

STUDIES ON
CYTOPATHOGENICITY OF
EGG DROP SYNDROME-76
VIRUS

Thesis

Submitted to the

**G. B. Pant University of Agriculture & Technology,
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By

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PANTNAGAR
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CERTIFICATE

This is to certify that the thesis entitled "**STUDIES ON CYTOPATHOGENICITY OF EGG DROP SYNDROME-76 VIRUS**" submitted in partial fulfilment of the requirements for the degree of **Master of Science** with major in **Molecular Biology and Biotechnology** of the college of post graduate studies, G. B. Pant University of Agriculture and Technology, Pantnagar, is a record of bona fide, research carried out by **Ms. Gaurangi Srivastava, ID. No. 30831** under my supervision and no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation has been acknowledged.

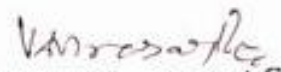
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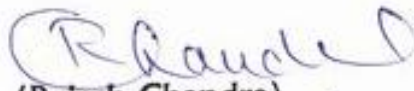


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CERTIFICATE

We, the undersigned, members of the advisory committee of Ms. Gaurangi Srivastava, Id No. 30831, a candidate for the degree of Master of Science with major in Molecular Biology and Biotechnology "STUDIES ON CYTOPATHOGENICITY OF EGG DROP SYNDROME-76 VIRUS" may be submitted in partial fulfilment of the requirements of above degree.


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Poultry production has gained momentum during last two decades in our country and changed from backyard poultry rearing to an industry. This commercialization has been achieved by intensification of poultry farming using high stocking density, multiage group rearing system, development of hybrid strains with high production potential and import of newer breeds and varieties, which have triggered several new diseases.

A considerable economic downfall to the poultry industry is caused by mortality of large proportion of birds, adversely affected growth and decreased egg production. Many microbial agents including egg drop syndrome (EDS-76) virus are responsible for drop in egg production.

EDS-76, an economically important viral disease of laying hens characterized by a drop in egg production and egg shell defects, was reported for the first time in the Netherlands in 1976 (**Van Eck, 1976**).

Since then, the virus has become almost worldwide in distribution (**Chandra et al., 1997**). The causative agent known

as EDS-76 virus was isolated from chickens in Australia, Belgium, Great Britain, Hungary, India, Israel, Italy Japan, Northern Ireland, Singapore and Taiwan,

In other countries like Bolivia, Brazil, Denmark, Germany, Mexico, New Zealand and Nigeria, serological evidence of EDS-76 virus infection has been well documented.

In addition to chickens, the infection also has been reported in many other avian species, viz., ducks, geese, Japanese quail, guinea fowl, turkeys, pheasants, house sparrows, *grebes* and marsh birds etc.

The occurrence of EDS-76 appears to be independent of seasonal variation but influenced by the age of the birds. The infection is most prevalent at the start of laying period (16-20 weeks) and continues upto 4-12 weeks.

The egg drop syndrome is characterized by diarrhoea at the onset of laying followed by drop in egg production and formation of incompletely calcified deformed eggs. The eggs laid by infected birds may be shell-less, soft-shelled and/or thin-shelled associated with loss of egg-shell pigment. In spite of abnormality in eggs laid by infected hens, the internal egg quality, fertility and hatchability of eggs remain unaffected.

In a flock, whenever there is a failure to reach peak production or there is a serious drop in production and the produced eggs have poor quality shells, EDS-76 virus almost should always become a potential possibility.

Definitive diagnosis of EDS, however, requires laboratory support. Certain serological tests such as haemagglutination inhibition (HI) test, ELISA, serum neutralization test (SNT), double immunodiffusion (DID) test, fluorescent antibody test (FAT) and plaque reduction test are found to be very helpful in confirmatory diagnosis of disease.

The disease comes in three different forms with the endemic form being more common than the classic or the sporadic form. The spread of the infection could occur both vertically and horizontally and thus the chances of infection of healthy birds in a flock are high.

The virus is still posing a serious threat to the economy of almost all the poultry producing countries of the world. Keeping above facts in view, the present study has been designed with the following objectives:

1. To propagate the virus in the allantoic fluid of duck eggs.
2. To adapt the virus from allantoic fluid of duck eggs in chicken embryo liver cell culture.

3. To study the cytopathic effects of virus in unstained and May-Grunwald Geimsa stained preparations.
4. To standardize haemagglutination (HA), haemagglutination inhibition (HI) and fluorescent antibody tests (FAT) for the diagnosis of EDS-76.

2.1 HISTORY AND DISTRIBUTION

Avian adenovirus was first reported by **Yates *et al.* (1954)** from chicken embryos, which has later been classified into three groups (I to III), some being associated with specific diseases. These are egg drop syndrome-76 (EDS-76) in hens (group III), haemorrhagic enteritis of turkeys and marble spleen virus disease of pheasants (group II) and conventional adenovirus associated with chickens (FAV 1-12), geese (DAV-2) and turkeys (TAVI-2) in group I.

Since the first description of the disease from the Netherlands (**Van Eck *et al.*, 1976**), the virus has almost become worldwide in distribution (**Chandra *et al.*, 1997**). EDS-76 virus has been isolated from chickens in France (**Picault, 1978**), Great Britain (**Baxendale, 1978**), Northern Ireland (**McFerran *et al.*, 1978**), Belgium (**Meulemans *et al.*, 1979**), Italy (**Zanella *et al.*, 1980**), Hungary (**Zsak and Kisary, 1981**), Australia (**Firth *et al.*, 1981**), Japan (**Yamaguchi, 1981**), Taiwan (**Lu *et al.*, 1985**) and South Africa (**Bragg *et al.*, 1991**).

In other countries like Denmark (**Badstue and Smidt, 1978**), Brazil (**Hwang et al., 1980**), Mexico (**Rosales et al., 1980**), Nigeria (**Nawathe and Abagunde, 1980**), New Zealand (**Howell, 1982**) Germany (**Hlinok et al., 1998**) serological evidence of EDS-76 virus infection has been found.

Though the virus infection seems to have worldwide distribution, only one serotype has been recognized (**Darbyshire and Peter, 1980; Yamaguchi et al., 1981; Chandra et al., 1997**).

In addition to chicken, the infection also has been reported in many other avian species, viz., ducks (**Badstue et al., 1978; Baxendale, 1978; McFerran, 1978; Villegas et al., 1979; Kaleta et al., 1980; Malkinson and Weisman, 1980**), geese (**Kaleta et al., 1980; Zsak and Kisary, 1982**), Japanese quail (**Das and Pradhan, 1992**), Turkeys, pheasants (**Parsons et al., 1980; Zanella et al., 1980**), house sparrows (**Rampin et al., 1978**), grebes and marsh birds (**Gulka et al., 1984**), cattle egrets (**Malkinson and Weisman, 1980**) and herring gulls (**Bartha et al., 1980**), but the disease was not observed in these birds.

A study conducted in Germany (**Kaleta et al., 1980**) and Japan (**Yamaguchi et al., 1982**) revealed that antibody against

EDS-76 virus existed in domesticated and wild ducks and geese before 1976 and in chickens, it first appeared in 1976 and the number increased suddenly in 1979 (**Kaleta et al., 1980; Yamaguchi et al., 1982**), hence called EDS-76.

Generally, all breeds of chicken are susceptible to experimental infection with EDS-76 virus. However, data from natural outbreaks suggest that broiler breeders and brown-egg producing heavy breeds are more severely affected than the white-egg laying birds. Susceptible birds may be infected by EDS-76 virus at any age but higher doses of virus are required to infect chicks with maternal antibodies (**Baxendale et al., 1980**). Since the disease occurs around the peak production age of a chicken, it is suggested that infection might just be the reactivation of a latent infection (**McFerran et al., 1978**),

The disease occurs throughout the year, i.e., from October to March (**Higashihara et al., 1983**) and from April to October (**Lu et al., 1985; Justacara et al., 1988**). It has been observed that from a peak of 92% production there may be descent to 70-75% (**McFerran et al., 1978**). In Quail flocks, decrease in egg production may reach 50.6% (**Das and Pradhan, 1992**). The production may or may not return to normal (**McFerran et al., 1978**).

2.2 ETIOLOGY

Egg drop syndrome-1976 is caused by an avian adenovirus (**Firth *et al.*, 1981; Higashihara *et al.*, 1993**). Typical adenovirus particles often aggregated in paracrystalline array forming a cubic lattice in the nuclei of thin sectioned cells (**Kraft *et al.*, 1979; Higashihara *et al.*, 1983**) have been observed.

The EDS-76 virus is filterable through membrane filter of 100 nm or greater pore sizes but not through 50 nm filters (**Ramkumar *et al.*, 1992**).

Different viral strains isolated from different geographical regions, viz., D61, BC 14 (**Darbyshire and Peters. 1980**), JBP (Pune strain), SPC (Banglore strain) (**Shakya and Dhawedkar, 1991**), E77, 3877 (**Zanella *et al.*, 1980**) and a Belgian strain behave similar to each other and are morphologically and serologically indistinguishable (**Meulemans *et al.*, 1979; Firth *et al.*, 1981**). However, U.K. and Belgian isolates forming one group could be well differentiated from duck isolates in U.K. The Australian isolate with a DNA deletion (0.4 kbp) at one end of genome (32.6 kbp) differed in this respect from European isolates and formed a third group (**Todd *et al.*, 1988**).

It is reported that no relationship was found between EDS-76 virus and the fowl adenovirus (FAV) using indirect

immunofluorescence, immunodiffusion or serum neutralization tests (**Adair et al., 1979 b**). The EDS-76 virus strain 127 shared an antigen with fowl adenovirus type-1 (FAV-1) (**McFerran et al., 1978**).

2.3 MORPHOLOGY

Avian adenoviruses are icosahedral particles. The size of EDS-76 virus was reported to range from 76 nm (**McFerran et al., 1978 a**) to 80 ± 5 nm (**Kraft et al., 1979**). **Kraft et al. (1979)** were able to demonstrate the typical adenovirus morphology, with triangular faces and six capsomeres on the edge, and a single 25 nm fiber projecting from each vertex.

Virus particles of 70-75 nm have been seen in the nucleus of the infected cells in thin-section preparation of EDS-76 virus infected chicken embryo liver cell cultures (**Adair et al., 1979a**). Particles of 68-80 nm in diameter have been described in nuclei of epithelial cells of oviduct mucosa (**Taniguchi et al., 1981**).

2.4 PHYSICO-CHEMICAL PROPERTIES

The EDS-76 virus is filterable through membrane filters of 100 nm or greater pore sizes (**Ramkumar et al., 1991**). There have been many discrepancies observed in the density of EDS-76 virus and the biological property of haemagglutination. **Kraft et al. (1979)** reported that the EDS-76 virus particles banded at

densities 1.30 g/ml and 1.32 g/ml, with variations in the ability to agglutinate chicken red blood cells (RBCs).

Zsak and Kisary (1981) showed that the methods used for virus propagation and virus purification influenced the density and haemagglutination ability of EDS-76 virus particles.

Swain *et al.* (1997) found that purification of the EDS-76 virus using potassium tartrate retained the infectivity and antigenicity better than the cesium chloride and sucrose.

Tritiated-thymidine labeling and inhibition with iododeoxy uridine showed that EDS-76 virus contained DNA (**Adair *et al.*, 1979b; Kraft *et al.*, 1979; Todd and McNulty, 1978; Yamaguchi *et al.*, 1981a**). The molecular weight of the DNA was estimated at 22.6×10^6 Daltons compared to 28.9×10^6 for fowl adenovirus type 1 (CELO) (**Zsak and Kisary, 1981**).

EDS-76 virus is a non-enveloped virus and hence virus infectivity remains unaffected by chloroform or ether treatment (**Adair *et al.*, 1979b, Meulemans *et al.*, 1979**) or by treatment with trypsin, α -chymotrypsin, neuraminidase, 2-mercaptoethanol and ethylenediamine tetra acetic acid (**Smyth *et al.*, 1988**).

The virus is stable in monovalent but not in divalent cations and is resistant to pH in the range of 3-10 (**Adair *et al.*,**

1979; Yamaguchi et al., 1981). However, **Zsak and Kisary (1981)** found that infectivity decreased outside pH 6-8 but that haemagglutinin was stable over a wide range.

The virus survives for 3 hours at 56°C, while it is inactivated by heating for 30 minutes at 60°C (**Adair et al., 1979; Yamaguchi et al., 1981).**

Treatment with 0.5% formaldehyde or 0.5% glutaraldehyde abolishes the infectivity of this virus (**Takai et al., 1984).**

2.5 HAEMAGGLUTINATING PROPERTY

The biological property of haemagglutination is a characteristic distinguishing feature of viruses belonging to the Adenoviridae family (**Zanella et al., 1980).** Erythrocytes of chicken, duck, turkey, goose, pigeon and peacock were agglutinated by all the strains of virus, while those of rabbit, horse, sheep, cattle, goat or pig were not agglutinated (**Adair et al., 1979b; Lu et al., 1985).** The virus, unlike the other fowl adenoviruses, agglutinates fowl erythrocytes to high titers (**Adair et al., 1979 b).**

The haemagglutinin is made up of two polypeptides of molecular weight 67,000 Daltons. It has density of 1.24 g/ml in cesium chloride and in purified soluble form, it is inactivated by trypsin, urea and pyridine (**Todd and McNulty, 1978).** However,

the HA is resistant to treatment with trypsin, 2-mercaptoethanol, EDTA, papain, ficin, sodium sulphite and 0.5% formaldehyde and 37°C for 1 hour (**Takai et al., 1984**). Electron microscopically, it is rod-like structure of 25-30 nm is length radiating from central area and measuring approximately 10 nm is diameter (**Todd and McNulty, 1978**). It is quite resistant to heat and is unchanged when exposed to 56°C for 3 hour (**Meulemans et al., 1979**).

The HA activity of virus is lost by pelleting and sonication (**Zsak and Kisary, 1981 a**) and is specifically inhibited with antiserum against the virus (**Higashihara et al., 1983**).

Virus titers is greatly reduced when HA was treated with potassium periodate and 0.5% glutaraldehyde. Though trypsin and neuraminidase did not have any effect on the virus receptors on chicken erythrocytes, α -chymotrypsin destroyed them (**Takai et al., 1984**).

2.6 ISOLATION AND CULTIVATION OF EDS-76 VIRUS

The EDS-76 virus can be isolated from cultures of white blood cells (WBC) collected one week after onset of disease instead of cloacal swabs (**Firth et al., 1981**). **Baxendale (1978)** reported high success rate on cultures of blood leucocytes within 15 days of the flock showing EDS.

2.6.1 Propagation in chicken/duck embryos

The EDS virus grows very well in 11-day-old duck embryos inoculated via amnioallantoic sac route (**Parsons *et al.*, 1980; Zanella *et al.*, 1980; Zsak and Kisary, 1983 and Ramkumar and Mohanty, 1995**) with maximum titer of 1,28,000 HA units/0.1 ml of allantoic fluid (**Firth *et al.*, 1981**). The virus also kills duck embryos in 7-10 days (**Zanella *et al.*, 1980**). However, in chick embryos, either no viral growth was seen (**Higashihara *et al.*, 1983**) or it only stunted the growth of embryos (**Zanella *et al.*, 1980**).

2.6.2 Propagation in Cell Cultures

Over the year, numerous *in vitro* systems have been employed for propagating EDS-76 virus in the laboratory, with varying degree of efficiencies. The virus grows to high titre in duck embryo liver (**Adair *et al.*, 1979**), goose embryo liver (**Zsak and Kisary, 1981a and b**) and chicken embryo liver cell cultures (**McFerran *et.*, 1978b; Todd and McNulty 1978; Parsons *et al.*, 1980 and Zanella *et al* 1981a and b**), less well in chicken kidney and duck embryo fibroblast cells, and poorly in chicken embryo fibroblast cells (**Adair *et al.*, 1979 b; Yamaguchi *et al.*, 1981**) with production of cytopathic effects (CPE) (**Zanella *et al.*, 1980**).

The virus also grows poorly in turkey cell cultures and do not grow in a variety of mammalian cell cultures and chicken embryos (**Adair et al., 1979b; Zsak and Kisary, 1982; Higashihara et al., 1987**). An Indian isolate of EDS-76 virus was observed to replicate best in primary chicken embryo liver cells, however, the allantoic fluid of duck embryos provided the highest haemagglutinating titer of the virus (**Swain et al., 1993**).

The onset of CPE usually occurs about 48 hours post infection (**Adair et al., 1979b**) but can also occur later or after several passages, particularly on primary isolations (**Heffels et al., 1982**). The virus replicates in the nucleus of infected cells and results in the formation of intranuclear eosinophilic inclusion bodies, which helps in histopathological diagnosis of the infection (**Zsak and Kisary et al., 1981a and b; Higashihara et al., 1983**). The CPE in DEF cell culture was first seen on day 4 and increased upto day 6 PI characterized by swollen (10-20 times normal size), rounded or ovoid and slightly refractile cells (**Firth et al., 1981**).

The cytopathic effects produced in chickens embryo liver cells are characterized by rounding, swelling and clustering of cells, intracytoplasmic vacuolation and detachment of cells from glass surface in 24-48 hrs PI with the evidence of intranuclear inclusion bodies in the affected cells (**Adair et al., 1979**). The

growth of virus in cell cultures would also be assessed by immunofluorescence positivity of the cells (**Zsak and Kisary, 1981a and b**), HA (**Adair et al., 1979; Firth et al., 1981; Ramkumar et al., 1993**), HI and virus neutralization tests (**Bragg et al., 1991**).

2.7 CLINICAL SIGNS

Hens infected vertically by EDS-76 virus may show one of the two syndromes depending on the antibody status of the flock at 20 weeks of age (**McFerran et al., 1978**). The flocks in which a percentage of the birds have antibody to EDS-76 virus at 20 weeks fail to reach predicted peak production and onset of lay is delayed.

On the other hand, chickens free of antibody at 20 weeks exhibit a sudden fall in production of 10-30% commencing from 26 to 35 weeks of age, production usually returns to normal within 4-10 weeks but in some cases remains depressed.

Pale eggs, eggs with poor shell strength and soft-shelled eggs are produced for 1 to 3 weeks and their appearance either precedes or coincides with the production drop. Cracked eggs have also been observed in Rhode Island Red layer birds following oral inoculation of JPA-1 strain of EDS-76 virus (**Yamaguchi et al., 1981**). Some investigators have observed

impairments in egg size (**McFerran *et al.*, 1978; Cook and Darbyshire, 1981**) and albumen quality (**Van Eck *et al.*, 1976; Meulemans *et al.*, 1979; Cook and Darbyshire, 1981**).

In spite of abnormality in eggs laid by infected hens, the internal egg quality, fertility and hatchability of eggs remain unaffected (**Darbyshire and Peters, 1980**).

Hens that acquire lateral infection during their laying period produce pale eggs, thin shelled eggs and/or shell less eggs and their production falls. The rate, percentage and duration of the fall depend on the rate of spread through the poultry house and the age of the birds. Many reports from field as well as experimental studies mentioned transient diarrhoea as a clinical symptom (**Van Eck *et al.*, 1976; Yamaguchi *et al.*, 1981**). It was suggested by **Smyth *et al.* (1988)** that the oviductal exudates could possibly be mistaken for diarrhoea.

Cook and Darbyshire (1980) reported an increased mortality in the first week of life in susceptible day-old chicks infected orally with EDS-76 virus. The surviving chicks laid apparently normal eggs, though the size and internal quality of eggs were reduced. Turkeys experimentally inoculated with EDS-76 virus both by nasal and oral routes did not exhibit any

clinical signs, although they showed good HI response (**Parsons et al., 1980**).

2.8 PATHOLOGY OF INFECTION

2.8.1 Macroscopic lesions

Lu et al. (1985) reported uterine oedema in an outbreak of EDS-76 virus in Taiwan. However, in experimental infections more prominent macroscopic lesions characterized by remarkable oedema and swelling of the uterine mucosa were observed by **Taniguchi et al. (1981)** in experimentally inoculated Rhode Island Red layers. The uterine cavity got filled with whitish exudates around 14 days PI. Mild splenomegaly was evident around 5-7 days PI. In addition, flaccid ovules and eggs in various stages of formation have been observed within the abdominal cavity (**Taniguchi et al., 1981; Smyth et al., 1988**).

Atrophy of surface and glandular epithelium in the uterus is generally seen but sometimes, glandular cells may show degeneration (**Gylstroff and Rolf, 1982**). The degenerative changes are followed by inflammatory reaction in the propria of uterus, infundibulum and vagina. Almost no reaction is seen in magnum and isthmus (**Gylstroff and Rolf, 1982**).

The infiltration of mononuclear cells leads to the formation of lymphoid follicles in all parts of oviduct in variable frequency **(Gylstroff and Rolf, 1982)**.

The lesions are first seen in the infundibulum 5 day PI and reach maximum in uterus between 11th and 13th days PI **(Adair et al., 1979)**. Haemorrhages and lymphoid aggregates are seen in the lungs, liver, kidney and proventriculus with a mild fibrosis and atrophy of oviduct and occasional haemorrhages in the ovary under experimental conditions **(Moorthy et al., 1987)**.

2.8.2 Microscopic lesions

The virus multiplies in the nucleus of cells leading to formation of intranuclear inclusion bodies (INIBs) containing typical adenovirus particles **(Adair et al., 1979)**. EDS-76 virus produces INIBs in the surface epithelium of the nasal cavity around 2-6 days PI **(Smyth et al., 1988)**.

The major pathological changes associated with the infection could be observed in pouch shell gland. The normal ciliated pseudo stratified columnar epithelium of the uterine folds is largely replaced by squamous and non-ciliated cuboidal epithelium and atrophy of the uterine glands is also noticed **(Todd and McNulty, 1978)**.

Thus it appears that reproductive tract is the most important source of virus for lateral spread in the laying hens, as

virus antigen was never detected in the surface epithelium of the alimentary tract (**Smyth *et al.*, 1988**).

2.9 DIAGNOSIS

2.9.1 Identification of the antigen

In a flock, whenever there is failure to reach peak production or there is a serious drop in production and the produced eggs have poor quality shells, EDS-76 virus should always be considered as a potential possibility. The egg shell changes usually coincide with production drops and the birds are apparently healthy although occasionally transient diarrhoea and dullness have been seen. Definitive diagnosis of EDS, however, requires laboratory support. Virus isolation and identification and serology are the main methods of diagnosing EDS-76 virus infection in a poultry flock.

Allantoic fluid or cell supernatant should be checked after each passage of virus for the presence of virus by HA test using fowl erythrocytes. Immunofluorescence could be performed to detect virus growth in cell cultures (**McFerran and Adair, 2003**). The isolation attempts may fail due to late stage of sampling but histopathological examination is strongly suggestive of this viral infection (**Justacara *et al.*, 1988**). Demonstration of specific viral antigen in the lining epithelium and glandular cells of

uterus by immunofluorescence (**Das and Pradhan, 1992**) provides strong evidence of disease.

Recently, various molecular biological techniques are being utilized to detect the presence of EDS-76 virus in poultry flock. Polymerase chain reaction (PCR) alone or in combination with restriction enzyme (RE) analysis, has been utilized to a large extent to detect the viral DNA (**Senthil Kumar et al., 2003**).

Zsak and Kisary (1981) utilized different restriction enzymes to cut the DNA from EDS-76 virus (strain B8/78) and CELO virus and observed different restriction patterns for both the viruses suggesting that RE analysis could also serve as a secondary diagnostic tool.

2.9.2 Serological tests

Demonstrating the presence of antibodies to EDS-76 virus by serology is the most common and generally recommended method for confirming the infection.

Several serological tests have been utilized, the haemagglutination inhibition (HI) test, ELISA, serum neutralization test (SNT), double immunodiffusion (DID) test, fluorescent antibody test (FAT) (**Adair et al., 1986**) and plaque reduction test (**Baxendale et al., 1980**).

Adair et al. (1986) compared the sensitivity of various serological tests and found that HI, ELISA, SN, DID and Indirect fluorescent antibody (IFA) tests had similar sensitivity.

HI test, using antigen prepared from cell culture or embryonated duck eggs, is considered to be the test of choice for serological diagnosis of EDS (**Eck et al., 1980**). This test also correlates very well with between serum and egg yolk titers. The test has an additional advantage that lyophilized HA antigen of duck embryo origin and inactivated with 0.2% formalin can be stored for at least a year at 4°C without any loss of titer (**Lu et al., 1985**).

2.10 PREVENTION AND CONTROL

Many therapeutic attempts have been made for the control of EDS-76 virus infection in experimental trials by adding higher levels of vitamins, calcium and proteins in the ration of the infected birds. However, no successful treatment is available for EDS virus infections in birds (**McFerran, 1997**).

The eradication of virus can effectively prevent the vertical spread of the disease. This can be achieved by intervening in the breeding programme.

A testing and culling programme was employed to eradicate the infection from primary breeders in Northern Ireland **(McFerran, 1997)**.

A beta-propiolactone inactivated vaccine adsorbed on aluminium hydroxide gel when injected s/c (0.5 and 1.0 ml) or i/m (1.0 and 2.0 ml) in 18 to 41-weeks-old birds was found harmless at all doses and produced no local reactions or changes in the behaviour of vaccinated birds. However, i/m injection produced higher and more persistent antibody titers **(Baxendale et al., 1980)**. The formalized or beta-propiolactone inactivated mineral oil adjuvant vaccines behaved similarly and produced no adverse effects particularly on egg production.

Both vaccines induced neutralizing and HI antibodies in serum and to a lesser degree in egg yolk **(Khalaf, 1981)**.

Double vaccination greatly enhances the antibody titer **(Salyom et al., 1982)**. Trials using both heat inactivated and formalin inactivated forms of an Indian isolate (EDS-76 virus strain 'N') offered good immunological responses to vaccination **(Chetty and Rao, 1988)**.

Intramuscularly administered vaccine produces a better antibody response than subcutaneous administration **(Solyom et**

al., 1982). However, the latter route of administration also provides adequate protection against the disease (**Baxendale, 1978**).

On the other hand, oral administration of the inactivated vaccine does not produce an antibody response (**Solyom et al., 1982**).

Oil adjuvants are found to offer a better antibody response than aluminium hydroxide gel adjuvant, though both have been found safe and effective (**Solyom et al., 1982**) but latter is more economic in large scale commercial manufacture (**Garg and Garg, 1994**).

Maximal post-vaccination HI-titers occur approximately 3 weeks after vaccination (**Khalaf et al., 1982**) but **Zanella et al. (1980)** reported that complete protection can be achieved as early as 10 days after vaccination of layers. Virus-neutralizing antibodies appear within 7 days of vaccination reaching a maximum 4 weeks later. These levels have been reported to be maintained in the blood stream for 24 weeks and then decline slowly (**Khalaf et al., 1982**). However, there are some reports that immunity due to vaccination can last upto one year (**Baxendale et al., 1980; Cook and Darbyshire, 1981; Khalaf et al., 1982; Solyom et al., 1982**).

3.1 EDS-76 VIRUS

The VN1 isolate of EDS-76 virus passaged five times in duck embryos was procured from Department of Vety. Microbiology, College of Veterinary Sciences, Tirupati and used in the present study.

3.2 EMBRYONATED EGGS

Fresh fertile White Leghorn (WLH) chicken eggs were obtained from Instructional Poultry Farm, Nagla and incubated at 37°C with 80% relative humidity to get the embryos of desired age.

3.3 PREPARATION OF HYPERIMMUNE SERUM IN CHICKEN

Hyperimmune serum against EDS-76 virus was prepared in seronegative chicken as per the method of **Yates et al. (1976)**. The purified virus in 0.5 ml quantity was emulsified with equal volume of Freund's complete adjuvant (FCA) and injected intramuscularly into the chicken. After one week, 0.5 ml of virus emulsified with equal volume of Freund's incomplete adjuvant (FIA) was given intramuscularly and tested for

haemagglutination inhibition (HI) antibodies. The serum was stored at -20°C after adding merthiolate at a final concentration of 1: 10,000.

3.4 PROPAGATION OF VIRUS

The virus originally procured as infected duck allantoic fluid was diluted 20 times with PBS (pH 7.2) containing 250 IU of benzyl penicillin and 250 µg of streptomycin per ml. The inoculum was kept for 30 min at room temperature and then filtered through 0.45 µm nitrocellulose membrane filter. The filtrate was inoculated in 9 to 10-day-old embryonated duck eggs via allantoic cavity route of inoculation @ 0.2 ml/embryo and incubated at 37°C for 4-5 days. The eggs dying within 24 hrs post inoculation were treated as nonspecific. After 4-5 days, the eggs were chilled for 12 hrs to avoid bleeding. The allantoic fluid was harvested and presence of virus was tested by spot HA test using 10 percent chicken RBCs. The harvested allantoic fluid was pooled, aliquoted and stored at -20°C until used for successive passaging or for other tests.

Same procedure was applied for the propagation of virus in the chicken and guinea fowl eggs.

3.5 PREPARATION OF PRIMARY CHICKEN EMBRYO LIVER (CEL) CELL CULTURE

Primary CEL cell culture was prepared using 14 to 16-day-old chicken embryos as per the method of **Adair et al. (1979)**

with slight modifications. The embryos were collected aseptically and livers taken out in HBSS. These were then cut into small pieces and passed through barrel of a 10 ml glass syringe. The minced tissue was washed with HBSS and trypsinized in 0.125% trypsin solution (Difco, USA, 1:250) for 15 minutes with constant stirring over magnetic stirrer. The cells were filtered through sterilized muslin cloth and centrifuged at 3000 rpm for 10 minutes at 4°C. The cells were washed in HBSS and finally in Medium-199 (sigma). The packed cells were diluted 1:200 (v/v) in medium-199 containing 15% foetal calf serum and 4% tryptose phosphate broth (1% v/v) to serve as growth media and dispensed in tissue culture bottles (25 cm²) and sterilized Leighton tubes @ 8 ml and 2ml, respectively. The seeded bottles were incubated at 37°C till confluent monolayers were formed. Complete monolayers were usually formed in 48-72 hrs post seeding.

3.6 ADAPTATION OF VIRUS IN CELL CULTURE

The growth media from tissue culture bottles and Leighton tubes with confluent monolayers were decanted and then inoculated with filtered allantoic fluid collected from infected duck eggs @ 1.0 ml and 0.1 ml, respectively. The culture bottles and Leighton tubes were incubated at 37°C for adsorption of virus. After 1 hr of incubation, the inocula were removed and 8 ml and 2 ml of maintenance media (M-199 + 2% FCS) were

poured in each bottle and Leighton tubes, respectively. The infected monolayers were observed daily for cytopathic effects (CPE) under inverted light microscope. After 4-5 days, the virus was harvested by freezing and thawing 3 times followed by centrifugation at 4000 rpm for 30 minutes. The supernatant was collected and passed through membrane filter of 0.22 µm and filtrate stored at -20°C for further successive passages of virus.

3.7 STUDY OF CYTOPATHIC EFFECTS (CPE)

The CPE produced by the virus in CEL cell culture were observed regularly in unstained as well as stained preparations.

3.7.1 Unstained monolayer

The infected cell culture bottles and Leighton tubes were observed twice daily in the morning and evening under inverted microscope up to 4 days PI. The changes were compared with the control bottles and tubes to detect the appearance of CPE.

3.7.2 May-Grunwald and Giemsa stained monolayer

The monolayers grown on coverslips were stained with May-Grunwald and Giemsa stains at 24, 48 and 72 hr post inoculation as per the technique described by **Merchant *et al.* (1960)** with some modifications. The medium from Leighton tubes was decanted and monolayers washed with

PBS (pH 7.2). The monolayers were then fixed in absolute methanol for 30 min at room temperature. The fixed monolayers were stained with filtered May-Grunwald stain for 30 min and then with filtered Geimsa stain (1:10 dilution of stock solution) for 30 min. The monolayers were washed with distilled water and dehydrated in two changes of acetone, acetone+xylene (1:1) and finally cleared in two changes of xylene and mounted on a clean glass slide with a drop of DPX mountant. The stained coverslips were examined for CPE under light microscope.

3.8 SEROLOGICAL TESTS

3.8.1 Agar gel precipitation test

Agar gel precipitation test was performed according to the method of **McFerran *et al.* (1978a)** to detect the presence of precipitating antibodies in infected duck allantoic fluid. Agar gel was prepared by dissolving 1 gm of noble agar in 100 ml of 8% sodium chloride solution in boiling water bath. The molten agar gel was poured on 75mm X 25mm glass slide and allowed to solidify. Slide was then kept at 4°C for 30 minutes. On the slide, one central and 2 peripheral wells of 3 mm diameter at a distance of 3 mm were punched and sealed with molten agar. The central well was charged with infected duck allantoic fluid and peripheral wells were filled with standard

and experimentally raised hyperimmune sera against EDS-76 virus.

The slide was then incubated at 37°C for 24 hrs in a humid chamber. After 24 hours, the slide was transferred to refrigerator at 4°C for further development of lines.

3.8.2 Counter immunoelectrophoresis (CIE)

The gel was prepared by dissolving 1% agarose in 0.2 M barbitone buffer (pH 8.6) in boiling water bath and poured on microscopic slides and allowed to solidify at room temperature. The slide was then kept at 4°C for 20 min. Four wells of 3 mm diameter towards anode and four wells towards cathode were punched and sealed with molten gel. The well to well distance was four mm. The first two wells towards cathode side were charged with infected duck allantoic fluid of 5th passage and other two wells with concentrated infected cell culture supernatant of 5th passage. The wells towards anode side were charged with standard and experimentally raised hyperimmune sera against EDS-76 virus alternatively.

The chambers of electrophoresis assembly were filled with 0.2 M barbitone buffer and the current of 50 mV per slide was passed for one hr and the results read after two hr.

3.8.3 Haemagglutination (HA) test

The HA test was performed in 'U' shaped microtiter plates as per the method of **Cunningham (1966)**. A serial two-fold dilution of the virus (50 µl) starting from 1:2 and an equal volume of one percent chicken RBCs was added to each well. The plates were shaken gently to enable the mixing of the virus and RBCs and then kept at room temperature for 45 min. Appropriate positive and negative controls were also included in the test. The reciprocal of the highest virus dilution showing mat formation was taken as the HA titre.

3.8.4 Haemagglutination inhibition (HI) test

The HI test was performed as per the method of **Cunningham (1966)**. A serial two-fold dilution of the test serum (50 µl) was made in PBS, pH 7.2, starting from 1:2. An equal volume of virus containing 4 HA units was then added to each well. After gentle shaking, one percent chicken RBCs were added to all the wells and incubated at room temperature. Appropriate positive and negative controls were also included in the test. The reciprocal of highest serum dilution at which there was clear button formation was taken as the end point. The HI titre was calculated by multiplying the reciprocal of the end point serum dilution by 4.

3.8.5 Fluorescent antibody technique (FAT)

Fluorescent antibody technique was performed for detection of viral antigen in infected CEL cell culture. Infected chicken embryo liver cell monolayers on coverslips were stained at 24, 48 and 72 hr PI. The media from Leighton tubes were decanted and coverslips washed with HBSS (pH 7.4) and then fixed in chilled acetone for 30 min. The coverslips were flooded with (1:200) diluted hyperimmune serum filtered through membrane filter of 0.45 μm pore size and incubated at 37°C for 2 hr in a moist chamber. The coverslips were washed with HBSS three times each for 5 min and flooded with anti-chicken FITC conjugate (1:20, Sigma) filtered through membrane filter of 0.45 μm pore size and again incubated for 2 hr at 37°C in moist chamber. The coverslips were washed 3 times as mentioned above with HBSS, air-dried and mounted in 50% glycerol saline. The stained coverslips were examined under fluorescent microscope (Olympus B-40 microscope).

4.1 PROPAGATION OF VIRUS

The VN1 isolate of EDS-76 virus passaged five times in duck embryos could be propagated only in embryonated duck and chicken embryos, as the harvested allantoic fluids of inoculated developing embryos gave positive spot HA test upto fifth and fourth passages, respectively. The guinea fowl embryos were found refractory to EDS-76 virus, as the allantoic fluid of developing guinea fowl embryos failed to show spot HA test from second passage onwards.

4.2 ADAPTATION OF VIRUS IN CHICKEN EMBRYO LIVER CELL CULTURE

The EDS-76 virus isolate was adapted to primary chicken embryo liver cell culture through successive passages. The multiplication of virus was assessed by HA activity (Table 1) in fluid collected from each passage and by appearance of CPE. The perusal of table 2 indicates that HA titre increased gradually and attained a titre of 1024 at the 4th passage level.

The CPE became evident in the first passage but it was prominent duly at 4th passage. However, the persistence of HA activity and appearance of CPE in infected cell culture indicated adaptation and multiplication of virus in CEL cell culture.

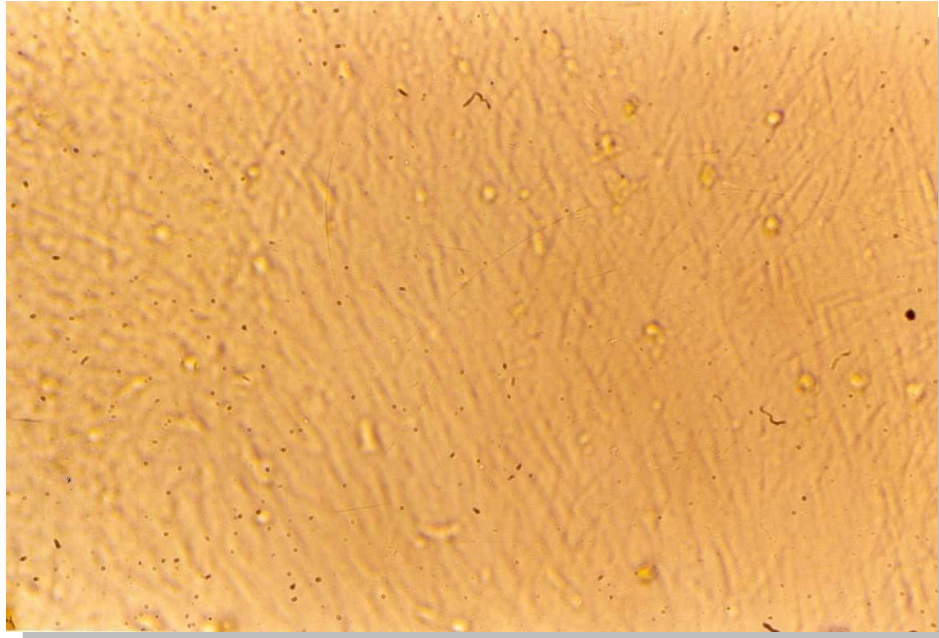
4.3 CYTOPATHIC EFFECTS (CPE)

4.3.1 Unstained cell monolayer

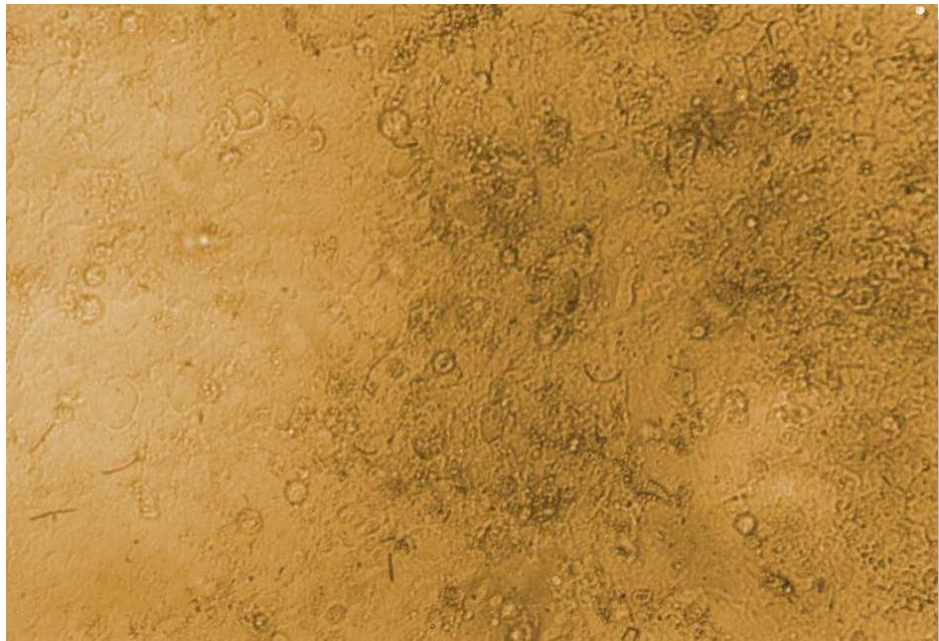
The CEL cell culture inoculated with infected allantoic fluid of duck eggs showed cytopathic effects characterized by rounding of cells at 72 hrs PI in the first passage itself. At second passage and onwards, the CPE were evident at 36 hrs and by 48 hrs PI and 70-80% of cells were involved. At 72 hrs PI, more than 80% of monolayer was affected. On successive passaging, the CPE were more stable and distinct. At 4th passage level, distinct micro-and macroplaques were observed. The uninfected control monolayer did not exhibit any change (Fig. 1-6).

4.3.2 May-Grunwald and Giemsa (MGG) staining of monolayer

Microscopic examination of MGG stained infected CEL cell culture showed enlargement of nuclei of cells at 3rd passage with occasional eosinophilic intranuclear inclusion bodies at 24 hrs PI. At 48 hrs PI, the number of nuclei having inclusions were increased filling almost whole nucleus. The infected cells also showed varying degree of cytoplasmic vacuolation. The shape of nuclei was also



**Fig. 1. Photomicrograph of uninfected CEL cell culture.
(Unstained 100 X)**



**Fig. 2. Photomicrograph of infected CEL cell culture
showing rounding of cells at 24 hrs. PI. (Unstained
100 X)**

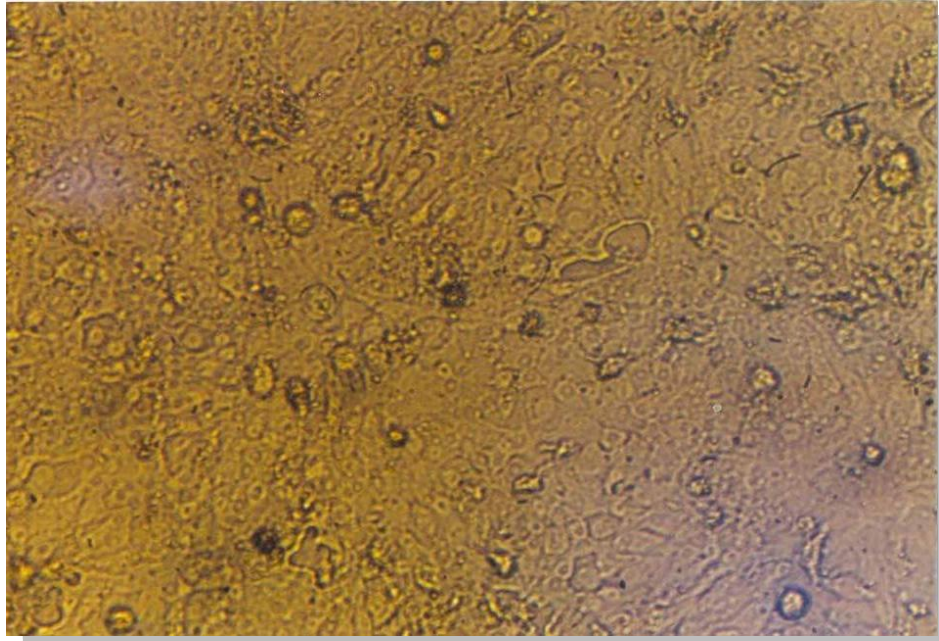


Fig. 3. Photomicrograph of infected CEL cell culture showing clumping of cells at 36 hrs. PI. (Unstained 100 X)

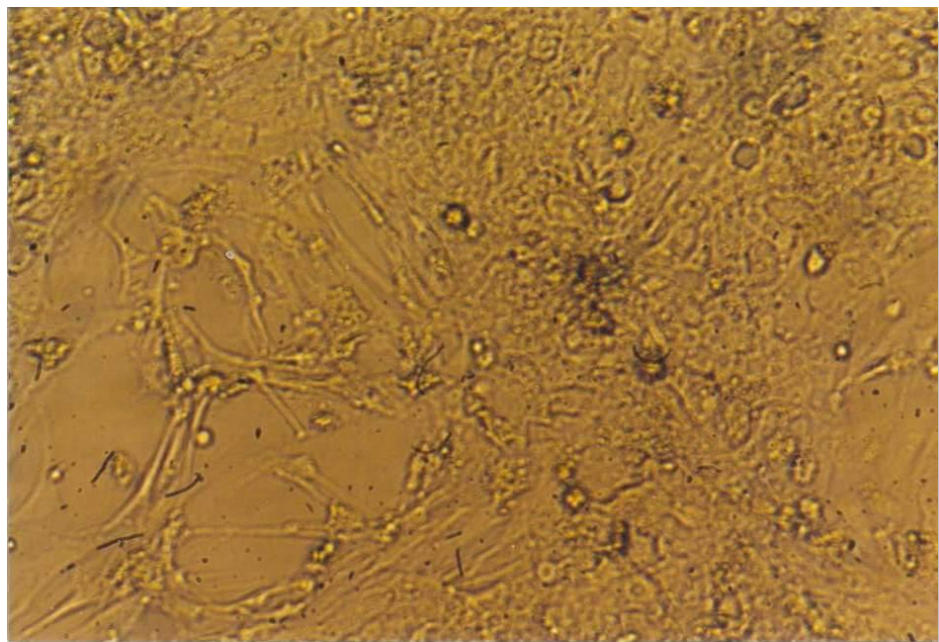


Fig. 4. Photomicrograph of infected CEL cell culture showing detachment of cells at 48 hrs. PI. (Unstained 100 X)

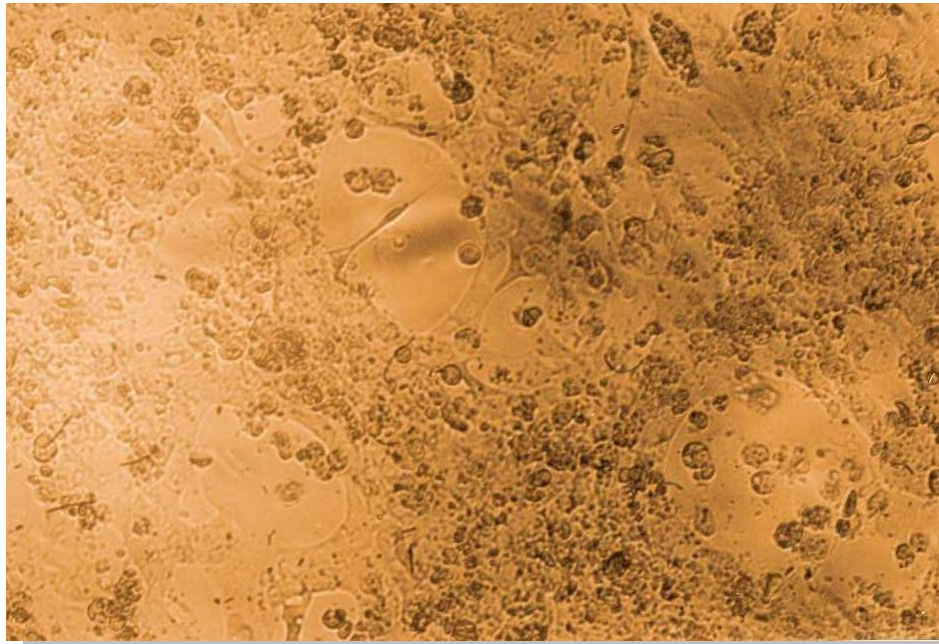


Fig. 5. Photomicrograph of infected CEL cell culture showing plaque formation at 72 hrs. PI. (Unstained 100 X)

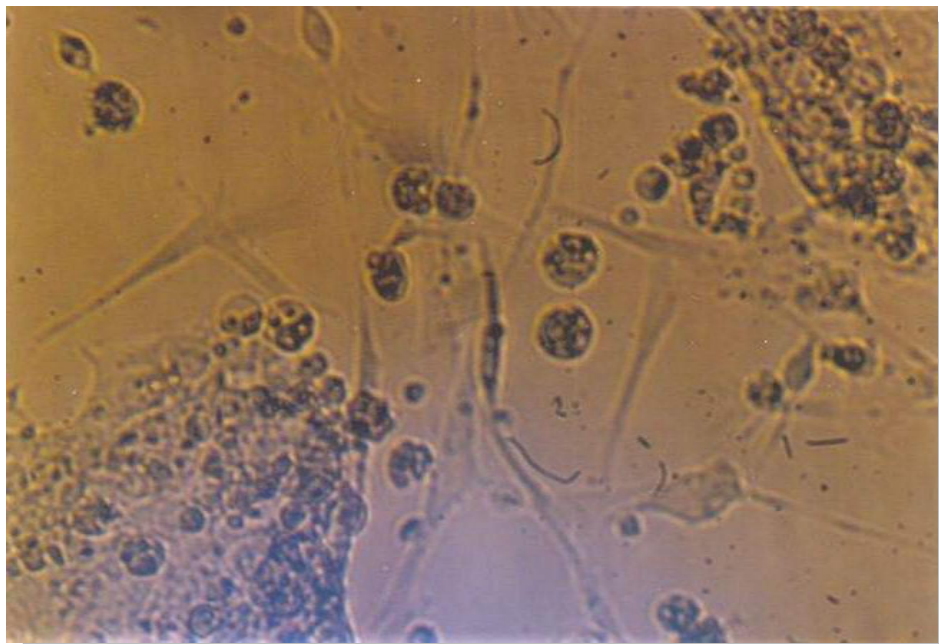
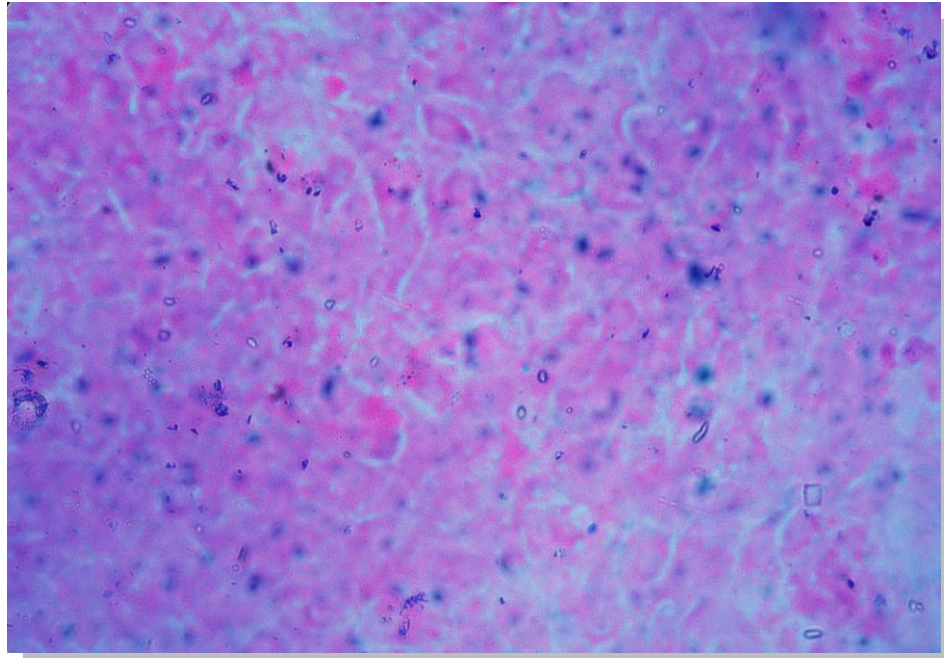
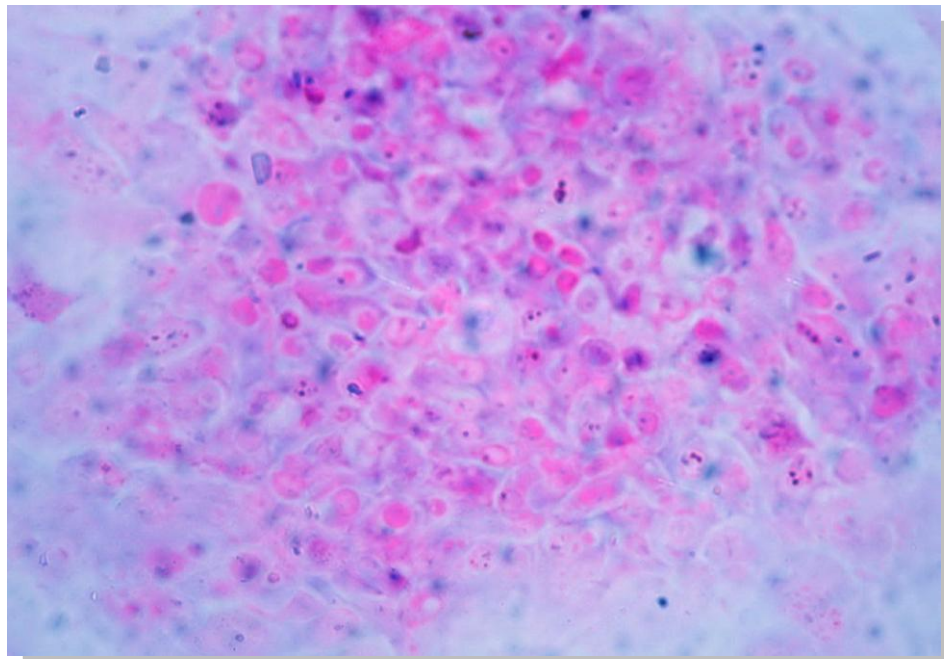


Fig. 6. Photomicrograph of infected CEL cell culture showing degeneration of cells at 96 hrs. PI. (Unstained 100 X)



**Fig. 7. Photomicrograph of uninfected CEL cell culture.
(MGG stained 1000 X)**



**Fig.8. Photomicrograph of infected CEL cell culture
showing eosinophilic inclusion bodies at 24 hrs.
PI. (MGG stained 400 X)**

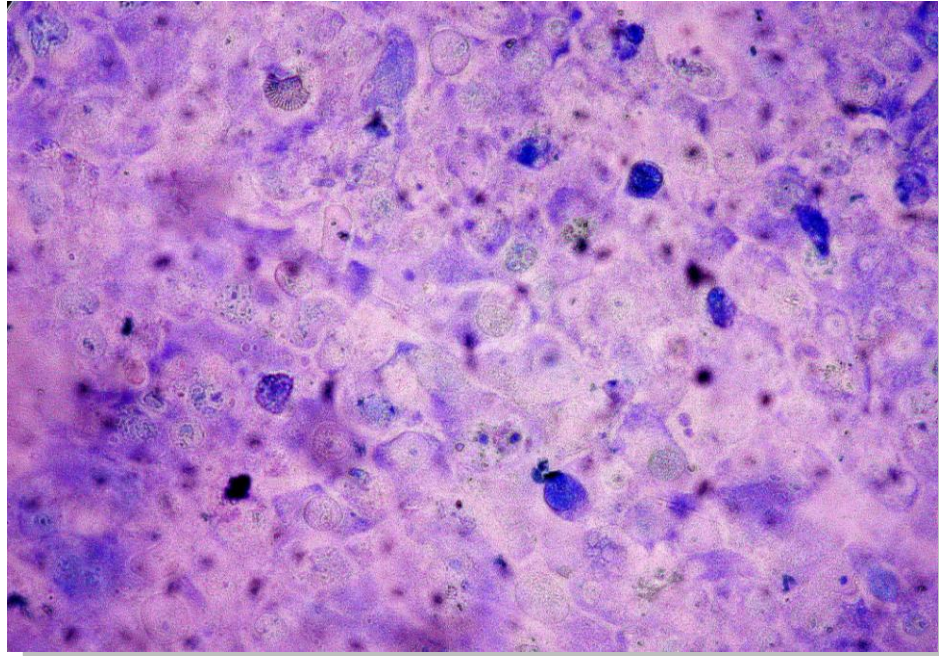


Fig.9. Photomicrograph of infected CEL cell culture showing eosinophilic inclusion bodies at 36 hrs. PI. (MGG stained 400 X)

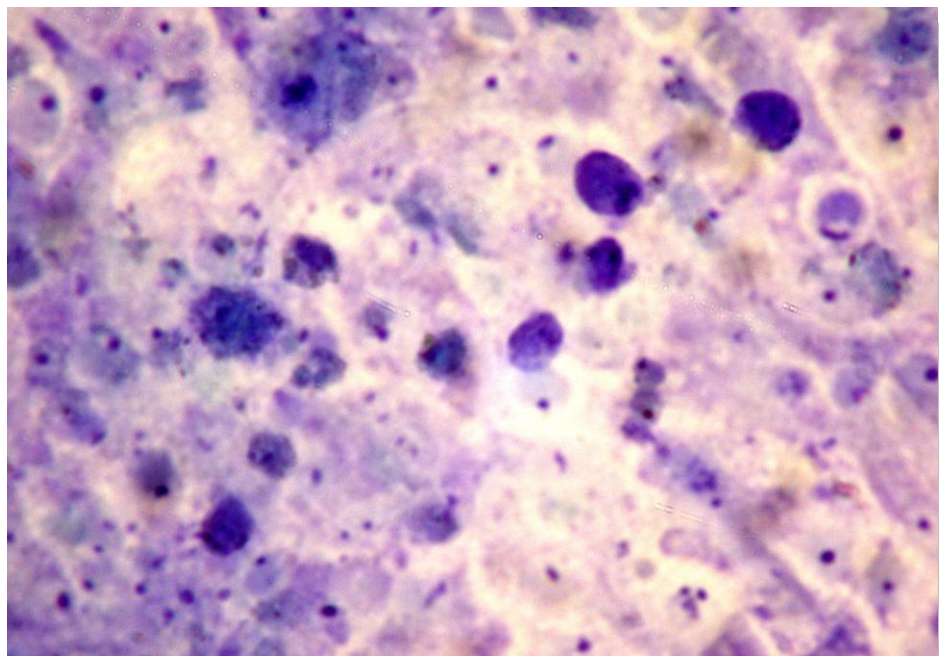


Fig.10. Photomicrograph of infected CEL cell culture showing eosinophilic inclusion bodies at 48 hrs. PI. (MGG stained 400 X)

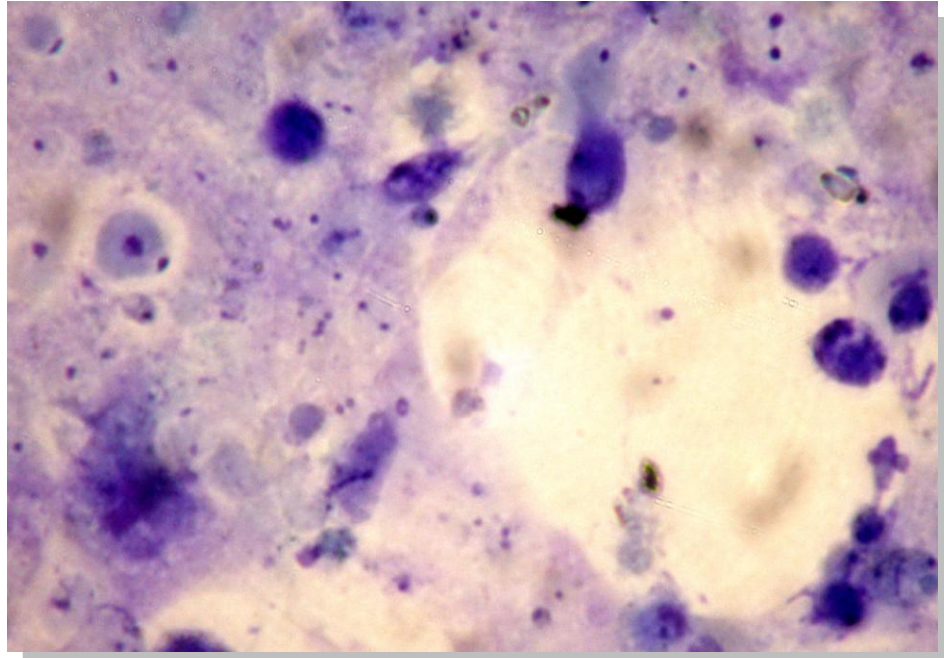


Fig.11. Photomicrograph of infected CEL cell culture showing eosinophilic inclusion bodies at 72 hrs. PI. (MGG stained 400 X)

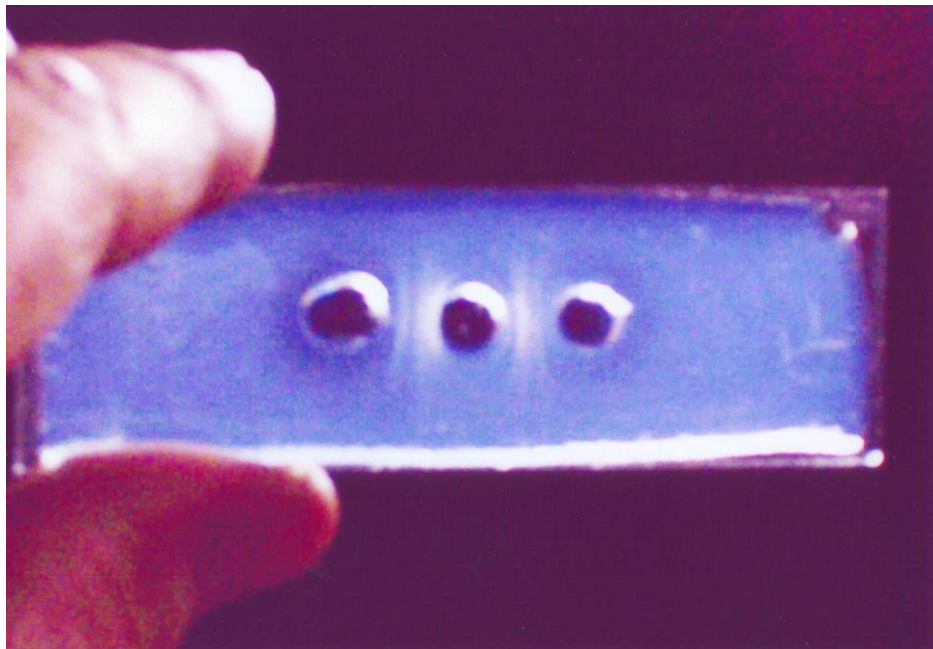


Fig.12. Photograph of agar gel precipitation test showing precipitin lines between standard and experimentally raised hyperimmune sera against EDS-76 virus and infected duck allantoic fluid.

distorted and enlarged. At 72 hrs PI, most of the cells detached from the surface and therefore, number of cells containing intranuclear inclusion bodies were less but percentage was higher. Uninfected MGG stained monolayers did not show these cytopathic effects (Fig. 7-11).

4.4 SEROLOGICAL TESTS

4.4.1 Agar gel precipitation test (AGPT)

The presence of viral antigen was detected in the allantoic fluid and cell culture harvest. By double immunodiffusion technique, the allantoic fluid of duck embryos at fifth passage level showed precipitin lines against both standard and experimentally raised hyperimmune sera after 24 hrs.

The unconcentrated cell culture harvest did not yield precipitin lines. When the cell culture harvest was concentrated using PEG-6000 to about half the original volume, even then no precipitin lines were observed (Fig. 12).

4.4.2 Counter immunoelectrophoresis (CIE)

CIE also failed to exhibit precipitin line between antigens prepared from infected duck allantoic fluid and cell culture harvest and standard and experimentally raised hyper immune sera even after incubation.

4.4.3 Haemagglutination (HA) test

HA test was carried out to know the titre of virus in the allantoic fluid and cell culture harvest. The HA titre of the allantoic fluid of infected duck embryos was initially 1:4096, which increased to 1:20,97,152 by fifth passage. In chicken embryos, the HA titre showed a decreasing pattern from 256 to 40 HA units. The guinea fowl embryos failed to show HA activity from second passage onwards (Table 1&2) (Fig. 13-15).

4.4.4 Haemagglutination inhibition (HI) test

The infected allantoic fluid, which revealed HA activity was further tested by HI test using hyperimmune sera raised in layer birds against known standard strain of EDS-76. The virus at its 5th passage level showed a HI titre of 1:20480 (Table 3).

4.4.5 Fluorescent antibody technique

Infected chicken embryo liver cells, examined for presence of virus by immunofluorescence at 4th passage level, revealed greenish yellow fluorescence of low intensity in the nuclei of some liver cells at 24 hr PI. At 36 and 48 hr PI, intensity of fluorescence increased and at 72 hr PI, almost all nuclei exhibited bright yellowish green fluorescence. There was no fluorescence in the cytoplasm of infected cells. The uninfected liver cells did not show any nuclear or cytoplasmic fluorescence (Fig. 16-18).

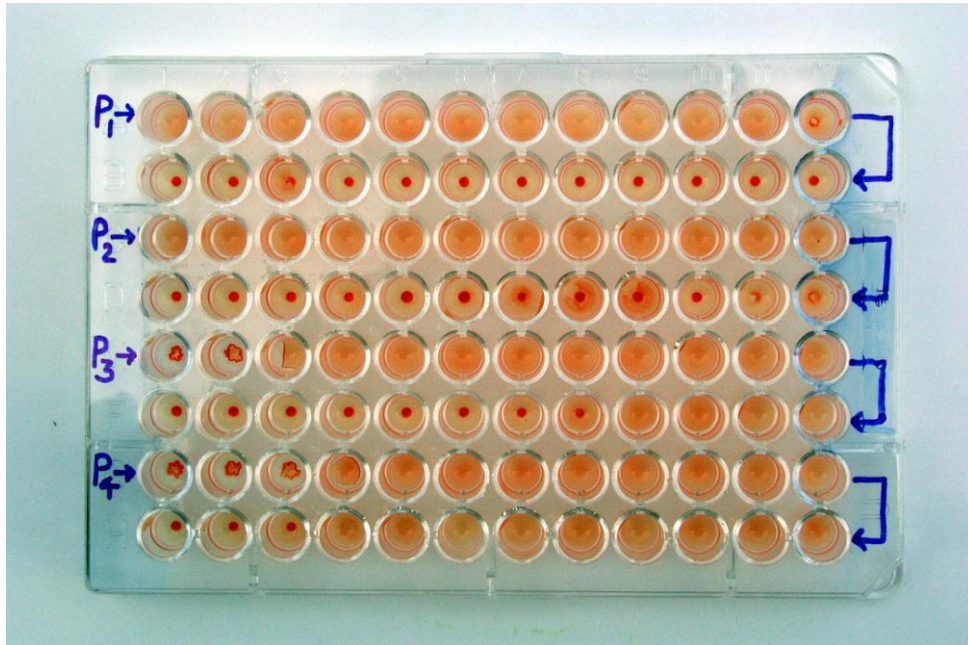


Fig. 13. Photograph showing HA titre in different passages in infected duck allantoic fluid



Fig. 14. Photograph showing HA titre in different passages cell culture

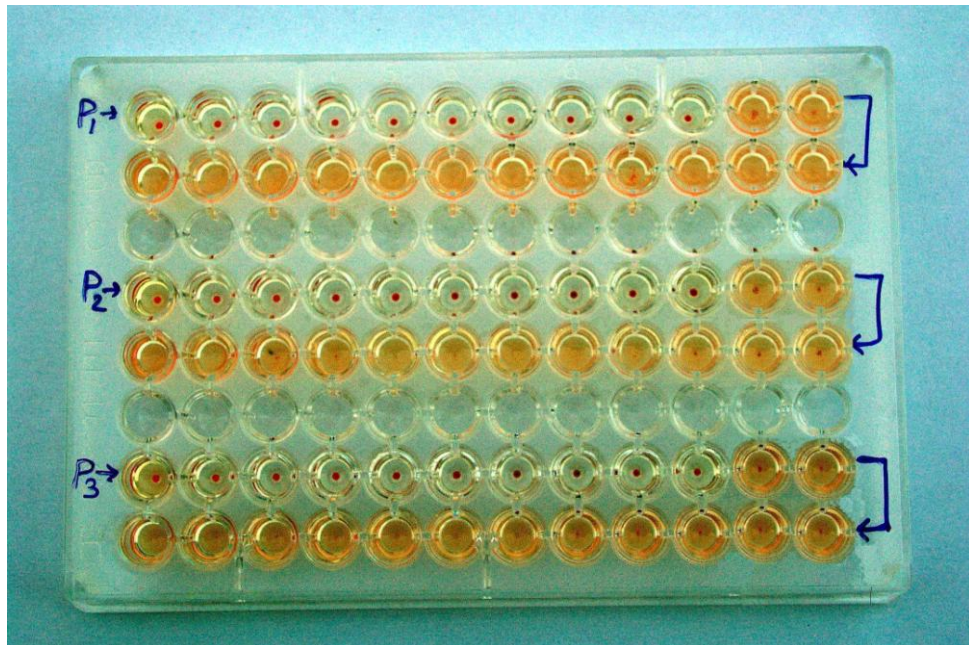


Fig. 15. Photograph showing haemagglutination inhibition pattern of virus with anti EDS-76 antibodies and fowl erythrocytes

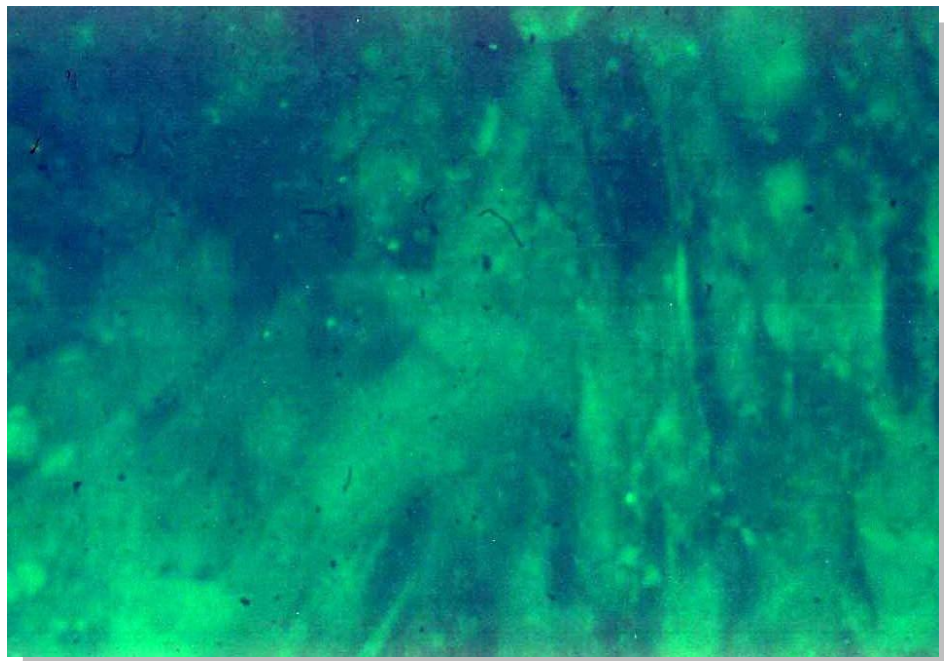


Fig. 16. Photomicrograph of uninfected CEL cell culture showing no fluorescence. (1000 X)

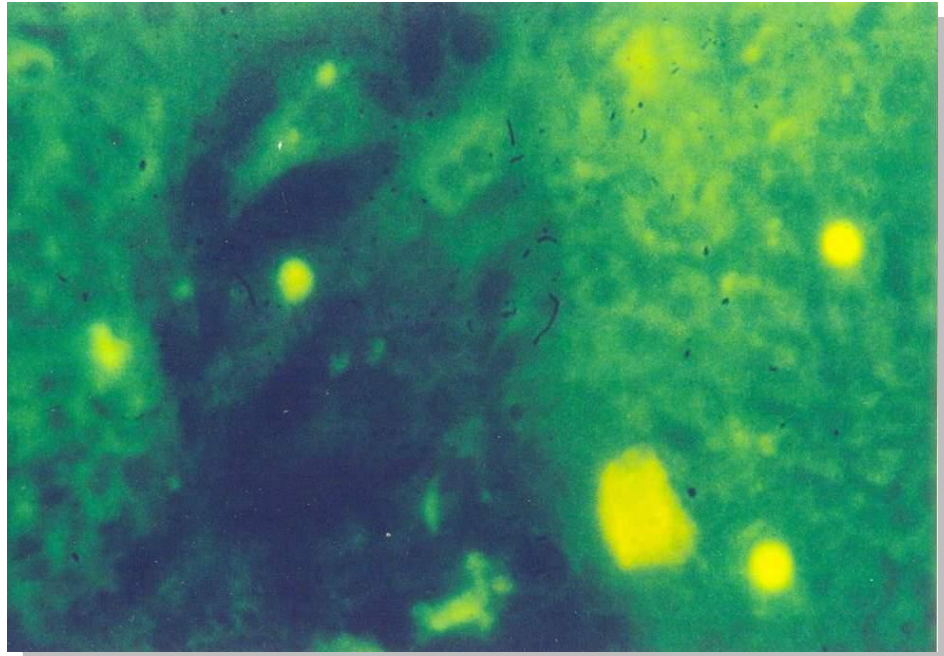


Fig.17. Photomicrograph of infected CEL cell culture showing intranuclear fluorescence. (1000 X)

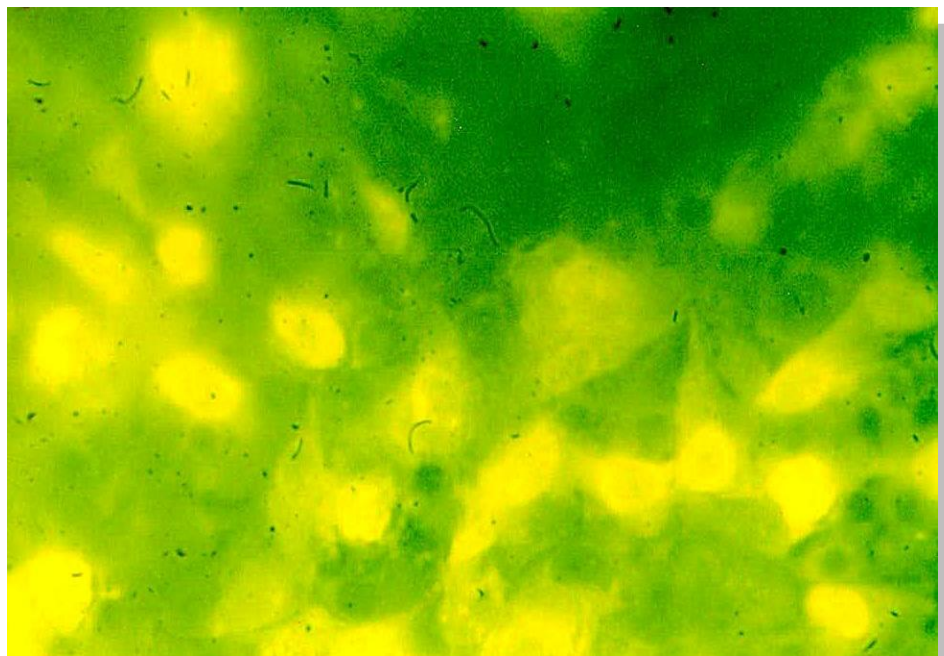


Fig. 18. Photomicrograph of infected CEL cell culture showing increase in the intensity of intranuclear fluorescence. (1000 X)

Table 1. HA titre of EDS-76 virus isolate in allantoic fluid

Passage number	HA titre		
	Duck eggs	Chicken eggs	Guinea fowl eggs
1	4096	256	16
2	16384	128	8
3	65536	40	ND
4	20,97,152	ND	ND

Table 2. HA titre of EDS-76 virus isolate in CEL cell culture

Passage number	HA titre
2	320
3	320
4	5120

Table 3. HI titre of EDS-76 virus isolate in allantoic fluid

Passage number	HI titre
1	20480
2	20480
3	20480

4.5 EXPERIMENTAL PATHOGENECITY IN BIRDS

4.5.1 *In ovo* pathogenicity

The infected allantoic fluid upon inoculation @ 0.2 ml/embryo into the allantoic cavity of embryonated duck eggs caused embryo mortality within 72 to 96 hrs post inoculation. The dead embryos appeared congested and haemorrhages were seen particularly on head. The virus was detected in allantoic fluid by spot HA.

4.5.2 *In vivo* pathogenicity (birds)

The clinical signs in experimentally infected susceptible birds appeared from third day of infection. The infected birds showed no inclination towards feed and water. They appeared drowsy, listless, weak and depressed. Besides these, diarrhoea and paralysis of wings and legs and drop in production of eggs with poor shell strength/soft shelled eggs were also evident the production of soft shelled eggs for 1 to 3 weeks preceded drop in production from 15 to 20%.

Egg drop syndrome-76 (EDS-76) of poultry, a viral disease of significant economic importance, mainly affects layers and breeding flocks in almost all poultry rearing countries of the world. The virus is still posing a serious threat to economy of poultry rearing countries, because of drop in egg production. The affected birds may remain healthy or show only transient dullness and diarrhoea followed by egg shell abnormalities. The disease is associated with either egg shell changes or failure to achieve predicted production levels (**Ecle *et al.*, 1976; McFerran *et al.*, 1978**).

In this study, VNI isolate of EDS-76 virus, earlier characterized and maintained in the laboratory, was tested serologically with antiserum against standard EDS-76 virus strain 127. Further, the EDS-76 virus strains isolated in different countries, viz., D61, BC14, JBP, SPC, 3877 and Belgian strain were serologically and morphologically identical to strain 127 (**Chandra *et al.*, 1997**). This suggested that the virus isolate used in the study was serologically similar to other strains of EDS-76 virus.

The virus on cultivation in 9 to 10-day-old duck embryos showed a HA titre of 1:4096, which increased to 1:20,97,152 at 4th passage. These observations were in agreement with the findings of **Zanella et al. (1980)** and **Firth et al. (1981)** who reported successful growth of EDS-76 virus in the 11-day-old duck embryos by allantoic cavity route.

The chicken and guinea fowl embryos did not support the growth of EDS-76 virus as was also reported by several investigators (**Adair et al., 1979, Zsak and Kisary 1981, Ramkumar, 1990**). The experimentally affected birds showed no inclination towards feed and water and appeared dull, weak and depressed. Besides these, transient diarrhoea was a clinical symptom similar to many reports from field and experimental studies (**Van Eck et al., 1976; Yamaguchi et al., 1981**); however such an observation was not made by other researchers (**McCracken and McFerran, 1978; Hiruma et al., 1981**).

All inoculated hens had abnormal egg production. Egg production was depressed and/or aberrant for 3 days or longer. Abnormal egg production began 3 to 14 days PI in hens. The eggs laid were soft-shelled or thin shelled and showed discolouration. Rough-ridged or misshapen eggs and eggs with watery or thin albumen/turbid or thick albumen were not

observed. These observations are in agreement with of **Van Eck (1983)**, **Lu et al. (1985)** and **Higashihara et al. (1986)**.

The etiological agent of EDS-76 was isolated in chicken embryo liver cell culture. Chicken embryos liver cell culture, duck embryo liver cell culture, duck embryo fibroblast cell culture and chicken embryo kidney cell culture are reported to support the growth of adenoviruses including EDS-76 virus (**Swain et al., 1993**). In CEL cells, the virus produced cytopathic effects characterized by rounding and swelling of cells, which were evident from the first passage itself. **Adair et al., (1979)** observed CPE characterized by appearance of retractile cells with swelling. The CPE comprising rounding, cell degeneration, microplaque formation and detachment of monolayer from glass surface within 96 hrs PI along with intranuclear inclusion bodies were also observed by **Swain et al. (1993)**. Development of micro-and macroplaques in infected monolayers is suggestive of cytolysis by virus. On successive passaging, the CPE were more stable and distinct. Therefore, CEL cell culture was found suitable for primary isolation of virus.

May-Grunwald and Giemsa (MGG) staining of infected CEL cell culture revealed eosinophilic intranuclear inclusion bodies in the infected cells at 36, 48 and 72 hrs PI. **Zsak and Kisary (1981)** and **Higashihara (1983)** also reported eosinophilic

intranuclear inclusion bodies accompanied by cytoplasmic vacuolar degeneration. The presence of intranuclear inclusion bodies and distorted and enlarged nuclei were suggestive of virus multiplication in the nucleus.

Since drop in egg production is also associated with number of other etiological agents. Hence, it is necessary to diagnose the disease either by detection of antigen or antibody.

EDS-76 virus infection is currently diagnosed by various immunological methods such as Haemagglutination (HA), Haemagglutination inhibition (HI), Agar gel precipitation tests, counter immunoelectrophoresis (CIE) and fluorescent antibody technique (FAT).

Piela and Yates (1983) compared the results of ELISA, HI and AGID test and found HI and AGID to be more sensitive than ELISA. The HI test is more reliable and a titre of 1:8 and above is considered positive (**Adair et al., 1986**). HI has become the accepted serological test for EDS virus.

Agar gel precipitation test (AGPT) performed using allantoic fluid from infected duck eggs showed precipitin lines with hyperimmune sera raised against EDS-76 virus, which proved the presence of adenovirus in the allantoic fluid. But no precipitin line was observed between 5th passage infected CEL

cell culture supernatant and hyperimmune sera raised in laboratory using infected allantoic fluid as antigen. This clearly indicated that causative agent was isolated in the allantoic fluid.

In counter immunoelectrophoresis (CIE), there was again no precipitin line between infected allantoic fluid and cell culture harvest and hyperimmune sera raised against EDS-76. However, a precipitin line was observed in CIE as reported by **Lakshmi Kavitha (2003)**. This difference may be attributed to changes in the physical conditions.

The fluorescent antibody technique (FAT) performed with EDS-76 virus grown in CEL cell culture revealed intense intranuclear fluorescence, which indicated presence of viral antigen inside the nucleus of liver cells. Therefore, FAT could be used for detection of EDS-76 virus in cell culture. Indirect immunofluorescence test could be used to detect adenovirus in tissues of affected birds. Immunofluorescent labeling methods can be employed depending on the nature of the tissue sections. **Ramkumar et al. (1991)** suggested that virus specific immunofluorescence in the nuclei of infected DEF cells is indicative of its multiplication in the nuclei of cells.

In view of significant economic importance of Egg drop syndrome-76 to poultry industry, the present investigation was undertaken to study its cytopathogenicity. The study mainly involved the propagation of virus in embryonated duck eggs and adaptation to primary chicken embryo liver cell culture.

In this study VN1 isolate of EDS-76 virus was cultivated in 9 to 10-day-old embryonated duck eggs, which was detected by spot HA test.

The inoculum on injection into embryonated duck eggs via allantoic cavity route resulted in mortality of embryos within 96 hrs post inoculation. The presence of virus in the allantoic fluid was demonstrated by HA test. The HA titre in first passage was 1:4096, which increased in the subsequent passages and by 4th passage, the titre was 1:20,97,152.

The specificity of virus isolate was further confirmed by haemagglutination inhibition (HI) test by employing hyperimmune serum raised against standard EDS-76 virus. The HI titre was found to be 1:20480. The chicken embryos were not

found suitable for cultivation of EDS-76 virus and Guinea fowl embryos were found refractory to virus infection.+

Attempts were made to adapt the virus isolate in primary chicken embryo liver (CEL) cell culture. The virus could be successfully adapted to CEL cell culture by 4th passage and produced cytopathic effects consisting of rounding of cells, micro-and macroplaque formation, degeneration of monolayer and intranuclear inclusion bodies. The changes were prominent by 48 hr to 72 hr PI. The HA titre increased gradually to 1:20,97,152 at 4th passage. May-Grunwald and Giemsa (MGG) staining of infected CEL cell culture revealed eosinophilic intranuclear inclusion bodies filling almost entire nucleus.

Agar gel precipitation test (AGPT) performed using infected duck allantoic fluid showed precipitin line with hyperimmune sera raised against EDS-76 virus. No precipitin line was observed between 5th passage infected CEL cell culture supernatant and standard hyperimmune sera and hyperimmune sera raised in laboratory using infected duck allantoic fluid as antigen.

In counter electrophoresis (CIE) again, no precipitin line was observed between infected duck allantoic fluid and 5th passage infected cell culture supernatant against standard and

experimentally raised hyperimmune sera raised against EDS-76 virus.

The fluorescent antibody technique (FAT) performed to detect EDS-76 virus in CEL culture revealed intense intranuclear fluorescence, which indicated presence of viral antigen inside the nucleus of liver cells.

The infected allantoic fluid when inoculated into the susceptible laying birds revealed characteristic clinical signs of disease on day 3rd post inoculation. The clinical signs included drowsy appearance, paralysis of wings and legs and drop in egg production coinciding with the production of soft shelled or thin-shelled eggs for 1 to 3 weeks.

- Adair, B.M.; Caurran, W.L. and McFerran, J.B. (1979a).** Ultrastructural studies of the replication of fowl adenoviruses in primary cell cultures *Avian Pathology*, 8:133-144.
- Adair, B.M.; McFerran, J.B.; Conner, T.J.; McNulty, M.S. and Mckillop, E.R. (1979b).** Biological and physical properties of a virus (Strain 127) associated with the Egg drop syndrome 1976. *Avian Pathology*, 8: 249-264.
- Adair, B.M.; Todd, D.; McFerran, J.B. and McKillop, E.R. (1986).** Comparative serological studies with Egg drop syndrome virus. *Avian Pathology*, 15:677-685.
- Badstue, P.B. and Schmidt, 1978.** Egg drop syndrome in Danish poultry. *Nordish Veteri.*, 30: 498-505.
- Bartha, A.; Meszaros, J. and Tanyi, J. (1982).** Antibodies against EDS-76 avian adenovirus in birds species before 1975, *Avian Pathology*, 11: 511-513.
- Baxendale, W. (1980).** Recent research on Egg drop syndrome 76 (EDS-76). Proceedings of 29th *Western Poultry Disease Conference, Acapulco, Gro, Mexico, April 1980*, p. 206-211.
- Baxendale, W. 1978.** Egg drop syndrome-76. *Veterinary Record*, 102: 285-286.
- Baxendale, W.; Lütticken, D.; Hein, R. and Orthel, F.W. (1978).** Vaccine against the Egg drop syndrome 76 of chicken. *International Virology IV* 119. Abstract of the fourth international congress for virology, The Hague.
- Bragg, R.R.; Allwright, O.M. and Coetzee, L. (1991).** Isolation and identification of adenovirus 127, the causative agent of egg drop syndrome (EDS), from commercially laying hens in South Africa. *Onderstepoort J. Vet. Res.*, 58: 309-310.
- Chandra, Rajesh; Dixit, V.P.; Verma, K.C.; Rao, V.D.P and Shukla, S.K. (1997).** Egg drop syndrome-1976: A Review.

Indians J. of Comp, Micro., Imm. and Infections Diseases, 18: 1-17.

Cook Jane, K.A. and Darbyshire, J.H. (1980). Epidemiological studies with egg drop syndrome 1976 (EDS-76) virus. *Avian Pathology*, 9: 437-443.

Cook Jane, K.A. and Darbyshire, J.H. (1981). Longitudinal studies on the egg drop syndrome (EDS-76) in the fowl following experimental infection at 1-day-old. *Avian Pathology*, 10: 449-459.

Cunningham, C.H. (1966). A laboratory guide in virology, 4th ed. Minneapolis, Minn, Burgess Publishing Company, Minnesota.

Darbyshire, J.H. and Peters, R.W. (1980). Studies on EDS-76 virus infection in laying chickens. *Avian Pathology*, 9: 277-270.

Das, B.B. and Pradhan, H.K. (1982). Outbreaks of egg drop syndrome due to EDS-76 virus in quail (*Coturnix coturnix japonica*). *Vet. Rec. J. Br. Vet. Assoc. London.*, 131: 264-265.

Firth, G.A.; Hall, M.H. and McFerran, J.B. (1981). Isolation of a haemagglutinating adeno like virus related to virus 127 from an Australian poultry flock with an Egg drop syndrome. *Australian Veterinary Journal*, 57: 239-242.

Garg, S.P. and Garg, R.P. (1994). Studies on egg drop syndrome-76 vaccines immunization with killed, adjuvanted vaccines. *Indian Veterinary Journal (India)*, 71: 325-328.

Gulka, C.M.; Piela, T.H.; Yates, V.J.; and Bagshaw, C. (1984). Evidence of exposure of water fowl and other aquatic birds to the haemagglutinating duck adenovirus identical to EDS-76 virus. *J. Wildlife disease*, 20: 1-5.

Gylstorff, I. and Rolf, J. (1982). Egg drop syndrome of hens. II Pathology and histopathology. *Deutsche Tierärztliche Wochenschrift*. 89: 14-16.

Heffels, U.; Khalaf, S.E.D. and Kaleta, E.F. (1982). Studies on the persistence and excretion of egg drop syndrome 1976 virus in chickens. *Avian Pathology*, 11: 441-452.

- Higashihara, M.; Takai, S.; Hidaka, A.; Houolatsu, T.; Hiruma, M.; Watanbe, Y. and Matsmoto, M. 1983.** Isolation of the virus of Egg drop syndrome-76 in a Japanese outbreak. *Japan Journal of Veterinary Science*, 45:605-612.
- Hiruma, M.; Hidaka, A.; Takai, S.; Sasaki, T. and Higashihara, M. (1980).** Pathological lesions of the oviducts in fowls experimentally infected with avian adenovirus strain H-162 associated with Egg drop syndrome-1976. Japanese Veterinary Poultry Association: English Summaries of the Studies on Poultry Diseases in Japan, P. 13-15.
- Hlinok, A.; Muller, T.; Kramer, M.; Muhle, R.U.; Liebner, H. and Ziedler, K. (1998).** Serological Survey of Viral Pathogens in bean and white fronted geese from Germany. *Journal of Wildlife Diseases*, 34: 479-486.
- Howell, J. (1982).** Egg drop syndrome in Ross Brown Hens: an interim report. *Surveillance*, New Zealand 9: 10-11.
- Hudson, L. and Hay, F.C. (1980).** Practical immunology, Blackwell scientific publication. Oxford, London.
- Hwang, M.H.; Lauras, J.M.; Hipolito, O. and Silva, E.M. (1980).** Egg drop syndrome. 1976, a serological survey in Brazil, Proceedings of VIth *European Poultry Conference WPSA, Hamburg*, Vol. II P. 313-320.
- Justacara, I.; Leal, S.; De. Noguera, C. and De. Rosa, M. (1988).** Detection of an outbreak of the egg drop syndrome by serological and histopathological examinations. *Primeras Jornadas Nacionales de Actualizacion Avicola*, SOVVEA, 1988: 66-71.
- Kaletka, E.F.; Khalaf, S.E.D. and Stegmann, O. (1980).** Antibodies to Egg drop syndrome 76 virus in wild birds in possible conjunction with Egg shell problems. *Avian Pathology*, 9: 587-590.
- Kataria, R.J.M and Mohanty, G.C. (1991).** Isolation and characterization of haemagglutinating viral agents resembling EDS-76 virus from poultry flocks. *Indian Journal of Virology*, 7: 53-58.
- Khalaf, S.E.D.; Kaletka, E.F. and Siegmann, O. (1982).** Comparative studies on the kinetics of haemagglutination

inhibition and virus neutralizing antibodies following vaccination of chickens against egg drop syndrome 1976 (EDS-76). *Dev. Bio. Stand. Basel.*, 51 127-137.

Khalaf, S.E.D.; Kaleta, E.F.; Siegmann, O. (1980). Untersuchungen zur Schutzimpfung von Legehennen gegen das Egg drop syndrome 1976 (EDS-76). Proceedings of the VI European Poultry Conference WPSA, Hamburg, Vol. II, P. 408-415.

Kraft, V.; Grund, S. and Monreal, G. (1979). Ultrastructural characterization of isolate 127 of egg drop syndrome 1976 virus as an antigen. *Avian Pathology*, 8: 353.

Lakshmi Kavitha, K. (2003). Preparation and application of a DNA probe for chicken egg drop syndrome-76 virus. M.V.Sc. Thesis. A.N.G. Ranga Agri. Univ. Hyderabad.

Lu, Y.S.; Lin, D.F.; Tsai, H.J.; Lee, Y.L.; Chiu, S.Y.; Lee, C. and Huang, S.T. (1985). Outbreaks of egg drop syndrome-1976 in Taiwan and isolation of the etiological agent. *J. Chinese Society of Vet. Sci.*, 11: 157-165.

Malkinson, M. and Weismann, Y. (1980). Serological survey for the prevalence of antibodies to Egg drop syndrome 1976 virus in domesticated and wild birds in Israel. *Avian pathology*, 9: 421-426

Mathews, R.E.F. (1982). Classification and nomenclature of viruses (Fourth report of ICTV) *Intervirology* 17: 1-199.

McCracken, R.M. and McFerran, J.B. (1978). Experimental reproduction of the egg drop syndrome 1976 with a haemagglutinating adenovirus. *Avian Pathology*. 7: 483-490.

McFerran, J.B. (1979). Egg drop syndrome 1976 (EDS-76) *Veterinary Quarterly*, 1: 176-180.

McFerran, J.B. (1997). Egg drop syndrome. In: Diseases of Poultry, 10th Edition, B.W. Calnek et al., (eds.) Iowa State Univ. Press. Ames. Iowa, pp. 633.

McFerran, J.B. and Adair, B.M. (2003). In: disease of Poultry. 11th edn. Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald and D.E. Swayne (eds). Iowa State Press, Ames, Iowa. pp: 227.

- McFerran, J.B.; Connor, T.J. and Adair, B.M. (1978 a).** Studies on the antigenic relationship between an isolate (127) from the Egg drop syndrome 1976 and a fowl adenovirus. *Avian Pathology*, 7: 629-623.
- McFerran, J.B.; McCracken, R.M.; McKillop Eileen, R.; McNulty, M.S. and Colcins, D.S. (1978 b).** Studies on a depressed Egg production syndrome in Northern Ireland. *Avian Pathology*, 7: 35-47.
- Merchant, D.J.; Khan, R.H. and Murphy, W.H.Jr. (1960).** Hand-book of cell and organ culture, Minnesota, Burgees, Pub. Co.
- Meulemans, G.; Froyman, R.; Peters, J. and Halen, P. (1979).** Causes of drop in Egg productions in Belgian laying flocks. *Vlaams Diegeneeskungid Tijdschrift*, 47: 292-290.
- Monreal, G. and Dorn R. (1981).** Comparative investigations on the development of antibodies in the course of chicken- adeno and egg drop syndrome 76 virus infections. *Dtsch. Tierarztl. Wochenschr.*, 88: 508-511.
- Moorthy, A.S.; Rao, P.R.; Hahendar, M.; Rao, A.S.; Babu, T.S.; Reddy, T.V.; Moorthy, K.G. and Chetty, M.S. (1978).** Experimental Studies on the pathology of EDS-76 in White Leghorn chicks and grower chicken infected with strain '127'. *Indian Veterinary Journal*, 64: 826-828.
- Newathe, D.R. and Abegunde, A. (1980).** Egg drop syndrome-76 in Nigeria: serological evidence in commercial forms. *Vet. Rec. London. British Vet. Asso.*, 107: 466-467.
- Parsons, D.G.; Bracewell, C.D. and Parsons, G. (1980).** Experimental infection of turkeys with egg drop syndrome 1976 virus and studies on the application of the haemagglutination inhibition test. *Res. Vet. Sci. London*, 29: 89-92.
- Picault, J.P. (1978).** Chutes de ponte asseiées à la Production d'oeufs sans coquille on à coquille fragile. *L' Aviculture*, 379: 57-60.
- Ramkumar (1990).** Immunopathological studies on egg drop syndrome in poultry associated with an adenovirus infection Ph.D. Thesis, Indian Veterinary Research Institute, Izatnagar.

- Ramkumar and Mohanty, G.C. (1995).** Embryopathic effects of egg drop syndrome (EDS-76) virus in duck embryos. *Indian Journal of Vet. Pathology*, 19:6-12.
- Rampin, T.; Enice, F. and Mandelli, G. (1978).** Anticorpi contro antigen "B/C14" in oviaiole e riproduttori di allevamenti italiani *Clinica Veterinaria*, 101: 265-272.
- Rosales, G.; Antillon, A.; Morales, C. and D.A. McMartin (1980).** Antibodies against avian adenovirus strain BC-14 in hens in Mexico. Proceedings of 29th Western Poultry Disease Conference, Acapulco, Mexico, 192-196.
- Shakya, S. and Dhawedkar, K.G. (1991).** Antigenic relationship among Egg drop syndrome- 1976 virus strains. *Indian Veterinary Journal* 68: 510-513.
- Smyth, J.A.; Platten, M.A. and McFerran, J.B. (1988).** A study of the pathogenesis of egg drop syndrome in laying hens. *Avian Pathology*, 17: 653-666.
- Solyom, F.; Nemesi, M.; Forgacs, A.; Balla, E. and Perenyi, T. (1982).** Studies on EDS-76 egg drop syndrome vaccine. *Dev. Bio. Stand. Basel.*, 51: 105-121.
- Swain, P.; Kataria, J.M. and Verma, K.C. (1993).** Biological characterization of an Indian isolate of egg drop syndrome-76 virus. *Research in Veterinary Science*, 55: 396-397.
- Swain, P.; Kataria, J.M.; Verma, K.C. and Satish, K. (1992).** Characterizations of a field isolate of Egg drop syndrome 76 (EDS-76) virus. *Indian Journal of virology* 8: 8-14.
- Swaiu, P.; Kataria, J.M. and Verma, K.C. (1993).** Biological characterization of an Indian isolate of egg drop syndrome-76 virus. *Res. Vet. Sci. London*, 55: 396-397.
- Takai, S.; Higashihara, M. and Matumoto, M. (1984).** Purification and haemagglutinating properties of Egg drop Syndrome 1976 virus. *Archives of Virology* 80: 59-67.
- Taniguchi, T.; Yamaguchi, S.; Maeda, M.; Kawamura, H. and Horiuchi, T. (1981).** Pathological changes in laying hens inoculated with the JPA-1 strain of Egg drop syndrome-1976 virus. *Natl. Inst. Anim. Health Q. (Japan)*. Vol. 2. 1981.

- Todd, D. and McNulty, M.S. (1978).** Biochemical studies on a virus associated with Egg drop syndrome-1976. *Journal of General Virology* 40: 63-75
- Van Eck, J.H.H. (1983).** Effects of experimental infection of fowl with EDS-76 virus, infectious bronchitis virus and for fowl adenovirus on laying performance, *Vet. Q.* 5:11-25.
- Van Eck, J.H.H.; Develaar, F.G.; Vanden Heuvel Plesman, T.A.M.; Vankol, N.; Kouwenhoven, B. and Guldie, F.H.M. (1976).** Dropped egg production, soft shelled and shell less eggs associated with the appearance of precipitins to adeno virus in flocks of laying fowls. *Avian Pathology* 5: 261-272.
- Villegas, P.; Kleven, S.H.; Eidson, L.S. and Trampel, D. (1979).** Isolation of a haemagglutinating adenovirus serologically related to adenovirus-127. *Avian diseases* 25: 507-514.
- Yamaguchi, S.; Imada, T.; Kawamura, H.; Taniguchi, S.; Saio, H. and Shimamtsu, K. (1981a).** Outbreak of Egg Drop Syndrome in Japan its etiological agent. *Avian diseases* 25: 628-641.
- Yamaguchi, S.; Imada, T.; Kawamura, H.; Taniguchi, T and Kawakami, M. (1981b).** Pathogenicity and distribution of Egg drop syndrome- 1976. Virus (JPA-1) in inoculated laying hens. *Avian diseases* 25: 642-649.
- Yates, V.J.; Rhee, Y.O.; Fry, D.E.; Mishad, E.I. and McCornick, K.T. (1976).** The presence of avian adenovirus and AAV in healthy chickens. *Avian Disease*, 20: 146-152.
- Zanella, A.; Donato, A.N.; Nigrellia, A. and Poli, G. (1980),** Etiopathogenesis, Epidemiology, Immunology and control of diseases. *Clinico. Vet.*, 103: 549-569 (Vet. Bulletin 51: 3219).
- Zsak, L. and Kisary, J. (1981a).** Studies on Egg drop syndrome (EDS) and chicken embryo lethal orphan (CELO) Avian adenovirus DNAs by restriction endonucleases. *Journal of General Virology* 56: 87-95.
- Zsak, L. and Kisary, J. (1981b).** Some biological and physicochemical properties of Egg drop syndrome (EDS) avian Adenovirus strain B8/78. *Archives of Virology* 68: 211-219.

Zsak, L.; Szekely, A. and Kisary, J. (1982). Experimental infection of young and laying geese with Egg drop syndrome 1976. Adenovirus strain B8/78. *Avian Pathology* 11: 555-562.

(A) REAGENTS FOR CELL CULTURE**1. Medium-199, pH 7.2**

M-199 (Sigma)	9.67 gm
Penicillin	1 Lac IU
Streptomycin	100 mg
TGDW	100 ml

- ☛ Sterilize the media by filtration through 0.22 μm membrane filter and store at 4°C.

a. Growth medium

M-199	83.0 ml
Fetal calf serum	15.0 ml
Tryptose phosphate broth (1%)	2.0 ml

b. Maintenance medium

M-199	98.0 ml
Fetal calf serum	2.0 ml

2. Trypsin glucose solution (pH 7.2, 0.125%)

Sodium chloride	8.0 g
Potassium chloride	0.2 gm
Disodium hydrogen phosphate	2.312 gm
Potassium dihydrogen orthophosphate	0.2 gm
Trypsin powder	1.25 gm
Glucose	2.0 gm
TGDW	1000 ml
Streptomycin	100 mg
Penicillin	1 Lac IU

- ☛ The pH was adjusted to 7.2 with addition of sodium bicarbonate and the solution was sterilized by filtration through seitz filter and stored at 4°C.

(B) MAY-GRUNWALD AND GIEMSA STAIN**1. May grunwald stain****(Stock solution)**

May-Grunwald stain	3.0 gm
Methanol	1000 ml

(Working solution)

Stock MGG stain	20 ml
Distilled water	10 ml

2. Giemsa stain

(Stock solution)

Giemsa powder	7.5 gm
Glycerin	500 ml
Methanol	500 ml

(Working solution)

Stock solution	10 ml
Distil water	90 ml

- ☞ Dissolve the powder in glycerin at 56°C for 1-2 hr in water bath and then add methanol and leave at least fir a week for maturation.

(C) REAGENTS FOR AGPT TEST**1. Agarose gel (1%) for AGPT**

Agarose	1.000 g
Sodium azide	0.020 g
Normal saline	100 ml

- ☞ Sterilize by autoclaving at 10 lbs pressure for 30 min.

(D) REAGENTS FOR HA TEST**1. Alsevers solution**

Dextrose	20.500 g
Sodium citrate	8.000 g
Sodium chloride	4.200 g
Citric Acid	0.550 g
Distilled water	1000 ml

- ☞ Sterilize by autoclaving at 10 lbs pressure and store at 4°C

2. Alsevers solution

Sodium chloride	0.850 g
Triple glass distilled water	100 ml

Vita

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