INFLUENCE OF ADAPTATION OF THE VACCINE STRAIN OF DUCK PLAGUE VIRUS IN CHICKEN EMBRYO FIBROBLAST ON ITS IMMUNOGENICITY

By

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THESIS

Submitted in partial fulfilment of the
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DECLARATION

I hereby declare that the thesis entitled "**INFLUENCE OF ADAPTATION OF THE VACCINE STRAIN OF DUCK PLAGUE VIRUS IN CHICKEN EMBRYO FIBROBLAST ON ITS IMMUNOGENICITY**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Mannuthy
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CERTIFICATE

Certified that the thesis, entitled **"INFLUENCE OF ADAPTATION OF THE VACCINE STRAIN OF DUCK PLAGUE VIRUS IN CHICKEN EMBRYO FIBROBLAST ON ITS IMMUNOGENICITY"** is a record of research work done independently by **Shri. K. Senthil Kumar**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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K. SENTHIL KUMAR

To

My Beloved

Parents

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LIST OF ABBREVIATIONS

AAF	-	Allanto amniotic fluid
AEI	-	Acetylaziridine
BPL	-	ß-propiolactone
CAM	-	Chorio allantoic membrane
CAS	-	Chorio allantoic sac
CCL-141	-	Certified cell line - 141
CE	-	Chicken embryo
$CEID_{50}$	-	Chicken embryo infective $dose_{50}$
CEF	-	Chicken embryo fibroblast
CMF-PBS	-	Calcium magnesium free phosphate buffered saline
CPE	-	Cytopathic effects
DEF	-	Duck embryo fibroblast
$DELD_{50}$	-	Duck embryo lethal $dose_{50}$
DP	-	Duck plague
DPV	-	Duck plague virus
DVE	-	Duck viral enteritis
DVE-MLV	-	Duck viral enteritis - modified live vaccine
ELD_{50}	-	Embryo lethal $dose_{50}$
E-MEM	-	Eagle's minimum essential medium
FTE	-	Formalinised tanned erythrocyte
IVPM	-	Institute of Veterinary Preventive Medicine
PHA	-	Passave haemagglutination
PI	-	Post inoculation
rpm	-	revolutions per minute
SNT	-	Serum neutralization test
SPF	-	Specific pathogen free
SRBC	-	Sheep red blood cell
$TCID_{50}$	-	Tissue culture infective $dose_{50}$
TDW	-	Triple distilled water
VBI	-	Veterinary biological institute
VNI	-	Virus neutralization index

Introduction

1. INTRODUCTION

Duck rearing is popular among farmers and labourers in waterlogged areas and coastal states of India, particularly in West Bengal, Assam, Andhra Pradesh, Orissa and Kerala. The presence of lakes, ponds, rivers and streams, abound with fishes and snails and marshy lands unsuitable for cultivation in these areas, facilitate duck farming. Duck eggs and duck meat is preferred over hen's egg and meat in these areas because of low cost of production under free range system of management.

According to the 1994 census the total duck population and duck egg production in India comes to around 23.47 million and 320 million respectively (Panda *et al.*, 1994). Even though duck farming is second to poultry farming, ducks are easy to rear, comparatively less susceptible to various infectious diseases and produce more eggs than chicken. It contributes substantial income to agricultural labourers and farmers.

Good forage capacity, role in biological control of some insects and snails and ability to fertilize soil are added advantages of duck farming. Majority of farmers move their flocks from one place to another during harvesting season for ensuring ample availability of feed and for utilizing the

perennial water sources, during summer months. During such migrations, the ducks are exposed to various adverse conditions such as environmental, industrial pollutants, bacterial and viral agents.

Among the threats to the flourishing duck farming, a fatal disease, Duck Viral Enteritis (DVE), also known as Duck Plague (DP), caused by an alpha herpes virus is the most important. This causes heavy economic loss to duck farmers.

In Kerala, the disease was first reported in 1976 (Punnoose and Abdulla, 1976) and since then Kerala is endemic to this disease and mortality is being reported every year from various duck rearing areas in the state.

The conventional chicken embryo adapted vaccine is reported to have low titre and poor immunogenicity (Kulkarni, 1993; Bordolai *et al.*, 1994). It also possesses certain disadvantages of the possible presence of endogenous avian infectious agents such as Salmonella, Mycoplasma and Newcastle disease virus. As the chicken embryo vaccine is a live virus vaccine, there is every chance of the vaccine to act as a disseminator for the above infectious agents.

Kalaimathi and Janakiram (1991) and Bordolai *et al.* (1994) have shown that the chicken embryo adapted DP virus when serially passaged in Chicken Embryo Fibroblast (CEF) cell

cultures produce a high titred virus which was safer and more potent compared to the former. Moreover, cell cultures are cheaper, easier to handle and high titred viruses can be produced within a shorter period and in most cases, cell culture vaccine should be preferred over chicken embryo adapted vaccine (Rovozzo and Burke, 1973).

In the above circumstances the present study was undertaken to evolve a safe and potent CEF cell culture DP vaccine by:-

(i) Serial passage of the chicken embryo adapted seed DP vaccine strain in CEF cell cultures.

(ii) Physico-chemical characterisation and titration of the 5th and 10th CEF passaged virus.

(iii) Immunization of six to eight week old ducklings to evaluate the potency of the 5th and 10th passaged virus by antibody titration using passive haemagglutination and serum neutralisation test and by challenge test.

Review of Literature

2. REVIEW OF LITERATURE

Duck plague, also known as Duck Viral Enteritis is an acute, contagious herpes virus infection of ducks, geese and swans. It is characterized by vascular damage, with tissue haemorrhage and free blood in body cavities, digestive mucosal eruptions, lesions of lymphoid organs and degenerative changes in parenchymatous organs (Leibovitz, 1991).

2.1 History

The first outbreak of duck plague was recorded by Baudet (1923) in Netherlands, wherein high mortality, petechial lesions throughout the body and enteritis were observed. The disease was suspected to be fowl plague. Dezeeuw (1930) recorded a similar outbreak and opined that the agent was the same as that causing fowl plague.

Bos (1942) indicated that the disease was not the same as fowl plague since hens and pigeons could not be experimentally infected and concluded that the infectious agent was an independent virus. At the XIV International Veterinary Congress held in 1949, Jansen and Kunst proposed the name duck plague for the disease.

Jansen *et al.* (1952) reported another outbreak and found that the ducks surviving this outbreak were immune to the

virus of earlier outbreaks. Jansen (1961) reported several such outbreaks and distinguished the disease from Newcastle disease, fowl plague and duck hepatitis. Based on symptoms and pathological features, duck plague was renamed as Duck Viral Enteritis (DVE) (Leibovitz, 1991).

2.2 Incidence and distribution

In addition to the Netherlands, DVE had been reported from France (Lucam, 1949), India (Mukerji *et al.*, 1963), Belgium (Devos *et al.*, 1964), the American continents (Leibovitz and Hwang, 1968), Britain (Hall and Simmons, 1972), Bangladesh (Sarkar, 1980), Russia (Simonova *et al.*, 1984) and France (Fleury *et al.*, 1986).

The first official report of DVE in India was from West Bengal in 1963 (Mukerji *et al.*, 1963) and later on by Jansen (1964), Bhowmik and Chakrabarty (1985) and Bhowmik and Ray (1987). Outbreaks of DVE were also reported from several states of India. The disease was first reported from Kerala in 1976 (Punnoose and Abdulla, 1976; Nair, 1978). Duraiswami *et al.* (1979) reported the disease from Tamil Nadu, Chakrabarty *et al.* (1980) from Assam and Sreeramulu (1986) from Andhra Pradesh.

In Kerala heavy mortality was reported during the period from April 1976 to January 1977 in Alleppey district wherein

the ducks showed characteristic symptoms and lesions of duck plague (Nair, 1978). Since then several outbreaks have been reported from Kottayam, Pathanamthitta, Thrissur and other parts of Kerala. Kulkarni *et al.* (1995) isolated DPV from both vaccinated and unvaccinated flocks from several parts of Kerala.

2.3 Aetiological agent

Duck plague is caused by a DNA virus of the family Herpes viridae, sub family alpha herpes virinae (Roizman *et al.*, 1981).

2.4 Physiochemical and biological properties

The virus does not have any haemagglutination and haemadsorption properties (Jansen, 1961).

Duck plague virus was found to be sensitive to ether and chloroform. Infectivity of the virus could be destroyed by heating at 56°C for 30 min. The virus is inactivated by exposure to pH 3 and 11 for 45 minutes and by treatment with trypsin, chymotrypsin and pancreatic lipase for 18 hrs at 37°C (Hess and Dardiri, 1968).

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

2.4 Epizootiology

Duck Viral Enteritis is a disease of members of the family Anatidae which includes ducks, geese and swans. It has not been reported from other avian species, mammals or human beings (Jansen, 1964).

Burgess and Yuill (1981b) reported that carrier birds under stress shed more virus and experimental vertical transmission was possible in persistently infected water fowls. The effects of virus on fertility and hatchability depended upon strain of virus and breeds of ducks affected. But Prip *et al.* (1983) opined that transovarian transmission was not of great significance.

Burgess and Yuill (1982) also suspected a carrier status in wild ducks. Lin *et al.* (1984a) isolated a herpes virus from waterfowls dying of DVE and designated it as Sheridan-83 which was biologically and serologically related to Holland and Lake Andes strain of DVE.

Leibovitz (1991) observed that natural infection could occur by direct or indirect contact and opined that infected water fowls may play a significant role in transmission of infection. He also stated that the virus could be transmitted experimentally via oral, intranasal, intramuscular, intravenous, intraperitoneal and cloacal routes and by blood

sucking arthropods during the viremic phase. He also found Muscovy ducks to be more susceptible to DVE than White Pekins.

Natural infection occurs in all age groups ranging from seven days to mature ducks and the incubation period varies from three to seven days (Leibovitz, 1991).

2.5 Symptoms

Different symptoms are shown by birds suffering from DP. The affected birds showed photophobia, associated with half closed pasty eyelids, inappetence, extreme thirst, droopiness, ataxia, ruffled feathers, nasal discharge, soiled vent and greenish diarrhoea (Rajan *et al.*, 1980; Leibovitz, 1991). The birds died suddenly in good flesh, breeder male birds died with prolapse of penis. A 20 to 40 per cent drop in egg production was noticed in layers (Leibovitz, 1991). In experimentally infected birds respiratory signs predominated, leading to spasms and paralysis similar to Newcastle disease (Skalinskii and Borisovich, 1969). Dardiri (1971) reported heavy mortality as a result of dual infection of DP with various bacterial organisms.

2.6 Lesions

Pathologic lesions depended on species affected, age, sex, susceptibility of host, stage of infection, virulence of

the infective agent and intensity of virus exposure. The most striking lesions were multiple petechiae throughout the body, especially on the heart and mucosal surface of oesophagus. Red coloured haemorrhagic annular ring bands were also seen at regularly spaced intervals in ileum and jejunum (Leibovitz, 1971).

Jansen (1964) observed diphtheritic changes in the esophagus and cloaca as the disease progressed. The liver was moderately enlarged with scattered petechial haemorrhages and grayish white necrosis, while the spleen showed mottled appearance. Caseous necrotic exudates were noticed in the trachea and petechial haemorrhages were seen on the heart, giving a paint brush appearance to the pericardium. Necrotic foci was also noticed in gizzard muscle in chronic cases (Rajan *et al.*, 1980; Leibovitz, 1991).

On histopathological examination, acidophilic intra-nuclear type A inclusion bodies were seen in the hepatic cells, epithelium of oesophagus, bursa of Fabricius and Harderian glands (Ray *et al.*, 1983; Barr *et al.*, 1992).

2.7 Propagation of DPV

Propagation of DPV in various systems namely, ducklings, embryonated eggs and cell cultures have been reported by many

workers (Jansen, 1964; Kalaimathi and Janakiram, 1989; John *et al.*, 1990; Bordolai *et al.*, 1994).

2.7.1 Duckling

The most suitable model for propagation of DPV is day old ducklings. Specific pathogen free ducklings are preferred for inoculation. Inoculation of 0.5 ml of tissue suspension (liver and spleen) by intramuscular route resulted in death of ducklings with typical symptoms and lesions of DPV (Sarkar, 1982; John *et al.*, 1990; Leibovitz, 1991).

2.7.2 Embryonated eggs

Isolation of virus was done by inoculating tissue suspension onto chorio allantoic membrane (CAM) of embryonated duck eggs, wherein embryos died four days after inoculation, showing extensive haemorrhages throughout the body (Jansen, 1961; Kalaimathi and Janakiram, 1989; John *et al.*, 1990).

Jansen (1961) reported that the virus after 12 passages in duck embryos and three passages in chicken embryos was able to cause death of the chicken embryos within four to five days post inoculation (PI) with extensive haemorrhages. He further observed that DPV did not show any interference phenomenon with fowl plague and Newcastle disease, in embryonated chicken eggs. Skalinskii and Borisovich (1969) studied the pathogenicity of strain of DPV. They reported that the virus

grew readily in allantoic sac of developing duck and chicken embryos.

Attenuated duck plague virus inoculated via chorioallantoic sac (CAS) yielded higher titre of virus in amniotic fluid and allantoic fluid than the CAM route of inoculation (Butterfield *et al.*, 1969). Therefore CAS route was recommended as the most suitable route for large scale vaccine production (Kalaimathi *et al.*, 1985).

The attenuated strain of chicken embryo adapted DPV virus regained its virulence by repassaging in duck embryos (Bhattacharya *et al.*, 1977).

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

2.7.3 Cell cultures

Kunst (1967) first passaged the DPV in duck embryo fibroblast (DEF) cell cultures and observed specific cytopathic effects (CPE) three to four days PI. Breese and Dardiri (1968) infected DEF cell cultures with DPV and found inclusion bodies in nucleus and cytoplasm.

Cultivation of virulent DPV in DEF cell cultures had been reported (Dardiri, 1969; Kocam, 1976; Burgess and Yuill, 1981a). DPV passaged in duck embryos was propagated in CEF cell cultures (Kurochka *et al.*, 1983; Kalaimathi and Janakiram, 1989; John, 1988; Bordolai *et al.*, 1994). Kenwolf *et al.* (1974) propagated DPV in CCL-141 cell lines which originated from white pekin ducks.

The virus grew well in CCL-141 cell cultures and yielded 5.6 times more virus than by other methods. Plaques were easily detectable in the cell lines. This cell line has uniformity, convenient method of storage and known health history (Kenwolf *et al.*, 1976).

Muscovy and wood duck cell lines yielded high titres of virus and had given better plaque quality, leading to the recommendation of this cell line for diagnostic and research purpose (Kocam, 1976).

Duck plague virus infected cell cultures (DEF, CCL 141) showed CPE, 48 hr PI, characterised by rounding of cells and marked pyknosis, resulting in very small clusters leading to bridge formation (Kenwolf *et al.*, 1976; Nair, 1978).

Less virulent DP virus strains were propagated in cell cultures using an incubation temperatures higher than 37°C. The ability of DPV isolate to grow at higher temperature

ranging from 40.5°C to 41.5°C may be an indicator of its virulence. A less virulent isolate produced plaques at higher temperature (Burgess and Yuill, 1981a).

Vetesi *et al.* (1982) demonstrated CPE in duck and goose embryo fibroblasts and kidney cells. Solsich *et al.* (1983) demonstrated intra cytoplasmic inclusions in DEF cell cultures. Simonova *et al.* (1984) were also successful in propagation of attenuated Jansen strain of virus in chicken embryo monolayer cell cultures.

Gough (1984) reported nine isolations of duck enteritis virus in DEF cell cultures and observed CPE rarely at primary isolation. In most cases two or three passages were required before the demonstration of CPE in cell cultures.

Intracytoplasmic inclusion bodies were reported in CEF cell cultures stained with haematoxylin and eosin (Kalaimathi and Janakiram, 1990). They also observed fluorescing areas in the infected cell cultures when stained with acridine orange and indirect fluorescent antibody test.

Panisup *et al.* (1990) reported eosinophilic intranuclear inclusion bodies, extensive vacuolation of cytoplasm, dilatation of endoplasmic reticulum, mitochontrial degeneration with condensation and migration of

heterochromatin and nuclear bleb formation in DEF cell cultures infected with DPV.

2.8 Adaptation of DPV

2.8.1 Chicken embryos

Chicken and chicken embryos were found to be refractory to DPV (Bhowmik and Chakrabarty, 1985; Kulkarni, 1993). However, DPV could be adapted to grow in 9-11 day old chicken embryos by CAM route after its adaptation in embryonated duck eggs (Jansen, 1961; Kalaimathi *et al.*, 1985; John *et al.*, 1990; Bordolai *et al.*, 1994).

Mean titre of virus when inoculated via CAS route was higher than by the CAM route because of more number of cells lining the allantoic sac were exposed to virus (Kenneth and Lauffer, 1953).

Jansen (1961) reported that the pathogenicity of DPV for ducks rapidly decreased by 20 serial passages in chicken embryos. This chicken embryo adapted DPV killed the chicken embryos four to five days PI but hens were uninfected. Jansen *et al.* (1963) observed that chicken embryo adapted DPV was avirulent to ducks and that such virus induced virus neutralizing antibodies in vaccinated ducks.

Mukerji *et al.* (1965) also could not isolate DPV using chicken embryos primarily, but they could isolate it using

duck embryos. When DPV was serially passaged 15 times in duck embryos the virulence had not decreased. But on further serial passage in chicken embryos the virulence for ducks decreased and after the 25th passage in chicken embryos, the virus became completely avirulent to ducks.

Butterfield *et al.* (1969) reported titres of virus as high as $10^{6.5}$ $DELD_{50}$/ml in 144-158 hr PI in the CAM suspension of embryonated chicken eggs inoculated via CAS. They further stated that Allanto amniotic fluid (AAF) also yielded high titre of virus 96 hr PI. High titre of virus in CAM, embryo and AAF provided antigenic material for vaccine production.

Duck embryos inoculated with DPV were dead by four to six days PI in the first passage, whereas those inoculated with subsequent passage materials were dead within three to four days PI (Sarkar, 1982; John *et al.*, 1990).

2.8.2 Cell cultures

Duck plague virus isolated from infected ducks was propagated in DEF cell cultures and the 5th and 7th passaged material was further passaged in CEF cell cultures. The resulting virus elicited low levels of antibody production but was avirulent to ducks and afforded protection against challenge infection with virulent virus (Dardiri, 1969).

Chicken embryo adapted virus produced large sized plaques as that of virulent virus, which produced maximum number of plaques with 10 mm diameter at 14 days PI, but DPV passaged five times in DEF cell cultures produced minute plaques of one to two mm in diameter and small plaques of three to six mm diameter in DEF and CEF cell cultures (Dardiri, 1975).

The duck embryo adapted DPV at the 12th passage level was further passaged in CEF cell cultures. Adaptation of virus in cell culture was observed by degenerative changes at three to four days PI (John, 1988).

Kalaimathi and Janakiram (1989) passaged field isolate of DPV in CEF cell cultures and studied the degree of virulence of the virus at 5th, 10th and 12th passage level in ducklings and duck embryos. They observed a reduction in mortality rate and decrease in severity of reactions as passages increased indicating reduction in virulence of the virus.

When DPV was passaged serially in CEF cell cultures, the time taken for the onset of CPE decreased as the passages increased. CPE was observed at 60 hr PI at the first passage and by the 12th passage CPE occurred at 24 hr PI. Similarly time taken for complete desquamation of cells decreased from 120 hr at first passage to 60 to 72 hr at the 12th passage (Kalaimathi and Janakiram, 1990).

Kalaimathi and Janakiram (1990) also reported that the titre of virus steadily increased from $10^{4.8}$ $TCID_{50}$/ml at the first passage to $10^{6.83}$ $TCID_{50}$/ml at the 12th passage, indicating adaptation of field strain of DPV in CEF cell cultures.

Bordolai *et al.* (1994) serially propagated a local strain of DPV 15 times in duck embryos, 15 times in chicken embryos and further 12 times in CEF cell cultures. The 5th and 12th CEF passaged virus was titrated in chicken embryos and found that the titre of the virus increased from $10^{5.3}$ $CEID_{50}$/ml to $10^{6.5}$ $CEID_{50}$/ml respectively, indicating adaptation of the virus in CEF cell cultures. CPE was observed from fourth passage only. The time of onset of CPE was reduced from 72 hr PI at the fourth passage to 48 hr PI at the eighth passage.

2.9 Immunity

Jansen (1964) reported that chicken embryo adapted strains of DPV were completely avirulent to ducks and induced active immunity. Virus neutralizing antibodies could be demonstrated in the sera of majority of vaccinated birds, but not in all. Non-antibody defence could be due to the interference phenomenon. Interference phenomenon could be demonstrated by vaccinating birds with egg adapted DPV, following infection with virulent virus.

Chicken embryo adapted DPV when inactivated with 0.05 per cent 1-acetylaziridine (AEI) at 37°C for a period of six hr, induced a serologic response as that of attenuated virus and afforded protection to the challenge (Butterfield and Dardiri, 1969). They further observed that DPV inactivated with 0.4 per cent ß-propiolactone (BPL) for an hour did not protect ducks against challenge infection.

Toth (1970) tested the safety and immunogenicity of thirteen serially passaged egg adapted DPV vaccines in ducks. The titre was 4.2 $\log_{10}$ $CEID_{50}$/ml. One ml of undiluted vaccine was given to each of 260 ducklings and on challenge only two birds died, whereas mortality in control was 80 per cent. He opined that undiluted virus was effective in producing immunity and in warding off challenge infection.

Toth (1971) while studying the effect of single and double vaccination in ducks, observed that single vaccination did not induce any neutralizing antibodies and showed 12, 24 and 14 per cent mortality on challenge with virulent virus in the fifth, 24th and 36th week post vaccination, respectively, while controls showed 64, 74 and 50 per cent mortality in the respective weeks. In the double vaccinated group the mortality was eight and 10 per cent respectively, when challenged at 5th and 17th week, while controls showed 84 and 59 per cent mortality in the respective weeks. It was also

reported that vaccinated birds did not spread vaccine virus to unvaccinated birds.

Dardiri (1975) observed that there was field and laboratory evidence indicating inapparent infection prevailing among ducks infected with DPV. Virulent virus was isolated from certain tissues of waterfowls with high levels of serum neutralizing antibodies. He reported poor serological response in vaccinated waterfowls as the virus neutralizing serum index was 1.75 $\log_{10}$ in contrast to an index of 4 $\log_{10}$ in birds exposed to virulent virus. Injection of vaccine or presence of low level of neutralizing antibody would predispose waterfowls to secondary response.

Eventhough ducks that recovered from infection were immune to reinfection, Burgess and Yuill (1982) in an experimental study found that superinfection was able to cause death of persistently infected mallard ducks.

Zheng (1983) reported that DPV vaccine protected geese against DP.

Lin *et al.* (1984a) vaccinated White Pekin ducks intramuscularly with 4 $\log_{10}$ $TCID_{50}$/ml Sheridan 83 strain. It resulted in the production of antibodies that enabled the ducks to resist the challenge with 100 $TCID_{50}$/ml of the Lake Andes strain of DPV.

Lam and Lin (1986) reported that antiserum was able to transfer immunity to recipient birds against challenge infection, probably because of passive humoral immunity.

Sergeev *et al.* (1990) observed that live attenuated `AKV' strain of DPV with 10^2-10^3 $CEID_{50}$/ml protected 60 per cent ducklings when challenged five months post vaccination with a virulent virus.

Hong and Hwang (1991) cultivated DPV and geese parvo virus in duck embryos and produced a bivalent vaccine which induced protective antibodies in adult geese against DP.

2.10 Immunogenicity and vaccination

Jansen (1964) reported that when a local strain of DPV was passaged serially 20 times in chicken embryo, it became avirulent to ducks and was capable of producing immunity and that this chicken embryo adapted virus could be used as a reliable vaccine. He recommended use of this vaccine on farms where outbreaks of disease had occurred. This strain was named `Utrecht' strain of Netherlands.

Jensen and Kunst (1964) also showed that chicken embryo adapted DPV could withstand challenge with virulent DPV.

Mukerji *et al.* (1965) reported that 25 passages of the local strain of DPV in chicken embryos rendered it completely

safe and on testing the immunogenicity of the strain in ducks, it was found that the vaccinated birds withstood challenge, 14 months post vaccination.

Jansen and Kunst (1967) attempted to reactivate the attenuated strain of DPV by passaging in duck embryos but they could not reactivate the virus which indicated that the attenuated strains were stable and hence safe for vaccine production.

Dardiri (1975) reported that the chicken embryo and CEF cell culture adapted DPV was avirulent to ducks.

Balla (1984) vaccinated ducks with doses as low as 2.6 $\log_{10}$ $TCID_{50}$/ml instead of the recommended 4 $\log_{10}$ $TCID_{50}$/ml and observed that the vaccine protected 80-84.6 per cent of the ducks, whereas $10^{1.64}$ to $10^{2.2}$ $TCID_{50}$/ml gave only 55.6 to 69.2 per cent protection.

Lin *et al.* (1984b) reported that a dose, less than 10 $TCID_{50}$ of Sheridan-83 strain of DPV could protect the ducks but the immunity lasted only one to two months.

Local strain of DPV after 20 passages in chicken embryos and having a titre of $10^{3.5}$ $CEID_{50}$/ml or above induced immunity in ducklings (John *et al.*, 1990).

Serum neutralization test (SNT) and micro SNT were used to study the immunogenicity of the 5th and 12th CEF passaged virus in eight week old duckling (Kalaimathi and Janakiram, 1991). They observed that there was a gradual rise in SN titre over passages which suggested an enhancement of immunogenicity during passage in CEF. They further reported that survival rate of ducklings with 12th passage virus was 25 per cent; however the 5th and 10th passaged virus caused 100 per cent mortality in ducks when challenged with virulent virus. This indicated that the virus required few more passages to attain optimum immunogenicity to serve as an effective cell culture adapted DP vaccine.

Bordolai *et al.* (1994) passaged the chicken embryo adapted local strain of DPV in CEF cell cultures 12 times and used it for vaccination of ducks by intramuscular administration. This 12th passaged sample had a virus titre of 4.5 $\log_{10}$ $TCID_{50}$/ml. They observed that there was no untoward reactions in the vaccinated ducks and the ducks withstood challenge six weeks post vaccination. From this they concluded that cell culture DP vaccine was superior to commercial chicken embryo adapted vaccine.

Intervet has adapted the `Utrecht' strain of DPV to primary CEF and this is being marketed as DP vaccine `Nobillis' with 4.5 $\log_{10}$ $TCID_{50}$/dose. They recommended a schedule of vaccination, as follows: Initial vaccination at

four weeks by intramuscular or subcutaneous route, in high risk areas even at day old, followed by a booster dose at fourth week. In breeding stock, two vaccinations at six weeks interval and for the whole duck population annual revaccination was recommended (Anonymous, 1993).

In India, four laboratories produce DP vaccine. Most of them use seed virus from the same source. As per the information supplied by two manufacturing institutes (IVPM, Ranipet and VBI, Palode), the vaccine is a suspension of AAF, CAM and embryo, of modified live virus inoculated chicken eggs and is freeze dried. It is claimed that immunity developed within seven days and lasts for one year in case of vaccine from VBI, Palode. In case of IVPM Ranipet vaccine, immunity develop within 14 days and last for 18 months. The IVPM, Ranipet vaccine is reported to be safe for ducklings of eight to 12 weeks of age. DP vaccine from Palode is recommended at seventh week of age with a booster dose at fourth month. In high risk areas vaccination at two to four weeks of age, followed by two booster doses at eighth and 16th weeks and then annual revaccinations are recommended (Anonymous, 1991).

2.11 Diagnosis

Diagnosis is mainly based on the clinical signs, macroscopic and microscopic lesions and isolation and identification of virus using 12-14 day old embryonated duck

eggs and DEF cell cultures. Serological tests such as serum neutralization (Mukerji *et al.*, 1965; Dardiri, 1975 and Mukit *et al.*, 1988), plaque assay (Dardiri and Hess, 1967), agar gel diffusion (John, 1988), immunofluorescence (Erickson *et al.*, 1974), reverse passive haemagglutination (Deng *et al.*, 1984), passive haemagglutination test (Kulkarni, 1993) counter immunoelectrophoresis (John *et al.*, 1989), electronmicroscopy (Tantaswasdi *et al.*, 1988), immunoperoxidase test (Islam *et al.*, 1993) and enzyme linked immunosorbent assay (Chandrika, 1996) are valuable in diagnosis of DVE.

2.11.1 Serum neutralization test (SNT)

Serum neutralization test is employed to assess the immunogenicity of DPV. Serum of ducks immunised with chicken embryo passaged DPV, contained antibodies, which could neutralize the virulent virus. But virus neutralizing antibodies could not be detected in serum of all vaccinated birds (Jansen, 1964).

Serum neutralization test could be carried out in 9 to 11 day old embryonated eggs (Mukerji *et al.*, 1965, Dardiri and Hess, 1967; Mukit *et al.*, 1988 and Bordolai *et al.*, 1994) as well as in cell cultures (Lin *et al.*, 1984a and Kalaimathi and Janakiram, 1991).

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

Jansen and Wemmenhove (1966) observed a lack of positive correlation between the virus neutralising antibody and the ability of vaccinated birds to withstand the challenge.

I-Acetyl aziridine (AEI) inactivated virus elicited a virus neutralizing index (VNI) of $10^{1.4}$ compared to $10^{1.3}$ VNI by attenuated virus inoculated intramuscularly and $10^{0.4}$ for cell culture attenuated virus administered orally (Butterfield and Dardiri, 1969).

A neutralization index of 1.75 or higher was accepted as sign of significant neutralizing antibody development (Asplin, 1970).

Dardiri (1975) observed a lack of positive correlation between antibodies and ability to withstand challenge with virulent virus because ducks having low antibody titre of $10^{0.4}$ were protected against challenge. He opined that protection in such cases could be due to cellular type of immunity.

Mukit *et al.* (1988) have observed an average neutralization index of 1.5 in unvaccinated ducks, 2.3 in vaccinated and 3.8 in recovered ducks.

Kalaimathi and Janakiram (1991) observed an antibody titre of 40 and 80 by micro SNT in CEF cell culture, using sera of ducks immunized with fifth and 12th CEF passaged virus, respectively.

Bordolai *et al.* (1994) reported that ducklings vaccinated with chicken embryo adapted vaccine had a VNI of 1.22 whereas cell culture adapted vaccine had an index of 1.78, six weeks post vaccination.

2.11.2 Passive haemagglutination test (PHA)

Passive haemagglutination test is rapid, less cumbersome, accurate and economical when compared to neutralization test and is equally sensitive (Vengris and Marie, 1971; Zyambo *et al.*, 1973). Kulkarni (1993) demonstrated antibodies to DPV using this test. Vengris and Marie (1971) reported that the results were better when the plates were kept at 4°C for 12 to 14 hr than at 37°C.

2.12 Challenge test

Eight weeks old ducklings, immunized with cell culture adapted vaccine were challenged with virulent virus containing 100 $DELD_{50}$/ml. All the ducks vaccinated with fifth and eighth passaged virus succumbed to the challenge, while in ducklings vaccinated with 12th passage material, the mortality recorded

was 75 per cent. In control groups only 60 per cent mortality was noticed which was less than vaccinated group and could be attributed to the superinfection of ducklings (Kalaimathi and Janakiram, 1991).

Bordolai *et al.* (1994) reported that cell culture vaccine was superior to commercial chicken embryo adapted vaccine. The commercial chicken embryo vaccine protected 86.6 per cent, while cell culture adapted vaccine protected 100 per cent ducklings upon challenge with virulent virus.

2.13 Characterisation of propagated DPV

2.13.1 pH sensitivity

Duck plague virus remained stable at pH 7-9 for a period of six hours, but was inactivated at pH 6 and above pH 10. Rapid inactivation was noticed at pH 3 and 11 at 37°C (Hess and Dardiri, 1968). Kunst (1968) observed total inactivation of virus at pH 3, when kept at 4°C. Nair (1978) reported that virus samples were stable at pH 7.2, however complete inactivation of virus samples was noticed at pH 9.2. DPV-N (Nilambur) strain was less susceptible to inactivation at pH 4.7 compared to known strain of virus.

Sarmah *et al.* (1997) reported that there was considerable reduction in the 5th CEF passaged virus titre from 4.5 $\log_{10}$ $TCID_{50}$ to 0.66 $\log_{10}$ $TCID_{50}$ and 1.00 $\log_{10}$ $TCID_{50}$ when the virus

was exposed to pH 3 and 11 respectively. They further stated that there was no change in the infectivity titre when the virus was exposed to pH 9 for 3 hr.

2.13.2 Thermostability

Duck plague virus was inactivated at 56°C in 30 min. (Kunst, 1968; Nair, 1978). However, Hess and Dardiri (1968) reported complete inactivation of the virus in ten min. at 56°C and 90-120 min. at 50°C. At 22°C infectivity persisted even after 30 days. Jansen (1964) reported that when stored at -20°C all activities of the virus were retained and virulence remained unaltered for many years when kept in freeze dried form.

Sarmah *et al.* (1997) reported that the 5th CEF passaged DPV completely lost its infectivity when exposed to 56°C and 60°C respectively for 15 minutes.

Materials and Methods

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Duck plague vaccine virus

Lyophilized chicken embryo adapted duck plague vaccine was procured from Veterinary Biological Institute (VBI), Palode, Kerala, revived in embryonated chicken eggs and propagated in chicken embryo fibroblast cell culture.

3.1.2 Chicken eggs

Ten to twelve day old embryonated chicken eggs obtained from the University Poultry Farm, Mannuthy were used for the revival of vaccine virus, its titration and preparation of CEF cell cultures.

3.1.3 Ducklings

Healthy day old unvaccinated (White Pekin/desi) ducklings obtained from a private agency were used for immunisation trials.

3.1.4 Virulent virus

The field strain of DPV isolated from birds in an outbreak of DP in Kuttanad area of Alleppey district was revived in ducklings and employed for challenge test.

3.1.5 Sheep red blood cell (SRBC)

Sheep blood was collected in Alsever's solution (1:1). After removing the plasma and buffy coat, the RBCs were washed thrice in PBS of pH 7.2 and stored at 4°C until use.

3.1.6 Cell culture

3.1.6.1 Glassware

Borosil brand of glasswares and Laxbro plastic vials were used for laboratory work.

3.1.6.2 Triple glass distilled water (TDW)

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

3.1.6.3 Calcium, magnesium free phosphate buffered saline (CMF-PBS)

Calcium, magnesium free phosphate buffered saline was prepared from readymade dehydrated powder obtained from Hi-Media laboratories. One vial was dissolved in 1000 ml TDW and sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure.

3.1.6.4 Sodium bicarbonate solution (7.5 per cent)

Sodium bicarbonate	-	7.5 g
TDW	-	100 ml

This was sterilized by filtration using membrane filter, pore size 0.45 μm and stored in sterile vials at 4°C until use.

3.1.6.5 Trypsin solution (5 per cent)

Trypsin	-	5 g
CMF-PBS	-	100 ml

The solution was sterilized by filtration using membrane filter and stored at -20°C. The working solution of trypsin (0.25 per cent) was prepared by diluting with CMF-PBS at the time of use.

3.1.6.6 Calf serum

Blood collected from healthy bull-calves about six months to one year old was used for serum separation. Serum was separated, clarified, inactivated at 56°C for 30 min., sterilized by filtration using membrane filter and stored in 10 ml aliquotes at -20°C.

3.1.6.7 Antibiotic solution

Streptomycin	-	1 g
Benzyl penicilin	-	1,00,000 IU
Sterile TDW	-	40 ml

One ml of this solution was added to 100 ml of medium to obtain the final concentration of 250 μg streptomycin and 250 IU of benzyl penicillin per ml of medium.

3.1.6.8 Cell culture medium

Dehydrated Eagle's Minimum essential medium (E-MEM) supplied by Hi-Media laboratories, Bombay was used through out the study.

Working solution

Eagle's Minimum essential medium	-	1 vial
TDW	-	1000 ml
Yeast extract	-	2 g
Lactalbumin hydrolysate	-	5 g

The media was sterilized by filtration using membrane filter and stored at 4°C until use.

Growth medium

E-MEM working solution	-	94 ml
Calf serum	-	5 ml
Antibiotics	-	1 ml

The pH was adjusted to 7.2 to 7.3 with sterile 7.5 per cent sodium bicarbonate solution under aseptic conditions.

Maintenance medium

E-MEM working solution	-	96 ml
Calf serum	-	3 ml
Antibiotic solution	-	1 ml

The pH was adjusted to 7.2 to 7.3 as above.

3.1.7 Microtitre plate

Microtitre plate obtained from Laxbro Pvt. Ltd., was used in passive haemagglutination test.

3.1.8 Citrate phosphate buffer pH 3

The stock solutions A (0.1 M solution of citric acid) and B (0.2 M solution of dibasic sodium phosphate) were prepared in TDW. Then 39.8 ml of solution A was mixed with 10.2 ml of solution B and diluted to make a total volume of 100 ml.

3.1.9 Phosphate buffer pH 11

CMF-PBS - 10 ml

The pH was adjusted to 11 with 1N NaOH.

3.1.10 May-Grunwald - Giemsa stain

May-Grunwald stain

The stain powder 2.5 g was dissolved in 1000 ml absolute methanol and allowed to age for one month.

Giemsa stain

One gram of giemsa powder was dissolved in 66 ml of glycerol at 55°C overnight and 66 ml of absolute methanol was added.

3.2 Methods

3.2.1 Revival of vaccine virus

The lyophilised vaccine procured from VBI, Palode was reconstituted in five ml of CMF-PBS and used as inoculum. This was inoculated into 11 day old embryonated chicken eggs, at the rate of 0.1 ml per embryo by CAM route. The inoculated eggs were incubated at 37°C. Embryo mortality was monitored at 24 hr interval. The dead embryos were collected and chilled at 4°C overnight.

Embryos were harvested and examined for the DP lesions. The AAF, CAM, liver and spleen were collected. The tissues and CAM were homogenized in AAF and centrifuged at 5000 rpm for five min. The supernatent was collected, antibiotics added at the rate of 500 IU benzyl penicillin, 500 μg streptomycin per ml of fluid and preserved at -70°C. This was used as antigen (virus) for further passages in the CEF cell cultures.

3.2.2 Propagation of virus in cell cultures

3.2.2.1 Preparation of primary CEF cell culture.

Twelve day old embryonated chicken eggs were used for preparation of CEF cell culture. The cell cultures were prepared using standard procedures (Burleson *et al.*, 1992). The embryos were collected aseptically into a petridish containing CMF-PBS. The embryos were decapitated, limbs removed eviscerated and cut into pieces of one to two mm size.

The fragments were washed in PBS and trypsinized with 0.25 per cent chilled trypsin. The supernatant was discarded. The cells were trypsinized with activated 0.25 per cent trypsin thrice, 20 min each time. The suspensions were pooled, neutralised with two per cent calf serum, filtered through muslin cloth, centrifuged at 800 rpm for five min to pellet the cells and resuspended in growth medium. The cell concentration was adjusted to $1x10^6$ cells per ml, seeded into

prescription bottles and test tubes with coverslips at the rate of 10 ml and one ml respectively. These were incubated at 37°C.

The bottles and tubes showing confluent monolayers at 24 hr of incubation were inoculated with vaccine virus mentioned above at the rate of one ml per bottle and 0.1 ml per tube. The infected monolayers were incubated at 37°C for 45 min for virus adsorption. After the adsorption period the monolayers were washed with CMF-PBS, re-fed with maintenance medium and incubated at 38.5°C with few monolayers being incubated at 37°C. Suitable controls were also maintained. The monolayers were examined under inverted microscope at 12 hr intervals for the appearance of cytopathic effects (CPE).

The bottles and tubes showing complete CPE were frozen at -70°C and thawed at 37°C. The freezing and thawing was repeated thrice to release the virus from the cells. The contents were pooled, centrifuged at 3000 rpm for 30 min in refrigerated centrifuge at 4°C. The supernatent was collected and preserved at -70°C until use. It was used as inoculum for the subsequent passages.

3.2.3 Cytological studies

After seeding the virus, the cell cultures grown in bottles were examined at 12 hr intervals to study the CPE. However, cell culture grown on coverslips infected with viruses of different passages were examined at different time intervals (12, 24, 48, 64, 72 and 96 hr), after fixing the coverslips in ten per cent formol saline and stained by May-Grunwald Giemsa staining method.

3.2.4 Adaptation of vaccine virus

Adaptation of vaccine virus was achieved by serial passages in CEF cell cultures. The confluent monolayers were infected with virus and the materials received from this passage was used as inoculum for the next passage. This process was repeated upto the tenth passage.

3.2.5 Virus titration

Chicken embryo fibroblast cell culture passaged vaccine strain of DPV at 5th and 10th passages were titrated in both developing chicken embryos and CEF cell cultures, in order to evaluate the concentration of virus in the suspension. The titre was calculated as per the method described by Reed and Muench (1938).

3.2.5.1 In embryonated chicken eggs (ELD_{50})

Ten fold serial dilutions of vaccine strain of DPV, 5th and 10th CEF passaged viruses were made in chilled CMF-PBS starting from 10^{-1} to 10^{-6}, using separate sterile pipette for each dilution. The virus inoculated into 11 day old embryonated chicken eggs at the rate of 0.1 ml by CAM route. For each dilution, four embryos were used. The inoculated eggs were incubated at 37°C and candled daily. The embryos that died after 24 hr PI were chilled at 4°C overnight, harvested and examined for specific lesions of DP. The 50 per cent end point of embryolethal dose (ELD_{50}) was calculated as per the method described above.

3.2.5.2 In cell culture ($TCID_{50}$)

The cell cultures were prepared in test tubes. Serial ten fold dilutions of 5th and 10th passaged viruses were prepared as above. From each dilution 0.1 ml of the virus was inoculated into each tube, four tubes being used per dilution. The tubes were incubated at 38.5°C and examined at 12 hr intervals for appearance of CPE. The tubes showing CPE were noted and 50 per cent end point of tissue culture infective dose ($TCID_{50}$) was calculated at the end of 4th day as per the method described by Reed and Muench (1938).

3.2.6 Immunization trials

Immunization trial was conducted, for 5th and 10th CEF passaged virus separately.

Twenty day old ducklings were purchased from a private agency. The ducks were reared upto six weeks of age. Then each of the fifteen ducklings were immunised intramuscularly (i/m) with 0.5 ml of 3.5 $\log_{10}$ $TCID_{50}$ of 5th passaged virus and five ducklings were kept as control. On 20th day post vaccination the ducklings were bled and serum was separated and stored at -20°C until used.

Similarly, another group of 15 ducklings at six week old were vaccinated with 10th CEF passaged virus having 3.5 $\log_{10}$ $TCID_{50}$ at the rate of 0.5 ml each by i/m route and serum collected on 20th day post vaccination was stored at -20°C and five ducklings were kept as control.

3.2.7 Titration of antibody

The serum samples collected from birds vaccinated with the 5th and 10th passaged viruses were used for serum neutralization test (SNT) after heat inactivation at 56°C for 30 min. Beta method of SNT was conducted in CEF cultures as per the method described by Hoskin (1967).

3.2.7.1 Serum neutralization test

Serial two fold dilutions of serum (1:2 to 1:128) were made in maintenance medium at pH 7.2. Equal quantity of 100 $TCID_{50}$ virus per 0.1 ml was added to each dilution and incubated at 37°C for 60 min for neutralization of virus. From each dilution 0.1 ml of serum virus mixture was inoculated into three CEF cell culture tubes and incubated at 38.5°C. The inoculated tubes were examined at 24 hr intervals until the virus control tubes showed CPE. The neutralization titre and virus neutralisation index were calculated as per the method described by Hitchner *et al.* (1975). Suitable antigen controls and uninfected cell controls were also incorporated in this test.

3.2.7.2 Passive haemagglutination test (PHA)

This test was standardised as per the method described by Vengris and Marie (1971) and Zyambo *et al.* (1973), with some modifications.

Sheep erythrocytes were formalinized by adding equal volume of ten per cent SRBC and three per cent formol saline and incubating this mixture at 37°C for 18 hr. The formalinized sheep erythrocyte was washed thrice and stored as ten per cent suspension in CMF-PBS. Tannic acid 1:20000 dilution was used for tanning the formalized erythrocyte. For

coating the antigen, two per cent formalinised tanned erythrocyte (FTE) was mixed with virus suspension containing 3 $\log_{10}$ $TCID_{50}$ per ml in CMF-PBS, pH 6.4.

Serum samples of 5th and 10th passage immunization trials were heat inactivated at 56°C for 30 min and adsorbed with 0.1 ml of ten per cent FTE per ml of serum to eliminate non specific agglutinin. After 30 min incubation at 37°C, the serum was recovered by centrifugation.

The test was performed in micro titre plate. Serum samples of 5th and 10th passaged immunization trials which were treated as above were serially diluted in diluent (one per cent inactivated rabbit serum in CMF-PBS) from 1:2 to 1:1028 in microtitre plates and equal quantity (50 μl) of two per cent antigen coated FTE were added to each well. The plates were kept at 4°C for 12-14 hr and results recorded. The highest dilution of serum giving 50 per cent agglutination of the antigen coated FTE was taken as the PHA titer of the serum. The controls incorporated in this tests were

1. Known +ve serum + Uncoated FTE
2. Known +ve serum + Antigen coated FTE
3. Known -ve serum + Antigen coated FTE
4. Diluent + Antigen coated FTE

3.2.7.3 Challenge test

Ducklings immunized with 5th and 10th passaged virus and respective control groups were challenged with 0.2 ml virulent virus by i/m route on 22nd day post vaccination. The ducklings were observed for 20 days post challenge for clinical signs and dead birds were necropsied and examined for specific lesions of DP.

3.2.8 Characterisation of 5th and 10th passage virus

3.2.8.1 pH sensitivity

The pH sensitivity of the 5th and 10th passage cell culture fluid was studied at the pH levels of 3, 7.2 and 11. One in four dilutions of the 5th and 10th passage viruses were prepared in citrate phosphate buffer (pH 3) and phosphate buffer (pH 7.2 and pH 11). These diluted samples were kept at room temperature for 3 hr. The samples were then neutralized with either 0.1 N sodium hydroxide or 0.1 N hydrochloric acid as the case may be. The degree of inactivation of the 5th and 10th passaged virus at different pH levels were determined by titrating the residual infectivity in CEF cell culture tubes. The inoculated tubes were incubated at 38.5°C and examined for presence of CPE at 24 hr intervals. The infectivity titre was calculated (Reed and Muench, 1938) after 96 hr. Suitable uninoculated cell culture tubes and untreated virus controls were incorporated in this test.

3.2.8.2 Thermostability

Fifth and tenth CEF passaged cell culture fluids were heated at 56°C for 30 min and then rapidly cooled at -20°C. The infectivity of both the samples were determined by inoculating the serial dilutions of the treated fluids into CEF cell culture tubes at the rate of 0.1 ml per tube, four tubes for each dilution. Suitable untreated virus control and uninoculated controls were also kept simultaneously. The tubes were examined for the presence of CPE at 24 hr intervals and infectivity titre was calculated (Reed and Muench, 1938).

Results

4. RESULTS

The DPV vaccine obtained from VBI, Palode was utilized in the study.

4.1 Revival of vaccine virus

Ten, 11 day old embryonated chicken eggs were inoculated with reconstituted vaccine virus via CAM route. The death of embryo was noticed 70-120 hr PI. The dead embryos showed extensive congestion all over the body and on CAM (Plate 1), necrotic areas in the liver and congestion on the spleen. The whole embryo along with AAF and CAM was collected and used for further passages in CEF cell culture.

4.2 Chicken embryo fibroblast cell culture

A confluent monolayer of CEF cell culture was observed at 20-24 hr. The monolayer showed elongated, spindle shaped fibroblast cells (Plate 2). The monolayer stained by May-Grunwald giemsa showed cells containing acidophilic cytoplasm and oval shaped, centrally located nuclei (Plate 3).

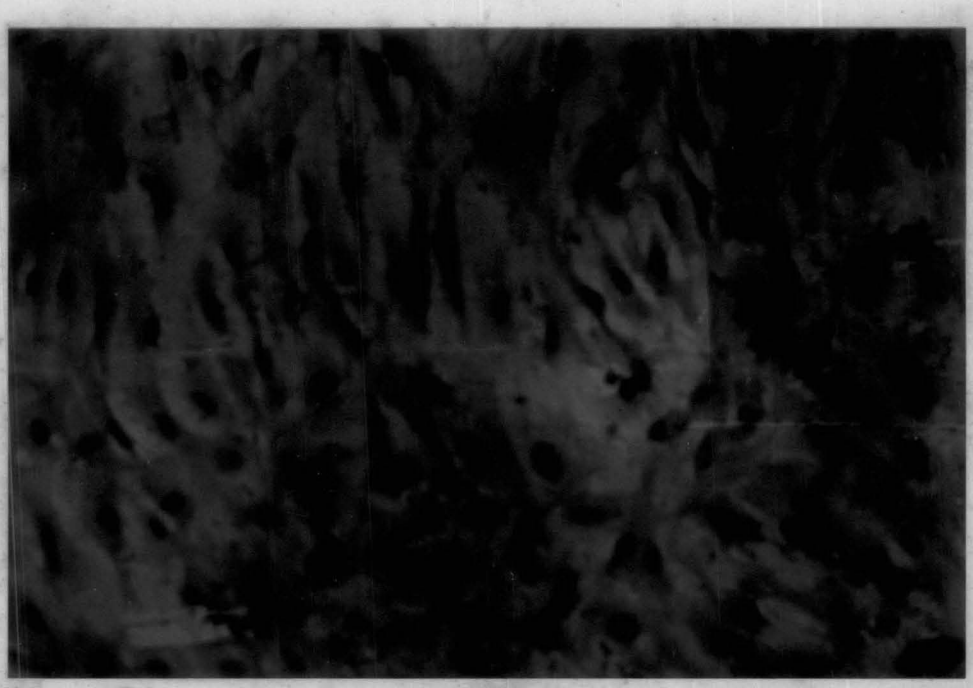
4.3 Cytopathic effects on CEF cell culture

The CEF cell cultures infected with DPV vaccine virus showed CPE at 48 hr post inoculation. The production of CPE

Plate 1. **Congestion on chicken embryo and CAM (inoculated with vaccine strain of DPV)**

Plate 2. **Normal monolayer of chicken embryo fibroblast (Unstained x 100)**

Plate 3. **Normal monolayer of chicken embryo fibroblast (May-Grunwald Giemsa stain x 400)**



was more pronounced at 38.5°C than at 37°C. The CPE was characterised by rounding and clumping of cells at 48 hr PI, vacuolation of cells at 72 hr PI, bridge formation and syncytium formation at 96 hr PI (Plate 4) and desquamation of cells was noticed at 120 hr PI.

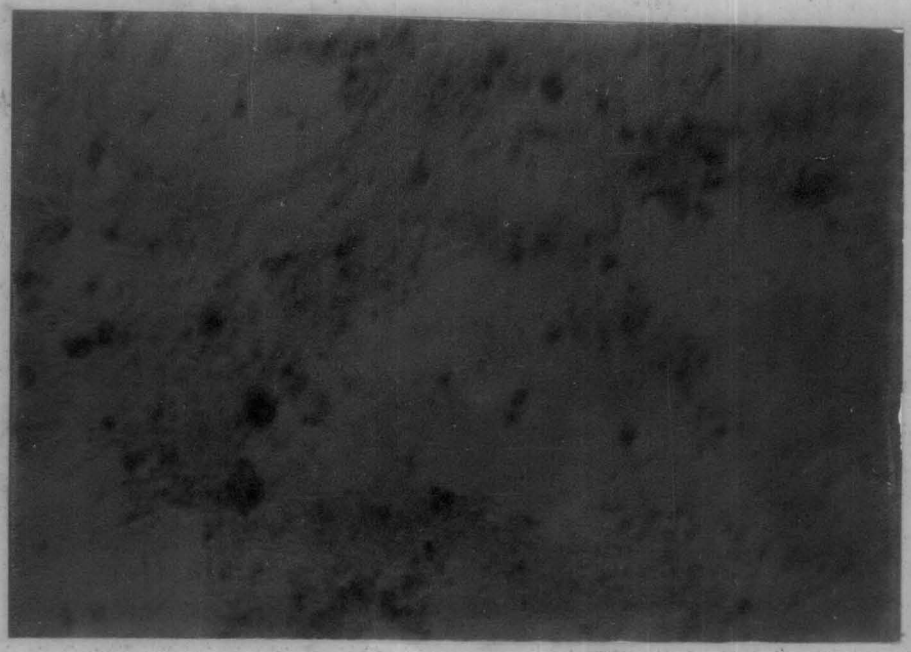
The infected monolayer stained with May Grunwald-Giemsa, showed syncytium formation (Plate 5). The cells showed extensive cytoplasmic vacuolations (Plate 6). and eosinophilic intranuclear inclusion bodies (Plate 7). Desquamation of cells were also observed (Plate 8).

4.4 Adaptation of virus

When the virus was serially passaged in CEF cell culture, the virus produced similar type of CPE. But the time taken for appearance of the CPE and complete desquamation of the cells varied with different passage levels (Table 1). The results obtained in the present study showed that the time required for onset of CPE and complete desquamation of cells reduced with increase in passages. The time taken for appearance of CPE reduced from 48 hr at first passage to 30 hr at 5th passage and 24 hr at 10th passage. Similarly time taken for complete desquamation of cells also reduced from 120 hr at first passage to 90 hr and 80 hr at 5th and 10th passages respectively.

Plate 4. **CPE of CEF infected with DPV vaccine strain at 96 hr - Bridge Formation and syncytium formation (Unstained x 100)**

Plate 5. **CPE of CEF infected with DPV vaccine strain at 96 hr - Syncytium formation (May-Grunwald Giemsa stain x 1000)**



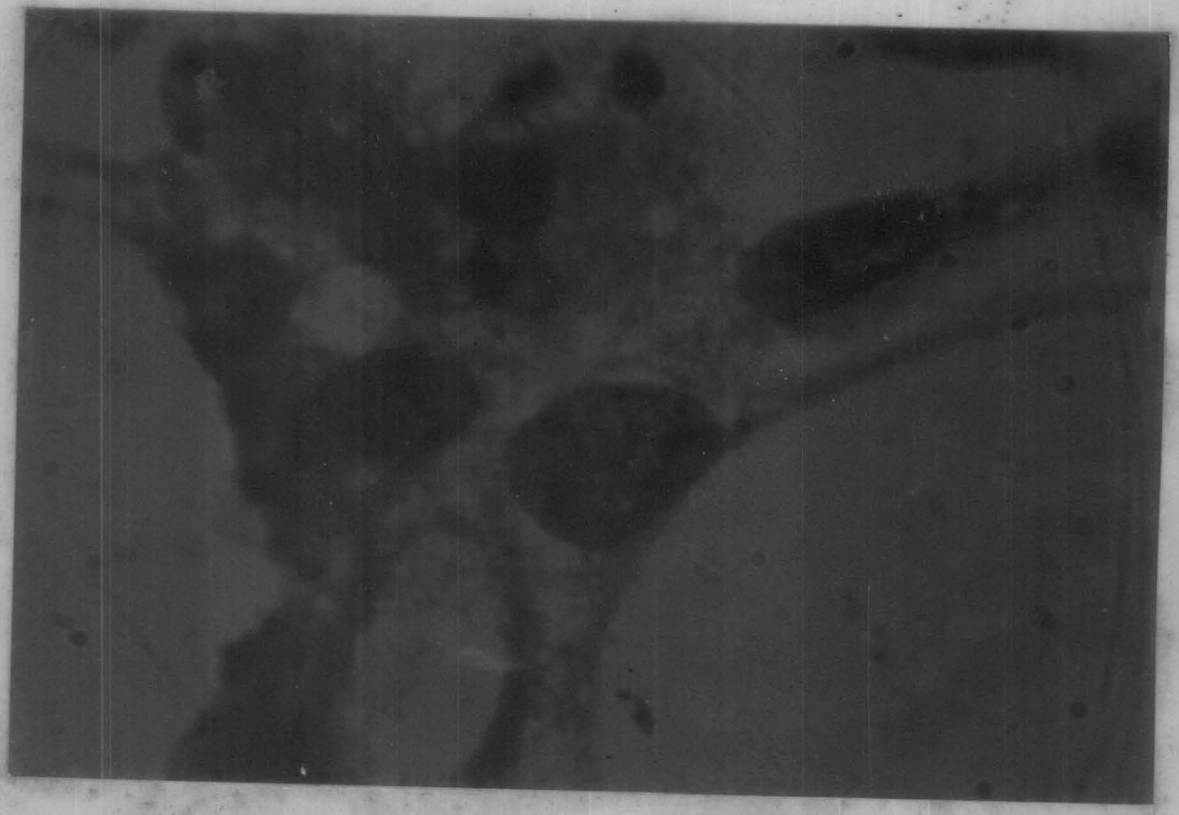
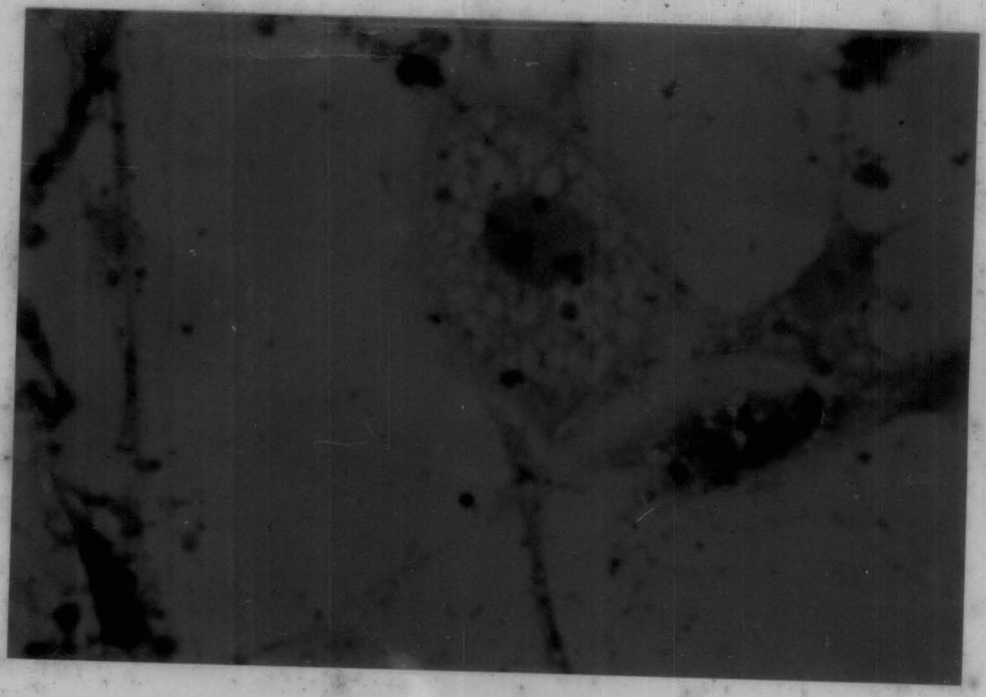


Plate 6. **CPE of CEF infected with DPV vaccine strain at 72 hr - Rounding of cells with extensive cytoplasmic vacuolations (May-Grunwald Giemsa stain x 1000)**

Plate 7. **Intra-nuclear inclusion bodies in CEF infected with DPV-vaccine strain (May Grunwald Giemsa stain x 1000)**



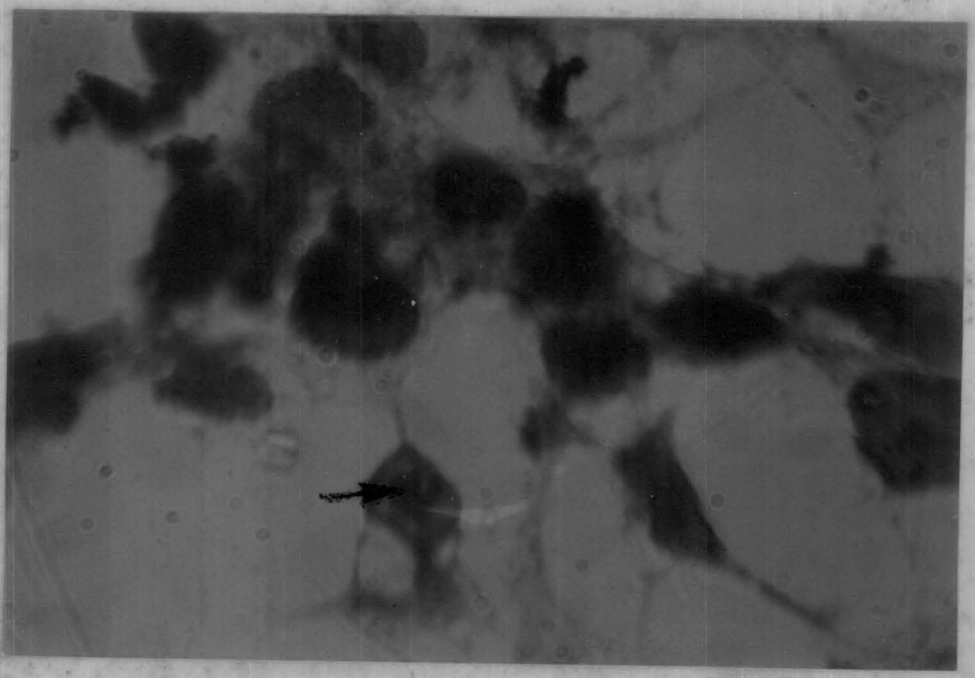


Plate 8. **CPE of CEF infected with DPV vaccine strain at 120 hr - Desquamation of cells (May Grunwald Giemsa stain x 1000)**

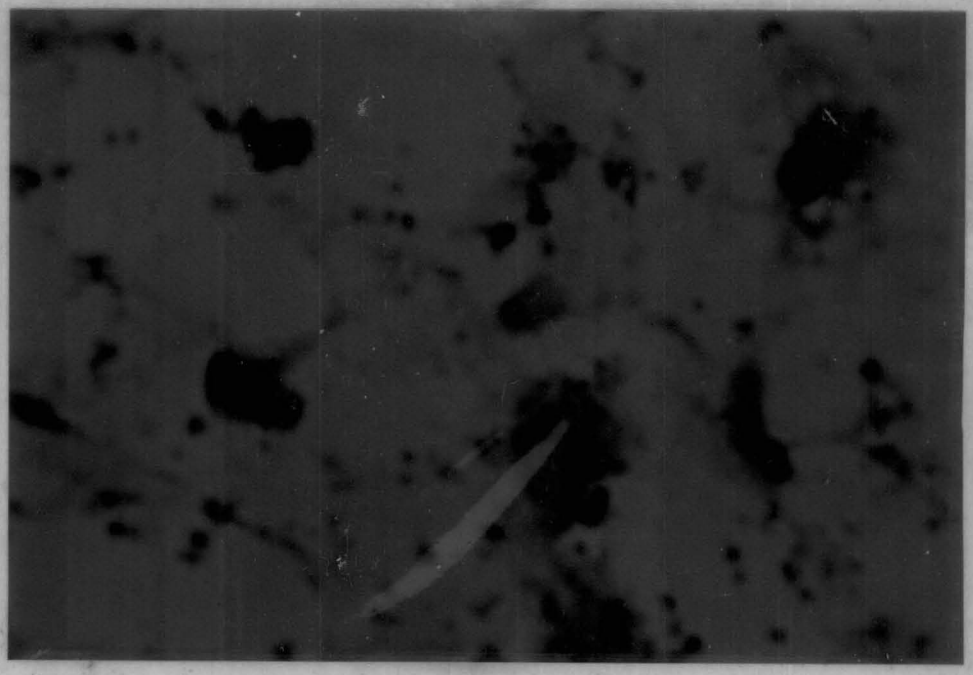


Table 1. Time of appearance of CPE and desquamation of cells at different passage levels

Serial passage	Appearance of CPE (in hr PI)	Desquamation of cells (in hr PI)
1	48	120
2	40	100
3	36	100
4	36	90
5	30	90
6	30	90
7	28	85
8	26	85
9	26	80
10	24	80

4.5 Titration of the virus

The vaccine strain, 5th and 10th passage viruses were titrated in chicken embryos. The 5th and 10th passage viruses were also titrated in CEF cell culture.

4.5.1 Vaccine virus

4.5.1.1 In developing chicken embryos

Ten fold dilutions of DP vaccine virus prepared in CMF-PBS was inoculated via CAM route at the rate of 0.2 ml per egg. The 50 per cent embryo lethal dose (ELD_{50}) was calculated as per Reed and Muench Method (1938). It was found to be 10^4/ml of inoculum (Table 2).

4.5.2 Fifth passage virus

4.5.2.1 In developing chicken embryo

The 5th passage virus was titrated in eleven day old embryonated eggs by inoculating virus by CAM route at the rate of 0.1 ml per egg. The titre was found to be $10^{4.75}$/ml of inoculum (Table 3).

4.5.2.2 In CEF cell culture

The tissue culture infective dose ($TCID_{50}$) of 5th passage virus was assessed by examining the coverslip cultures infected with ten fold dilutions of virus at the rate of

Table 2. Titration of DPV-vaccine strain in chicken embryo (ELD_{50})

Dilution	No. of eggs per dilution	No. of		Cumulative		+ve ratio	Percentage infected
		infected	non-infected	+ve	-ve		
10^{0}	3	3	0	12	0	12/12	100
10^{-1}	3	3	0	9	0	9/9	100
10^{-2}	3	3	0	6	0	6/6	100
10^{-3}	3	2	1	3	1	3/4	75
10^{-4}	3	1	2	1	3	1/4	25
10^{-5}	3	0	3	0	6	0/6	0
10^{-6}	3	0	3	0	9	0/9	0

$$\text{Proportionate distance} = \frac{\%\text{ of infectivity just above 50\% - 50}}{\%\text{ of infectivity just above 50\% - \% of infectivity just below 50\%}}$$

$$= \frac{75-50}{75-25} = \frac{25}{50} = 0.5$$

$$\text{Titre } (ELD_{50}) = 1\text{x}10^{3.5}/0.2 \text{ ml}$$

$$= 1\text{x}10^{4}/\text{ml}$$

Table 3. Titration of 5th CEF passaged virus in chicken embryo (ELD_{50})

Dilution	No. of eggs per dilution	No. of		Cumulative		+ve ratio	Percentage infected
		infected	non-infected	+ve	-ve		
10^{-1}	3	3	0	10	0	10/10	100
10^{-2}	3	3	0	7	0	7/7	100
10^{-3}	3	3	0	4	0	4/4	100
10^{-4}	3	1	2	1	2	1/3	33
10^{-5}	3	0	3	0	5	0/5	0
10^{-6}	3	0	3	0	8	0/8	0

$$\text{Proportionate distance} = \frac{100-50}{100-33} = \frac{50}{67} = 0.75$$

$$\text{Titre } (ELD_{50}) = 10^{3.75}/0.1 \text{ ml}$$

$$= 10^{4.75}/\text{ml}$$

0.1 ml per tube. Four tubes were used for each dilution. The infective dose was found to be $10^{5.67}$/ml of inoculum (Table 4).

4.5.3 Tenth passage virus

4.5.3.1 In developing chicken embryo

The tenth passage virus titrated in 11 day old embryonated eggs had a titre (ELD_{50}) of $10^{5.77}$/ml (Table 5).

4.5.3.2 In CEF cell culture

The $TCID_{50}$ of the tenth passage virus was assessed by infecting three CEF cell culture tubes per dilution at the rate of 0.1 ml per tube. It was found to be $10^{6.77}$/ml of inoculum (Table 6).

4.6 Immunogenicity of CEF passaged virus

The 5th and 10th passage viruses having titre of 3.5 $\log_{10}$ $TCID_{50}$ were used as vaccine. Two groups of ducklings comprising 15 each were vaccinated with 0.5 ml of either 5th or 10th CEF passaged virus by i/m route. The vaccinated birds did not show any untoward reaction during the observation period of 20 days.

Table 4. Titration of 5th CEF passaged virus in chicken embryo fibroblast cell culture ($TCID_{50}$)

Dilution	No. of tubes per dilution	No. of		Cumulative		+ve ratio	Percentage infected
		infected	non-infected	+ve	-ve		
10^{-1}	4	4	0	17	0	17/17	100
10^{-2}	4	4	0	13	0	13/13	100
10^{-3}	4	4	0	9	0	9/9	100
10^{-4}	4	4	0	5	0	5/5	100
10^{-5}	4	1	3	1	3	1/4	25
10^{-6}	4	0	4	0	7	0/7	0

$$\text{Proportionate distance} = \frac{100-50}{100-25} = \frac{50}{75} = 0.67$$

$$\text{Titre } (TCID_{50}) = 10^{4.67}/0.1 \text{ ml}$$

$$= 10^{5.67}/\text{ml}$$

Table 5. Titration of 10th CEF passaged virus in chicken embryo (ELD_{50})

Dilution	No. of eggs per dilution	No. of		Cumulative		+ve ratio	Percentage infected
		infected	non-infected	+ve	-ve		
10^{-1}	4	4	-	17	0	17/17	100
10^{-2}	4	4	-	13	0	13/13	100
10^{-3}	4	4	-	9	0	9/9	100
10^{-4}	4	3	1	5	1	5/6	83
10^{-5}	4	2	2	2	3	2/5	40
10^{-6}	4	0	4	0	7	0/7	0

$$\text{Proportionate distance} = \frac{83-50}{83-40} = \frac{33}{43} = 0.77$$

$$\text{Titre } (ELD_{50}) = 10^{4.77}/0.1 \text{ ml}$$

$$= 10^{5.77}/\text{ml}$$

Table 6. Titration of 10th CEF passaged virus in CEF cell culture ($TCID_{50}$)

Dilution	No. of tubes per dilution	No. of		Cumulative		+ve ratio	Percentage infected
		CPE +ve	CPE -ve	+ve	-ve		
10^{-1}	4	4	0	21	0	21/21	100
10^{-2}	4	4	0	17	0	17/17	100
10^{-3}	4	4	0	13	0	13/13	100
10^{-4}	4	4	0	9	0	9/9	100
10^{-5}	4	3	1	5	1	5/6	83
10^{-6}	4	2	2	2	3	2/5	40
10^{-7}	4	0	4	0	7	0/7	0

$$\text{Proportionate distance} = \frac{83-50}{83-40} = \frac{33}{43} = 0.77$$

$$\text{Titre } (TCID_{50}) = 10^{5.77}/0.1 \text{ ml}$$

$$= 10^{6.77}/\text{ml}$$

4.7 Titration of antibody

4.7.1 Serum neutralization test

The results of serum neutralization test with the sera collected from the ducks vaccinated with 5th and 10th CEF cell culture passaged viruses are shown in the Table 7 and 8. The SNT was performed in CEF cell culture. The results were recorded when the antigen control tubes showed marked CPE, usually at 72 hr PI. Neutralization was confirmed by the absence of CPE in the tubes inoculated with serum virus mixtures.

The mean SN titre of the ducks vaccinated with 5th passage virus was found to be 64 with virus neutralization index (VNI) of 1.8 on 20th day post vaccination.

However the 10th passage virus vaccinated group had a mean titre of 54. This serum had neutralization index of 1.73 on 20th day post vaccination.

4.7.2 Passive haemagglutination test

Passive haemagglutination test was standardised using tannic acid in 1:20000 dilution for tanning sheep erythrocytes. Cell culture adapted virus having 3 $\log_{10}$ $TCID_{50}$ was used as antigen. PBS pH 6.4 was used as buffer for

Table 7. Serum neutralization titre and index of ducks vaccinated with 5th CEF passaged virus

Dilution	No. of tubes per dilution	No. of		Cumulative		+ve ratio	Percentage infected
		CPE	non-CPE	+ve	-ve		
1:2 ($10^{-0.3}$)	4	0	4	0	22	0/22	0
1:4 ($10^{-0.6}$)	4	0	4	0	18	0/18	0
1:8 ($10^{-0.9}$)	4	0	4	0	14	0/14	0
1:16 ($10^{-1.2}$)	4	0	4	0	10	0/10	0
1:32 ($10^{-1.5}$)	4	0	4	0	6	0/6	0
1:64 ($10^{-1.8}$)	4	2	2	2	2	2/4	50
1:128 ($10^{-2.1}$)	4	4	0	6	0	6/6	100

Log_{50} per cent neutralization end point = -1.8 + (proportionate distance x log dilution factor)

= -1.8 + (0 x 0.3)

= -1.8

50 per cent neutralization end point = $10^{-1.8}$

= 1/64

50 per cent neutralization titre of serum = 1/64

Virus neutralization index = 1.8

Table 8. Serum neutralization titre and index of ducks vaccinated with 10th CEF passaged virus

Dilution	No. of tubes per dilution	No. of		Cumulative		+ve ratio	Percentage infected
		CPE	non-CPE	+ve	-ve		
1:2 ($10^{-0.3}$)	3	0	3	0	16	0/16	0
1:4 ($10^{-0.6}$)	3	0	3	0	13	0/13	0
1:8 ($10^{-0.9}$)	3	0	3	0	10	0/10	0
1:16 ($10^{-1.2}$)	3	0	3	0	7	0/7	0
1:32 ($10^{-1.5}$)	3	0	3	0	4	0/4	0
1:64 ($10^{-1.8}$)	3	2	1	2	1	2/3	66
1:128 ($10^{-2.1}$)	3	3	0	5	0	5/5	100

$$\text{Proportionate distance} = \frac{50\% - (\text{CPE at dilution next below } 50\%)}{\text{CPE at dilution next above } 50\% - \text{CPE at dilution next below } 50\%}$$

$$= \frac{50-0}{66-0} = \frac{50}{66} = 0.75$$

50 per cent neutralization end point = -1.5 + (proportionate distance x log dilution factor)

$$= -1.5 + (0.75 \times 0.3)$$

$$= -1.5 + 0.225$$

$$= -1.725 \text{ or } -1.73$$

$$= 10^{-1.73}$$

50 per cent neutralization titre of serum = 1/54

Virus neutralization index of serum = 1.73

coating the antigen to the formalinized tanned erythrocyte (FTE).

Hyperimmune serum that had titre of 64 was used as positive control. The PHA titres of sera of ducks vaccinated with 5th and 10th passaged viruses are presented in Table 9 and in Plate 9.

The mean PHA titre in birds vaccinated with 5th passaged virus was found to be 32, while the 10th passage vaccination group had a mean titre of 22. However the control birds did not show any PHA antibodies.

4.7.3 Challenge test

Two groups of six week old ducks (five each nos.) vaccinated with 5th or 10th passage viruses and another group of unvaccinated control birds were challenged with virulent DPV at the rate of 0.2 ml per bird by i/m route on 22nd day post vaccination. The results are presented in the Table 10.

All the birds vaccinated with 5th passage virus survived the challenge infection. They did not show any untoward reaction/symptoms during the observation period of 20 days. Similarly duck vaccinated with 10th passage virus also showed 100 per cent protection on challenge.

Plate 9. **Passive haemagglutination test**

A-E vaccinated duck sera
F - Known positive serum
G - Known negative serum
H - Diluent and antigen coated FTE

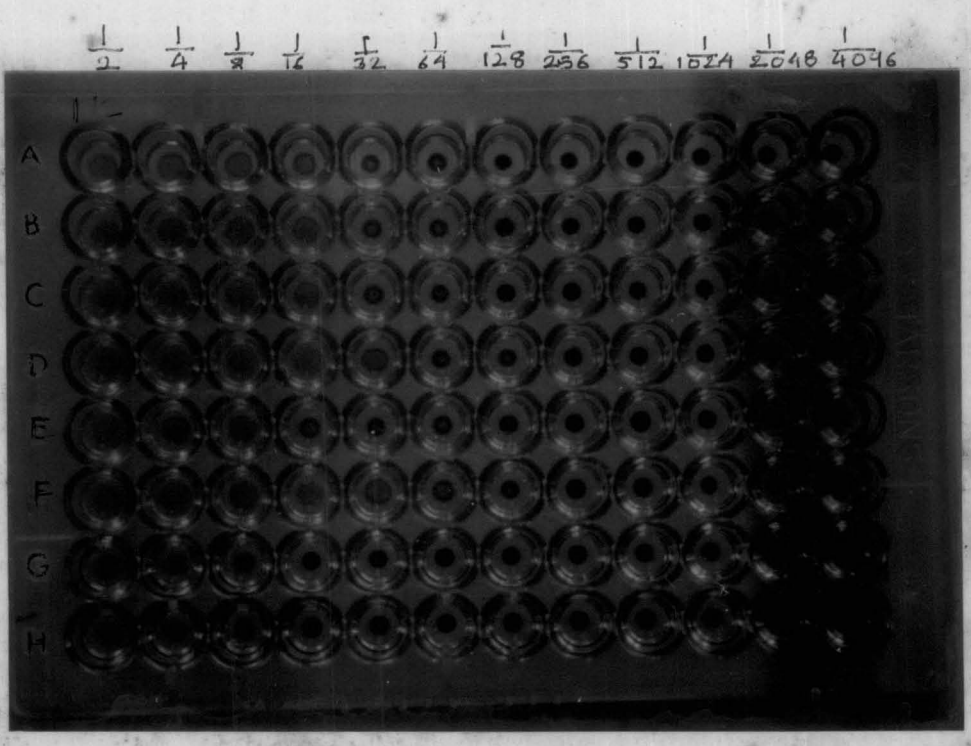

1/2
1/4
1/8
1/16
1/32
1/64
1/128
1/256
1/512
1/1024
1/2048
1/4096
A
B
C
D
E
F
G
H

Table 9. Passive haemagglutination titre of sera of ducks vaccinated with 5th and 10th CEF cell culture passaged virus

Group	PHA titre (Average)
5th passage vaccination	32
10th passage vaccination	22
Control group	Nil

Table 10. Protection percentage of 5th and 10th passage viruses after challenge

Groups	No. of birds	Survived	Dead	Percentage of protection
5th passage vaccination	15	15	0	100
10th passage vaccination	15	15	0	100
Unvaccinated	10	0	10	0

However the ducks in unvaccinated control group showed clinical signs such as anorexia, ocular discharge with pasty eyelids, nasal discharge, whitish diarrhoea, breast sitting posture and soiled vent. All the birds died during seven to nine day post challenge. On necropsy the dead birds showed necrotic areas and petechial haemorrhages on liver (Plate 10), enlargement of spleen with congestion, haemorrhagic enteritis and foci of necrosis on gizzard muscle. Some birds showed diphtheritic changes in oesophageal mucous membrane.

4.8 Characterisation of CEF passaged virus

4.8.1 pH sensitivity

The pH sensitivity of 5th and 10th passaged viruses were determined at three different pH viz. 3, 7.2 and 11. The results are shown in Table 11. The results obtained in the present study indicated that the 5th and 10th passage viruses were completely inactivated at the pH 3 and 11. The pH sensitivity was confirmed by failure of the virus to produce CPE in CEF cell culture. This was more evident as the virus could not produce CPE even at 10^0 dilution when compared to untreated virus samples.

However both 5th and 10th passaged viruses were unaffected when exposed to pH 7.2. This was confirmed by the

Plate 10. Experimentally infected duck with DP lesions

pH 7.2 exposed virus showing same type of CPE and same titre as that of control.

4.8.2 Thermostability

The results of thermostability test of 5th and 10th CEF passaged viruses are presented in Table 12. It is seen in the result that the 5th and 10th passaged viruses were completely inactivated when subjected to 56°C for 30 min, as shown by failure of the virus to produce CPE in CEF cell culture even at 10° dilution compared to untreated control.

Table 11. Effects of pH on the cell culture titre of 5th and 10th CEF passaged viruses

pH	$TCID_{50}$ of 5th passage virus	$TCID_{50}$ of 10th passage virus
3	Nil	Nil
7.2	$10^{5.67}$	$10^{6.77}$
11	Nil	Nil
Control	$10^{5.67}$	$10^{6.77}$

Table 12. Effects of temperature in cell culture titre of 5th and 10th CEF passaged viruses

Treatment	$TCID_{50}$ of 5th passage virus	$TCID_{50}$ of 10th passage virus
Heated at 56°C for 30 min	Nil	Nil
Control	$10^{5.67}$	$10^{6.77}$

Discussion

5. DISCUSSION

Since 1976 Kerala is endemic for duck plague. A chicken embryo adapted vaccine is routinely used in this state and elsewhere to control this disease. In spite of regular vaccination, outbreaks are being reported every year, both in vaccinated and unvaccinated flocks. However antigenic variation of isolates from the above outbreaks have not so far been reported by the conventional serological tests (Kulkarni, 1993) and even at the molecular level (Vijaysri, 1996; Taylor, 1997).

The chicken embryo vaccine presently used in Kerala and other parts of the country is prepared in conventional chicken embryos and not in SPF eggs. Hence there is a possibility of transmission of organisms causing egg borne diseases to the vaccines. More over in chicken embryos only low titres are obtained, while high titres are obtained in cell cultures. (Kalaimathi and Janakiram, 1990, Bordolai *et al.*, 1994).

This study was undertaken to adapt the chicken embryo vaccine strain of DPV in CEF cell cultures and to study the changes in the cell culture passaged virus if any, including its immunogenicity at various passage levels.

5.1 Revival of vaccine virus

Eleven day old chicken embryos inoculated with the vaccine strain of DPV died within 70-120 hr PI with extensive haemorrhages all over the body, congestion of CAM and spleen and necrotic areas in liver. Similar lesions were observed by Jansen (1961) and Mukerji *et al.*, (1965) when they passaged DP virus in chicken embryos.

5.2 Cytopathic effects in CEF cell culture

Chicken embryo fibroblast cultures, when infected with the vaccine strain revealed rounding and clumping of cells at 48 hr PI similar to the CPE described by Kunst (1967), Nair (1978), Kenwolf *et al.* (1974) and Taylor (1997) in DEF cell culture and Kalaimathi and Janakiram (1990) and Vijaysri (1996) in CEF cell culture. Syncytium formation noticed at 96 hr PI was in agreement with the observation made by Gough (1984) and Kalaimathi and Janakiram (1990). Vacuolation noticed in cytoplasm was similar to the findings of Panisup *et al.* (1990) under electron microscope and Vijaysri (1996) under light microscope. Desquamation of cells noticed at 120 hr PI in this study, was reported earlier by John (1988) and Kalaimathi and Janakiram (1990) in the same cell culture system.

In the present study the production of CPE was more pronounced at 38.5°C than at 37°C. Burgess and Yuill (1981a) recorded higher incubation temperature for cultivation of less virulent DPV. So the result of the present study with vaccine strain of DPV, agrees with the findings of Burgess and Yuill (1981a).

Breese and Dardiri (1968), Bergmann and Kinder (1982) and Barr *et al.* (1992) observed inclusion bodies in both nucleus and cytoplasm of the infected cells under electron microscope. However in the present study eosinophilic inclusion bodies were observed only in nucleus, which is in agreement with the findings of Gough (1984), Panisup *et al.* (1990) and Vijaysri (1996) who reported inclusion bodies only in the nucleus of infected DEF cells. Vijaysri (1996) and Taylor (1997) also reported intranuclear inclusion bodies in infected CEF cells. Leibovitz (1971) and Rajan *et al.* (1980) have demonstrated eosinophilic intranuclear inclusions in tissue sections of infected cells stained with haemotoxylin and eosin under light microscope.

5.3 Adaptation of virus

As the number of passages increased the time taken for appearance of CPE reduced from 48 hr at first passage to 30 hr at fifth passage and 24 hr at 10th passage. This corroborated with the findings of Kalaimathi and Janakiram

(1990) and Bordolai *et al.* (1994) who reported a reduction in time for appearance of CPE with increase in the number of passages.

The time taken for complete detachment of cells was also reduced from 120 hr at first passage to 90 hr and 80 hr at fifth and 10th passages respectively. This concurs with the findings of Kalaimathi and Janakiram (1990) and Bordolai *et al.* (1994) who reported rapid detachment of cells as the number of passages increased.

Kalaimathi and Janakiram (1990) and Bordolai *et al.* (1994) have opined that decrease in the time taken for production of CPE and desquamation of cells indicated adaptation of the virus into the CEF cell culture, which substantiates the findings of the present study where, there was reduction in time taken for appearance of CPE and detachment of cells.

5.4 Titration of virus

5.4.1 In chicken embryo (ELD_{50})

Fifth and 10th passage viruses were titrated in both chicken embryo and cell culture. This virus gave higher titre in cell culture ($TCID_{50}$) than in embryonated chicken eggs (ELD_{50}).

The present study showed an increase in ELD_{50} from 10^4/ml for the vaccine virus to $10^{4.75}$/ml for fifth passage virus and $10^{5.77}$ for 10th passage virus. Vijaysri (1996) obtained ELD_{50} of 10^4/ml for the vaccine virus, which is in agreement with our finding. Bordolai *et al.* (1994) reported an increase in titre of virus from $10^{5.3}$ ELD_{50}/ml on fifth passage to $10^{6.5}$ ELD_{50}/ml on 12th passage and attributed it to the adaptation of the virus to the CEF cell culture system. Thus the increase in titre as passages advanced observed in the present study could be attributed to adaptation of the virus to the cell culture system as observed by Bordolai *et al.* (1994).

5.4.2 In CEF cell culture

The titres of 5th and 10th passaged viruses were $10^{5.67}$ and $10^{6.77}$ $TCID_{50}$/ml respectively. Vijaysri (1996) obtained a titre of 10^5/ml for the vaccine strain in CEF cell culture. In this study the titre increased by $10^{0.67}$ in the 5th passage and by $10^{1.77}$ in the 10th passage as compared to the vaccine virus (10^5/ml). Kalaimathi and Janakiram (1990) obtained an increased titre from $10^{4.8}$ at the first passage to $10^{6.83}$ at the 12th passage which they attributed to adaptation of the virus to the cell. An increased titre recorded in the present study could be due to the adaptation of the virus to CEF cell culture system as suggested by Kalaimathi and Janakiram (1990). The results of the present study also showed an increase in virus titre compared to chicken embryos.

5.5 Immunogenicity of CEF passaged virus

The two groups of ducklings vaccinated with either 5th or 10th CEF passaged virus did not show any untoward reaction during the observation period of 20 days, indicating that the cell culture adapted vaccine was safe to the ducks.

5.6 Titration of antibody

5.6.1 Serum neutralization test

The birds vaccinated with 5th passage virus showed an average SN titre of 64 (VNI of 1.8) and 10th passage virus vaccinated ducks showed SN titre of 54 (VNI of 1.73) on 20th day post vaccination. A reduction in SN titre and VNI was noticed as passages increased. Bordolai *et al.* (1994) used a virulent field isolate of DPV for CEF passage after initially passaging it for 12 times in duck embryos and subsequently 20 times in chicken embryos. This virus after 12 passages in CEF cultures was safe and produced protective immunity and the VNI ranged between non detectable to 1.31 at the third week and 1.78 by the sixth week.

Toth (1971) had observed an average VNI of 1.7 only after second vaccination using DVE-MLV while Kulkarni (1993) reported VN indices of 0.70 and 1.19 for single commercial vaccine and single lab adapted vaccine respectively. In the present study higher VNI of 1.8 was obtained using 5th CEF

passaged virus after first vaccination. This indicates that CEF passaged virus is better suited than CE adapted virus in inducing higher neutralising antibodies.

Dardiri (1975) and Butterfield and Dardiri (1969) have reported VNI of more than 2.5 in ducks recovered from the natural infection. Mukit *et al.* (1988) obtained VNI of 3.8 in ducks which withstood the challenge infection. These findings indicated that higher VNI could be expected in birds surviving natural/challenge infection.

Viral interference (Toth, 1971) and humoral immune response (Dardiri, 1975) are considered to be of prime importance in immunity against DP. But the low SN titre reported in the present study even when the birds were withstanding challenge indicate that immunity to DP may be a combined effect of both humoral and cell mediated immunity.

5.6.2 Passive haemagglutination test

The 5th and 10th CEF passaged virus vaccinated ducks had titres of 32 and 22 respectively on 20th day post vaccination. Kulkarni (1993) reported mean PHA titre of 12.66 for single CE lab adapted DPV vaccinated ducks and 9.33 for single commercial CE vaccine immunized ducks, on the fourth week of vaccination. This shows that the cell culture adapted vaccine has given higher PHA titre than that of lab adapted and

commercial vaccines. Kulkarni (1993) had observed an average PHA titre of 34.66 in double vaccinated ducks, but in the present study a PHA titre of 32 was observed in single vaccinated group with fifth CEF passaged virus.

The use of PHA test for assessing the immunogenicity of the CEF cell culture adapted DPV vaccine strain is the first attempt of its kind and hence no comparison could be made. The higher PHA titre obtained in the present study as compared to the titres obtained by Kulkarni (1993) using CE adapted vaccine could be probably due to the low titre and lower immunogenicity of the vaccine virus used in his study.

The higher PHA titre of 32 obtained in the present study using 5th passage virus compared to 22 obtained with 10th passage virus suggest that immunogenicity decreases after the optimum number of passages. This is to be confirmed by further studies. It can be concluded that passaging of the virus in cell cultures will be beneficial only upto certain level and thereafter the immunogenicity is likely to wane. PHA has added advantages of simplicity, specificity and rapidity, compared to the SNT.

5.6.3 Challenge test

The 5th and 10th CEF passaged virus vaccinated ducks showed 100 per cent protection when challenged on 22nd day

post vaccination and these ducks did not show any untoward reaction during the observation period. This concurs with findings of Bordolai *et al.* (1994) who reported that the cell culture attenuated vaccine gave 100 per cent protection to the vaccinated ducks on challenge and further stated that vaccinated ducks did not show any untoward reaction.

All the control ducks in the study died on seven to nine days post challenge. Similarly Kalaimathi and Janakiram (1991) and Bordolai *et al.* (1994) have also reported death of the control ducks on challenge while the vaccinated ones remained healthy.

The control ducks showed clinical signs such as anorexia, ocular discharge with pasty eyelids, nasal discharge, whitish diarrhoea and squatting posture which were typical of DP and were in agreement with findings of Rajan *et al.* (1980) and Leibovitz (1991). On necropsy the dead birds showed necrotic areas and petechial haemorrhages on liver, congestion of spleen, haemorrhagic enteritis and diphtheritic changes on oesophagus. These observations were similar to the lesions of DP recorded by Leibovitz (1971), Duraiswami *et al.* (1979) and Rajan *et al.* (1980).

5.7 Characterisation of CEF passaged virus

5.7.1 pH sensitivity

pH sensitivity is considered to be a criterion for characterisation of virus. The results presented here showed that 5th and 10th passage viruses were completely inactivated when they were exposed to pH 3 and 11 at room temperature for 3 hr. This is in agreement with the findings of Hess and Dardiri (1968), who reported complete inactivation of DPV at pH 3 and 11 at 37°C. Kunst (1968) also found that the virus was completely inactivated when it was exposed to pH 3 for a period of three hr.

Similarly Sarmah *et al.* (1997) also observed reduction in titre of CEF passaged virus when exposed to pH 3 and 11. These findings indicated that the virus is sensitive to pH 3 and 11, irrespective of temperature.

The results obtained in the study showed both 5th and 10th passage viruses are stable at pH 7.2. This concurs with the findings of Nair (1978) who reported that the DPV was stable at pH 7.2. This indicates that CEF passage of the virus did not influence the pH sensitivity of the virus.

5.7.2 Thermostability

Thermostability is also one of the physical characters used in the characterisation of viruses. The results showed that the 5th and 10th passage viruses were completely inactivated when exposed to the temperature of 56°C for 30 min. These findings coincide with those of Hess and Dardiri (1968), Kunst (1968) and Nair (1978), who also observed that the virus was completely inactivated when subjected to 56°C for 30 min.

Similarly Sarmah *et al.* (1997) reported that fifth CEF passaged DPV completely lost its infectivity when heated to 56°C and 60°C for 15 min. These findings indicated that there was no change in the property of the virus eventhough it was passaged in CEF cell culture incubated at 38.5°C.

5.8 Conclusion

The vaccine strain of duck plague virus produced specific lesions in chicken embryos, indicating that it was well adapted to chicken embryo cells.

The CPE produced by vaccine strain of DPV in chicken embryo fibroblast cell culture was similar to the CPE in DEF cell cultures. On serial passage in CEF cell culture the time taken for the appearance of CPE and total detachment of

infected cells were reduced as the number of passages increased. But the titre of the virus increased as the number of passages progressed from $10^{5.67}$ at 5th passage to $10^{6.77}$ $TCID_{50}$ per ml at 10th passage. This might be due to the adaptation of the virus to CEF cell culture and its rapid replication. Hence CEF cell system could be recommended for cultivation of vaccine strain of DPV for large scale production.

Ducklings vaccinated with 5th and 10th passage virus had SN titre of 64 (VNI of 1.8) and 54 (VNI of 1.73) and mean PHA titre of 32 and 22 respectively on 20th day post vaccination. The 5th passage virus, induced higher antibody titre than 10th passage virus indicating that the optimum adaptation of the virus without compromising immunogenicity might have occurred at or around fifth passage. Lower antibody titres in the 10th passage virus vaccinated ducks suggested that the virus might have lost some of its immunogenicity. However a detailed study is required to confirm this.

The 5th and 10th passage virus induced enough antibody titres in ducks, enabling them to withstand the challenge.

The 5th and 10th CEF passaged viruses were sensitive to pH 3 and 11, but stable at pH 7.2. Likewise these viruses were completely inactivated when exposed to 56°C for 30 min.

The results of the present study using 5th and 10th CEF passaged DP virus indicated that these induced enough antibody titres in ducklings, evidenced by higher SN titre, PHA titre and cent per cent protection, when challenged with virulent virus. Thus the cell culture passaged virus at its fifth passage level is ideal for vaccination as it induces higher SN and PHA titres and afforded 100 per cent protection to ducks challenged with virulent virus.

Summary

SUMMARY

Chicken embryo adapted vaccine strain of duck plague virus received from VBI, Palode was revived by inoculation into 11 day old embryonated chicken eggs by the CAM route. The infected embryos died 70 to 120 hr PI, with congestion all over the body, enlarged liver and spleen and necrotic areas in the liver. The allanto amniotic fluid, CAM and embryo were collected and processed for CEF passages.

The confluent CEF cell culture infected with the embryo passaged virus showed rounding and clumping of cells, syncytium and bridge formation with extensive vacuolation in the cytoplasm and eosinophilic intra nuclear inclusion bodies. The cells got detached from the glass surface by 120 hr PI.

The virus was serially passaged in CEF cell culture upto 10th passage to adapt the virus. The virus produced similar types of CPE at every passage, but the time taken for the appearance of CPE and desquamation of cells was reduced as the number of passages increased. Adaptation of the virus in CEF cell culture was evident from the time required for the appearance of CPE and desquamation of cells and by titration of virus of different passages.

The time taken for the appearance of CPE was reduced from 48 hr at first passage to 30 hr at fifth passage and 24 hr at

10th passage. Similarly the time required for complete desquamation of cells was also reduced from 120 hr at first passage to 90 hr and 80 hr at fifth and 10th passages respectively.

The titre of the virus increased as the passages progressed. The ELD_{50} of the fifth and 10th passage virus in chicken embryos were $10^{4.75}$ and $10^{5.77}$ per ml respectively, which was higher than the vaccine virus (10^4/ml). Similarly fifth and 10th passage CEF passaged virus had $TCID_{50}$ of $10^{5.67}$ and $10^{6.77}$ per ml respectively in CEF cell culture.

The rapid onset of CPE and increasing virus titre as the passages progressed in CEF cell culture, indicated adaptation of the virus into the cell system.

The immunogenicity of the CEF passaged virus was studied by immunising ducks and quantitation of antibodies by SNT and PHA test and also by challenge test.

Two groups of six weeks old ducklings were immunised intramuscularly either with 5th or 10th passage virus at the rate of 3.5 $\log_{10}$ $TCID_{50}$ per bird. The ducks did not show any untoward reaction during the observation period of 20 days. The average antibody titre of the sera of birds vaccinated with the fifth passage virus collected on the 20th day post vaccination were 64 (VNI of 1.8) and 32 by SNT and PHA

respectively. The above values in birds vaccinated with the 10th passage virus were 54 (VNI of 1.73) and 22.

The higher titre induced by fifth passage virus compared to 10th passage virus indicated that the optimum adaptation might be at or around the 5th passage and there after it is losing its immunogenicity.

On challenge, ducks in both the groups showed 100 per cent protection. The vaccinated ducks did not show any untoward reaction during the observation period. The unvaccinated control ducks showed anorexia, ocular discharge, whitish diarrhoea and died seven to nine day post challenge. On necropsy, the dead birds showed necrotic areas in liver, congestion and enlargement of spleen and haemorrhagic enteritis.

The pH sensitivity and thermostability of CEF passaged virus was done to characterise the virus. The 5th and 10th passaged viruses were completely inactivated, when heated at 56°C for 30 min. Similarly the 5th and 10th passaged viruses lost their infectivity when exposed to pH 3 and 11, though they were stable at pH 7.2. The results of these studies indicated that there was no change in these characters when the virus was serially passaged in CEF cell culture at 38.5°C.

From the results of this study, it was concluded that the vaccine strain of DPV when passaged in CEF culture retained its immunogenicity upto fifth passage and it could be tried for large scale vaccine production.

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* Originals not seen

INFLUENCE OF ADAPTATION OF THE VACCINE STRAIN OF DUCK PLAGUE VIRUS IN CHICKEN EMBRYO FIBROBLAST ON ITS IMMUNOGENICITY

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ABSTRACT OF A THESIS

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ABSTRACT

A chicken embryo adapted vaccine strain of duck plague virus was serially passaged in chicken embryo fibroblast cell cultures and its immunogenicity was evaluated at different passage levels.

The vaccine strain of DPV received from VBI, Palode was revived in 11 day old chicken embryos by CAM route. The infected embryos died in 70 to 120 hr PI with lesions of congestion on the embryo and CAM and enlargement of liver and spleen. This embryo passaged virus was propagated in CEF cell culture, prepared from 12 day old embryonated chicken eggs. The virus produced CPE, characterised by rounding and clumping of cells, syncytium formation, vacuolation of cytoplasm and eosinophilic intranuclear inclusion bodies.

The virus was adapted in CEF cultures by serial passage. It was passaged for ten times and the various characters of the fifth and 10th passaged viruses were studied. There was no change in the CPE but the time required for the appearance of CPE and total detachment of the cells decreased as the passages increased. The CPE appeared at 48 hr, 30 hr and 24 hr for first, fifth and 10th passages respectively. Similarly the time required for total detachment of cells also reduced

from 120 hr at first passage to 90 hr at fifth passage and 80 hr at 10th passage. The rapid onset of CPE and desquamation of cells indicated the adaptation of the virus in CEF cell culture.

The titres of fifth and 10th passage viruses in chicken embryos were $10^{4.75}$ and $10^{5.77}$ ELD_{50}/ml respectively. The titres in CEF cultures were slightly higher. The values were $10^{5.67}$ and $10^{6.77}$ $TCID_{50}$/ml respectively for the fifth and 10th passaged samples.

The immunogenicity of the fifth and 10th passage viruses were studied by vaccinating six weeks old ducklings. Each duckling received 3.5 $\log_{10}$ $TCID_{50}$ of either fifth or 10th passaged virus intramuscularly. The birds remained normal till the 20th day and when challenged with virulent virus.

Birds that received the fifth passaged virus showed mean antibody titres of 64 and 32 by SNT and PHA respectively. All the birds withstood challenge indicating the effectiveness of fifth CEF passaged virus as a vaccine. In birds that received the 10th passaged virus, the antibody titres were low both by the SNT (1:54) and PHA (1:22). However all the ducks survived without manifesting any clinical signs. All the control ducks developed clinical signs of DP and died in seven to nine days time.

The fifth and 10th CEF passaged viruses were sensitive to pH 3 and 11, but stable at pH 7.2. They were completely inactivated at 56°C in 30 min. These indicated that there was no change in the above physical characters of the virus though it was passaged in CEF cultures incubated at 38.5°C.

Though the efficacy of the 10th passage virus was slightly lower as it was evident from the low antibody level, a detailed study is required to establish the present findings that an increase in the number of passages would result in decreased immunogenicity of the DPV vaccine strain. However from the results obtained during this study, it is evident that cell culture adapted DP vaccine strain could be recommended for production of vaccine against DP.