ASSESSMENT OF IMMUNITY TO DUCK PLAGUE VIRUS (DUCK VIRUS ENTERITIS) ON VACCINATION

BY

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THESIS

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DECLARATION

I hereby declare that this thesis entitled **Assessment of immunity to duck plague virus (duck virus enteritis) on vaccination** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other university or society.

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Certified that this thesis entitled **Assessment of immunity to duck plague virus (duck virus enteritis) on vaccination** is a record of research work done independently by **Dr. Diwakar Dattatrayrao Kulkarni** 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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To

LATE DR. M. N. KULKARNI

the constant source of my inspiration

CERTIFICATE

We, the undersigned members of the advisory committee of **Dr. Diwakar Dattatrayrao Kulkarni**, a candidate for the degree of Ph.D. in Microbiology agree that the thesis entitled **Assessment of immunity to duck plague virus (duck virus enteritis) on vaccination** may be submitted by Dr. D.D. Kulkarni in partial fulfilment of the requirement for the degree.

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ABBREVIATIONS USED IN THE THESIS

AAF	-	Allanto-amniotic fluid
CAM	-	Chorio-allantoic membrane
CEF	-	Chicken-embryo-fibroblast
DAV	-	The group of ducks receiving one dose of lab-adapted vaccine at 6th week and the second at 10th week of age.
DCE	-	Developing chicken embryo
DCV	-	The group of ducks receiving one dose of commercial vaccine at 6th week and the second at 10th week of age.
ELD 50	-	Embryo lethal dose 50
FTE	-	Formalinized-tanned sheep erythrocytes
HBSS	-	Hank's balanced salt solution (Hi-media)
LM	-	Leucocyte migration
LMI	-	Leucocyte migration inhibition
LMIT	-	Leucocyte migration inhibition test
MDT	-	Mean death time
MI	-	Migration index
ND	-	Not done
NS	-	Non-significant
NT	-	Not tested
NVC	-	Group of unvaccinated ducks used as controls
pdv	-	Post-double vaccination

PEG	-	Polyethylene glycol 6000
PHa	-	Phytohaemagglutinin
PHA	-	Passive haemagglutination
PI	-	Post-infection/Post-inoculation
p.i.	-	Pathogenicity index
PTA	-	Phosphotungstic acid
PV	-	Post-vaccination
SAV	-	Group of ducks receiving only one dose of lab-adapted vaccine at 6th week of age
SCV	-	Group of ducks receiving only one dose of commercial vaccine at 6th week of age
VN Ab	-	Virus neutralizing antibody
VNI	-	Virus neutralizing index

Introduction

INTRODUCTION

Asia is considered to be the homeland of domesticated ducks. In most of the watershed areas and coastal states in India duck rearing is popularly practised. Lately, the duck farming is catching the attention of the progressive farmers in rural areas as a diversion to the over-concentrated poultry industry. According to the 1984 census, the duck population in the country was around 15 millions and the egg production 5 millions (Sreenivasaiah, 1987).

The duck and chicken supplement the protein requirement of the human population. While organized poultry sector is largely dependent upon the food-grains, thus competing for human food, the advantages of duck farming are manyfold. The cost of production of eggs from ducks under free-range system of management is relatively very less. The ducks are endowed with good foraging capacity and are generally resistant to many avian diseases. Their role in biological control of some invertebrate vectors is an additional advantage.

The major infectious diseases with considerable economic losses in ducks in India are duck plague (DP) and pasteurellosis. While the bacterial diseases can be controlled by treatment, viral diseases like DP are the cause

of worry among duck farmers. Several DP outbreaks have resulted in heavy mortality in ducks despite regular vaccinations. The carrier status and excretion of large quantities of the virus under stress probably perpetuates the situation (Burgess, 1981).

Majority of the duck-farmers follow those managemental practices which will necessitate them to move the flocks from one place to another for at least 6 months in a year. During harvesting season, they migrate for ensuring ample availability of feed; during summer months, for utilizing the perinneal water sources of different areas,not only in the state but very often outside the state. During such migrations, the ducks get exposed to various adverse conditions including environmental and industrial pollutants, bacterial and viral agents etc. During recent outbreaks it has been clearly seen that while almost all migrating duck population was affected with DP, the nonmigrating closed flocks in organized farms, remained free of any infection.

The role of immunity in conferring protection against DP is not very clear. Several reports have indicated that there is no positive correlation between humoral antibodies and protection against the disease. On the other hand, Lam (1984) and Lam and Lin (1986) have demonstrated that the resistance can be transferred to a susceptible duck by

inoculating hyperimmune serum. Looking at the nature of herpes virus, which modify the antigens on the cell membrane and bud off from the surface as the infectious particles, the local or circulating antibodies alone would be inadequate to control the virus infection (Roitt, 1977). Not much work has been done on cell-mediated immune response of the ducks against duck plague.

Many of the reports from field regarding DP incidence are based on clinical findings. In the absence of systematic studies relating to epidemiological observations supported by virus isolation, it is difficult to indicate that all the mortality is solely due to duck plague. Dardiri (1975) had incriminated the mortality during DP outbreaks to latent and secondary microbial invaders. It was not known whether the outbreaks even among vaccinated lots were due to immunosuppressive effects of the virus, or any hypervirulent strain or any technical fault with vaccine or vaccination procedure. To have a better understanding of these aspects the present study was undertaken with the following objectives.

1. Isolation and characterization of DP virus from field outbreaks.

2. Assessment of immune responses to DP vaccine and duration of immunity in vaccinated ducks.

Review of Literature

REVIEW OF LITERATURE

This chapter comprises a comprehensive review of publications on duck plague (DP) relevant to the areas of present study.

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

2.1 HISTORY

The recognition of duck plague as a specific disease evolved from repeated observations of occurrences in domesticated ducks over an extended period in the limited geographical area of the Netherlands. The description of each occurrence was a segment of continuous commentary and reappraisal of the cause of the disease (Jansen, 1961).

The first outbreak of DP was recorded by Baudet (1923) in the Netherlands. He observed an acute disease among ducks in a village called Bruekelen with cent per cent mortality. It was believed to be due to a specific duck adapted strain of

fowl plague (influenza virus). The second outbreak (DeZeeuw, 1930) occurred seven years later. Being identical with that of earlier one, the author too made the diagnosis as fowl plague. In both cases the specimens were not preserved for further study. It was Bos (1942) who, on observing new outbreaks, re-examined the earlier findings and concluded that the disease was not due to fowl plague virus, but a new distinct viral entity, and termed it as 'duck plague'. He did not study this infectious agent but preserved the material. The fourth outbreak was observed eleven years later by Jansen et al. (1952). The ducks surviving this outbreak were immune to the earlier virus preserved by Bos. An account of these initial eight outbreaks in the Netherlands during 1923-1960 has been given by Jansen (1961).

In the XIV International Veterinary Congress at London, the name 'duck plague' was approved as suggested by Jansen and Kunst in 1949. The disease was continued to be recognised as DP till its first report from the United states of America in 1967 (Leibovitz and Hwang, 1968), when the name duck virus enteritis (DVE) was introduced. This change was motivated by the fact that the clinical signs were predominantly in the gastro-intestinal tract and the consumers were scared by the word 'Plague' (Dardiri, 1975). Based on the principle features of the disease, and also to distinguish

it from fowl plague, DVE has become preferred term in the USA (Leibovitz, 1991).

2.2 INCIDENCE AND DISTRIBUTION

Apart from the Netherlands, DP has been reported from France (Lucam, 1949), Belgium (Devos et al., 1964), India (Mukerjee et al., 1963a), USA (Leibovitz and Hwang, 1968), Britain (Hall and Simmons, 1972), Canada (Hanson and Willis, 1976), Thailand (Suwatviroj et al., 1977), Bangladesh (Sarkar, 1982), Hungari (Vetesi et al., 1982), Denmark (Prip et al., 1983), Germany (Ziedler et al., 1984), China (Kunst, 1958; Wang et al., 1984), Russia (Simonova et al., 1984), and Austria (Pechan et al., 1985).

The first official report of DP in India was from West Bengal in 1963 (Mukerjee et al., 1963a, b; Jansen and Kunst, 1964). The serum samples from recovered ducks were sent to Dr. Jansen in Netherlands, which were neutralized by DP virus (Dutch strain). Earlier to this report, a severe duck mortality occurred in Tamil Nadu during 1944-45 and in the opinion of Jansen (1964) the cause was duck plague. Later, Jansen and Kunst (1964) reported isolation of DP virus (DPV) from India. The reports of DP are still coming from West Bengal (Bhowmik and Chakrabarty, 1985; Bhowmik and Ray, 1987).

In Kerala, heavy mortality in ducks was reported in Alleppy district during 1976-77 (Punnoose and Abdulla, 1976; Nair, 1978; Rajan et al., 1980). Since then, Kerala state has become endemic for DP and regular reports are received from Alleppy, Kottayam, Pattanamthitta, Thrissur and other waterlogged areas (Punnoose et al., 1993).

In Tamil Nadu DP outbreaks were confirmed by Duraiswamy et al. (1979) from North Arcot and Thanjavur district.

The first outbreak of DP in Assam in 1978 was reported by Chakrabarty et al. (1980). Similarly the virus isolation from an outbreak was reported by John et al. (1992).

In Andhra Pradesh, DP has been reported only in adult birds with high mortality (Sreeramulu, 1986).

2.2.1 Factors influencing the disease incidence

Leibovitz (1991) has pointed out that thick population of susceptible domestic ducks and geese enhance the probability of disease detection. In contrast, lack of information on DP in wild waterfowls, small domestic flocks, ornamental and zoo birds results from limited surveillance and inadequate sampling. The migratory habits of most of the duck flocks do not allow the investigators to follow up the disease patterns. Accordingly, the reported incidence of DP in

domestic ducks may be misleading, when compared to its natural occurrence in other anseriforms.

2.2.1.1 Species and breed differences

Van Dorssen and Kunst (1955) showed that Khaki Campbell, Indian runner and Pekin ducks were equally susceptible to DP when compared with wild duck, wild goose and mute swan. Dardiri and Gailiunas (1969) observed high mortality in Pekins with comparative resistance of mallard ducks. Similarly, during an outbreak in Denmark, Prip et al. (1983) noted 51 per cent mortality in Pekin ducks but very low in mallards.

Kocam (1976) compared duck embryo-fibroblast (DEF) culture from seven species of ducks for virus yield, plaque quantity as well as senitivity to infection and showed that muscovy duck cells were most superior. Confirming these findings, Gough (1984) found muscovy ducks more susceptible.

While studying 58 outbreaks during 1984-87, Gough and Alexander (1987) found that only one was in commercial flocks of ducks and geese and the others in captive waterfowls including muscovy ducks.

2.2.1.2 Differences in virulence among virus strains

Burgess and Yuill (1982) reported two non-lethal and

one lethal strains of DP virus. Lin et al. (1984a) isolated a non-pathogenic strain in USA and designated it as Sheridan-83. Mauris-Jestin et al. (1987) isolated a hypervirulent strain from mule ducks. The isolation of DP viruses of different virulence indicated that there are many different biotypes of viruses (Lin et al., 1984b).

Jansen (1964) observed that in any area, initial outbreaks were more severe and caused 100 per cent mortality, which later came down considerably. Indian isolates caused high mortality. The outbreaks in Assam during 1978 resulted in 49 per cent mortality in adults and 100 per cent in ducklings (Chakrabarty et al., 1980). In Tamil Nadu it was 100 per cent (Duraiswamy et al., 1979) and in Andhra Pradesh, 95 per cent (Sreeramulu, 1986).

A DPV strain isolated from France caused cent per cent mortality in Barbary ducks but only 20-40 per cent mortality in Khaki-Campbell, Khaki-Pekin and Pekin ducks, thus differing from Dutch strain which caused 80-95 per cent mortality in all breeds (Gaudry et al., 1970).

2.2.1.3 Age of the birds

Natural infection has been observed in ages ranging from 7-day-old ducklings to mature breeder ducks (Leibovitz, 1991). Suwatviroj et al. (1977) recorded the disease in

Thailand affecting ducks from 28 days to two years of age, with highest mortality in young ducklings. Vetesi (1982) observed DP outbreaks in Hungry from third week of life onwards. Bhowmik and Chakraborty (1985) described two DP epidemics in ducklings between ages of one to three weeks.

2.2.1.4 Season

Most of the DP outbreaks occur during the period from January to June (spring season or after winter), however the incidence is not uncommon during the remaining months (Jansen, 1963). In Netherlands, out of the first 26 outbreaks, 20 were during the months of January to May (Jansen, 1964). Similar observations were made by Gough and Alexander (1987), 21 of the 58 epidemics were between April to June. In another study, Gough and Alexander (1990) noted 92.5 per cent of the confirmed cases of DP occurred in the months of April to June in Great Britain. However, Vuillaume (1989) reported two DP outbreaks in France between September and January. General incidence in India suggests DP occurs most commonly during March to May (Punnoose et al., 1993).

2.2.1.5 Synergistic effect of other infections

Dardiri (1975) stated that the severity of the disease depended upon secondary microbial invaders in the host at the

time of DP infection. These secondary or latent microbial invaders might jeopardize the resistance of the infected bird.

Proctor and Matthews (1976) studied DP in gnotobiotic and conventional ducks and found no difference in lesions or mortality, indicating synergic action of species of Salmonella or Pasteurella was not essential for development of lesions. Fleury et al., (1986) isolated DPV and influenza A virus simultaneously from commercial ducks in France. Mo and Burgess (1987) observed no synergism between DPV and Pasteurella strains. However the authors admitted that their results did not prove that DPV could not enhance bacterial infections under different conditions. A paramyxovirus type-6 isolated alongwith DPV had no synergistic effect on DP infection (Marius-Jestin et al., 1987). Recently, several duck flocks were simultaneously affected with duck plague and Pasteurella anatipestifer in Kerala, resulting in high mortality (Punnoose et al., 1993).

2.2.2 Host range

The disease occurs naturally only in ducks, geese and swans (birds of family-Anatidae, order-Anseriformes). Among domestic ducks, (Anas platyrhynchos domesticus), the common breeds which are affected include White Pekins, Khaki-Campbell, Indian runners, hybrids, and local non-descript

breeds (Leibovitz, 1991). Outbreaks have also been noted in muscovy ducks (Cairina moschata) and domestic geese (Anser anser) (Jansen, 1961; Leibovitz and Hwang, 1968; Gough, 1984).

In case of wild waterfowls, the disease has been recorded in mallards (Anas platyrhynchos platyrhynchos), black ducks (Anas rubripes), bufflehead (Bycephala albeola), greater scaup (Aythya marila), lesser scaup (Aythya affinis), Canada goose (Branta canadensis) and mute swan (Cygnus olor) (Leibovitz, 1969; Dardiri and Butterfield, 1969). DP has also been seen in pintail-mallard hybrids, red heads (Aythya americana), common mergansers, common goldeneyes (Bucephala clanquala), canvas blacks (Aythya valisineria), American widegeons (Mareca americana) and woodducks (Aix sponsa) (Leibovitz, 1969; 1991; Kaleta et al., 1983).

In addition to these naturally occurring species, DP can be produced experimentally in garganey teals (Anas querquedula), Gadwals (Anas strepera), Europian widegeons (Anas penelope), Shovelers (Spatula clypeata), Common pochards (Aythya ferina), common eiders (Somateria mollisima), white frontal geese (Anser albefrons), bean geese (Anser fabalis) (Van Dorssen and Kunst, 1955) and bluewinged teals (Anas discors) (Wobeser, 1987).

Van Dorssen and Kunst (1955) found European teals (Anas crecca) and pintails (Anas acuta) to be resistant to DP

infection, however they produced antibodies as a result of experimental infection. Certain birds of the order Charadriformes like herring gulls (Larus argentatus) and black headed gulls (Larus ridibudus) were neither susceptible nor produced antibodies to DP virus and gray call ducks were resistant to lethal infection (Van Dorssen and Kunst, 1955). Similarly the virus was not fatal to cowbirds (Molothrus ater) when administered orally (Dardiri and Buttertield, 1969).

Bos (1942) was unable to infect chickens, pigeons, rabbits, guineapigs, rats or mice experimentally. Adult poultry birds were totally resistant to DP infection (Jansen, 1961). Kunst (1958) conducted experiments with DPV in day-old chicks and found them susceptible. Even after 15 passages in chicken, the virus was still virulent for ducks. The virus could be adapted by serial passages in chickens upto two weeks and embryonating chicken eggs (Jansen, 1968). The infection has not been reported in avian species other than anseriforms (Leibovitz, 1991).

2.3 AETIOLOGY

The causative agent is a herpesvirus in subfamily Alphaherpesvirinae (Mohanty and Dutta, 1981) and provisionally designated as Anatid herpes virus I (Roizman, 1982).

2.3.1 Virus structure

In a negatively stained preparation, Proctor et al. (1976) described DPV capsids, when free in suspension, were usually coated with extraneous substances, partially obscuring their morphological features. The most common forms found in cell culture supernatent were virions with diameter 120-160 nm. Intact virions were impermeable to the negative stain. In damaged particles, it was usually possible to observe a central herpesvirus-like capsid and blebs of the envelope.

In a morphogenesis study by Bergmann and Kinder (1982), spherical nucleocapsids of 93 nm diameter with core about 61 nm were recorded in cell nuclei. Encasement of nucleocapsid by interior nuclear membrane was followed by occurrence of 126-129 nm particles in perinuclear spaces and in cytoplasm,definitive virus forms (156-384 nm, mean 200 nm) were deposited through a tubular system in cytoplasmic vacuoles, before being discharged into extracellular spaces. They consisted of enveloped nucleocapsids surrounded by osmiophilic masses and delimited by an additional membrane. On the basis of these structural properties, DPV was differentiated from many other animal herpesviruses.

Tantaswasdi et al. (1988) described two types of virus particles in the cytoplasm of the hepatic cells three to four

days post-infection (DPI). One was naked nucleocapsid measuring approximately 80 nm in diameter. These consisted of an envelope with surface projections approximately 10 nm long, a nucleocapsid 80 nm in diameter and an electron-dense zone existing between the envelope and capsid. In the cytoplasmic matrix,the naked nucleocapsids were either associated with or were within the accumulation of electron-dense amorphous material, which appeared to be coated with the dense material to form a viral tegument. The envelopment of cytoplasmic nucleocapsid was observed to occur by budding process at the membrane-bounding cytoplasmic spaces such as tubules, vesicles or vacuoles. Subsequently, they became enveloped by those portions of the membrane and the mature virions were produced at the terminal stages of development.

Hess and Dardiri (1968) noted the size of the virus particles to be more than 100 nm but less than 220 nm. In an another study, Simonova et al. (1984) observed the spherical particles measuring upto 394 nm (average 150-250 nm), with a nucleoid 20-25 nm, icosahedral internal capsid 50-60 nm and an external capsid 90-100 nm.

2.3.2 Biological properties of the virus

Duck plague is caused by a herpesvirus whose biologic characteristics are similar to those described for the group of herpesviruses (Dardiri, 1975).

2.3.2.1 General properties

Duck plague virus did not agglutinate erythrocytes of chicken, duck, horse, or sheep when saline or citrate buffer was used as diluent (Jansen, 1961). It was non-haemagglutinating and non-haemadsorbing (Dardiri and Hess, 1968). It did not stimulate precipitating antibodies in ducks (Panisup and Verma, 1989).

2.3.2.2 Inclusion bodies

Proctor et al. (1975) noted that the virus produced intranuclear inclusions in stratified squamous epithelium of the oesophagus and cloaca, crypt epithelium of the intestine, hepatocytes, bile duct epithelium, cells of Hasssel's corpuscles, splenic periarteriolar reticular cells and epithelial cells in bursa of Fabricius. Bergmann and Kinder (1982) also reported Cowdry type A inclusions consisting of an osmiophilic amorphous matrix in infected tissues. The intranuclear inclusions in DPV-infected cells of many organs like liver, spleen, bursa, lining epithelial tissues of oesophagus etc. were eosinophilic and granular and seen in degenerative and necrotic cells from day three to day seven post-infection (Tantaswasdi et al., 1988). Panisup and Verma (1989) reported the production of pinkish intranuclear inclusions in liver, oesophagus, oeso-proventricular junction,

intestine, cloaca, bursa, trachea, harderian gland and spleen. In hepatic cells it was seen from four days post-infection.

Gailiunas and Dardiri (1970) reported the inclusion bodies in duck embryo hepatocytes. In infected chicken-and duck-embryo-fibroblast cell cultures they appeared as early as 12 hours post-infection (Dardiri, 1975).

2.3.2.3 Plaque formation

The virus was able to form plaques in cell culture, which could be inhibited by specific anti-serum (Dardiri and Hess, 1968).

2.3.2.4 Interference

Duck plaque virus was reported to produce interference for infection and was supposed to provoke a non-antibody defense in very early stage of infection (Jansen, 1961). Effective cell blockage by Ranikhet disease virus against multiplication of DPV at the face of epidemic was hinted by Mukerji et al. (1963 b). Attempts to halt DP outbreaks using RD vaccine gained success. However, reversely, DPV did not interfere with RD virus multiplication (Mukit et al., 1989). These workers suggested that ducks should then be vaccinated as early as possible with DP vaccine as the interference phenomenon existed for a very short duration.

2.3.2.5 Virus strain classification

The DPV is immunologically distinct from other avian viruses including fowl plague, Newcastle disease virus, duck hepatitis virus (Jansen and Kunst, 1949; Levine and Fabricant, 1950; Dardiri and Hess, 1968) and herpesviruses (Roizman et al., 1981). Studies conducted by Jansen (1961) demonstrated that all isolates during initial outbreaks in Netherlands were serologically homologous. There was no antigenic variation between Dutch strains and Indian strains (Jansen, 1964). Dardiri and Hess (1968) compared Holland strain with an American strain (Long Island DPV) by plaque-inhibition assay and indicated that there was no antigenic difference. Spieker (1977) also confirmed the serologic homogenecity of various DPV isolates. Lin et al. (1984 a) isolated a non-pathogenic strain and designated it as Sheridan-83, which was serologically indistinguishable from Holland and Long Island strains (Lin et al., 1984 b).

Deng et al. (1984) employed reverse passive haemagglutination test and immunofluorescence test and detected 6 strains of DPV, where conventional plaque assay failed. Lin et al. (1984 b) concluded that the various isolates of different virulence were all serologically related biotypes of the duck plague virus.

2.3.2.6 Experimentation under laboratory host system

Primary isolation of DPV was done on 9-12 - day-old duck embryos inoculated via chorio-allantoic membrane (CAM) route. When blood or tissue suspension from field cases were used as inoculum, the embryos showed extensive haemorrhages and died between 5-15 days post-inoculation. After subsequent passages in 12-day-old duck embryos, regular deaths were observed after 4 days with extensive haemorrhages (Jansen, 1961).

Jansen (1964) stated that repeated attempts to cultivate DPV on CAM, allantoic cavity, amniotic sac of chicken embryos using morbid materials were unsuccessful. After 12 duck embryo passages and three blind passages in chicken embryos, the virus became lethal to chicken embryos. The pathogenicity of this chicken-embryo passaged virus for ducks decreased rapidly. It was virulent after 10 passages in chicken embryos but attenuated enough after 20 passages to protect the ducks against challenge DP infection.

Dardiri (1975) noted that the mortality rate of the chicken embryo after inoculation with chicken embryo-adapted DPV was maximum between 72 h and 192 h post-inoculation. There was a lag phase of 48 h before the death of the embryo. The viral concentration was highest in the CAM at 144 h and

amnio-allantoic fluid (AAF) at 96 hours. The virus titre was highest in CAM followed by AAF and low in embryo and yolk-suspensions. Butterfield and Dardiri (1969) stated that the addition of minced chicken embryo to CAM and AAF decreased virus titre of the material. The mortality of embryos depended upon the virulence and the virus concentration of the inoculum (Dardiri, 1975).

While earlier studies (Jansen, 1961; 1964) indicated CAM as a route of inoculation for DPV cultivation in embryos, Butterfield et al. (1969) and Dardiri (1975) preferred chorio-allantoic sac (CAS) route. Kalaimathi et al. (1985) compared various routes of inoculation and CAM from virulent virus-infected embryonating duck/chicken eggs yielded high virus titres when inoculated via CAS route. The advantages of CAS route over CAM as stated were - reduction in mortality in lag phase, harvesting the embryos a day earlier than that for CAM route and the higher yield of virus.

The virulent DPV was also propogated in duck embryo-fibroblast-cell culture (Kunst, 1967; Dardiri, 1969), chicken embryo-cell culture (Dardiri and Hess, 1968) and muscovy duck fibroblast-cell culture (Kocam, 1976). A cell culture-plaque-assay method for titrating DPV concentration and plaque-inhibition assay by neutralizing antiserum was developed by Dardiri and Hess (1968).

Burgess and Yuill (1981 a) found that less virulent DPV isolates could be isolated on muscovy duck embryo fibroblast cell culture using higher incubation temperature i.e., 39.5°C to 41.5°C instead of 37°C. They correlated the range of temperature as an indicator for virulence of DP virus.

The virus could also grow equally well on a cell line originated from Pekin duck fibroblast, certified cell line-141 (CCL-141) of the American Type Culture Collection (Wolf et al., 1976).

2.4. EPIZOOTIOLOGY

2.4.1 Transmission

Since waterfowl are dependent on aquatic medium which provides a common vehicle for feeding, drinking and body support, water appears to be the natural means of virus transmission from infected to susceptible individuals. Duck plague can also be transmitted by direct contact between infected and susceptible birds or indirectly by contact with contaminated environment (Leibovitz, 1991).

Jansen (1963) reported that the outbreaks of DP in Netherlands were observed only in ducks allowed to swim in free waterbodies and not on farms with drinking troughs instead of ponds.

In Kerala, all the flocks affected with DP during the period 1990-93 had a migratory history whereas on duck farms, not having the access to external water source, outbreaks were not recorded (Punnoose et al., 1993).

While virus had been recovered from an egg removed from the oviduct of an infected domestic duck (Jansen, 1964), it had not been recovered from eggs laid during a natural outbreak (Ziedler et al., 1984). Under experimental conditions, vertical transmission of DPV has been found to occur in persistantly infected Pekin, muscovy and mallard ducks (Burgess, 1981). She observed that the effect of egg fertility and hatchability depended on the isolate and duck species, but Prip et al. (1983) stated that transovarian transmission was not of much importance.

Blood sucking inects may transmit the infection mechanically (Leibovitz, 1991).

2.4.2 Carrier state and reservoirs

Burgess et al. (1979) found healthy waterfowls to be carriers of DP virus. Black ducks and Canada geese surviving a natural outbreak of DP at Coloma, Wisconsin in 1973, yielded the virus from cloacal swabs taken four years after infection. Experimental infection of previously unexposed mallard ducks with DPV also produced cloacal virus-shedding for upto four

years after infection. The frequency of swabs positive for DPV varied between individuals within each of the tested species. The amount of detectable DPV shed was about 100 plaque-forming units/cloacal swab.

A carrier state has been suspected in wild ducks (De Zeeuw, 1930; Van Dorssen and Kunst, 1955; Burgess et al., 1979). Contact between wild anseriforms and domestic ducks is common and frequently mediated by the use of open waterbodies for duck production (Leibovitz, 1991).

Jansen and Künst (1964) experimentally proved that ducklings vaccinated with DP vaccine did not excrete DPV to immunize the in-contact ducks.

Mallard ducks are considered as a possible natural reservoir of DPV infection (Leibovitz, 1991). It was as early as 1955, when Van Dorssen and Kunst had suggested the involvement of mallards. Gough (1984) as well as Gough and Alexander (1987) supported this fact based on their observation of arrival of mallard drakes (free-flying waterfowls) one to two weeks before the losses occurred due to DP outbreaks.

Burgess (1981) noted that the DPV carrier mallard ducks could be stimulated under experimental stress conditions

to excrete large quantities of DPV (upto 10^8 fluorescent focus unit-FFU/swab/day) while unstimulated carriers excreted only 10^3 FFU/swab/day. The stress of reproductive state and exercise augmented oral excretion in carrier birds.

Kapp et al. (1984) studied the liver lesions of naturally and experimentally infected mallards and stated that the acute disease occurring in younger birds was associated with circumscribed focal and haemorrhagic changes and acute dystrophy directly elicited by the virus. The chronic disease occurred in older birds as interstitial hepatitis, which was not always fatal. These surviving birds were considered as potential symptomless carriers.

Brand and Docherty (1988) found no evidence of exposure to or shedding of DPV in migratory waterfowl found in two regions where DP appeared enzootic. They disapproved earlier conclusions that the exposed birds could become carriers and periodically shed the virus upto five years and argued that shedding of DPV was sporadic and unpredictable. The ability to detect such birds was limited to sampling from cloacal and oral swabs. They also cautioned about the interpretation of such surveys.

2.5 PATHOLOGY

2.5.1 Pathogenesis

Proctor et al. (1976) inoculated 6-week-old white Pekin ducks intravenously with DP virus. The virus replicated in hepatic macrophages, hepatocytes and bile-duct epithelium. This was contrary to earlier findings by Dardiri and Gailiunas (1969), who observed that the hepatic macrophages were a major pathway for the removal of the viruses from blood and a primary defense against hepatocyte invasion. Proctor et al. (1976) concluded that in white Pekins, hepatic macrophages like those of suckling mice (Stevens and Cook, 1971) were unable to destroy DPV and subsequent invasion of hepatocytes and bile-duct epithelium occurred.

The lymphoid depletion in the cortex of the thymus and cortex and medulla of bursal follicles was seen on three days post-infection (DPI). On 4th and 5th DPI, most of the medullary lymphocytes, epithelial cells and small foci of cortical lymphocytes contained DPV antigens in thymus, whereas diffuse necrosis of medullary lymphocytes of bursal follicles with majority of viral antigens concentrating in epithelial cells and macrophages was the characteristic picture in bursa. The viral antigens and nucleocapsids were found in both T- and B-lymphocytes before necrosis occurred. In spleen,

scattered macrophages contained phagocytized lymphocytes. Most of the spleenic lymphocytes and sinusoid-lining epithelial cells were necrotic (Proctor, 1976). Lymphocytic necrosis in the bursa, thymus and spleen of the duck infected with DPV was similar to that after acute irradiation in mice or following IBD infection in chicken (Breese and Dardiri, 1968).

Tantaswasdi et al. (1988) observed that when given orally, DPV entered the epithelial cells of alimentary tract and associated lymphatic structures. Primary multiplication occurred in these cells as evidenced by detection of viral antigen in nucleus and cytoplasm of mucosal epithelium by immunofluorescence from day 3-7 post-infection. With viraemia developing, involvement of liver was seen.

Glavits et al. (1990) inoculated geese embryos with DPV and demonstrated pathomorphological changes indicative of virus replication in liver, kidney, myocardium,gizzard and CAM of the embryos.

2.5.2 Symptoms

In domestic ducks, the incubation period ranges from 3-7 days. Once overt signs appear, death usually occurs within 1-5 days (Leibovitz, 1991). Egg production may drop 20-100 per cent (Newcomb, 1968). An inapparent disease form

may prevail in some flocks. Also, mortality may be severe as a result of dual infection of DP and latent bacterial infection (Dardiri, 1971).

The symptoms shown by naturally infected and experimentally inoculated ducks are similar. Jansen (1961 and 1964) described that three or four days after infection, ducks became listless, lost appetite but became thirsty, sat most of the time with drooping wings and moved only with difficulty. They showed no desire to swim and there was evidence of photophobia. The feathers were ruffled and dull, the eyes were moist, the discharge from eyes and nostrils became sticky, and in dead ducks, the swollen eyelids appeared glued. During the course of the disease, the call became hoarse and breathing laboured, especially when the ducks were frightened. There was a watery diarrhoea.

2.5.3 Lesions

The disease was too acute to cause emaciation, consequently the carcass was usually in good condition. The most striking lesions were the multiple petechiae throughout the body particularly on serous membranes, heart and mucosa of the oesophagus. The ovary in production showed the blood vessels distended, the follicles congested and containing blood or ruptured. There was egg-peritonitis, distention and

degeneration of the heart and friable liver. The mucosa of the intestines was inflamed and showed petechiation (Jansen, 1964).

At the oesophageal-proventricular junction and at regularly spaced intervals within the small intestine, there were red annular bands or discs on the mucosal surface (Leibovitz, 1969). Catarrhal proventriculitis, blood-stained contents of gizzard and haemorrhagic enteritis were pronounced. The lumen of digestive tract was frequently filled with free blood. In young birds, there was either complete redness or multiple haemorrhages of the mucosal lining of the bursa of Fabricius. Later diphtheroid masses were found in it (Leibovitz, 1971). He further described the lesion in heart of the breeder ducks as closely spaced petechial epicardial haemorrhages which gave the cardiac surface, a red 'paintbrushed' appearance. In ducklings less than five weeks of age, cardiac and mesenteric haemorrhages were uncommon. The spleen was usually mottled, darker and smaller than normal. Early lesions in liver showed the pale, copper-coloured friable surface covered with a heterogenous mixture of pinpoint haemorrhages and white spots. During later stages the hepatic surface became dark bronze and bile-stained with greater contrasting, large merging white spots.

Dardiri (1975) noted that the oesophageal mucosa in

the initial stages showed tiny haemorrhagic spots which were arranged in longitudinal rows and later stages showed yellow or gray diphtheritic crusty plaques or eruptions on oesophagus and cloaca. These were considered as pathognomonic lesions of duck plague. Extravasation of blood in abdominal and thoracic cavities were associated with disease.

2.6 IMMUNITY

Ducks survived from natural or experimental infection are solidly immune (Jansen, 1961; Mukerji et al., 1963 b; Jansen and Wemmenhove, 1966; Butterfield and Dardiri, 1969; Dardiri, 1975).

Burgess and Yuill (1982) observed that the superinfection of persistantly infected mallard ducks resulted in death and indicated that protection against mortality was dependent on route of exposure, strain of the initial virus and strain of the superinfecting virus.

2.6.1 Neutralizing antibodies and protective immunity

Butterfield and Dardiri (1969) in an experiment, found the highest mean antibody level at post vaccination stage as 1.4 log 10. However ducks with a pre-challenge mean antibody level as low as 0.4 log 10 were protected against challenge.

In a study of 2936 sera samples, from infected and

non-infected duck flocks, Dardiri (1975) recorded that non-infected flocks had a virus-neutralization index (VNI) in the range of 0.0 to 1.2 log 10 and approximately 90 per cent of these had a VNI of 0.75 log 10 whereas clinically affected ducks had a VNI of 1.75 to 3.0 log 10. Comparison of the VNI of sera obtained at 0, 21, and 42 DPI from ducks that survived experimental exposure indicated the increase in 1 to 3 logs in VNI. The serum antibody titre increased substantially in 21 DPI but reached a high level (2 to 3.5 logs) at 42 DPI. It was concluded that an antibody level of log 1.75 or more signified infection of ducks with virulent DP virus.

Dardiri (1975) further studied the response of some species of waterfowls including mallards and Pekins. Each of these birds was given 2 x (6 log 10) chicken embryo lethal dose 50 (CELD50) virus orally, and challenged after 85 days. The level of antibody in post-vaccinal serum ranged between 0.9 to 2.6 log 10. There was no correlation between antibody level and protection. One of the mallard duck having 2.1 log 10 antibody titre succumbed to challenge whereas six mallard with no or negligible antibody levels withstood it. A high level of neutralizing antibody developed in vaccinated birds after challenge infection. The neutralization indices declined within 10-12 weeks, however, ducks were resistant to challenge even after one year.

Such lack of positive correlation between VN antibodies and the ability to withstand challenge infection had been reported by many workers (Jansen et al., 1963; Jansen and Wemmemhove, 1966; Butterfield and Dardiri, 1969). Dardiri (1969) gave possible explanation by pointing out the presence of secondary or latent microbial infections.

Toth (1971 a) reported VNI from vaccinated breeder ducks between 0.2 to 0.9 whereas from double vaccinated, the indices were between 0.7 to 1.7. A high level of VNI i.e., 3.1 to 4.0 was seen in challenged birds. He also described DP virus VN antibody with a short life expectancy in breeders. Low-positive titres of twice vaccinated breeders became negative in 12 weeks but the high post-challenge VNI also decreased significantly often by more than 1.5 log 10 in 10 weeks. The breeders were less reactive to DPV than the ducklings.

Mukit et al. (1988) found VN indices in adult ducks against DPV as 1.5, 2.3 and 3.8 in unvaccinated, vaccinated and recovered ducks respectively.

2.6.2 Antibody-mediated and cell-mediated immunity

Lam (1984) described a general biological phenomenon - immune lysis - applicable to DP virus. Anti-DPV antibody and guinea pig complement lysed DPV infected cells. According to

him, this might be due to the expression of large number of viral antigens on the surface of plasma membranes. In another study, Lam and Lin (1986) could transfer the resistance against the challenge strain from vaccinated ducks to recipient susceptible ducks via serum transfer. They postulated that the mechanism of protection in DP, based upon in-vivo and in-vitro studies, was probably mediated by the humoral immune system.

Dardiri (1969) referring to the apparent lack of correlation of detectable circulating antibodies with protection raised the question that whether such protection was due to a cellular type of immunity apart from the humoral neutralizing antibody.

Li et al. (1988) studied phyto-haemagglutinin (PHa) response of peripheral blood lymphocytes in thirty - two 10-month-old ducks. They were divided into four equal groups. Group I was inoculated with virulent DPV, group II with attenuated DPV, group III with virulent DPV two weeks after attenuated DPV and group IV as uninfected control. A depression in PHa response was observed in all infected groups at three days. No depression was noted in group III when challenged with virulent DPV showing significant improvement in PHa response after attenuated DPV vaccination.

2.6.3 Passive immunity

The progeny of breeder ducks whose serum had 3.5 log 10 or more VN antibody level was provided with passive maternal immunity. This protection was of very short duration as 50 per cent of the 13-day-old ducklings died when exposed to challenge with virulent DP virus (Toth, 1971 b).

Burgess (1981) suggested the possibility of immunosuppression in hatchlings after the vertical transmission of DP virus.

In one experiment, Lin et al. (1984 b) inoculated the ducks with one ml of hyper-immune sera intramuscularly on the day before; on the same day; the day after or both one and two days after challenge infection. Most ducks receiving hyper-immune sera one day before or one day after challenge were significantly protected, whereas the protection was negligible when the virus and hyperimmune sera were given a few minutes apart.

Glavits et al. (1990) inoculated goose embryos with virulent DPV and attenuated vaccine virus. The surviving goslings were allowed to hatch. No VN antibodies were demonstrated in the yolk or serum of these goslings. The lymphocytes separated from the blood of these goslings were

used for rosette formation and lymphocyte stimulation tests, wherein the lymphocytes recognised the virus antigens, and responded to blastogenic transformation.

2.7 DIAGNOSIS

Traditionally, gross lesions are diagnostic of DP virus infection. Histopathological studies could further support these findings (Leibovitz, 1991). Jansen (1961; 1964) had described differential diagnosis of DP from Newcastle disease, fowl cholera, duck hepatitis and toxicities. The virus isolation from a morbid specimen showing characteristic lesions as well as mortality in duck embryos and absence of it in chicken embryos made the difference. It was also suggested to use rabbit, hen and duck for differential diagnosis with the above conditions. Duck hepatitis is essentially a disease of young ducklings. In duck plague, intra-nuclear inclusions in hepatocytes could support the diagnosis.

Once the virus was isolated on duck embryos, it could be adapted to grow on duck embryos and chicken embryos and could be neutralized by anti-DP sera. The neutralization test could be done in duck embryo, chicken embryo, duck embryo fibroblast or chicken embryo-fibroblast-cell cultures (Dardiri and Hess, 1967).

Dardiri and Hess (1968) developed a plaque assay for chicken embryo-adapted virus and a duck-lethal virus and used it to determine the identity of these viruses. This assay could also be used for inhibition of plaque formation by specific antiserum.

Wolf et al. (1974) inoculated morbid specimens on a microtitre plate system for direct isolation and concurrent virus identification by serum-neutralization test (SNT), wherein definitive virological results could be obtained within 60 to 72 hours.

Erickson et al. (1974) used immunofluorescence technique (IFT) for diagnostic purpose. Tantaswasdi et al. (1988) compared IFT with electron microscopy.

Cottral (1978) reported agar gel diffusion test (AGDT) for identification of DP viral antigen or antibody. Using 20 per cent trichurated CAM suspension as an antigen and hyperimmune sera raised in rabbits, John et al. (1989) employed counterimmunoelectrophoresis test. Kalaimathi and Jankiram (1990) also used AGDT for duck plaque.

Deng et al. (1984) developed a reverse passive haemagglutination test (RPHA) to detect duck plague virus. The technique used sheep erythrocytes stabilized with formaldehyde and pyruvaldehyde and coated with

sheep anti-DPV IgG. The high specificity of the test was indicated by the absence of cross-reaction with heterologous virus strains, host materials and by haemagglutination inhibition only with DPV antiserum. The test was less sensitive than the conventional plaque assay, or IFT; however, there was positive correlation in the titres of DPV antigens between all three tests. The RPHA was stated to be rapid, simple procedure sufficiently sensitive for diagnostic detection of DPV in acute infections.

It is a known fact now that in-vitro antigen-induced inhibition of leucocyte migration could be used to assess cell-mediated immunity (Buening, 1973; Carson et al, 1977; Nyindo et al., 1980; Nagaraja et al., 1982; Jayaprakasan, 1986). Leucocyte-migration-inhibition test (LMIT) had been successfully employed for assessment of CMI in various diseases, viz. tuberculosis (Little and Naylor, 1977); Measles and mumps (Kantoch et al., 1979), infectious bovine rhinotracheitis (IBR) (Hussain and Mohanty, 1979), brucellosis (Azadegan et al., 1981), ethmoid carcinoma (Sulochana et al., 1982 b), Sheep pox (Karpe, 1982) and fowl pox (Farshid, 1992).

Tims (1979) compared LMIT and lymphocytic transformation (LT) test and stated that the primary and secondary immune responses could be detected by LMIT for longer period of time and on more occasions than by LT test.

Ziambo et al. (1973 a;b) standardized PHA test for IBR antibody detection and stated that it had got some advantages over neutralization test in that it was rapid, less cumbersome and equally sensitive. Moreover the latter test did not detect antibody in known carrier animals. Sulochana and Rajan (1981) used PHA for detection of antibodies against ethmoid carcinoma in cattle. Sulochana et al. (1982 a) conducted a serological survey on the occurrence of IBR in Kerala, whereas Suresh (1992) studied the seroprevalence in Tamilnadu State using this test.

2.8. PREVENTION AND CONTROL

Leibovitz (1991) has stated that there is no specific treatment for duck plague. Prevention is achieved by maintaining susceptible birds in environment free from exposure to the virus. These measures include addition of stock known to be free from infection and avoiding direct and indirect contact with possibly contaminated material. Introduction of the disease by free-flying birds and contaminated environments must be prevented. Once DP has been introduced, control can be effected by depopulation, removal of birds from the contaminated environments, sanitation and disinfection. All possible measures should be taken to prevent spread of the virus by free-flowing water.

2.8.1 Vaccine, vaccination and protection

On adaptation of DPV to chicken embryos, after 20 passages, Jansen and Kunst (1949) showed that the virus was no longer virulent for ducks but was capable of immunizing ducks via intramuscular or oral route. Jansen (1964), after field trials demonstrated that the vaccination was completely harmless and resulted in a rapid and reliable resistance. This adapted DPV strain was named as 'Utrecht' strain of Netherlands. Later it was propogated in chicken embryo and chiken embryo-fibroblast-cell culture by Plum Island Animal Disease Laboratory of USA. After its assay for immunization of ducks and freedom from extraneous microbial and viral agents, it was released as 20 per cent CAM suspension in allanto-amniotic fluid (AAF) as a vaccine. Ducklings given this vaccine withstood the challenge with 5.8 log 10 duck lethal dose 50 (Dardiri, 1975).

In a study conducted by Butterfield and Dardiri, (1969), DCE adapted DPV was inactivated in three hours with 0.05 per cent 1-acetylaziridine (AEI) and in half an hour with 0.4 per cent beta-propiolactone (BPL). Intramuscular inoculation of AEI-inactivated antigen and live attenuated virus produced comparable levels of antibody, both higher than the level attained with orally administered cell-culture-propogated virus. In second experiment, a concentrated

AEI-inactivated CAM and AAF preparations was used. It produced a low serologic response but protected ducks against challenge infection whereas BPL-inactivated virus gave no protection.

Thirteen serials of an experimental DCE-adapted live DP virus vaccine were tested by Toth (1970) for their safety and immunogenicity in susceptible ducklings. Titres of serials varied between 3.2 and 4.6 (Average 4.2) log 10 median CELD50/ml. The dose of one ml of undiluted vaccine did not cause disease or mortality among 260 inoculated ducklings. After challenge, in vaccinated group, only two (0.77 per cent) ducks died for nonspecific reasons whereas in controls, mortality was 88.0 per cent. The affected birds died between 3-8 days post-challenge infection, peaking on fourth day. Immunity was manifested in two ways - in the positive correlation between increasing concentration of vaccine and resistance against challenge; as well as in delayed peak of mortality.

In an another study, Toth (1971 a) vaccinated white Pekin breeder ducks with DCE-adapted DP vaccine, one group received single vaccination and the other received second vaccination 19 weeks later. Single vaccination did not induce any development, whereas two vaccinations did induce a low

level of neutralizing antibody. On challenge, one-time vaccinated birds showed 12.0, 24.0 and 15.0 per cent mortality (Av. 17.0) at intervals of 5 weeks, 24 weeks and 36 weeks post vaccination respectively, whereas controls showed 64.0, 70.0 and 50.0 per cent mortality (Av. 60.0) at the corresponding intervals. In double vaccinated group, mortality was 8.0 and 10.0 per cent (Av. 9.0) at 5 weeks and 17 weeks post double vaccination respectively, whereas control mortality was 84.0 and 59.0 per cent (Av. 74.0) at the corresponding intervals.

Lin et al. (1984 a) reported isolation of an apathogenic DPV strain-Sheridan-83 from waterfowls in California. Lin et al. (1984 b) used this strain as vaccine virus and 4 log 10 tissue culture-infective dose 50 (TCID 50) of it when given to ducks resulted in the production of antibodies that enabled the ducks to resist challenge with virulent DP virus.

Balla (1984) inoculated ducks with commercial vaccine in Hungary at 30 to 124 days of age, the protection to challenge infection was 89 to 100 per cent after 8 to 14 days, whereas for ducklings vaccinated at two weeks of age, it was upto 76.5 per cent and vaccinated younger ducklings had no protection on challenge infection.

Further studies indicated that the vaccine doses as

low as 2.6 log10 TCID50 instead of recommended 4 log10 TCID50 protected 80 to 84.6 per cent ducks whereas 1.64 to 2.2 log10 TCID 50 gave 55.6 to 69.2 per cent protection in birds older than three weeks (Balla, 1984). Sergeev et al. (1990) used live vaccine in former USSR for inoculation into 10- to 15-day-old duckling at 2 to 3 log 10 TCID 50, which protected 60 per cent of ducks from challenge infection at five months post vaccination.

The minimum protective dose of DPV vaccine virus strain (Sheridan-83) was determined to be less than 10 TCID 50 wherein immunity lasted for one to two months (Lin et al., 1984 b).

Balla and Kelemenne (1985) studied the duration of protective immunity in ducks vaccinated at 36 to 60 days of age against DP and found that vaccine having 2.5 to 3.5 log 10 TCID 50 elicited 100 per cent protection on eighth day whereas vaccine containing 2.4 log 10 TCID 50 gave 93.3 per cent protection. In another group of ducks vaccinated twice, at 74 and 91 days, the protection was 81.3 per cent on 63rd day after second vaccination, 66.7 per cent after 21 weeks and 64.7 per cent after 31 weeks.

Due to interference phenomenon, live vaccine is recommended at the face of outbreaks (Jansen, 1964).

In Netherlands, commercial vaccine available is the Utrecht strain adapted to primary chicken embryo fibroblasts from SPF eggs and this lyophilized vaccine contains a minimum 4.5 log 10 TCID 50 per dose. The recommended schedule is as follows: initial vaccination at four weeks by intramuscular or subcutaneous route; in areas of high risk, even at day one followed by four weeks later; breeding stock twice at six weeks interval and for all duck population, annual revaccination (Intervet, 1993).

2.8.2 Duck plague vaccines in India

In India four laboratories produce DP vaccine regularly. Most of them use the same source of seed virus. As per the information supplied by the manufacturing institutes (IVPM, 1991 and VBI, 1991) the vaccine is a suspension of AAF, CAM and embryo of modified-live-virus-inoculated chicken embryos and is freeze-dried. It is claimed to develop the immunity within seven days and lasts for one year in case of DP vaccine from Veterinary Biological Insitute (VBI) Palode (Kerala) and in case of DP vaccine from Institute of Veterinary Preventive Medicine, Ranipet (Tamil Nadu) the immunity is claimed to be developed within 14 days and lasts for 18 months. DP vaccine from VBI Palode is recommended to be used at seventh week of age in ducks with booster dose given in the fourth month of age. In high risk areas it should

be given from third week of age followed by a second vaccination after four weeks and booster after four months. DP vaccine from IVPM Ranipet is safe in the age group of 8 to 12 weeks in ducks.

Kalaimathi and Janakiram (1989 and 1991) adapted a virulent strain of DPV to CEF and its immunogenicity was studied by serum neutralization test (SNT) in chicken eggs, counterimmunoelectrophoresis, micro SNT and challenge test. The findings indicated that the virus required a few more passages to reach optimum attenuation and immunogenicity to serve as an effective cell culture adapted DP vaccine.

Materials and Methods

MATERIALS AND METHODS

A. MATERIALS

Reference duck plague vaccine virus: The strain was obtained from Division of Avian Diseases, Indian Veterinary Research Institute, Izatnagar, in the form of freeze-dried chorio-allantoic membrane suspension in allantoic fluid harvested from infected duck embryos. The seed virus was originally procured from West Bengal Vaccine Institute, Calcutta by IVRI and undergone five passages in developing duck embryos.

Reference duck plague virulent virus: This strain (Repository number - (Vir) AD/87-1) was also obtained from Division of Avian Diseases, IVRI Izatnagar. The virus originally isolated from an outbreak in Assam, had undergone one passage in duckling and its liver suspension was lyophilized.

Commercial duck plague vaccine: Freeze-dried DP Vaccine (200 doses vial) of batch No.1 (mfg 5/92) was purchased from Institute of Preventive Veterinary Medicine, Ranipet, Tamil Nadu.

Ducklings: Healthy day-old unvaccinated male ducklings of White Pekins and Khaki Campbell breed were purchased from

Government Duck Farm, Niranam for main experiment. Their parent stock was vaccinated against DP before one year. Four-to eight-week-old ducklings and adult ducks, as and when required for virus passage or hyperimmunization, were procured from Duck Unit, University Poultry Farm, Mannuthy. Neither these ducklings nor their parents received DP vaccine.

Fertile duck/chicken eggs and chicken: These were obtained from University Poultry Farm, Mannuthy.

Sheep red blood cells (SRBC): Pooled blood from 3-4 healthy adult sheep was collected in Alsevers' solution in 1:1 (V/V) proportion and kept at 4°C for a week. After removal of plasma and buffy coat, three washings were given in phosphate buffered saline (PBS) pH 7.2 and if not used immediately, resuspended again in Alsevers' solution (1:4 V/V) and stored at 4°C upto two weeks.

Growth medium for leucocyte migration inhibiton test: Minimum Essential Medium (Eagle's) with Hanks' Salts (HiMedia) added with 10 per cent neonatal calf serum collected before colostrum-feeding, pH adjusted finally to 7.2 with sodium bicarbonate 7.5 per cent solution.

B. METHODS

3.1 PRELIMINARY INVESTIGATIONS

3.1.1. Collection of morbid specimens from field outbreaks

Ailing and dead ducks received from various outbreaks (Table 1) were utilized for virus isolation. In each case, history and essential epidemiological data were recorded. Ailing birds sacrificed by exsanguination and freshly dead ones were subjected to detail post-mortem examination.

3.1.2 Laboratory investigations

The following procedure was used in each case. The heart-blood was cultured on bacteriological and mycological media and the blood smears were examined after staining by Giemsa's technique. The tissues collected for further examination were liver, lung, and spleen. One portion of these tissues was preserved at -20°C after adding 3-4 ml tryptose phosphate broth and remaining portion pooled together, homogenised in a mechanical blender, centrifuged at 1000 rpm for 10 minutes and 0.2 ml supernatent each inoculated into a pair of mice and a three-week-old chicken subcutaneously to rule out the involvement bacterial or other viral agents.

Using the preserved tissues, 20 per cent (W/V)

Table 1. Details of duck plague (DP) outbreaks investigated during 1991

Sl. No.	Month	Name of the place	Age of ducks	No. of ducks in the flocks	Vaccination status against DP	Number of ducks affected	Number of ducks died	Duration of the ailment
1	2	3	4	5	6	7	8	9
1.	May	West Koratty	Adults	250	Two times vaccinated	60	50	7 days
2.	May-June	Shornur	Adults	240	Two times vaccinated	92	80	15 days
3.	May-June	Nellai	Adults	550	Once vaccinated before 4 months	75	60	10 days
4.	June	Kanjani	Adults	370	Not vaccinated	150	125	15 days
5.	September	Parappanangadi	1 mo.Old	700	Not vaccinated	150	47	10 days
6.	September-October	Eswaramangalam	3 mo.Old	1000	Not vaccinated	450	400	20 days

suspension was prepared in chilled Hank's balanced salt solution (HBSS, HiMedia) pH 7.2, centrifuged at 3000 rpm for 20 minutes and the supernatent treated with 600 IU penicillin G, 600 mcg of streptomycin sulfate and 300 mcg of gentamicin per ml and incubated for 45 minutes at 37°C. It was then inoculated into two susceptible ducklings of six weeks of age, which were observed for 10 days post-inoculation.

3.2 VIRUS ISOLATION

From the experimentally inoculated ducklings, when died, the tissues were collected and processed again in a similar way as described earlier. Finally, the supernatent treated with antibiotics was inoculated into five 12-day-old duck embryos via chorio-allantoic membrane (CAM) route as described by Betts (1967).

The inoculated eggs were candled twice daily for six days. Any mortality upto 36 h post-inoculation (PI) was considered as nonspecific and discarded. The embryos dying between 36 h and six days PI were chilled at 4°C for 4 h and those surviving upto sixth day were chilled for 12 h before being harvested. The procedure for inoculation into chicken or duck embryo was essentially the same except for the age of the embryo. Chicken embryos were inoculated on 10th day of incubation.

Harvesting of embryos was done as described by Nair (1978). Each embryo was carefully examined for the lesions and after noting the changes, liver, spleen and CAM were collected in allanto-amniotic fluid (AAF) and stored at -20°C until used.

3.2.1 Titration of virus

The titration of virus suspension was done as per the method described by Villegas and Purchase (1980).

Ten-fold serial dilutions of the suspension starting from 1 to 6 log 10 in chilled diluent (consisting of HBSS added with penicillin G 600 units, streptomycin sulfate 600 mcg and gentamicin 300 mcg) were made using separate sterile pipette for each dilution. From each dilution, 0.1 ml dose per embryo was inoculated into five embryos by CAM route. After recording the deaths resulting between 36 h and six days, the 50 per cent endpoint of embryo lethal dose (ELD50) was calculated as per the method illustrated by Reed and Muench (1938).

3.3 PREPARATION OF HYPERIMMUNE SERUM

Two 8-week-old healthy ducks were given three injections each of one ml DP vaccine strain virus having 2.5 log 10 ELD 50/ml at weekly intervals followed by two

injections of one ml virulent virus 3.0 log 10 ELD 50/ml intramuscularly at fortnightly intervals. Both the ducks were bled on 14th day after last injection, their sera pooled together, heat-inactivated at 56°C for 30 minutes and preserved in small aliquotes at -20°C until used.

3. 4 VIRUS NEUTRALIZATION TEST

All the virus neutralization (VN) tests were conducted in chicken embryos. Beta neutralization (constant virus-varying serum dilution) procedure was used as described and recommended by Beard (1980) for low-titred viruses.

The serum samples were collected aseptically, heat-inactivated at 56°C for 30 minutes and stored at -20°C until used.

Two-fold serum dilutions (starting from 1:2 to 1:128) were made in HBSS pH 7.2 added with 600 IU of penicillin G, 600 mcg of streptomycin sulfate and 300 mcg of gentamicin. One hundred ELD 50 units of recently titrated DPV (Vaccine strain) were added in each serum dilution and incubated at 30°C for one hour. From each dilution tube, 0.1 ml serum-virus mixture was then inoculated into each of the five chicken embryos via CAM route. The observations and harvesting of embryos were done as described earlier. The residual virus

was assayed for neutralization index (VNI) calculated as per the method described by Reed and Muench (1938).

3.5 IDENTIFICATION, CHARACTERIZATION AND ATTENUATION OF THE LOCAL ISOLATE

3.5.1 Identification

All the six isolates from local outbreaks were given one passage each in 6-8-week-old ducklings followed by 10 serial passages in duck embryos after which they were adapted to grow in developing chicken embryos by passaging three times.

All the isolates were then subjected to spot haemagglutination tests using chicken, duck, sheep and bovine erythrocytes as well as neutralization tests against known antiserum to DP virus.

3.5.2 Attenuation and characterization of the virus isolate

One of these isolates labelled as DP-S was used for attenuation. It was serially passaged in duck embryos 20 times (including earlier 10 passages) and labelled as DP-S20, further adapted in chicken embryos and passaged in them for 10 times and labelled DP-S20/10.

The isolate DP-S20 was inoculated into two ducks and

DP-S20/10 into three ducks (one ml each) by subcutaneous route to assess the degree of attenuation. Similarly at both passage levels, minimum lethal dose (MLD) and mean death time (MDT) in duck embryo as well as chicken embryo; mortality rate and pathogenicity index (p.i.) in ducks were calculated as per the methods illustrated in Poultry Biologics (1963).

3.5.2.1 Concentration of the virus

Step I: Harvesting of infected embryos: Several duck embryos infected with DP-S20 were harvested, AAF collected separately; liver, spleen and CAM pooled together and were homogenized with minimum of allanto-amniotic fluid. This homogenized material was frozen and thawed 3 times, centrifuged at 3000 rpm for one hour and the clear supernatent collected which was used for concentration by precipitation with polyethylene glycol 6000 (PEG).

Step II: Concentration by PEG-6000 treatment: The method described by Inglot et al. (1973) was followed. Two hundred ml supernatent was added with 14 g of PEG-6000 and 4.4 g of sodium chloride with constant stirring for one hour. The mixture was kept overnight at 4°C,then centrifuged at 3000 rpm for one hour, the supernatent discarded and sediment redissolved in sterile PBS, pH 7.2 to make a total volume to 20 ml. One half of this PEG-concentrated antigen was

preserved and used for agar gel diffusion test and remaining half was further concentrated by ultracentrifugation.

Step III: Concentration by ultracentrifugation: Ten ml of PEG-concentrated antigen was spun at 10000x g for one hour, the supernatent further centrifuged at 35000x g for two hours, the pellet resuspended in 0.5 ml PBS, pH 7.2 and centrifuged at 5000x g for 20 minutes. The clear supernatent was collected in a small siliconised vial and preserved at 4°C till use.

3.5.2.2 Electron microscopy of concentrated virus suspension

Copper grids (200 mesh) were coated with 0.33 per cent formvar in chloroform as described by Horne (1967). After overnight drying at 30°C, the concentrated virus suspension was charged on the grid, and after about two hours, stained with two per cent phosphotungstic acid (pH 7.0) as per the method detailed by Labzoffsky (1974). The grid was screened under 60000x to 120000x magnification in transmission electron microscope (Hitachi, Japan).

3.5.2.3 Agar gel diffusion test (AGDT)

The gel medium containing sodium azide (final concentration 0.001 per cent) for AGDT test was prepared in distilled water by adding the ingradients in following combinations.

A	-	1% agarose + 0.85% sodium chloride pH 7.4
B	-	1% agarose + 2.0% sodium chloride pH 7.4
C	-	1% agarose + 4.0% sodium chloride pH 7.4
D_1	-	1% agarose + 8.0% sodium chloride pH 7.4
D_2	-	1% agarose + 8.0% sodium chloride pH 6.0

Approximately 2.5 ml melted gel was slowly layered on precoated slides. Wells of 6 mm diameter and 4 mm interwell distance were cut. The test was conducted in duplicate sets. On each slide, the well in centre was loaded with DP hyperimmune serum, the well on right side with crude (unconcentrated) antigen and the other side, PEG-concentrated antigen. The tests were incubated for 18 h in humid chamber at 37°C and then at 4°C for 24 hours.

3.6 PREPARATION OF VACCINE FROM LABORATORY-ADAPTED STRAIN

The attenuated strain of DPV obtained from IVRI was passaged three times in duck embryos and then five times in chicken embryos. After the last passage, one ml each of 4 log 10 ELD50/ml was inoculated into two ducks which were observed closely for 12 days for detection of symptoms of disease. From the last embryo passaged material 20 per cent suspension of CAM, liver and spleen in AAF was prepared and stored in small aliquotes at -20°C until used.

Before use, this vaccine was titrated to know the ELD50 and then diluted suitably to get final virus concentration as 3.5 log 10/ml.

Its safety and sterility was tested as described in the information brochure supplied with commercial vaccine (IVPM, 1991).

3.7 TITRATION OF COMMERCIAL DP VACCINE

Freshly received two vials of freeze-dried DP vaccine (from IVPM Ranipet) of the same batch were selected. One was used for the vaccination of ducklings as per manufacturer's instructions and the other for titration.

For vaccine virus titration, the contents of the vial were suspended into two ml chilled HBSS pH 7.2 and then serially diluted, 10^{-1} to 10^{-5}. Each dilution including undiluted virus was inoculated in five developing chicken embryos and the ELD50 calculated as described earlier.

3.8 CHALLENGE VIRUS PREPARATION

The freeze-dried virulent DPV received from IVRI was reconstituted in one ml chilled HBSS pH 7.2 and inoculated into two healthy susceptible six-week-old ducklings. After their death, liver and spleen were collected and processed to make 20 per cent homogenate in chilled HBSS pH 7.2,

distributed in small aliquotes and preserved at -20°C till use. Just before challenge, one aliquote was opened and the virus was titrated.

3.9 LEUCOCYTE MIGRATION-INHIBITION TEST (LMIT)

3.9.1 Standardization of antigen concentration

From each of six ducks (three exposed and three unexposed to DPV) 10 ml blood in heparin (20 IU/ml) was collected. The leucocyte separation in each case was done as follows -

The blood was centrifuged at 200 x g for 20 minutes, the leucocyte-rich plasma along with top portion of erythrocyte column was collected, mixed and gently layered over 5 ml Ficoll-paque (Pharmacia, Sweden) and centrifuged at 400x g for 30 minutes. The buffy layer above the packed erythrocyte column aspirated with sterile Pasteur-pipette and transferred to four ml chilled HBSS, pH 7.2. After two washings in HBSS, they were finally suspended in growth medium. The concentration of viable cells was adjusted to 1.5×10^8/ml.

The allanto-amniotic fluid from DP-infected chicken embryos having ELD50 2.8 log 10/ml was used as the antigen. Each leucocyte sample was divided into three sets. Set A and B

were treated with 1:10 and 1:20 dilution of the antigen in growth medium respectively and set C was treated with growth medium containing 10 per cent AAF collected from uninoculated normal chicken embryo.

3.9.2 Test proper

The method was similar to that described by Karpe (1982) with following modifications.

a. Leucocyte separation: Four ml of blood was collected from jugular vein of ducks in heparin (20 IU/ml). The separation of leucocytes was done using two ml Ficoll-paque per sample as described earlier.

b. The antigen concentration 1:10 in growth medium was used after standardization.

c. LMI plates (Laxbro, Pune) were used.

d. The area of migration of each sample was measured under microscope using 5x eyepiece equipped with ocular micrometer and 4x objective lens (total magnification 20x).

The area of migration zone in antigen-treated chamber was compared with that of the corresponding control

and migration index as well as percentage of inhibition were calculated by the following formulae

Migration index (MI)

$$= \left(\frac{\text{Average area of leucocyte migration with antigen}}{\text{Average area of leucocyte migration without antigen}} \right) \times 100$$

Per cent inhibition

$$= \left\{ \left(1 - \frac{\text{Average area of leucocyte migration with antigen}}{\text{Average are of leucocyte migration without antigen}} \right) \times 100 \right\}\%$$

3.10 PASSIVE HAEMAGGLUTINATION (PHA) TEST

This test was standardized as per the method followed by Ziambo *et al*. (1973 a) with some modifications.

Tannic acid (Merck) 1:20000 was used for coating formalinised sheep erythrocytes after making trials with 1:10000, 1:20000 and 1:30000. For antigen coating, two per cent tanned sheep erythrocytes (FTE) were mixed with 1:10 diluted AAF having virus titre 2.8 log 10 ELD50/ml after making trials for undiluted AAF, 1:10 and 1:20. Similarly for antigen coating of FTE, PBS of different pH, viz. 4.5, 6.0 and

7.2 were tried and PBS, pH 6.0 was used after obtaining optimum results.

All the sera samples to be tested were heat-inactivated at 56°C for 30 minutes, after cooling, adsorbed with 0.1 ml of 10 per cent FTE/ml of serum, incubated at 37°C for 30 minutes and then the cells harvested by centrifugation.

The tests were performed in microtitre plates (Laxbro, Pune) with serial two-fold serum dilutions in diluent (comprising of one per cent inactivated rabbit serum in PBS pH 7.2) starting from 1:2 to 1:256 and equal quantity of one per cent antigen-coated FTE were added to each cup. The results were read after three hours incubation at 30°C in humid chamber.

Appropriate controls were added with each batch of tests, viz.

1. Known positive serum + Uncoated FTE
2. Known positive serum + Antigen coated FTE
3. Known negative serum + Antigen coated FTE
4. Diluent only + Antigen coated FTE

After the standardization of test, five test serum samples were used in triplicate sets, one without

heat-inactivation and without FTE adsorption, second heat-inactivated but without FTE adsorption and the third set heat-inactivated and FTE adsorbed, to know the difference in PHA titres due to nonspecific agglutinins.

3.11 ESTIMATION OF LEVEL OF PROTECTION

Each bird receiving challenge infection was keenly observed for clinical signs of the disease daily upto 10 days.

A method to know the intravenous pathogenicity index of Newcastle disease virus in a comparative study of various strains has been described in Poultry Biologics (1963). An attempt was made in this study to apply the method for assessment of protection due to vaccination in challenged birds.

If a comparison is made between the pathogenicity indices (p.i.) of groups receiving different type of vaccine, the reduction in p.i. would be more prominent in effective vaccine whereas in case of less effective vaccine, the p.i. would be much higher.

The method used was as follows:

Four probable conditions of the ducks in each group were considered to catagorise their resistance to challenge infection.

	Condition (Clinical status)	Scoring factor
a.	Death	3
b.	Severe (lesions in buccal cavity, swelling around eyes, oculonasal discharge, diarrhoea and prostration)	2
c.	Mild (Transient anorexia, dullness)	1
d.	Normal (Healthy and active)	0

Each duck in each group was observed for the clinical status. A total of 10 days observation was called as sum, which was multiplied by scoring factor of that catagory. The total score thus arrived was divided by total number of observations i.e. Number of ducks challenged x number of days observation, to obtain the p.i. An illustration of this method has been presented in Table 2.

Table 2. Model for calculation of pathogenicity index (p.i.)

Example Group A. Vaccinated with commercial vaccine (Single) on 6th week of age and challenged at 4 weeks after vaccination

Clinical status	Days of observation	1	2	3	4	5	6	7	8	9	10	Sum	x	Scoring Factor	=	Total
1. Death		0	0	0	0	0	0	0	0	0	0	0	x	3	=	0
2. Severe ailment with oral lesions		0	0	0	0	1	3	3	3	3	3	16	x	2	=	32
3. Mild signs of disease		0	0	1	2	2	0	0	0	0	0	5	x	1	=	5
4. Normal		3	3	2	1	0	0	0	0	0	0	9	x	0	=	0
																37

$$\text{Pathogenecity index} = \frac{\text{Total Score}}{\text{Total number of observation}} = \frac{37}{30} = 1.23$$

3.12 ASSESSMENT OF IMMUNE RESPONSE TO VACCIATION

One hundred healthy, day-old male ducklings (randomly selected from among White Pekins and Khaki Campbell in equal number) were procured from Government Duck Breeding Farm, Nirnam. They were maintained under standard managemental conditions in cage system without any medication and fed commercial duck feed.

At the age of sixth week, 63 of these ducklings were divided into five groups, each group being maintained in separate cages placed sufficiently wide apart to prevent the contact between the groups. Four groups, namely, SCV (receiving single commercial vaccine), SAV (receiving single laboratory-adapted vaccine), DCV (receiving double commercial vaccine) and DAV (receiving double laboratory adapted vaccine) comprised 12 ducklings each and the fifth group NVC (unvaccinated control), 15 ducklings.

In order to establish the duck plague-free status of the experimental ducklings, they were screened for neutralizing antibodies to DPV, passive haemagglutination titre of the serum, leucocyte migration-inhibition in presence of DP antigen and shedding of virus as assessed by rectal swab-processing before the commencement of experiment.

Ducks in each group (except control-NVC) were given DP vaccine. The details of type of vaccine, age at vaccination etc. are shown in Table 3.

To assess the immune responses to these vaccines, the following parameters were studied at regular intervals -

a. Virus neutralization (VN) indices
b. Leucocyte migration-inhibition (LMI)
c. Passive haemagglutination (PHA) titre of the serum
d. Protection to challenge infection

3.12.1 Test intervals

Collection of blood from ducks for VN, PHA and LMI tests was done simultaneously at the same intervals. The first collection was done before the commencement of experiment (pre-test). For single vaccinated groups, bleeding was done every week upto four weeks, every fortnight upto 12 weeks and then every month upto 24 weeks post vaccination. The ducks in other three groups (i.e., double vaccinated with either commercial or lab adapted vaccine and unvaccinated controls) were bled regularly every week upto six weeks post single vaccination, thereafter at fortnightly interval upto eight weeks post double vaccination and last three bleedings were done at monthly interval. The details are shown in Table 4.

Table 3. Assessment of immune response: Plan of vaccination

Sl. No.	Group	No.of ducks	Age of ducks at first vaccination	Age of ducks at second vaccination
1.	Single commercial vaccine (SCV)	12	6 weeks	--
2.	Single laboratory-adapted vaccine (SAV)	12	6 weeks	--
3.	Double commercial vaccine (DCV)	12	6 weeks	10 weeks
4.	Double laboratory-adapted vaccine (DAV)	12	6 weeks	10 weeks
5.	Unvaccinated controls (NVC)	15	--	--

Table 4. Assessment of immune response: Plan of experiment at a glance

Operation	Age of ducks	Weeks post vaccination		Groups				
		Single	Double	1 SCV	2 SAV	3 DCV	4 DAV	5 NVC
Pre test observation	6 weeks	0	-	(12)	(12)	(12)	(12)	(12)
LMIT/VN/PHA								
Vaccination	"			+(12)	+(12)	+(12)	+(12)	+(12)
LMIT/VN/PHA	7	1	-	+	+	+	+	+
"	8	2	-	+	+	+	+	+
"	9	2	-	+	+	+	+	+
"	10	4	-	+	+	+	+	+
Challenge infection	10	4	-	+(3)*	+(3)*	+(3)*	+(3)*	+(3)*
Revaccination	10	4	0	-	-	+(9)	+(9)	-
LMIT/VN/PHA	11	5	1	-	-	+	+	+
"	12	6	2	+	+	+	+	+
"	14	8	4	+	+	+	+	+
Challenge infection	14	8	4	+(3)*	+(3)*	+(3)*	+(3)*	+(3)*
LMIT/VN/PHA	16	10	6	+	+	+	+	+
"	18	12	8	+	+	+	+	+
"	22	16	12	+	+	+	+	+
"	26	20	16	+	+	+	+	+
Challenge infection	26	20	-	+(3)*	+(3)*	-	-	+(3)*
Challenge	30	24	20	-	-	+(3)*	+(3)*	+(3)*
LMIT/VN/PHA	30	24	20	+	+	+	+	+

Figures in parenthesis indicate number of birds used

* Birds eliminated after challenge experiment

\+ Groups tested/used

\- Not done

1. Single commercial vaccine
2. Single lab-adapted vaccine
3. Double commercial vaccine
4. Double lab-adapted vaccine
5. Unvaccinated controls

3.12.2 Challenge infection

Ducks in each group were subjected to challenge infection at regular intervals. The details are presented in Table 5. Each duck to be challenged was inoculated one ml virulent virus having 3.5 log 10 ELD50/ml intramuscularly and observed for two weeks thereafter for mortality and pathogenicity indices. They were also tested for VN indices, LM inhibition and PHA titres at 30 days post-challenge.

3.12.3 Sampling details of in-vitro tests

For VN test, equal quantity of serum sample from each bird was pooled to make two pool samples from each group e.g. Initial 12 serum samples constituted two pools of six each. Such 10 pooled serum samples from five groups were subjected to virus neutralization at every test interval except the last, when three ducks remained in each group constituting only one pool.

For LMI test, from every group four ducks were randomly selected on every test interval except the last, when only three duck remained in each group and all them were tested.

For PHA test, all the birds were individually tested at every test interval.

Table 5. Assessment of immune response: Plan of challenge infection

Sl. No.	Group	Weeks after single vaccination			Weeks after double vaccination	
		4	8	20	4	20
1.	SCV	3	3	3	-	-
2.	SAV	3	3	3	-	-
3.	NVC	(3)	(3)*	(3)	(3)*	(3)
4.	DCV	3	-	-	3	3
5.	DAV	3	-	-	3	3

* Challenge after 8 weeks of single vaccination and 4 weeks of double vaccination was on the same day and hence the control group was common for both challenges

SCV - Single commercial vaccine DCV - Double commercial vaccine

SAV - Single lab-adapted vaccine DAV - Double lab-adapted vaccine

NVC - Unvaccinated control

3.13 ASSESSMENT OF CARRIER STATUS

Rectal swabs from randomly selected three ducks from each group were collected on prevaccination stage, and on seventh, 14th and 30th day post-vaccination.

Similarly from each of the challenged and survived bird, rectal swabs were collected on seventh, 14th and 30th day post challenge.

The rectal swabs from three ducks in each group were collected separately and added with 1.5 ml HBSS pH 7.2 and after 2 treatments of freezing and thawing, the washings were pooled together from each group, centrifuged at 3000 rpm for 20 minutes. The supernatent was added with antibiotics at the rate of 600 units of penicillin G, 600 mcg of streptomycin sulfate and 300 mcg of gentamicin per ml, incubated for 45 minutes at 37°C. Each pooled sample was then inoculated into five duck embryos by CAM route for DP virus isolation.

3.14 VACCINE TRIAL IN AN ORGANIZED DUCK FARM

University Duck Farm, Mannuthy having a mixed population of 180 White Pekin and Khaki Campbell adult breeder ducks, maintained under semi-intensive system of management, was selected for vaccine trial. The ducks had no earlier exposure to DP either through disease or vaccination.

All the 180 ducks were vaccinated with commercial DP vaccine at the age of one year. Just before vaccination and again after eight weeks of vaccination randomly selected 10 per cent ducks (18 each) were bled, their sera samples screened for VN and PHA test. For neutralization, six sera samples were pooled to make one test sample and such three samples were tested each time. For passive haemagglutination test, two samples were pooled in each case and nine such samples were tested each time.

After six weeks of vaccination each of the randomly selected two ducks from this flock were challenged with one ml virulent DPV having titre 3.5 log 10/ml to assess the protection level after vaccination.

The eggs laid by the vaccinated ducks were screened for presence of antibodies in the yolk. Nine eggs from the ducks of this farm were collected six weeks post-vaccination. The yolks from these eggs were processed as per the method described by Sulochana (1988). The nine yolks were pooled to make three samples of three each which were then diluted 1:2, 1:4 and 1:8 and were tested for virus neutralization antibodies.

Three ducklings hatched from eggs collected before and three to 6 weeks after vaccination from the eggs collected

were challenged on third day of hatching with 0.1 ml of 2.5 log 10 ELD50/ml virulent DP virus.

3.15 RECORDING AND HANDLING OF DATA

The results of passive haemagglutination and leucocyte migration-inhibition tests were analysed statistically to determine the mean titres, mean migration indices and standard error. The complete randamised design was applied to demonstrate the significant differences in the responses of vaccinated ducks. The methods described by Snedecor and Cochran (1967) were followed.

Results

RESULTS

4.1 PRELIMINARY INVESTIGATIONS

The history, nature and the clinical picture of six outbreaks investigated during 1991 indicated duck plague virus as the causative agent. The vaccinated as well as unvaccinated ducks were affected. The outbreaks occurred between May and October. The details of the outbreaks are presented in Table 1 and the abstract is shown below -

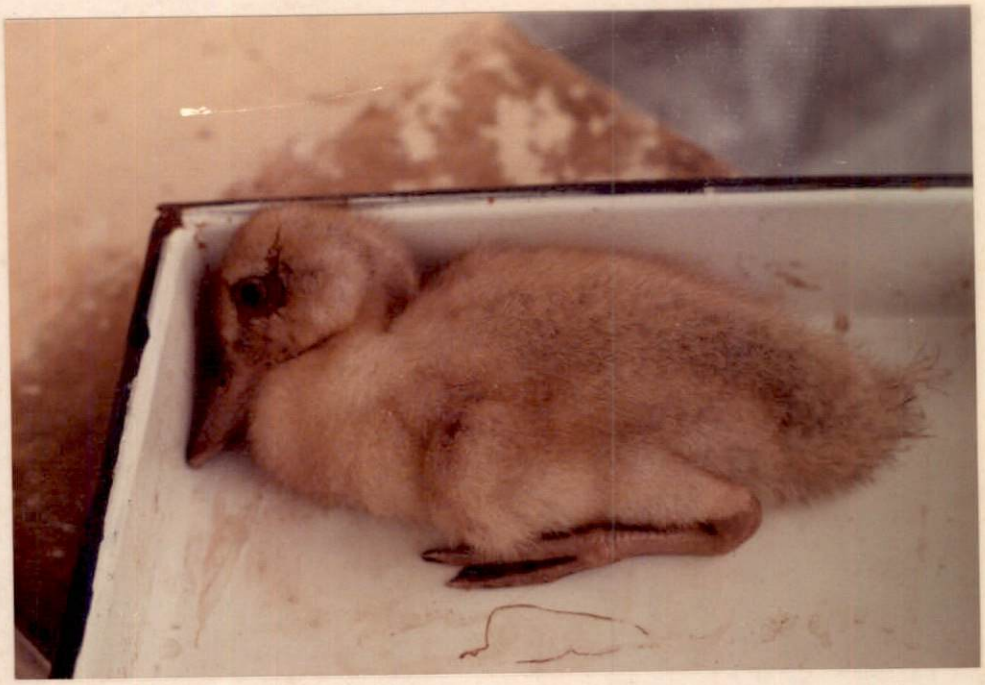
Morbidity and mortality in DP outbreaks—

Age group	Status of vaccination against DP	Morbidity %	Mortality %
Adults	Vaccinated	25	22
Adults	Unvaccinated	40	33
Ducklings 1-3 mo	Unvaccinated	33	23

During these outbreaks, the ailing ducks were keenly observed for the clinical signs, which were more or less similar as listed below - dullness, loss of appetite, increased thirst, disinclination to move, hyperthermia, lachrymation, nasal discharge initially watery becoming thick and mucoid at later stages, swelling of the face (Plates I and II), difficulty in opening the mouth, hoarse call, laboured breathing and whitish diarrhoea. A few hours before death,

Plate I Naturally infected duck. Note swelling of the head, glueing of the eyes and thick mucus nasal discharge

Plate II Experimentally infected duckling showing severe symptoms on 3rd day PI. Note wetting around the eyes and ruffled feathers



ducks preferred to sit with beak rested on some object or on back in the wings. The affected birds usually died and the recovery percentage was very low.

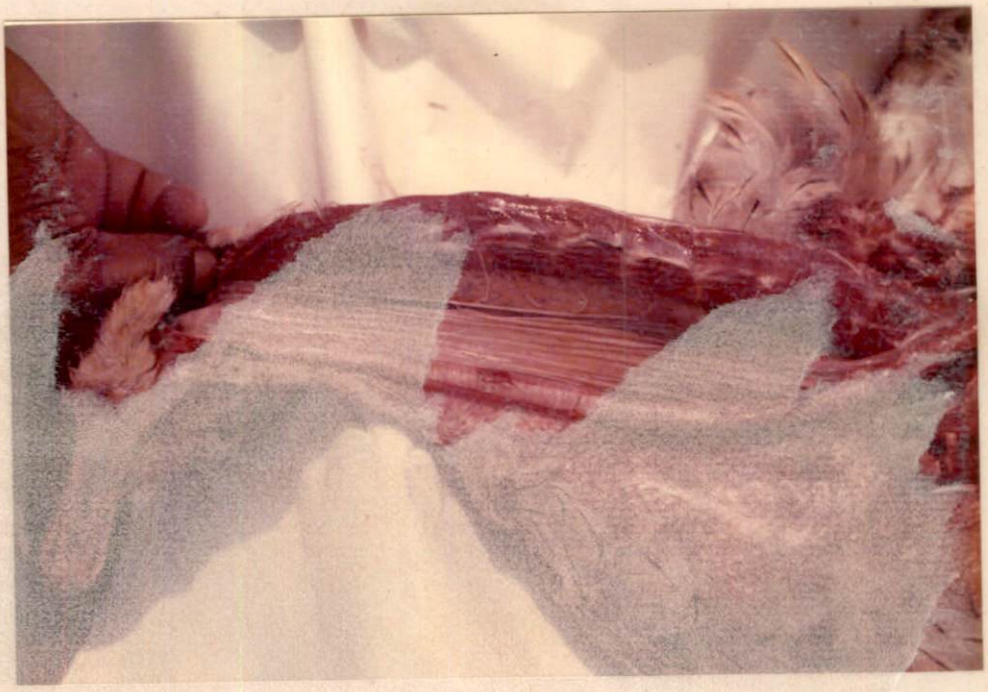
During the investigation of these outbreaks, 27 dead and eight sacrificed ducks were subjected to detailed necropsy examination. The lesions seen were:

Petechial or ecchymotic haemorrhages on visceral organs, particularly on heart, serous membranes and abdominal walls; necrotic foci and petechial haemorrhages on liver; enlargement and congestion of liver and spleen; haemorrhages on ovary in case of adult female ducks and variable extent of enteritis and peritonitis. In case of birds at recovery stage or in chronic cases, the liver and spleen showed significant atrophy.

The pathognomonic lesions were seen in majority of the cases, viz., yellow, greyish, greenish crusty diphtheritic plaques on the inner wall of the oesophagus arranged in longitudinal rows with petechiation, sometimes extending upto buccal cavity. In many cases, tiny haemorrhagic spots or generalized congestion of proventriculus and gizzard walls, gizzard muscle necrosis and haemorrhages in musculature were observed (Plates III to VII). In a few cases, red annular thickened bands at regularly spaced intervals in the small intestines were seen.

Plate III Initial lesions of DP in oesophagus. Yellowish diphtheritic crust on longitudinal rows (Ailing duck sacrificed on 3rd day PI)

Plate IV Excessive necrosis and greenish discolouration of oesophageal lesions in an adult duck died on 6th day PI. Note the necrotic lesions on tongue and throat



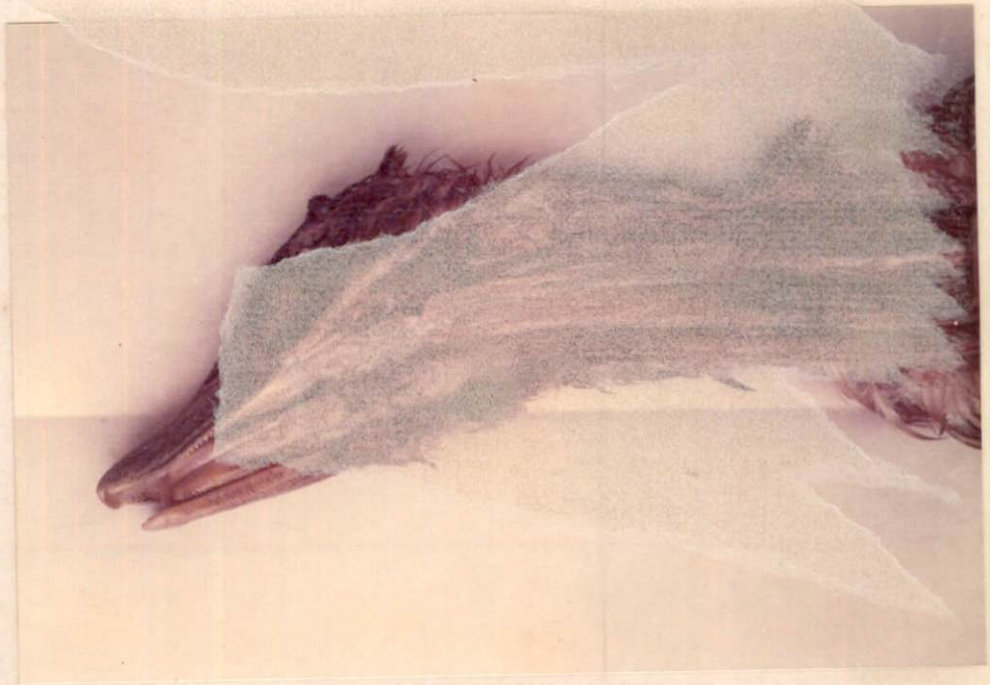
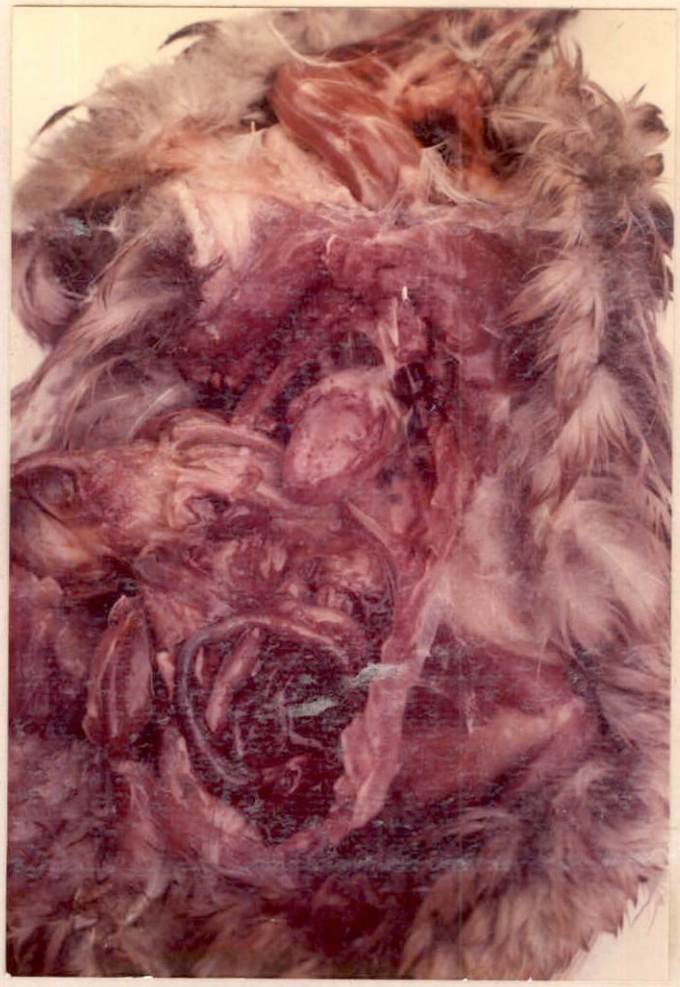


Plate V Viscera of a duck died of DP. Note petechial haemorrhages on heart, necrotic foci on spleen, peritonitis and muscular haemorrhages on neck muscles (arrow)

Plate VI Erosion of papillae and haemorrhages on proventriculus, congestion of ovary with misshapen ova



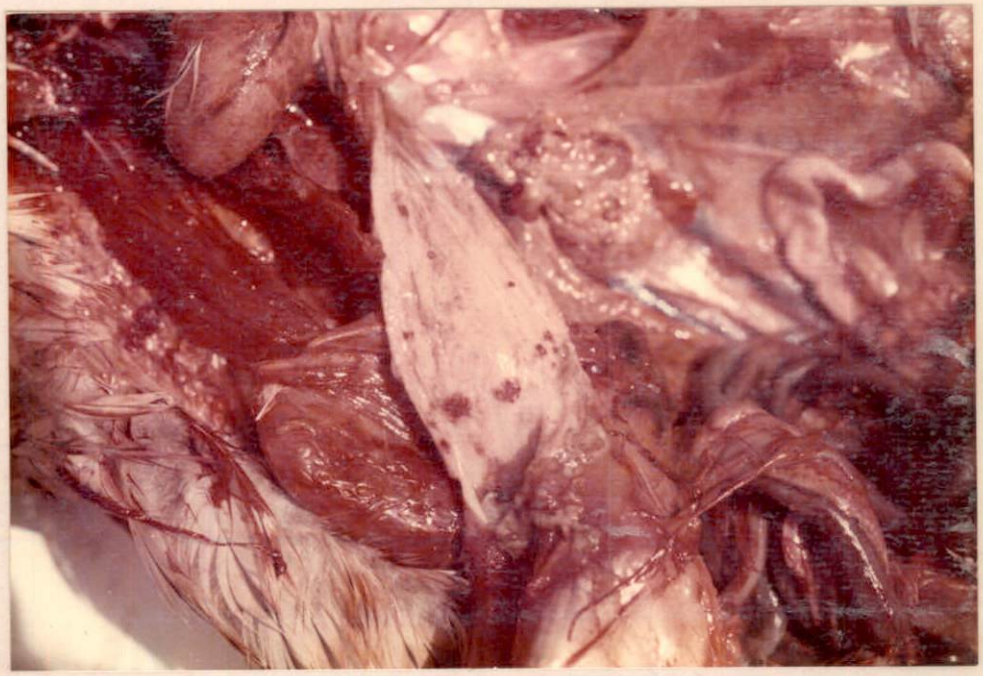
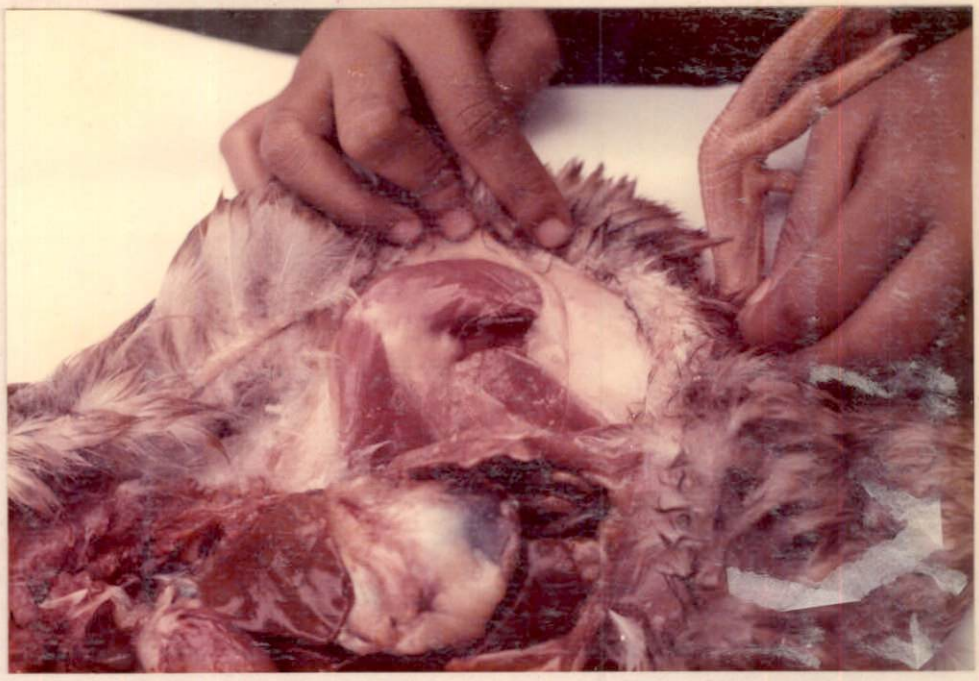
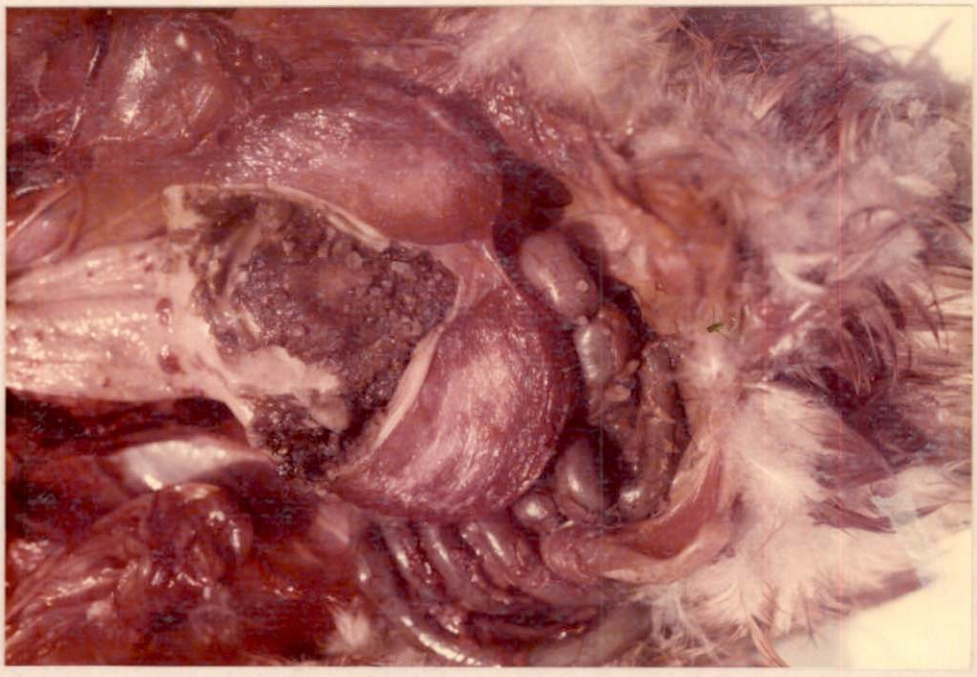


Plate VII Muscular haemorrhages in thigh muscle in DP

Plate VIII Gizzard muscle necrosis



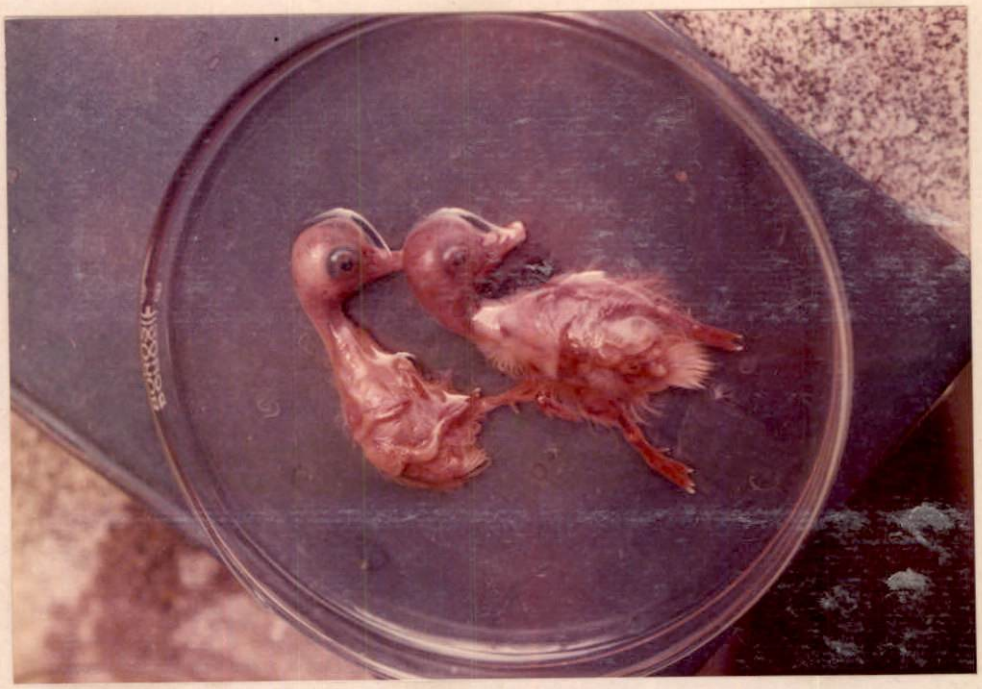


4.2 ISOLATION, CHARACTERIZATION AND ATTENUATION OF AETIOLOGICAL AGENT

The heart blood from freshly dead and sacrificed ducks cultured on bacteriological and mycological media yielded no growth. The blood smears stained with Giemsa's stain did not reveal any specific organism. All the mice and chicks inoculated with fresh tissue homogenate without addition of antibiotics remained healthy throughout the observation period of two weeks, whereas the ducklings inoculated with similar material (for each of the six outbreaks) showed symptoms of duck plague on third or fourth day and died between fourth and sixth day post-inoculation (PI). On postmortem examination of these ducklings, typical lesions as described earlier were seen.

Twelve-day-old duck embryos inoculated with 20 per cent suspension of liver and spleen from these ducklings died between fourth and seventh day PI showing oedema, haemorrhages all over the body (Plate IX), congestion of chorio-allantoic membrane (CAM) and enlargement of spleen and liver with petechial haemorrhages and necrotic foci. The same material inoculated into 10-day-old chicken embryos did not kill them even after two blind passages.

Plate IX Duck embryos infected with field sample, died on 6th day PI. Note the congestion particularly on extremities



4.2.1 Identification and characterization of the isolates

The allanto-amniotic fluid (AAF) or tissue homogenate from duck or chicken embryos inoculated with field samples did not agglutinate chicken, duck, sheep or bovine erythrocytes.

The log 10 values of embryo lethal dose 50 (ELD 50) of these six isolates ranged between 2.6 to 3.3 per ml. Each isolate was neutralized by specific hyperimmune serum raised against DPV in ducks having neutralization index (VNI) 2.4 log 10.

These six isolates were named according to the place of outbreak. One of the isolates from Shornur, named DP-S was further studied for biological characterization. After initial two passages in duck embryos DP-S caused even pattern of mortality and uniformity of lesions between day four and six post-inoculation.

4.2.2 Attenuation of the isolate DP-S

In an attempt to attenuate the virus, DP-S was passaged 20 times in duck embryos. After undergoing 20 passages (DP-S20), when inoculated into two ducklings, the virus caused their death on fifth day PI showing symptoms and lesions of duck plague.

It was then adapted to grow in chicken embryos

resulting in death of the embryos between third and fifth day PI and produced similar lesions as in duck embryos (Plates X to XIII). The DP-S20 was given 10 chicken embryos passages (DP-S20/10) and inoculated into three ducklings resulting in cent per cent mortality between day six and eight PI showing symptoms and lesions typical of duck plague. Its pathogenicity index (p.i.) was 1.23 (Table 6).

The minimum lethal dose (MLD) for DP-S20 and DP-S20/10 were the same i.e. 10^{-2}/0.1 ml dose. The mean death time of DP-S20 and DP-S20/10 were 106.3 h and 85.0 hours respectively.

After last passage in chicken embryo, the ELD 50 of 20 per cent suspension of liver, spleen and CAM in AAF was 4.8 log 10/ml whereas that of AAF alone, it was 2.8 log 10/ml. The results are presented in Table 6.

4.2.3 Electron microscopy of the isolate DP-S

The concentrated DP-S isolate when examined under transmission electron microscope after negative staining, revealed characteristic herpesvirus like capsids. They were seen in suspension coated with extraneous substances, partially obscuring their morphology. However, most of the virion particles had damaged structure permeable to PTA stain. The virion diameter was around 100 nm and enveloped virions measured upto 380 nm (Plate XIV).

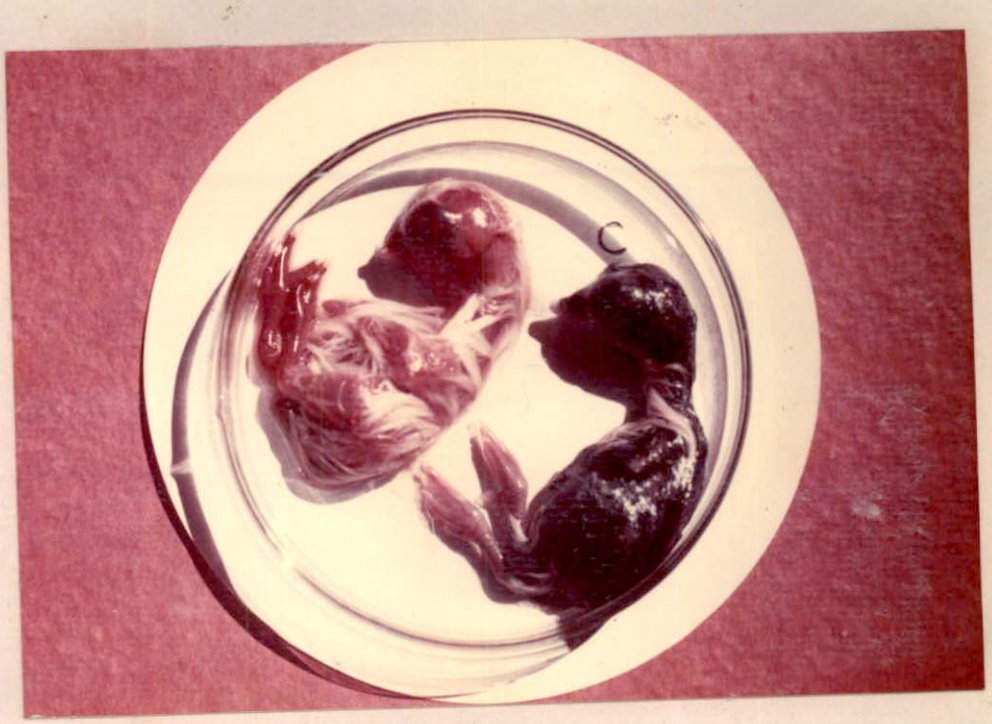
Table 6. Biological characters of DP virus (DP-S) after partial attenuation

Sl. No.	Character	After 20 passages in duck embryo (DP-S20)	After 20 passages in duck embryo + 10 passages in chicken embryo (DP-S20/10)
1.	Haemagglutination with duck, chicken, sheep or bovine erythrocytes	-	-
2.	Minimum lethal dose in embryos	10^{-2}/0.1 ml	10^{-2}/0.1 ml
3.	Mean death time of embryos after inoculation	106.3 h	85.0 h
4.	Pathogenicity index in susceptible 6-week-old ducklings	ND	1.23
5.	Mortality in susceptible 6-week-old ducklings	2/2	3/3
6.	Embryo lethal dose 50 (ELD 50) of 20 per cent suspension of CAM, liver and spleen in allanto-amniotic fluid	ND	4.8 log 10/ml
7.	ELD 50 of allantoic fluid alone	ND	2.8 log 10/ml
8.	Characteristic lesions in ducks	+	+
9.	Characteristic lesions in ducks embryos	+	ND
10.	Characteristic lesions in chicken embryos	ND	+

ND - Not done + present - absent

Plate X Chicken embryo infected with DP virus (DP-S20).
Right side (C) - Uninfected control

Plate XI Congested chorio-allantoic membrane of DP-infected chicken embyro.
Left side (C) - Uninfected control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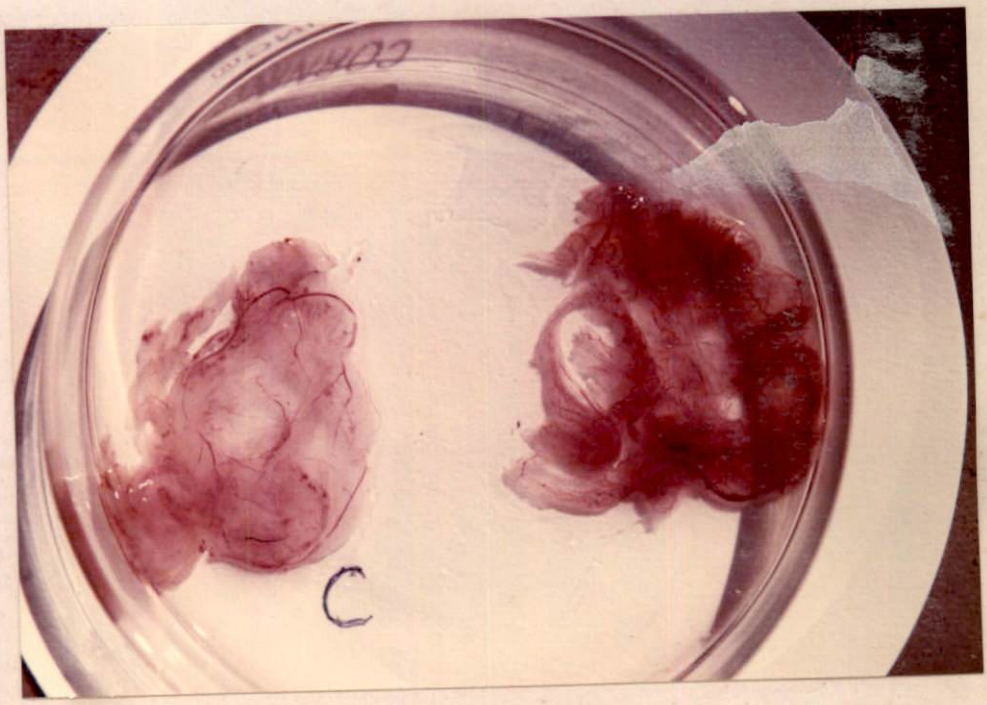

C

Plate XII Chicken embryos infected with DPV, 10th passage. showing severe congestion. Left side small petridish - Uninfected control

Plate XIII DPV infected chicken embryo showing petechial haemorrhages and necrotic foci on liver

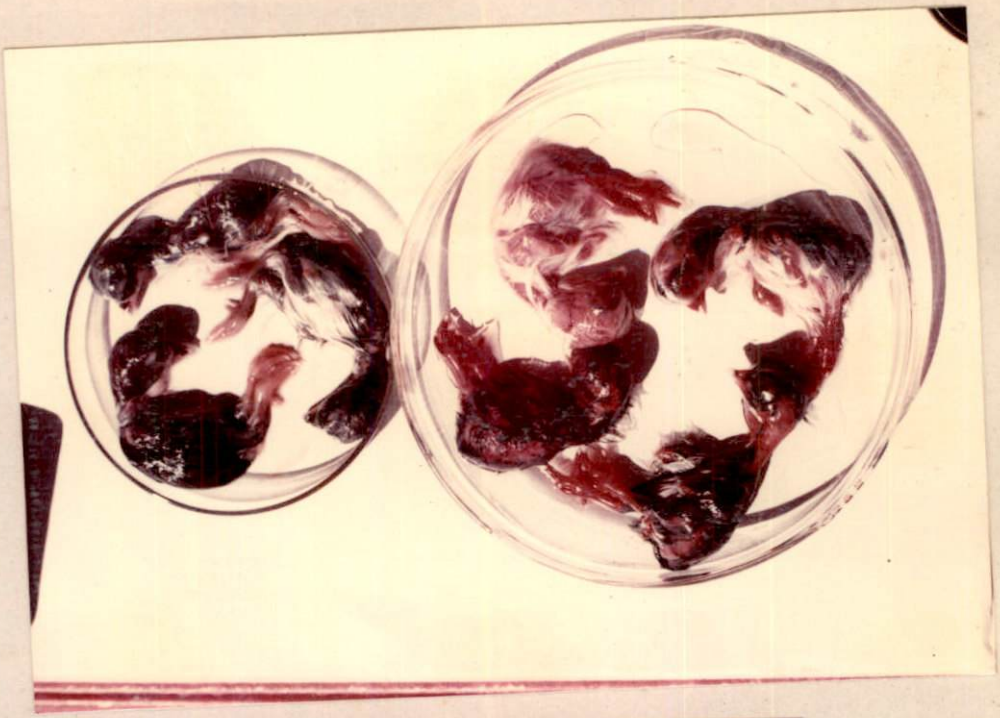


Plate XIV Electron micrograph of negatively stained DPV (80000x)

4.2.4 Agar gel diffusion test

The PEG-concentrated DPV or crude DPV (infected embryo-tissue extract) did not show any precipitation reaction at any of the concentration of sodium chloride tried at pH 6.0 or 7.2.

4.3 TITRATION OF LABORATORY-ADAPTED VACCINE

The log 10 value of ELD 50 for laboratory-adapted vaccine was found to be 4.5 per ml, which was then diluted suitably in HBSS pH 7.2 to contain 3.5 log 10 ELD 50/ml dose before vaccination.

4.4 TITRATION OF COMMERCIAL VACCINE

The vaccine vial contained 0.74 log 10 ELD 50/ml when diluted as per manufacturers instruction. The reconstituted vaccine was not homogenous due to particulate material which settled down after resuspension.

4.5 ASSESSMENT OF IMMUNE RESPONSES

4.5.1 Detection of virus neutralizing antibodies (VN Abs) to DPV in experimental ducks (Table 7, Fig.1,2)

None of the 10 pools of serum samples collected from ducklings before vaccination had any VN Abs to DP virus.

The ducks receiving only one dose of commercial vaccine (SCV) at six weeks of age showed a very low level of VN Abs throughout the observation period. One week after vaccination, VN Ab titres were between 2 and 4 (mean VN index 0.46) which slowly increased to 8 by third week and for another four weeks remained almost at the same level. Thereafter VN indices were fast declining and by 16th week post-vaccination (pv), they were negligible. For the last two tests, i.e., 20th and 24th week pv, the indices were nil.

In ducks vaccinated with single dose of laboratory-adapted vaccine (SAV), the increase in VN Abs was faster. There was increase upto four weeks when the maximum titre recorded was 16 (mean VN index 1.19). It remained almost at the same level for another four weeks. Thereafter slow decline in mean VN indices was noticed. However, even at the end of observation period i.e., 24th week pv the level of VN Abs was comparable to the peak VN Abs seen in ducks from SCV group.

Table 7. Mean neutralization indices of ducks vaccinated against DP

Observation No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Age of ducks in weeks	6	7	8	9	10	11	12	14	16	18	22	26	30
Weeks after 1st vaccination	Pre-vaccine	1	2	3	4	5	6	8	10	12	16	20	24
Weeks after 2nd vaccination	-	-	-	-	-	1	2	4	6	8	12	16	20
SCV (Single (comm. vaccine)	0	0.46	0.54	0.71	0.70	NT	0.68	0.61	0.60	0.48	0.30	0	0
SAV (Single lab-adapted vaccine)	0	0.68	0.90	1.13	1.19	NT	1.16	1.01	0.91	0.90	0.90	0.78	0.70
DCV (Double commercial vaccine)	0	0.48	0.62	0.71	0.77	1.38	1.26	1.21	1.07	0.98	0.85	0.81	0.75
DAV (Double lab-adapted vaccine)	0	0.65	0.91	1.11	1.19	1.59	1.61	1.64	1.64	1.45	1.23	1.20	1.06
NVC (Unvaccinated control)	0	0	0	0	0	0	0	0	0	0	0	0	0

NT = Not tested

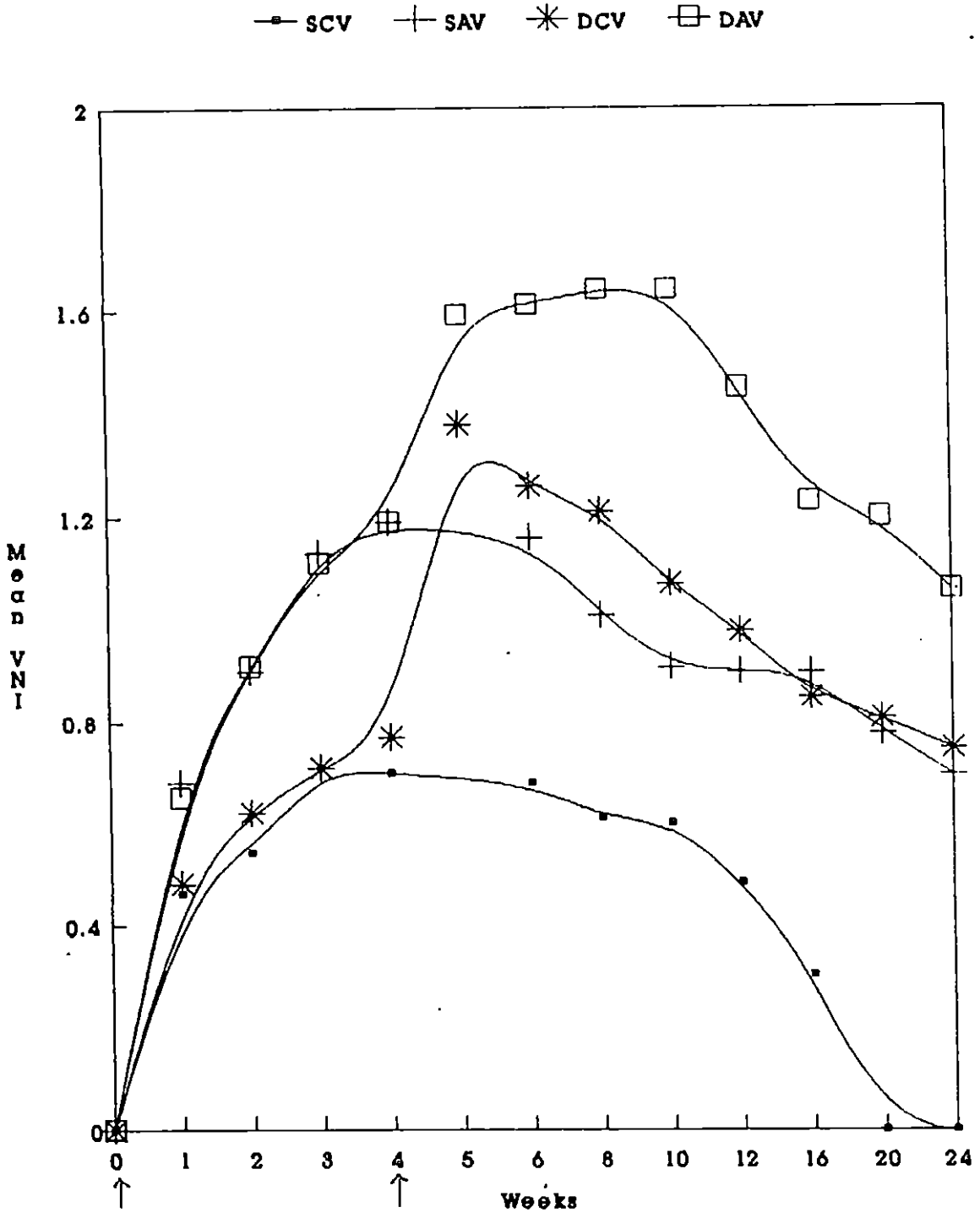
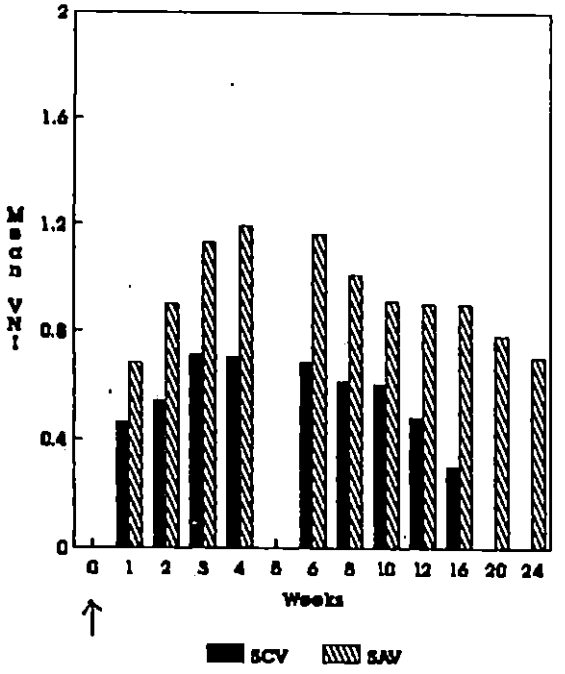


Fig.1 Mean virus neutralizing indices of vaccinated groups. Control titres were nil.
Arrows indicates day of vaccination.

SINGLE VACCINATION GROUPS



DOUBLE VACCINATION GROUPS

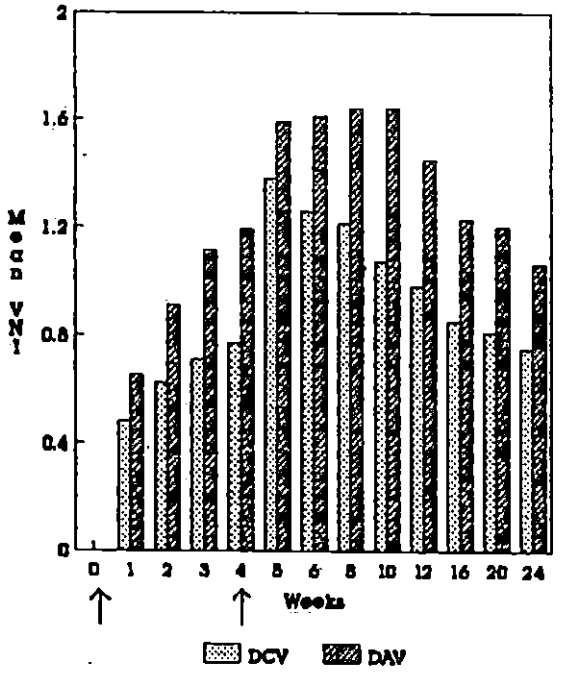


Fig.2 Comparison of mean virus neutralizing indices (VNI) among single and double vaccination groups. Control titres were nil. SCV & SAV not tested on week 5. Arrows indicate day of vaccination

The trend and levels of VN Abs in double vaccinated groups were similar to that of single vaccinated groups before receiving second dose of vaccine (i.e., upto four weeks pv).

The ducks receiving second dose of commercial vaccine (DCV) at 10th week of age (i.e., four weeks after first vaccination) showed a better increase in VN Abs. At second week post-double vaccination (pdv), VN indices rose upto 1.44 (mean VN index 1.38) but could not maintain the peak for more than one week. Although, significant level of titres remained till the end of observation, they were less than half of the peak obtained in this group.

As compared to the groups SCV, SAV and DCV, ducks of the DAV group which received laboratory adapted vaccine twice at an interval of four weeks showed the highest titres of VN Ab and longer persistance period. During the period of one to six weeks pdv, the VN titres were in between 32 and 64 (VN indices 1.59 to 1.64). The decline thereafter was very slow, at the end of observation period, i.e., 16 and 20 weeks pdv, VN indices were 1.20 and 1.06 respectively.

The VN Ab titres in unvaccinated ducks (NVC) remained nil throughout the experiment period.

4.5.2 Leucocyte migration-inhibition test (LMIT) (Plate XV and XVI)

On standardization of LMIT using two antigen concentrations (i.e., 1:10 and 1:20), 1:10 was found more suitable. The results of the trial are presented in Table 8.

While recording the individual leucocyte migration (LM) indices in healthy controls, it was found that the lowest LM index was 81.0 hence values between 80 and 100 were considered as normal migration, whereas those below 80 were treated as inhibition of migration.

None of the control samples at any stage of observation showed inhibition of LM in presence of DP antigen. Similarly leucocytes from all DP-vaccinated ducks migrated to the normal extent without adding any antigen or with 10 per cent sterile allantoic fluid (from healthy uninfected chicken embryos) in the growth medium.

The mean LM indices of 16 randomly selected ducks (four from each of the four vaccinated groups) on the day before vaccination were comparable to that of unvaccinated controls (NVC) which ranged between 94.9 and 100.0. The results are furnished in Table 9, Fig. 3 and 4.

In group of ducks vaccinated with single dose of

Table 8. Standardization of LMIT using two concentrations of DP antigen

Duck No.	Mean migration indices	
	with 1:20 DP antigen	with 1:10 DP antigen
DP exposed ducks		
1	78	51
2	80	47
3	77	45
Control ducks		
4	101	101
5	91	95
6	87	94

Table 9. Mean leucocyte - migration indices in experimental ducks

Sl. No.	Weeks		Groups of ducks					Significant difference			
	pv	pdv	SCV	SAV	DCV	DAV	NVC	SCV& SAV	DCV& DAV	SCV& DCV	SAV& DAV
1.	0	-	99.6±4.8	95.8±2.7	98.7±3.2	101.9±4.5	95.4±4.5	NS	NS	NS	NS
Vaccination			Yes	Yes	Yes	Yes	No				
2.	1	-	88.4±3.1	78.2±6.9	87.7±4.4	77.4±4.4	104.4±5.2	NS	NS	NS	NS
3.	2	-	68.3±4.6	52.7±5.2	65.5±2.6	55.9±6.3	103.4±3.3	*	NS	NS	NS
4.	3	-	58.3±3.8	39.0±5.6	56.0±1.8	40.5±1.8	95.9±3.1	*	*	NS	NS
5.	4	-	58.3±2.8	35.3±2.0	58.5±1.6	36.6±4.3	99.9±3.0	**	**	NS	NS
-	4	Revacci-nation	No	No	Yes	Yes	No				
6.	5	1	NT	NT	40.3±2.9	21.4±1.5	98.6±1.7	NT	**	NT	NT
7.	6	2	61.9±3.9	35.7±1.0	31.6±2.7	7.2±3.9	94.9±2.2	**	**	**	**
8.	8	4	65.7±1.4	46.0±1.4	35.8±2.3	17.9±2.6	96.2±4.3	**	**	**	**
9.	10	6	75.0±3.6	56.8±4.1	50.5±5.3	25.9±4.6	98.2±2.0	**	**	**	**
10.	12	8	77.2±1.2	61.5±2.5	60.6±2.9	40.0±1.3	96.2±5.0	**	**	**	**
11.	16	12	86.1±1.2	73.7±3.1	68.8±0.9	59.9±4.1	100.7±1.4	**	NS	**	**
12.	20	16	89.4±1.3	84.0±3.9	82.9±2.6	65.5±2.5	98.6±0.3	NS	NS	NS	**
13.	24	20	89.1±1.9	84.5±1.4	81.3±0.5	74.6±2.2	98.2±0.7	NS	**	**	**

pv = post-vaccination; pdv = post double vaccination; NT = Not tested

SCV = Single commercial vaccine; SAV = Single lab-adapted vaccine; DCV = Double commercial vaccine;
DAV = Double lab-adapted vaccine; NVC = Unvaccinated controls

* Significant (P<0.05); ** Highly significant (P<0.01); NS = Non-significant

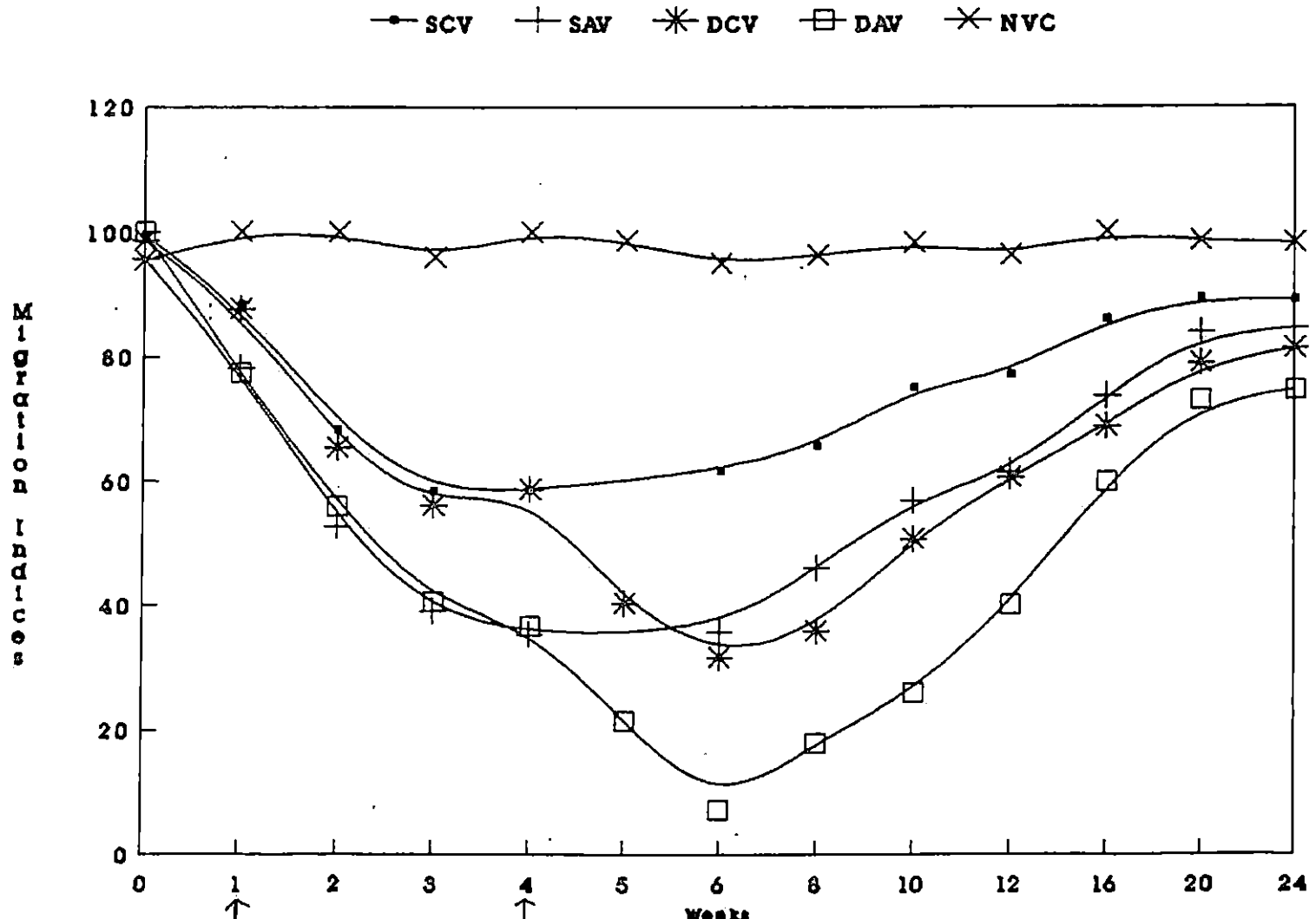


Fig. 3 MEAN LM INDICES OF GROUPS VACCINATED AGAINST DPV

Arrows indicate day of vaccination

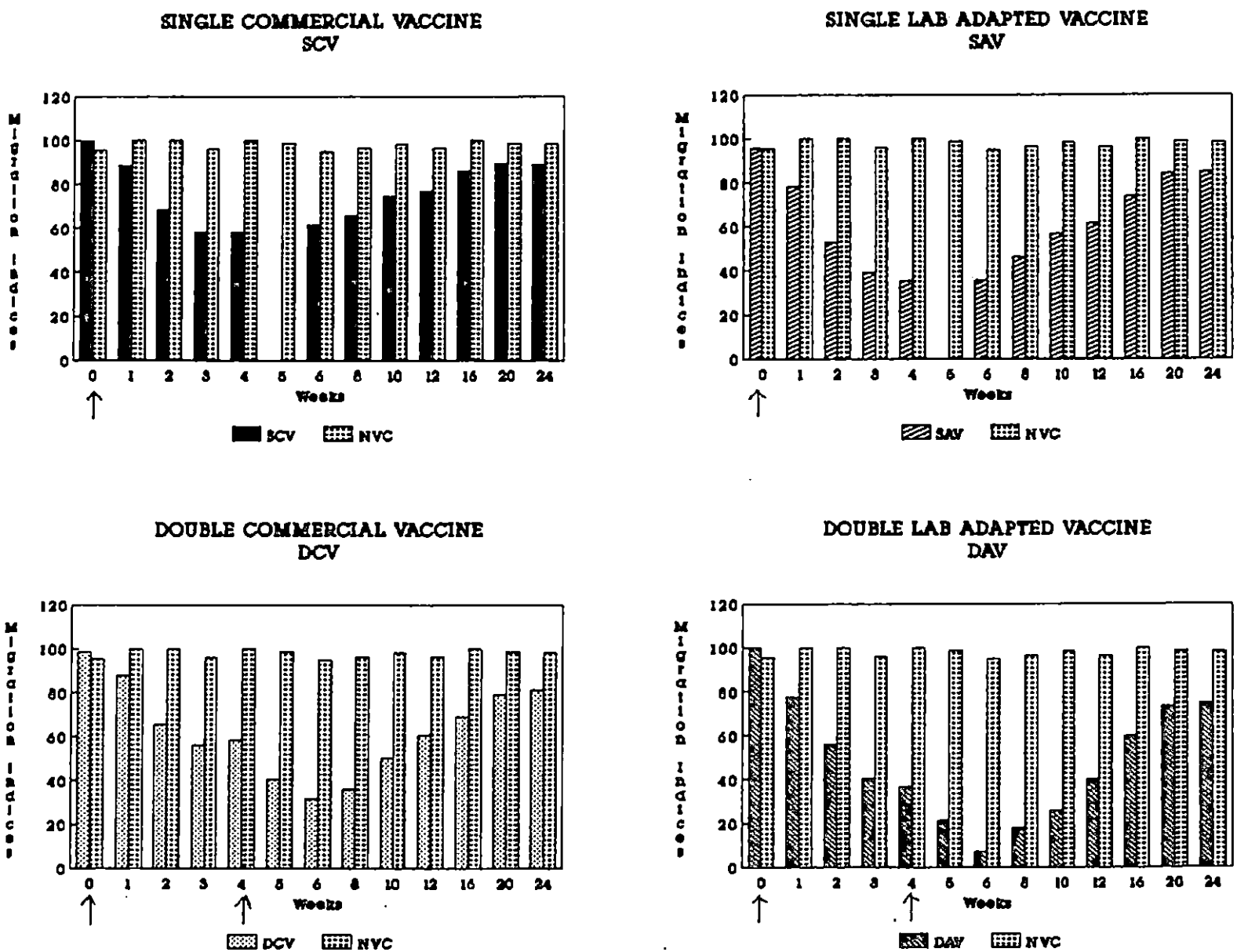
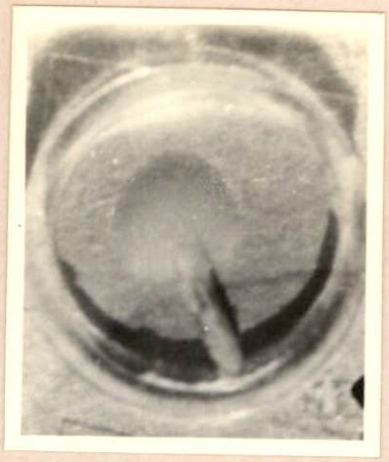
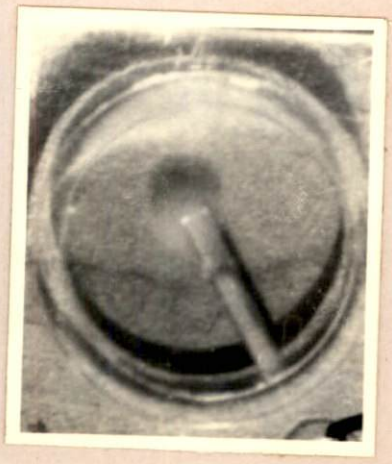


Fig.4 Mean leucocyte-migration (LM) indices of each vaccinated group compared with unvaccinated control (NVC). Arrows indicate day of vaccination. Single vaccinated were not tested on week 5.

Plate XV Leucocyte migration in normal duck

Plate XVI Leucocyte migration-inhibition in DP-exposed duck





commercial vaccine (SCV), the LM indices started declining significantly from second week pv, although, marginal reduction was seen in first week. The inhibition of LM was more prominent in third week pv (42.7 per cent), thereafter it declined slowly and by 16th week pv, returned to almost normal.

Ducks vaccinated with single laboratory-adapted vaccine (SAV) showed inhibition of migration on seventh day pv, which increased to its peak at fourth week to 64.7 per cent and continued at the same level for another two weeks. Thereafter the inhibiton decreased to a considerable extent upto 12th week. For the last two observations, i.e., 20th and 24th week pv, the LM indices were in normal range.

The two groups receiving single vaccinations (SCV and SAV) were compared at every test intervals and their LM indices did not show any significant differences in first week pv and later 16th and 20th week pv. These differences were significant ($P < 0.05$) for second and third week pv and highly significant ($P < 0.01$) for rest of the period.

In double-vaccinated groups, the trend and levels of LM indices were almost similar to those of single vaccinated ducks in their corresponding groups upto fourth week pv, i.e. upto second vaccination.

In group of duck receiving two doses of commercial vaccine (DCV), four weeks apart, LM inhibition was evident from one week pdv. The effect of two doses of vaccine in DCV group lowered the LM index to 31.6 in two weeks pdv. However the peak was not maintained for more than two weeks pdv and as observed in SCV and SAV, this group too returned to normal migration in 16th and 20th week pdv.

The mean LM indices were compared in single and double commercial vaccine-receiving groups (SCV and DCV) from second to 20th weeks pdv wherein except on 16th week pdv, the differences were highly significant ($P < 0.01$) at all test intervals. In another double vaccinated group receiving laboratory-adapted DP vaccine (DAV), the inhibition of LM was the maximum. In second week pdv, of four ducks tested, one showed complete inhibition whereas others also had high percentage of LM inhibition. However, this inhibition declined very fast and on 16th and 20th week pdv, it was around 30 and 25 per cent respectively.

The two groups receiving double vaccination (DCV and DAV) when compared , showed no significant difference in their mean LM indices on first three observations and then on 12th and 16th week pdv. At third week pv, the difference was significant ($P < 0.05$) and from fourth to eighth week pdv, the

mean LM indices were different at highly significant level ($P < 0.01$).

When the two groups receiving single and double laboratory adapted vaccines (SAV and DAV) were compared, the mean LM indices were similar upto four weeks pv but they were different at highly significant levels ($P < 0.01$) from second week pdv onwards at all test intervals till the end of the observation period.

4.5.3 Passive haemagglutination (PHA) test (Plate XVII)

Standardization of PHA was done by using tannic acid in 1:20000 dilution, allanto-amniotic fluid-antigen diluted to 1:10 and coating buffer, PBS pH 6.0 for coating the antigen to formalinized tanned sheep erythrocytes (FTE).

Similarly the serum samples given heat-inactivation and FTE adsorption treatment were used for optimum results. The findings of these trials are presented in Table 10 and the results of PHA test are furnished in Table 11, Fig. 5 and 6.

All the 63 pre-vaccination sera samples tested individually had nil PHA titres at all dilutions starting from 1:2 except two samples which showed non-specific clumping at the lowest dilution.

Table 10. Standardization of passive haemagglutination test

	PHA titre of Hyperimmune serum	PHA titre of Known negative serum
A. Tannic acid (TA) concentration for formalinized SRBC-tanning		
TA 1 : 10000	256	Nil
TA 1 : 20000*	256	Nil
TA 1 : 30000	128	NIl
B. Antigen concentration for coating formalinized tanned sheep erythrocytes (FTE)		
Neat antigen	1:128	1:2 ±
1 : 10*	1:256	Nil
1 : 20	1:64	Nil
C. pH requirement of PBS for antigen coating to FTE		
PBS pH 4.5	1:8	Nil
PBS pH 6.0*	1:256	Nil
PBS pH 7.2	1:128	Nil

D. Effect of different serum treatments on PHA titre

Test serum	Raw (untreated)	Heat inactivated (HI)	HI&FTE* absorbed
No. 1	256	64	16
No. 2	32	8	2
No. 3	128	32	8
Hyperimmune sera	1024	1024	256
Known negative serum	32	8	0

* Treatment selected

Table 11. Mean passive haemagglutination titres in vaccinated ducks

Sl. No.	Weeks		Groups of ducks					Significant difference			
	pv	pdv	SCV	SAV	DCV	DAV	NVC	SCV& SAV	DCV& DAV	SCV& DCV	SAV& DAV
1.	0	-	0	0	0	0	0	NT	NT	NT	NT
	Vaccination		Yes	Yes	Yes	Yes	No				
2.	1	-	0.50	0.50	0.66	0.50	0	NT	NT	NT	NT
3.	2	-	4.83±0.57	6.00±0.60	4.33±0.54	5.66±0.59	0	**	**	NS	NS
4.	3	-	9.33±1.24	12.00±1.20	9.00±1.31	12.00±1.20	0	**	**	NS	NS
5.	4	-	9.33±1.24	12.66±1.19	9.33±1.24	12.66±1.19	0	**	**	NS	NS
-	4	Revacci-nation	No	No	Yes	Yes	No				
6.	5	1	NT	NT	20.44±3.01	32.00±6.53	0	NT	**	NT	NT
7.	6	2	7.11±0.58	11.55±1.40	27.55±5.51	48.00±6.53	0	**	**	**	**
8.	8	4	6.66±0.66	11.11±1.60	21.33±2.66	40.88±6.02	0	**	**	**	**
9.	10	6	5.33±0.84	10.66±1.69	17.33±3.22	40.00±8.03	0	**	**	**	**
10.	12	8	4.33±0.80	8.00±1.79	13.33±1.69	34.66±6.44	0	**	**	**	**
11.	16	12	3.33±0.42	6.00±0.89	12.66±2.17	24.00±3.59	0	**	**	**	**
12.	20	16	1.66±0.56	5.00±1.00	11.33±2.17	21.33±3.38	0	**	**	**	**
13.	24	20	1.33±0.63	3.33±0.66	10.66±2.66	21.33±5.33	0	**	**	**	**

pv = post-vaccination; pdv = post double vaccination; NT = Not tested

SCV = Single commercial vaccine; SAV = Single lab-adapted vaccine; DCV = Double commercial vaccine; DAV = Double lab-adapted vaccine; NVC = Unvaccinated controls

* Significant (P<0.05); ** Highly significant (P<0.01); NS = Non-significant

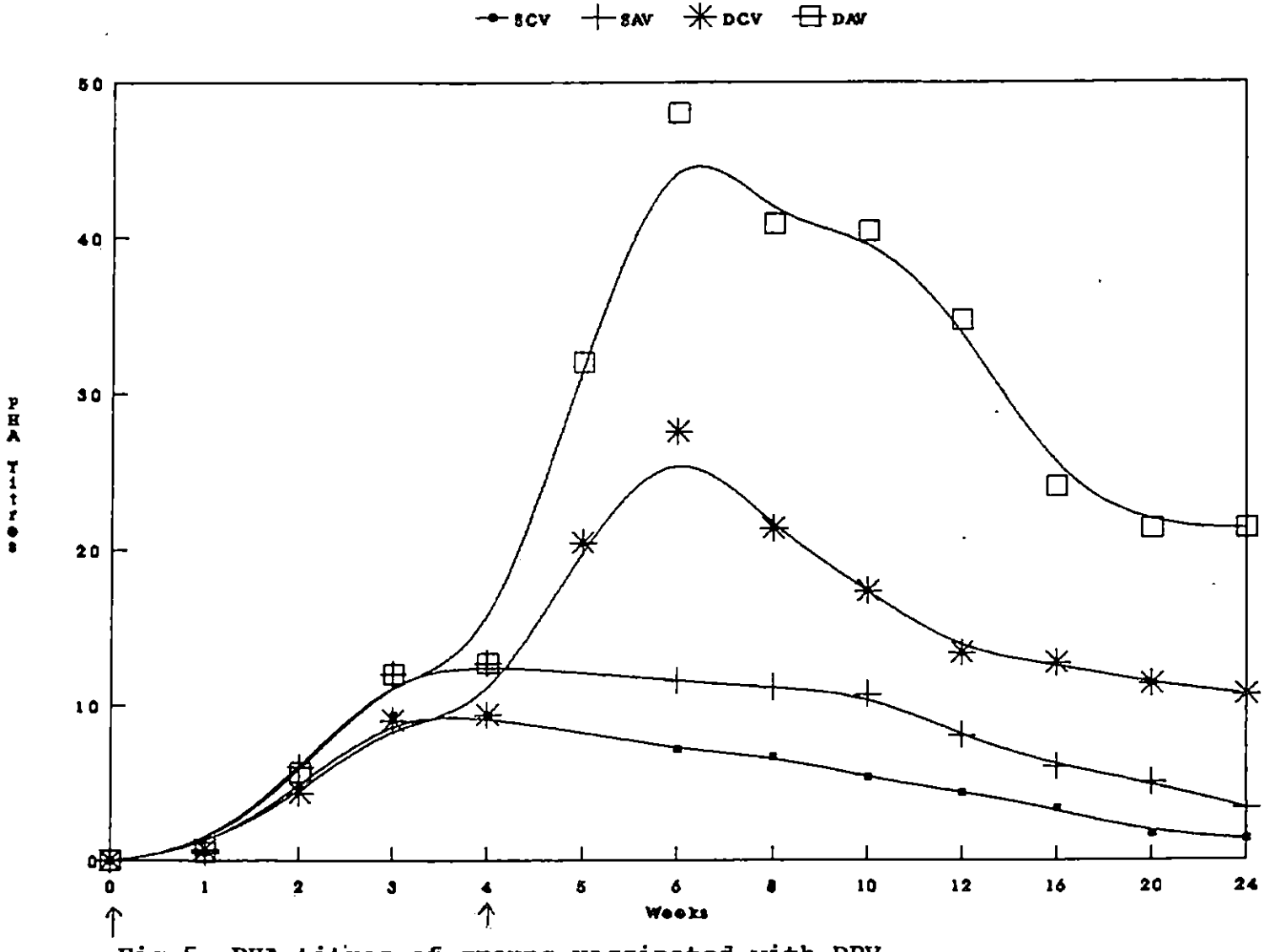
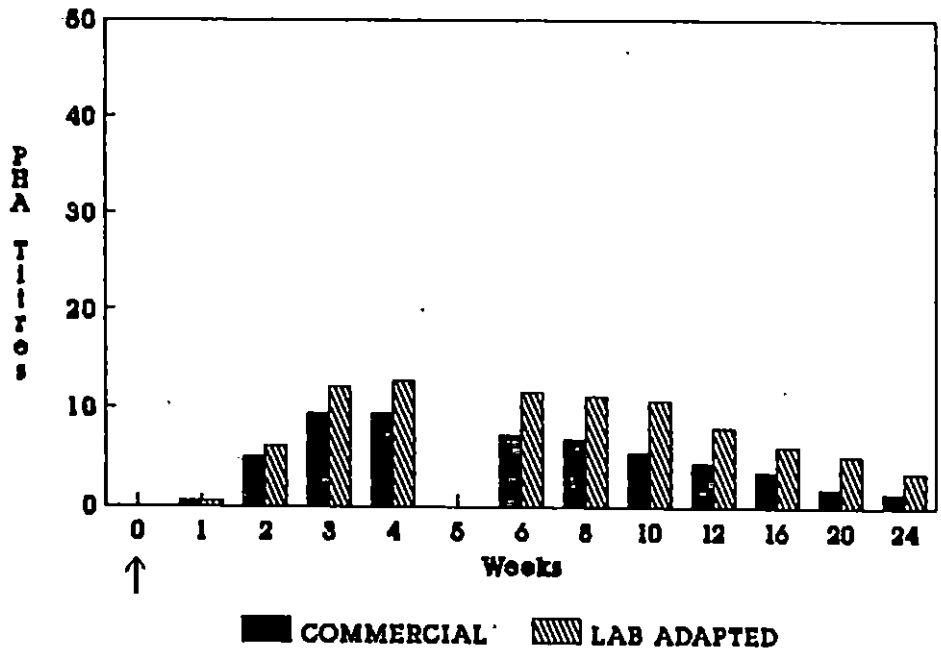


Fig.5 PHA titres of groups vaccinated with DPV
Control titres were nil. Arrows indicate day of vaccination

SINGLE VACCINATED GROUPS



DOUBLE VACCINATED GROUPS

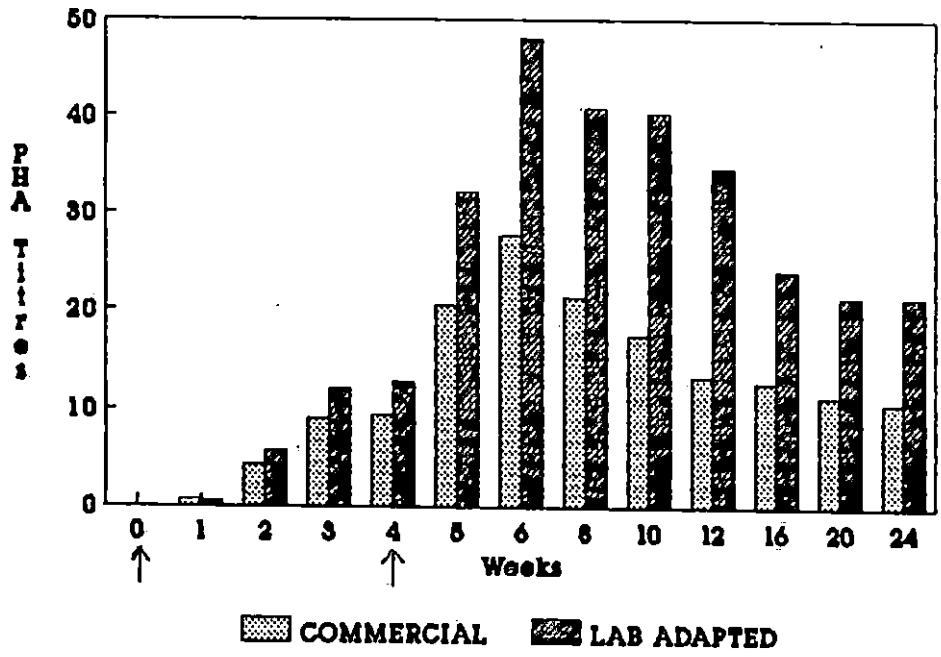
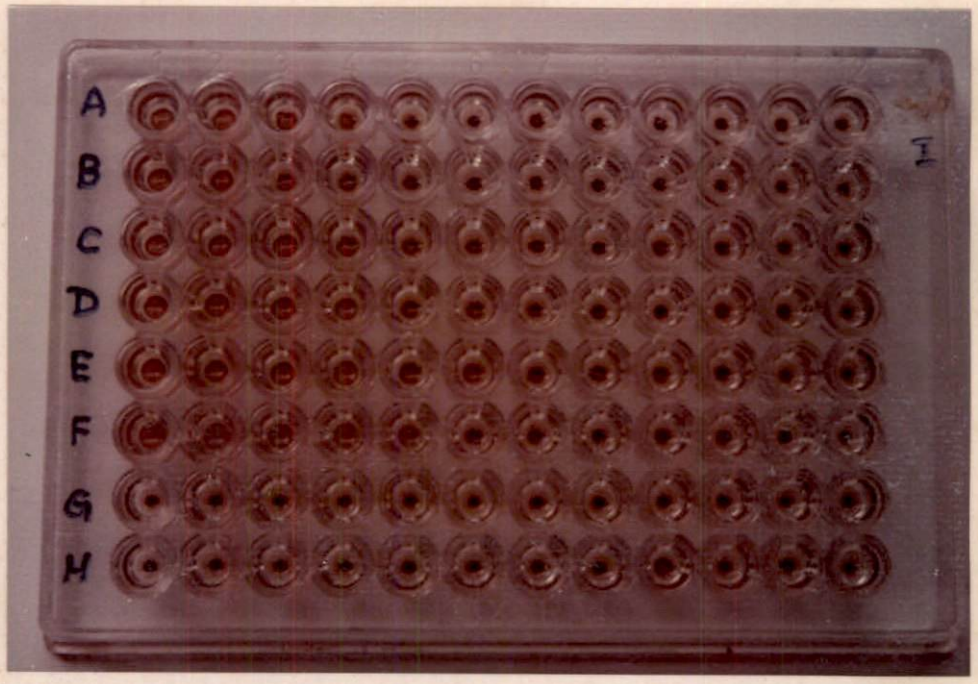


Fig.6 Comparison of mean passive haemagglutination (PHA) titres among vaccinated groups. Controls were nil. Arrows indicate day of vaccination.
Single vaccinated were not tested in week 5.

Plate XVII Passive haemagglutination test.

A to E test sera
F - known positive serum
G - known negative serum
H - known positive serum and uncoated FTE


A
B
C
D
E
F
G
H
I

The PHA titres of all unvaccinated control ducks (NVC) were nil throughout the observation period.

On seventh day post-vaccination (pv), the PHA titres did not show appreciable increase. Of the 48 ducks screened, only 14 showed low titre i.e., 2. The significant response was seen from second week post-vaccination.

In group of ducks receiving one dose of commercial vaccine (SCV), the maximum PHA titres were 16 at third week pv (mean 9.33 $\pm$ 1.2) which declined within two weeks, and became nonsignificant after 16 weeks pv. Throughout the observation period, this group had very low titre range.

In ducks given one dose of laboratory-adapted vaccine (SAV), the PHA titres showed an increase upto fourth week pv (mean 12.66 $\pm$ 1.19) and were almost at the same level for another five weeks. Thereafter titres decreased slowly but were maintained between 4 and 8 upto 20th week pv and became nonsignificant in the last observation at 24th week.

When the PHA titres between SCV and SAV groups were compared, the differences were highly significant ($P < 0.01$) throughout the observation period except for first week pv, when the response was nonsignificant.

The mean PHA titres in single and double vaccinated birds in their corresponding groups were at the similar level and showed same trend till fourth week, when the second dose of vaccine was given to groups DCV and DAV.

Ducks receiving double commercial vaccine, showed a good rise in titres within one week and at two weeks pdv, the titres were nearing 32 (mean 27.55 $\pm$ 5.51). However, this peak could not be maintained and within six weeks thereafter, the mean titres were less than half the peak. Till 20 weeks pdv, the titres were between 8 and 16.

There were significant differences between the PHA titres of single and double commercial vaccine and at all testings except first five, they were highly significant ($P < 0.01$).

The group vaccinated twice with lab-adapted vaccine (DAV) showed the highest titres among all the groups at all testings. After second vaccination, the PHA titres rose to three-fold in first week and four-fold in second week when the peak titres in a few ducks were around 64. The mean titre at second week pdv was 48.0 $\pm$ 6.53. The range of 32 and 64 was maintained upto eight week pdv, and even during last two observations, i.e., 16th and 20th week pdv, the titres were almost double than the peak obtained on single vaccination.

On comparison between these two double-vaccinated groups (DCV and DAV), it was seen that from second week pv to 20th week pdv (24th week pv), the differences were highly significant ($P < 0.01$). Similarly, when the effect of single and double laboratory adapted vaccines was compared, there were highly significant ($P < 0.01$) differences from second to 20th week pdv.

4.5.4 Comparison between VN indices, PHA titres and LM indices in experimental ducks (Tables 7, 9 and 11)

The VN and PHA titres of the ducks vaccinated with single vaccines (groups SCV and SAV), when compared, it was found that VN had a far better and stable response in case of laboratory-adapted vaccine. In case of double vaccinated groups (DCV and DAV) the trend was parallel, however at the end of observation period i.e., 20 weeks pdv, VN titres were persistantly higher.

When compared with LMI response, VN titres at the initial and later stage of observation period were higher than LMI percentages, however, LMI response showed higher peak in single vaccinated groups between two and eight weeks pv and in double vaccinated groups between one and four weeks pdv. The graphic illustration of these comparison is presented in Fig.7 and 8.

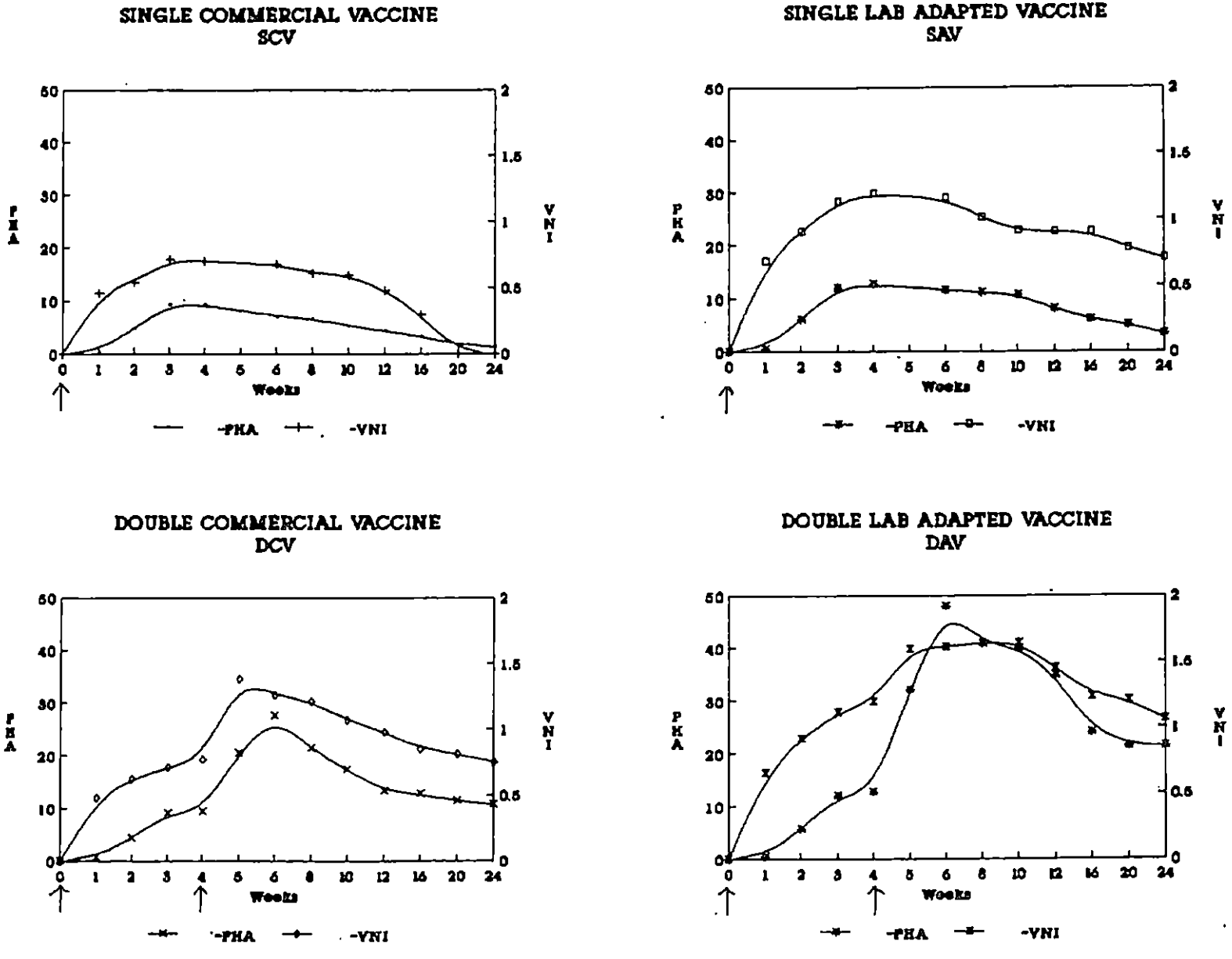


Fig.7 Comparison of trends of passive haemagglutination (PHA) titres and virus neutralizing indices (VNI). Control titres were nil in both the tests. Arrows indicate day of vaccination

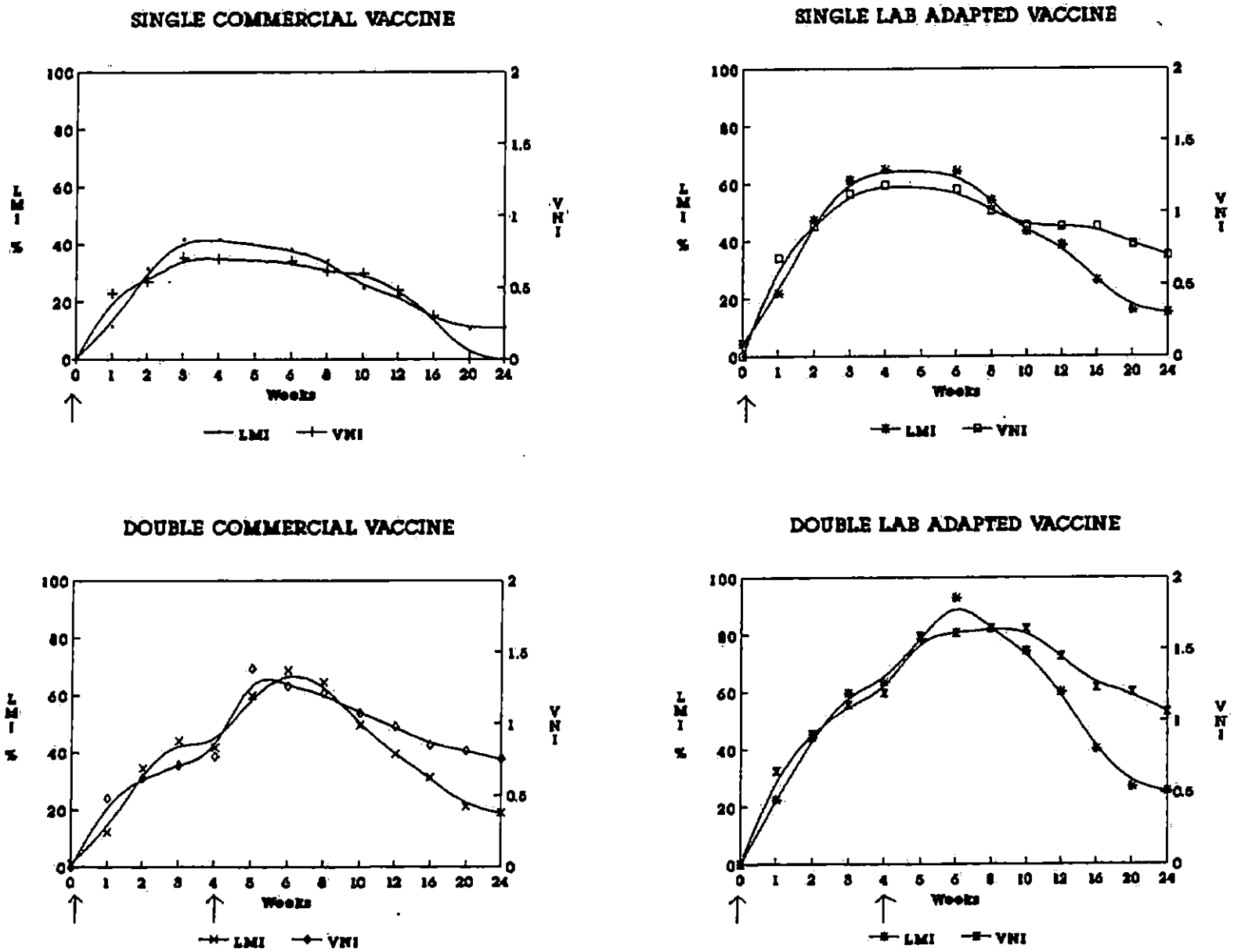


Fig.8 Comparison of trends of leucocyte migration inhibition (LMI) percentage and virus neutralizing indices (VNI) between various vaccinated groups. Arrows indicate day of vaccination.

The percentages of leucocyte migration inhibition were almost parallel to the VN indices whereas those of PHA were in a lower range. The LM indices were showing appreciable inhibition in first week whereas the PHA response was seen only after two weeks. However, in the double vaccinated groups, at the end of observation period (i.e., at 20 weeks pdv) the persistance of PHA titres was seen at higher level than LM inhibition percentages.

4.5.5 Challenge experiment (Tables 12, 13 and 14)

As shown in Table 5, ducks in all the groups including controls, were challenged with virulent DP virus to know their level of protection (at that stage) conferred by DP vaccine(s).

All the ducks inoculated with virulent DP virus at four weeks post-vaccination, survived. However, there was variation in symptoms and survival pattern. The ducks receiving single dose of commercial vaccine (SCV) showed more severe reaction to DP challenge. all the three birds showed clinical symptoms of disease for 4-6 days PI and recovered whereas those vaccinated with laboratory adapted vaccine (SAV) were healthy and active throughout the observation period. The pathogenecity index (pi) of SCV group was 1.23 whereas that of SAV, zero.

Table 12 Mortality in challenge experiment

Groups of ducks	Weeks after single vaccination			Weeks after double vaccination	
	4	8	20	4	20
SCV[1]	0/3	1/3	2/3	--	--
SAV[2]	0/3	0/3	0/3	--	--
DCV[3]	0/3	--	--	0/3	0/3
DAV[4]	0/3	--	--	0/3	0/3
NVC[5]	3/3	3/3*	3/3	3/3*	3/3

1 - Single commercial vaccine 2 - Single lab-adapted vaccine

3 - Double commercial vaccine 4 - Double lab-adapted vaccine

5 - Unvaccinated control

* 8 weeks pv and 4 weeks pdv (NVC) common group challenged on the same day

Table 13. Pathogenicity indices of challenged ducks

Groups of ducks	Weeks after single vaccination			Weeks after double vaccination	
	4	8	20	4	20
SCV[1]	1.23	1.30	1.50	--	--
SAV[2]	0.00	0.07	0.40	--	--
DCV[3]	1.27	--	--	0.14	1.17
DAV[4]	0.00	--	--	0.00	0.17
NVC[5]	1.80	1.90*	1.87	1.90*	2.13

1 - Single commercial vaccine 2 - Single lab-adapted vaccine

3 - Double commercial vaccine 4 - Double lab-adapted vaccine

5 - Unvaccinated control

* 8 weeks pv and 4 weeks pdv (NVC) common group challenged on the same day

Table 14. Immune response, mortality and pathogenicity indices of ducks after challenge infections

Group	Weeks post vaccination	Average VN indices		Mean LM inhibition %		Mean PHA titre		Mortality		Pathogenicity index
		pre-challenge	Post-challenge	Pre-challenge	Post-challenge	pre-challenge	Post-challenge	No. of ducks challenged	No. of ducks died	
SCV	4 weeks (pv)	0.70	2.12	41.7	35.0	9.33	53.0	3	0	1.23
SAV	4 weeks (pv)	1.19	2.72	64.7	55.0	12.66	64.0	3	0	0.00
DCV	4 weeks (pv)	0.77	2.36	42.5	50.0	9.33	64.0	3	0	1.27
DAV	4 weeks (pv)	1.19	2.95	63.4	68.0	12.66	64.00	3	0	0.00
SCV	8 weeks (pv)	0.61	1.96	34.3	27.0	6.66	48.0	3	1	1.30
SAV	8 weeks (pv)	1.01	2.90	54.0	54.0	11.11	53.0	3	0	0.07
DCV	4 weeks pdv	1.21	2.72	64.2	61.0	13.33	75.0	3	0	0.14
DAV	4 weeks pdv	1.64	3.27	82.1	71.0	34.66	85.0	3	0	0.00
SCV	20 weeks (pv)	0	1.80	10.6	25.0	1.33	32.0	3	2	1.50
SAV	20 weeks (pv)	0.78	2.52	16.0	40.0	3.33	64.0	3	0	0.40
DCV	20 weeks pdv	0.81	3.50	17.1	65.0	10.66	85.0	3	0	1.17
DAV	20 weeks pdv	1.20	3.50	30.5	70.0	21.33	107.0	3	0	0.17

SCV - Single commercial vaccine SAV - Single lab-adapted vaccine pv - post vaccination

DCV - Double commercial vaccine DAV - Double lab-adapted vaccine pdv - post double vaccination

On challenge at eight weeks pv the SCV group of ducks suffered from the disease, and on eighth day, one out of three ducks succumbed to DP infection. The remaining two also showed severe symptoms for 4-6 days before recovery and their p.i. was 1.3. The ducks in SAV group withstood the challenge and, of the three challenged, only one showed mild signs on day four and five PI before being recovered completely. The p.i. was 0.07.

Both the groups receiving two doses of DP vaccine, withstood the challenge in a better way. The ducks in group DCV showed mild symptoms and out of three, only two showed the signs for four days and then became normal when challenged at fourth week pdv. The p.i. was 0.14. Ducks in group DAV remained healthy throughout the observation period.

At the time of the challenge given on 20th week post vaccination, the ducks in group SCV suffered very severely and of the three challenged, one each died on seventh and eighth day respectively. The one which survived was ailing for six days post-challenge and the recovery was very slow. The p.i. was 1.50.

The laboratory-adapted vaccine receiving group could sustain the challenge and none of the three died. However, one showed severe symptoms for 8 to 10 days post-challenge and

two ducks showed milder signs on fifth to seventh day post-challenge. The p.i. was 0.40.

Group DCV withstood the challenge but all the three were severely affected for four days and then slowly recovered. The p.i. was 1.17. The other group, DAV showed high resistance to challenge at 20th week and although two out of three suffered mild signs for three days, recovery was fast and p.i. was only 0.17.

In case of unvaccinated controls, three ducks receiving challenge infection each time, succumbed to clinical DP infection and none of them survived. The p.i. were 1.8, 1.9, 1.87 and 2.13 (Tables 13 and 14).

Out of 12 ducks challenged from control group at four stages, two died on fourth day, five on fifth day, three on sixth day and two on seventh day. The mean death time for the control ducks was 5.4 days.

All the surviving ducks after challenge were screened for their VN antibody-titres, LM inhibition and PHA titres on 30th day post-challenge. The results are furnished in Table 14.

The single commercial vaccine-receiving group had the lowest values as regards to the VN indices, LM inhibition

percentages and PHA titres at all the three intervals when challenged. The ducks receiving laboratory adapted vaccine showed better response. The VN indices were highest i.e., 3.5 in both double vaccinated groups challenged after 20 weeks. Their LMI and PHA responses were also higher. However, the differences in PHA response among various challenged groups were not as sharp unlike VN indices.

4.5.6 Vaccine trial on an organized duck farm

The pre-test sera from 18 unvaccinated adult ducks (pooled two each to make nine samples)tested for PHA showed nil titres, similarly these nine samples pooled three each to make three pools, tested for virus neutralization did not reveal any neutralizing antibody against DP virus.

Subsequent testing of these birds in the similar way was done eight weeks after giving single dose of commercial vaccine. Of nine pooled serum samples from these ducks tested for PHA titres, only one had titre 16, four had 8 and four had 4. Three samples screened for VN antibodies had very low VN indices. The results are furnished in Table 15.

The yolk samples from the eggs collected from DP-vaccinated layer ducks six weeks post-vaccination when screened for DPV neutralizing antibodies, did not reveal any titre and the VN indices were nil.

Table 15. PHA and VN antibody-titres in vaccinated ducks from an organized farm

Serum pool for PHA test	PHA titre		Serum pool for VN test	Pre vaccination VN titre/ VN index	Post vaccination	
	Pre-vaccination	Post-vaccination			VN titres between	VN index
P 1	0	16				
P 2	0	8	p1	0	4-8	0.85
P 3	0	4				
P 4	0	4				
P 5	0	4	p2	0	4-8	0.60
P 6	0	8				
P 7	0	4				
P 8	0	8	p3	0	4-8	0.76
P 9	0	8				
Average	0	7.11		0	-	0.74

Two randomly selected ducks from this flock were challenged on sixth week. One of the two died, the other suffered from DP for seven days and recovered.

Three ducklings each hatched from the eggs collected from this flock before and 30 days after vaccination were challenged and all six died of DP.

4.6 ASSESSMENT OF THE CARRIER STATUS (Table 16)

None of the rectal swabs collected from vaccinated ducks against DP resulted in virus isolation at any stage, when processed and inoculated into duck embryos. Similarly rectal swab processing from vaccinated and challenged ducks did not yield any virus even after two blind passages in duck embryos.

Table 16. Assessment of carrier status of experimental birds

Status of the ducks at	Age of ducklings	SCV	SAV	DCV	DAV	NVC
Pre vaccination		-(3)	-(3)	-(3)	-(3)	-(3)
7 day post single vaccination		-(3)	-(3)	-(3)	-(3)	-(3)
7 day post challenge (single vaccinated)		-(3)	-(3)	NT	NT	NT
7 day post double vaccination		NT	NT	-(3)	-(3)	NT
14 day post challenge (single vaccinated)		-(3)	-(3)	NT	NT	NT (No survivals)
30 day post challenge (single vaccinated)		-(3)	-(3)	NT	NT	NT
7 day post (2nd) challenge (double vaccinated)		NT	NT	-(3)	-(3)	No survivals hence not tested
14 day post (2nd) challenge (double vaccinated)		NT	NT	-(3)	-(3)	NT
30 day post (2nd) challenge (double vaccinated)		NT	NT	-(3)	-(3)	NT

Figures in parenthesis indicate number of birds tested for carrier status

SCV - Singe Commercial Vaccine; DCV - Duble Commercial Vaccine
SAV - Single Lab-adapted Vaccine; DAV - Double Lab-adapted Vaccine;
NVC - Unvaccinated Control NT - Not tested; - No isolation

Discussion

DISCUSSION

Ever since the first report on duck plague (DP) in India (Mukerji et al., 1963 a), innumerable outbreaks have been recorded during last 3 decades from West Bengal, Assam, Kerala, Tamil Nadu and Andhra Pradesh and as of today, DP has become an established disease in the country in most of the duck-populated areas.

A high rate of mortality due to DP had been reported in Kerala despite undertaking regular vaccinations. However, many of the reports were based on clinical findings. During discussions with field veterinarians, doubts were raised about unresponsiveness to and/or failure of vaccinations resulting from either immunosuppression or technical faults with vaccine or vaccination procedures.

Dardiri (1975) has stated that the role of immunity to protect the ducks against DP is not clear. While humoral immunity studies have been amply documented, not much work has been done on cell-mediated immunity (CMI), which generally affords protection in infections caused by herpes virus group.

With this background, the present work was undertaken to confirm the incidence of DP and to assess antibody-mediated

and cell-mediated immune responses to vaccination alongwith protection studies.

5.1 Preliminary investigations

During 1991, DP outbreaks were recorded in adult ducks as well as the young ones. The mortality in ducklings was found to be less than that in the adults but data available were limited to draw a definite inference on influence of age factor on susceptibility to duck plague. However, several reports (Suwatviroj et al., 1977; Vetesi, et al., 1982. Bhowmik and Chakrabarty, 1985) regarding susceptibility to DP over a wide range of age-groups indicate that age may be a less important factor than the immune status of the population and stress.

Many affected flocks had been vaccinated against DP before the onset of outbreaks. Probably the vaccine failed to protect them, nevertheless average morbidity and mortality figures (Table 1) in vaccinated birds were significantly less than those of unvaccinated ones. This might be due to the partial protection afforded by less effective vaccine.

All the outbreaks in 1991 were observed between May and October. Generally majority of the DP outbreaks occur between January and June (Jansen, 1963; 1964; Gough and Alexander, 1987; 1990). Vuillaume (1989) had noted the incidence of DP in France in September and January. The

epidemiological factors for this difference in the season of incidence are not understood clearly. Probably the period of vaccination, which provokes partial protection for short period might be a factor for change in the season of outbreaks.

From all the six outbreaks investigated, the virus was isolated, thus proving that the vaccination with the vaccine available in the market could not prevent the disease.

The clinical picture and postmortem lesions observed in the present investigation were similar to what has been described by earlier workers and summarised by Leibovitz (1991). The pathognomonic lesions in oesophagus were seen in almost all cases.

5.2 Virus isolation, characterization and attenuation

At initial stages of isolation, there were differences in the period of duck embryo mortality and in the lesions in embryos. After two serial passages, uniformity in embryo mortality was seen between day four and six post-inoculation. Initially the virus could not produce lesions or kill the chicken embryos but after 10 duck embryo passages, the virus isolates were adapted to grow in chicken embryos with mortality and lesions similar to those in duck embryo. Similar observations have been made by Jansen (1961, 1964),

who failed to cultivate DPV on chicken embryo using morbid materials but after 12 duck embryo-passages and three blind passages in chicken embryo, the virus became lethal to them.

All the virus isolates were neutralized by hyperimmune serum raised against vaccine strain and virulent strain obtained from IVRI. This finding indicates that there is no antigenic variation in DPV strains isolated from various sources.

None of the isolates agglutinated chicken, duck, sheep or bovine erythrocytes. Leibovitz (1991) has stated that the virus is non-haemagglutinating.

One of the isolate (DP-S) concentrated 100 times by PEG-6000 precipitation and ultracentrifugation when viewed under transmission electron microscope revealed characteristic herpes virus-like capsids coated with extraneous substances partially obscuring morphological details. Moreover, damaged particles permeable to the negative stain were more in number. The viral nucleocapsid around 100 nm and enveloped virion around 380 nm were observed. Except for a little larger size of enveloped virions, the morphological features were similar to those described in literature. Proctor et al. (1976) and Simonova et al. (1984) have quoted the range of virion size as 150-384 nm with mean 220 nm. The size of the naked

nucleocapsids ranged between 95 and 100 nm. Our findings are in agreement with these observations.

The duck embryos were harvested for virus yield instead of cell culture supernatent and the virus preparation was partially purified. These factors might have been responsible for extraneous coating over the envelope.

The agar gel diffusion test (AGDT) using crude as well as concentrated antigen against hyperimmune sera over a range of sodium chloride concentrations and pH 6.0 and 7.4 of gel medium failed to show any reaction. Nair (1978) did not get AGD line with 2 DPV isolates tested. Toth and Norcross (1981) and Higgins (1989) had demonstrated poor ability of ducks to produce precipitating antibodies. Panisup and Verma (1989) showed that DPV did not stimulate precipitating antibodies. However, Kalaimathi and Janakiram (1990) and John et al. (1992) employed AGDT for duck plague successfully.

The virus isolate DP-S was not attenuated sufficiently after 20 duck embryo passages followed by 10 chicken embryo passages. The pathogenicity index (p.i.) of the virulent virus in susceptible ducks was 1.95; after passages through duck embryos and chicken embryos, it was reduced to 1.23. The reduction in p.i. indicated that the virulence of the virus was being decreased. Probably it would require a few more

passages in chicken embryos to achieve complete attenuation. Jansen and Kunst (1949) attenuated the virus after 20 chicken embryo passages. John et al. (1990) had demonstrated that the virus produced symptoms and death in ducks when inoculated after 10 passages, symptoms but not death after 15 passages and no symptoms or death in ducks after 20 passages in chicken embryo.

The embryo lethal dose 50 (ELD50) of 20 per cent suspension of liver, spleen and CAM in allanto-amniotic fluid (AAF) was found to be 4.8 log 10/ml, whereas that of AAF alone was 2.8 log 10/ml. This observation gives clear indication that the highest virus titre is in CAM, liver and spleen. Butterfield and Dardiri (1969) stated that the addition of minced chicken embryos to CAM and AAF decreased the virus titre. Our findings are also in agreement with Dardiri (1975) who found the highest virus titre in CAM followed by AAF and low in embryos.

5.3 ASSESSMENT OF IMMUNE RESPONSE AND CARRIER STATUS

For assessing the immune responses, the experiment was planned in such a way, that a comparison could be made between the immune response elicited by vaccination with commercial vaccine and laboratory adapted one. The former was used as per the manufacturer's instructions whereas the latter was

prepared as per the protocol. Similarly the effects of single and double vaccination were compared.

The age of the ducks for initial vaccination (6 weeks) was decided after considering two facts:

1. In Government farms ducks are vaccinated against DP between six to nine weeks whereas private breeders and farmers carry out the vaccinations between three to seven weeks (Punnoose et al., 1993).

2. Duck lymphoid system functions actively only after 3 to 5 weeks (Hashimoto and Sugimura, 1976).

The humoral immune response was studied by virus neutralization (VN) test and passive haemagglutination (PHA) test and the cell-mediated immune response by leucocyte migration inhibition (LMI) test at different specified time intervals. The birds were challenged with virulent DPV to know the level and duration of protection imparted by vaccine.

5.3.1 Post-vaccination VN indices

The virus-neutralizing titres in general were very low (Table 6). The highest VN indices recorded at two weeks after two injections of vaccines were 1.36 for commercial vaccine and 1.64 for laboratory adapted vaccine. Dardiri and Hess (1967) had reported the highest VN index in vaccinated ducks

as 1.3. In another study Toth (1971a) recorded VN indices as 1.7 five weeks after two vaccinations (19 weeks apart) and 0.7 after 17 weeks. Our findings are fully comparable to both of these observations.

The effect of single vaccine in terms of VN indices was still lower. At its peak after three weeks post-vaccination, the mean VN indices were 0.7 and 1.2 for commercial and laboratory-adapted vaccine respectively. When 180 ducks in an organized farm were vaccinated once with commercial vaccine, the titres were similar to that of the experimental ducks. The mean VN indices ranged between 0.6 to 0.85 at six weeks post vaccination. Toth (1971 a) found nil titres in almost all one-time vaccinated ducks. This might have happened because he had diluted the serum samples to 1:5 before using them for neutralization test.

5.3.2 Post-challenge VN indices

The virus-neutralizing indices increased substantially after challenge infection. On 30th day of each challenge, group SCV (receiving single commercial vaccine) had 2.12 (all survived), 1.96 (1/3 died) and 1.80 (2/3 died) VN indices at 4th, 8th, and 20th week post vaccination respectively. On the same intervals group SAV (receiving single lab-adapted vaccine) had VN indices 2.72, 2.9 and 2.52 (all survived at

all intervals) respectively. Twenty weeks after double vaccination, challenged birds showed more uniform VN indices on 30th day post-challenge and both the groups (receiving commercial as well as lab-adapted vaccine) had the same VN index of 3.5 (Table 14).

Dardiri and Hess (1967), Butterfield and Dardiri (1969) and Dardiri (1975) have shown that the exposure to virulent virus results in high levels of antibody and a VN index of 1.75 or more, indicates 'infection experience' with the virus. The present findings are in conformity with this observation. Toth (1971 a) noted higher level of VN antibodies i.e., VN indices 3.1 to 4.0 after challenge. Mukit et al., (1988) found VN index 3.8 in DP-recovered ducks.

In the present experiment, the ducks receiving single vaccination when challenged, suffered from the disease and developed low level of VN antibodies as compared to those of double vaccinated groups and as reported by Toth (1971 a) as well as Mukit et al. (1988). The possible explanation for this discrepancy could be as given below -

> The pathogenesis studies of DPV indicated that the virus under active infection cause massive destruction of T- and B- lymphocytes in bursa, thymus and spleen (Proctor et al., 1976). This effect is similar to that after acute irradiation in mice or following infectious

bursal disease in chicken (Breese and Dardiri, 1968). In the present experiemnt, there seemed to be a generalized immunosuppression after challenge and in partially protected birds of group SCV the VN titres were low. The scale of immunosuppression might be far less in well protected (effectively vaccinated) birds, like those in DAV, resulting in normal response and higher levels of VN antibodies.

5.3.3 Passive haemagglutination test

The passive haemagglutination test was standardized for duck plague system. No other work could be traced for employment of this test to DP virus.

The trends of PHA titres showed positive correlation with VN indices. The titres rose from second week post vaccination and maintained upto three to four months. In general they were low, and none of the control sera showed any titre. The specificity and simplicity of the test has been its advantage particularly when large battery of sera samples are to be screened. It has been sucessfully employed earlier for another herpes virus i.e., infectious bovine rhinotracheitis (Vengris and Mare, 1971; Ziambo et al., 1973a, Sulochana et al., 1982 a; Suresh, 1992).

5.3.4 Role of cell-mediated immunity in DP

The leucocyte migration inhibition results indicated that there was significant levels of LM-inhibition following vaccinations and post-challenge. This observation points at the triggering of cell-mediated immune response to duck plague virus antigen. Li et al. (1988) demonstrated the effect of DPV on phytohaemagglutinin response of peripheral blood lymphocytes. Glavits et al. (1990) found no antibodies to DPV in yolk or serum of goslings hatched from DPV inoculated embryos but their lymphocytes recognised the virus antigens and the stimulated lymphocytes responded to blastogenic transformation.

The ducks in group SCV when challenged at four weeks post-vaccination, survived. Their VN index was as low as 0.7 log 10 but LM inhibition was significantly high (42 per cent). It meant that the protection was having correlation with LM inhibition better than VN index. The lack of correlation between VN antibody responses and the ability of ducks to withstand the challenge was highlighted by Jansen et al. (1963), Butterfield and Dardiri (1969) and Toth (1971 a).

The duck plague virus has physical, chemical and biologic properties of herpes virus. The factors common to

the group of herpes viruses and also shared by DPV are restricted host range (Jansen, 1964), events of replicative cycle (Breese and Dardiri, 1968) and cytopathogenicity (Proctor et al., 1976). There is field and laboratory evidence to indicate that the inapparent infection prevails among ducks infected with the virus (Burgess, 1981). The immunity in case of herpes group of viruses is predominantly cell-mediated type (Roitt, 1977). In the light of these observations and our findings, it can be stated that CMI also plays an important role in protecting the birds against DP in addition to the humoral immune response.

5.3.5 Comparison between VN, PHA and LMIT

The post-vaccinal response was noticed as early as seventh day by virus neutralization, showing by significant increase in indices, whereas by PHA, it took two weeks to show appreciable rise. After single and double vaccinations, the rise seen in VN and PHA titres was similar but both the responses were related more to their corresponding rates of increase or decrease rather than with each other. In short, there was general correlation in trends but not in titres.

Unlike PHA, LM inhibition in group SAV and DAV was seen on seventh day post-vaccination. The increase in LM inhibition percentage was parallel with both PHA and VN

titres. However, in SAV, which received only one vaccine, VN titres persisted upto 24 weeks (till last observation), PHA upto 20 weeks and LMI upto 16 weeks. Even in DAV group, VN indices, PHA titres and LMI percentages were appreciably pronounced after second vaccination and retained at the peak for longer duration but the LM inhibition disappeared very fast and was marginal at 20 weeks post-vaccination.

Based on these results, it can be stated that the earlier immune responses were noticed by VN or LMIT but persistance was more in VN followed by PHA.

5.3.6 Post-challenge mortality and VN indices

On challenge, mortality and symptoms of DP were seen in groups having VN indices 0.8 or less whereas those having VN indices 1.0 and above remained healthy. Since the number of birds used for challenge was limited, it is difficult to establish a definite positive correlation between VN index and mortality. However it was seen that changes in VN indices and pathogenicity indices were parallel. In this respect, the observations of Toth (1971 a) and Dardiri (1975) are not in agreement with our findings. The possible reasons which could be attributed to this difference are stated below -

1. In the study conducted by Toth, the mortality in control group was not cent per cent. The dose and virulence of

the challenge virus might have been less to find uniform results.

Secondly, the author himself agreed that the vaccinated breeders and the respective monitors were kept under less spacious conditions. The greater social stress might have been responsible for the variation in mortality.

2. Dardiri (1975) had attributed some mortality in ducks to secondary microbial invaders and latent infections which naturally would not have correlation with immune status of the bird.

5.3.7 Vaccinations, challenge and protection

At four weeks post-vaccination, all the ducks except unvaccinated controls, survived the challenge infection. However, those received commercial vaccine suffered from the disease for four to six days. On challenge at eight weeks pv, one of three from this group died and remaining two survived after ailment. On challenge at 20 weeks pv from the same group, two of three died and remaining one suffered severely for 11 days before recovering.

In the ducks receiving lab-adapted vaccine the challenge at fourth week pv did not produce the disease; after

challenge at eighth week pv, mild symptoms were seen and at 20th week pv, these birds suffered but recovered within two days of challenge. At this challenge, birds from DCV group suffered more severely and recovered slowly.

The ducks receiving two doses of laboratory-adapted vaccine remained healthy after every challenge and did not show any sign of ailment.

The above findings proved that with the elapse of the time after vaccination, the protection level diminished. The protection period by single dose of commercial vaccine was very short (one month), that by lab adapted vaccine also did not exceed two months beyond which the partial protection was provided upto five months. Even double vaccination with the commercial vaccine was not much better to confer the protection, but double vaccination with lab-adapted vaccine conferred complete protection at least for six months.

5.3.8 Pathogenicity index

A method to calculate the pathogenicity index (p.i.) was adapted in this study to get some idea about the degree of protection offered by a particular vaccine. The method originally used for differentiating the Newcastle disease virus strains based on their virulence can be successfully used for in-vivo assessment of immunity in birds on

vaccination. The current model system available for this purpose is the challenge and mortality wherein death or no death are the only degrees and may not show the exact level of protection unless large number of birds/animals are used and hence in the present experiment, this method was used. The only pre-requisite for the use of p.i. is that there should be clear distinction in degree of severity of symptoms to score-mark the condition (Table 2).

Each group after challenge was evaluated for calculation of p.i. which ranged between 0.0 to 2.13. The control group naturally had the highest p.i. values at all challenge infections. viz., 1.80, 1.87, 1.90 and 2.13, whereas the groups completely resisting challenge even without showing symptoms had nil pathogenicity index (Table 13).

The degree of protection afforded by the commercial vaccine was very less as either the birds died, or if survived, suffered with severe infection on challenge and hence p.i. was more, whereas the double lab adapted vaccine gave complete protection against challenge resulting in almost nil p.i. even after 20 weeks post-vaccination. In short, the values of p.i. of more than 1.8 showed cent per cent mortality, less than 1.0 indicated more survival chances than those between 1.3 and 1.8 and p.i. less than 0.2 showed total

protection (Table 14). This way, the p.i. had a definite correlation with mortality due to challenge.

Thus determination of p.i. provides a better and preferable substitute method to assess the degree of protection of birds against diseases like DP and for that matter, this method can be adapted for any similar situation. Incidentally, the calculation of p.i. for DPV as done in the present study happens to be the first of its kind and hence the results obtained could not be correlated with the findings of earlier workers.

5.3.9 Carrier status

The rectal swabs from all groups of vaccinated ducks were collected on seventh, 14th and 30th day pv, processed and inoculated into duck embryos. None of the samples yielded DP vaccine virus, indicating that the vaccinated birds did not excrete the virus. Similar observation on the failure to isolate the virus after vaccination have been reported by Jansen and Kunst (1964), Toth (1971b) and Wang et al. (1984).

The rectal swabs were also collected from the groups receiving vaccine followed by the challenge with virulent virus. After processing them in the similar way, none yielded DP virus. The excretion of the virus from naturally recovered birds has been reported by Burgess et al. (1979) and Burgess

(1981), however there are no reports of excretion of the virus in experimentally infected birds which had earlier been vaccinated.

5.3.10 Comparison between single and double vaccination (Tables 7, 9, 11, 13 and 14)

As regards the antibody titres, single vaccination did not induce satisfactory response. In commercial as well as lab adapted vaccine, the immunity did not last long beyond eight to 10 weeks of vaccination, eventhough LM inhibition was seen upto 16 weeks in case of lab adapted vaccine.

The second vaccination extended the duration of immunity upto at least 20 weeks and boosted the antibodies as well as LM inhibition percentages to the higher levels and gave better protection after challenge infection with lower levels of pathogenicity indices.

5.3.11 Comparison between the immune responses elicited by commercial vaccine and laboratory-adapted vaccine

In the present study, two duck plague vaccines were used. Both were chicken embryo-attenuated-live virus vaccines and as there is no immunological variation between strains

(Leibovitz, 1991), the only difference was in the quality of vaccines.

Before carrying out vaccinations, both the vaccines were subjected to estimation of the virus contents in them. It was found that the commercial vaccine was non-homogenous crude embryo extract having virus titre 0.74 log 10 ELD 50/ml dose whereas the laboratory adapted vaccine was a homogenous material having virus titre 3.5 log 10 ELD 50/ml dose.

In case of duck vaccinated only once, the difference in the immune responses among these two vaccines was seen in terms of VN indices, PHA titres, LM indices as well as p.i. and mortality after challenge infection. These differences became more prominent when the second dose (of the corresponding vaccine) was repeated after four weeks.

The commercial vaccine when used for vaccinating the ducks in an organized farm also yielded poor response. Within six weeks of vaccination, one of two birds could not withstand the challenge and succumbed. There were no neutralizing antibodies in the yolk from eggs collected at four weeks post vaccination and the ducklings hatched out from these eggs succumbed to challenge infection. These findings corraborated the results of the experiment in which the ducklings vaccinated with the same commercial vaccine also showed poor

response. It indicates that the concentration of antigen injected per bird is very vital factor to impart protection. Toth (1970) demonstrated this effect by using 13 serials of the chicken embryo-adapted DP vaccine and showed that the mortality in vaccinated ducklings due to challenge was directly proportional to the concentration of the virus in the vaccine. Leibovitz (1980) has stated that the ideal DP vaccine should contain minimum 3.5 log 10 ELD50/ml (vaccine dose) to be effective. The virus concentration in vaccine was also studied by John et al. (1990). The vaccine having virus titre 2.5 log 10 ELD50/ml gave 50 per cent protection against DP challenge whereas titre 3.5 to 4.5 log 10 offered 100 per cent protection. In these circumstances, eventhough the vaccine failed to induce adequate immune response, question arises as to why such a low titred vaccine was able to elicit at least some immune response. The possible explanations could be-

1. Owing to its nonhomogenous nature, some doses might be having more virus titre than others and a few birds who received them were protected.

2. Being a live vaccine, the surviving virus particles act in the body to develop immunity (Toth, 1970).

3. Survivals to challenge might be due to the interference phenomenon as described by Jansen (1964).

4. Lin et al. (1984) has stated that as little as 10 TCID50 of a virulent virus protected the ducks upto one month. Two months post-vaccination, only two out of five ducks survived challenge.

5.3.12 Assessment of immunity in general

The results of the present study have amply highlighted the fact that humoral immune response in duck plague is characterized by low antibody titres. The findings have also proved that single dose of the vaccine can have only marginal effect with short duration.

Due to migratory habits of ducks, and seasonal uncertainty of the outbreaks, the protection from the disease is required at least for one year and the results indicated that it could only be achieved by giving two doses of vaccines on sixth and 10th week. The vaccine should be well homogenized and should contain the virus concentration to a minimum of 3.5 log 10 ELD50/ml dose.

In the present study, the humoral as well as cellular factors got stimulated with the antigen exposure indicating that both antibody-mediated and cell-mediated immunity are involved in protection from duck plague. The exact elucidation of the extent of roles played by these responses warrants further studies.

Summary

SUMMARY

During 1991, six duck plague (DP) outbreaks in vaccinated as well as unvaccinated ducks in Kerala were investigated. The average morbidity was 33 per cent and mortality 26 per cent. In most of the cases, the typical symptoms and pathognomonic lesions of DP were seen. The virus was isolated in all six outbreaks, which could produce death of ducks and duck embryos, when inoculated. All these isolates failed to kill chicken embryos or produce any type of lesions in them on initial passages. They were not able to agglutinate chicken, duck, sheep or bovine erythrocytes.

The log 10 values of ELD50 of these six isolates ranged between 2.6 to 3.3 per ml. Each isolate was neutralized by anti-DPV serum.

One of these isolate DP-S was further studied for biological characterization. It was partially attenuated by passaging 20 times in duck embryos followed by 10 times in chicken embryos. Even after these passages, the virus caused the death of inoculated ducklings. However, its pathogenecity was reduced as evidenced by decrease in pathogenecity index from 1.9 to 1.23. The mean death time in duck embryos was 106.3 h and in chicken embryos, 85.0 hours. The ELD50 of 20

per cent suspension of embryo tissues and chorio-allantoic membranes (CAM) in allanto-amniotic fluid (AAF) was 4.8 log 10/ml whereas that of AAF alone was 2.8 log 10/ml.

The isolate DP-S was concentrated to 100 times by polyethylene glycol 6000 (PEG) and ultracentrifugation and was screened under transmission electron microscope after negative staining. The herpesvirus like capsids of around 100 nm virion diameter and enveloped virions measuring upto 380 nm were seen.

The PEG-concentrated or crude (unconcentrated) DPV did not show any precipitation reaction on agar gel with hyperimmune sera raised against reference DP virus.

The log 10 values of ELD50 for laboratory-adapted vaccine prepared from attenuated DPV strain obtained from IVRI, Izatnagar and commercial DP vaccine of IVPM Ranipet were 3.5 and 0.74 per ml respectively. The reconstituted commercial vaccine was in a particulate suspension and was not homogenous in nature.

In an experiment conducted for assessment of immune response, 63 six-week-old ducklings were divided into five groups with 12 ducklings each in groups SCV, SAV, DCV and DAV and 15 in group NVC. The ducks from group SCV and DCV were given commercial vaccine and SAV and DAV, laboratory-adapted

vaccine at sixth week of age. Groups DCV and DAV were revaccinated with their corresponding vaccine four weeks after first vaccination i.e., at 10th week of age. Group NVC was maintained as unvaccinated control throughout the experiment. The birds in all these groups were screened for the immune responses against DPV by virus neutralization (VN), leucocyte migration-inhibition (LM inhibition) and passive haemagglutination (PHA) tests before vaccination, every week upto six weeks after vaccination (except group SCV and SAV which were not tested at 5th week), every fortnight upto 12 weeks and thereafter every month upto 24 weeks.

In control group throughout the experiment and in the pre-vaccination screening of all the groups, none of the birds was found to have any VN antibody or PHA titre against DP virus, similarly leucocyte migration was not inhibited.

The virus neutralization indices for SCV and DCV groups were quite low upto four weeks of age as compared to those of SAV and DAV groups. After second dose of vaccine, ducks in DCV group showed significant increase but the mean VN indices were far low as compared to the group receiving lab-adapted vaccine. The highest VN index was 1.64 obtained among all vaccinated group in second week after double lab-adapted vaccine. Similarly, at the end of the observation period, i.e., 24 weeks post-vaccination, VN indices of group SCV were

nil; SAV and DCV were almost equal and those of DAV were above 1.0. The results of PHA also had similar trend and except for the first two weeks, the PHA titres of SCV and SAV as well as DCV and DAV were having highly significant ($P < 0.01$) differences.

Three ducks from each group each time were subjected to challenge with virulent virus at four, eight and 20 weeks post-vaccination for groups SCV, SAV and NVC and four and 20 weeks post-double vaccination for groups DCV and DAV. All the challenged birds from unvaccinated control group NVC died with the mean death time 5.4 days whereas all the double vaccinated ducks and those vaccinated once with laboratory-adopted vaccine survived the challenge. In single commercial vaccine receiving groups, at four weeks, all survived, at eight weeks one of three and at 20 weeks two of three (challenged) succumbed to duck plague. The pathogenicity indices (p.i.) of DAV and SAV groups were negligible, SCV had 1.23, 1.30 and 1.50 at four, eight and 20 weeks post-vaccination respectively and DCV had 0.14 and 1.17 at four and 20 weeks post-double vaccination respectively. The p.i. of controls were in the range of 1.80 to 2.13.

Three rectal swabs were collected from each group at each interval of seven, 14 and 30 days post-vaccination, similarly at the same intervals after challenge in survived

birds for studying the carrier status. None of the rectal swabs from vaccinated or challenged ducks yielded virus isolation even after two blind passages in duck embryos.

All the 180 ducks from University Duck Farm, Mannuthy were vaccinated with single dose of commercial vaccine and the serum samples of randomly selected 10 per cent ducks from this farm were screened for VN antibody and PHA titres before and 8 weeks after vaccination. These samples showed low level of antibody response and of two randomly selected ducks subjected to challenge, one died of the infection and other suffered with disease but recovered. The eggs collected from this flock had no antibody to DPV in their yolk and three ducklings hatched from these eggs, when challenged on third day of hatching, succumbed to DP infection.

In brief the salient features of the study are indicated below:

* Six duck plague outbreaks were investigated, the virus isolated, and characterized. It was partially attenuated in duck and chicken embryo.

* The effect of single vaccine was found to be negligible. The commercial vaccine failed due to its low virus contents. The laboratory-adapted vaccine yielded good

results in terms of VN titres, LM inhibition percentage and PHA titres and the duration of immunity after use of this vaccine was found to be more than six months.

* There was positive correlation between the trends of VNI, LMI and PHA titres. However, in general, titres were very low even with double vaccination which rose to a greater extent after challenge infection.

* Apart from antibody mediated immunity, cell mediated immune response was also seen to be involved in protection.

* The carrier status was not seen in the vaccinated or vaccinated and challenged ducks.

* PHA test was standardized for diagnostic purpose.

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ABSTRACT

During 1991, six outbreaks, clinically suspected to be duck plague (DP) with 33 per cent morbidity and 26 per cent mortality were investigated. Duck plague virus was isolated from each outbreak. The isolates were able to produce the lesions and death of the duck embryos but failed to kill the chicken embryos during initial passages.

One of the strains named DP-S was partially attenuated by 10 passages in chicken embryos following 20 passages in duck embryos. Though the attenuated strain did kill ducks, its pathogenicity index was reduced from 1.9 to 1.23. The isolate DP-S under transmission electron microscope revealed virions of herpes virus morphology.

Two DP vaccines - commercial vaccine and lab-adapted vaccine having virus titres 0.74 and 3.5 log 10 ELD 50/ml respectively, were separately inoculated into four groups of ducklings respectively, two groups receiving single dose and two receiving double dose of corresponding vaccines at an interval of four weeks. Another group of ducklings was kept as control without vaccination.

Three ducks in each group were challenged with virulent DPV at four,eight and 20 weeks post-vaccination. The birds in all the five groups were screened at regular intervals for studying the immune response by virus neutralization (VN), leucocyte migration-inhibition (LMI) and passive haemagglutination (PHA) test.

The challenged and survived birds were screened for the carrier status of DPV by examination of their rectal swabs for virus isolation.

In an organized farm, 180 ducks were given commercial vaccine at one year of age and were screened for VN antibodies, LMI response and PHA titres before and eight weeks post-vaccination. Randomly selected two birds were challenged six weeks post-vaccination.

The findings of the study are briefly listed as under:

* Six duck plague outbreaks were investigated, the virus isolated, and characterized. It was partially attenuated in duck and chicken embryos.

* The commercial vaccine could elicit very poor immune response as compared to laboratory adapted vaccine. The immunity could not last long even upto eight weeks in single vaccination and 20 weeks in double vaccination.

* Single vaccination is not effective as compared to double vaccination given four weeks apart.

* The assessment of antibody-mediated (AMI) and cell-mediated (CMI) immune responses indicated that both AMI and CMI are involved in protection of ducks against duck plague.

* The vaccinated or vaccinated and infected birds did not show carrier status as attempts to isolate the virus from rectal swabs collected after vaccination and challenge were unsuccessful.

* The PHA has been standardized for diagnosis of duck plague.