EVALUATION OF ENZYME IMMUNOASSAYS IN THE DIAGNOSIS OF DUCK PLAGUE

BY

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THESIS

Submitted in partial fulfilment of the
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Faculty of Veterinary and Animal Sciences
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DECLARATION

I hereby declare that the thesis entitled "**EVALUATION OF ENZYME IMMUNOASSAYS IN THE DIAGNOSIS OF DUCKPLAGUE**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Certified that the thesis, entitled "**EVALUATION OF ENZYME IMMUNOASSAYS IN THE DIAGNOSIS OF DUCKPLAGUE**" is a record of research work done independently by **Shri. S. Malmarugan**, 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

Amma, Appa and Teachers

CONTENTS

LIST OF ABBREVIATIONS

ABC	-	Streptavidin-biotin peroxidase complex
ABTS	-	2'-2 azino-di-ethyl-benzthiazoline-6-sulfonic acid
ADS	-	Antiduck serum
ADV	-	Aujeszky's disease virus
AGPT	-	Agargel precipitation test
AHV	-	Anatid herpes virus
AI	-	Avian Influenza
ASS	-	Ammonium sulphate solution
BHV	-	Bovine herpes virus
BRS	-	Bovine respiratory syncytial virus
BSA	-	Bovine Serum Albumin
CAM	-	Chorio allantoic membrane
CCL-141	-	Certified cell line 141
CEF	-	Chicken embryo fibroblast
CF	-	Complement Fixation
CIEP	-	Counter immuno electrophoresis
CI-ELISA	-	Competitive inhibition enzyme linked immunosorbent assay
CPE	-	Cytopathic effect
DAB	-	3'3-diaminobenzidine tetrachloride
DP	-	Duck plague
DPV	-	Duck plague virus
DVE	-	Duck viral enteritis
EHV	-	Equine herpes virus
EIA	-	Enzyme immuno assays
ELD_{50}	-	Embryo lethal dose 50
ELISA	-	Enzyme linked immunosorbent assay
FAT	-	Fluorescence antibody test
fg	-	femto gram
FTE	-	Formalized tanned erythrocytes
HI	-	Haemagglutination inhibition test

HRPO	-	Horse radish peroxidase
HVT	-	Herpes virus of turkeys
IBRT	-	Infectious bovine rhino tracheitis
IBV	-	Infectious bronchitis virus
IEP	-	Immuno electrophoresis
IFA	-	Immuno fluorescence antibody test
IFT	-	Immuno fluorescence test
IgG	-	Immunoglobulin G
IgM	-	Immunoglobulin M
ILT	-	Infectious laryngo tracheitis
IPT	-	Immunoperoxidase test
LAB-ELISA	-	Labelled avidin-biotin enzyme linked immunosorbent assay
LAT	-	latex agglutination test
MCFV	-	Malignant catarrhal fever virus
MDV	-	Marek's disease virus
mg	-	Milligram
NC	-	Nitrocellulose
NDV	-	Newcastle disease virus
OD	-	Optical density
PBS	-	Phosphate buffered saline
PBS-T	-	Phosphate buffered saline-Tween 20
PCR	-	Polymerase chain reaction
PEV	-	Porcine enterovirus
PHA	-	Passive haemagglutination
PM	-	Post-mortem
PRV	-	Pseudorabies virus
RIA	-	Radio immuno assay
RPHA	-	Reverse passive haemagglutination
SAS	-	Saturated Ammonium Sulphate Solution
SNT	-	Serum neutralization test
SPF	-	Specific pathogen free
SRBC	-	Sheep red blood cells
$TCID_{50}$	-	Tissue culture infective $dose_{50}$
VNT	-	Virus neutralization test

LIST OF TABLES

LIST OF PLATES

Introduction

INTRODUCTION

Duck farming plays an important role in the rural economy of back water areas of the eastern and southern states of India. Duck stand second to chicken as far as population and egg production are concerned. Nearly 23.5 millions ducks contribute four crore rupees to national economy and provide ample employment opportunities in the animal husbandry sector.

Compared to chicken, ducks offer many advantages like foraging capacity, biological control of certain invertebrate vectors, higher average egg production and good protein source. Of late, these advantages have been recognised as evidenced by the rapid strides in the growth of duck industry.

In India, Kerala ranks fourth in total duck population. Of these 88.5 per cent is concentrated in rural areas. The state is endowed with the ideal climatic, topographic and waterlogging conditions essential for duck farming.

The managemental and rearing practices like free range system and movement of flocks during harvesting seasons and summer months to paddy fields and available water sources predispose them to many infectious diseases of which the most important one is duck plague (DP) or duck viral enteritis (DVE).

In India, the occurrence of the disease was first reported by Mukerji *et al.* (1963), from West Bengal. Since this first report, duck plague is known to exist in various parts of the country (Duraiswamy, 1979, Chakraborty *et al.*, 1980, Sreeramulu, 1986).

In Kerala, the disease outbreak was first observed in Alleppey district by Punoose and Abdulla in 1976. Apart from regular vaccination, outbreaks of this disease have occurred almost every year in various parts of the State.

As is the case with many infectious diseases, diagnosis of DP has been traditionally based on the clinical findings, PM lesions and isolation and identification of the virus which is laborious and time consuming.

Several techniques like Agar gel precipitation test (AGPT), Counter immunoelectrophoresis (CIEP) (Cottral, 1978) histopathology (Culling, 1974), immunofluorescence (Proctor, 1975, Tantaswasdi *et al.*, 1988) and Virus neutralization test (VNT) (Chakraborty *et al.*, 1980) are available for diagnosis of duck plague viral antigen and antibody.

The two widely accepted assays like FAT and RIA's have its own advantages and disadvantages. In practice, immunofluorescence is not easy to be used for antibody

assays, since it depends on subjective visual assessments of fluorescence and the results are usually expressed as the serial dilution of serum that given the least fluorescence.

Radio immunoassay on the otherhand is highly sensitive and permits precise quantification, however the isotope labels may decay rapidly and require complex equipment. Moreover it is hazardous to handle these isotopes, and must be handled only by trained personnel.

The introduction of enzyme immunoassays (EIA), pioneered by Engvall and Perlmann (1971) offered an attractive, alternative to the labelled antibody/antigen methods. It is simple, sensitive, inexpensive and reagents have a relatively long shelf life.

ELISA is more sensitive than the immunodiffusion and haemagglutination tests and is of comparable sensitivity to the VNT. This test is capable of detecting both neutralizing and haemagglutinating antibodies (Nicholas and Thronton, 1986).

Several forms of ELISA viz., microwells, dot, dipsticks and membrane devices are commercially available and they have been widely used in various fields like Endocrinology, Immunopathology, Parasitology and Microbiology.

Reports on the use of EIA for duck plague virus is scanty. However these techniques for other herpes viruses are available (Pearson *et al.*, 1979; Guy *et al.*, 1992; Pandita and Srivastava, 1995).

The use of immunoparoxidase test (IPT) in place of FAT for the identification and localization of viral antigens in infected cells and in various cell cultures was introduced by Avrameas and Uriel (1966). This test is highly sensitive, can be performed on paraffin embedded tissue sections and gives a permanent preparation which can be examined by conventional light microscopy. Recently IP staining has been developed for the detection of DPV antigen in paraffin embedded tissue sections (Islam *et al.*, 1993).

There are many possible applications of enzyme immunoassay's in Veterinary Virology. Among avian diseases, indirect ELISA have been reported for antibodies to most avian viruses, with the exception of duck viral hepatitis, duck plague, fowl pox and the pigeon viruses (Nicholas and Thronton, 1986).

This necessiate to evolve a rapid method of diagnosis of duck plague. So, the present study was undertaken with the following objectives.

1. Standardization of immunoperoxidase technique for the detection of duck plague viral antigen in tissues.

2. Standardization of enzyme linked immuno-sorbent assay for the detection of antibodies to duck plague virus.

3. Comparison of ELISA test with passive haemagglutination test in the quantitation of antibodies to duck plague virus.

Review of Literature

REVIEW OF LITERATURE

Duck viral enteritis more commonly known as duck plague is an acute, contagious herpes virus infection of ducks, geese and swans, characterised by vascular damage with petechial haemorrhages in mucous membranes and tissues, especially of the gastro intestinal tract, free blood in the body cavities, lesions in lymphoid organ and degenerative changes in parenchymatous organs (Leibovitz, 1991).

2.1 History

An acute haemorrhagic disease of domestic ducks was first reported in Netherland by Baudet (1923) and he suspected the causative agent to be a specific duck adapted strain of fowl plague virus. Bos (1942) critically evaluated the above findings and concluded that the disease was caused by an independent virus with high affinity for ducks and termed it as duck plague (DP). However only Jansen and Kunst (1949) had proposed DP as the official name, which was later approved by the XIV International Veterinary Congress. They distinguished this disease from other viral diseases like duckling hepatitis, fowl plague and Newcastle disease.

Outbreaks of DP during 1923-1960 had been cited by Jansen (1961). The name, duck viral enteritis (DVE) was first

introduced by Leibovitz (1991) based on the Pathological features and clinical symptoms.

2.2 Incidence and distribution

Occurrence of duck plague had been reported from France (Lucam, 1949), Belgium (Devos *et al.*, 1964), USA (Leibovitz and Hwang, 1968), Britain (Hall and Simmons, 1972), Canada (Hanson and Willis, 1976), Thailand (Suwatviroj *et al.*, 1977), Denmark (Prip *et al.*, 1983), Germany (Zielder *et al.*, 1984), China (Kunst, 1958; Wang *et al.*, 1984), Russia (Simonova *et al.*, 1984), Austria (Pechan *et al.*, 1985), Malaysia (Sharifah *et al.*, 1994) and East Anglia (Gough *et al.*, 1987).

In India, the first official report of duck plague was by Mukerji *et al.* (1963) from West Bengal. Since then, the disease has been reported from Tamil Nadu (Duraiswamy *et al.*, 1979), Assam (Chakraborty *et al.*, 1980; John *et al.*, 1990), West Bengal (Bhowmik and Chakraborty, 1985) and Andhra Pradesh (Sreeramulu, 1986) indicating the endemic nature of this disease in India.

In Kerala, the disease outbreaks were reported by Punnoose and Abdulla (1976) and Nair (1978). Since then, outbreaks have been quite regular with incurrence of heavy losses to the farming community in duck rearing areas of Alleppey, Kottayam, Pathanamthitta, Thrissur and other coastal

areas. The incidence of disease was found to occur mostly during March to May (Kulkarni, 1993).

2.3 Aetiology

The virus of DP is a herpes virus belonging to the sub family alpha herpes virinae designated as Anatid herpes virus-I (AHV-I) by the herpes virus study group (Roizman, 1982).

Duck plague virus is a non-haemagglutinating non-haemadsorbing virus which produces plaques and intranuclear inclusions in cell cultures (Jansen, 1961, Dardiri and Hess, 1968). This virus is sensitive to the usual disinfectants and at a pH <3 or >10, it is rapidly inactivated (Hess and Dardiri, 1968).

In the laboratory the virus has been cultivated in 12-14 day embryonated duck eggs (Jansen, 1961), duck embryofibroblasts (Kunst, 1967) and CCL-141 (Wolf *et al.*, 1976). The cytopathic changes described were rounding and clumping of infected cells, syncytium formation with intranuclear inclusion bodies (Nair, 1978).

Lam (1984) reported, Duck embryofibroblast infected with Duck plague virus was lysed by homologus antibodies in the

presence of complement. This virus did not stimulate precipitating antibodies in ducks (Panisup and Verma, 1989).

2.5 Symptoms

Natural infection of duck plague was restricted only to anseriformes, not in other species of birds, mammals or man (Jansen, 1968). Leibovitz (1971a) found that the incubation period ranged from 3 to 7 days in domestic ducks.

Characteristic signs of DP included high persistent flock mortality, drop in egg yield by 25 per cent, extreme thirst, droopiness, ataxia, lachrymation, photophobia, gluing of eyelids, nasal discharge, ruffled feathers, soiled vent with greenish watery diarrhoea, and weakness along with tremors. Ailing birds stayed away from water, assuming a squatting posture with droopy wings (Leibovitz, 1991).

During the course of the disease, the call became hoarse and breathing laboured, especially when the ducks were frightened. Mortality may be severe as a result of dual infection of DP and latent bacterial infection (Dardiri, 1971). Symptoms with 78 to 100 per cent mortality among young ducks were also recorded (Suwatviroj *et al.*, 1977). Death even within three days post infection was reported by Roy *et al.*, (1980).

2.6 Lesions

The most striking lesions of the disease were, catarrhal proventriculitis, gizzard muscle necrosis, erythematous lesions of digestive tract, haemorrhages and necrosis of visceral organs and gonads, serosal haemorrhages and collection of free blood within body cavities (Leibovitz, 1969), closely packed petichiae giving a red "paint brush" appearance over the pericardium and liver with dark mottled spleen of reduced size (Leibovitz, 1971b; Ray *et al.*, 1983; Bhowmik and Chakraborty, 1985) were the other lesions described. Dardiri (1975) and Das *et al.* (1990) opined that longitudinal rows of haemorrhage in the oesophagus and cloaca, which later became yellow or gray with haemorrhagic diptheritic pseudomembranes were pathognomonic lesions of DP.

Chennakesavalu *et al.* (1987) demonstrated intranuclear inclusion bodies and also cowdry type-A inclusion bodies in the hepatocytes and epithelium of oesophagus and cloaca. The presence of intranuclear inclusion bodies in epithelial cells of bursa of Fabricius, stratified squamous epithelium of oesophagus, intestine, spleenic periarticular reticular cells and harderian glands were demonstrated by Barr *et al.* (1992).

2.7 Diagnosis

Traditionally diagnosis of DP was based on symptoms and lesions, supported by histopathological studies. Differential diagnosis of DP from Newcastle disease, Fowl cholera, Duck hepatitis and toxicities was made by virus isolation from morbid specimens showing characteristic lesions as well as mortality in duck embryos and absence of these in chicken embryos (Jansen, 1961, 1964). Experimental animals like hen and dayold ducklings are suitable for making differential diagnosis with the above conditions. Duck hepatitis is essentially a disease of young ducklings. In duck plague, intranuclear inclusions in hepatocytes could support the diagnosis (Rajan *et al.*, 1980).

Isolation and identification of the virus, in 12-14 day old embryonated duck eggs or ducklings or duck embryo fibroblast cell cultures provided confirmation even in the absence of diagnostic morphological alterations. The characteristic changes produced by DP virus were rounding of cells, syncytia granulation and vacuolation of the cytoplasm with or without intranuclear inclusion bodies (Kunst, 1967).

Dardiri and Hess (1968) developed a plaque assay for chicken embryo adapted virus and a virulent DP virus. These authors opined that this could very well be used for

differentiation of avirulent and virulent DP virus and for titration of antibody by plaque neutralization inhibition test.

Wolf *et al.* (1974) developed microtitre plate system for direct isolations and concurrent virus identification by serum neutralization test (SNT), using duck embryo fibroblast cell line CC1-141, wherein definitive virological results could be obtained within 60 to 72 hrs. However, they cautioned the use of microtitre system for DPV detection in subclinical cases as the area of cell sheet is limited. In low level of infections they recommended cell cultures in flasks, bottles or Petridishes, which will provide larger areas of cell sheet.

Erickson *et al.* (1974) used immunofluorescence technique (IFT) for diagnostic purpose. Light immunofluorescent and electron microscopy were used by Tantaswadi *et al.* (1988) in the detection of DP viral antigens in the tissues of experimentally infected ducklings. From the immunofluorescent and electronmicroscopic observations they have concluded that the lesions of DP developed in close association with the appearance and distribution of viral antigens/virions. The authors could also demonstrate cytoplasmic inclusions in the oesophageal epithelial cells of infected ducklings. Specific fluorescence was described in the nucleus and cytoplasm of digestive mucosal epithelial cells, hepatocytes, bile duct

epithelial cells and reticular cells of various organs and tissues.

An indirect fluorescent antibody test (IFA) was developed as a rapid serological test for duck plague virus infection (DPV) by Lim *et al.* (1994). They compared IFA with serum neutralization test (SNT) and stated that the IFA test was able to detect virulent DPV infections in duck flocks irrespective of vaccination, and clinical histories.

Agar gel precipitation (AGPT) was developed for identification of DP viral antigen or antibody (Cottral, 1978). He used 20 per cent trichurated CAM suspension as the antigen and hyperimmune sera raised in rabbits as known positive sera for detection of antigen. Kalaimathi and Janakiram (1990) also used AGPT for diagnosis of duck plague.

For rapid detection of DP viral antigen and antibody, John *et al.* (1989) recommended counter immunoelectrophoresis (CIEP) using 20 per cent trichurated CAM suspension as the antigen and hyperimmune sera raised in 3 to 4 month old ducks as the antibody.

Rao and Reo (1994) in their studies to evaluate the deficiency of functional bivalency of the antibody molecules of duck, in precipitation tests observed that DP antibody could be detected by AGPT, when barbital buffer (pH 7.4) with

0.15 M NaCl was used to prepare the agarose and sonicated infected liver tissue was used as the antigen.

Deng *et al.* (1984) developed a reverse passive haemagglutination test (RPHA) to detect duck plague virus. They utilized sheep erythrocytes stabilized with formaldehyde and pyruvaldehyde, coated with sheep anti DPV IgG in the test. 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 in all the three tests. The RPHA was stated to be rapid, simple and sensitive for diagnostic detection of DPV in acute infections.

2.7.1 Passive haemagglutination test: (PHA)

Zyambo *et al.* (1973 a,b) standardized PHA test for IBR antibody detection and stated that it had some advantages over neutralization test, namely it was rapid, less cumbersome and equally sensitive, moreover the latter test did not detect antibody in known carrier animals. Sulochana *et al.* (1982) and Suresh (1992) conducted a serological survey on the occurrence of IBR in Kerala, and Tamil Nadu respectively using this test.

Kulkarni (1993) standardized PHA for DPV and stated that the 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 were its advantages particularly when large number of sera samples are to be screened.

2.7.2 Enzyme linked immunosorbent assay (ELISA)

The new analytical technique enzyme linked immunosorbent assay commonly termed ELISA was introduced by Engvall and Perlmann (1971). The research on simple and sensitive method for detecting and quantitating antigen and antibody without using particulate agglutination, fluorescent or radio-labelled antigen has led to the development of a versatile immunodiagnostic technique, enzyme immuno assay (EIA).

ELISA is a type of immuno assay that uses an enzyme chromogen read out system to enhance test sensitivity. Now-a-days, several forms of ELISA and its modifications are routinely used as diagnostic tools in human and veterinary medicine.

Reports of enzyme immuno assays for detection of duck plague viral antigen and antibodies are scantly. However,

application of these techniques for other herpes viruses are available.

2.7.2.1 Cytomegalovirus

Tsueminglin *et al.* (1986) developed rapid Dot enzyme immuno assay (Dot-ELISA) for the detection of antibodies to cytomegalovirus. They used white opaque plastic card for antigen coating and found that the test was highly reproducible, required no instruments. The reagents including coated antigen dots, are stable at room temperature for atleast two months and can be used readily.

2.7.2.2 Bovine herpes virus (BHV)

A micro-enzyme linked immunosorbent assay (micro-ELISA) was developed by Herring *et al.* (1980) for the detection of infectious bovine rhino tracheitis (IBRT) viral antibodies compared with serum neutralization test (SNT), it was showed that the sensitivity of antibody detection by the micro-ELISA was atleast as good as that of the SNT. Advantages of the test described were simplicity in the preparation of the antigen, the antigen is required only in small quantities, and it is quite stable atleast for two months at -70°C. Moreover the antigen coated plates could be stored for three days at -20°C. They have also observed that virus from disrupted

cells was suitable as antigen without further purification and extra cellular virus always gave better results.

Bolton *et al.* (1981) developed a sensitive ELISA test for detection of antibodies to IBRT virus. Equilibrium density gradient purified IBR virus was used as antigen (at an optimal concentration of 0.60 ug/cuvette). At this concentration of the antigen it was possible to test sera even at dilutions of 1:10 without any non-specific reactions. They have also reported that the conditions of conjugate dilution, substrate concentration and reaction time had significant effects on the ELISA test and when standardized properly, ELISA procedure was as much as 1000 fold more sensitive than the serum neutralization plaque assay. They also remarked that the increased sensitivity was greatest with sera having SN titres 1:4 or less thus helping to detect immune status at low levels of antibody.

The ELISA for detection of antibodies to bovid herpes virus-4 (BHV-4) was developed using antigen prepared by detergent lysis of infected cell cultures. The assay was used to study the immune responses of experimentally immunised calves. The results correlated well with the indirect fluorescent antibody method (Edwards and Newman, 1984).

Collins *et al.* (1985) compared serological assays like virus neutralization (VN), complement fixation (CF) and ELISA

for the detection of specific BHV-1 antibody titres, sero-conversions and early antibody response in experimentally infected cattle. They found, that the ELISA end point titres and single dilution values were more sensitive than the CF or VN assays for specific antibody quantitation.

Suribabu *et al.* (1984) opined that the ELISA can be used as an efficacious diagnostic tool as well as to detect very small quantities of IBRT antibodies from the cross bred and indigenous cattle and buffaloes.

Edwards *et al.* (1986) evaluated five serological tests. ELISA for IgM and IgG, passive haemagglutination (PHA) and two methods of virus neutralization for the detection of antibody to BHV-1 in vaccinated and experimentally infected cattle. On statistical comparison of ELISA (IgG), PHA and VN results, the assays showed highly significant correlation ($P<0.01$). The sensitivities of ELISA and 24 hour neutralisation tests were similar, in contrast to PHA and one hour neutralisation, which failed to detect BHV-1 antibodies in some low titre sera.

A capture ELISA for the detection of IgM antibodies to IBRT and to Bovine Respiratory syncytial virus (BRS) was developed by Florent and Wiseman (1990). In these assays the first monoclonal antibody to bovine IgM was used as the catching antibody. While the second monoclonal detected specific antiviral antibodies. It was shown that primary IBR and BRS

virus infections can be confirmed using serum samples collected 5-10 days after the appearance of clinical signs of disease.

Indirect ELISA for detection of IgM antibodies to BHV-1 was developed by Ungar-Waron and Abraham (1991). From the results obtained in their study it was concluded that IgM-ELISA was of particular value in diagnosing recent infection with BHV-1 in calves.

Li *et al.* (1994) developed a competitive inhibition ELISA (Cl-ELISA) for the detection of antibody to malignant catarrhal fever virus (MCFV) in ruminants based on a monoclonal antibody.

Hongli *et al.* (1995) investigated sheep associated MCFV infection in ruminants by polymerase chain reaction (PCR) and Cl-ELISA and opined PCR to be more accurate than Cl-ELISA.

Dot immunobinding assays (Dot-ELISA) for detection of BHV-1 was developed by Pandita and Srivastava (1995). They compared Dot-ELISA with plate ELISA, but no differences either in sensitivity or in specificity could be detected. Of the two tests, Dot-ELISA was recommended as the field test, because it was easier and more economical to perform.

2.7.2.3 Equine Herpes Virus (EHV)

Dutta *et al.* (1983) used a four layer ELISA for the detection of EHV-1 viral antigen and its specific antibody.

Huhdatsu *et al.* (1986) in their studies on the detection of antibodies to EHV-1 by ELISA test had reported that the values of the test were almost constant in the antigen protein concentration range of 5-10 ug/well. They compared the ELISA values and neutralizing antibody titres and reported that the detection sensitivity of the ELISA was excellent.

Vulcano *et al.* (1988) compared serum neutralization, complement fixation and ELISA for the detection of antibodies to Equine rhinopneumonitis virus and opined that the ELISA test was the most sensitive, with the greatest agreement with the serum neutralization test (70% agreement for positive and negative results combined). In a similar study Singh *et al.* (1995) reported that though ELISA was more sensitive, its was less specific compared to VNT and CFT.

Galosi *et al.* (1993) concluded that the indirect ELISA was a specific and sensitive test for the diagnosis of EHV-1 infection and can be preferred for large scale use.

2.7.2.4 Swine herpes virus

A rapid method for the detection of antibodies in whole blood of pigs infected with Aujeszky's disease by ELISA disc was introduced by Banks (1985). Here, a punched filter paper disc was loaded with blood, dried and used directly for the ELISA with minimal pre-treatment. According to him the rapidity and sensitivity were equal to that of the serum ELISA. It was possible to store the discs at 4°C for almost a year without loss of its activity.

Filter paper ELISA for detection of antibodies to AD was also described by Shibata *et al.* (1988). They have also described this technique as simple and reliable and recommended for large scale epidemiological survey.

Afshar *et al.* (1987) developed an indirect ELISA for detection of pseudorabies (PRV) antibodies in pigs exposed to BHV-1. They found that the specificity of the ELISA can detect anti-PRV antibody in pigs only, when they were challenged with PRV, not after exposure to BHV-1.

ELISA was used to detect the cross reaction of sheep and dog herpes virus to swine herpes virus by Banks (1989). He could observe cross reaction only with antibodies to BHV-1. None of the ovine, canine or bovine sera had neutralizing antibody to swine herpes virus.

2.7.2.5 Avian herpes virus

An indirect ELISA with modified solid phase and improved specificity was developed and optimized to assay antibodies to adeno associated virus, adenovirus, Infectious laryngotracheitis virus (ILT), Marek's disease virus (MDV), Herpes virus of turkeys (HVT), Reovirus, Infectious bursal disease virus, Newcastle disease virus (NDV) and Infectious bronchitis virus (IBV) (Block, 1988). He used small strips of nitrocellulose (NC) membrane as a solid phase and reported that the NC-ELISA was highly specific, sensitive, reproducible, rapid, and simple to perform. In nearly all cases it was more sensitive than agar-gel precipitation and indirect-immunofluorescence, and was of comparable sensitivity to serum neutralization and microtiter plate ELISA and some times more sensitive.

ELISA systems incorporating pooled viral and mycoplasma antigen preparations for antibody screening of avian sera were described by Adair *et al.* (1990). A triple antigen test, comprising NDV, ILT and avian influenza (AI) antigens for screening sera normally negative for antibodies to these viruses, was shown to be as sensitive as the corresponding single antigen ELISA in detecting seroconversion in experimentally inoculated birds and was also as sensitive as the HI test for NDV and AI, and SNT for ILT virus.

An indirect ELISA was developed by Heider *et al.* (1990) to verify the freedom of SPF chicks from viral diseases like ILT, pox, encephalomyelitis, ND, influenza, leukosis and reticuloendotheliosis. They used polyethylene glycol precipitation method for antigen purification.

2.7.2.6 Infectious laryngo tracheitis (ILT) virus

Meulemans and Halen (1982) developed ELISA for detecting ILT viral antibodies in samples collected from ILT vaccinated chicken.

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

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

Fuchs *et al.* (1985) compared different serological tests (IFA, SNT, indirect ELISA, AGPT) for the detection of ILT antibodies in samples collected from ILT vaccinated chickens and from flocks recently infected or suspected as infected. In qualitative assay micro SN test was slightly more sensitive than FA and ELISA but all the three tests were equally sensitive in quantitative assay. AGPT was unsuitable for detecting ILT antibodies in individual birds because of its low sensitivity.

Ohkubo *et al.* (1988) compared a labelled avidin-biotin enzyme linked immunosorbent assay (LAB-ELISA) for antibody to ILT virus with an ordinary ELISA. Purified ILT virus, biotin-labelled anti-chicken IgG rabbit IgG conjugate, and horse radish-peroxidase-labelled avidin were used in the LAB-ELISA. The correlation rate between SN and LAB-ELISA was 100 per cent and that between SN and ordinary ELISA was 78 per cent. These workers observed much lower non specific reactions with LAB-ELISA than ordinary ELISA. It was also possible to detect ILT antibody after vaccination by this test.

Hopkins *et al.* (1990) conducted a survey of selected infectious diseases and parasites in wild turkeys, trapped as part of a relocation programme from Arkansas. The serum

samples from these birds were negative for ILT, MD and Avian encephalomyelitis by ELISA.

Serum samples collected from commercial hens were tested by ELISA for antibodies against ILT virus, and the data were analysed statistically (Leong *et al.*, 1994). They found that ELISA was useful to detect and quantitate ILT vaccine induced antibody but it could not identify older flocks that were vaccinated at young age with a live vaccine of chick-tissue-culture-origin.

2.7.2.7 Marek's disease virus (MDV)

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

Antigen requirements and specificity of a micro plate ELISA for MD viral antibodies in chicken, mouse and rabbit sera were analysed by Coudert and Ahluwalia (1984). They concluded that the optimal sensitivity was obtained by using 10 fg/ml of partially purified viral proteins.

A reproducible ELISA using MDV infected cells for the detection of antibodies to MDV was described by You-Quancheng *et al.* (1985). They compared two antigens-purified virus preparation and MDV infected CEFs. The latter produced highly specific reactions with low non-specific reactivities. According to them ELISA was 20-40 fold more sensitive than indirect immunofluorescene.

An ELISA and dot-blot hybridization were developed to detect and measure the antigens and DNA of MDV in feather tips from infected chickens. Buffered extracts of the feathers was the test material. Antigen detection by AGPT and ELISA was compared with DNA detection of dot-blot hybridization. (Davidson *et al.*, 1986a).

Davidson *et al.* (1986b) developed a streptavidin-biotin peroxidase complex (ABC) system to improve the sensitivity of MDV antigen detection in the feather tip extracts from infected chickens. ELISA test was more sensitive at lower dilutions though at higher concentration ABC system and standard ELISA gave the same sensitivity. Both the tests were superior to AGPT.

Lukina *et al.* (1989a) used immuno enzyme assay as a rapid screening test for potency of Turkey herpes virus (THV) vaccines.

Lukina *et al.* (1989b) developed a double sandwich ELISA for assessing the serological status of fowls vaccinated against MD. This technique was capable of distinguishing antibodies to turkey herpes virus antigen from antibodies to the non-protective viral glycoprotein antigen. They concluded that maternal antibody had suppressed multiplication of the vaccine virus administered to 1-day-old chicks, in cases of increased mortality from MD.

Scholten *et al.* (1990) standardized an immuno assay to detect MDV antigen on the tips of feathers obtained from MDV-infected chickens using a specific monoclonal antibody. The principle of an indirect ELISA was employed and feather tip was used as the solid phase. Presence of MDV was shown by a dark brown precipitate on the feather tip which could be observed by the naked eye. This test system was more sensitive than AGPT.

Adeniran and Oyejide (1995) standardized an indirect ELISA for the detection of MD antibodies in chicken and opined that the test could be used for screening antibodies to MD in chicken flocks.

2.7.2.8 Duck plague virus (DPV)

Chandrika (1996) developed a field diagnostic kit for duck plague virus. She inferred that DOT-ELISA and Latex

agglutination test (LAT) would serve as alternatives for VN test because of their sensitivity, rapidity and simplicity and could very well be used even in geographically remote areas.

2.7.3 Immunoperoxidase test (IPT)

The immunoperoxidase technique has been widely used as a rapid method for the identification and localization of viral antigens in infected host cells and in various cell cultures. In this technique, the viral antigens are detected with conjugates of their specific antibodies coupled to enzymes. Use of enzyme labelled antibodies in the localization of viral antigens by both light and electron microscopy has been described by various workers (Nakane and Pierce, 1967; Wicker and Avrameas, 1969).

The fluorescent antibody technique is capable of localizing and identifying viral antigens. Certain disadvantages, such as the influence of the pH and the ratio of fluorescent dye to antibody on the intensity of fluorescence, which can give rise to non-specific reactions, interference of naturally occurring autofluorescence with the specificity of the reaction, failure to obtain a permanent preparation, the need for a fluorescence microscope and the requirement of experienced and expert persons for the interpretation of the results make it unacceptable in some situations (Nakane and Pierce, 1966; Shabo *et al.*, 1972).

Some characteristics of the immunoperoxidase test make it more acceptable than immunofluorescence (IF) test. Thus, unlike conjugation with a fluorescein dye, an exact ratio of peroxidase/protein is not essential, since differences in the ratio do not markedly alter specificity or reactivity. Preparation of the conjugate is relatively easy; the Horse radish peroxidase (HRPO) conjugated viral antibodies have the ability to retain their enzymatic and immunological activity; the results are easily interpreted and permanent preparations can be made and examined with an ordinary light microscope with very sensitive and reproducible results (Nakane and Pierce, 1966; 1967).

The immunoperoxidase procedures have been successfully applied to the diagnosis of several viral infections.

Wilke and Noronha, 1970 used HRPO labelled antibody for light and electron microscope localization of reovirus antigen, in tissue culture. They reported that the reaction product in infected cells was easily detected in cytoplasm, and the procedure was sensitive as the fluorescent antibody technique.

Inclusion bodies of fowl pox virus demonstrated by hematoxylin and eosin, acridine orange, and giemsa, and by fuelgen reaction in formalin fixed paraffin embedded infected chicken embryo chorio allantoic membranes were identified by an immunoperoxidase method (Tripathy *et al.*, 1973). They

found that the fowl pox virus inclusions stained pale to dark brown when reacted with a peroxidase labelled fowl pox antibody. Specificity of the reaction was confirmed by blocking with unlabelled fowl pox antiserum.

Benjamin and Georgeroy (1974) used IPT for the rapid identification of human myxoviruses and paramyxo viruses in tissue culture. They concluded that the IPT was ideally suited for the rapid identification and typing of common human respiratory viruses on a routine basis.

Sutmoller and Cowan (1974) compared three immuno-peroxidase techniques (direct, indirect and peroxidase anti-peroxidase) for the detection of foot and mouth disease virus antigens in infected cell cultures. They found that each technique was simple and efficient in detecting FMD virus and its virus infection associated antigen in infected cells.

The indirect immunoperoxidase test with horse radish peroxidase labelled goat anti rabbit globulin was found to be a specific, sensitive, simple and rapid technique for the localization and identification of Porcine entero viral (PEV) antigens in infected PK 15 cell cultures. An antigenic relationship between type I and type II CPE strains of PEV was also recognised by this test (Sulochana and Derbyshire, 1978).

Immunoperoxidase staining has been used for the detection of virus antigen in several herpes virus infections.

Matsuoka *et al.* (1987) demonstrated the Aujeszky's diseases virus (ADV) antigen in formalin fixed and paraffin embedded brain tissue sections. They opined that immunoperoxidase staining along with routine histologic procedure was effective in the diagnosis of ADV.

Guy *et al.* (1992) developed indirect immunoperoxidase (IP) test for detection of ILT viral antigen in frozen tissue sections. They compared IP with an indirect immunofluorescent antibody (IFA) test, histopathology and virus isolation for detection of ILT virus in the tracheas of experimentally infected chickens. In this study the sensitivity and specificity of IP was 72 and 93 per cent and FA 53 and 90 per cent respectively. Histopathological detection of ILT virus infection was highly specific (98%) but sensitivity was poor (42%).

Islam *et al.* (1993) successfully adapted avidin-biotin-peroxidase method of IPT for detection of duck plague virus antigen in formalin fixed paraffin embedded tissue sections of the liver and spleen of experimentally infected domestic ducks. It was observed that the positive reactions were localized mostly in the nucleus and less frequently in the

cytoplasm of few hepatocytes and kuffer cells of the liver and lymphocytes and reticular cells of the spleen.

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

Ogino *et al.* (1996) demonstrated IBRT virus antigen by ABC method of IPT in tissues of aborted bovine fetuses, which had been stored for 25 years after fixing in formalin and embedding in paraffin.

Materials and Methods

3. MATERIALS AND METHODS

3.1 Glassware and reagents

In this study Borosil brand of glassware, laxbro plastics and analytical or guaranteed reagent grade of chemicals were only used.

The materials were processed using standard methods (Hoskins, 1967) and sterilized either in hot air oven or in autoclave depending upon the material to be sterilized.

3.2 Collection of blood and separation of serum

Blood samples were collected in sterile containers from healthy ducks, either by cardiac puncture or from saphenous vein and pooled.

The blood was then allowed to stand for half to one hour at room temperature for clot formation, in a slanting position. Subsequently the clot was carefully dislodged from the wall of the flasks with sterile rod and allowed to stand at 37°C for one hour and transferred to the refrigerator overnight for serum separation. The next day the separated serum further clarified by centrifugation at 2000 rpm for 5 mt, transferred to sterile 30 ml glass or plastic vials, and stored at -20°C.

3.3 Production of antiduck whole serum

Three healthy rabbits weighing 1-1 1/2 kg, procured from the small animal breeding station (SABS), Mannuthy, K.A.U. were used for this purpose.

The animals were immunized by the following schedule.

One ml of whole duck serum having a protein concentration of 60 mg per ml was homogenized with one ml (1:1) of freund's complete adjuvant and one ml of this emulsion was given intramuscularly to each rabbit. Three booster doses of 0.5 ml each, without adjuvants were given at weekly intervals by the same route.

Ten days following the last injection, test bleeding was done to assess the antibody response by AGPT and immuno-electrophoresis. When the results were found satisfactory, the animals were bled, the serum separated, and stored at -50°C in small quantities to be used as antiduck serum.

3.3.1 AGAR GEL precipitation test (AGPT)

AGPT was done as per the method described by Vengadabady (1995) with slight modifications.

3.3.1.a Reagents

Agarose	-	0.8 gm
Sodium chloride	-	0.8 gm
Phenol	-	one drop
Distilled water	-	100 ml

Staining solutions

Amidoblack 10 B	-	1 gm
Sodium chloride	-	8.5 gm
Distilled water	-	1000 ml

Decolouriser-I

Methanol	-	40 parts
Acetic acid	-	10 parts
Distilled water	-	10 parts

Decolouriser-II

Absolute alcohol	-	35 parts
Acetic acid	-	5 parts
Distilled water	-	10 parts

3.3.1.b Test proper

The melted agarose was poured on to glass slides and wells were cut in a three well pattern.

The central well was loaded with antiduck whole serum raised in rabbit and the peripheral wells were loaded with duck gamma globulins and duck whole serum respectively. After charging, the slides were kept in a humid chamber at room temperature for 48 hr and examined against light for the development of any precipitin line.

3.3.1.c Staining

The slids were washed after soaking in two changes of normal saline for 24 hr each and then in distilled water for further 24 hr to remove unreacted proteins. The slides were dried slowly, stained with amidoblack for 15 mts, and decolourised in solutions I and II for 20 min each. The slides were dried at 37°C for 1 hr and mounted in DPX.

3.3.2 Immunoelectrophoresis

It was done as per the method described by Nair (1990).

3.3.2.a Reagents

1. Tris-barbital buffer

Barbitone sodium	-	9.9 gm
Tris (hydroxy methyl amino methane)	-	17.7 gm
Sodium azide	-	0.3 gm

Distilled water - 2000 ml

pH adjusted to 8.6 with 1N HCl.

2. Agar coated slides

Clean microscopic slides (2.5 x 7.5 cm) were dipped in one per cent melted agar in distilled water and dried in air by keeping the slides horizontally over glass rods. Dried slides were stored at room temperature until used.

3. Melted agarose

Agarose (0.8 gm) was boiled in 100 ml tris-barbital buffer until the agar was dissolved completely and then stored at room temperature until used.

4. Duck serum samples

5. Antiduck serum raised in rabbits

3.3.2.b Test proper

The 0.8 per cent agarose in tris-barbiturate buffer was melted and 3 ml of agarose at about 50°C was poured onto each slide kept on a levelled surface. The agarose was allowed to solidify initially at room temperature and subsequently at 4°C. Wells and troughs in between the wells were cut on each slide. The wells were always cut towards one end of the slide. After removing the agarose, the wells were filled with

antigens. A drop of bromophenol blue dye was added to the side of the well as an indicator. The slides were then placed in the electrophoresis chamber in such a way that the antigen wells were nearer to the cathode than to anode. Contact between the slides and the buffer was effected by filter paper wicks one on each end of the slide so that each covered about 1/2 cm of the agarose on either side of the slide. Power supply at the rate of 3 mA per slide was given and the electrophoresis was continued till the indicator dye reached 1 cm away from the anode end of the slide.

The power supply was disconnected, slides were taken and the agarose in the troughs were removed carefully. The troughs were then filled with the respective antisera (Antiduck whole serum) and left at room temperature in the electrophoretic chamber itself for 20-24 hrs.

The slides were examined against a light for the development of precipitin arcs and then the slides were washed and stained as for AGPT.

3.4 Separation of duck gamma globulins

Gamma globulins from the duck serum was separated using the procedure described by Garvey (1977).

3.4.1 Reagents

Saturated ammonium sulphate (SAS) solution:

SAS was prepared by adding 760 g of ammonium sulphate to one litre of triple distilled water and heating to 50°C for 30 min in a water bath, with continuous stirring. It was filtered while still hot to remove insoluble impurities and then cooled to room temperature. The pH was adjusted to 7.0 with ammonium hydroxide solution just prior to use.

Working SAS solution:

Solution of 66 per cent and 80 per cent strength were prepared (v/v) freshly from the stock SAS.

2. Ammonium hydroxide solution
3. Physiological saline
4. Ten per cent barium chloride solution
5. Borate-buffered saline, pH 8.5

Five parts of borate buffer was added to ninety five parts of saline.

Borate buffer

Boric acid	-	6.184 g
Borax	-	9.536 g

NaCl	-	4.384 g
Distilled water	-	1000 ml

The above reagents were added to one litre volumetric flask containing 600-800 ml of distilled water and shaken until contents were completely dissolved. To this, distilled water was added to make the volume to one litre and the pH was adjusted to 8.5.

3.4.2 Method

Fifty ml of 66 or 80 per cent ASS was added dropwise to a 50 ml of serum sample while stirring. The stirring of serum ASS mixture was continued for 30 min after the addition of the last drop of ASS and the precipitate was allowed to stand overnight at 4°C. Next day the suspension was centrifuged in a refrigerated centrifuge at 3000 rpm for 30 min. The precipitate so obtained was dissolved in enough saline to restore the original volume of serum and reprecipitated twice following the above procedure, omitting the overnight keeping of the suspension at 4°C. The precipitate from the third precipitation was dissolved in borate buffered saline to a final volume of 20 ml. The ammonium sulphate was removed from the precipitate by dialysing against borate buffered saline at 4°C.

3.4.3 Estimation of globulins

Total protein and albumin kit (Qualigens fine chemicals) was used to estimate the globulins.

To obtain the required concentration of 10 to 15 mg/ml of protein the globulin was subjected to dialysing against heavy materials like polyvinyl pyrolidone (or) polyethylene glycol.

3.4.4 Purity of gamma globulins

The purity of gamma globulins was tested by Agar gel immunodiffusion test and immunoelectrophoresis as described earlier.

3.5 Preparation of antiduck gamma globulins

Antiduck gamma globulins was raised by the same procedure as for ADS in rabbits, using ammonium sulphate precipitated serum globulins dissolved in borate buffered saline and having an approximate protein concentration of 10 to 15 mg/ml. Six rabbits were used for this purpose.

Ten days following the last injection rabbits were bled, serum separated, pooled and tested by AGPT. Gamma globulin fraction was separated and used for HRPO conjugation after checking its purity and concentration.

3.6 Conjugation

The labelling of antiduck gamma globulins with horse radish peroxidase was done as per the procedure described by Avrameas's (1969) with slight modifications.

3.6.1 Reagents

1. 0.1 M potassium phosphate solution

   Potassium phosphate (KH_2PO_4) - 1.36 gm

   Distilled water - 100 ml

2. 1 per cent gluteraldehyde solution

   Gluteraldehyde (50% solution) - 0.2 ml

   Distilled water - 9.8 ml

3. Physiological saline, pH 7.4

3.6.2 Method

1. The antiduck gamma globulins was reconstituted with the borate buffer to obtain 5-7 mg of globulins/ml.

2. pH was adjusted to 6.9 by addition of 0.1 M solution of potassium phosphate.

3. For each ml of the above solution, 10-14 mg of HRPO enzyme added, after its complete dissolution, 0.05 ml of glutaraldehyde was added.

4. The mixture was shaken for 2 hr at room temperature by end-over-end rotation.

5. The product thus obtained was then dialysed overnight at 4°C against physiological saline, pH 7.4.

6. Next day the solution was centrifuged for 15 min at 1500-2000 g.

7. The supernatent was collected and stored in small aliquot at -20°C.

3.7 Preparation of hyperimmune serum to duck plague virus

Live attenuated duck plague vaccine, obtained from the Institute of Animal Health and Veterinary Biologicals, Palode was used.

Three injections each of one ml duck plague vaccine virus having 3.5 $\log_{10}$ ELD_{50}/0.5 ml was given to 6 numbers of 8 week old healthy ducklings at weekly intervals. Ten days following last injection the birds were bled and the serum tested for the presence of antibodies.

The ducks were sacrificed, the serum was separated and subjected to heat inactivation at 56°C for 30 min. The serum

was distributed in small quantities and preserved at -50°C to be used as a positive control.

Sera from normal, non-infected and non-vaccinated birds were used as negative controls.

3.8 Serum neutralization test

Serial two fold dilutions of hyperimmune serum (1:2 to 1:128) were made in maintenance medium at pH 7.2. Equal quantity of 100 $TCID_{50}$ virus per 0.1 ml was added to each dilution and incubated at 37°C for 60 min for neutralization of virus. From each dilution 0.1 ml of serum virus mixture was inoculated into three CEF cell culture tubes and incubated at 38.5°C. The inoculated tubes were examined at 24 hr interval until the virus control tubes showed CPE. The neutralization titres were calculated as per the method described by Reed and Munch (1938). Suitable antigen controls and uninfected cell controls were also incorporated in this test.

3.9 Collection of samples

A total of 200 serum samples were collected from ducks of different localities and used for DP viral antibody detection. The sera were collected from

1. University Poultry Farm, Mannuthy
2. Government Duck Farm, Niranam
3. Private farms in Kuttanad area, Alleppy
4. Slaughter house, Mannuthy

Tissue samples, 35 liver and 30 spleen were collected in 10 per cent buffered formalin from birds suspected to have died of duck plague in Niranam, Alleppy, Chenam, Mannuthy and also from those ducks brought for post-mortem examination in the Department of Pathology.

3.10 Detection of duck plague viral antibodies

3.10.1 Passive haemagglutination test

The test was carried out as per the method described by Kulkarni (1993) with slight modifications.

3.10.1.a Reagents

Sheep red blood cells (SRBC)

The blood was collected from healthy adult sheep in Alsever's solution in 1:1 (v/v) proportion and kept at 4°C. 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 Alsever's solution (1:4 v/v) and stored at 4°C upto two weeks.

3.10.1.b Method

1. Formalised tanned erythrocyte (FTE) preparation

Sheep erythrocytes collected in Alsever's solution and washed three times in phosphate buffered saline (pH 7.2) were made into a 10 per cent suspension in phosphate buffered saline (pH 7.2). An equal volume of 3 per cent formalised saline was added and the mixture was incubated at 37°C for 20 hours. These formalised erythrocytes were then washed three times in PBS, pH 7.2 and stored at 10 per cent concentration in PBS, pH 7.2 at 4°C until required for tanning.

Tannic acid dilutions (1:20000) in physiological saline were added to an equal volume of 10 per cent formalised erythrocytes in PBS (pH 7.2) and kept in variable temperatures, times and shaking frequencies.

2. Sensitisation of FTE by antigen

Three volumes of 1:35 diluted virus were mixed with two volumes of PBS (pH 6.4) and one volume of 10 per cent FTE at pH 7.2, in that order. The mixture was incubated at 37°C for 45 min, with and without agitation. The resulting antigen coated erythrocytes were washed three times in PBS, pH 7.2 and resuspended as a 2 per cent suspension in normal rabbit serum diluent.

3. Rabbit serum diluent preparation

Rabbit serum was heat inactivated at 56°C for 30 mts and adsorbed with packed formalised erythrocytes to remove serum haemagglutinins for sheep erythrocytes. The absorbed serum was then incorporated at two per cent concentration in physiological saline.

4. Treatment of test serums

All the sera samples to be tested were heat inactivated at 56°C for 30 mts, 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 were removed by centrifugation.

The tests were performed in microtitre plates by adapting following procedure.

Fifty microlitre of rabbit serum diluent was added to all wells. Fifty microlitre of test serum was added to first well, then serial two fold dilutions were made. Then fifty ul of antigen coated FTE was added to each well. The results were read after three hr incubation at 30°C in humid chamber.

Appropriate known negative, and positive controls were made.

3.10.2 Dot ELISA

3.10.2.a Materials

1. Duck plague virus antigen

Chicken embryo fibroplast (CEF) adapted duck plague vaccine strain at the fifth passage and having the virus titre of $10^{5.7}$ $TCID_{50}$ was used as a antigen.

2. Phosphate buffered saline (PBS) 10 x

Sodium chloride	-	80.0 gm
Disodium hydrogen phosphate	-	11.5 gm
Potassium chloride	-	2.0 gm
Potassium dihydrogen phosphate	-	2.0 gm
Distilled water to make	-	1000 ml

pH was adjusted to 7.2 and sterilised by autoclaving at 121°C for 15 min at 15 lb pressure.

3. PBS - Tween 20 (PBST) solution (pH 7.2)

PBS 10x solution	-	100 ml
Tween 20	-	500 μl
Distilled to make	-	1000 ml

pH was adjusted to 7.2.

4. Antiduck gamma globulins - horse radish peroxidase conjugate.

5. Substrate solution

Five milligram of 3-3-diamino benzidine tetrachloride (DAB) was dissolved in 10 ml of PBS-T solution to which 30 ul of 30 per cent hydrogen peroxide was added and used as substrate solution.

6. 5 per cent skim milk powder

Milk powder	- 5 gm
PBST to make	- 100 ml

3.10.2.b Test proper

The test was conducted as per the method described by Maity *et al.* (1993) with minor modifications.

One ul quantities of the duck plague viral antigen was dotted onto a nitrocellulose membrane (NC). After air drying the antigen was fixed to the membrane by baking at 80°C for 30 min. The membrane was then incubated at 37°C for 30 min in 5 per cent dried milk powder in PBS-T for blocking non-specific reaction. The membrane was then washed thrice in PBS-T for 10 min and incubated with lin 10 dilution of duck

plague hyperimmune serum/field samples/known negative samples at 37°C for 45 min and washed thrice in PBS-T. After washing, the membrane was treated with 1:100 diluted labelled antiduck gamma globulins and incubated at 37°C for 30 min. The NC membrane was then washed thrice in PBS-T and treated with the freshly prepared substrate solution. After 3-5 mts, the reaction was stopped by rinsing the membrane in tap water. Positive reaction was evidenced by the appearance of brown dots.

3.10.3 Plate ELISA

3.10.3.a Reagents

1. Duck plague virus: Fifth passage, chicken embryo fibroblast (CEF) adapted DP vaccine strain having the virus titre of $10^{5.7}TCID_{50}$ was used as the antigen. The dilution 1in 10 was determined by checkerboard titration.

2. ELISA Plates: ELISA plates with 96 U bottomed wells (Tarson Pvt. Ltd.) were used.

3. 2% Bovine serum albumin (BSA)

   BSA - 2 gm

   PBST - 100 ml

4. PBST, pH 7.2

PBS 10 x	- 100 ml
Tween 20	- 500 μl
Distilled water to make upto	- 1 liter

pH was adjusted to pH 7.2

5. Carbonate - bicarbonate buffer, pH 9.6

Sodium carbonate (Na_2CO_3)	- 1.59 gm
Sodium bicarbonate ($NaHCo_3$)	- 2.93 gm
Distilled water	- 1 litre

pH was adjusted to pH 9.6

6. Sodium citrate buffer, 0.05 m, pH 4.2

Sodium citrate	- 14.71 gm
Distilled water	- 1 litre

pH was adjusted to 4.2

7. Substrate solution

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

8. Hydrofluiric acid

0.1 M hydrofluiric acid

3.10.3.b Method

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

Optimum concentrations of coating antigen, serum samples and HRP conjugates were arrived at by preliminary checker board titration.

Each well of the ELISA plate was coated with 100 µl of lin 10 dilution of antigen in ELISA coating buffer, pH 9.6 and kept at 4°C overnight. The plate was then washed by emptying, filling with PBS-T from a wash bottle and leaving for three minutes. This process was repeated three times, and blocked with 2 per cent BSA for 60 min at 37°C. The wells were washed as above and incubated with two fold diluted sera samples (lin 80 to lin 2560) collected from ducks, in different rows of wells at 37°C for one hour. The wells were again washed with PBST and incubated with antiduck globulin peroxidase conjugate (lin 1000 dilution) at 37°C for 1 hour. The wells were washed with PBS-T and 100 µl of ABTS substrate was added and incubated at room temperature in dark for 30 min for the development of color reaction. Hundred µl 0.1 M hydrofluiric

acid was added to all the wells to stop the reaction and the plates were read in a multiskan ELISA reader at 405 nm. Substrate control well was blanked to read the samples.

The sera samples of control ducks were taken as negative control.

The optical density (OD) which is nearest to the sum of mean of the negative control and single standard error was taken as the titre of the particular sample.

3.10.4 Filter paper strip method

The method described by Banks (1985) was followed with slight modifications.

3.10.4.a Preparation of filter paper strip

Filter paper strips were made by the method described by MaxBrugh and Beard (1980).

Whatman No.1 filter paper was cut into strips approximately 1.3 x 10.0 cm and 3 strips were overlapped in the middle and stapled together (Plate 7). This cluster of 3 strips was used to collect 6 samples because blood was collected on both ends of each strip.

The paper strip sample was collected from the small pool of blood formed on the wing surface after puncture of a wing vein with a hypodermic needle. The distal 1.0 to 2.0 cm of the strip was saturated with blood, and complete saturation was evidenced by equal blood staining on both surfaces.

The end of each strip was folded up slightly after saturation with blood to give each cluster of 6 samples a concave shape. The cluster of samples was placed with the concave side up on flat surface for temporary storage and drying. In the laboratory they were dried at 37°C for 2 hour, sealed in plastic bags and stored at 4°C.

3.10.4.b Elution of dried blood

Filter paper pieces with blood samples were put in separate test tubes containing 0.5 ml of phosphate buffered saline, pH 7.2 and kept for 10 minutes. They were taken out leaving a straw coloured elute.

3.10.4.c Test proper

Eluted sera samples were used as source of antibodies instead of serum for their detection by ELISA-test as per the method described for indirect ELISA using conventional serum samples.

3.11 Detection of duck plague viral antigen

3.11.1 Indirect immunoperoxidase test

3.11.1.a Reagents

PBS, pH 7.4

PBS 10x —— 100 ml

Distilled water to make —— 1000 ml

pH was adjusted to pH 7.4

2. Tris-Hcl, pH 7.6

A. 0.2 M Tris (2.42 g/100 ml)

B. 0.2 N HCl

50 ml of A + 38.4 ml of B

to make up to 200 ml, add distilled water.

3. 0.25% Trypsin

Trypsin (1:250) —— 0.25 gm

Distilled water —— 100 ml

4. Substrate solution

0.05 per cent 3, 3 diaminobenzidine (DAB) was made by 5 mg DAB was dissolved in 10 ml Tris-HCl buffer, and 30 ul of 33 per cent H_2O_2 (0.01% H_2O_2) was added.

3.11.1.b Method

Tissues (liver and spleen) fixed in 10 per cent buffered formalin were subjected to paraffin embedding and the sections of 4 to 6 micrometer thickness were made and mounted onto the slide. Sections were deparaffinised and subjected to 0.25 per cent trypsin treatment for one hour. Then the sections were treated with hyperimmune serum (1 in 10 dilution) incubated at 37°C for 45 min in a humid chamber.

The slides were then washed in PBS twice for 5 min and kept in methanol bath containing 0.5% H_2O_2 at room temperature for 30 min, for removing the endogenous peroxidase. These sections were washed in PBS and treated with HRPO labelled antiduck gamma globulins (1:100) for 45 min at 37°C. Then the excess conjugate was removed by washing in PBS. Freshly prepared substrate solution (DAB) in Tris-HCl buffer in the presence of 0.01% H_2O_2 was added and kept for 2-5 min at room temperature.

Finally the slides were washed in buffer and dehydrated through a graded series of ethanol, followed by xylol and mounted in DPX mountant/50% glycerol phosphate buffer saline.

The slides were examined under a light microscope, and the peroxidase activity was observed as dark brown precipitate.

Results

4. RESULTS

4.1 Preparation of antiduck whole serum

Antisera to whole serum of ducks were raised in rabbits and the antiduck serum was tested against duck serum by immunoelectrophoresis. The duck serum produced 9 precipitation arcs against antiduck serum (Plate 1).

The high molecular weight slow moving globulin fraction was seen close to the antigen well slightly towards the cathode. This globulin line was very marked as in the case of the fast moving albumin fraction.

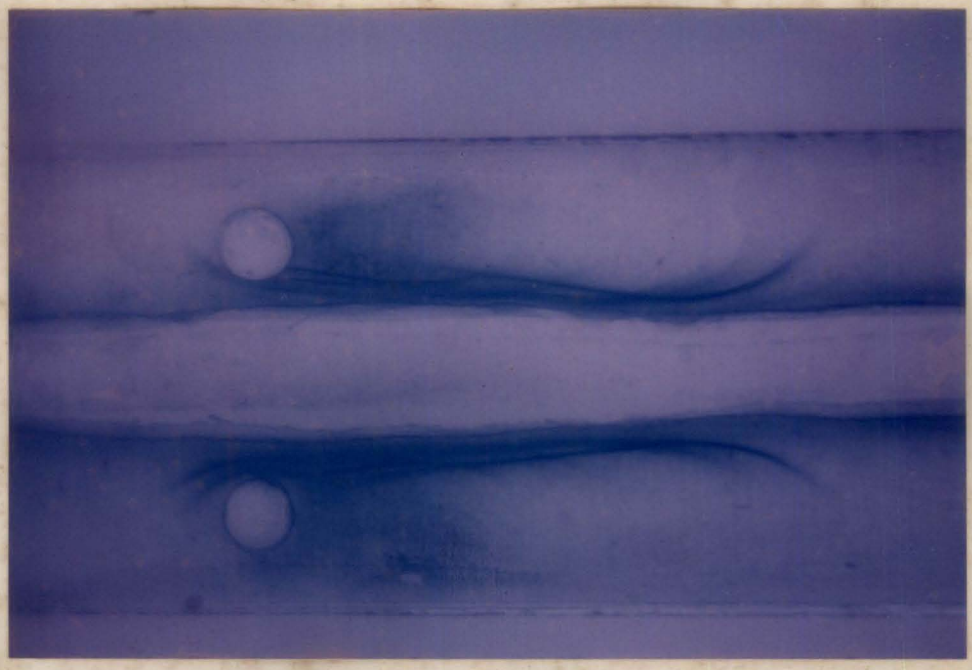
4.2 Separation of duck gamma globulins

The protein concentration of pooled serum was 82 mg/ml, and the protein concentration of ammonium sulphate precipitated globulin was 34 mg/ml. It was reconstituted with borate buffer to the required concentration of 10 to 15 mg/ml.

The purity of the globulin fraction separated from the whole serum was tested by Agar gel precipitation test (AGPT) and Immunoelectrophoresis.

In AGPT the separated globulin produced three precipitin lines. One of these, was very sharp and close to the antigen

Plate 1. **Immunoelectrophoresis**
(Duck serum against antiduck serum)



well. Of the remaining two, one was faint but sharp while the other one was diffuse in nature (Plate 2a).

Immunoelectrophorogram of the separated globulin revealed one sharp precipitin arc between the antigen well and antiserum trough extending towards anode. Two additional arcs were also visible but very faint parallel to the first sharp line (Plate 3a).

4.3 Antiduck gamma globulin

Antisera to the separated gamma globulins were raised in rabbits and was tested by AGPT. The antiduck globulin so prepared was separated by precipitation with 33 per cent ammonium sulphate. The concentration of antiduck globulin was 15 mg/ml.

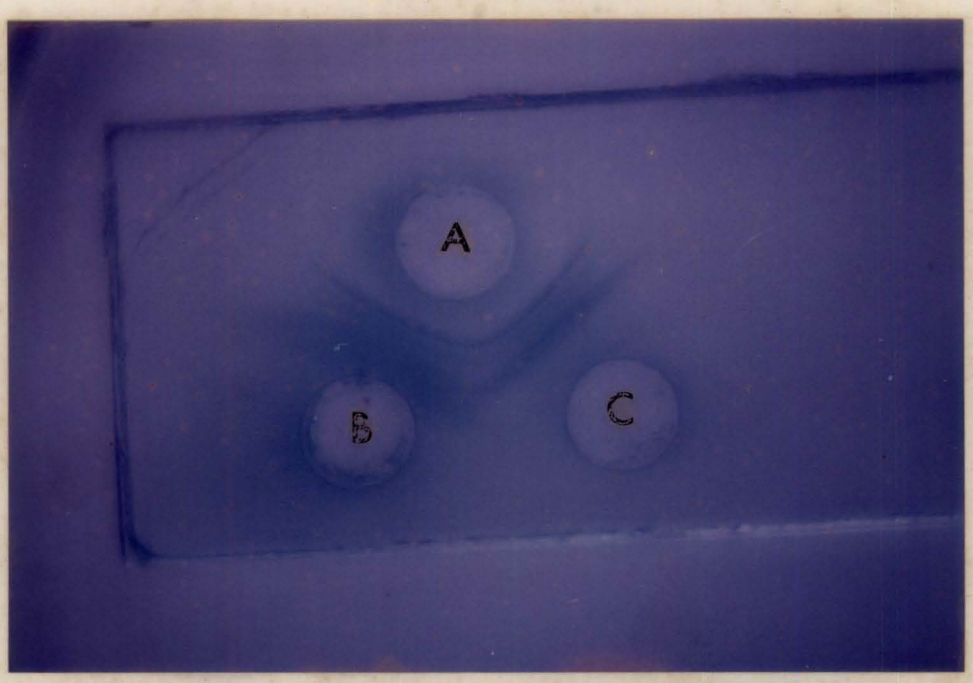
AGPT of antiduck globulin against duck globulin produced one thick and sharp precipitin line, close to antigen well and two faint lines nearer to the antiserum well. When tested against duck serum produced one sharp line and two additional lines, one was sharp but faint while the third one was diffuse in nature (Plate 2b).

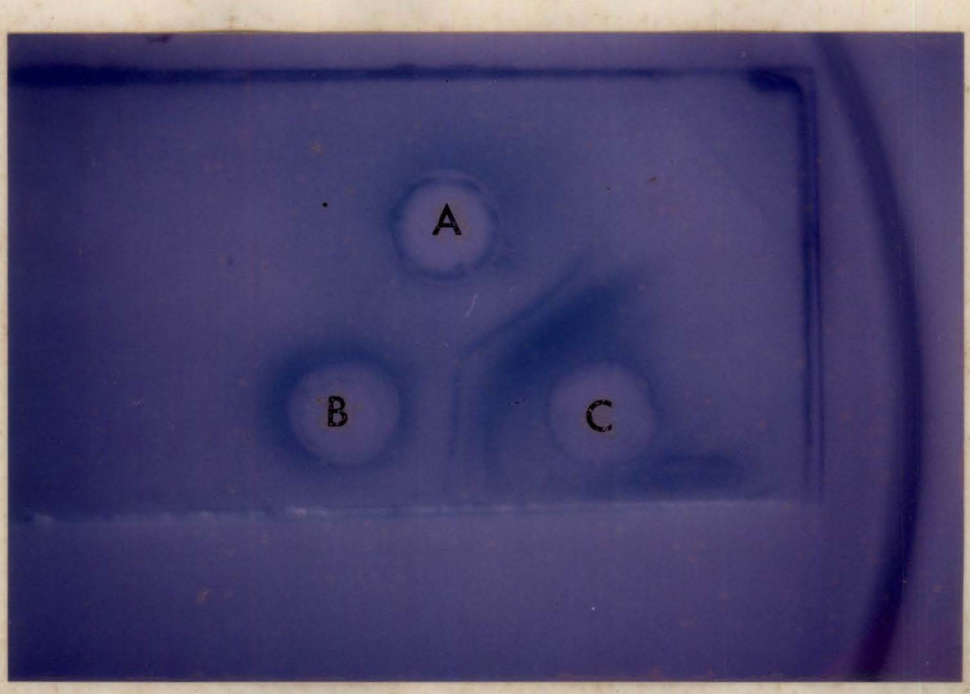
On immunoelectrophoresis, three precipitin arcs were obtained. Two of them were between the antigen well and antiserum trough. The third was extending towards the anode. (Plate 3b).

2a. Agar gel precipitation test

A. Duck globulin
B. Antiduck serum
C. Antiduck globulin

b. A. Duck serum
B. Duck globulin
C. Antiduck globulin

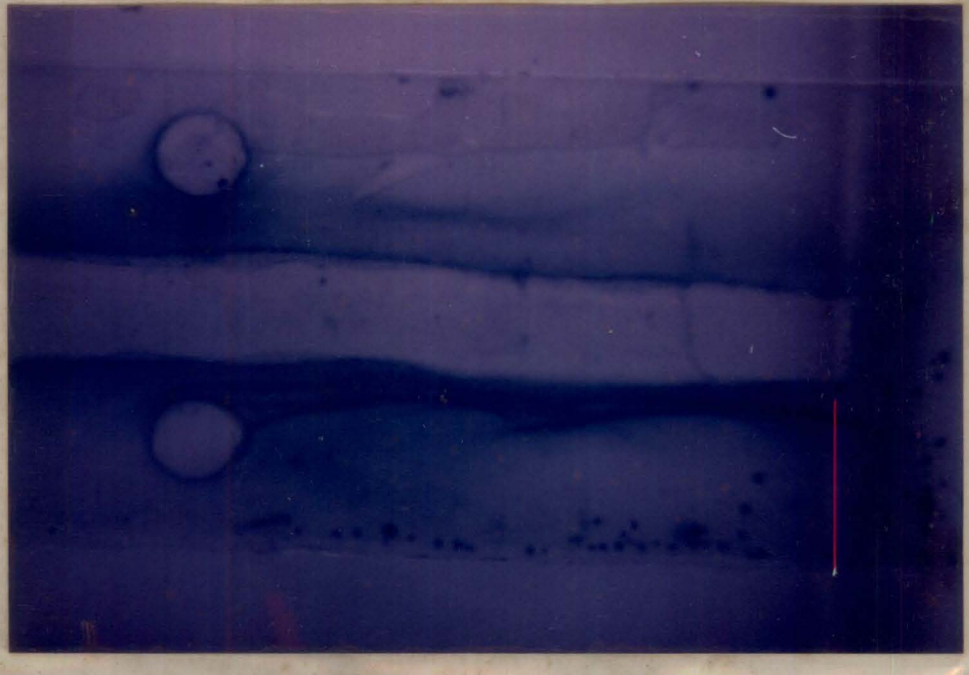

A
B
C


A
B
C

3. **Immunoelectrophoresis**

a. **Duck globulin against antiduck serum**

b. **Antiduck globulin against duck serum**



4.4 Conjugation

The protein concentration of the antiduck globulin was adjusted to 5-6 mg/ml before conjugation. The conjugated globulins was used for IPT, dot ELISA and plate ELISA.

4.5 Hyperimmune serum to duck plague virus

The hyperimmune serum to DPV was raised in ducklings using attenuated DPV having the virus titre of 3.5 $\log_{10}$ ELD_{50}/0.5 ml. The serum was assayed by passive haemagglutination and serum neutralization test. The titres were lin 32 and lin 64 respectively.

4.6 Passive haemagglutination test (PHA)

All the 200 samples collected from field cases were screened by PHA for DP viral antibodies. Agglutination of antigen coated FTE was read within three hours. Only samples giving titres 1 in 8 and above were taken as positive (Plate 4). Out of 200 samples tested, 128 samples were found to be positive. The titers ranged between 1 in 8 to 1 in 64. The percentage of positivity by PHA was 64. The sensitivity and specificity of PHA were 71.87 per cent and 38.88 per cent respectively (Table 1).

4. Passive Haemagglutination

A,B, D to H (1 to 7) row	———	Test serum samples (Serially diluted from 1:2 to 1:128)
C row	———	Positive serum contro (Serially diluted from 1:2 to 1:128)
B row (10 to 12)	———	Antigen control
C row (10 to 12)	———	Serum control

5. Dot ELISA

A. Positive control

B. Negative control


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

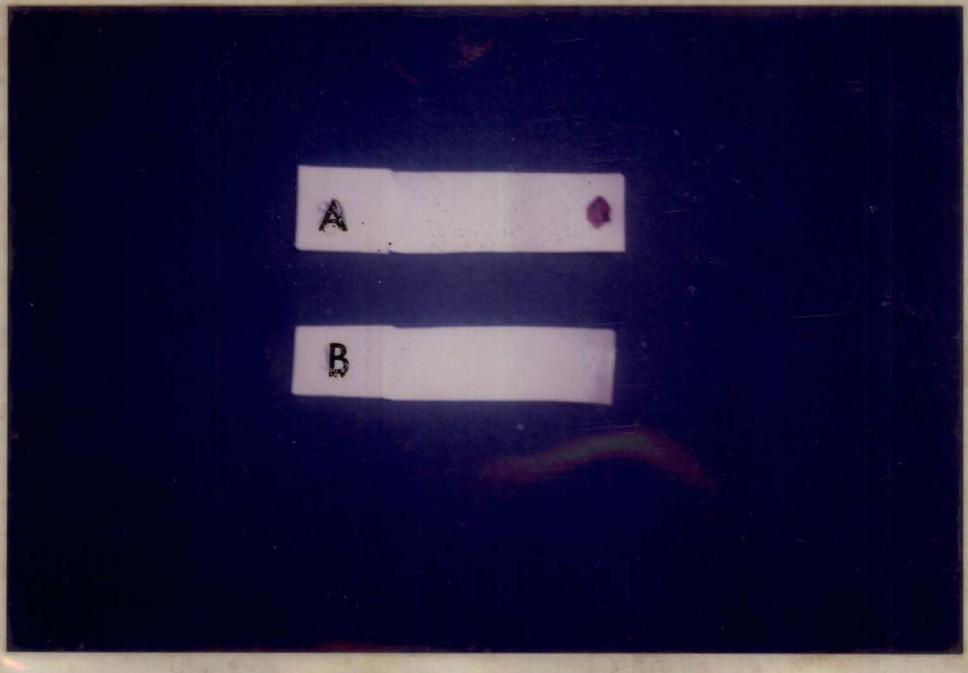

A
B

Table 1. Comparative efficacy of DOT-ELISA and PHA

Test		DOT-ELISA		Total
		Positive	Negative	
PHA	Positive	92	36	128
	Negative	44	28	72
Total		136	64	200

$X^2 = 2.45^{**}$

** - High significant association ($P<0.05$)

Concordance - 60%

DOT-ELISA

Sensitivity - 67.64%
Specificity - 43.75%

PHA

Sensitivity - 71.87%
Specificity - 38.88%

4.7 Dot-ELISA

The samples screened by PHA were subjected to Dot-ELISA. Out of 200 samples tested 136 were found to be positive which was indicated by the development of a brown dot at the site of application of the sample on the nitrocellulose membrane (Plate 5). The percentage of positivity by Dot-ELISA was 68. The sensitivity, specificity and concordance of Dot-ELISA with PHA are furnished in Table 1. The sensitivity and specificity of Dot-ELISA were 67.64 per cent and 43.75 per cent respectively.

4.8 Plate ELISA

The samples screened by DOT-ELISA and PHA were subjected to Plate ELISA. Only samples giving titres lin 160 and above were taken as positive (Plate 6). Out of 200 samples tested, 145 samples were found to be positive. The titres ranged between lin 160 to lin 2560. The percentage of positivity by plate ELISA was 72.5 per cent. The sensitivity and specificity of plate ELISA were 77.24 per cent and 70.90 per cent respectively (Table 2).

4.9 Filter paper strip method

Two hundred whole blood samples dried on filter paper were correspondingly evaluated against whole serum samples using PHA and plate ELISA for the detection of antibodies.

Table 2. Comparative efficacy of plate ELISA and PHA

Test		Plate-ELISA		Total
		Positive	Negative	
PHA	Positive	112	16	128
	Negative	33	39	72
Total		145	55	200

$X^2 = 38.06^{**}$

** - High significant difference (P>0.05)

Concordance - 75.5%

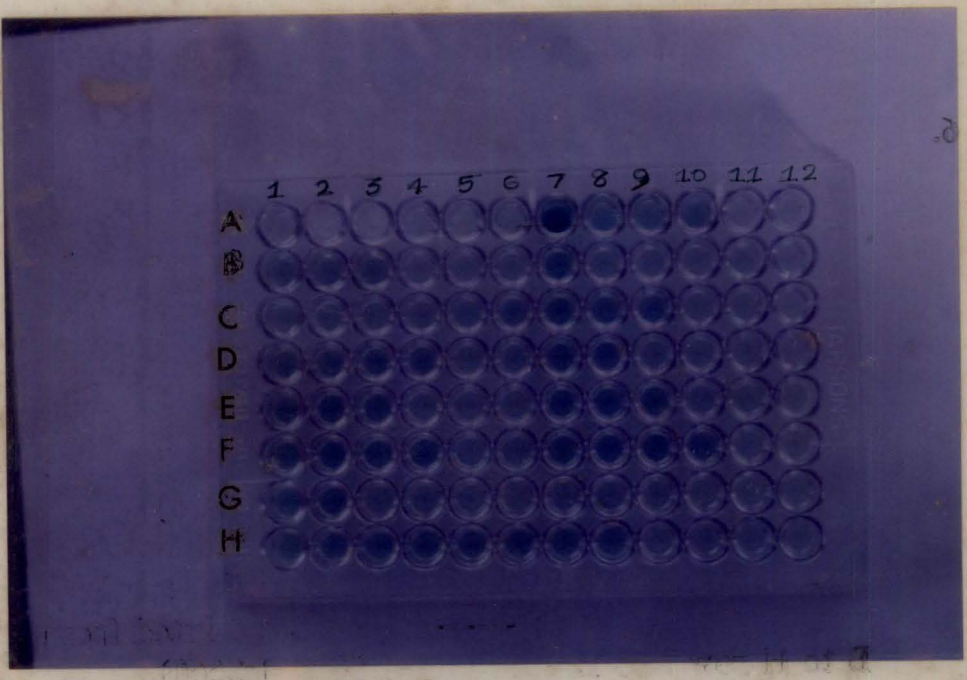
Plate-ELISA

Sensitivity - 77.24%
Specificity - 70.90%

6. Plate ELISA

A row 1	———	Substrate control
2,3	———	HRPO control
4 to 6	———	Negative control
7 to 12	———	Positive serum control (Serially diluted from 1:80 to 1:2560)
B to H row 1 to 6	¦	Test serum samples (Serially diluted from 1:80 to 1:2560)
B to H row 7 to 12	¦ ——	

7. Filter paper strips
(Method of blood collection)


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

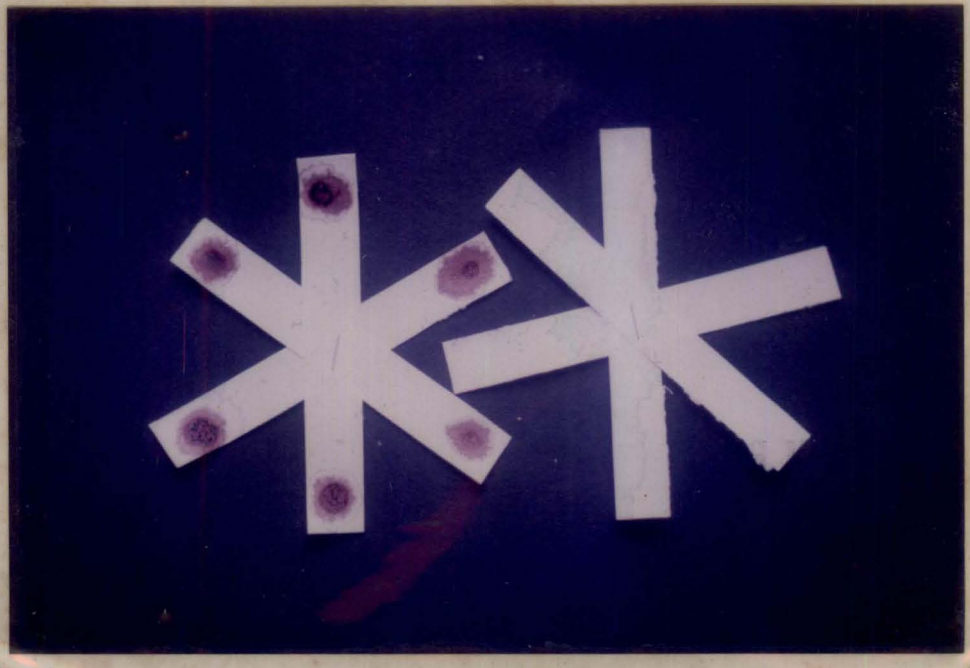


Table 3. Comparative efficacy of filter paper strip method and PHA

Test		Filter paper strip method		Total
		Positive	Negative	
PHA	Positive	109	19	128
	Negative	32	40	72
Total		141	59	200

$X^2 = 34.79^{**}$

** - High significant difference (P>0.05)

Sensitivity - 77.30%
Specificity - 67.79%
Concordance - 74.5%

Table 4. Comparative efficacy of Plate ELISA and filter paper strip method

Test		Plate ELISA		Total
		Positive	Negative	
Filter paper strip method	Positive	111	30	141
	Negative	34	25	59
Total		145	55	200

X^2 = 7.289**

** - High significant association (P<0.05)

Concordance - 68%

Plate ELISA	Filter paper strip method
Sensitivity - 76.55%	Sensitivity - 78.72%
Specificity - 45.45%	Specificity - 42.37%

Out of these, 141 samples were found to be positive. The percentage of positivity was 70.5 per cent. The sensitivity and specificity of filter paper strip method were 77.30 per cent and 67.79 per cent (Table 3).

The elute corresponds to 1:100 dilution of the serum. Thus, the samples were serially diluted from 1 in 40 to 1 in 1280. Only samples giving titres 1 in 40 and above were taken as positive. The sensitivity sepcificity and concordance of filter paper strip method with PHA and plate ELISA were furnished in Table 3, Table 4 respectively.

4.10 Immunoperoxidase test (IPT)

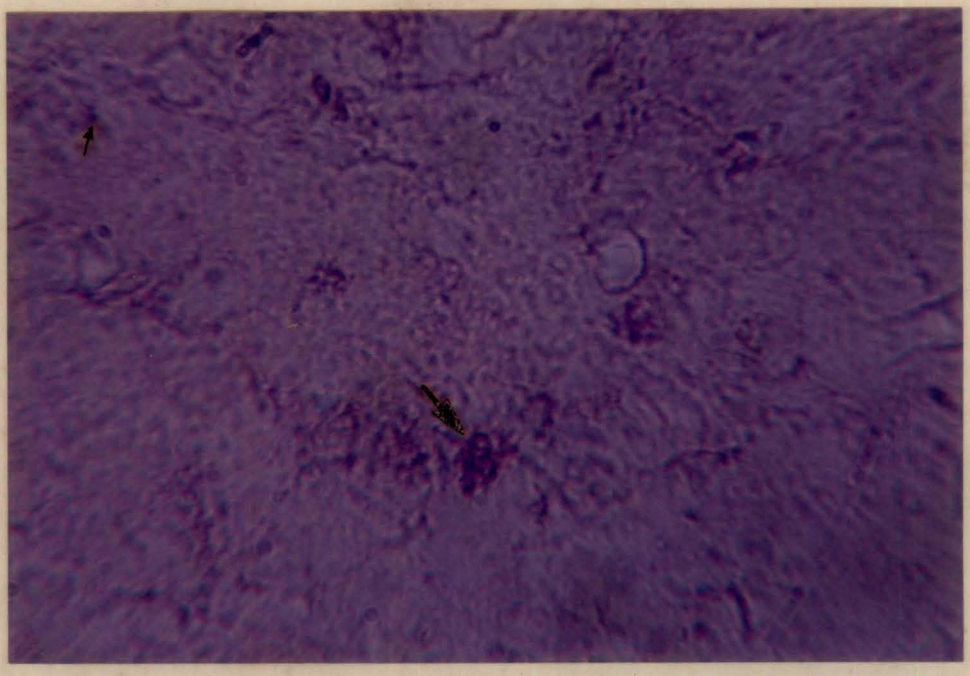
Thirty five liver and 30 spleen sections were tested for DPV antigens by IPT. These sections were from formalin fixed, paraffin embedded tissues originating from DP affected birds from field outbreaks.

Sections showing dark brown precipitate either in the cytoplasm or nucleus of the cells (Plate 8a, b) were considered as positive for DP viral antigens. Out of the 51 liver sections 31 (85%) was positive. While only 24 out of 42 (80%) spleen revealed positive reaction (Table 5).

8. Immunoperoxidase test

a. Liver

b. Spleen



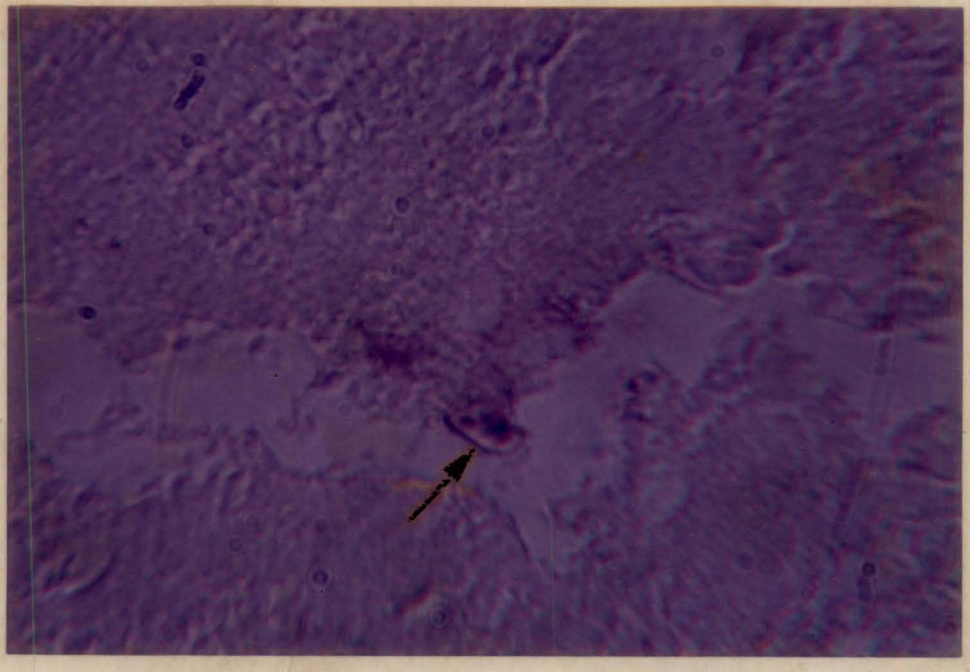


Table 5. Results of various tests in the detection of antibodies to DPV and DPV antigen

Test	Positive		Negative	
	No.	%	No.	%
PHA[a]	128	(64.0)	72	(36.0)
Dot-ELISA[a]	136	(68.0)	64	(32.0)
Plate-ELISA[a]	145	(72.5)	55	(27.5)
Filter paper strip method[a]	141	(70.5)	59	(29.5)
IPT				
Liver[b]	30	(85.7)	5	(14.3)
Spleen[c]	24	(80.0)	6	(20.0)

a. n = 200
b. n = 35
c. n = 30

Discussion

5. DISCUSSION

Diagnosis of field outbreaks of DP has been traditionally based on the clinical findings, PM lesions and isolation and identification of the virus which is laborious and time consuming. Because DVE produces heavy economic loss, development of a rapid and sensitive method for diagnosis of duck plague is warranted. In the present study such an attempt was made by standardizing enzyme immuno assays for the diagnosis of DP under various conditions.

5.1 Antiduck whole serum

Antiduck serum produced in rabbits revealed 9 precipitin arcs when it was subjected to IEP and AGID against homologous antigen. In a similar study Nair (1990) observed 13 precipitin arcs.

5.2 Duck gammaglobulins

Pooled duck serum having a protein concentration of 82 mg/ml was subjected to 33 per cent ASS precipitation for separation of duck gamma globulins. The protein concentration of the precipitated globulin was 34 mg/ml.

Nair (1990) reported a protein concentration of 16.88 mg/ml at 33 per cent ASS and 59.69 mg/ml at 40 per cent ASS

precipitin. Based on IEP analysis he opined that 33 per cent ASS precipitin is superior to 40 per cent ASS. He had reported six precipitin arcs for the separated gamma globulins when tested against rabbit antiduck serum. In the present study, the separated globulin produced one bold and two faint arcs on IEP and three precipitin lines by AGPT.

The higher concentration of globulins in the present study could be due to the high concentration of protein in the sera used for separation. The less number of precipitin arcs in IEP and precipitin lines in AGID indicate that the globulin fraction in this study was more pure compared to the preparation of Nair (1990) who had reported 6 arcs by IEP.

5.3 Anti-duck gamma globulins

Duck gamma globulins having the protein concentration of 10 to 15 mg/ml was used to prepare antiduck globulin in rabbits. The antiduck globulin was then precipitated as before and the purity checked by IEP and AGPT. On IEP analysis the separated globulins produced three precipitin arcs and in AGID one thick and two faint precipitin lines.

Engvall (1980) reported that if the antiserum was raised in rabbits, the most convenient purification procedure for gamma globulins is affinity chromatography on protein A sepharose.

5.4 Conjugation

In the present study, for conjugation, the protein concentration of antiduck globulin was adjusted to 6 to 7 mg/ml and the method described by Avrameas (1969) was followed with slight modifications. The conjugate was then stored in small aliquots at 4°C.

Voller *et al.* (1976) reported that peroxidase conjugate stored in a lyophilized state had retained its full, activity for one and half years. However, they opined that this technique of storage of conjugate leads to wastage of some conjugate as it is usually stored in larger amounts than required at a time.

In this study the enzyme Horse radish peroxidase, (SRL, Bombay) was used for conjugation. Voller *et al.* (1976) suggested that the enzyme to be used for conjugation should be stable, highly reactive, cheap, readily available, safe, easy to prepare and detect. Several workers suggested peroxidase as a good choice for conjugation (Avrameas, 1969; Nakane and Pierce, 1966), because it has higher activity, cheaper than alkaline phosphatase, and yields a visible (brown) reaction products.

Engvall (1980) preferred purified antibodies for conjugation. He had opined that use of purified antibodies

results in more efficient conjugates than those prepared from whole IgG. More over it also saves enzyme.

Voller *et al.* (1976) reported that Ammonium sulphate precipitation of the serum provided adequate material for the enzyme labelling and affinity chromatography purified antibody gave slightly lower background reactions in indirect tests and the conjugate could be used at a higher dilution. It was also observed that Fab fragments derived from the immunoglobulin part of the antisera and labelled with the enzyme gave high affinity with a marked reduction in background staining in indirect ELISA systems.

5.5 Passive haemagglutination test (PHA)

PHA test is rapid, less cumbersome, accurate and economical when compared to neutralization test and is equally sensitive (Vengris and Marie, 1971; Zyambo *et al.*, 1973b). In this study, serum samples collected from ducks in different localities were subjected to PHA. The samples showing titres 1 in 8 and above were considered as positive. Out of the 200 samples screened, 128 (64%) samples were positive.

The sensitivity, specificity and concordance of PHA with Dot ELISA, Plate ELISA and filter paper method are furnished in Tables 2, 3 and 4 respectively. The sensitivity of this test was comparable to other tests. This test is useful in

detection of DPV antibodies. But unlike plate ELISA this test was not useful to detect low antibody titres.

5.6 Dot ELISA

Dot-enzyme linked immunosorbent assay (Dot ELISA) has become one of the most rapid simple and sensitive diagnostic tool for the detection of viral antigens and antibody, since its development by Pappas *et al.* (1983). In Dot ELISA,the antigen or antibody is dotted onto the nitro cellulose membranes. The antigen dotted membranes were sequentially treated with the blocking reagents, positive serum labelled antibody and the chromogenic substrate leading to the development of a coloured dot at the site of impregnation of antigen in positive cases (Towbin and Gordon, 1984). All the samples screened by PHA were tested by Dot-ELISA. Out of the 200 samples tested, 136 samples (68%) were found to be positive. The sensitivity, specificity and concordance of Dot-ELISA and PHA are furnished in Table 2. The estimated sensitivity was 67.64 per cent. On analysis, no significant difference was observed between Dot-ELISA and PHA. A high degree of association ($P<0.05$) was also noticed between Dot-ELISA and PHA.

The sensitivity of Dot-ELISA in the diagnosis of DPV antigen has been reported to be as high as 93.21 per cent

(Chandrika, 1996). Although there is no report on the use of Dot ELISA for the detection of DPV antibody, information regarding its use in the detection of other herpes virus is available (Pandita and Srivastava, 1995).

Pandita and Srivastava (1995) in a study observed that 48.5 per cent of the cases were positive in the detection of BHV-1 antibody by using Dot ELISA. In the present study, the positivity was 68 per cent and the sensitivity was 67.64 per cent.

Engvall and Perlmann (1971) reported that the purity of the antibody used to prepare conjugate can influence the sensitivity of the test. They opined that the greater the purity of antigen more the sensitivity and diagnostic specificity of the test. This could be one of the reason for the low sensitivity of the test, as in the present study the antigen and conjugate used were not very pure. However, it was higher than that was reported for BHV-1 (Pandita and Srivastava 1995).

It is also possible that use of milk powder as a blocking agent in this study could have affected the sensitivity of the test as reported by Maiti *et al.* (1993). These authors found that when milk powder was used as the blocking agent the colour reaction produced was slightly less intense. They attributed this to the hindrance caused by some milk protein

which can bind the antigen and mask the epitopes from the reaction of antibody or due to the non specific loss of protein from nitro cellulose membrane.

Compared to other tests, a positive reaction in Dot-ELISA can be read by the naked eye without difficulty. Dot ELISA also facilitates easy method of transport of specimens from remote corners by just dotting the suspected materials onto the nitrocellulose membrane. Moreover the antigen dotted strips can be stored in the dark for years without a significant loss of colour thus maintaining a permanent record of the results (Walton *et al.*, 1986). Hence, though the sensitivity was low to PHA, Dot ELISA can be used for detection of duck plague viral antibodies even in geographically remote areas.

5.7 Plate ELISA

ELISA technique is a welcome method for the serodiagnosis of several virus infections. But report on the use of this technique for detection of duck plague viral antibodies or antigen are scanty. In this study, ELISA was used to detect antibody to DPV. Bolton *et al.* (1981) reported that conditions of conjugate dilution, antigen concentration, substrate concentration and reaction time have significant effects on the ELISA test. In this study, 1 in 10 dilution of

antigen, 1 in 1000 dilution of conjugate with a reaction time of 30 min was found to give better results.

Out of the 200 serum samples screened, 145 samples (72.5%) showed positive reaction. On statistical analysis highly significant difference was observed between plate ELISA and PHA. The sensitivity, specificity and concordance of plate ELISA and PHA are furnished in Table 2. Concordance between these two test was 75.5 per cent. This agreement was higher than the results obtained by Vulcano *et al.* (1988) in detection of anibodies for equine rhino pneumonitis virus. He opined that, the ELISA test was most sensitive, with the greatest agreement with the serum neutralization test (70 per cent agreement for negative and positive results combined).

In this study compared to PHA, ELISA showed higer specificity. Singh *et al.* (1995) reported that ELISA was more sensitive and less specific compared to VNT and CFT in the detection of equine rhino pneumonitis virus antibodies. Fuchs *et al.* (1985) compared different serological tests for detection of ILT and reported that in qualitative assay micro SN test was slightly more sensitive than FAT and ELISA. However, all the three tests were equally sensitive in quantitative assay.

The titres obtained in ELISA were 20 to 40 fold higher than titres obtained by PHA. Similar observation was also made by York *et al.* (1983). He reported that the titres obtained by the ELISA test were usually 16 to 32 fold higher than those obtained by SNT.

In this study, compared to PHA, ELISA could detect DPV antibodies in some low titred sera. Edwards *et al.* (1986) also reported similar findings. In their studies in contrast to PHA and one hour neutralization test, ELISA could detect BHV-1 antibodies in low titred sera.

Lower sensitivity obtained with ELISA, in this study may be due to use of unpurified viral antigen. According to Bolton *et al.* (1981) when equilibrium density gradient purified IBR virus was used as antigen the sensitivity of ELISA was 1000 fold more than the serum neutralization plaque assay.

Due to the low titre of the virus in cell cultures/embryo materials and its association with cellular materials during purification, it was very difficult to get high titred purified virus for coating. Similar observations were also made by York *et al.* (1983) with ILT virus. To overcome this difficulty the authors suggested initial coating of microtitre plates with antibody prepared against purified ILT virus and subsequent coating with relatively crude virus preparation.

They have also tried treatment of serum or plate and changes in the composition of diluent, but was ineffective in increasing the sensitivity of ELISA.

Considering the fact that ELISA is simple, cheap, rapid, highly specific and can detect DPV antibodies even in low titred sera, the test can be recommended for diagnosis of DPV particularly in inapparent and atypical conditions and also to determine the immune status of vaccinated birds. However, the test needs further refinement with monoclonal antibodies and purified antigens to enhance the sensitivity of the test.

5.8 Filter paper strip method

Blood samples dried on filter paper strips or discs have been used in a variety of laboratory test including ELISA (Maxbrugh and Beard, 1980; Shibata *et al.*, 1988; Garg *et al.*, 1993). The elutes of whole blood samples dried on filter paper strip were subjected to Plate ELISA for detection of DPV antibodies. A 1 in 5 dilution of initial elute corresponds to 1 in 20 dilution of the whole serum samples, then the initial dilution was made at 1 in 40 and the samples showing titres 1 in 40 and above are considered as positive.

Out of the 200 samples tested, 141 samples (70.5%) showed positive reaction. the percentage of positive reaction with whole serum was 72.5. On statistical analysis no significant

difference was observed between the ELISA test done with whole serum or filter paper elutes. A high degree of association was also noticed between these two tests. Similar observations were also made by Lana *et al.* (1984). In their studies on comparison of whole blood dried on filter paper and serum for the detection of antibodies to avian infectious bronchitis virus. These authors reported a slightly higher sensitivity (78.01%) with filter paper elutes compared to serum ELISA.

According to Banks (1985) though rapidity and sensitivity were equal, the reproducibility of serum ELISA was slightly better than filter paper elute ELISA. Antibody detection by PHA in the filter paper elute had revealed 64 per cent positivity. This was significantly different ($P<0.05$) from that of ELISA. Sensitivity and specificity was also slightly lower in PHA.

From the results obtained, it is clear that there is no significant difference in the antibody titer of DPV in the whole serum and whole blood dried on filter paper strips. However, the latter had added advantages, such as simplicity, easiness, reliability and rapidity. Moreover no expertise or special equipment is required for blood collection and only a small quantity (0.1 ml) of blood is required. Collecting serum by this technique also avoid bacterial contamination and

problems in despatching the samples to the laboratory. Hence this method can be considered as an alternative to serum in ELISA test for detection of antibodies to DPV.

5.9 Immunoperoxidase test (IPT)

Immunoperoxidase staining (IPT) in which the antibodies are conjugated with the enzyme HRPO have been used as a rapid method for the identification and localization of viral antigens in infected tissues and cell cultures (Ledue *et al.*, 1969, Herrmann and Morse, 1973; Gerna *et al.*, 1976; Silamban, 1996). Though fluorescent antibody staining technique has been widely employed for the above purpose, IPT has certain characteristics which make it more acceptable than the former. Some of such advantages are:

(i) For conjugation an exact ratio of peroxidase/protein is not essential, since differences in the ratio do not markedly alter specificity and reactivity.

(ii) Preparation of conjugate is relatively easy.

(iii) The HRPO conjugated antibodies have the capacity to retain their enzymatic and immunologic activity.

(iv) The results can be easily interpreted.

(v) Preparations can be made permanent and examined in an ordinary light microscope

(vi) The test is sensitive and results are reproducible (Nakane and Pierce, 1966; 1967; Herrmann *et al.*, 1974)

(vii) Could retain the histological details of tissues

(viii Paraffin blocks as old as 25 years could be used for detection of antigens (Ogino *et al.*, 1996).

This test has been used for the diagnosis of several herpes virus infections (Pearson *et al.*, 1979, Guy *et al.*, 1992). Recently IPT has been developed for the detection of DPV antigen in paraffin embedded tissue sections (Islam *et al.*, 1993).

In the present study, an indirect immunoperoxidase test, in which the antigen was reacted first with whole serum containing antibody and then with antigammaglobulin conjugated with the enzyme HRPO was employed for the localization and identification of DPV antigens. The positive staining was characterised by the deposition of dark brown granular precipitates at the site of antigen localization. No such staining was observed in the control sections. Out of 35 liver sections 30 (85.7%) was positive, while only 24 out of 30 (80%) spleen samples revealed positive reaction.

The reaction was found mostly in the nucleus and also in the cytoplasm. This observation was similar to the findings of Islam and Khan (1995). Duck plague virus usually produces intranuclear inclusions, although intracytoplasmic epithelial inclusions also have been reported (Barr *et al.*, 1992).

Laboratory diagnosis of duck plague often requires virus isolation and identification either in developing duck embryos or in duck embryo fibroblasts. The chance of getting a positive isolation depends on various factors. Moreover, this is cumbersome and takes a minimum of 7 to 15 days. But a positive diagnosis can be made within 2 to 3 days time by IPT, if the specimens - liver and spleen - from suspected cases are despatched to the laboratory in formal saline.

Some amount of non specific reactions and background staining was observed during the present study. This could be due to the use of polyclonal antiserum.

Guy *et al.* (1992) opined that use of monoclonal antibody to the virus could not only increase the sensitivity but could reduce non specific reactions.

Though HRPO is reputed to produce equal sensitivity to FA test, Guy *et al.* (1992) reported that streptavidin peroxidase conjugate IP procedure gives greater sensitivity to commonly

used IP procedures including ABC and peroxidase antiperoxidase techniques.

From the observations made in the present study it was felt that IPT can be recommended for diagnosis of DPV. Refinement of the technique using monoclonal antibody to the virus will definitely enhance the sensitivity and specificity of this test. Moreover, this test can also provide an alternative procedure for those laboratories which lack ELISA facilities.

5.10 Conclusions

1. Satisfactory level of gammaglobulin separation was achieved from whole duck serum by 33 per cent Ammonium sulphate precipitation.

2. Dot ELISA has various advantages over the other tests (PHA and plate ELISA) and may be recommended for detection of antibodies to DPV even under field conditions. However this test has to be tried under field conditions before such be recommendation is made.

3. PHA for titration of DPV antibodies has been standardized. This test was unsuitable for detection of antibodies in low titred sera, as a significant number of samples (33/72) were positive by plate ELISA.

4. Of the three tests, Plate ELISA was more sensitive than dot ELISA and PHA.

5. A technique of blood collection on filter paper strip for titration of DP antibodies has been standardized.

6. The results of antibody titration in sera collected by the conventional and the filter paper techniques were comparable.

7. Indirect immunoperoxidase test in paraffin embedded liver and spleen was performed. Liver sections showed higher percentage of positive reactions compared to spleen. In this test background staining of tissue sections was minimum. This test can be recommended for detection of DP viral antigen.

Summary

SUMMARY

Enzyme immuno assays were evaluated in the detection of DP viral antibodies in the sera samples collected by the conventional method and by filter paper strip technique. It was also used to detect DP viral antigens in paraffin embedded tissues.

Pooled duck serum collected from adult ducks was used for preparation of antiduck serum in rabbits. Immuno-electrophoretic analysis of the whole duck serum with antiduck serum revealed 9 precipitin arcs.

Gammaglobulins from the whole serum was separated by precipitation with 33 per cent ammonium sulphate. The precipitated gamma globulins was tested for its purity by AGID and IEP against antiduck serum. This gamma-globulin containing 15 mg/ml of protein was used for production of antiduck gammaglobulins in rabbits. The antiduck gammaglobulins separated by 33 per cent ammonium sulphate precipitation, revealed 3 precipitin lines in AGPT and 3 precipitin arcs in IEP against duck gammaglobulins. One of the precipitin arcs in IEP was bold while the other two were faint. The concentration of antiduck gamma globulin was adjusted to 5-7 mg/ml for HRPO conjugation.

Live attenuated duck plague vaccine having a virus titre of 3.5 $\log_{10}$ ELD_{50}/0.5 ml was used to prepare hyperimmune serum to duck plague virus in ducklings and this serum was used as the positive control.

A total of 200 serum samples collected from different localities in Kerala were used for detection of DP viral antibodies. All the samples were screened by PHA, DOT ELISA and plate ELISA.

For PHA sheep red blood cells treated with formalin and tannic acid were used for coating the antigen. Out of the 200 samples tested 128 (64%) were found to be positive. A high degree of association was seen between PHA and DOT-ELISA. When compared to plate ELISA the PHA was unsuitable for detection of antibodies in low titered sera.

Dot ELISA was standardized to detect DPV antibodies. One hundred and thirty six samples (68%) was found to be positive. The sensitivity of this test was 67.64 per cent. On statistical analysis, a high degree of association was noticed with PHA, and dot ELISA, however the sensitivity of the latter was lower than the former.

The quantitative ELISA was also standardized to detect DPV antibodies. Sera samples showing titres 160 and above was considered as positive. The percentage of positive samples

was 72.5 per cent with a 77.24 per cent sensitivity. Antibody titres in this test were 20 to 40 fold greater than the titres obtained by PHA. In addition, the test was able to detect low titred sera.

Whole blood dried on filter paper strip was evaluated for its suitability as an alternate method for conventional serum collections. Elutes from such strips were tested by plate ELISA and the results compared. Among 200 samples, 141 (70.5%) samples showed positive reaction. The sensitivity was 77.30 per cent when compared to whole serum ELISA, a high degree of association ($P<0.05$) was noticed and this test showed high degree of significant difference ($P>0.05$) as like plate ELISA, when compared with PHA.

Indirect immunoperoxidase staining technique was used to detect DP viral antigen in tissue samples collected from different localities. Out of 35 liver sections 30 (85.7%) showed positive reaction. The number of spleen samples showing positive reaction was 24 (80%). The reaction sites were seen both in the nucleus and cytoplasm.

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EVALUATION OF ENZYME IMMUNOASSAYS IN THE DIAGNOSIS OF DUCK PLAGUE

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ABSTRACT OF A THESIS

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ABSTRACT

Use of Enzyme immunoassays namely dot ELISA and plate ELISA were evaluated to detect DP viral antibodies in serum samples and in whole blood dried on filter paper strips and their efficacy was compared with standard passive haemagglutination test.

Indirect immunoperoxidase test was also used to detect DP viral antigen in paraffin embedded liver and spleen.

ASS at 33 per cent level was used for separation of duck globulins and antiduck globulins. The protein concentration of these globulins were 34 mg/ml and 15 mg/ml respectively. The purity of these globulins were tested by IEP and AGPT using antiduck whole serum raised in rabbits.

Duck plague hyperimmune serum was raised in healthy ducklings with live attenuated DP vaccine having a virus titre of 3.5 $\log_{10}$ ELD_{50}/0.5 ml. This serum was used as the positive control.

A total of 200 serum samples, 35 liver and 30 spleen samples were collected from different localities for the detection of duck plague viral antibodies and antigen. Corresponding blood samples were also collected on filter paper strips and the serum was eluted and ELISA was carried

out. The results in this test were then compared with whole serum ELISA.

The percentage of positive reaction in PHA, Dot ELISA, plate ELISA and filter paper strip method are 64 per cent, 68 per cent 72.5 per cent and 70.5 per cent respectively.

Comparative efficacy of PHA with Dot ELISA, plate ELISA and filter paper strip method were carried out and sensitivity of the tests are 71.87, 67.64 and 77.30 per cent respectively. The specificity of these tests were 38.88, 43.75, 70.90 and 67.79 per cent respectively. The concordance of PHA with these tests were 60, 75.5 and 74.5 per cent respectively.

On statistical analysis high degree of association ($P<0.05$) was observed between PHA and Dot ELISA, plate ELISA and filter paper strip method. Highly significant different ($P>0.05$) was observed between PHA and plate ELISA, and PHA and filter paper strip method.

Based on the results, it was concluded that because of the simplicity, easiness and accuracy, Dot ELISA is suitable for detection of DPV antibodies under field conditions.

But plate ELISA was highly sensitive, specific and able to detect low titred sera. Hence this test may be recommended for titration of DPV antibodies in the laboratories,

particularly when the potency of the vaccine is to be checked and the immune status of a flock is to be evaluated.

Because of various advantages filter paper strip method will serve as an alternative to collection of whole serum for detection of DPV antibodies. For the detection of DPV antigen, IPT was considered as suitable one because of its ability to detect high positive (83%) cases and less non specific reactions.