DPV-A and DPV-I respectively.

ELISA titres recorded in the present study with homologous strain using polyclonal serum and with heterologous strain were very high. York *et al.*(1983) reported that ELISA titres were usually 16 to 32 fold higher than those obtained by SNT. Malmarugan (1997) also observed 20 to 40 fold higher ELISA titres compared to PHA. It was futher reported that ELISA was able to detect DPV antibodies in low titred sera. The author further recommended the test for diagnosis of DPV in inapparent and atypical conditions and to evaluate vaccinal immunity. In the present study as with the polyclonal sera we have the observed the test to be highly sensitive. The titres recorded in the present study are also similar to the reference.

5.4.2 Immunoperoxidase test

IPT was used in the present study for the detection of viral antigens in CEF cell cultures and in liver and spleen tissue from the birds experimentally infected with Alleppy strain and IVRI strain of DPV.

The test has been used for the rapid diagnosis of the viral antigens in infected tissue and in cell cultures. It has many advantages over immuno fluorescence and immuno electron microscopy (Malmarugan 1997).

Polyclonal sera raised against the three strains of DPV i.e. DPV-V, A and I were used to study the cross reactivity in detecting the viral antigens. DPV-V polyclonal serum detected homologous as well as heterologous viruses in CEF cell cultures and in liver and spleen sections from the birds died of experimental infection with DPV-A and DPV-I.

Similarly other two polyclonal sera raised against DPV-A and DPV-I also detected the homologous as well as heterologous strains.

Islam *et al.* (1993) used IPT for the detection of tissue antigens in liver and spleen . They observed positive staining reaction as dark brown deposits under light microscope. Islam and Khan (1995) reported such deposits in nucleus as well as in the cytoplasm of the cell. In the present investigation similar deposits were observed in the stained preparations. Our findings are in agreement with the above reports.

While reporting IPT with polyclonal serum in DPV Malmarugan (1997) stated that the background tissue was also stained dark brown and non specific reactions could be noticed under the light microscope. This dark brown staining of the tissue may be because of the use of polyclonal serum. Similar dark brown staining was noticed in our staining reaction.

Though the positive reaction could be detected nonspecific reaction and dark staining complicated the diagnosis.

Guy *et al.* (1992) also observed dark staining reaction of the tissue and attributed it to the use of polyclonal serum. It was further opined that use of a Mab will not only increase the specificity but also reduce the back ground staining.

It can be emphasized that, the IPT with its many advantages over similar tests can be recommended for the diagnosis of DPV. The test can be refined to enhance its sensitivity and specificity by appropriate reagents .

5.4.3 Virus Neutralization test

Polyclonal serum raised against the three strains of DPV was employed for testing the cross reactivity of the viruses under study.

The polyclonal serum raised against DPV-V showed VN titre of 64 and VNI 1.8 , however it showed a virus neutralization titre of 48 with heterologous strains and VNI of 1.65.

Toth *et al.* (1971) observed an average VNI of 1.7 in ducks only after second vaccination. Senthilkumar (1997) also observed a VNT of 64 and a VNI of 1.8 in birds vaccinated with tissue culture adapted 5th passage virus. In the present study VNT and VNI observed are in full agreement with this. However Kulkarni (1993) observed VNI of 0.79 in ducks vaccinated with commercial vaccine and 1.19 in lab adapted vaccine.. Higher VN titres and VNI observed in the present study can be attributed to the multiple injections of the antigen and to the tissue culture adapted strain of DPV.

Bordolai *et al.* (1994) reported that ducklings vaccinated with chicken adapted vaccine had a VNI of 1.22 whereas cell culture adapted vaccine had a VNI of 1.78 six weeks post vaccination. A VN antibody titre of 40 and 80 was observed in ducks vaccinated with 5th and 12th passage virus by Kaliamathi and Janakiram (1990). Findings in the present study were similer with the references quoted.

Polyclonal serum raised against Alleppy strain of DPV had neutralized homologous virus with a titre of 32 and VNI 1.5. It also cross

reacted with hetrologous strain of DPV with a titre of 32 and 24 and VNI 1.5 and 1.35 for DPV-A and DPV-I respectively.

Bordolai *et al.* (1994) used a virulent isolate of DPV and passaged serially in duck embryo, chick embryo and CEF cell culture. It was reported that the vaccination with this virus produced a VNI ranging between non detectable to 1.31 in 3rd week and it increased up to 1.78 in 6 th week. The VNI observed in the present study are almost similar with those reported.

SenthilKumar (1997) observed slightly higher titre (64) in ducklings vaccinated with 5th CEF passaged vaccine virus. He further reported that as the passages increased there was reduction in VNT and VNI and in 10 th passage vaccinated birds VNT was 54 with a VNI of 1.73. Lower titre observed in the present study with DPV-A may be because of more number of passages the virus had undergone before adaption to CEF cell culture system.

Polyclonal serum raised against IVRI strain of DPV reacted with homologous virus and had VN titre of 48 with a VNI of 1.65. It also cross reacted with other two strains and had VN titre of 32 and VNI 1.5 for both heterologous strains.

In the present study, it has been clearly observed that the polyclonal serum raised against the three strains DPV neutralised the homologous virus as well as the other two strains of the virus with almost same titre in the same log dilution. The VNI observed was also similar in cross reactivity test with heterologous virus.

Mukerji *et al.* (1965) used SNT and cross immunity test and reported that the Dutch and Indian strains of DPV were identical in all aspects. .

Dardiri and Hess (1968) studied serological relationship between the two viruses using Plaque inhibition test. Hyper immune serum raised against the virus and from the recovered birds was used in the neutralisation test .It was reported that the two viruses were antigenically homologous.

The findings in the present study substantiate the above reports that the three strains of DPV i.e. DPV-V, DPV-A and DPV-I employed in the study are serologically and antigenically related as is evident from VNT. It can be further concluded that the three strains may be biotypes of DPV as suggested by Lin *et al.* (1984a).

The cross reactivity trials conducted with polyclonal serum employing ELISA and IPT further confirm our finding about the antigenic similarity of the strains.

5.5 Monoclonal antibody production

5.5.1 Procedure

There is vast variation in the procedure adapted for the Mab production. Various workers have developed different procedures depending upon the virus involved.

5.5.2 Immunization of mice

In the present study female BALB /c mice were immunised with DPV vaccine stain. The immunisation was done with a schedule of four fortnightly inoculations by i.p. route and a final i.v.inoculation four days prior to fusion . Same schedule was used for raising polyclonal serum. The titres were ascertained by ELISA.

Similar procedure has been reported by Anonymous (1992).a. However, the procedure of Mab production in herpes viruses differ considerably. Ikuta *et al.* (1983a); Lee *et al.* (1983a); Lee *et al.* (1983b); and Nakajima *et al.* (1989) used totally different protocol for mice inoculation, route of inoculation and the inoculum.

It may be stressed that, the procedure of fortnightly inoculation and an i.v. inoculation prior to fusion works well as is evident from our studies. Therefore, the procedure can be recommended for Mab production in future.

5.5.3 Fusion

Fusion of Myeloma cells and the splenocytes from the donor mice was undertaken in the presence of PEG 4000. The procedure of fusion, dilution of the PEG and seeding of the fused cells was done as per the procedure suggested by Anonymous (1992). The procedure works well and is evident from the results obtained in our studies.

The procedure of fusion of myeloma cells and splenocytes from the donor mice, addition of the PEG , Molecular weight of PEG used, dilution of PEG and further seeding of the fused hybrids differ from laboratories to laboratories. We have used a procedure reported by Galfre and Milstein (1981), Zola and Brooks (1989) and Anonymous (1992) and from the fusions undertaken in the present study it can be stated that, the procedure is perfect and works well.

However, Goding (1980) reported that the precise details of the fusion protocol vary greatly between laboratories and different protocols have been

adapted by Lee *et al.* (1983a); Lee *et al.* (1983b); Chang *et al.* (1986) and Nakajima *et al.* (1989). It was further reported that precise ratio of spleen cells to myeloma cells used was also differed between laboratories.

The procedure adapted for maintenance of hybrids, change of the medium and total changes in HAT and HT medium was similar to that reported by Anonymous (1992a). However, Zola and Brooks (1989) suggested changing the medium after seven days of seeding of the hybrids in the plates and further changes according to the change in the pH of the medium. It has been observed that, the pH in the wells containing the fused hybrids changes very fast and a medium change in every 3 to 4 days becomes inevitable. Hence, the results in the present study indicate that procedure adapted is appropriate and should be used for fusion work.

5.6 Monoclonal Antibodies

A total of seven Mabs produced by seven different clones were used in the present study. These Mabs were tested for cross reactivity by utilizing ELISA, IPT and VNT.

5.7 Cross reactivity by Mab

5.7.1 ELISA

ELISA was performed on the same lines as that used for polyclonal serum except, that the polyclonal serum was replaced by Mab in the test.

Out of the 7 Mabs tested , Mab 1 reacted with homologous virus and a titre of 1: 32 was recorded. it also reacted with the hetrologous strain with the same titre.

Mab 3,4 and 6 had a titre of 1:16 with the homologous virus as well as with the hetrologous virus strains of DPV. and Mab 2, 5 and 7 showed a titre of 1:8 with all the three strains of DPV.

Chang *et al.* (1986) also used ELISA to screen the hybridoma supernatant and used these high titred Mab to study the virus neutralization. Reactivity of Mabs was studied with the glycoproteins of IBR virus and it was observed that the Mabs reacted with all the GP tested.

Layaku *et al.* (1992) also reported similar finding that the Mab produced against Cervine herpes virus reacted with other 4 alpha herpes viruses from ruminants and a close antigenic relationship of all the viruses was established. We have also observed cross reactivity in the three stains of DPV which suggest a close antigenic relationship.

Nakajima *et al.* (1989) also observed similar findings with VVMDV. The cross reactivity observed in the present study can be correlated with the report.

However, Lee *et al.* (1983 a and b) were also able to differentiate the stains of MDV and HVT with the help of Mab.

Moer *et al.* (1993) could also differentiate EHV-1 and EHV -4 sera samples with a type specific Mab and observed that the Mab reacted with all the strains of EHV-1 but not with the EHV-4. As regards the reactivity of Mab with EHV-1 strains, our findings can be correlated.

Thus the seven Mabs tested in the present study could not detect the differences in the three strains of DPV.

5.7.2 Immuno peroxidase test

Detection of the viral antigens in the CEF cell cultures and in the liver and spleen tissue from the birds died of the experimental infection with Alleppy and IVRI strain was done by employing Mab based IPT. The procedure of the test was same as the one used for polyclonal IPT staining except the polyclonal serum was replaced by Mab.

All the seven Mabs under study detected homologous virus in the CEF cell culture infected with vaccine strain of DPV. These also detected heterologous virus in the liver and spleen tissue sections. The staining reaction was very clear and the background staining was almost negligible. The viral deposits could be detected as red brown deposits in the cytoplasm as well as in the nucleus of the cell. The results were confirmed by negative controls which did not showed any deposits.

Moer *et al.* (1993) used Mab based IPT for the detection of viral antigens in EHV-1 and reported that all the strains of EHV-1 could be detected in IPT staining with Mab.

Winkler *et al.*(1995) also reported similar observation about the red brown staining reaction in the tissue sections The findings in the present study are in complete agreement with the above reports.

Guy *et al.* (1992) observed that IPT staining with hyper immune serum resulted in nonspecific background staining and false positive reaction. A high sensitivity and high quality reaction without background staining was observed in Mab based IPT and thus the detection of ILT antigens was easy and accurate.

Malmarugan (1997) also reported dark brown staining of the background tissue in IPT with polyclonal serum in DPV and opined that Mab may refine the detection.

In the present investigation we have observed that Mab based IPT staining reduced the background staining and thus false positive detection of the antigen, thereby increasing the accuracy in the diagnosis.

Thus it can be concluded that in situations where the detection of the viral antigens is complicated by non specific reaction, Mab can be the tool to refine the test and increase the accuracy.

5.7.3 Virus neutralization

VNT was performed by using 7 Mab on the same lines as that of polyclonal serum. The results obtained in the present study indicated that all the Mab reacted with homologous as well as hetrologous strains in VNT.

Mab 1 had neutralized the homologous virus and showed a VNT 1:8 and a VNI of 0.9. The Mab also reacted with heterologous strains with similar titre and VNI.

Mab 2, 4 and 6 had a VNT of 1:6 with homologous virus with a VNI of 0.79. It also neutralized the heterologous virus with a VNT of 1:4 and VNI of 0.75.

Mab 3 had a VNT of 1:2.4 with homologous virus with a VNI of 0.39. However, it showed a VNT 1:2 and VNI 0.30 with other two heterologous strains. Mab 5 and 7 did not neutralize the virus and may be non neutralizing.

Lee *et al.* (1983a) also reported low VNT with 11 Mab tested. The hybridoma supernatant tested had a VNT of less than 2. Ten Mabs specific for MDV reacted with homologous virus with a very low titre. However one Mab specific for HVT showed highest VNT of 10. Our findings regarding the VNT are similar to those reported.

Abdel Magid *et al* .(1992) also reported that out of four Mabs produced against BHV-1 , one Mab was nonneutralizing and three could neutralize the virus

Karaku and Nagio (1993) used Mab based VNT to detect specific antibodies to IBV and reported that Mab produced during secondary response reacted more strongly with homologous virus but also exhibited some level of cross reactivity with heterologous antigen.

The procedure adapted was deviating from the one suggested by Anonymous (1992). However, though there was recovery of cells after thawing , the percentage of non viable cells was very high and because of this there was a chance of losing of the cells as well as the hybrids. It can be therefore suggested that the procedure needs a through review and then only should be adapted in future.

5.9 Survivability and longevity trial

The trials conducted for studying survivability and longevity in the present experiment suggest that the parameters traditionally used for collection of the supernatant for maximum antibody production like change in pH and 80 per cent coverage of the well are sufficient for knowing the time of collection of the hybridoma supernatant. However, if the titres in the hybridoma supernatant are required then such a trial may be of use.

For routine collection of the hybridoma supernatant from the clones it is suggested that the traditional parameters are sufficient to fulfill the need.

5.10 Conclusions

The present investigation was aimed at producing Mab against Vaccine strain of DPV and compare the efficacy of these Mab as with the polyclonal serum in diagnosis of DPV and detection of viral antigens.

The procedure adapted was deviating from the one suggested by Anonymous (1992). However, though there was recovery of cells after thawing , the percentage of non viable cells was very high and because of this there was a chance of losing of the cells as well as the hybrids. It can be therefore suggested that the procedure needs a through review and then only should be adapted in future.

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5.10 Conclusions

The present investigation was aimed at producing Mab against Vaccine strain of DPV and compare the efficacy of these Mab as with the polyclonal serum in diagnosis of DPV and detection of viral antigens.

It was also envisaged to evaluate the strain variation if any, in the different isolates of DPV available. The present study has pinpointed the following.

In comparison with polyclonal serum,

The Mabs are highly specific in the detection of the viral antigens in tissue sections and in CEF cell cultures infected with virus.

Mabs have considerably reduced the background staining thereby refining the detection and ultimately the diagnosis.

As regards the ELISA test for the diagnosis of the DPV , it is highly sensitive, easy to perform, accurate and less time consuming with polyclonal serum as well as with Mab.As it detects low titred serum also, it should be recommended as routine diagnostic test for DPV.Moreover no marked difference was observed in present investigation with the use of Mab.

VNT is highly sensitive and specific and its specificity can be enhanced with the Mab. However the hybridoma supernatant always shows very low titres and thus may be used to study the VN with neat supernatant.

5.11 Strain variation

Polyclonal serum conventionally used for detection of strain variation usually fails to detect the minor differences in the antigen. On the other hand Mabs detect differences in closely related antigens .

It has been observed from the present study that polyclonal serum reacted with all the three strains of DPV with almost same specificity and intensity, thereby making it difficult to differentiate the viruses.

Of the panel of seven Mabs tested, all reacted with the three strains of DPV suggesting no possible strain variation in DPV isolates.

The question of vaccination failure in the field therefore may not be due to strain variation.

The cross reactivity tests with ELISA, SNT and IPT has proved it beyond doubt.

It can be suggested that, the strains might have increased its virulence by sub clinical passage in the ducks which might have resulted in the breakdown of immunity and vaccination failure, thereby causing outbreaks in the field.

As reported, a potent tissue culture adapted vaccine against DPV in place of embryo adapted vaccine may prove effective.

SUMMARY

SUMMARY

Three strains of DPV Viz., Vaccine (DPV-V), IVRI (DPV-I) and a local isolate from Alleppy district of Kerala state, Alleppy strain (DPV-A) were used in the present study.

DPV-V and DPV-A were revived and produced in bulk in CEF cell culture. The cytopathy in CEF cell culture observed was rounding and clumping of the cells , Syncytium formation and bridge formation with extensive vacuolation in the cytoplasm. Intranuclear inclusion bodies were noticed and the detachment of the cells was observed at 120 h PI.

The virulent strain of DPV received from IVRI, Izatnagar U.P. i.e. DPV-I was inoculated in the ducklings. The ducklings showed the symptoms of duck plague. All the inoculated birds died with extensive hemorrhage on serous membranes, muscles and visceral organs. Necrotic foci on liver, enlargement and congestion of liver ,spleen white necrotic foci in the gizzard were evident. The virus was further passeged in DDE and embryo death was recorded 4 to 5 days PI. The lesions observed in the dead embryo were similar those observed in DCE during cultivation of DPV-V.

The virus was further grown in bulk in DEF cell culture showed similar CPE in CEF cell culture.

The DPV-V and DPV-A were titrated in CEF cell culture to ascertain the tissue culture infective dose $TCID_{50}$. The $TCID_{50}$ was calculated and it was $4.7 X 10^5$ per ml of the inoculum for DPV-V and $3.2 X 10^4$ for DPV-A.

DPV-I cultivated in DEF cell culture and pooled over passages was titrated in the same system. The $TCID_{50}$ of the virus sample was 10^5 per ml of the inoculum.

The virus produced in the bulk was partially purified at 40,000 rpm for 4.5 h at 4 ° C in Beckman ultra centrifuge. The small pellet received after centrifugation was dissolved in small amount of TNE buffer and was preserved at -20 ° C for further use.

The protein concentration of the partially purified virus was estimated by biurate method and was found to be 11 mg, 8 mg and 7 mg for DPV-V, A and I respectively.

Polyclonal serum against all the three stains of DPV was raised in mice. Four mice out of 5 inoculated with DPV-V showed ELISA titres more than 1:12800, one mice showed a titre of 1:6400. The mice inoculated with DPV-A showed a titre of more than 1:12800 and those inoculated with DPV-I, 1:6400 ELISA was used to test the sera samples of the mice inoculated with DPV strains. The test was found to be highly sensitive, easy to perform and less time consuming. The test therefore can be recommended for routine diagnosis of DPV.

Cross reactivity of the three strains of DPV was studied by using the polyclonal serum. DPV-V polyclonal serum reacted with the homologous virus with a titre of 1:12800 . It also showed similar titer with other two heterologous strains i.e. DPV-A and DPV-I. Polyclonal serum raised against DPV-A had a titre of 1:12800 with homologous and heterologous strains of DPV and DPV-I reacted with homologous strain at a titre of 1:6400. Similar titres were observed with hertologous strains.

Immuno peroxidase test was used for the detection of the tissue antigens in DPV infected CEF monolayers and in Liver and spleen sections. Polyclonal serum raised against DPV-V detected homologous virus in the CEF monolayers and in the liver and spleen sections of the birds died of experimental infection with virulent Alleppy and IVRI strains of virus. The staining

reaction was observed as dark brown deposits at the virus localization, however a brown to faint yellow background tissue reaction was also visible with false positive deposits in the stained preparation.

The virus neutralization test was used to study the cross neutralization by employing polyclonal serum raised against the three strains of DPV. Polyclonal serum raised against DPV-V showed a VNT of 64 with a VNI of 1.8 with homologous virus and a VNT 48 with VNI 1.65 with other two strains of DPV.

Polyclonal serum raised against DPV-A neutralized the homologous virus at a titre of 32 with a VNI of 1.5. It also neutralized the vaccine strain of DPV with same VNT and VNI. However neutralized DPV-I with VNT 24 and VNI 1.35.

DPV-I polyclonal serum neutralized the homologous virus at a titre of 45 with a VNI of 1.65 . The same neutralized the the two strains i.e. DPV-V and DPV-A with a VNT 32 and VNI 1.5.

Monoclonal Antibody Production

The mice primed with DPV vaccine strain showed an ELISA titre of more than 1:12800. The mice showing high titres in ELISA were only used in the fusion experiment.

Viable cell count for myeloma cells , feeder cells and for the splenocytes on the day of fusion was ascertained by dye exclusion method..The cell viability observed in all the fusions was above 90 per cent.

Fusion of the myeloma cells and the splenocytes from the donor mice primed with the vaccine strain of DPV was under taken in the presence of poly ethylene glycol (PEG 4000). Fused clumps of the cells were observed on the fusion day in the wells.

The plates were monitored every day for the development of the hybrid colonies. The hybrid supernatant was tested for desired antibodies with the help of indirect ELISA.The positive hybrids were selected based upon the color reaction in the test.

The positive clones were then shifted to 24 well cluster plate and supernatant collected and preserved for further testing at -20 o C.

Cloning of the positive hybrids was done by limiting dilution method to ascertain monoclonality. The cells from the 24 well cluster plate were diluted to achieve 1, 5, 10 , 100 and 1000 cells per ml and seeded in the plates. Four to eight wells in the plate having 1 cell showed the development of small colonies which was monitored and the supernatant was tested for the antibody production. The clones showing the desired antibodies were only further developed and grown.

A total of 7 hybrids producing antibodies against DPV vaccine strain were obtained from the fusions undertaken. These Mabs were then used for testing cross reactivity of the three strains of DPV .

Out of 7 Mabs tested Mab 1 reacted with homologous virus with a titre of 32. it also reacted with other two hetrologous strains with the similar titre.

Mab 3, 4 and 6 had a titre of16with homologous as well as hetrologous virus strains of DPV and Mab 2, 5 and 7 showed a titre of 8 with all the strains of DPV.No differences were recorded in the ELISA titres of the 7 Mabs with homologous as well as hetrologous strains of DPV.This suggested that there

may be a close antigenic and serologic relationship within the three strains of DPV under study.

Detection of the viral antigens in the DPV infected CEF and in the liver and spleen tissue sections from the birds infected with virulent Alleppy and IVRI was attempted with the help of Immuno peroxidase test.

All the seven Mabs under study detected homologous as well as hetrologous virus in the infected CEF and in the liver and spleen sections. The reaction was very clear and background staining was almost negligible. The viral antigen deposits could be detected as dark red brown deposits in the cytoplasm and nucleus of the cells. The results were confirmed by negative controls where in no such deposits were observed.

Virus neutralization test was performed on the same lines as that of polyclonal serum. the results in the present study indicated that, Mab 1 neutralized the homologous virus and had a VNT of 8 with VNI 0.9. It also neutralized the hetrologous strains with similar VNT and VNI.

Mab 2 , 4 and 6 had a VNT of 6 with homologous virus and a VNI 0.79. These neutralized the other two virus strains with a VNT 4 and VNI 0.75.

Mab 3 had a VNT of 2.0 with VNI 0.39 with homologous virus and a VNT of 2 with VNI of 0.35 with the heterologous strains. However the Mab 5 and 7 did not neutralize the homologous as well as heterologous viruses and may be non neutralizing.

The survivability and longevity trial was conducted. It was observed that the titres increased up to 96 h of seeding of the cells. The pH of the medium changes very fast after 72 h and the cell growth also reaches maximum at that stage. II can be therefore suggested that the criterion designated for the collection of the hybridoma supernatant like change of pH and 80 per cent coverage of the well seems to be appropriate for deciding the medium change and collection of the supernatant.

The conclusions from the present study are, in comparison with the polyclonal serum Mabs are highly specific in detection of the viral antigens in the tissue sections infected with the virus. The background staining reaction was completely reduced there by refining the test and making the diagnosis very easy. The test can be recommended for the diagnosis of DPV.

As regards the ELISA test it has been observed that the test is highly sensitive , easy to perform and less time consuming. The test should be recommended for routine diagnosis of DPV.

VNT is highly specific with polyclonal sera as well as Mab . The specificity of the test is increased fairly by using Mabs.

As regards the possible strain variation of the DPV strains under study it can be concluded that the , results obtained in the present study indicated no strain variation in thestrains tested. The probable reason for the break down of immunity and vaccination failure may be because of low titered vaccine used in the field for vaccination of the birds or the virus may be acquiring virulence by sub clinical passage in the ducks in the field which might result in the breakdown of immunity and failure of the vaccine.

A potent tissue culture adapted vaccine may be useful in solving the problem.

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PRODUCTION AND APPLICATION OF MONOCLONAL ANTIBODIES AGAINST DUCK PLAGUE VIRUS

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ABSTRACT OF A THESIS

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ABSTRACT

Monoclonal antibodies (Mabs) were raised against the vaccine strain of DPV and three strains of DPV *Viz,* Vaccine (DPV-V), IVRI (DPV-I) and Alleppy strain (DPV-A) were used to raise polyclonal serum in the present investigation.

DPV-V was revived in 11 day old chicken embryo and the embryo death was recorded four to five days PI with congestion all over the body and spleen and necrotic foci in liver. The cytopathy in CEF cell culture observed was rounding and clumping of the cells, syncytium formation and bridge formation with extensive vacuolation in the Cytoplasm. The detachment of the cells was observed at 120 h PI.

DPV-I a virulent strain was inoculated in the ducklings, death was recorded in all the inoculated birds with extensive hemorrhages on serous membranes, muscles and visceral organs. Necrotic foci on liver,' enlargement and congestion of liver and spleen, and white necrotic foci in the gizzard were evident. The virus was further passaged in DDE and cultivated in bulk in DEF cell culture.

The DPV-V and DPV-A were titrated in CEF cell culture and the $TCID_{50}$ was 4.7 X 10^5 per ml of the inoculum for DPV-V and 3.2 X 10^4 for DPV-A. DPV-I cultivated in DEF cell culture had a $TCID_{50}$ of 1X10 ^{5} per ml of the inoculum

All the strains were partially purified at 100000 g for 4.5 h at 4° C in Beckman ultra centrifuge and the protein concentration of the virus was estimated by biuret method and was found to be 11 mg, 8 mg and 7 mg for DPV-V, A and I respectively.

All the three strains of DPV were inoculated in mice to raise polyclonal serum. Four mice out of five inoculated with DPV-V showed ELISA titres more than 1:12800, one mouse showed a titre of 1:6400. The mice inoculated with DPV-A showed a titre of more than 1:12800 and those inoculated with DPV-I, 1:6400

ELISA was used to test the sera samples of the mice inoculated with DPV strains. The test was found to be highly sensitive, easy to perform and less time consuming. The test therefore can be recommended for routine diagnosis of DPV

Polyclonal serum was used to study the cross reactivity of the three strains of DPV with ELISA. DPV-V polyclonal serum reacted with the homologous virus with a titre of 1:12800. It also showed similar titer with other two heterologous strains. Polyclonal serum raised against DPV-A had a titre of 1:12800 with homologous and heterologous strains of DPV: DPV-I reacted with homologous strain at a titre of 1:6400. Similar titres were observed with hertologous strains.

Immuno peroxidase test was used for the detection of the tissue antigens in DPV infected CEF monolayers and in liver and spleen sections. Polyclonal serum raised against DPV-V detected homologous virus in the CEF monolayers and hetrologous virus in the liver and spleen sections. The staining reaction was observed as dark brown deposits at the virus localization. However background tissue was also stained brown to faint yellow in the stained preparation.

The virus neutralization test was used to study the cross neutralization by employing polyclonal serum raised against the three strains of DPV. Polyclonal serum raised against DPV-V showed a VNT of 64 with a VNI of 1.8 with homologous virus and a VNT 45 with VNI 1.65 with other two strains of DPV. Polyclonal serum raised against DPV-A neutralized the homologous virus at a titre of 32 with a VNI of 1.5. It also neutralized the

vaccine strain of DPV with same VNT and VNI, However it neutralized DPV-I with VNT 24 and VNI 1.35. DPV-I polyclonal serum neutralized the homologous virus at a titre of 45 with a VNI of 1.65 . The same neutralized DPV-V and DPV-A with a VNT 32 and VNI 1.5.

Monoclonal Antibody Production

The plates were seeded with the feeder cells by collecting the spleen cells from a normal healthy mouse. The same plates were used for seeding the fused cells.

Fusion of the myeloma cells and the splenocytes from the donor mice primed with the vaccine strain of DPV was under aken in presence of poly ethylene glycol (PEG 4000). Small colonies of the hybrids were observed in the wells and the growth of these was monitored. As soon as the hybrids attained a satisfactory growth i.e. 80 per cent coverage of the well the hybrid supernatant was tested for antibody production with the help of indirect ELISA and the positive hybrids were selected. Cloning of the positive hybrids was done by limiting dilution method to ascertain mono-clonality.

A total of seven clones producing desired antibodies against DPV vaccine strains were grown and Mabs were used for testing the cross

reactivity of the three strains of DPV. Out of 7 Mabs tested Mab 1 reacted with homologous virus with a titre of 32. It also reacted with other two heterologous strains with the similar titre.

Mab 3, 4 and 6 had a titre of 16 with homologous as well as heterologous virus strains of DPV and Mab 2, 5 and 7 showed a titre of 8 with all the strains of DPV. No differences were recorded in the ELISA titres of the 7 Mabs with homologous as well as heterologous strains of DPV. This suggested that there may be a close antigenic and serologic relationship within the three strains of DPV under study.

Immuno peroxidase test was used to detect the viral antigens in the DPV infected CEF and in the liver and spleen tissue sections from the birds infected with virulent Alleppy and IVRI viruses. All the seven Mabs detected homologous as well as heterologous virus in the infected CEF and in the liver and spleen sections. The reaction was very clear and background staining was almost negligible. The viral antigen deposits could be detected as dark red brown deposits in the cytoplasm and nucleus of the cells.

All the seven Mabs were tested for cross neutralization and the results indicated that, Mab 1 neutralized the homologous virus and had a VNT of 8 with VNI 0.9. It also neutralized the heterologous strains with similar VNT

and VNI. Mab 2, 4 and 6 had a VNT of 6 with homologous virus and a VNI 0.79. These neutralized the other two virus strains with a VNT 4 and VNI 0.75.

Mab 3 had a VNT 0f 2.4 with VNI 0.39 with homologous virus and a VNT of 2 with VNI of 0.35 with the heterologous strains. However, Mab 5 and 7 did not neutralize the homologous as well as hetrologous viruses and may be a non neutralizing.

The survivability and longevity trial was conducted to ascertain the appropriate time of collection of supernatant along with the usual parameters like change in the pH and 80 per cent coverage of the well. The criterion designated for the collection of the hybridoma supernatant like change of pH and 80 per cent coverage of the well seems to be appropriate for deciding the medium change and collection of the supernatant.

The conclusions from the present study are , in comparison with the polyclonal serum Mabs are highly specific in detection of the viral antigens in the tissue sections infected with the virus and the background staining reaction was completely reduced, thereby refining the test and making the diagnosis very easy. The test can be recommended for the diagnosis of DPV.

As regards the ELISA test it has been observed that the test is highly sensitive , easy to perform and less time consuming and should be recommended as routine diagnostic test for DPV.V

VNT is highly specific with polyclonal sera as well as with Mab. The specificity of the test is increased fairly by using Mabs.

As regards the possible strain variation of the DPV it can be concluded that there may not be any strain variation in the strains tested. The probable reason for the break down of *immunity* and vaccination failure may be because of low titered vaccine used in the field for vaccination of the birds or the virus may be acquiring virulence by sub clinical passage *in* the ducks in the field which might result in the breakdown of immunity and failure of the vaccine.

A potent tissue culture adapted vaccine may be useful in solving the problem.