SEROPREVALENCE AND RESTRICTION ENZYME ANALYSIS OF EGG DROP SYNDROME VIRUS

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"SEROPREVALENCE AND RESTRICTION ENZYME ANALYSIS OF EGG DROP SYNDROME VIRUS"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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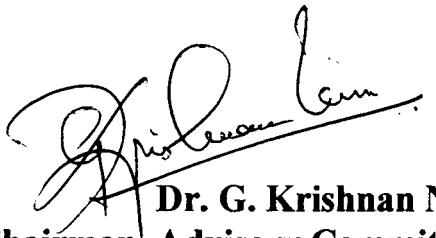
Certified that this thesis entitled **"SEROPREVALENCE AND RESTRICTION ENZYME ANALYSIS OF EGG DROP SYNDROME VIRUS"** is a record of research work done independently by **Miss. Priya. P.M.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.



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EXTERNAL EXAMINER

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Priya, P.M.

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INTRODUCTION

1. INTRODUCTION

The success of poultry industry heavily depends upon the achievement of production targets. Inspite of all the efforts made to augment the health of the birds, poultry industry in Kerala suffers economic loss due to significant drop in egg production. Many workers have pointed out the involvement of an aviadenovirus in apparently healthy flocks which upsets the expected levels of peak production. Incidence of infectious bronchitis (IB) and infectious laryngotracheitis (ILT) was ruled out by Mahalingam *et al.* (1973) and George (1979) in Kerala. These studies throw light on the possibility of an inapparent infection with Egg drop syndrome -76 (EDS-76) virus in poultry flocks.

Unlike other infections which cripple the poultry industry through heavy mortality, EDS causes a paralysing effect on the poultry sector. This is because drop in egg production and laying of abnormal eggs are the only signs suggestive of EDS infection. By the time the bird has developed antibody, the flock is already infected and many a time it goes unnoticed. So the infected birds are maintained and fed with poor

returns. Hence early detection is a must and it becomes easy only if the test used for diagnosis is a simple one. Several serological techniques like haemagglutination inhibition (HI) test, enzyme linked immunosorbent assay (ELISA), virus neutralisation (VN), agar gel precipitation test (AGPT) and counter immunoelectrophoresis (CIE) have been employed for the diagnosis of EDS-76 infection. Among these HI and ELISA are more sensitive (Monreal and Dorn, 1981) and the latter is used for differential diagnosis from other avian adenoviruses.

Ducks are considered to be asymptomatic carriers of EDS-76 virus. In Kerala, the practice of backyard system of poultry keeping is very common wherein the ducks are commonly found mingling with chicken, sharing feeders and waterers, which could result in horizontal transmission of the disease among these birds. This necessitates the screening of available duck population.

Serologically similar but genetically different strains can be differentiated by restriction enzyme (RE) analysis. In India, though many indigenous EDS-76 virus isolates have been characterised with respect to

their physicochemical features, data on the molecular organisation of the viral genome is scanty. Hence molecular diagnostic techniques that are quick, sensitive and specific, such as RE analysis, is the need of the hour for rapid detection of EDS-76 virus.

Considering the situation and facts mentioned above and understanding the possible involvement of EDS-76 virus in chicken and duck diseases in the state, the present study was undertaken with the following objectives.

1. To assess the seroprevalence of EDS-76 in birds which fail to reach peak production, as well as in healthy flocks, employing HI test and Indirect ELISA.
2. To compare the efficacy of the above tests in detecting antibodies against EDS-76 virus.
3. To differentiate EDS-76 virus strains by restriction enzyme analysis.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Egg drop syndrome-1976 (EDS-76) of poultry, a viral disease of significant economic importance, has been reported from all parts of the world including India.

2.1 Classification

During the mid-seventies, an economically devastating syndrome affecting layers with alarming drop in egg production was reported for the first time in Netherlands (Van Eck *et al.*, 1976). This disease has since become known as EDS-76 and is caused by an Aviadenovirus (McFerran *et al.*, 1978b).

Egg drop syndrome-76 virus belongs to the family *Adenoviridae* and is classified under the genus, *Aviadenovirus*. But it was considered as an "atypical avian adenovirus", because of its unique biological and biochemical characteristics (Wigand *et al.*,1982). As this virus had originated from ducks, it was classified as duck adenovirus type I (Russell *et al.*,1995).

The phylogenetic analysis of the protease gene revealed that EDS-76 virus was more closely related to bovine adenovirus type-7 (BAV-7) and ovine adenovirus-287 (OAV-287) and so it was opined that it should be classified along with BAV-7 and OAV-287 in a separate taxon (Harrach *et al.*, 1997). Hess *et al.* (1997) also reported similar results after analysing the complete nucleotide sequence of EDS-76 viral genome and these support the assignment of a new Adenovirus genus or subgenus within the *Adenoviridae* family.

2.2 Strain classification

The Egg drop syndrome virus is the sole member of group III avian adenoviruses. It is serologically unrelated to group I and group II avian adenoviruses .

Only one serotype of EDS-76 virus has been recognised (Yamaguchi *et al.*, 1981b). But based on the restriction enzyme analysis of the EDS-76 virus genome, three groups have been formed. The isolates obtained from infected European chickens formed the first group. The second group consisted of viruses isolated from the ducks in U.K. One virus isolated from chicken in Australia formed the third group. (Todd *et al.*, 1988).

2.3 Incidence and distribution

Egg drop syndrome is most commonly seen in heavier egg laying and broiler breeding flocks. The occurrence of EDS-76 appears to be independent of seasonal variation. The disease occurs throughout the year. Birds of all ages are susceptible to infection, and if EDS-76 virus is introduced onto a site, effects on egg production can be seen in all ages of laying hens. However, when birds apparently become infected around peak egg production (McFerran *et al.*, 1978a), this may be due to reactivation of the latent virus. The course of the disease varies from two to six weeks.

Transmission of disease is mainly by vertical route. At one time these viruses were spread by the contamination of Marek's disease vaccine, which was produced in duck embryo fibroblasts. This led to the infection of the breeding flocks and the virus spread widely through fertile eggs. Droppings also contain virus and contaminated fomites such as crates or trucks can spread virus. This virus is also transmitted by needles used for vaccinations.

Egg drop syndrome -76 virus has been isolated from chicken in Great Britain (Baxendale, 1978a), Northern

Ireland (McFerran *et al.*, 1978b), Belgium (Meulemans *et al.*, 1979a), India (Mohanty *et al.*, 1980), Italy (Zanella *et al.*, 1980), Australia (Firth *et al.*, 1981), Singapore (Sing and Chew-Lim, 1981) Japan (Yamaguchi *et al.*, 1981b), Hungary (Zsak and Kisary, 1981a), Taiwan (Lu *et al.*, 1985a) and Egypt (Mahmoud and Sami, 1989).

Serological evidence of infection has been found in chicken in Denmark (Badstue and Smidt, 1978), Brazil (Hwang *et al.*, 1980), Nigeria (Nawathe and Abegunde, 1980), New Zealand (Howell, 1982), Czech republic (Packova and Pospisilova, 1992), New California (Lambert and Kabar, 1994) and Bolivia (Bishop and Cardozo, 1996).

2.4 Serological relationship

The strains 127 and BC14 were considered as the representative strains.(Calnek, 1978). Different strains of EDS virus isolated in different countries viz. D61 and BC14 from United Kingdom; JBP (Pune strain) and SPC (Bangalore strain) from India; E77, BC14 and 3877 from Italy and a Belgium strain were serologically and morphologically identical to reference strain 127 (Meulemans *et al.*, 1979a). The Australian strain also showed cross reaction in HI with

strain 127 (Firth *et al.*, 1981). The same was true with Korian strains A and D which cross reacted with strain BC14 (Rhee *et al.*, 1983). The Japanese strain (H162) was also serologically identical to strain 127 (Higashihara *et al.*,1983), whereas TN strain from Taiwan was identical to Japanese strain (JPA-1) (Lu *et al.*, 1985b).

The EDS-76 virus strain 127 also shared an antigen with fowl adenovirus type 1 (FAV-I) (McFerran *et al.*, 1978a) but there was no cross immunofluorescence between them and also there was no cross reaction in neutralization and HI tests between strain 127 and eleven other adenoviruses (Adair *et al.*, 1979).

2.5 Seroprevalence of EDS-76

Serological investigation of the affected flock will indicate the relationship between the development of antibody to the virus and the syndrome.

2.5.1 Seroprevalence in countries other than India

The serological surveys conducted by several workers from different parts of the world revealed the presence of specific antibodies to EDS-76 virus in chicken, ducks, quails, geese and in a few wild birds

(McFerran *et al.*,1977; Baxendale, 1978a; Calnek, 1978; Muelemans *et al.*, 1979b; Van Eck *et al.*, 1980; Bidin *et al.*, 1981; Wilcox *et al.*, 1983; Khafagi and Hamouda, 1991; WenXian *et al.*, 1996 and Hasegawa *et al.*, 1999).

2.5.2 Seroprevalence of EDS-76 in India

In India, Mohanty *et al.* (1980) reported the egg drop syndrome in chicken associated with EDS-76 virus.

Mohanty *et al.* (1985) conducted a serological survey in exotic and indigenous breeds of ducks, quails, turkeys, pheasants and guinea fowls to determine the prevalence of EDS-76 virus infection. Most of the indigenous ducks and one quail showed HI antibody to EDS-76 virus. Sera from turkeys, pheasants and guinea fowls were negative.

Seroprevalence of EDS-76 virus in some of the domesticated and free flying birds in Kerala was studied by Sulochana and Sudharma (1987) using HI test. Out of 268 duck sera screened, 128 (47.8 per cent) had HI titre ranging from 10 to 640. Only nine out of 219 (4.1 per cent) chicken sera were positive and it was noted that the positive birds were from areas where duck and chicken were reared in close vicinity. All the

125 Japanese quails and five love birds examined were negative. Both the pigeons and two out of five crows screened were positive.

Reddy and Raghavan (1987) reported the incidence of EDS-76 in 16 flocks of White Leghorn experiencing drop in egg production. Out of 770 poultry sera screened, an overall incidence of 27.3 per cent was recorded. Birds of 44 to 60 weeks of age had the highest incidence, compared to 20, 28, 35 and 36 week old birds. A similar survey in five flocks of Khaki Campbell ducks revealed an overall incidence of 37.6 per cent. Antibodies were present in different age groups from 20 to 52 weeks.

Seroprevalence study conducted in commercial layer and broiler birds by HI test revealed highest positive reactors (22.41 per cent) in chicken of five to ten weeks of age, while the adult birds of 21 to 30 weeks of age showed the lowest (4.34 per cent) positivity (Shakya and Dhawedkar, 1991).

Das and Pradhan (1992) detected HI antibodies to EDS-76 virus in two different outbreaks, both in quail and chicken flocks with decrease in egg production. The

egg drop ranged between 10.6 and 50.6 per cent, and the number of soft-shelled eggs increased with the decline in egg production.

Serological screening of 323 sera samples received from different states revealed seroprevalence of EDS-76 infection in several poultry flocks. The titre of HI antibody level ranged between 1:4 to 1:32 and production remained aberrant for two to seven weeks, without return to preinfection level, following recovery (Ramkumar *et al.*, 1992).

Rangnekar and Kher (1995) reported that 68.38 per cent of birds were positive for antibodies to EDS-76 in Anand area of Gujarat. Maximum prevalence rate was seen in birds aged 46 to 55 weeks.

2.6 Diagnosis

2.6.1 Clinical signs and lesions

The combination of sudden fall in egg production associated with soft-shelled and shell-less eggs in a flock of apparently healthy birds, is almost diagnostic of EDS-76 infection.

Major lesions are seen in the pouch shell gland and oviduct, where epithelial cells become necrotic and contain intranuclear inclusion bodies. Though these lesions are virtually pathognomonic, diagnosis should be confirmed by either virus isolation or serology.

2.6.2 Isolation and identification of causative agent

Adair *et al.* (1979) reported in decreasing order of sensitivity as an indicator system, embryonated duck or goose eggs from a flock free of EDS-76 virus infection, duck or goose cell cultures, chicken embryo liver cells, followed by chicken kidney cells. Chicken embryo fibroblasts were found to be insensitive. Embryonated chicken eggs were also not found suitable (Higashihara *et al.*, 1983).

EDS-76 virus was successfully propagated in embryonated duck eggs via allantoic route. After four to five days of incubation, the allantoic fluid was screened for HA activity and found to be positive by several workers (Adair *et al.*, 1979; Gulka *et al.*, 1981; Gough *et al.*, 1982; Bartha, 1984; Ramkumar *et al.*, 1992). Either the virus or cloacal swabs along with faecal materials were used as inoculum by Adair *et al.* (1979) and Ramkumar *et al.* (1992) respectively.

Swain *et al.* (1993) found that allantoic fluid was having the highest HA activity, followed in order by chorioallantoic membrane, skin and internal organs. Chicken and quail embryos did not support the growth of the virus.

Egg drop syndrome-76 virus was inoculated at different dilutions into three batches of 9, 10 and 11 days old duck embryos. The HA titre of the allantoic fluid was found to be highest with 1:10 dilution of the inoculum and the yield of the virus was maximum in nine day old embryos inoculated with this dilution of the virus (Xue-HuaQing *et al.*, 1995).

2.6.3 Haemagglutination Inhibition (HI) test

Egg drop syndrome -76 virus was found to agglutinate erythrocytes of chicken, ducks, turkeys, geese, pigeons and peacocks, at 4°C, room temperature and 37°C. No agglutination was observed with erythrocytes of rat, rabbit, horse, sheep, cattle, goat and pig. The haemagglutinin was stable to heating and freezing (Adair *et al.*, 1979). Haemagglutination inhibition test had an additional advantage that lyophilized haemagglutination antigen of duck embryo origin, inactivated with 0.2 per cent formalin, could

be stored for at least a year at 4°C without any loss in titre (Lu *et al.*, 1985b).

Several workers employed HI test to detect antibodies to EDS-76 virus (Rampin *et al.*,1978; Schloer, 1980; Kaleta *et al.*, 1980 a, b; Ng *et al.*, 1980; Bartha *et al.*, 1982; Adair *et al.*, 1986; Sukumar and Babu, 1986; Sheriff *et al.*, 1987; Sulochana and Sudharma 1987; Chetty *et al.*, 1988; Justacara *et al.*,1988; Antarasena *et al.*, 1989; Oberoi *et al.*, 1990; Shakya and Dhawedkar, 1991; Ramkumar *et al.*, 1992; Rao *et al.*, 1992; Das *et al.*, 1995 and AnChun *et al.*,1997).

Akay *et al.* (1988) described HI antibody titre against EDS-76 virus in vaccinated hens and yolk material of hen's eggs. EDS-76 antibodies were not detected in the egg yolk and sera of unvaccinated hens. A good correlation existed between the sera and egg yolk HI titres.

Garg *et al.* (1993) employed filter paper strip method for collection of blood samples for demonstration of EDS-76 antibodies. They observed that

the HI titres of sera were ten times more than those of filter paper elutes.

2.6.4 Enzyme linked immunosorbent assay (ELISA)

The new analytical technique Enzyme linked immunosorbent assay (ELISA) was introduced by Engvall and Perlmann (1971). ELISA was comparable in sensitivity to radio-immunoassay (RIA) (Walter *et al.*, 1977) and the indirect fluorescent -antibody procedures (Bullock and Walls, 1977).

Enzyme linked immunosorbent assay was considered as an alternative to VN test in diagnosis of avian diseases such as infectious bronchitis virus (Garcia *et al.*, 1980), infectious bursal disease virus (Marquardt *et al.*, 1980), avian adenovirus (CELO), and the avian adeno- associated virus (AAV) (Dawson *et al.*, 1980).

Mesanjaz *et al.* (1982) reported that ELISA and HI tests were comparable for detecting antibodies to EDS-76 virus.

Piela and Yates (1983) concluded that ELISA was found to be a sensitive and reliable method for

detecting antibody, although positive titres did not agree with HI and immunodiffusion results at one week post inoculation.

Lal *et al.* (1992) compared the ELISA and VN tests to detect antibodies againt fowl adenovirus (FAV) type-I and found that ELISA could readily be used to screen sera for antibodies against FAV with higher specificity and sensitivity.

Oberoi *et al.* (1993) described a rapid indirect Dot-ELISA for detection of avian viruses. They opined that nitrocellulose strips coated with tannic acid in phosphate buffer containing PEG 6000, egg albumin and high NaCl gave enhanced antigen-antibody reaction at 40°C in 10 minutes.

2.6.5 Preparation of conjugate for ELISA

The three major steps for the preparation of conjugate are

1. Purification of immunoglobulins (Igs)
2. Raising antisera against these Igs
3. Coupling/ labelling / conjugation of Igs with the enzymes.

2.6.5.1 Purification of Igs.

A plethora of methods exist to purify Igs from sera such as precipitation with neutral salts or organic solvents, chromatography, electrophoresis, isoelectricfocussing, isotachophoresis, ion-exchange chromatography and affinity chromatography (Kurstak and Kurstak, 1974; Kurstak *et al.*, 1984).

2.6.5.1a Precipitation by neutral salts

This is the oldest and simplest method and is based on the principle of increasing the ioning strength (Salting out). Two commonly used salts are ammonium sulphate and sodium sulphate.

Benedict(1967) observed that Igs of chicken could be precipitated from serum at room temperature in three successive steps viz, by the addition of 18, 14 and 14 per cent respectively of crystalline sodium sulphate. Later on various workers have precipitated chicken globulins (Higgins, 1976; Goel *et al.*, 1980; Nandapalan *et al.*, 1983) and turkey globulins (Saif and Dohms, 1976) using this technique. Globulins of ducks were fractionated using sodium sulphate at two successive concentrations of 50 per cent and 33 per cent respectively (Toth and Norcross, 1981).

Separation of duck globulins was carried out by Nair (1990) employing ammonium sulphate (at 33 and 40 per cent levels) and sodium sulphate (in three stages of 18, 14 and 14 per cent final concentration). He opined that 33 per cent ammonium sulphate precipitated fraction was more pure, compared to the other two.

Sodium sulphate saturation varied considerably between 0°C to 20°C, whereas ammonium sulphate saturation had little dependence on temperature (Goers,1993).

Two methods were used to achieve the high concentration of ammonium sulphate required for protein precipitation. In the first method, solid ammonium sulphate was used and in the second method, an amount of SAS was added to the stirred protein solution (Goers, 1993).

Malmarugan (1997) and Singh (1997) also precipitated duck globulins using 33 per cent ammonium sulphate solution.

2.6.5.2 Conjugation of Igs with enzymes

In ELISA, the conjugates usually consist of proteins coupled to enzymes. The coupling has been satisfactorily carried out using glutaraldehyde in either one step (Avrameas, 1969) or two steps (Avrameas and Ternyck, 1971) or by using periodate (Nakane and Kawaoi, 1974).

The three enzymes most commonly used in ELISA are horse radish peroxidase (HRPO), alkaline phosphatase (AP) and β-D- galactosidase (β-GAL)(Kemeny, 1991).

Enzymes may be attached to antibodies by direct or indirect methods (Goers, 1993).

In direct method, the antibodies are directly coupled with enzymes in such a way as to preserve the activity of both the enzyme and the antibody. Although there is no single method that is equally successful for all enzyme-antibody systems, glutaraldehyde has been reasonably successful for most enzymes.

Indirect method often resulted in the attachment of several enzyme molecules to each primary antibody

molecule. Two of the most popular secondary molecules are streptavidin and protein A (or protein G).

2.7 Comparison of serological tests for detection of antibody to EDS-76 virus.

Kaleta *et al.* (1980b) studied and compared the kinetics of antibody formation against EDS virus in pigeon, turkey and fowls and reported that the VN test was more sensitive than HI test in wild birds.

Monreal and Dorn (1981) made comparative studies between VN, ELISA and HI for demonstration of antibodies to avian adenovirus and EDS-76 virus. It was concluded that ELISA was more sensitive and recommended as a routine method for the detection of avian adenovirus and EDS-76 antibodies.

Piela and Yates (1983) compared ELISA, HI and immunodiffusion for detection of antibodies to a duck adenovirus in experimentally infected chicken. The ELISA was found to be a sensitive and reliable method for detecting antibody, although positive titres did not agree with HI and immunodiffusion results at one week after inoculation, probably reflecting the different classes of antibodies being detected.

Adair *et al.* (1986) compared the sensitivity of five serological tests, viz., HI, ELISA, VN, AGPT and Fluorescent antibody technique (FAT) for the detection of EDS-76 virus antibody. They concluded that HI or VN test could be used for the detection of infection in commercial birds.

The competitive ELISA had a higher sensitivity (71.8 per cent versus 50 per cent detection rates) and was more specific than HI test when used for detection of antibodies to EDS-76 in 30 week old hens (Ma *et al.*, 1991).

The sero-epidemiological study undertaken by Shaw *et al.* (1995) in eight districts of Tamil Nadu, employing HI and Dot immunoassay (DIA), revealed a wide-spread distribution of antibodies to EDS-76 virus in chicken. Birds of all ages were found to be susceptible. The per cent positive by DIA was 16.78 as against 14.89 by HI, out of the total 584 sera samples tested. These workers were of the opinion that there was no significant difference between the two tests and they favoured the use of simple HI, which was as sensitive and specific as the latest test like DIA for seromonitoring purposes.

Gang *et al.* (1996) compared the HI and AGPT for detecting EDS-76 virus using egg yolk as a substitute for serum. The results indicated that although AGPT was more simple, it was less sensitive, rapid and specific than HI. Haemagglutination inhibition titres of the yolk were lower than serum antibody titres initially, but both titres were the same after 20 days.

2.8 Purification of the virus

Todd and McNulty (1978) and Takai *et al.* (1984) purified the EDS-76 virus after propagation in chicken embryo liver (CEL) cells, by CsCl equilibrium density gradient centrifugation.

Kisary and Zsak (1980) purified the EDS-76 virus (strain B8/78) by two cycles of isopycnic centrifugation in CsCl.

Yamaguchi *et al.* (1981a) purified JPA-1 strain from infected cells and fluid of CEL and chicken kidney (CK) cell cultures by CsCl equilibrium density gradient centrifugation. Two bands with densities 1.33 g/ml and 1.30 g/ml respectively were obtained.

Polyethylene glycol (PEG) concentrated EDS-76 virus after density gradient centrifugation on potassium tartarate revealed two bands. The virus banded at a density of 1.25 g/ml whereas the tissue contaminants banded at a density of 1.21 g/ml (Swain *et al.*, 1992).

Chandramohan (1994) adopted 36 per cent sucrose cushion ultracentrifugation technique to purify indigenous strains of EDS-76 virus.

Swain *et al.* (1995) carried out a simple method of purification and isolation of nucleic acid of EDS-76 virus of chicken and quail origin. The virus was concentrated by PEG 6000 and purified by detergent treatment (Genetron 113). Of the three different concentrations namely four, six and eight per cent, six per cent PEG was found to precipitate maximum virus particles.

By using CsCl gradient ultracentrifugation, EDS-76 virus particles were purified from allantoic fluids of goose embryos inoculated with H91 strain (YuYou *et al.*, 1995).

Gang *et al.* (1997) purified the EDS-76 viruses using chloroform extraction, PEG-6000 dialysis and concentration and Sephadex G-200 chromatography. The purified virus was observed by electron microscopy. They concluded that chromatographic method for purifying EDS-76 viruses was both simple and practical.

Maiti and Sarkar (1997) purified the virus from the infected allantoic fluid by CsCl equilibrium density gradient centrifugation. Two opaque bands at approximately one third (minor band) and two thirds (major band) of the distance from the top of the gradient were obtained.

JinPing *et al.* (1999) purified the chicken (strain NE4) and duck (strain JE1) egg drop syndrome virus isolates by differential centrifugation.

2.9 Nucleic acid profile of EDS-76 virus

2.9.1 Isolation of viral DNA

The DNA from the purified virions of EDS virus (strain B8/78) was extracted by three cycles of phenol extraction method. The estimated molecular weight of the whole undigested genome was about 22.9 x 10^6 Da (Kisary and Zsak, 1980).

Zsak and Kisary (1981b) isolated DNA from EDS-76 strain B8/78 after purification employing phenol extraction method. The average total molecular weight of the undigested DNA was found to be 22.6 x 10^6 Da (about 34.2 Kbpp).

Egg drop syndrome -76 (strain 127) was purified by centrifugation in CsCl gradient and the viral DNA was extracted from the purified virions by sodium dodecyl sulphate (SDS)-proteinase K treatment and phenol extraction (Zakharchuk *et al.*, 1993).

The viral DNA was extracted from the purified EDS-76 virus particles by phenol:chloroform extraction method and the purity of the DNA was checked by comparing the optical density (OD) at 260 and 280 nm in a Spectrophotometer (Chandramohan, 1994).

Swain *et al.* (1995) employed a new method which was more suitable, easy and less time consuming without the use of ultracentrifugation and other sophisticated techniques. The nucleic acid was extracted from PEG-6000 concentrated EDS-76 viruses of quail and chicken origin by phenol:chloroform extraction method. The DNA of EDS-76 virus of quail origin had almost the

same molecular weight of 22.9x 10^6 Da as that of EDS-76 virus of chicken origin.

The complete nucleotide sequence of EDS-76 virus was reported by Hess *et al.* (1997). The total genome length was 33,213 nucleotides, with a molecular weight of 21.9 x 10^6 Da.

2.9.2 Restriction enzyme analysis

The restriction endonuclease R-EcoRI cleaved at two sites of the EDS -76 viral (strain B8/78) DNA, generating 3 fragments with molecular weights of 13.5 x 10^6, 5.0 x 10^6 and 4.4 x 10^6 Da. respectively (Kisary and Zsak, 1980).

On doing restriction enzyme analysis of the above strain using the enzymes EcoRI, BamHI, HindIII, BglI, BglII, HpaI and PstI, 4, 4, 10, 8, 7, 6 and 8 numbers of fragments respectively were obtained (Zsak and Kisary, 1981b).

Based upon the restriction patterns of DNAs generated by restriction endonucleases BamHI and HindIII, 17 fowl adenovirus strains, representing 11 serotypes, were placed into five groups and the

molecular weights of the fragments were calculated with reference to EDS-76 virus DNA fragments generated by various restriction enzymes (Zsak and Kisary, 1984).

Restriction enzyme analysis of 13 isolates of EDS virus revealed that UK and Belgium isolates formed one group and could be well differentiated from duck isolates of U.K. The Australian isolate with a DNA deletion (0.4 Kbp) at one end of the genome (32.6 Kbp), differed in this respect from European isolates and formed a third group (Todd *et al.*, 1988).

The restriction enzyme analysis of five local isolates and reference strain 127 of EDS-76 virus with PstI enzyme revealed identical banding patterns, thereby confirming the genetic similarity among the local isolates and the European strain (Chandramohan, 1994).

The phenol-extracted DNA, from the purified EDS-76 virus suspension when cleaved with BamHI enzyme yielded four fragments of 17Kbp, 10Kbp, 4Kbp and 2Kbp and with the Eco RI enzyme, it was cut into four fragments of sizes 17Kbp, 8Kbp, 6.5Kbp and 1.2Kbp (Duan-Yuyou *et al.*, 1995).

YuYou *et al.* (1995) studied the RE analysis of EDS H91 viral DNA. There were some differences between EDS H91 and EDS 127 strains in the number and lengths of fragments produced by digestion of genomic DNA with HindIII and SmaI.

The viral genomic DNA of WPD V205 strain was cleaved into 4,4 and 7 fragments respectively with EcoRI, BamHI and BamHI + EcoRI and compared with reference strain 127 and strain B8/78, there were some differences in the lengths of the smaller fragments with BamHI and EcoRI adhesive ends (Huang *et al.*, 1996).

XueMin *et al.* (1998) reported that the molecular weights and lengths of restriction fragments of isolates NE4 and GC2 DNA were similar to AV127. From these observations they opined that NE4, GC2 and AV127 were of the same genotype.

Digestion of chicken (strain NE4) and duck (strain JE1) egg drop syndrome virus isolates with the restriction endonucleases EcoRI, BamHI, BgII, BglII, KpnI, PstI and HindIII resulted in 4,4,6,8,5,9 and 10

fragments for NE4 and 4,4,5,7,9,10 and 8 fragments for JE1 respectively. The replication maps differed between the two isolates, except with EcoRI digestion. It was concluded that EDS virus strains from different hosts, but with the same serotype, had different genomes (JinPing *et al.*, 1999).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

In this study, Borosil brand of glass ware and Laxbro brand plastics were used. All the chemicals used in this study were of analar or molecular biology grade.

3.1 Materials

3.1.1 Buffers and Reagents

3.1.1.1 Alsever's solution

Glucose	-	2.05 g
Sodium citrate	-	0.8 g
Citric acid	-	0.055 g
Sodium chloride	-	0.42 g
Triple distilled water	-	100 ml

Sterilised by autoclaving at 10 lbs pressure for 10 min.

3.1.1.2 Globulin separation

3.1.1.2a Saturated Ammonium Sulphate (SAS) solution

This was prepared by adding 760 g of ammonium sulphate to one litre of triple distilled water and heating at 56°C for 30 min in a waterbath with continuous stirring. The solution was filtered to

remove insoluble impurities and then cooled to room temperature. The pH of the solution was adjusted to 7.0 with ammonia solution just prior to use.

3.1.1.2b Working Ammonium Sulphate Solution (ASS)

Solution of 66 per cent strength was prepared (v/v), freshly from stock SAS (3.1.1.2a).

3.1.1.2c Borate buffer (Stock solution)

Boric acid	-	6.184 g
Borax	-	9.536 g
Sodium chloride	-	4.384 g
Triple distilled water	-	1000 ml

3.1.1.2d Borate Buffered Saline (BBS) [Working solution]

Borate buffer (3.1.1.2c)	-	5 ml
Physiological Saline	-	95 ml

The pH of the solution was adjusted to 8.5 using 1N NaOH.

3.1.1.2e Ten per cent barium chloride solution

3.1.1.3 Agar Gel Precipitation Test. (AGPT)

3.1.1.3a Gel for AGPT

Agarose	-	0.8 g

Sodium chloride	-	0.85 g
Phenol	-	one drop
Distilled water	-	100 ml

To dissolve agarose in saline the solution was boiled for five minutes.

3.1.1.3b Stain for AGPT and IEP

Amidoblack 10B	-	0.1 g
Sodium chloride	-	0.9 g
Distilled water	-	100 ml

3.1.1.4 Immuno electrophoresis (IEP)

3.1.1.4a Tris-Barbital Buffer (TBB)

Barbitone sodium	-	9.9 g
Tris (hydroxy methylamino methane)	-	17.7 g
Sodium azide	-	0.3 g
Triple distilled water	-	2000 ml

pH adjusted to 8.6 with 1N HCl.

3.1.1.4b 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.

3.1.1.4c Gel for IEP

Agarose	-	0.7 g
TBB (3.1.1.4a)	-	100 ml

The solution was kept in a boiling water bath till agarose got dissolved completely.

3.1.1.4d Stain for IEP

Amido black	-	0.1 g
Sodium acetate- acetic acid buffer (0.2M, pH- 3.2)	-	100 ml

3.1.1.5 Decolouriser for AGPT and IEP

3.1.1.5a Decolouriser I

Methanol	-	120 ml
Acetic acid	-	30 ml
Distilled water	-	30 ml

3.1.1.5b Decolouriser II

Absolute alcohol	-	140 ml
Acetic acid	-	20 ml
Distilled water	-	40 ml

3.1.1.6 Horse radish peroxidase (HRPO) conjugation

3.1.1.6a 0.1 M Potassium Phosphate Solution

Potassium dihydrogen phosphate (KH_2PO_4)	-	1.36g

Triple distilled water - 100 ml

3.1.1.6b One per cent glutaraldehyde solution

Glutaraldehyde (25 per cent) - 0.4 ml

Triple distilled water - 9.6 ml

3.1.1.6c Physiological saline, pH 7.4

3.1.1.6d Horse radish peroxidase (HRPO) - 252 units/mg powder (Obtained from Bangalore Genei, Pvt. Ltd.)

3.1.1.7 Plate ELISA

3.1.1.7a ELISA plates

ELISA plates with 96 flat bottomed wells (Tarsons Pvt. Ltd) were used.

3.1.1.7b Phosphate Buffered Saline (PBS) [10x Stock solution]

Sodium chloride - 80 g

Potassium chloride - 2 g

Disodium hydrogen phosphate

($Na_2HPO_4.12H_2O$) - 11.33 g

Potassium dihydrogen phosphate

(KH_2PO_4) - 2 g

Distilled water - 1000 ml

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

3.1.1.7c PBS-Tween-20(PBS-T)

PBS (10x) (3.1.1.7b)	-	100 ml
Tween 20	-	500 µl
Distilled water to make	-	1000 ml

pH was adjusted to 7.2 using 1N HCl

3.1.1.7d PBS-T with Bovine serum albumin (2 per cent)

Bovine serum albumin	-	2 g
PBS-T (3.1.1.7c)	-	100 ml

It was always prepared just before use.

3.1.1.7e Carbonate-bicarbonate buffer, pH 9.6

Sodium carbonate (Na_2CO_3)	-	1.59 g
Sodium bicarbonate ($NaHCO_3$)	-	2.93 g
Triple distilled water	-	1000 ml

pH was adjusted to 9.6 using IN HCl and the solution was kept at 4°C.

3.1.1.7f Sodium citrate buffer, 0.05 M, pH 4.2

Sodium citrate	-	14.71 g
Distilled water	-	1000 ml

pH was adjusted to 4.2 using 1N HCl and the solution was kept at 4°C.

3.1.1.7g Substrate solution

ABTS (2'-2 azino-di-ethyl benz-
thiazoline 6-sulfonic acid) - 11 mg

Sodium citrate buffer (3.1.1.7f) - 50 ml

Just before use, 35 µl of 30 per cent Hydrogen peroxide was added to freshly prepared substrate solution.

3.1.1.7h Stopping reagent (0.1 M Hydrofluric acid)

3.1.1.8 Restriction enzyme analysis

3.1.1.8a 1M Tris -HCl (Stock solution, pH 8.0)

Tris base - 12.11 g

Dissolved in 70ml triple distilled water and pH was adjusted to 8.0 using 1N HCl and then made upto 100 ml with triple distilled water and autoclaved at 121°C for 15 min at 15 lbs pressure. It was stored at 4°C until use.

3.1.1.8b 0.5M EDTA (Stock solution, pH 8.0)

Dissolved 18.61 g of disodium EDTA in 70 ml triple distilled water. Approximately 2 g of NaOH was added

and solution was heated till the EDTA dissolved completely. Made upto 100 ml with triple distilled water and autoclaved at 121°C for 15 min at 15 lbs pressure and stored at 4°C.

3.1.1.8c 1 M NaCl. (Stock solution, pH 8.0)

Dissolved 5.84 g of NaCl in 100 ml triple distilled water and pH was adjusted to 8.0 and autoclaved at 121°C for 15 min at 15 lbs pressure and stored at 4°C.

3.1.1.8d Tris sodium EDTA (TME) buffer [Working solution]

10 mM Tris	-	1ml (3.1.1.8a)
1 mM EDTA	-	0.2ml(3.1.1.8b)
100 mM NaCl	-	10ml(3.1.1.8c)

Made upto 100 ml with triple distilled water and pH was adjusted to 8.0 and autoclaved at 121°C for 15 min at 15 lbs pressure and stored at 4°C.

3.1.1.8e Sucrose solution (36 per cent)

Sucrose	-	36 g
Triple distilled water to make	-	100 ml

3.1.1.8f Proteinase K solution

Proteinase K (Sigma) - 10 mg

Triple distilled water - 1 ml

The solution was incubated at 37°C for 1h and then stored at -20°C.

3.1.1.8g Equilibration of phenol

Equal volume of saturated phenol ("Bangalore Genei" Pvt. Ltd.) and 0.5 M Tris HCl (pH- 8.0) was taken in a separating funnel and mixed well and left for five min. When the two phases were separated, the lower phenolic phase was collected. Then an equal volume of 0.1 M Tris-HCl (pH- 8.0) was added to the phenol. Extractions were repeated until the pH of the phenolic phase was greater than 7.8 (measured with pH paper) (Sambrook *et al.*,1989).

3.1.1.8h Chloroform, ultra pure grade (SRL) was used.

3.1.1.8i Isoamyl alcohol (SRL) was used.

3.1.1.8j Absolute alcohol

Commercially available rectified spirit was distilled twice with acid and alkali and stored at -20°C in glass stoppered bottle.

3.1.1.8k Alcohol (70 per cent)

Absolute alcohol (3.1.1.8j), 70 ml was added to 30 ml of triple distilled water and was used chilled by storing at 4°C.

3.1.1.8l Tris EDTA (TE) buffer

10 mM Tris	-	1 ml (3.1.1.8a)
1mM EDTA	-	0.2ml (3.1.1.8b)

Made upto 100 ml with triple distilled water and pH was adjusted to 8.0 and then autoclaved at 121°C for 15 min at 15lbs pressure.

3.1.1.8m Restriction enzymes (RE)

EcoRI ("Bangalore Genei")-	4000Units(20U/µl)
BamHI ("Bangalore Genei")-	2000Units(10U/µl)
HindIII("Bangalore Genei")-	4000Units(20U/µl)

3.1.1.8n DNA size marker

Lambda DNA HindIII digest- 250 µg / ml

(Obtained from "Bangalore Genei")

3.1.1.9 Agarose gel electrophoresis

3.1.1.9a Agarose (SRL) was used

3.1.1.9b Tris Borate EDTA (TBE) Buffer [Stock solution 10x, pH 8.2]

Tris Base	-	108 g
Boric acid	-	55 g
Disodium EDTA	-	9.3 g
Triple distilled water	-	1000 ml

3.1.1.9c Working solution (1x)

Mixed stock solution of TBE buffer (3.1.1.9b) 100 ml with 900 ml of triple distilled water to prepare the working solution of 1x TBE buffer and pH was adjusted to 8.2.

3.1.1.9d Gel loading buffer

Sucrose	-	40 g
Bromophenol blue	-	0.25 g
TBE buffer (1x) (3.1.1.9c) to make	-	100 ml

3.1.1.9e Ethidium bromide (Stock solution 10 mg / ml)

One gram of ethidium bromide (Boehringer Mannheim, Germany) was dissolved in 100 ml of triple distilled water with constant stirring in a magnetic stirrer for one to two hours. The solution was then transferred to

an amber coloured bottle, wrapped in aluminium foil and stored at 4°C.

3.2 Biologicals

3.2.1 Reference virus

The reference virus (strain 127 of UK) of EDS-76 was obtained from the Department of Animal Biotechnology, Madras Veterinary College, Chennai.

3.2.2 Virus strains

Three different strains of EDS-76 virus were used in the research work (i) Madras strain, obtained from Department of Microbiology, Madras Veterinary College, Chennai, (ii) Namakkal strain obtained from Department of Microbiology, Veterinary College and Research Institute, Namakkal and (iii) Hyderabad Vaccine strain which was maintained in the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy.

3.2.3 Duck eggs

Nine day old embryonated duck eggs obtained from University Poultry Farm, Mannuthy, were used for revival of stock viral isolates.

3.2.4 Rabbits

Ten healthy rabbits each weighing about 1- 1½ kg, procured from the Small Animal Breeding Station (SABS), Mannuthy, were used for the production of antiserum, antiglobulin and hyperimmune serum against EDS-76 virus.

3.2.5 Cockerels

Two healthy cockerels of 15 weeks of age, purchased from Revolving Fund Hatchery, Mannuthy, were used for the production of hyperimmune serum against EDS-76 virus.

3.2.6 Sera samples

A total of 603 sera samples were collected, 281 from ducks and 322 from chicken of 28 to 30 weeks of age.

3.2.7 Chicken erythrocytes

Blood from chicken was collected in Alsever's solution (3.1.1.1) in the proportion of 1:4 for the preparation of 0.8 per cent erythrocytes for HI test.

3.3 METHODS

3.3.1 Collection of blood and separation of duck and chicken sera

Non haemolysed sera were collected separately by sacrificing ten ducks (20 to 22 weeks of age) and fifteen chicken (7 to 8 weeks of age) and stored as small aliquots at -20°C.

3.3.2 Estimation of protein concentration

The total protein content in the duck and chicken sera was estimated by Biuret method using total protein kit.

3.3.3 Production of antiduck and antichicken sera

Antiduck and antichicken sera were separately raised in two healthy rabbits each and they were immunised by the following schedule.

One millilitre of whole duck serum having a protein concentration of 68 mg per ml was homogenised with one millilitre of Freund's complete adjuvant (1:1) and one millilitre of this emulsion was given intramuscularly to each rabbit. Thereafter, at weekly intervals they were given three booster doses of 0.5 ml of serum without adjuvant by the same route. Meanwhile

the rabbits were monitored for the pesence of specific antibodies at periodic intervals by AGPT (3.1.1.3) and IEP (3.1.1.4). When sufficient level of antibody was found in the serum, which was about ten days after the third booster injection, 20 ml of blood was collected from each rabbit by cardiac puncture. Serum was separated and stored at -20°C until used.

Antichicken serum was raised by the same procedure as for antiduck serum in rabbits.

3.3.4 Separation of globulins from duck and chicken sera

Ammonium sulphate precipitation of globulins from duck and chicken sera was done as per the procedure described by Garvey *et al.* (1977).

Fifty millilitre of 66 per cent ASS (3.1.1.2b) was added dropwise to 50 ml of serum sample with constant stirring using magnetic stirrer. 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 900 x g 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 (3.1.1.2d) to a final volume of 20 ml. The ammonium sulphate was removed from the precipitate by dialysing against borate buffered saline at 4°C. The saline was changed frequently until there was no ammonium sulphate in the dialysate as evidenced by the absence of turbidity on testing with ten per cent barium chloride solution.

The concentration of the precipitated proteins was determined by Biuret method using total protein kit (3.1.1.2e).

3.3.5 Purity of gammaglobulins

The purity of gamma globulins was tested by AGPT and IEP.

3.3.5.1a Agar gel precipitation test (AGPT)

Agar gel precipitation test was done as per the method of Williams and Chase (1971) with suitable modifications. Melted agarose was poured onto glass

slides and then wells were cut at equidistance so as to get one central well and two peripheral wells. The purity of duck gammaglobulin fraction separated was checked by separately charging the peripheral wells with duck serum and duck gamma globulin, and central well with antiduck whole serum. The slides were incubated at 37°C in humid chamber for 48 h and examined against light for the presence of precipitin lines.

Chicken globulin was also tested by the same procedure.

3.3.5.1b Staining

The slides were washed after soaking in two changes of normal saline for 24h each and then in distilled water for further 24h to remove unreacted proteins. The slides were dried slowly, stained with amidoblack (3.1.1.3b) for 15 min and decolourised in Solution I and II for 20 min. each. The slides were dried at 37°C for 1h and mounted in DPX.

3.3.5.2 Immunoelectrophoresis (IEP)

Immunoelectrophoresis was done as per the method of Williams and Chase (1971) with slight modifications. Three millilitre of buffered agarose (3.1.1.4c) at

about 50°C was poured onto each slide (3.1.1.4b) kept on a levelled surface. The agarose was allowed to solidify initially at room temperature and subsequently at 4°C. Wells were cut towards one end of the slide with the troughs in between them. After removing the agarose the wells were filled with antigens. A drop of bromophenol blue dye was added to the anode 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 side so that each covered about ½ 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 1cm away from the anode end of the slide.

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

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

3.3.6 Preparation of antiduck and antichicken gammaglobulins

Antiduck and antichicken gammaglobulins were separately raised by the same procedure as for antiduck serum in rabbits, using ammonium sulphate precipitated serum globulins dissolved in borate buffered saline (3.1.1.2d) and having an approximate protein concentration of 10 to 15 mg per ml. Two rabbits each were used for this purpose.

Ten days following the last injection the rabbits were bled, serum was separated and pooled. Gamma globulin fraction was separated and used for HRPO conjugation.

3.3.7 Conjugation of antigammaglobulins with HRPO

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

1. The antiduck and antichicken gamma globulin was reconstituted with the borate buffer (3.1.1.2d) to obtain 5 to 7 mg of globulins / ml.

2. pH was adjusted to 6.9 by addition of 0.1 M solution of potassium phosphate (3.1.1.5a).

3. For each ml of the above solution, 10 to 14 mg of HRPO enzyme (3.1.1.5d) was added and after its complete dissolution, 0.05 ml of glutaraldehyde (3.1.1.5b) was added.

4. The mixture was shaken for 2h 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 2000 x g

7. The supernatant was collected and stored in small aliquots at -20°C.

3.3.8 Preparation of hyperimmune serum to EDS-76 virus

Antisera against EDS-76 reference strain (127) was prepared in rabbits and apparently healthy cockerels free from EDS viral antibodies as per the method of Chetty (1985) and Polly (1977) respectively with slight modifications.

Two rabbits and two cockerels were used. The EDS-76 reference virus grown in embryonated duck eggs having a HA titre of 2 $\log_{13}$ was used as a source of antigen. One millilitre of the virus suspension was mixed thoroughly with one millilitre of Freund's complete adjuvant and injected intramuscularly at the rate of one millilitre per rabbit and 0.6 ml per cockerel. The second and third doses of antigen were given without adjuvant at weekly intervals. Ten days after the third injection, the rabbits and cockerels were bled and checked for HI titre and the sera samples collected were stored in aliquots at -20°C.

3.3.9 Collection of sera samples

A total of 603 sera samples were collected by conventional (402) / filter paper strip method (201) from chicken and ducks aged between 28 to 30 weeks from different poultry farms belonging to both public and

private sectors with history of sudden fall in egg production and also from apparently healthy birds. The sera samples and filter paper strips were stored at -20°C and 4°C respectively until used.

3.3.10 Filter paper strip method

3.3.10a Preparation of filter paper strip

Filter paper strips were made following 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 three strips were overlapped in the middle and stapled together. This cluster of three strips was used to collect six samples because blood was collected on both ends of each strip.

3.3.10b Blood collection

The wing vein of the birds was punctured 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 absorbed on both surfaces.

The end of each strip was folded up slightly after saturation with blood to give each cluster of six 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 h, sealed in plastic bags and stored at 4°C.

3.3.10c Elution of dried blood

The portion of the paper strips with dried blood was cut into small pieces and placed in sterile vials. 100 µl of normal saline was added to each vial and the paper pieces were agitated properly to ensure thorough moistening. It was kept at 4°C overnight for complete elution. Next day 20 µl of eluted sample was pipetted out from each vial and used for HI.

3.3.11 Haemagglutination(HA)test and Haemagglutination Inhibition(HI)test

Haemagglutination (HA) and HI tests were carried out as per the procedures of Sulochana and Sudharma (1987) with slight modifications. Both the tests were performed by the microtitre method in 96 well U bottom microtitre plates (Laxbro, Pvt. Ltd.).

3.3.11a Haemagglutination (HA) test

Two fold dilutions of the virus were made in 60 µl per volumes starting from 1:4 dilution using physiological saline (pH 7.4). To each dilution of the virus, 40 µl of 0.8 per cent washed chicken erythrocytes were added and kept at room temperature for 30 min. The reciprocal of highest dilution of the virus showing complete haemagglutination was taken as HA titre.

3.3.11b Haemagglutination Inhibition (HI) test

The beta method of HI test was followed. Before using for serological tests, the sera samples were inactivated at 56°C for 30 min. The eluted sample was considered to be equivalent to 1:2 serum dilution. EDS virus (strain 127) in 4HA units was used as the antigen.

Test proper

1. Added 20 µl of physiological saline (pH - 7.4) to all the wells
2. Added 20 µl or sera/eluted sample to the first well. Serial dilution was made and 20 µl was discarded from the last well.

3. Added 20 µl of antigen to each well.
4. Allowed to react for 15 to 30 min.
5. Added 40 µl 0.8 per cent chicken erythrocytes to all the wells.
6. Mixed gently and the results were read after 45 min when the erythrocytes in the control wells settled. Simultaneous controls were run with known positive and negative sera for comparison.

The HI titre was expressed as the reciprocal of the highest dilution producing 100 per cent inhibition of HA activity. Titres of 3 $\log_2$ and above were taken as positive.

3.3.12 Plate ELISA

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

The purified EDS reference virus (vide 3.3.13b) was suspended in PBS and used as antigen.

Optimum concentration of coating antigen (1:200), positive control serum (1:200) and HRPO conjugate

(1:1000) were arrived at by preliminary checker board titrations.

While standardising the test, the optimum dilution of the test sera was found to be 1:40. This dilution of serum was used for screening the field sera samples.

Each well of the ELISA plate was coated with 100 µl of 1:200 dilution of antigen in carbonate-bicarbonate buffer, pH 9.6 (3.1.1.7e) and kept at 4°C overnight. The plate was then washed by emptying, filling with PBS-T (3.1.1.7c) from a wash bottle and leaving for three minutes. This process was repeated three times. Unreacted sites were blocked by addition of 100 µl of two per cent bovine serum albumin (3.1.1.7d) to each well and incubated for one hour at 37°C. The wells were washed as above and incubated with field sera/eluted samples (1:40 dilution made in PBS-T) in duplicates collected from ducks, in different rows of wells. Plates were incubated for one hour at 37°C. The wells were again washed with PBS-T three times.

Antiduck globulin peroxidase conjugate (3.3.7) (1:1000 dilutions in PBS-T) was added to all the wells

except in the substrate control and incubated for one hour at 37°C. The wells were washed with PBS-T and freshly prepared substrate was added and incubated at room temperature in dark for 30 min for the development of colour reaction.

After 30 min incubation, the reaction was stopped by adding 50 µl of hydrofluric acid to each well. Reading was taken in a Multiscan ELISA reader at 405 nm. The sera samples of control ducks were taken as negative control.

Samples with more than twice the mean optical density (OD) value of the negative serum was taken as positive (Garret *et al.*, 1984).

Same procedure was employed to screen sera from chicken. Optimum concentration of coating antigen (1:200) and positive control serum (1:200) and HRPO conjugate (1:2000) were arrived at by preliminary checker board titrations.

3.3.13 Restriction enzyme analysis

3.3.13a Propagation of the virus

The four strains of EDS-76 virus under study were revived by inoculation into allantoic cavity of nine-day-old embryonated duck eggs.

The procedure described by Bishai *et al.*(1974) was followed. The duck eggs were candled and the position of the air cell and embryo was marked. The air cell region was swabbed with tincture iodine and a hole was drilled 0.5 cm towards the centre from the rim of air cell. Using a sterile tuberculin syringe and a 22 gauge needle, 0.2 ml of inoculum was introduced into the allantoic cavity. The hole was then sealed with melted paraffin and the eggs were incubated at 37°C in an upright position. All the eggs were candled daily. Those embryos which died within 24 h of inoculation were discarded. The embryos which died after 24 h and those which were alive after five days were transferred to a refrigerator for chilling. Allantoic fluid was harvested and clarified at 5000 x g for 15 min. The harvested fluid of each passage was screened for haemagglutination activity.

3.3.13b Virus concentration and purification

Egg drop syndrome-1976 virus strain after passaging in duck embryos were purified as per the method of Chandramohan (1994).

The clarified infected allantoic fluid was subjected to ultracentrifugation in a Beckmann ultracentrifuge (Model L7-80) 70 Ti rotor at 80,000 x g for 2h at 4°C. The crude virus pellet obtained was resuspended in minimum quantity of TNE buffer (3.1.1.8a). The crude viral suspension was overlaid on a 36 per cent sucrose cushion and was then centrifuged at 1,00,000 x g for 4h at 4°C in a 60 Ti swingout rotor. The purified virus pellet was then suspended in TNE buffer.

3.3.13c Isolation of DNA

The DNA of all the four strains of EDS-76 virus was extracted from the purified virus samples by the phenol:chloroform method as described by Sambrook *et al.* (1989) with some modifications.

Hundred microlitres of Proteinase K (10 mg /ml) (3.1.1.8f) was added to 200 µl of purified virus and

mixed thoroughly. The mixture was then incubated at 57°C for 2h. To this 400µl of phenol : chloroform : isoamylalcohol (25:24:1) mixture was added and centrifuged at 19000 x g for 10 min. The upper aqueous phase was separated and treated with pure chloroform (3.1.1.8h). Subsequently, the mixture was centrifuged at 19000 x g for 10 min at 15°C. The aqueous phase was then precipitated with 600 µl of chilled ethanol (3.1.1.8j) and kept overnight at -20°C. It was then centrifuged at 19000 x g for 20 min. The pelleted DNA was washed with 70 per cent ethanol and dried in 37°C incubator for 1h. The DNA was then suspended in minimum quantity of TE buffer (3.1.1.8l).

3.3.13d Analysis of the viral DNA

Three microlitre of viral DNA suspension was analysed electrophoretically in 0.7 per cent agarose gel (w/v) containing ethidium bromide with 1X TBE buffer (3.1.1.9c). Lambda DNA HindIII digest (3.1.1.8l) was used as the DNA size marker. The gel was photographed under UV light (Fotodyne, USA).

3.3.13e Assessment of cencentration and purity of DNA

The concentration and purity of DNA were assessed by spectrophotometry. Ten microlitre of DNA sample was diluted to one ml with distilled water. The spectrophotometer (SL 159, UV-VIS Spectrophotometer) was set to zero with distilled water at 260 and 280 nm wavelength. The DNA samples from each of the four strains were taken in turn in the cuvette and OD was measured at 260 nm and 280 nm. One OD at 260 nm was taken as 50 µg per ml of double stranded DNA. Ratio of OD 260/ OD 280 indicated the purity of the DNA samples. DNA samples having ratio of 1.8 and more were subjected to RE analysis.

3.3.13f Restriction enzyme digestion

Restriction digestion of the whole viral genome was carried out in 20µl volumes. The following components were added to sterile microfuge tubes in the below mentioned order

Distilled water	-	6 µl
RE buffer, 10x	-	2 µl
Viral DNA	-	10µl
Restriction enzyme	-	2 µl

The restriction enzyme was used with the appropriate enzyme buffer supplied by the manufacturer.

The digestion mixture was incubated at 37°C waterbath for 12 h. the enzyme reaction was stopped by incubating the digest at 56°C for 10 min. The samples were prepared for agarose gel electrophoresis by mixing 20 µl of the RE digest with 5 µl of sample buffer.

3.3.13g Agarose gel electrophoresis

Two hundred and ten milligram of agarose was added to 30 ml of 1 X TBE and was kept in a boiling water bath to melt the agarose. This was then cooled to 60°C. Ethidium bromide was added to a final concentration of 0.5 µg per ml.

Agarose was poured onto a tray 15 cm x 7 cm size with combs fitted for formation of wells at one end and allowed to solidify. After solidification the combs were removed, and the gel was transferred into a submarine gel electrophoresis tank containing 1x TBE buffer, with wells at the cathode end.

The DNA digests of the four strains mixed with sample buffer were loaded into the wells. Lambda DNA HindIII digest, which was used as the molecular weight marker was loaded in a separate well.

Electrophoresis was done at 90 V for one to two hours from cathode to anode. The electrophoresis was stopped when the sample dye migrated more than half the length of the gel. At the end of the electrophoresis, the gel was visualised under UV transilluminator (Fotodyne) and the photograph was obtained using poloroid camera with wratten gelatin filter.

3.3.13h Estimation of total molecular size and molecular weight of the DNA

The molecular sizes of restriction enzyme digests of DNA were estimated by comparison of the distance migrated by restriction fragments to that of the standard molecular weight marker. Lambda DNA fragments obtained by digestion with HindIII was used as standard. By plotting the values of distance migrated by Lambda HindIII digested DNA fragments (X-axis), versus the $\log_{10}$ Kbp values of their molecular size (Y- axis) on graph paper, a linear curve was obtained. The molecular size of restriction fragment of EDS virus

with each RE was determined by interpolation of the curve from the values of distance migrated (cm) by them. The $\log_{10}$ Kbp values so obtained on Y-axis was calculated to antilog, so as to obtain the molecular size in Kbp of the restriction fragment.

The molecular weight (Daltons) of the restriction fragments were computed based on the relation 1.475 Kbp = 10^6Daltons (Sambrook *et al.*, 1989).

RESULTS

4. RESULTS

4.1 Duck and chicken sera

The protein concentration of the pooled duck sera obtained from ten healthy ducks was 68 mg per ml and that of chicken sera obtained from 15 chicken was 72 mg per ml.

4.2 Antiduck and antichicken sera

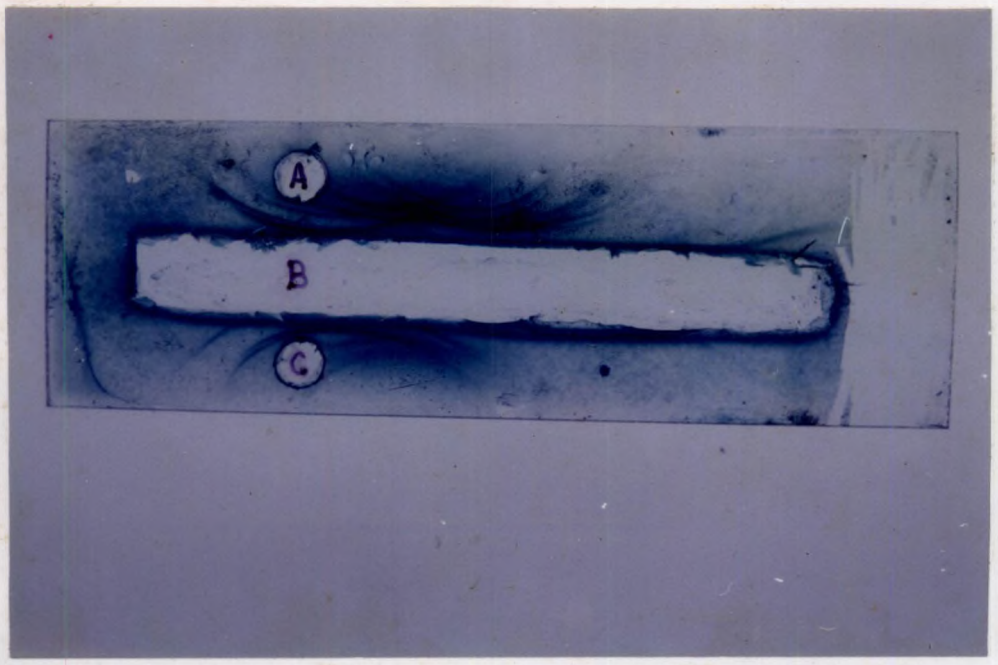
Antiduck and antichicken sera which were produced in rabbits when tested against their respective sera by IEP, developed 11 and 13 precipitin arcs respectively, extending to both the cathode and anode sides of the antigen well. The precipitin arcs seen towards the cathode side of the well were identified as globulins, considering their nature and positions (Fig. 1 and Fig.2).

4.3 Globulin precipitation by ammonium sulphate

The duck and chicken gammaglobulins were successfully precipitated separately from their respective sera using 33 per cent saturated ammonium sulphate solution (pH- 7.0). The protein concentration

Fig. 1. Immunoelectrophorogram of antiduck serum against duck gammaglobulin and duck serum

A- Duck serum
B- Antiduck serum
C- Duck gammaglobulin


A
B
C

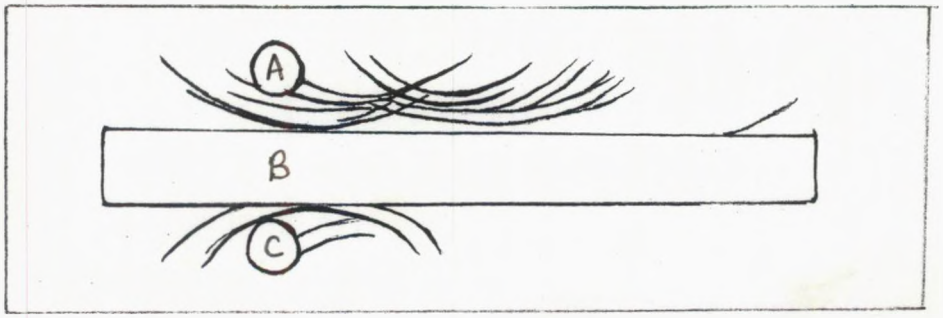
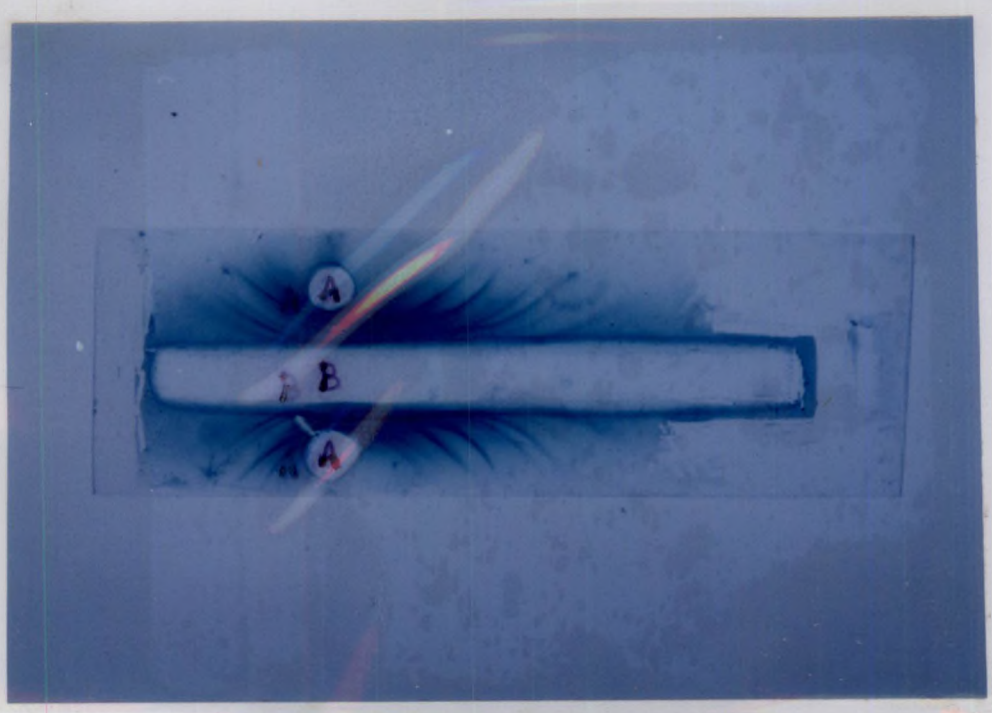

A
B
C

Fig. 2. Immunoelectrophorogram of antichicken serum against chicken serum

A- Chicken serum
B- Antichicken serum


A
B
A


A
B
A

of duck and chicken gammaglobulins was estimated to be 15 and 18 mg per ml respectively by Biuret method.

The purity of duck and chicken gammaglobulin was checked by AGPT and IEP.

On doing AGPT, one bold precipitin line and few other diffused lines were observed between duck serum and its antiserum. When duck globulin was tested against antiduck serum, the bold line seen in the previous case was absent and two other clear lines were observed (Fig. 3).

Five precipitin arcs were produced in IEP by duck gammaglobulin and 11 precipitin arcs in case of duck serum, when reacted against antiduck serum (Fig. 1).

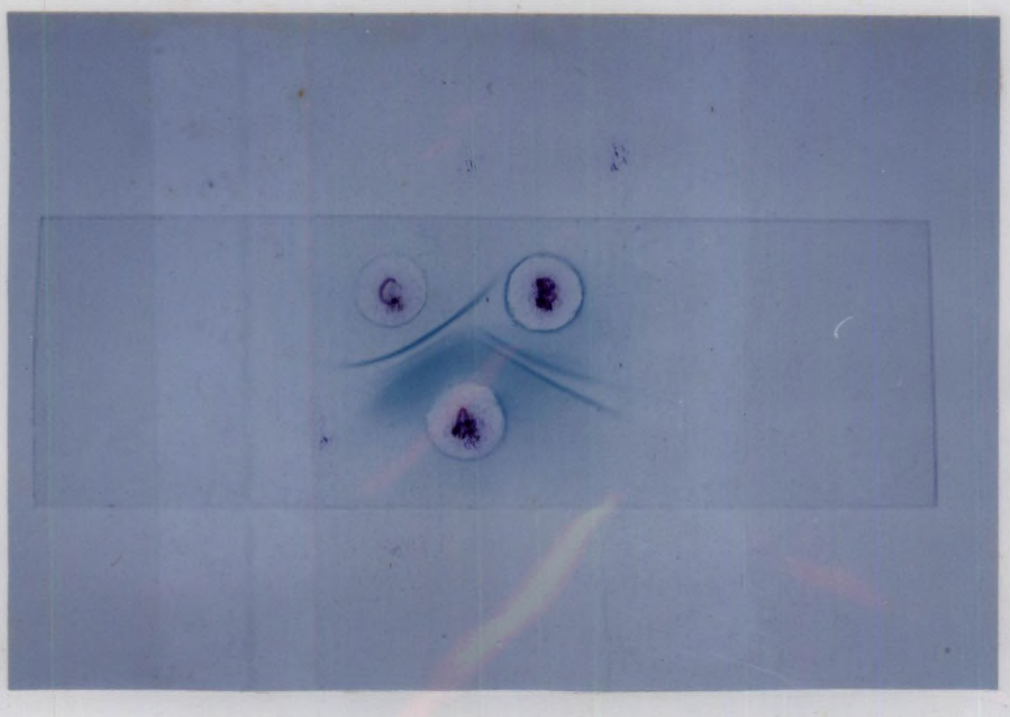
When AGPT was done with chicken serum and chicken globulin against antichicken serum, similar pattern of lines were formed as in the case of duck serum and its globulins. In addition, a line of identity was seen between chicken serum and chicken globulin, which was not clear with duck serum (Fig. 4).

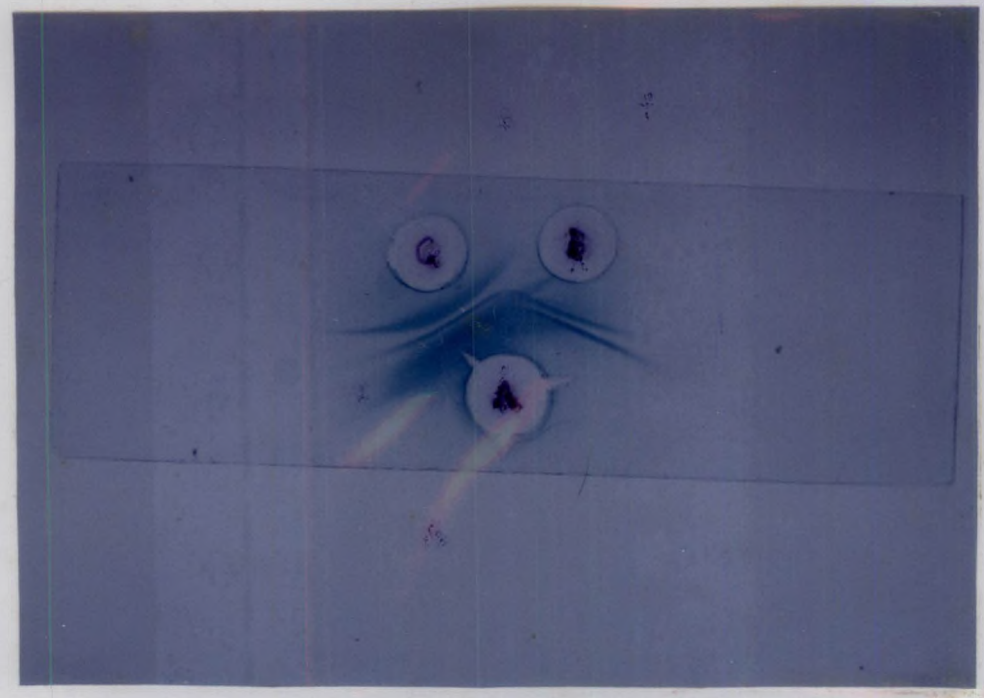
Fig. 3. Agar gel precipitation test of antiduck serum against duck globulin and duck serum

A- Antiduck serum
B- Duck gammaglobulin
C- Duck serum

Fig. 4. Agar gel precipitation test of antichicken serum against chicken globulin and chicken serum

A. Antichicken serum
B. Chicken gammaglobulin
C. Chicken serum


C
B
A


C
B
A

Two bold and one thin precipitin arcs extending on either side of the antigen well were produced in IEP by chicken gammaglobulin and 13 precipitin arcs were observed in case of chicken serum, when reacted against its antiserum (Fig. 5).

4.4 Antiduck and antichicken gammaglobulins

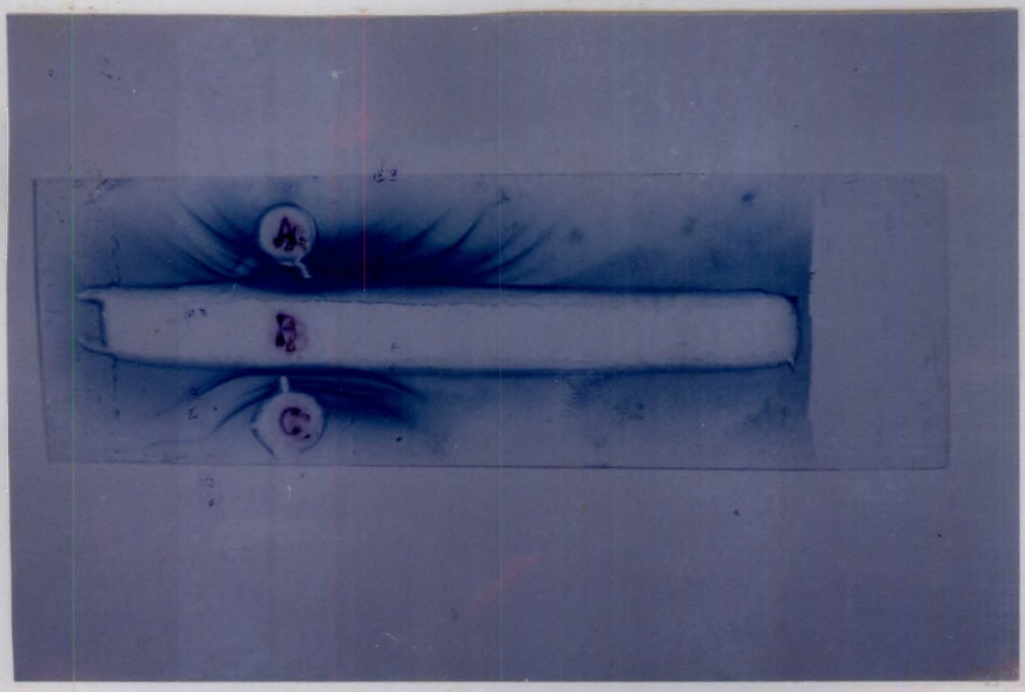
The procedure followed for raising antisera against duck and chicken gammaglobulin in rabbit gave sufficient antibody by AGPT in the serum of rabbit. The gammaglobulin fraction of the rabbit serum was separated by precipitation with 33 per cent ammonium sulphate and its purity was checked by IEP.

Immunoelectrophoresis of duck globulin against antiduckglobulin produced two bold and two faint precipitin arcs, whereas, one bold and one faint precipitin arc was obtained between chicken globulin and its antiglobulin(Fig. 6 and Fig. 7).

The protein concentration of the antiduck and antichicken gammaglobulins ranged between 12 to 14 mg per ml.

Fig. 5. Immunoelectrophorogram of antichicken serum against chicken gammaglobulin and chicken serum

A- Chicken serum
B- Antichicken serum
C- Chicken gammaglobulin


A
B
C

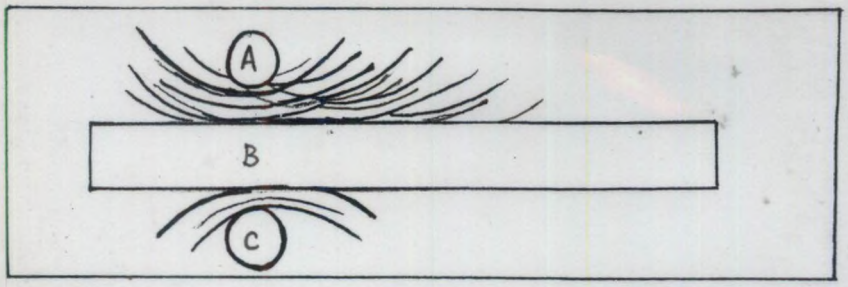
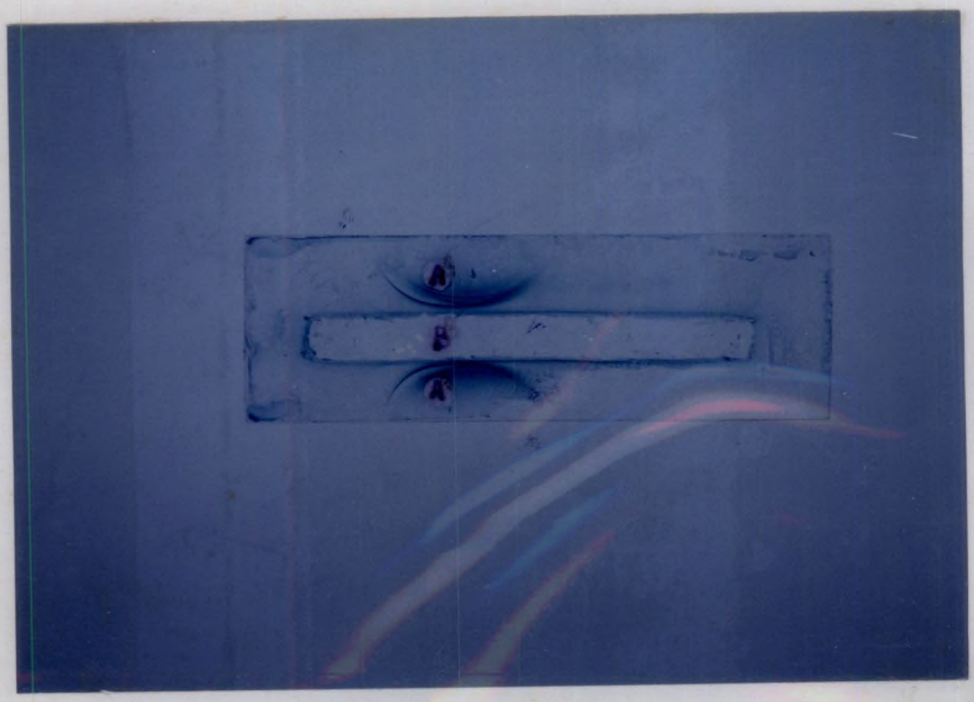

A
B
C

Fig. 6. Immunoelectrophorogram of antiduck gammaglobulin against duck gammaglobulin

A- Duck gammaglobulin
B- Antiduck gammaglobulin


A
B
A

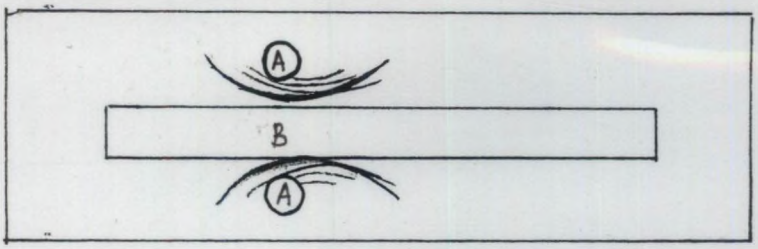
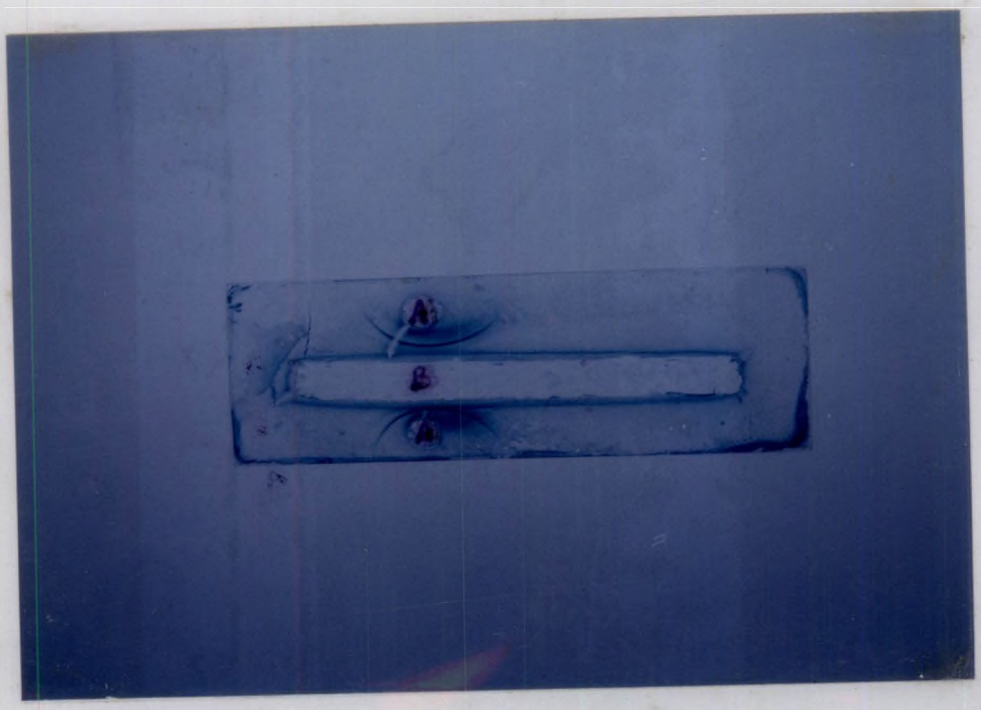
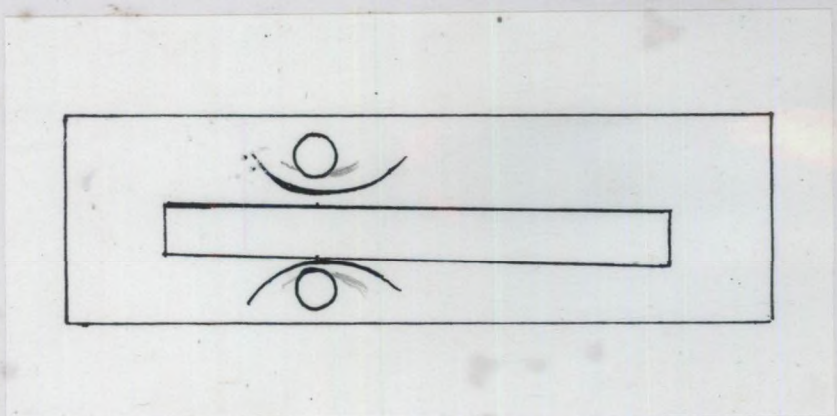

A
B
A

Fig. 7. Immunoelectrophorogram of antichicken gammaglobulin against chicken gammaglobulin

A- Chicken gammaglobulin
B- Antichicken gammaglobulin


A
B
A



4.5 Labelling of antigammaglobulins

The protein concentration of the duck and chicken gammaglobulins was adjusted to 5 to 7 mg per ml before conjugation. Two-step glutaraldehyde method employed in the present study yielded a stable enzyme conjugate. The enzyme HRPO was added at the rate of 2500 units per ml of antigammaglobulin. Glutaraldehyde was directly coupled to antigammaglobulins instead of using the indirect method. The conjugated globulins were used for indirect ELISA.

4.6 Seroprevalence

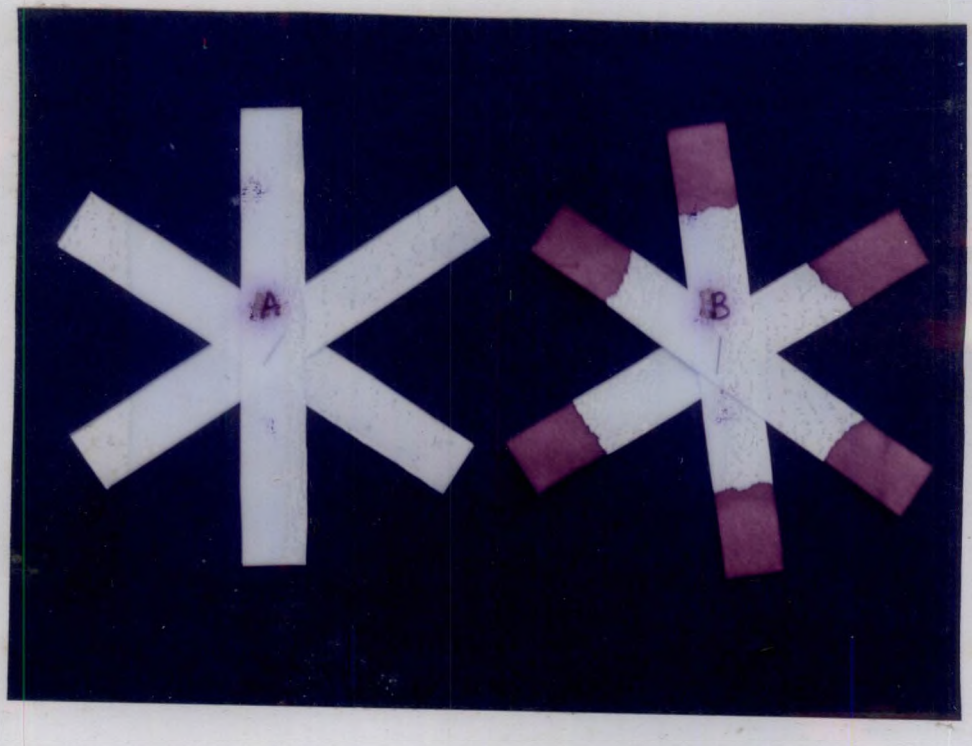
4.6.1 Collection of sera samples

A total of 603 sera samples were randomly collected from ducks and chicken from five districts in Kerala viz. Alleppey, Kottayam, Palakkad, Thrissur and Trivandrum. In Kottayam most of the birds had a history of significant drop in egg production from the peak at the time of collection.

Either conventional or paper strip method (Fig. 8) were used for sample collection. From 281 ducks, 177 samples were collected by conventional method and 104 samples by paper strip method.

Fig. 8. Filter paper strips for blood collection

A- Before collection
B- After collection


A
B

Out of 322 chicken sera samples, 225 were by conventional method and 97 were by paper strip method (Table 1).

4.6.2 Haemagglutination inhibition (HI) test

All the 603 sera samples collected from field cases were screened by HI test for EDS-76 antibodies. Only samples giving titres 3 $\log_2$ and above were taken as positive. Out of 322 chicken sera samples tested, 48 samples were found to be positive. The percentage of positivity being 14.91. Among 281 duck sera samples screened, 75 samples (26.69 per cent) were positive (Fig. 9).

4.6.3 Enzyme Linked Immunosorbent Assay (ELISA) test

All the samples screened by HI were subjected to ELISA. Samples having more than twice the mean OD value of the negative serum was considered as positive. Among 322 chicken sera samples screened, 59 were found to be positive. The percentage of positivity was 18.32. Of 281 duck sera samples screened, 93 samples (33.10 per cent) were found to be positive (Fig. 10).

Table 1. Collection of sera samples and testing by HI and ELISA

Type of serum	Total number of samples collected	Number of samples collected		Number of samples positive by HI		Total number of samples positive by HI	No of samples positive by ELISA		Total number of samples positive by ELISA
		conventional	Filter paper strip	Conventional method	Filter paper strip method		Conventional method	Filter paper strip method	
Chicken	322	225	97	40	8	48	49	10	59
Duck	281	177	104	46	29	75	57	36	93

Fig. 9. Haemagglutination inhibition (HI) test

Rows A to F (1 to 11)	:	Test sera/eluted samples (serially diluted from 1: 2 to 1:2048)
Rows A to F (12)	:	Sera controls
Row G	:	Positive serum control
Row H (1 to 4)	:	Virus control
(9 to 12)	:	RBC control

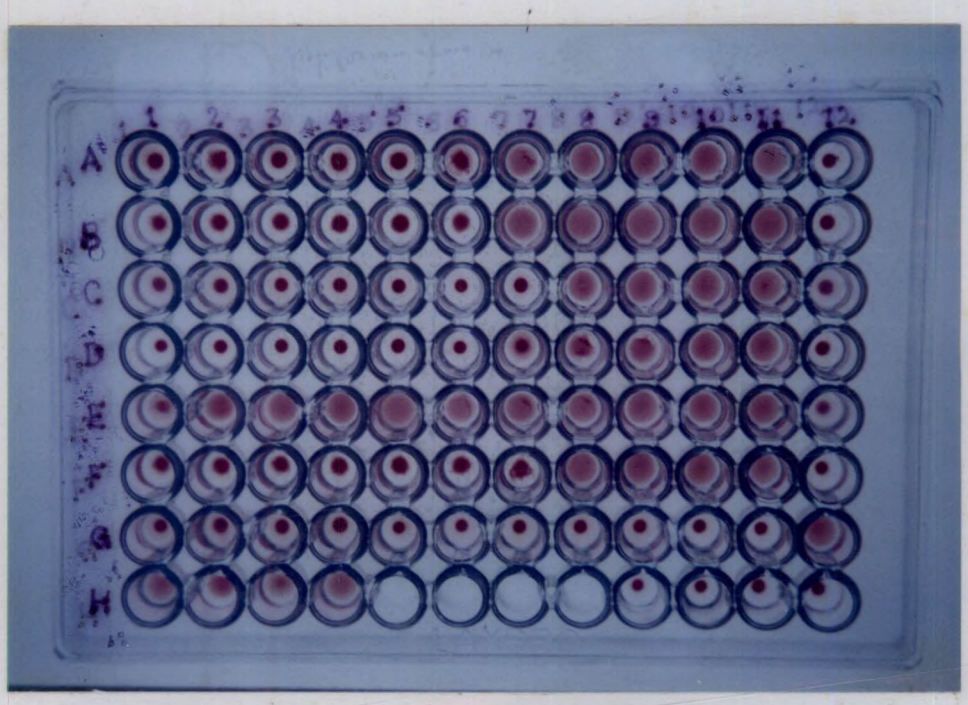
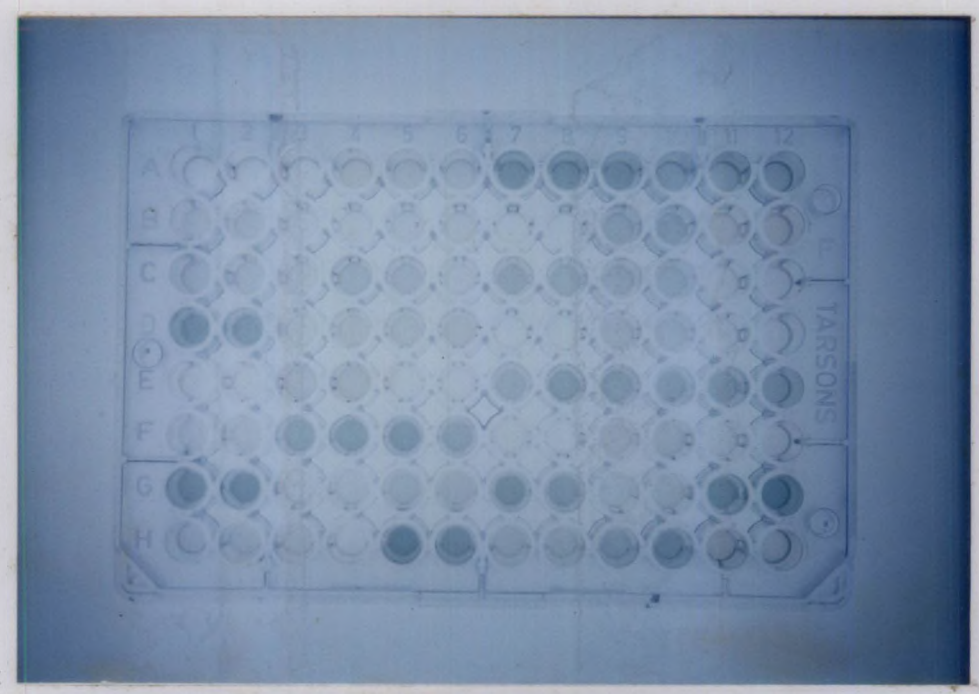

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

Fig. 10. Enzyme linked immunosorbent assay (ELISA)

Row A	(1)	: Substrate control
	(2 and 3)	: HRPO control
	(4,5 and 6)	: Negative sera controls
	(7 to 12)	: Positive sera controls
Row B to H	(1 to 12)	: Test sera samples in duplicates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4.6.4 District-wise distribution of EDS-76 antibodies

While screening chicken sera samples, Kottayam recorded the highest positive percentage (23.47) followed by Thrissur (16.5 per cent), Palakkad (8.57 per cent), Alleppey (6.90 per cent) and Trivandrum (3.57 per cent). In case of duck sera samples, Thrissur recorded the highest positive percentage (38.89), followed by Kottayam (32.86 per cent), Palakkad (22.22 per cent), Alleppey (18.46 per cent) and Trivandrum (10.53 per cent). District-wise distribution of EDS-76 viral antibodies are presented in Table 2 and Table 3.

4.6.5 Comparison of HI and ELISA

In this study, the percentage of positivity of HI is found to be lower when compared to that of ELISA (Fig. 11). But when the percentage of values were tested by 't' test for proportion(Snedecor and Cochran, 1989), it was found that $t = 1.66$ NS, $P<0.05$ and $t=1.16$ NS, $P<0.05$ for duck and chicken sera respectively, showing that there was no significant difference between these two tests. The comparative results of both the serological tests are presented in Table 4.

Table 2. Seroprevalence of EDS-76 virus in chicken in Kerala

District	Total number of samples screened	HI		ELISA	
		Number positive	Percentage positive	Number positive	Percentage positive
Alleppey	58	4	6.90	6	10.34
Kottayam	98	23	23.47	25	25.51
Palakkad	35	3	8.57	5	14.29
Thrissur	103	17	16.50	21	20.39
Trivandrum	28	1	3.57	2	7.14
Total	322	48		59	

Fig. 11. Comparison of HI and ELISA to detect EDS-76 antibodies in duck and chicken sera samples

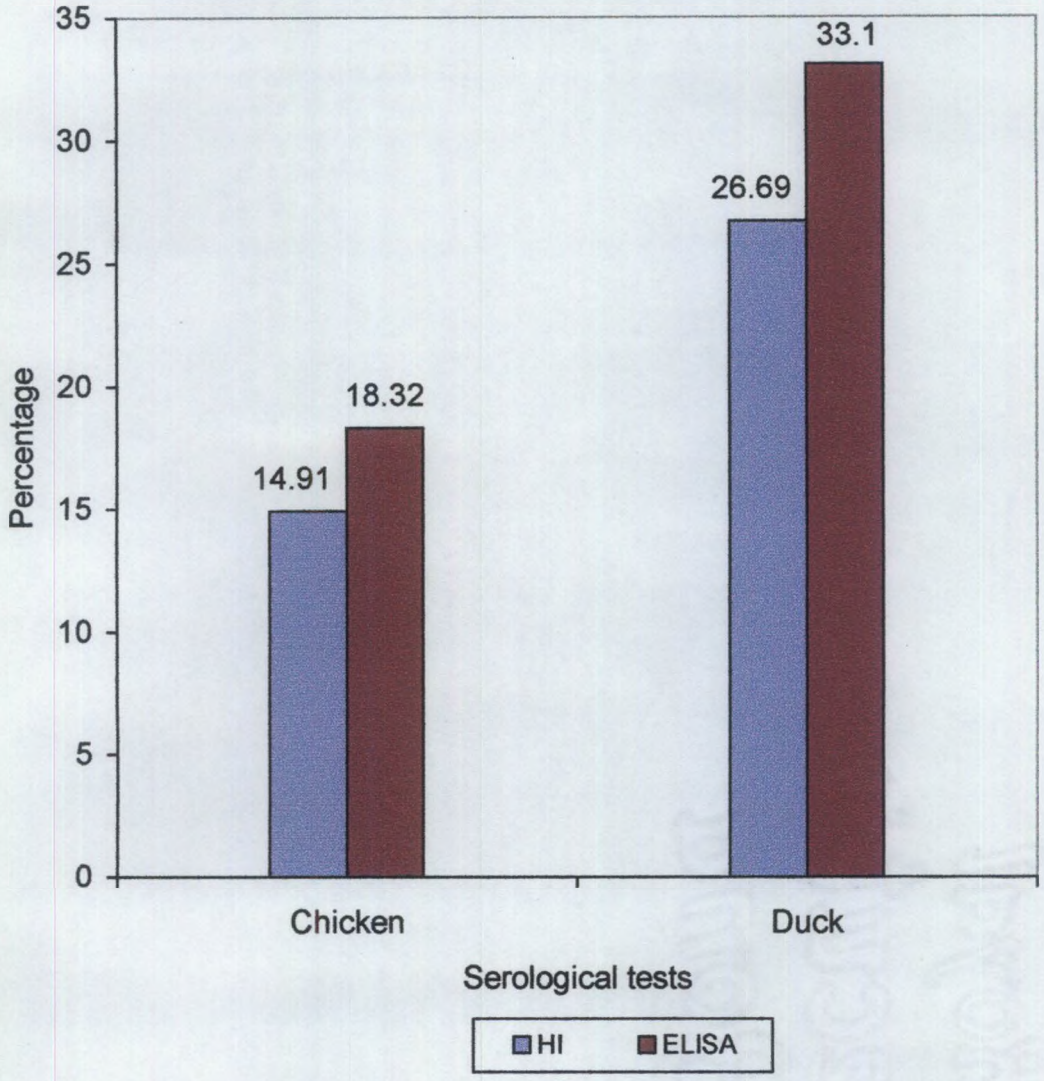


Table 3. Seroprevalence of EDS-76 virus in ducks in Kerala

District	Total number of samples screened	HI		ELISA	
		Number positive	Percentage positive	Number positive	Percentage positive
Alleppey	65	12	18.46	14	21.54
Kottayam	70	23	32.86	29	41.43
Palakkad	36	8	22.22	11	30.56
Thrissur	72	28	38.89	33	45.83
Trivandrum	38	4	10.53	6	15.79
Total	281	75		93	

Fig. 11. Comparison of HI and ELISA to detect EDS-76 antibodies in duck and chicken sera samples

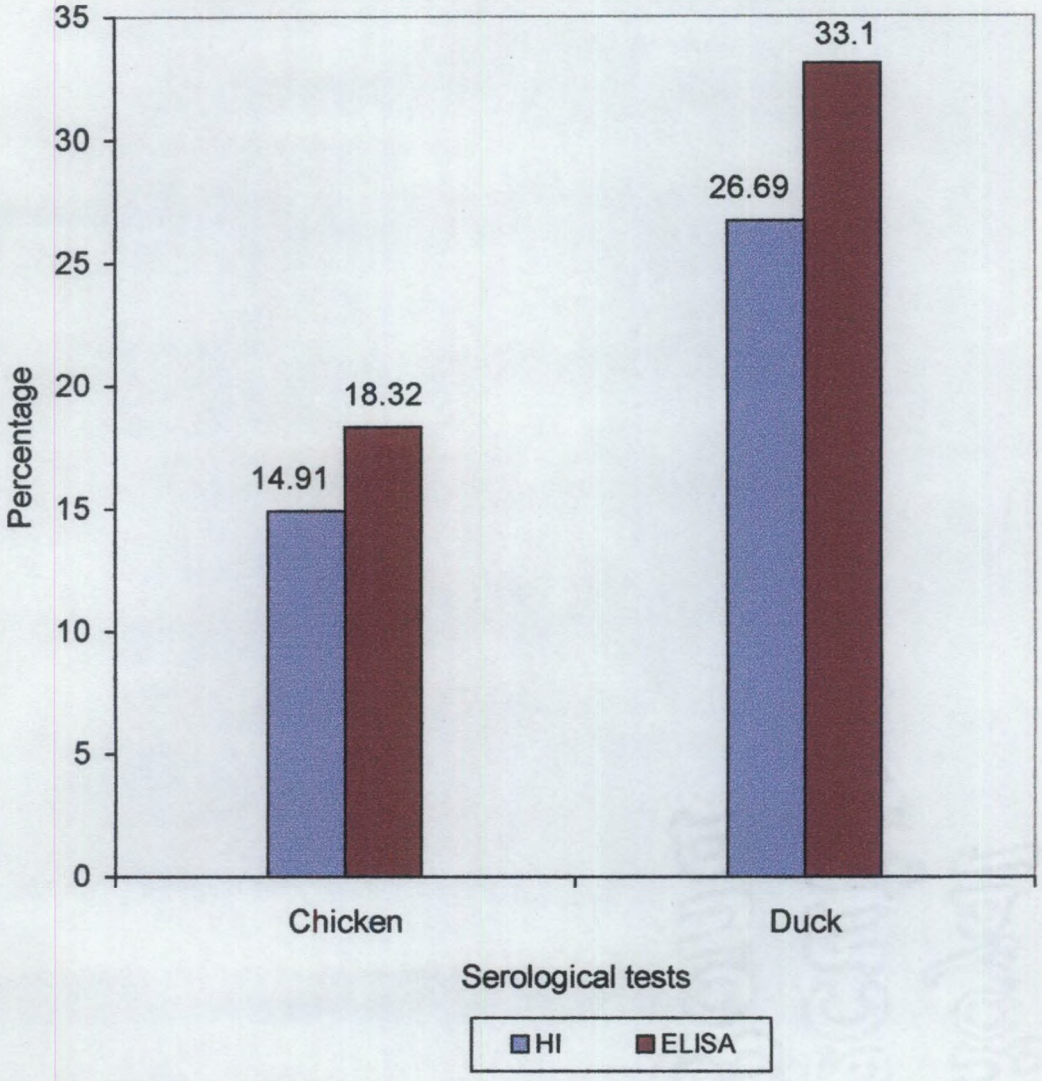


Table 4. Comparative sensitivity of HI and ELISA tests

Type of serum	Total number of samples screened	Total positive				T value (at 5% level)	Inference
		HI	Percentage	ELISA	percentage		
Chicken	322	48	14.91	59	18.32	1.16	NS*
Duck	281	75	26.69	93	33.10	1.66	NS*

*- Non significant

4.7 Revival of the viral strains

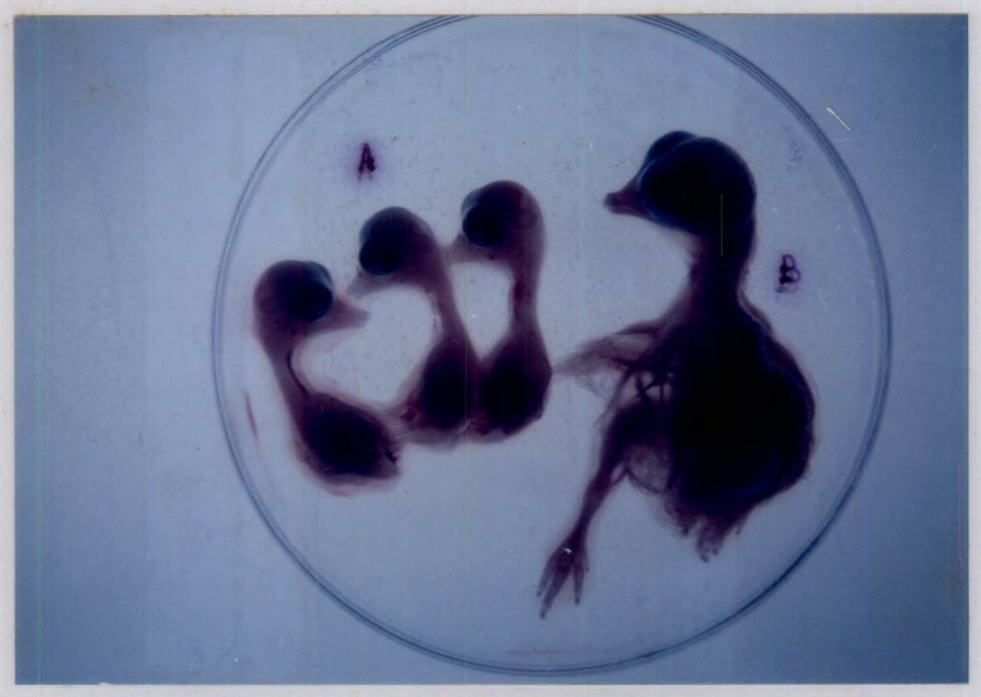
Three strains of EDS virus viz, reference strain, Madras strain and Namakkal strain were revived soon after their procurement from different laboratories. Along with them, Hyderabad strain which was preserved in the Department of Microbiology was also revived. The initial HA titre ranged from 3 $\log_2$ to 8 $\log_2$ HA units for different strains.

All the virus strains were revived successfully by inoculating into allantoic cavity of nine-day-old embryonated duck eggs. The reference strain and Hyderabad strain killed the embryos within two to three days of inoculation. There was also congestion of chorio-allantoic membrane (CAM). The embryos appeared stunted when compared with the controls (Fig. 12).

The above mentioned changes were comparatively less for Madras strain. Namakkal strain did not produce any change in embryos initially and had to be passaged for six times. Thereafter it started producing congestion of CAM which was less severe compared to the reference strain. Embryos did not reveal stunting.

Fig. 12. Nine-day old duck embryo infected with Hyderabad strain.

A- infected with Hyderabad strain
B. Control


A
B

After passaging in duck embryos, there was a significant increase in the HA titre (Table 5). Higher titres were obtained in three initial passages in the case of Hyderabad and reference strains, whereas higher titres were obtained after fourth and sixth passages respectively for Madras and Namakkal strains. On further passages, no more increase in titre was found for all the four strains.

4.8 Comparison of HI titre of EDS-76 strains using reference antiserum

The HA activity of all the four strains were inhibited specifically by the reference EDS-76 antiserum conforming their identity. The HI titres ranged from 8 $\log_2$ to 11 $\log_2$ HI units (Table 6). A high HI titre of 11 $\log_2$ was observed for homologous strain. Compared to the reference strain, the HI titre of Namakkal and Madras strains were low being 8 $\log_2$ and 9 $\log_2$ respectively. Only 1$\log_2$ difference was noticed between Hyderabad and reference strain.

4.9 Isolation of DNA

The DNA was extracted from the purified virus samples of all the four strains by phenol : chloroform method. They were then checked with 0.7 per cent

Table 5. Haemagglutination (HA) titre of EDS-76 viral strains (Log_2)

Sl. No.	Strain	Number of passages					
		1	2	3	4	5	6
1	Reference (strain 127)	8	11	14	14	14	14
2	Hyderabad	6	10	13	13	13	13
3	Madras	7	10	11	13	13	13
4	Namakkal	3	5	7	9	11	12

Table 6. Comparison of HI titre of EDS-76 strains using reference antiserum.

Sl. No.	Strain	HI titre (Log_2)
1	Reference (strain 127)	11
2	Hyderabad	10
3	Madras	9
4	Namakkal	8

agarose gel. A distinct DNA band of more than 21 Kbp was observed with all the four strains.

4.10 Restriction enzyme analysis

Restriction endonuclease Hind III, Bam HI and EcoRI were used to digest the DNA of the four strains. The size of the whole genome was estimated by adding the size of the fragments obtained with each enzyme.

4.10.1 DNA restriction pattern with Hind III

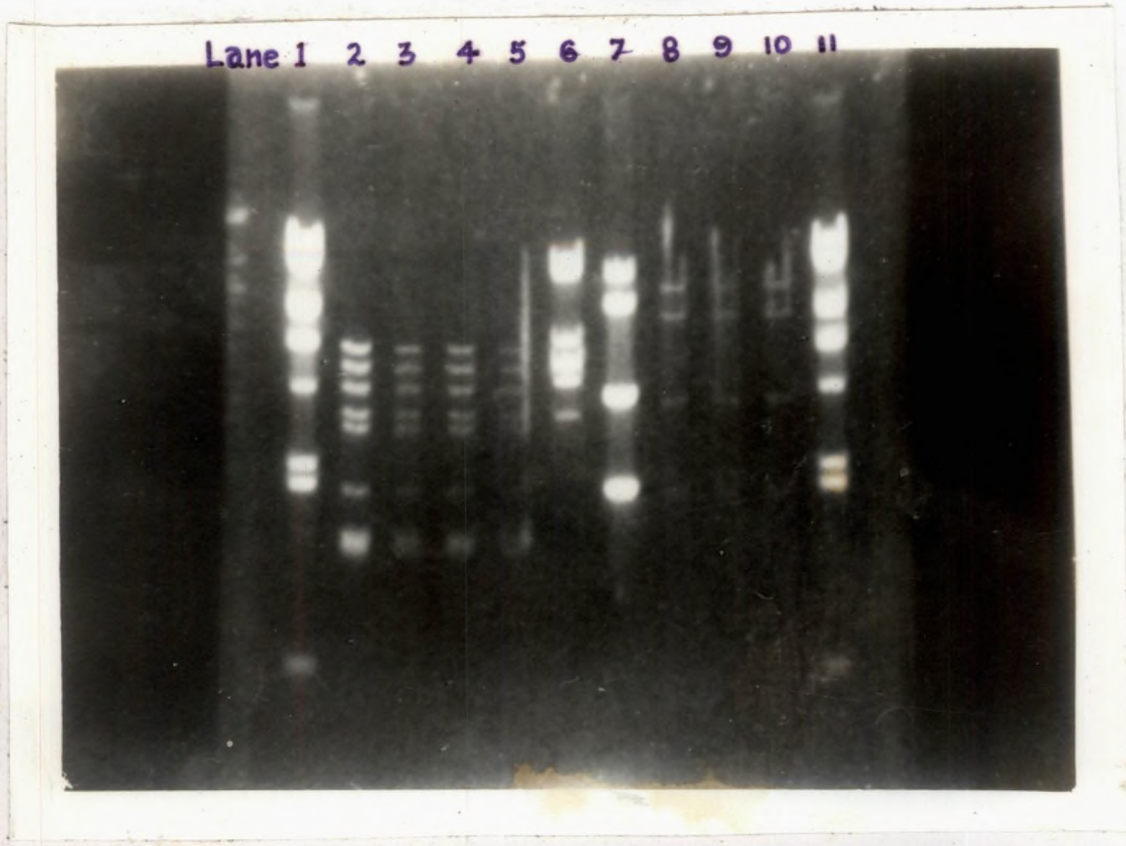
Restriction endonuclease Hind III cleaved all the four strains of EDS-76 viral genome into ten fragments (A,B,C,D,E,F,G,H,I and J). The size of the fragments ranged from 1.45 Kbp to 6.9 Kbp (Fig. 13). There was no difference between the different strains in the restriction digestion pattern (Table 7). The size of the whole viral DNA was estimated as 32.8 Kbp and the molecular weight as 21.8 MDa.

4.10.2 DNA restriction pattern with Bam HI

Four fragments each (A,B,C and D) were noted for all the EDS viral strains after restriction with Bam HI (Fig. 13). The fragments corresponding in size to 16.2 Kbp and 10 Kbp seen in case of all the three strains were missing in the reference strain. Instead

Fig. 13. Restriction pattern of four strains of EDS-76 viral DNA on digestion with HindIII and BamHI

Lane 1 and 11	:	λ DNA HindIII digest with eight fragments of standard size.
Lane 2,3,4 and 5	:	HindIII digested DNA of Reference, Hyderabad, Madras and Namakkal strains showing 10 fragments each
Lane 6	:	λ DNA EcoRI digest with six fragments of standard size.
Lane 7,8,9 and 10	:	BamHI digested DNA of Reference, Hyderabad, Madras and Namakkal strains showing four fragments each.


Lane 1 2 3 4 5 6 7 8 9 10 11

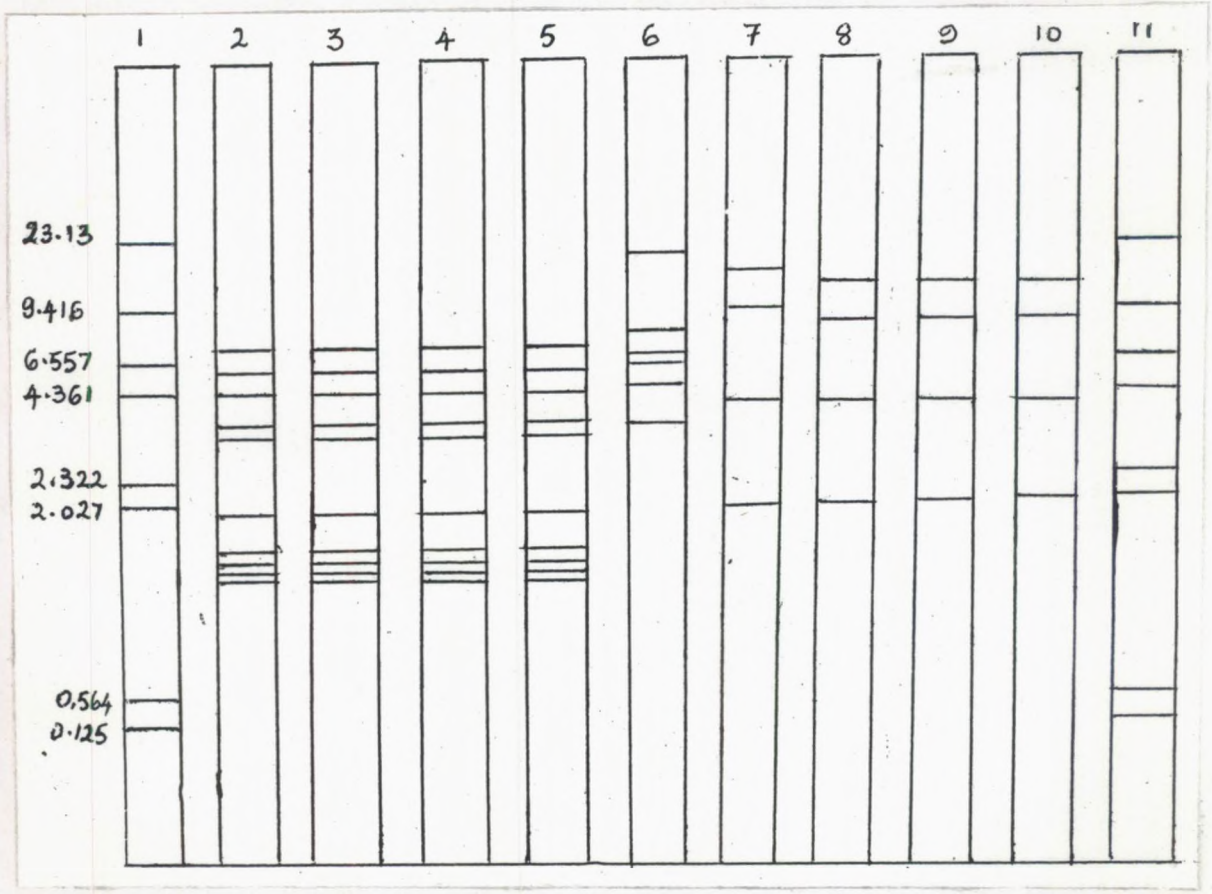

1 2 3 4 5 6 7 8 9 10 11
23.13
9.416
6.557
4.361
2.322
2.027
0.564
0.125

Table 7. Restriction fragments of EDS-76 viral genome with Hind III enzyme

Sl. No.	Fragment	Size (Kbp)	Molecular Weight (MDa)
1	A	6.9	4.6
2	B	5.7	3.8
3	C	4.5	3.0
4	D	3.6	2.4
5	E	3.4	2.3
6	F	2.2	1.5
7	G	1.8	1.2
8	H	1.7	1.1
9	I	1.5	1.0
10	J	1.45	0.9
Total		32.8	21.8

Table 8. Restriction fragments of EDS-76 viral genome with Bam HI enzyme

Sl. No.	Fragment	Size (Kbp)		Molecular weight (MDa)	
		For reference strain	For other three strains	For reference strain	For other three strains
1	A	17.2	16.2	11.5	10.8
2	B	11.0	10.0	7.3	6.7
3	C	4.1	4.1	2.7	2.7
4	D	2.0	2.0	1.3	1.3
	Total	34.3	32.3	22.8	21.5

Table 9. Restriction fragments of EDS-76 viral genome with EcoRI enzyme

Sl. No.	Fragment	Size (Kbp)	Molecular weight (MDa)
1	A	17.8	11.9
2	B	9.4	6.3
3	C	4.9	3.3
4	D	1.3	0.9
	Total	33.4	22.4

Fig. 14. Restriction pattern of four strains of EDS-76 viral DNA on digestion with EcoRI

Lane 1,2,3 and 4 : EcoRI digested DNA of Reference, Hyderabad, Madras and Namakkal strains showing four fragments each.

Lane 5 : λ DNA HindIII digest of standard size


1
2
3
4
5
1
2
3
4
5
23.13
9.416
6.557
4.361
2.322
2.027
0.564
0.125

of that, two fragments of sizes 17.2 Kbp and 11 Kbp were detected (Table 8). Difference in the size and molecular weight of the whole genome was also noticed between the reference strain and other three isolates. The size of the whole genome of reference strain was found to be approximately 34.3 Kbp and the molecular weight, 22.8 MDa. For the other three strains, the size and molecular weight of the whole genome was 32.3 Kbp and 21.5 MDa respectively. The fragments of 4.1 Kbp and 2 Kbp were of the same size for all the strains.

4.10.3 DNA restriction pattern with EcoRI

The DNA of all the four strains of EDS-76 virus, yielded four identical fragments (A,B,C and D) when cleaved with EcoRI enzyme (Fig.14). The sizes of the fragments ranged from 1.3 Kbp to 17.8 Kbp. The size of the whole genome was found to be approximately 33.4 Kbp and the molecular weight, 22.4 MDa (Table 9).

DISCUSSION

5. DISCUSSION

The economy of poultry industry mainly depends upon egg production. In Kerala, there are often reports from farmers about a sudden drop in egg production from the peak in their poultry flocks. Hence a detailed serological study was undertaken to unearth the involvement of EDS-76 virus using HI and ELISA. Also in the present study, three Indian strains of EDS-76 virus were compared with the reference virus (strain 127) at the molecular level by RE analysis.

5.1 Antiduck and antichicken sera

The antisera raised against duck and chicken sera produced 11 and 13 distinct precipitin arcs respectively by IEP. Only nine precipitin arcs were obtained by Malmarugan (1997) and Singh (1997) for duck serum following the same schedule. In a similar study, Nair (1990) observed 13 precipitin arcs when a lengthier immunization protocol was used to raise antiduck serum.

5.2 Globulin precipitation by ammonium sulphate

Salt precipitation remains most convenient, though some important precautions should be taken to obtain excellent instead of mediocre results. Some of the precautions taken in this study were proper choice of the salt and its concentration, and the correct measurement of the pH of the concentrated salt solution.

Saturated solution of ammonium sulphate (33 per cent, pH 7.0) was chosen to precipitate the duck and chicken globulins from their respective sera. Unlike sodium sulphate saturation, ammonium sulphate saturation was temperature independent. Hence, it was not necessary to control the temperature carefully during precipitation, as opined by Goers (1993).

The use of solid ammonium sulphate results in localized high concentration of the salt, leading to precipitation of non-immunoglobulins. In this investigation, addition of SAS drop by drop to the stirred serum avoids the above defects.

The globulin recovery from duck and chicken serum was estimated to be 15 mg and 18 mg of protein

per ml respectively. The present observations are in conformity with that of Nair (1990) who reported a protein concentration of 16.88 mg per ml at 33 per cent ASS and 59.69 mg per ml at 40 per cent ASS precipitin. Based on IEP analysis, he opined that 33 per cent ASS precipitin was superior to 40 per cent ASS.

The bold line observed by AGPT with duck serum against its antiserum was absent in the case of separated globulin. This indicates that this bold line is of albumin which was removed by salting out. The presence of two clear lines between duck globulin and antiserum indicates the concentration of the globulin fraction, while the occurrence of only a diffused line between duck serum and its antiserum is suggestive of low globulin concentration in the duck serum.

Nair (1990) had reported six precipitin arcs by IEP for the separated duck gammaglobulins when tested against antiduck serum. In the present study, the separated duck gammaglobulins produced only five precipitin arcs.

Similar pattern of lines were observed by AGPT with chicken serum against its antiserum. In addition,

the presence of line of identity between the chicken serum and its globulin indicates the higher concentration of globulins in chicken serum compared to that of duck serum.

Chicken gammaglobulin produced only three precipitin arcs by IEP and this less number of arcs indicates that it was more pure when compared to that of duck globulin fraction.

5.3 Antiduck and antichicken gammaglobulins

Duck and chicken gammaglobulins having 15 mg and 18 mg of protein per ml respectively when repeatedly injected into rabbit at weekly interval, produced sufficient level of antiglobulins by 31st day. The gammaglobulin fraction of the rabbit serum was separated as in case of duck and chicken globulin and the purity was checked by IEP.

On IEP analysis, the separated duck globulin produced two bold and two faint precipitin arcs. In this study only salting out was done to separate the globulins and further purification was not attempted. This has resulted in the production of four precipitin arcs against antiduck globulin, while the chicken

globulin showed only one bold and one faint arc against its antiglobulin. The lesser number of lines in case of chicken globulin indicates it was more pure when compared to that of duck globulin.

5.4 Conjugation of duck and chicken antiglobulins with HRPO

The choice of the enzyme and method of conjugation depend upon a number of factors. The enzyme Horse radish peroxidase ("Bangalore Genei" Pvt. Ltd.) was used for conjugation. Several workers suggested peroxidase as a good choice for conjugation among the three most commonly used enzymes (Avrameas, 1969; Nakane and Pierce, 1966). Because it was more stable, cheapest and had a much faster turnover time than other enzymes.

By one step glutaraldehyde method, HRPO could not be conjugated very well. For this reason, a two-step glutaraldehyde procedure was employed in which the protein was reacted first with glutaraldehyde and then, after dialysis, it was coupled with the enzyme. Kemeny (1991) opined that this method was best as it lowered the background binding which was essential for very sensitive assays.

The prepared conjugate was then stored in small aliquots at -20°C. Voller *et al.* (1976) reported that peroxidase conjugate stored in a lyophilised state had retained its full activity for one and a 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.

5.5 Seroprevalence

The present investigation revealed the presence of haemagglutination inhibiting and immunobinding antibodies against EDS-76 reference virus in five districts of Kerala (Table 1 and 2).

District wise distribution of EDS-76 antibodies in chicken sera samples showed that Kottayam recorded the highest positive percentage (23.47) than the other districts. In Kottayam, the samples were collected mostly from birds with history of a significant drop in egg production, and consequently a higher percentage of incidence of seropositivity. Though Kottayam recorded the maximum incidence of EDS-76, the mean HI titre was found to be low during the phase of reduced egg

production. This finding is in accordance with that of earlier workers (Mohanty *et al.*, 1984) where they observed a low HI titre during the phase of drop in egg production.

While screening duck sera samples, Thrissur recorded the highest positive percentage (38.89) followed by Kottayam (32.86 per cent), Palakkad (22.22 per cent), Alleppey (18.46 per cent) and Trivandrum (10.53 per cent).

The overall incidence of seropositivity in chicken in Kerala was 14.91 per cent by HI test. In Kerala, Karnataka, Andhra Pradesh and Tamil Nadu, earlier workers have reported an overall incidence of 4.1 per cent, 27.3 per cent, 28.15 per cent and 15.57 per cent respectively (Sulochana and Sudharma, 1987; Reddy and Raghavan, 1987; Chetty *et al.*, 1988 and Shaw *et al.*, 1995). This may be suggestive of the fact that the virus could have gained entry into poultry populations of Kerala as inapparent infection from the neighbouring states.

Apart from Kottayam and Thrissur, apparently healthy birds showing seropositivity,(3.57 per cent to

8.57 per cent)in other districts without manifestation of classical forms is suggestive of the widespread prevalence of inapparent infection with EDS-76 virus. The occurrence of inapparent infection with EDS-76 has been reported by many researchers (Rampin *et al.*, 1978; Gupta *et al.*, 1985; Shakya and Dhawedkar, 1991 and Sekar *et al.*, 1992).

Of the two tests, HI and ELISA, employed for the detection of EDS-76 antibodies with 322 chicken sera samples, 48 samples were positive by HI (14.91 per cent) as compared to 59 positive samples detected by ELISA (18.32 per cent). With 281 duck sera samples, 75 were positive by HI (26.69 per cent) as compared to 93 positive samples by ELISA (33.10 per cent) (Table 3). In the present study, it has been possible to detect more number of positive cases with ELISA compared to HI. A HI titre of 3 $\log_2$ and above has been taken as positive in this investigation in accordance with earlier workers (Bartha *et al.*, 1982; Shakya and Dhawedkar, 1991 and Sekar *et al.*, 1992). Mohanty *et al.* (1984) have taken HI titre of 1 $\log_2$ as positive HI titre. In this study, if 1 $\log_2$ HI titre was taken as positive, the percentage positivity for chicken and duck sera samples would be 18.01 per cent and 32.03

per cent respectively. In such case, it is also possible that the HI test is as sensitive as ELISA. Therefore, higher HI titre considered in this study could be the reason attributed to the lower incidence of detection of EDS-76 by HI in comparison with ELISA.

Even though more positive samples were detected by ELISA, statistically there was no significant difference between the two tests employed. The probable reason for this lower efficacy of ELISA might be due to the use of prepared conjugate rather than a commercial one. Further attempts in purification of globulins or use of ELISA kits might have resulted in statistically significant difference.

Hence, HI test could be employed as the serological test of choice since it was simple to perform and to quantify the antibody level, sensitive, specific and economical as opined by Adair *et al.* (1986) and Shaw *et al.* (1995).

In the present study, the overall incidence of seropositivity in chicken was 14.91 per cent as against 4.1 per cent reported earlier by Sulochana and Sudharma (1987) in Kerala. The high proportion of birds showing

antibodies to EDS-76 reveals, that the infection has spread state-wise since then and may be the major etiological factor associated with drop in egg production in poultry.

Presence of HI antibodies to EDS-76 virus in ducks indicated that the normally apathogenic virus for ducks had gained virulence on infecting chicken, thereby affecting their egg production.

5.6 Restriction endonuclease analysis of EDS-76 viral isolates

DNA fingerprinting is a modern technique for the present day molecular biologists to distinguish genetically different, yet serologically similar strains of viruses. In general, adenoviruses contain more number of inverted repeat sequences of the viral DNA. Considerable fingerprint variations occur, since sequences may have been added to or deleted from the existing fragments, resulting in mobility differences in the corresponding fragments from different isolates. This type of interstrain fingerprint variations occur more frequently in restriction fragments containing inverted repeat sequences of viral DNA (Zhang and Nagaraja, 1989).

In the present study the restriction endonuclease analysis of the DNA of the four strains of EDS-76 virus, was carried out using three restriction endonucleases, namely BamHI, EcoRI and Hind III.

5.6.1 DNA restriction pattern with Hind III

The endonuclease, Hind III, six base cutter, cleaved EDS-76 viral genome of the four strains into 10 identical fragments. The result obtained in the present study is in agreement with the reports of Zsak and Kisary (1981b), Todd *et al.* (1988), Zakharchuk *et al.* (1993), and Gagi (1995) with regard to both the number and size of the fragments.

5.6.2 DNA restriction pattern with BamHI

The enzyme BamHI generated four fragments when used to digest the genome of all the four strains of EDS-76 virus. The number of fragments obtained in this study were comparable to the results of Zsak and Kisary (1981b), Todd *et al.* (1988), Zakharchuk *et al.* (1993), Duan-YuYou (1995) and Gagi (1995). Out of the four fragments, first two fragments exhibited difference between the reference strain and other three isolates. No fragments corresponding to the size of 16.2 Kbp and 10 Kbp was found after the cleavage of reference strain

DNA with BamHI. This difference may be due to the genome changes in the BamHI recognition sequences, that must have occurred on repeated passages of the reference virus. This reasoning is in conformity with Hammarskjold and Winberg (1980) and Robinson and Winberg (1984) who had worked on human adenovirus prototypes 15 and 3 respectively.

5.6.3 DNA restriction pattern with EcoRI

The enzyme EcoRI, that recognises DNA sequences of 6 Kbp, cleaved the DNA into four identical fragments. The number of fragments obtained in this study is in agreement with the reports of Zsak and Kisary (1981b), Todd *et al.* (1988), Zakharchuk *et al.* (1993), Duan-YuYou (1995) and Gagi (1995).

Comparison of the DNA fingerprint of all the four strains digested with restriction endonucleases HindIII, BamHI and EcoRI revealed identical banding pattern, thereby conforming the genetic similarity of the strains. EDS-76 virus isolates, from different regions of the world have been classified into three groups, I, II and III based on the RE analysis. Among this the strain 127 is classified under group I. As the three indigenous strains had the same restriction

pattern as that of the reference virus, it is concluded that these three strains also could be classified under group I.

Even though no difference could be observed between four strains by RE analysis, an appreciable difference was noticed in the HI titre. This is indicative of genomic difference between the strains. This investigation was made employing the commonly used enzymes viz. HindIII, Bam HI and EcoRI. The use of rare cutting enzymes would have established the genomic difference between the strains.

The present study reveals widespread infection of EDS-76 in chicken in Kerala. To control this disease, in addition to raising the standards of hygiene and sanitisation, vaccination of the breeder stocks is a must. In this situation, attempts should be made to isolate the virus and preparation of vaccine using the local isolate. Experimentally Baxendale (1978b) and Chetty and Rao (1988) obtained good immune response with inactivated EDS vaccine prepared from field isolates. Though inactivated EDS vaccine is available commercially, it is not in vogue in Kerala. Hence it is

recommended to vaccinate the parent breeder stock at 14 to 16 weeks of age.

Further research is needed to study the sequencing of Indian isolates and to develop recombinant vaccine using adenovirus as vectors. Attempts should also be made to apply nucleic acid hybridisation techniques using probes for the development of field kits.

SUMMARY

6. SUMMARY

The drop in egg yield in poultry is due to multiple etiological factors of which Egg drop syndrome (EDS-76) virus is of major importance. As the infection remained latent till the bird reached sexual maturity, many a time it goes unnoticed. In Kerala, there are often complaints from farmers about a sudden drop in egg production in their poultry flocks. Hence this study was undertaken to assess the seroprevalence of EDS-76 by HI and ELISA. Also restriction enzyme analysis was done to characterise the indigenous strains based on genomic properties.

Duck and chicken antisera were raised in rabbits and the production of antibody and its purity was checked by AGPT and IEP. When sufficient level of antibodies were obtained , rabbits were bled, serum separated and stored at -20°C.

Duck and chicken globulins were separated from their respective sera and its purity was checked as above. Hyperimmune sera against duck and chicken globulins were raised in rabbits.

The duck and chicken antigammaglobulins with a protein concentration of 5-7 mg per ml were used for labelling with HRPO. Such conjugates were stored at -20°C, till used.

Hyperimmune sera against EDS-76 reference strain was raised separately in cockerels and rabbits and were used for HI and ELISA.

In the present study, seroprevalence of EDS-76 in five districts of Kerala was taken up both in duck and chicken flocks. Inspite of the fact that birds screened in this study were apparently normal, many had history of drop in egg production.

The seroconversion study was conducted by employing both HI and ELISA as serological tests using EDS-76 reference virus (Strain 127).

Out of 322 chicken sera samples screened, an overall incidence of 14.91 per cent was recorded. Kottayam district recorded the highest percentage of incidence, where there was a significant drop in egg production. Apparently healthy birds showing positivity in other districts indicates the prevalence of

inapparent infection. Among 281 ducks screened, an overall incidence of 26.69 per cent was recorded.

Comparative efficacy of the two tests employed revealed no significant difference statistically. Hence it is recommended that HI test can be used for the detection of EDS-76 infection as it is simple, sensitive and reliable.

Four strains of EDS-76 virus namely, strain 127, Hyderabad strain, Madras strain and Namakkal strain, were acquired from different laboratories. The strains were identified with HA test and confirmed with HI test employing chicken erythrocytes against EDS-76 reference virus antiserum.

The different strains were propagated in nine-day-old embryonated duck eggs. The allantoic fluid was collected after five days' post-inoculation and the virus strains were purified by sucrose cushion ultracentrifugation.

The DNA was extracted from the purified virus samples of all four strains by phenol : chloroform method. It was then subjected to RE analysis using

three restriction endonucleases, BamHI, EcoRI and HindIII which yielded 4, 4 and 10 numbers of identical restriction fragments respectively with all four strains.

EDS-76 virus isolates, from different regions of the world have been classified into three groups, I, II and III based on the RE analysis. Among this the strain 127 is classified under group I. As the three indigenous strains had the same restriction pattern as that of the reference virus, it is concluded that these three strains also could be classified under group I.

Based on size of the restriction fragments obtained with three enzymes, the average size of the DNA was estimated to be about 32.8 Kbp and the molecular weight as 21.9 MDa.

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SEROPREVALENCE AND RESTRICTION ENZYME ANALYSIS OF EGG DROP SYNDROME VIRUS

By

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ABSTRACT OF A THESIS

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ABSTRACT

In the present study, seroprevalence of EDS-76 was conducted in five districts of Kerala in duck and chicken flocks using HI and ELISA. Out of 322 chicken and 281 duck sera samples screened, an overall incidence of 14.91 per cent and 26.69 per cent respectively were recorded. The high proportion of birds showing antibodies to EDS-76 reveals that the infection is widespread in Kerala and may be the major etiological factor associated with drop in egg production in poultry.

Among the two serological tests namely, HI and ELISA employed for the detection of EDS-76 viral antibody, HI was found to be simple, sensitive and reliable. It is concluded that HI test could be used for the detection of EDS-76 infection in poultry flocks.

Restriction DNA fingerprinting of the three indigenous strains were carried out in conjunction with the reference strain to check for any genetic variation between the strains. Comparison of the DNA fingerprint

of all the four strains digested with restriction endonucleases BamHI, EcoRI and HindIII revealed identical banding pattern thereby conforming the genetic similarity of the strains.