MOLECULAR CHARACTERIZATION OF EGG DROP SYNDROME-76 (EDS-76) VIRUS

By

S. VIJAYA KRISHNA

M.V.Sc.,

THESIS SUBMITTED TO
ACHARYA N.G.RANGA AGRICULTURAL UNIVERSITY
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF DEGREE OF

DOCTOR OF PHILOSOPHY

(VETERINARY MICROBIOLOGY)

DEPARTMENT OF MICROBIOLOGY
COLLEGE OF VETERINARY SCIENCE, TIRUPATI
ACHARYA N.G. RANGA AGRICULTURAL UNIVERSITY
HYDERABAD - 500 030

MAY, 2000

CERTIFICATE

Dr. S. Vijaya Krishna *has satisfactorily prosecuted the course of research and that the thesis entitled* **"MOLECULAR CHARACTERIZATION OF EGG DROP SYNDROME - 76 (EDS - 76) VIRUS"** *submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any university.*

Date :

Major Advisor

(M.V. SUBBA RAO)
Associate Dean & University Head
Department of Microbiology
College of Veterinary Science
Gannavaram- 521 102

CERTIFICATE

This is to certify that the thesis entitled "**MOLECULAR CHARACTERIZATION OF EGG DROP SYNDROME - 76 (EDS - 76) VIRUS**" submitted in partial fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** of the Acharya N.G.Ranga Agricultural University, Hyderabad is a record of the bonafide research work carried out by **Dr. S. Vijaya Krishna** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of the investigation have been duly acknowledged by the author of the thesis.

(M. V. SUBBA RAO)
Chairman of the Advisory Committee

Thesis approved by the Student's Advisory Committee

Chairman : **(M.V. SUBBA RAO)**
Associate Dean & University Head
Department of Microbiology
College of Veterinary Science
Gannavaram - 521 102

Member : **(T. VENKATA REDDY)**
Associate Professor & Uni.Head
Department of Epidemiology and Preventive Medicine
College of Veterinary Science
Tirupati - 517 502

Member : **(P. C. CHOUDHURI)**
Professor & University Head
Department of Medicine
College of Veterinary Science
Tirupati - 517 502

Member : **(N. ALAHA SINGARI)**
Associate Professor
Department of Medicine
College of Veterinary Science
Tirupati - 517 502

Member : **(P. SREENIVASULU)**
Professor & Head
Department of Virology
Sri Venkateswara University
Tirupati - 517 502

CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ACKNOWLEDGEMENTS

Diction is not enough to express my deep sense of gratitude and I wish to place on record my sincere and heartfelt regards to **Prof. M.V. Subba Rao,** Associate Dean and Uni. Head, Department of Microbiology, College of Veterinary Science, Gannavaram for his inspiring and affectionate guidance, constant encouragement and constructive criticism in planning and presentation of the investigation reported in the thesis.

I am grateful to **Dr.T. Suribabu** , Associate Professor (retired), Department of Microbiology for his advice, help and suggestions during the course of the work.

I am greatly beholden beyond words to express my sense of gratitude to **Prof. P. Sreenivasulu**, Professor & Head, Department of Virology, S.V.University, Tirupati. His stupendous, splendid and stimulating guidance during this investigation are gratefully acknowledged.

I am highly indebted to **Dr. T. Venkata Reddy,** Associate Professor & University Head, Department of Epidemiology and Preventive Medicine, **Prof. P.C.Choudhuri**, Professor and University Head and **Dr. Alaha Singhari**, Associate Professor, Dept. of Medicine, College of Veterinary Science, Tirupati for their kind help and valuable suggestions during the research work.

I am very glad to place my sincere thanks to **Dr. D. Sreenivasulu**, Assistant Professor & i/c Head and **Dr. Y.N.Reddy**, Assistant Professor, Department of Microbiology, College of Veterinary Science, Tirupati, for their help and co-operation during my research work.

I deem it my privilege to express my fidelity to **Dr. D.V.R. Sai Gopal**, Assistant Professor and **Mr. M. Venkata Ramana,** Research Scholar, Dept. of Virology,

S.V.University, Tirupati for there stupendous co-operation in the laboratory during this investigation.

I would like to express my thanks to **Dr. T.S. Chandrasekhara Rao,** Associate Professor of Anatomy, **Dr. V.H.Rao.** Associate Professor of Physiology and **Dr. K.Subramanyam Reddy**, Assistant Professor of Statistics, for their kind help and encouragement during the present study.

I feel it is my duty to acknowledge the pleasant co-operation extended by **Smt. K. Varalakshmi,** SRF, **Smt. Sridevi,** JRF of DBT Scheme and **Mr. Sudheer** M.Sc., Student.

I am thankful to **Sri. K. Subramanyam Reddy, Sri. Balakrishna, Sri. G. Krishnaiah,** non teaching staff of Dept. of Microbiology, College of Veterinary Science, Tirupati for their help during the course of the present investigation.

I am in deep concern to express my great sense of honor, love, heartfelt regards and earnest gratitude towards my beloved parents **S. Munisundaram, Smt. S. Lakshmi Rajyam,** Aunty **Smt. P. Padmavathi,** Uncle **Dr. P. Chalapathi Naidu,** my wife **Mrs. P. Sujatha**, and my daughter **Ms. Sravani** for their ever lasting love unmatchable encouragement, needy inspiration and efforts throughout my educational career.

I wish to thank Government of Andhra Pradesh for providing the financial support in the form of stipend during my postgraduate programme.

(S. VIJAYA KRISHNA)

Name of the Author	:	**S. VIJAYA KRISHNA**
Title of the Thesis	:	**MOLECULAR CHARACTERISATION OF EGG DROP SYNDROME - 76 (EDS-76) VIRUS ISOLATES**
Degree to which it is submitted	:	DOCTOR OF PHILOSOPHY
Faculty	:	FACULTY OF VETERINARY SCIENCE
Major Advisor	:	**Dr. M.V. SUBBA RAO** Associate Dean and Univ. Head Department of Microbiology College of Veterinary Science Gannavaram - 521 102
University	:	ACHARYA N.G.RANGA AGRICULTURAL UNIVERSITY, HYDERABAD
Year of Submission	:	2000

ABSTRACT

The present investigation was taken up with a view to understand the antigenic and molecular characters of the indigenous Egg Drop Syndrome-76 (EDS-76) virus isolates which aids in better understanding to develop an effective vaccine which is very much essential now in India due to frequent outbreaks.

In this study, the virus isolates were procured from different geographical regions of India and were propagated in 9-10 day old duck embryos and were purified by ultracentrifugation in 10-40 per cent sucrose density gradients. The U.V. light absorption spectrum of purified virus has maximum and minimum absorption between 258.9-260. nm and 242.3 - 243.4 nm, respectively. The $A_{260/280}$ ratio was observed as 1.42.

The virus isolates were identified by analyzing the antigens using immunodiffusion test and comparison of their patterns with standard EDS-76 virus, 127 strain and its antiserum. All the isolates revealed lines of identity with the standard EDS-76, strain 127 and its antiserum within 24-48 h.

The electron microscopic observation of the purified virus preparations of the VN_1, isolate revealed the presence of complete hexagonal virus particles scattering singly with small number of disrupted and penetrated particles. The virus particle measured approximately 70 nm in diameter.

The viral polypeptides of six isolates were identified and characterized. The virion contained 13 polypeptides, which varied from 18 to 124 kDa, many of which appeared as major polypeptides. The immunogenic polypeptide(s) were identified among the 13 polypeptides by electroblot immunoassay. Only three polypeptides of molecular weights 124, 84 and 54 kDa appear to be immunogenic.

The virion polypeptide(s) responsible for protection / capable of absorbing HI antibodies and neutralizing antibody was isolated and characterized. The present study revealed that the polypeptide having molecular weight 84 kDa is responsible for production of neutralizing antibody as well as haemagglutinating antibody.

The cell mediated immune response in chicks experimentally infected with EDS-76 (VN_1 isolate) viral polypeptides was studied using the lymphocyte stimulation test by MTT assay. The results indicated that the polypeptide 84 kDa sensitized spleen cells stimulated actively more in numbers when compared to spleen cells sensitized with other polypeptides. Similarly a noticeable splenomegaly was also observed 5 days after sensitizing the chicken with 84 kDa polypeptide.

Cytotoxic assay revealed that the percentage of dead cells are more in the 84 kDa polypeptide introduced wells when compared to 54 and 124 kDa polypeptide treated

wells. Similarly, 84 kDa polypeptide induced more number of antibody forming cells when compared to other polypeptides in plaque assay.

Viral nucleic acid was extracted from the purified virus and the molecular weight of the genome was calculated as 22.6 x 10^6 daltons (34.2 kb). The DNA's of 3 isolates were compared with the DNA of the referral strain, 127 by *Eco RI* restriction enzyme and found that all the three isolates were found to have genetic similarity. The restriction enzymes *Hind III, Pvu II, Eco RI* and *Hae III* cleaved the VN_I virus genome at a total of 7, 12, 4 and 12 restriction sites respectively.

The physical map of EDS-76, VN_I, isolate obtained for *Eco RI* enzyme suggests that the genome of EDS virus is a linear duplex molecule.

In conclusion, the present investigation on molecular and antigenic characterization of EDS-76 virus isolates helped in identifying the immunogenic polypeptides. These results would be critical while designing a molecularly defined non infectious EDS-76 virus vaccine. It would be interesting to identify the genomic fragment (s) coding for the major immunogenic polypeptide (84 kDa) which will pave the way for molecularly designed vaccines.

ABBREVIATIONS

General

°C	:	degree celcius
Fig.	:	Figure
x g	:	centrifugal force equal to gravitational
DNA	:	deoxyribo nucleic acid
g	:	gram
h	:	hours
kDa	:	kilodaltons
M	:	molar
min	:	minutes
µl	:	microlitres
µg	:	microgram
ml	:	milliliters
mM	:	millimolar
mg	:	milligram
nm	:	nanometer
OD	:	Optical density
pH	:	hydrogen ion concentration
%	:	per cent
RBC	:	Red blood corpuscles
rpm	:	revolutions per minute
sec	:	seconds
~	:	Approximately
PFU	:	Plat forming units

Virus

EDSV : Egg Drop Syndrome virus

IBDV : Infectious bursal disease virus

IBV : Infectious bronchitis virus

Tests and Reagents

ID : Immunodiffusion test

HA : Haemagglutination test

HI : Haemagglutination inhibition

FCA : Freund's complete adjuvant

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Egg drop syndrome is currently one of the important economic problems faced by the poultry industry. The syndrome is caused by multiple agents viz., bacteria, mycoplasma, fungi and viruses. However, the major cause of this syndrome is a haemagglutinating aviadenovirus.

2.1 General Properties of EDS-76 virus

EDS-76 virus was classified in the genus *aviadenovirus* of the family *Adenoviridae* on the basis of its morphology, replication and chemical composition (Mathews, 1982). The size of the virus observed in negatively stained preparations has been reported as ranging from 76 nm to 80 ± 5 nm by McFerran *et al.*(1978). Kraft *et al.*(1979) were able to demonstrate typical adenovirus morphology with triangular faces with six capsomeres on the edge and a single 25 nm fibre projecting from each vertex.

Todd and McNulty (1978) found that infectious virus particles banded at densities of 1.32 and 1.30 g/ml in cesium chloride density gradients. Kraft *et al.* (1979) reported the presence of two bands of infectious haemagglutinating particles at 1.32 g/ml with non-infectious disrupted particles banded at 1.30 g/ml in cesium chloride. Zsak and Kisary (1981) reported that EDS-76 virus particles banded at densities of 1.36 and 1.31 g/ml.

Adair *et al.* (1979) by using ^{3}H thymidine labelling and inhibition with iododeoxyuridine showed that EDS-76 virus contains DNA. The molecular weight of DNA was estimated to be 22.6 x 10^{6} daltons.

The virus agglutinates erythrocytes of chicken, duck, turkey, goose, pigeon and peacock but does not agglutinate rat, rabbit, mouse, sheep, cattle, goat or pig erythrocytes. The virus is stable when treated with chloroform and variations in pH between 3 and 10. The virus was inactivated by heating for 30 min. at 60°C. The virus survives for 3 h. at 56°C and is stable in monovalent but not in divalent cations (Adair *et al.*, 1979). Takai *et al.* (1984) reported that the infectivity of the virus was lost after treatment with 0.5 per cent formaldehyde or 0.5 per cent glutaraldehyde.

Wigand *et al.* (1982) reported that the EDS appears to deviate in various properties from other avian adenoviruses and suggested to place the EDS-76 virus in a separate genus.

Wilson (1989) isolated and identified the polypeptide responsible for protection / capable of absorbing neutralizing and haemagglutination inhibiting antibodies. The virus polypeptide responsible for absorbing neutralizing and the haemagglutination inhibiting antibodies had a molecular weight of 44,000 daltons.

Shakya and Dhawedkar (1991) studied the antigenic relationship among EDS-76 virus strains using cross haemagglutination inhibition tests and showed that the strains are indistinguishable.

Swain *et al.* (1992) reported physicochemical properties of an EDS-76 virus isolated from a flock experiencing drop in egg production. They have also determined the viral nucleic acid as DNA. The purified virus was also analyzed for proteins and reported that the virus contains 12 polypeptides of 10-126 kDa.

RamKumar *et al.* (1992) studied the filterability of EDS-76 virus and reported that the virus is filterable through membrane filters of 100 nm greater pore sizes but not through 50 nm filters. These workers also reported that the virus is resistant to chloroform.

Harrach *et al.* (1997) reported the close phylogenetic relationship between EDS virus, bovine adenovirus serotype,7 and ovine adenovirus strain, 287.

Hess *et al.* (1997) studied the complete nucleotide sequence of the egg drop syndrome virus and reported that the virus is non-enveloped, icosahedral, 70-90 nm particles containing a double stranded linear, 30 to 45 kb DNA genome.

2.1.1 Physico-chemical characterization of EDS -76 virus

Adair *et al.* (1979) studied biological and physical properties of avian adenovirus strain 127 associated with EDS-76. The infectivity of virus was found to be stable in monovalent, but not in divalent cations, ether treatment and to extremes of pH, whereas IUDR inhibited the replication of virion.

The duck adenovirus isolate EDS-127 of Villegas *et al.* (1979) was resistant to chloroform, replicated in nucleus and contained DNA as its genome. An avian adenovirus strain E-77 isolated by Zanella *et al.* (1980) had similar physico-chemical properties as described by Adair *et al.* (1979) and Villegas *et al.* (1979).

An isolate of avian adenovirus namely, JPA-1 was cloned and found similar to BC-14 biologically and physico-chemically. The virus was stable at pH 3.0, inhibited by IUDR and resistant to heat at 50°C but not at 60°C (Yamaguchi *et al.*, 1981). The duck adenovirus isolate B8/78 isolated by Zsak *et al.* (1981) had similar physico-chemical properties as described by Adair *et al.* (1979) and Zanella *et al.* (1980).

Thermal inactivation of a duck adenovirus (DAV) serologically indistinguishable from adenovirus 127 was studied by Walker *et al.* (1982). They compared the virus infectivity with its HA activity. The infectivity of the virus declined rapidly at 56°C and non infectivity of the virus was detected after 90 min. However, HA activity declined gradually and was detectable at a dilution of 1:64 even after 90 min. of inactivation.

Takai *et al.* (1984) purified the three populations of EDS- 76 virus particles F_7, F_9 and F_{17} with buoyant densities of 1.34, 1.33 and 1.29 g /ml respectively in cesium chloride equilibrium density gradients. The three types of particles found to be infectious complete virions.

Rozhdestvenskii (1984) inactivated avian adenovirus strain B8/78 by exposure to 60 - 70°C for 10 to 40 min. formaldehyde solution (0.05-1.0 per cent) for 24 to 48 h. and ethyleneamine (0.01-0.03 per cent) for 3 - 72 h. at 37°C. Exposure to 0.2 per cent ethyleneamine for 3 h. was considered best method of inactivation to preserve intact HA activity.

Reddy (1984), studied the physico-chemical properties of local Indian EDS virus isolates namely, SPC, GEC and DFH and found similar results as described by Villegas *et al.* (1979) and Yamaguchi *et al.* (1981).

Swain *et al.* (1997) purified the EDS-76 virus by velocity density gradient centrifugation in continuous density gradients of cesium chloride, sodium potassium tartarate and sucrose. The band obtained with sodium potassium tartarate was found to have the highest infectivity and haemagglutinating activity.

2.2 Isolation of EDS-76 virus

In Northern-Ireland McFerran *et al.* (1978) first isolated adenovirus from birds of depressed egg production syndrome. Six of the isolates which agglutinated fowl erythrocytes at very high titres were recovered from six affected flocks by cultivating in chicken embryo liver cells. Out of six isolates, three were from oviduct, two from the respiratory tract and one from faeces. These agents were adenoviruses but not neutralized by antisera of the 11 known prototypes of avian adenoviruses.

Baxendale (1978) isolated BC-14 adenovirus strain from cases of EDS by inoculating blood leucocytes of affected birds in chicken embryo liver cell cultures. The affected flock failed to reach peak egg production. The birds laid soft shelled eggs and depigmented eggs.

Todd *et al.* (1988) isolated virus similar to EDS-127 from a syndrome of depressed egg production in broiler breeder flocks by inoculating in chicken embryo liver cells.

A strain of avian adenovirus was recovered by Meulemans *et al.* (1979) in Belgium from a flock suffering from egg drop syndrome. The isolate was serologically and morphologically identical with the virus isolated by McFerran *et al.*(1978) from Ireland.

Villegas *et al.* (1979) isolated a virus resembling adenovirus 127 from clinically normal ducks. The isolate haemagglutinated chicken RBCs which was specifically inhibited by antiserum against adenovirus 127. These workers employed chicken embryo liver cells for isolation of virus from cloacal swabs.

Bennejean *et al.* (1980) isolated a haemagglutinating viral agent from livers of hens in France from birds exhibiting typical clinical egg drop syndrome symptoms and reproduced the syndrome experimentally with viral isolates.

In Italy, Zanella *et al.* (1980) isolated a viral strain E-77 from cloacal swabs using chicken hepatocytes from hens affected with depressed egg production. The isolate was indistinguishable from adenovirus strains 127, BC-14 and 3877. Similarly, Firth *et al.* (1981) isolated two haemagglutinating viruses from broiler poultry flock having depressed egg production. These workers confirmed the isolates by electron microscopy as adenovirus.

Yamaguchi *et al.* (1981) isolated 11 haemagglutinating avian adenoviruses from conditions similar to EDS-76. These authors recovered the viruses from cloacal swabs and uterus in chicken kidney cells. One isolate was cloned and was named as JPA-1 which was biologically and physico-chemically similar to BC-14 strain of EDS-76.

Gough *et al.* (1982) isolated a haemagglutinating adenovirus PD-1917 from commercial ducks suffering from keratoconjunctivitis. The isolation was made in duck embryos.

In Japan, Higashihara *et al.* (1983) isolated a haemagglutinating virus H-162 from faeces of a hen in broiler breeding flock and shown to be serologically identical to EDS-76 virus by the immunodiffusion and HI tests.

Reddy (1984), isolated haemagglutinating adenoviruses from serologically positive poultry and duck flocks. Out of 33 pooled faecal samples of chicken tested, two isolates and from 9 pooled faecal samples of ducks tested, one isolate was obtained.

Das *et al.* (1996) isolated and identified the EDS-76 virus strains from organised poultry and duck breeding farms in Orissa.

2.3 Morphological characterization of EDS-76 Virus

Electron microscopy of purified soluble haemagglutinin showed rod-like structures of 25-30 nm x 10 nm and suggested it as an adenovirus (Todd and McNulty, 1978).

Electron microscopic studies carried out by several workers (Adair *et al.*, 1979, Firth *et al.*, 1981, Higashihara *et al.*, 1983, and Swain *et al.*, 1992) revealed hexogonal particles which is a typical characteristics of adenovirus. The virions measured about 70 nm in diameter. The EDS-76 virus particles appeared as large clumps in electron microscope and will occasionally occur singly (ZSak and Kisary, 1981)

Yamaguchi *et al.* (1981) reported the electron microscopy of EDS-76 purified viral particles. Both the B_1 and B_2 opalescent bands having densities of 1.33 and 1.30 g / ml respectively were negatively stained and observed virus particles in both the bands. These authors reported that B_1 consisted of complete particles and B_2 consisted of disrupted particles.

EDS-127 virus isolate was purified and examined by electronmicroscopy. In cesium chloride equilibrium density gradients three bands were visualised. The first two bands showed high HA activity and infectivity whereas third band showed only HA activity but not infectivity. Electron microscopic examination of the bands revealed typical adenovirus (Kraft *et al.*, 1979 and Firth *et al.*, 1981).

Higashihara *et al.* (1983) observed the typical adenovirus morphology of EDS-76 virus by electron microscopy. They found that the virus particles often aggregated in paracrystalline array forming a cubic lattice in the nuclei of thin sections of chicken embryo liver cells, fibroblasts and chicken kidney cells.

Takai *et al.* (1984) reported the electron microscopy of EDS-76 virus on carbon grids and negatively stained with 2 per cent phosphotungstic acid. The results revealed that the EDS-76 virus is possessing similar characteristics as other members of adenovirus family.

Electron and immunoelectron microscopic investigations on EDS-76 virus was studied by Chandramohan *et al.* (1997). They reported that the time taken to identify the viral clumps under immunoelectron microscope was much shorter than under direct negative contrast electron microscope. The number of virions observed were 50-100 times greater as compared to that of direct negative contrast electron microscope.

2.4 Immunological characterization of EDS-76 virus

2.4.1 Polyacrylamide gel electrophoresis and Electroblot immuno assay

Todd and McNulty (1978) studied the biochemical characters of a virus associated with EDS. The polypeptide profiles revealed 13 distinct polypeptide bands and these were in the molecular weight range of 126 to 11 kDa.

Chetty (1985) studied the polypeptide profiles of EDS-76 indigenous virus isolates with reference to EDS-76 virus and observed a total of 13 polypeptide bands in all the isolates. However, the authors used an unpurified virus preparation.

Manjuvani (1986) used PAGE to study the various antigenic fractions and differences present between the local isolates. The slab gel with SDS treatment revealed different pattern of polypeptide profiles for different isolates. However, the virus used was not a purified preparation.

Wilson (1989) isolated and identified the polypeptide responsible for protection or absorbing neutralizing and haemagglutination inhibiting antibodies using purified EDS-76 virus. The virus polypeptide responsible for absorbing neutralizing and the haemagglutination inhibition antibodies was having a molecular weight of 44 kDa. He also reported that EDS-76 virus contain 13 structural polypeptides.

Swain *et al.* (1992) characterised the field isolates of EDS-76 virus. The polypeptide profiles by SDS-PAGE revealed 12 viral proteins of 10-126 kDa.

Yishan *et al.* (1995) studied the protein characteristics of GC_2 strain of EDS-76 virus and revealed 13 distinct polypeptide bands ranging in molecular weights from 126 kDa to 11 kDa.

The monoclonal antibody (Mab) addition test and western blotting were applied by Kejun *et al.* (1996) to analyze the EDS-76 viral recognition sites of 10 monoclonal antibodies. They have reported that all the 10 Mab's recognised the 122,

85 and 150 kDa bands and the positive mouse-anti-EDS-76 virus serum reacted with viral polypeptides with bands of 141, 122, 109, 85, 47, 27 and 15 kDa.

Chandramohan *et al.* (1998) studied the protein profiles of EDS-76 viral isolates and observed 13 polypeptides with molecular weights ranging from 125 kDa to 11 kDa which were identical to those of the reference strain of the egg drop syndrome aviadenovirus.

Virion core polypeptides of EDS virus was analyzed by Yurov *et al.* (1998) and found 3 polypeptides (24,10.5 and 6.5 kDa) in the core by SDS-PAGE, whereas they found that the 40 kDa is not a core component.

2.4.2 Lymphocyte stimulation test and Cytotoxicity test

Thompson *et al.* (1980) evaluated nitrogen induced cellular cytotoxicity with peripheral blood leukocytes of chicken. They found that chickens from low plasma corticosterone line showed a greater lymphocyte transformation response than those from the higher plasma corticosterone line.

Mosmann (1983) developed a rapid colorimetric assay for cellular growth and survival and applied this technique to proliferation and cytotoxicity assays. The assay detects living cells but not dead cells. They used this assay to measure proliferative lymphokines, nitrogen stimulation and complement mediated lysis.

Mullbacher *et al.* (1984) reported an improved colorimetric assay for 'T' cell cytotoxicity in vitro. They introduced neutral red as an indicator in cytotoxicity assays to replace ^{51}Cr.

Green *et al.* (1984) developed a rapid colorimetric microtiter assay to detect cytotoxic lymphokines produced by human lymphocytes activated with lectins or tumor cells. The viability of lymphotoxin treated target cells was detected using a tetrazolium dye that is reduced to a blue formazan by living but not dead cells. The amount of dye formed was quantitated using a microplate spectrophotometer (ELISA plate reader) and their visual observations confirmed the amount of formazan dye produced was directly proportional to the number of viable target cells.

Eck and Van (1986) studied the involvement of spleen components of mature fowl in the primary and secondary humoral antibody response following experimental infection with EDS-76 virus and observed enhanced lymphoblast formation and splenomegaly, 4-12 days after infection.

Denizot and Lang (1986) estimated the number of surviving cells growing in microtiter tray wells by using a rapid colorimetric assay. These authors modified the original tetrazolium dye procedure and with these modification the reliability and sensitivity of the test have been increased to the point where it can replace the ^{3}H thymidine uptake assay to measure cell proliferation or survival in growth factor or cytotoxicity assay.

Gerlier and Thomasset (1986) used MTT colorimetric assay to measure cytotoxicity assay and cell proliferation. Their analysis of MTT cleavage by viable cells suggests that the colorimetric MTT test can be useful to quantify the activation of level of cells and independently of proliferation.

The importance of specific cytotoxic T lymphocytes in cell mediated immune response in case of viral infections was studied by Cannon and Russell (1986).

Chubb *et al.* (1987) detected the cytotoxic lymphocyte activity in chickens infected with infectious bronchitis virus or fowl pox virus. They used both target and effector cells from the same animal to overcome histoincompatibility.

Umesh Kumar *et al.* (1989) made studies on cell mediated immune response in chicks experimentally infected with EDS-76 virus using the lymphocyte stimulation test and the leucocyte migration inhibition test using ^{3}H thymidine uptake assay.

Bounous *et al.* (1992) compared the MTT colorimetric assay and tritiated thymidine uptake for lymphocyte proliferation using chicken splenocytes. The results from stimulated cells in both assay methods were significantly different from results from the control cells, and the MTT assay results regressed in a significantly linear manner on count from ^{3}H thymidine incorporation. On this basis they concluded that

the MTT assay is a valid test for evaluation of lymphocyte proliferation of chicken splenocytes.

Thomsson and Naqi (1997) studied the cytotoxic activity of cells recovered from the respiratory tracts of chickens inoculated with infectious bronchitis virus. They reported that the cytotoxic activity attributed to natural killer cells was statistically more pronounced ($P < 0.05$) in IBDV plus IBV - infected chicken than in chickens inoculated with IBV alone.

A colorimetric test employing MTT was used to determine the mitogenic response of intestinal intraepithelial lymphocytes (i-IEL) of chicken to T and B cell mitogen was studies by Agrawal and Reynolds (1999). This technique was useful in evaluating and studying the role of i-IEL in local cell mediated immune response.

Vickery *et al.* (1999) reported the duck hepatitis B virus antigen specific blastogenesis assay to measure the cellular immune response of ducks to duck hepatitis B virus infection and found that immune group was significantly different to other groups.

2.4.3 Plaque assay

Jerne's plaque assay is generally used to detect and to quantify antibody forming cells in response to stimulation by an antigen. Friedman and Goldner (1970)

reported an immediate 50 per cent decrease in the sheep erythrocyte plaque forming cell (PFC) response in new born hamsters infected with adeno 12 and SV_{40} virus.

Nagaraja *et al.* (1982) used hemolytic plaque forming technique to detect antibody forming cells in turkeys infected with haemorrhagic enteritis (HE) virus and observed the decreased capability of HE virus infected turkeys to produce antibodies to sheep RBC.

2.5 Molecular characterization of EDS-76 virus

2.5.1 Restriction enzyme analysis of EDS-76 virus

Kisary and Zsak (1980) reported the molecular weight of EDS avian adenovirus (strain B8/78) DNA as 22.9 x 10^6 daltons after digestion with *EcoR* I. This enzyme cleaved at 2 sites of the EDS adenovirus DNA generating 3 fragments with molecular weights of 13.5 x 10^6, 5 x 10^6 and 4.4 x 10^6 daltons respectively.

Zsak and Kisary (1981) studied on the EDS and CELO avian adenovirus DNA's by restriction endonucleases like *EcoRI, Bam HI, Hind III, Bgl I, Bgl II, Hpa I or Pst I.* These workers have recognized 48 cleavage sites in EDS virus strain B8/78 DNA, whereas CELO virus DNA was cut into 61 fragments by the same enzymes. They have also physically mapped the fragments generated from B8/78 DNA by *EcoRI and BamHI.*

Zsak and Kisary (1984) grouped the 17 fowl adenovirus strains representing 11 serotypes based upon the restriction patterns of DNA's generated by restriction endonucleases *Bam* HI and *Hind* III.

Todd *et al.* (1988) differentiated 13 EDS virus isolates by restriction endonuclease analysis of viral DNA. Genome map for *EcoRI, BamHI, Kpnl or Pstl* was also constructed for EDS virus, strain 127.

Ramkumar *et al.* (1991) studied restriction endonuclease analysis of EDSV-IVRI/AD-86 isolate and reported that the isolate resembles the classical European fowl EDS isolate typified by the Northern Ireland virus strain 127. These authors used *Hae III* enzyme to compare these two isolates.

Zakharchuk *et al.* (1993) reported the physical mapping and homology of EDS-76 virus DNA. The data reported by them suggests a genetic similarity between EDS-76 virus and bovine adenoviruses. They have physically mapped the fragments generated from EDS-76 virus DNA by 8 restriction endonucleases.

Chandramohan (1994) studied the Restriction endonuclease analytic patterns of EDS-76 virus DNA with 6 restriction enzymes and found that *Pst I* generated more number of DNA fragments.

Restriction endonuclease anlaysis of viral genomic DNA of chicken EDS H_{91} virus was studied by Yugou *et al.* (1995). They observed that some difference is there between EDS H_{91} and EDS AV_{127} strains in number and length of fragments produced by digestion of genomic DNA with *Hind* III and *Sma* I.

Yu You *et al.* (1995) developed a non radioactive labelled probe for detection of EDS virus. The 2 kb fragment was cloned into the PUC 18 plasmid and recombinant was labelled with digoxigenin and used as a probe to detect EDS-76 specific DNA in cloacal swabs.

Zijun *et al.* (1995) screened the recombinant (pTEZ-3) by restriction enzyme analysis, dot hybridization and southern hybridization and showed that the insert fragment of pTEZ- P_3 consisted of 318 bp and it was the J fragment of egg drop syndrome virus DNA.

Restriction endonuclease maps of the genome of fowl adenovirus serotype -9 were constructed by Erny *et al.* (1996) using *Xba I*, *Nde I* and *Not I* enzymes which cleaved FAV-9 DNA 7,4 and 3 times respectively.

2.5.2. Molecular identification of virus isolates by Polymerase chain reaction

Zijun *et al.* (1995) detected EDS virus from cloacal swabs of infected fowls by the polymerase chain reaction and they have also reported that PCR is markedly more sensitive than haemagglutination inhibition and nucleic acid hybridization tests.

They have also detected the egg drop syndrome virus using biotinylated recombinant.

Muzhi *et al.* (1996) studied on the analysis of nucleic acid sequence and gene amplification of EDS-76 virus. They have successfully amplified the 209 bp nucleic acid fragment from EDS-76 virus DNA.

Huang *et al.* (1996) reported the restriction endonuclease analysis of the EDS -76 virus DNA. They have found that the viral genome DNA was cleaved into 4, 4 and 7 fragments respectively by digestion with *EcoRI, BamHI* and *Bam* H_I + *EcoRI.*

Xuemin *et al.* (1998) analyzed the NE 4 and GC 2 isolates of EDS virus DNA by using 13 different restriction endonucleases and concluded that the molecular weights and lengths of restriction fragments of isolates were similar to those of the standard AV127 strain.

Hexon based PCR's combined with restriction enzyme analysis for rapid detection and differentiation of fowl adenoviruses and EDS virus was reported by Raue and Hess (1998) and concluded that these tests were useful to distinguish all 12 fowl adenoviruses and were also useful to differentiate fowl adenoviruses from the EDS virus.

The structure of the major protein of the 32,838 bp long EDSV genome was analyzed by Zeng *et al.*(1998) and reported that EDS viral proteins share unusually high homology with ovine adenovirus proteins.

Ping *et al.* (1999) used the PCR for the detection of fowl adenoviruses and the same conditions were applied for extraction of nucleic acid from infected cells.

An avian adenovirus specific PCR was developed by Zhixum *et al.* (1999). These workers have amplified the specific 421-bp DNA product from group I of adenovirus containing 12 serotypes and serotypes of adenovirus from group II and group III. The adenovirus specific DNA product was also amplified from the 19 field isolates of avian adenoviruses but not from the mammalian adenovirus.

MATERIAL AND METHODS

III. MATERIAL AND METHODS

3.1 Virus

Aviadenovirus, EDS-76, strain 127 was originally supplied by J.B.McFerran, Veterinary Research Laboratory, Starmont, Belfast, Northern Ireland in the year 1983, three isolates from different outbreaks in Andhra Pradesh (KC,VN_1, DFH) and two isolates obtained from IVRI, Izatnagar (A and C) were used in this study.

3.1.1 Inactivation of virus with hydroxylamine

The virus was inactivated as per the method described by Fellowes (1966).

Briefly, Hydroxylamine hydrochloride (H_3NOHcl;F.W.69.491:cat No:H98676,Sigma) was used as a inactivation agent. A 4 M solution of hydroxylamine and a 14 per cent solution of sodium hydroxide were prepared as described by Franklin and Wecker (1959) and by Schafer and Rott (1962). Equal parts of 4 M hydroxylamine and 14 per cent sodium hydroxide were mixed, so that the concentration of solution becomes 2 M. Then the pH of the preparation was adjusted to 7.5. This solution was added to the virus suspension at 37°C to achieve a final concentration of 0.25 M hydroxylamine and virus (2.1 ml virus +0.3 ml of 2 M hydroxylamine mixtures) and incubated at

23°C for 9 h. Later the samples were taken and action of hydroxylamine was stopped by adding 8 ml of sterile cold PBS (1:5). After dialyzing twice against PBS, Penicillin (1000 I.U/ml) and Streptomycin sulphate (1000 mg/ml) were added. These inactivated virus samples were inoculated to embryonated duck eggs to see for the residual infectivity.

3.2 Embryonated duck eggs

Fertile duck eggs were collected from nearby duck rearing farms. The eggs were cleaned and wiped with 70 per cent alcohol. The eggs were incubated at 38.5^0 C with 85 per cent humidity for 9-10 days in egg incubator before using them for cultivation of the EDS-76 virus.

3.3 Chicken

One hundred day-old male chick were procured from a commercial hatchery in Chittoor. The chicks were maintained in the animal house of the department. They were provided with unmedicated broiler starter mix and water *ad libitum* and exposed to continuous light from a 100 watt bulb. Chickens after attaining 6 weeks of age were used in different studies.

3.3.1 Chicken red blood cells (RBC)

Chicken blood was collected in an equal volume of Alsever's solution (Appendix V:1) and stored at 4^0C. The RBC were washed thrice with phosphate

buffer saline (PBS), pH 7.2 (Appendix V:2). The concentration of RBC (1 per cent and 10 per cent) was adjusted with PBS as and when required.

3.4 Rabbits

Young adult rabbits weighing one to two kg were procured from the rabittery maintained by the Department of Microbiology of this college. The rabbits were used for production of hyperimmune serum to Virus.

3.5 Hyperimmune serum

3.5.1 Preparation in Chicken

The method described by Yates *et al.* (1976) was followed. Briefly the method was as follows. Hyperimmune serum against purified EDS-76 virus was prepared in sero negative chicken by injecting 0.5 ml of purified virus (100 µg of protein) emulsified with equal volume of Freund's complete adjuvant (FCA) intramuscularly at weekly intervals. Four such injections were given. Ten days after the fourth injection the birds were bled. Serum was separated and tested for HI antibodies. The HI titer of the serum was found to be 256. The serum was stored at -20°C after adding merthiolate (1:10,000).

3.5.2 Preparation in Rabbit

Hyperimmune serum against EDS-76 virus was prepared in two rabbits as described above. The HI titer of the serum was found to be 64. The serum was stored at -20°C after adding merthiolate (1:10,000).

3.6. Isolation of the virus

3.6.1 Collection of fecal samples

Fecal samples collected from different outbreakk farms were diluted with PBS and centrifuged at 5000 rpm for 10 min. After centrifugation the supernatant was collected and mixed with antibiotics (penicillin 1000 I.U/ml; streptomycin 1000mg/ml) and incubated for 1/2 h. at 37°C.

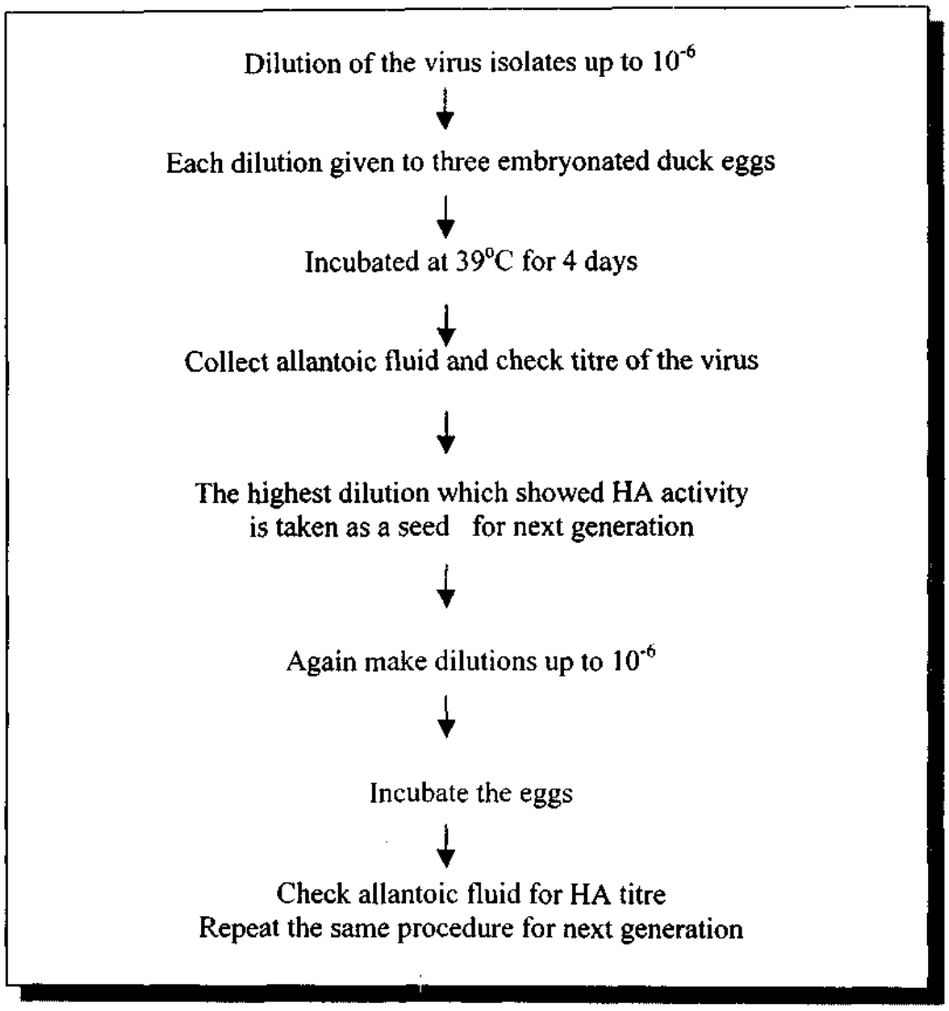
3.6.2 Isolation and Propagation of Virus isolates

Virus was diluted 20 times with PBS (pH 7.2) containing 250 IU of benzyl penicillin and 250 mg of streptomycin per ml. The inoculum was kept for 30 min at room temperature and was then filtered through a 0.45 nm membrane filter. Two-tenths milliliter of the inoculum was injected into the allantoic cavity of 9-10 day old embryonated duck eggs. The eggs were incubated at 38.5°C for five days. Embryos died during the first 24 h. were discarded considering them as non-specific deaths. The allantoic fluid was collected from embryos died after 24 h. The live and dead embryos after five days were chilled for 6 to 7 h. to reduce bleeding and to avoid RBC contamination in the virus harvest. The allantoic fluid from each embryo was tested for the presence of the virus by spot HA test using 10 per cent chicken RBC. The harvested allantoic fluid was pooled, aliquoted and stored at -20°C until use.

3.6.3 Clonal purification of virus isolates

Each of the virus isolates were clonally purified by employing the protocol given in the Flow chart I. Briefly, in this method serial dilutions of the virus isolates

Flow Chart I : Terminal dilution method for clonal purification of virus isolates



were made in the PBS and the titre of the virus was recognized by adding 1 per cent fowl RBC after keeping the test for 30 min. incubation. After incubation, the highest titre of the virus was taken as the seed for the next inoculation and continued this for three propagations and after that the virus was grown in bulk.

3.7. Virus purification

EDS-76 virus isolates were purified using sucrose density gradient centrifugation (Brakke, 1967). The allantoic fluid containing the virus was diluted twice with PBS. The diluted virus was clarified by centrifugation at 6,000 rpm for 15 min. at 4°C. The supernatant was collected and the virus was pelleted by centrifugation at 27,000 rpm for 2 h. at 4°C (Sorvall, Ultra centrifuge, OTD-75, rotor, AH-629). The pellet was suspended in desired volume of PBS and was again centrifuged at 3,000 rpm for 10 min. at 4°C. The supernatant was collected and layered on 10-40 per cent preformed linear sucrose density gradients prepared in 0.1M PBS (Appendix VI:1). This was centrifuged at 27,000 rpm for 2 h. at 4°C in a swing out rotor (Sorvall, rotor AH-629). After centrifugation, the light scattering zone was obtained between 20 and 30 per cent of sucrose gradients. The virus containing zone was collected with a bent needle and was suspended in equal volume of PBS. The virus was pelleted at 37,000 rpm for 2 h. at 4°C in an angle rotor (Sorvall, rotor, T-865). The virus pellet was suspended in 0.5 ml of PBS and stored at -20°C until use.

3.8 Infectivity assay

Serial ten-fold dilutions of the virus were made in PBS, pH 7.2. Two - tenth ml of each virus dilution was inoculated into three 10-day old embryonated duck eggs via allantoic cavity route. After 5 days of incubation at 37°C, the eggs were harvested and allantoic fluid was examined for presence of virus by spot HA test. The 50 per cent egg infectivity dose (EID_{50}) was calculated according to Reed and Muench (1938).

3.9 Identification of virus isolates

3.9.1 Electronmicroscopy (EM)

The method of Swain et al.(1993) was followed with slight modification using purified virus for particle morphology studies. A drop of virus preparation was placed on a piece of parafilm. The formwar coated grid was placed on the virus sample (formwar film facing the virus sample). After 5 min. the grid was removed using fine forceps and was washed with a few drops of distilled water. Then the grid was transferred immediately onto a drop of one per cent aqueous uranyl acetate After 2 min. the excess stain was removed from the grid, by touching the edge of the grid to filter paper. After drying, the grids were observed with a Phillips 201 C transmission electron microscope and the pictures were taken at 1,60,000 magnification.

3.9.2 Haemagglutination Test (HA)

The HA test was performed in 'V' shaped microtiter plates (Laxbro) as per Cunningham (1966). Serial two-fold dilutions of the virus (50 µl)were made in PBS (50 µl) starting from 1:2. An equal volume of one per cent chicken RBC was added to each well. The plates were shaken gently to enable the mixing of virus and RBC. The plates were kept at room temperature for 30 min. Appropriate positive and negative controls were included in the test. The reciprocal of the highest virus dilution showing mat formation was taken as the HA titre.

3.9.3 Haemagglutination inhibition (HI) Test

HI test was performed as per the method of Cunningham (1966). Serial two fold dilutions of the test serum (50 µl) were made in PBS, pH 7.2, starting from 1:2. An equal volume of virus containing 4 HA units was added to each well. After gentle shaking, the plates were kept at room temperature for 30 min. for antigen and antibody reaction. Then 50 µl of 1 per cent chicken RBC was added to all the wells and incubated at room temperature for settling of RBC. Appropriate positive and negative controls were included in the test. The reciprocal of the highest serum dilution at which there is a clear button formation was taken as the HI titer.

3.9.4 Immunodiffusion (ID) test

Immunodiffusion test was carried out on 75 x 25 mm microscopic glass slides according to the method described by Crowle (1961). Agarose gel was prepared by dissolving 0.8 grams of agarose in 100 ml of 0.15 M sodium chloride, pH 7.2.

Merthiolate was added at final concentration of 1:10,000 to prevent bacterial growth. Immunodiffusion slides were prepared by pouring 3.5 ml of molten agar over clean glass slides avoiding air bubbles. The gel was then allowed to solidify and the slides were transferred to refrigerator to harden the gel before cutting the wells. The pattern used consisted of one central and six peripheral wells having a diameter of 4 mm. The distance between the wells was also uniformly kept at 4 mm. The bottom of wells was then sealed with molten agar.

For antigenic analysis, the central well was charged with the hyperimmune serum raised against a standard EDS-76 virus strain 127 and the peripheral wells were charged with the different EDS-76 viral isolates. The slide was then placed in a humid chamber and incubated at 4°C for 48 h. The slide was observed after 48 h. for the development of precipitation lines. The slide was washed with normal saline, dried and stained with 0.2 per cent Coomassie brilliant blue R-250 before taking photographs.

3.10 Immunological characterization of viral isolates

3.10.1 SDS- Polyacrylamide gel electrophoresis (SDS-PAGE)

The virion polypeptides of EDS-virus isolates were fractionated by SDS-PAGE according to the procedure described by Laemmli (1970). The molecular weights of different fractions of the virus isolates were estimated as per the method of Shapiro *et al.* (1967).

Gel preparation: Gel (1 mm) was cast in the gel mold consisting of two glass plates, spacers and well forming comb. The 12 per cent resolving gel (Appendix I:10) solution was prepared and pipetted immediately into the gel mold gently from sides. Distilled water was overlaid on the top of the resolving gel to eliminate the air bubbles and to get a uniform gel surface. After polymerization the water layer was decanted carefully. Freshly prepared 4 per cent stacking gel (Appendix I:10) was poured without air bubbles and the comb was inserted into the stacking gel. The stacking gel solution was allowed to polymerize at room temperature for 30 min. Afterwards the comb was removed gently and the wells were marked.

Sample preparation : Purified virus suspended in 0.01 M potassium phosphate buffer, pH 7.0 was diluted with sample buffer (1:5 v/v) (Appendix I:5). The samples were heated at 100°C for 3 min. and then cooled rapidly on ice-bath.

Sample application and electrophoresis : The purified virus samples equivalent to 10 - 12 µg were loaded onto the gel slots. Protein molecular weight marker (Genei , Banglore) was loaded along with the samples. A constant 80 V current was applied and electrophoresed for 2 h. at room temperature. The run was terminated when tracking dye reached the bottom of the gel.

The gel was removed from the mold and stained for 3 h. using coomassie Brilliant Blue R250 stain (Appendix I:8). The gel was destained in destaining solution (Appendix I:9) till the background is clear.

The distance migrated by virus proteins and marker proteins was recorded . The molecular weights (Mr) of virus proteins was calculated by plotting a calibration curve for marker proteins using distance migration versus their molecular weights.

3.10.2 Electroblot immunoassay

The basic procedure followed for electroblotting and for detection of immunogenic viral proteins was the same as described by Burgermeister and Koenning (1984). Virus protein polypeptides were electrophoresed in SDS-PAGE as described earlier.

Method : The virus polypeptides were resolved by SDS-polyacrylamide gel electrophoresis on 12 per cent resolving gel. Gel holder was placed in a glass dish so that one panel is flat on the bottom of the vessel. Whattman No. 1 filter papers were cut to the size of the panel. Sponge pads, the gel and nitrocellulose membrane were saturated with transfer buffer for 30 min. Sponge pad was placed on the panel which is flat on the bottom of the vessel. Saturated piece of Whattman No. 1 paper was placed on the top of the pad. Then the pre-equilibrated gel was placed on top of the paper. Pre-wetted nitrocellulose membrane (Amersham, 0.45 nm) was placed on the gel and the air bubbles were removed. Again a piece of saturated Whattman filter paper was arranged on the system. Then the saturated sponge pad was placed on top of filter paper. The gel holder was closed on both sides. The gel holder was placed in the half-filled trans-blot tank so that the membrane is towards the anode side. The buffer tank was filled with transfer buffer (Appendix II:1) and electroblotting was

done for 3 h. at 50 V. The gel holder was opened and the membrane was taken out for further processing.

After electrophoretic transfer of proteins, the nitrocellulose membrane was incubated on a shaker with 5 per cent commercial milk powder in Tris - buffered saline (Appendix II:2) for 1 h. at room temperature. Afterwards, the nitrocellulose membrane was incubated for 1 h. in a 1 : 500 dilution of antisera in TBS with 0.05 per cent tween - 20 (TBS-T) (Appendix II:3) containing 5 per cent milk powder (TBS-TM). After three washings of 30 min. each in TBS-T the membrane was incubated for 1 h. in a 1:500 dilution of horseradish peroxidase labelled goat anti-rabbit antibodies in TBS-TM. The nitrocellulose membrane was washed three times for 30 min. each in TBS-T and transferred to a substrate solution (Appendix II:8). Colour development was recorded visually and stopped by washing the membrane in distilled water.

3.10.3 Antigenicity and immunogenicity of viral polypeptides

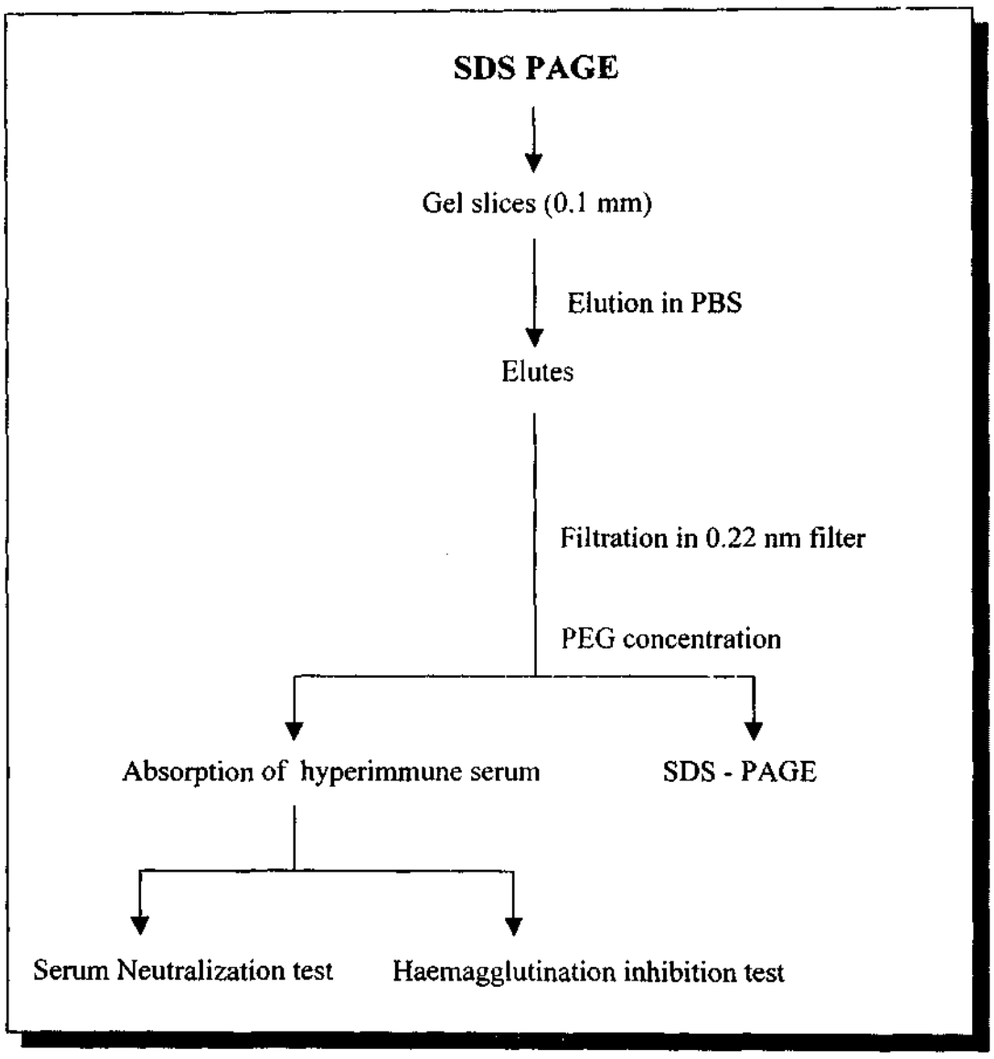
Recovery of Immunogenic polypeptides from polyacrylamide gels: Ten gels were run on preparative scale according to the procedure described in 3.10.1. After procuring the gels, they were washed thrice with 0.1 M PBS, pH 7.2 and gel slices of 0.2 mm were cut. The corresponding gel slices of the ten gels were pooled, crushed and triturated using pestle and mortar. The triturated material was collected in 2 ml of 0.1 M PBS, pH 7.2. They were frozen overnight to crystallize the residual SDS. Each homogenate was taken into a clean washed muslin cloth and was squeezed to collect the elute. The elutes were stored at -20°C until analyzed. The virus polypeptide(s)

responsible for protection / capable of absorbing HI antibody was isolated and characterized employing the protocol given in the Flow chart II (Wilson, 1989).

Absortion of hyperimmne serum against EDS-76 virus protein elutes : To identify the neutralizing antigen and / or haemagglutinin of EDS-76 virus, hyperimmune serum was absorbed with each of the protein elutes recovered from the gels. For this, rabbit hyperimmuneserum against EDS-76 was diluted 1:5 with 0.1 M PBS, pH 7.2 and filtered through 0.22 nm membrane filter. The elutes from the SDS gels were filtered through 0.22 nm membrane filter. Diluted hyperimmune serum in 0.5 ml quantity was added to 0.5 ml of elute fractions. The mixture was incubated at room temperature for 1 h. and then at 4°C overnight. Then the mixture was centrifuged at 10,000 rpm for 30 min. in a refrigerated centrifuge (Sorvall, RC 5C rotor, SS 34) to remove the antigen-antibody complexes. The supernatant was separated.

Serum neutralization test in embryonated duck eggs: This supernatant of the absorbed hyperimmune serum was used in serum neutralization and HI tests to identify the polypeptide containing neutralizing and / or HA activity . To the 0.5 ml of 1:10 dilution of the absorbed hyperimmune serum an equal amount of (0.5 ml) 100 EID_{50} of virus was added and incubated at room temperature for 1 h. Two-tenths ml of the above mixture was inoculated into 10 day old duck embryos via allantoic sac route. A total of three embryos were inoculated with each fraction. The inoculated embryos were incubated at 37°C for 5 days and examined for residual viral activity

Flow Chart II : **Isolation and Characterization of Immunogen /Neutralizing antigen of EDS-76 virus (Wilson 1989)**



by spot HA test. The virus-unabsorbed hyperimmune serum mixtures served as positive control while the neat virus served as negative control.

3.10.4 Lymphocyte stimulation test - MTT assay

The LST was performed using MTT assay method as described by Bounous et al.(1992). Briefly the method used was as follows. After first week of inoculation, Chickens were euthanized by cervical disarticulation and spleens were removed aseptically into PBS and maintained on ice until cell harvest. Spleen cells were isolated by gentle maceration of spleen after washing twice with PBS and removing the capsule. Spleen cells were aspirated into a 10 ml syringe through a 20 gauge needle; the needle was exchanged for 23-gauge needle, and the cells were then expelled into a sterile 15 ml conical polypropylene tube. The suspension was maintained on ice for 10-15 min. to allow non-cellular debris to settle. The cellular supernatant was transferred to a new tube with 0.1 ml cushion of fetal calf serum (FCS) placed at the bottom of the suspension and centrifuged at 585 x g for 10 min. at 4°C. Following two more washes as described above, the cells were resuspended in 3 ml of medium (RPMI 1640 with 3 per cent FCS, 4 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10,000 U penicillin / ml, 10 mg streptomycin / ml) for use in the assays.

Assay: MTT assays were performed using a concentration of 2.0×10^5 viable cells per well as determined by exclusion of 0.2 per cent trypan blue dye

(Mosmann,1983). Wright's stained preparations showed that suspensions consisted of greater than 90 per cent lymphocytes.

The sensitized cells for MTT colorimeter assay were incubated in 5 per cent CO_2 at 40°C for 72 h. After incubation, serum - containing medium was removed from the cells in the flat - bottomed plates by centrifugation for 10 min. at 1000 x g and replaced by 150 ml of serum free RPMI. When high serum concentrations were used in the assay, an alcohol - induced protein precipitate formed that caused light scattering, which interfered with dye measurement by spectrophotometry. After the addition of 15 µl of stock MTT (Appendix IV:1) to each well, the plates were incubated for 4 h. Media and untransformed MTT were then removed by centrifuging for 10 min. at 1000 x g, followed by careful inverting and blotting of the plate. Isopropanol acid (0.04 N HCl in 1 litre isopropanol) was added to each well and mixed thoroughly by pipetting to solubilize MTT formazan. The mean optical density was read on a microplate reader (Titertek, Lab systems,U.K.) using a test wavelength of 570 nm and a reference wavelength of 630 nm. The mean optical density was determined for quadruplicate samples and converted to cell numbers by comparison with a simultaneously generated standard curve of known numbers of cells. Stimulation index (SI) was calculated using the formula:

$$SI_{MTT} = \frac{(\text{Cell number}_S) - (\text{cell number}_U)}{(\text{cell number}_U)}$$

where,

cell number_S = Stimulated cells

cell number_U = Unstimulated cells

3.10.5 Cytotoxicity cell assay

The method used was essentially that described by Green *et al.* (1984) with slight modifications. The sensitized spleen cells were used as source of effector cells. Chicken liver cells which were coupled to hydroxylamine inactivated EDS-76 virus were suspended at $2x10^5$ cells/ml in RPMI 1640 medium containing 0.5µg/ml mitomycin c.

Coupling of inactivated virus to the liver cells: Freshly collected chicken embryo liver cells were washed thrice in RPMI 1640 medium and $2x10^5$ cells/ml were made. Two ml of purified EDS-76 virus with a protein concentration of 10 mg/ml was added to 2 ml of RPMI 1640 medium containing liver cells. The suspension was incubated at room temperature for 2 h. after adding two drops of 2.5 per cent glutaraldehyde with intermittent shaking. Then the traces of glutaraldehyde were removed by washing the suspension thrice with RPMI 1640 medium.

Assay: An equal quantity (100µl) of target cells and effector cells were dispensed in to 96 well flat bottomed microtiter plate using a multichannel pipette and were incubated 72 h. at 40°C under 5 per cent CO_2 tension. After incubation, serum containing medium was removed from the cells in the flat - bottomed plates by centrifugation for 10 min. at 1000 x g and replaced by 150 ml of serum free RPMI. When high serum concentrations were used in the assay, an alcohol - induced protein precipitate formed that caused light scattering, which interfered with dye

measurement by spectrophotometer. After the addition of 15 µl of stock MTT to each well, the plates were incubated for 4 h. Media and untransformed MTT were then removed by centrifuging for 10 min. at 1000 x g, followed by careful inverting and blotting of the plate. Isopropanol acid (0.04 N HCl in 1 litre isopropanol) was added to each well and mixed thoroughly by pipetting to solubilize MTT formazan. The mean optical density (OD) was read on a microplate reader (Titertek, Lab systems,U.K.) using a test wavelength of 570 nm. The optical density of control wells containing $2x10^4$ and $2x10^5$ cells/ml was determined at 570 nm wave length.

3.10.6 Plaque assay

The method used was essentially described by Nagaraja *et al.* (1982) to detect antibody forming cells with slight modification.

Chicken : Each group of chicken (four in each) was inoculated with respective polypeptide (124, 54, 84, 70, 40 and 26 kDa). The last group (four birds) served as un-inoculated control. After 5 days post inoculation of polypeptides, the spleen was collected aseptically.

Preparation of sheep red blood corpuscles (SRBC) : Sheep blood was collected in Alsever's solution. This was centrifuged at 500 x g for 10 min. to pellet the RBC. The RBC was resuspended in 0.9 per cent Sodium Chloride. After repeating this step twice, RBC were adjusted to the required concentration.

Coupling of EDS-76 Virus antigen to SRBC : The EDS-76 virus antigen was coupled to SRBC using glutaraldehyde method. (Prasad, 1992). Freshly collected SRBC in Alsever's solutions were washed thrice in stabilized saline (1 per cent rabbit serum) and 20 per cent suspension was made. Two ml of purified EDS-76 virus with a protein concentration of 10 mg/ml was added to 2 ml of 20 per cent SRBC. The suspension was incubated at room temperature for 2 h. after adding 2 drops of 2.5 per cent glutaraldehyde with intermittent shaking. Then the traces of glutaraldehyde was removed by washing the suspension thrice in stabilized normal saline. Finally 1 per cent suspension was made.

Preparation of spleen cell suspension: Spleens were aseptically removed, trimmed of their capsule, and placed into a sterile test tube containing 5 ml of chilled RPMI 1640 medium with added antibodies (Penicillin, 500 IU/ml and Streptomycin, 500 µg/ml). Each spleen was minced and placed into one of the two - 10 ml syringes. The contents were gently passed back and forth between the syringes until homogenized. The cell suspension was centrifuged at 500 x g for 10 min. at 4°C. The supernatant was discarded, and the pellet was suspended in cold RPMI 1640 medium by gentle mixing with a sterile Pasteur pipette. The number of viable lymphocytes was determined by trypan blue (0.25 per cent) dye exclusion technique. The concentration of spleen cells was adjusted to 40 x 10^3 cells / ml.

Complement: Pooled guinea pig serum was the source of complement.

Preparation of petri dishes with bottom layer for plaque assay: 2.8 per cent agar in water is prepared, autoclaved, cooled to 45°C and mixed with equal volume of pre warmed BSS (2x)(Appendix V:3). This agar is then poured in 20ml aliquots into each of a number of petri dishes (diameter 4"). The plates are prepared 2-4 days before use and stored at 4°C. Before use the plates are incubated without covers at 37°C for 1h. in order to evaporate water from agar.

Preparation of top layer: 1.4 per cent agar is prepared in pre warmed water and dissolved by controlled boiling of the suspension, avoiding excessive foaming. The suspension is continued to boil till no more foam is formed. At the time of use, equal volume of pre warmed BSS (2x) is added to this agar solution and kept warmed at 45°C. Five millilitres of DEAE dextran (1 per cent) was added to each 100 ml of 0.7 per cent agar solution just before use.

Assay procedure: The following were mixed in a sterile test tube (12 x 15 mm).

1. 75 µl of spleen cells (40 x 10^3 cells /ml)
2. 75 µl of EDS-76 viral antigen coupled to SRBC (1 x 10^7 cells / ml).

To this mixture, 225 µl of guinea pig complement was added and mixed gently. The sample was applied to the prepared petridishes. The petridishes were incubated for 3 h at 40°C under 5 per cent CO_2 tension. At the end of 3 h. plaques were counted under low power objective.

Detection of hemolytic PFC : The plaques were counted under low power objective of a microscope. Plaques were counted by counting 10 microscopic fields, and their average was determined. This was multiplied by the dilution factor to obtain the number of plaques / ml of spleen cells.

3.11 Molecular characterization virus isolates

3.11.1 Isolation of viral nucleic acid

The viral nucleic acid was isolated from purified virus preparations by the method of Sambrook *et al.* (1989). Briefly the method is as follows. The purified virus pellets were suspended in minimal volume of 0.01 M phosphate buffer, pH 7.0. To the purified virus suspension, 10 per cent SDS (Appendix III:1) and 200 mM EDTA (Appendix III:2 were added to 1 per cent and 20 mM, respectively. The contents of the tube were mixed and incubated at 65°C for 10 min. The tubes were cooled to room temperature and proteinase K was added to 1 mg/ml. Virus + SDS + EDTA + proteinase K mixture was incubated at 37°C for 10 min. Afterwards the suspension was extracted with an equal volume of phenol . The aqueous phase was collected and extracted with equal volume of phenol: chloroform - isoamyl alochol (24 : 1 v/v). The upper aqueous phase was collected and an equal volume of chloroform: isoamyl alcohol only was added and extracted .To the upper aqueous phase 2.5 M Sodium acetate was added to 250 mM and mixed gently. To this, 2.5 volumes of ice cold absolute ethanol was added. The tubes were covered with parafilm and kept at -20°C overnight. The nucleic acid was pelleted by centrifugation at 10,000 rpm for 15 min. The nucleic acid pellet was washed twice with 70 per cent

ethanol. The pellet was dried and dissolved in a minimal volume of TE buffer (Appendix V:4) .

Quantification of the nucleic acid : UV absorption spectrum of purified viral DNA was taken (200-300 nm) in a UV-visible spectrophotometer (Hitachi U-2000). The A260/280 ratio was calculated. DNA was quantified by assuming 1 0D = 50 µg/ml (Sambrook *et al.*, 1989) and stored at -20°C until use.

3.11.2 Determination of nucleic acid type

The type of nucleic acid present in the virus was determined by diphenylamine (Burton,1956), orcinol (Schneider,1957) and nuclease (DNase and RNase) sensitivity tests.

Diphenylamine test (Burton, 1956): Viral nucleic acid (100-300 µg) was taken into test tubes and made up to 20 ml with 5 per cent trichloroacetic acid. Four µl of diphenylamine reagent was added and boiled for 10 min. in a water bath and cooled rapidly. The colour of the solution in the tubes was recorded visually. Calf thymus DNA was used as reference. Appearance of blue color was regarded as positive.

Orcinol test (Schneider, 1957): This test was carried out for confirmation of the viral DNA. Viral nucleic acid (50-200 µg) was taken into test tubes and made upto 1 ml with 0.5 M perchloric acid. To the test tubes 1.5 ml of freshly prepared orcinol reagent (one gram orcinol dissolved in 100 ml of conc. HCl containing 0.5 µg

ferric chloride) was added and heated at 70°C for 20 min. The colour of solution in the tubes was recorded visually. Blank was prepared by taking 0.5 M perchloric acid. Appearance of green colour was regarded as positive for RNA.

Digestion of viral DNA with nucleases: Approximately one µg equivalent viral DNA was taken into eppendorf tubes and 1 µl of DNase I or RNase enzymes (0.5 µg equivalent) were added. The contents of tubes were made up to 20 µl with sterile water and incubated at 37°C for 30 min. The enzyme digested DNA samples were electrophoresed on 1 per cent agarose. Undigested viral DNA was included as control.

3.11.3 Setting up of digestions with restriction enzymes

Single digestion: One microgram equivalent EDS-76 viral DNA was taken into sterile eppendorf tubes and a sufficient amount of sterile water added to give a volume of 17 µl . Two µl of respective 10 x activity assay buffers were added, followed by 3 µl of (1 unit) restriction enzymes. The contents of tubes were mixed well on cyclomixture and microfuged at 6,000 rpm for 30 sec. The eppendorf tubes containing DNA and restriction enzymes were incubated at 37°C. After 1 $^{1}/_{2}$ h. the reaction was stopped by adding 20 mM EDTA. The restriction enzyme digested DNA samples were loaded on to the 1 per cent agarose gel in TAE buffer (Appendix V:5) by mixing with 5 µl of gel loading buffer (Appendix III:8). One µg of λ DNA / *Eco* RI *Hind* III - double digest (13 DNA fragments of 21 226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564 and 125 base pairs) was made up to 20 µl with

sterile water and heated at 65°C for 5 min. and rapidly cooled on ice bath. This was loaded onto the gel by adding 6 µl of gel loading buffer. Electrophoresis of restriction enzyme digested samples and λ DNA markers was carried out as described earlier. Molecular weight of viral DNA fragments was calculated by plotting distance of migration against molecular weights of λ DNA / *Eco* RI *Hind* III double digest fragments on a semi-log graph.

3.11.4 Restriction Enzyme Mapping of Viral DNA

Restriction endonucleases viz. *Eco* RI, *Hind* III, *Hae* III and *Pvu* II were obtained from Bangalore Genei Pvt., Limited. Bangalore. India. *Eco* RI digestions were performed in 100 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol ; *Hind* III digestions were in 10 mM Tris-HCl pH 7.8, 50 mM NaCl, 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol, *Pvu* II and *Hae* III enzyme digestions were performed in 10 mM Tris-HCl, pH 7.9, 150 mM NaCl, 7 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. All assay buffers contained 100 µg/ml nuclease free Bovine Serum Albumin (BSA).

3.11.5 Agarose gel electrophoresis of viral DNA

Horizontal agarose slab gel electrophoresis was done according to procedure of Sambrook *et al.* (1989).

The gel boat was prepared by covering the edges of the mold with cellophane tape and it was placed on a level surface. The comb was put on the gel boat by

adjusting the comb teeth without touching the gel bottom. Agarose (Bio-Rad) was dissolved in sterile TAE buffer. The agarose was cooled to 45^0C and poured into the gel boat until 3-5 mm thick. After solidification of the agarose (30-45 min. at room temperature), the comb and tape were removed carefully.

DNA samples approximately, 1 μg were mixed with 6 x gel loading buffer and loaded on to the gel wells using a micropipette (Amersham). After loading the DNA samples, the gel boat was transferred to a horizontal electrophoresis tank. TAE buffer was added slowly, just enough to cover the gel to a depth of about 1 mm. The tank was closed with a lid and the electrical leads were connected to an electrophoresis power pack. Electrophoresis was carried out at 60 volts for 2 h. After the run was over the gel was stained with ethidium bromide (0.5 μg/ml in water) for 20-30 min. at room temperature. The gel was viewed on UV-transilluminator (Upland, CA 91786 USA) and the results were recorded.

3.11.6 Constructionof physical map

The end fragments were determined by the method described by Winberg and Hammarskjold (1980). To find the A-T rich end of the VN_I isolate DNA molecule, an *EcoRI* digest of VN_I DNA was melted in 50 per cent formamide (Merck,Darmstadt, Germany) at various temperatures for 15 min. and subsequently electrophoresed. One of the end-fragments denatured by less heat was considered to be positioned at the right-hand end of the DNA molecule (Anonym,1977). The fragments between the ends were mapped by partial cleavage and combined digestion.

RESULTS

IV. RESULTS

In the present study, protein profiles of different isolates of EDS-76 virus were analyzed by polyacrylamide gel electrophoresis (PAGE). The immunogenic polypeptide was also identified in EDS-76 virus by using Electroblot immunoassay technique. In addition, the antigenicity and immunogenicity of viral polypeptides was studied by using lymphocyte stimulation test, cytotoxicity assay and plaque assay tests. The molecular characterization of EDS-76 virus was carried out by using restriction enzyme analysis and the genome map was also constructed for EDS-76 virus.

4.1 Virus

The different EDS-76 virus isolates obtained from different sources were collected and initially their titres were checked by haemagglutination test. The detailed history of the different isolates used in this study with their HA titres were shown in the Table1.

4.1.1 Clonal purification of virus isolates

All the isolates were propagated in 9-10 day old duck embryos. After four days of incubation, the allontoic fluid was tested for the presence of virus by spot haemagglutination test.

Table 1 : History of the isolates used in this study

S.No.	Name of the virus isolate	Place of collection	Per cent of egg drop	Age of the flock (in weeks)	Nature of the material collected	HA titre after 3rd passage into duck embryos (log^2)
1.	127 strain	Obtained from Dr. J.B. Mcferran, VRL, Starmont	—	—	—	11
2.	KC	Hyderabad, A.P.	40	50	Faecal samples cloacl and uterine swabs	11
3.	VN_1	Vijayanagaram, A.P.	30	45	-do-	10
4.	DFH	Hessargatta · Bangalore	20	35	-do-	10
5.	IVRI-A	Izatnagar, UP	30	40	-do-	13
6.	IVRI-C	Izatnagar, UP	40	50	-do-	12

All six isolates were purified by terminal dilution technique and the method followed was shown in the Flow chart 1. The highest dilution of the virus which shows haemagglutinating activity (based upon the infectivity assay (EID_{50})) was taken as seed for next inoculation and this was continued for three propagations and the results obtained were shown in the Table 2. After three such propagations, the finally obtained highest dilution was taken as a seed for bulk preparation.

4.1.2 Purification of virus isolates

EDS-76 viral isolates propagated by terminal dilution method were successfully purified using sucrose gradients. Single light scattering zone was observed as shown in the Fig. 1.

In U.V. light absorption spectrum purified virus has maximum and minimum absorption between 258.9 - 260.0 nm and 242.3 - 243.4 nm, respectively. $A_{260/280}$ ratios was 1.42 (Fig.2).

4.1.3 Infectivity assay of the virus

The egg infectivity dose (EID_{50} / ml) was calculated for the viral isolates and it was shown in Table 3. It can be seen from the table that the egg infectivity titers ranged from 5.3 to 7.2. The isolate obtained from Hessargatta duck rearing farm showed less infectivity assay (5.3).

Table 2 : Terminal dilution method of purification (based on EID_{50}) of six EDS-76 virus isolates

S.No.	Name of the virus isolate	Highest virus dilution in the 1st generation	Minimum HA titre	Highest virus dilution in the IInd generation	Minimum HA titre	Highest virus dilution in the IIIrd generation	Minimum HA titre
1.	KC	10^{-8}	64	10^{-4}	16	10^{-2}	16
2.	127 strain	10^{-6}	16	10^{-2}	4	10^{-3}	16
3.	VN_1	10^{-6}	256	10^{-4}	256	10^{-3}	256
4.	DFH	10^{-6}	256	10^{-3}	1024	10^{-4}	1024
5.	IVRI-A	10^{-10}	4096	10^{-8}	1024	10^{-5}	512
6.	IVRI-C	10^{-8}	1024	10^{-6}	64	10^{-3}	512

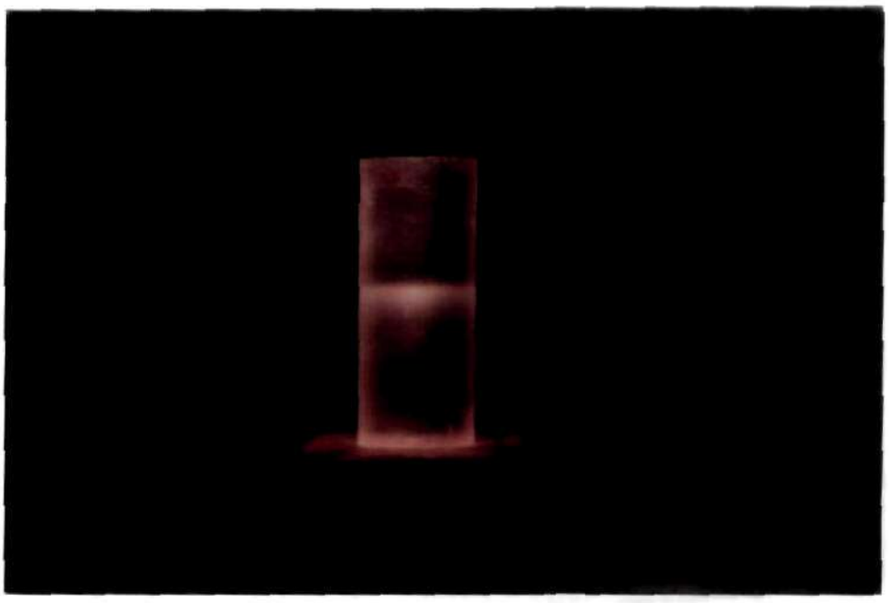


Fig. 1: Purification of EDS-76 virus (VN_1) isolate : Light scattering zone in 10-40 per cent sucrose gradients

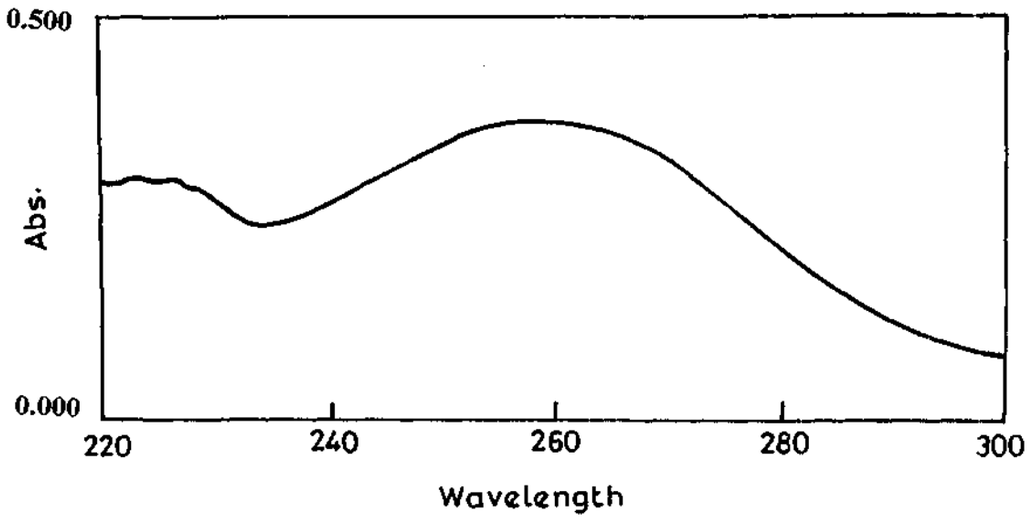


Fig. 2: U.V - visible absorption spectrum of purified EDS-76 virus (VN_1 isolate)

Table 3 : Infectivity assay of the Virus isolates

S.No	Name of the virus isolate	EID_{50} / ml (Log_{10})
1	EDS,127 Strain	6.9
2	KC	5.9
3	DFH	5.3
4	VNI	7.2
5	IVRI-A	6.5
6	IVRI-C	6.9

4.2 Identification of virus

4.2.1 Immunodiffusion (ID) test

The virus isolates were identified by analyzing the antigens using ID test and compared their patterns with standard EDS-76 virus, 127 strain and its antiserum. The ID results with all isolates revealed lines of identity with the standard EDS-76, strain 127 antigen and its antiserum with in 24-48 h. (Fig. 3).

4.2.2 Electron Microscopy

The electron microscopic observation of the purified virus preparations of VN_I isolate revealed the presence of complete hexagonal virus particles scattering singly with small number of disrupted and penetrated particles as shown in the Fig. 4. The virus particles measured approximately 70 nm in diameter. The capsomeres are distinctly visible but no viral fibers were seen.

4.3 Immunological characterization of EDS-76 virus isolates

4.3.1 Characterization of polypeptides of EDS-76 virus isolates by SDS-PAGE

After purification on 10-40 per cent sucrose density gradient centrifugation, the viral polypeptides of various EDS-76 virus isolates were analyzed on SDS-PAGE. The polypeptide profile of the different viral isolates are shown in Fig. 5. It is evident from the figure that all the isolates revealed a total of 13 polypeptide bands. Of these, 7 were major bands and 6 were minor bands. The molecular weights of these major and minor bands of VN_I isolate as calculated from the standard curve by using standard protein molecular weight markers were shown in Table 4. The

Fig. 3: Immunodiffusion of EDS-viral isolates

Central well : EDS-76 virus strain 127 hyperimmune serum

Peripheral well No. 1 : KC isolate
2 : DFH isolate
3 : VN1 isolate
4 : 127 isolate
5 : IVRI- A isolate
6 : IVRI -C isolate

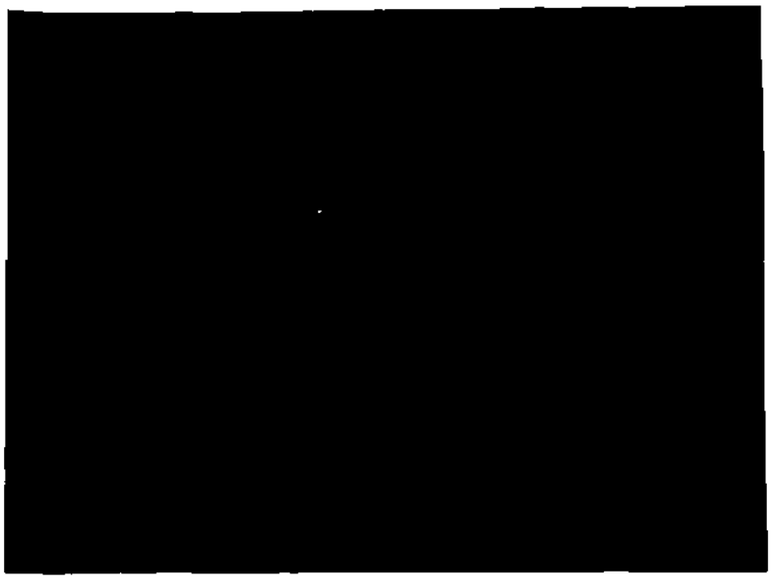


Fig. 4: Electron micrograph of EDS-76 virus, VN_1 isolate
(X 1,60,000)

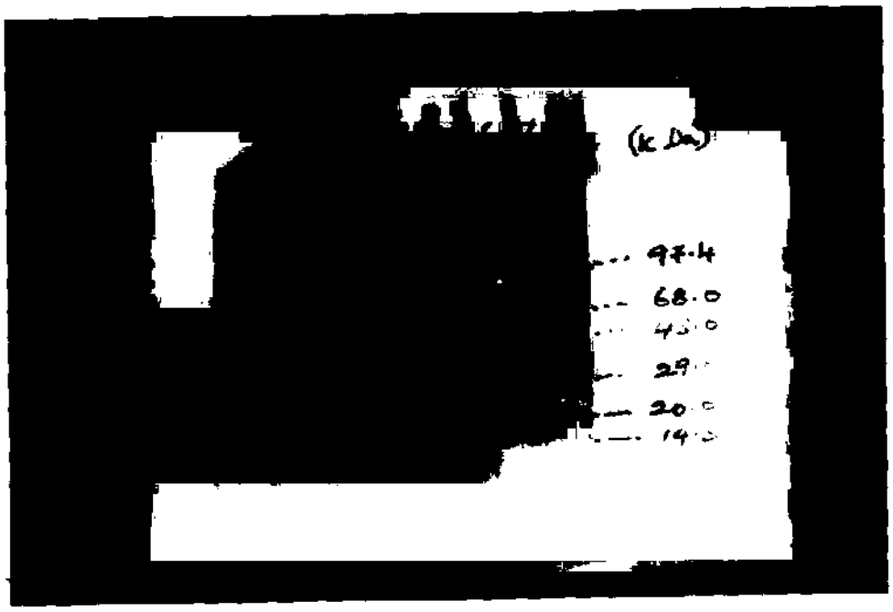


Fig. 5: Virion polypeptide profile of EDS-76 virus isolate in SDS-PAGE gels

Lane 1 : KC isolate

Lane 2 : DFH isolate

Lane 3 : VN_1 isolate

Lane 4 : Protein markers (from top to bottom - Phosphorylase b 97.4, Bovine serum albumin 68.0, Ovalbumin 43.0, Carbonic anhydrase 29.0, Trypsin inhibitor 20.0, Lysozyme 14.3).

Lane 5 : 127 isolate

Lane 6 : IVRI-A isolate

Lane 7 : IVRI-C isolate

Table 4 : Relative mobility and Molecular weight determination of EDS-76 virus (VN_1) isolate proteins by SDS-PAGE.

Polypeptide Number	Distance Migrated (cm)	Molecular Weight (kDa)
1	0.6	124
2	1.3	100
3	1.5	92
4	1.7	84
5	1.8	80
6	2.2	70
7	2.5	62
8	2.8	56
9	2.9	54
10	3.5	40
11	3.8	36
12	4.5	26
13	5.0	18

molecular weights of the major protein bands were 124, 100, 84, 70, 62, 26 and 18 kDa.

4.3.2 Electroblot immunoassay

SDS-PAGE separated viral polypeptides were transferred electrophoretically on to NC membrane with a pore size of 0.45nm (Amersham, U.S.A.) and reacted with hyperimmune rabbit serum prepared against EDS-76 virus (strain 127) and the results are shown in Fig.6. It can be seen from the figure that, a total of 3 immunogenic moieties are present. The molecular weights of these moieties as determined from the standard curve were found to be 124, 84 and 54 kDa and are shown in Table 5. Among these three, 84 kDa moiety reacted strongly with the antiserum. However, six polypeptides of higher molecular weight (100 and 92 kDa) were not observed in immunoblots.

4. 3.3 Antigenicity and immnogenicity of viral polypeptides

a. Characterization of polypeptide(s) responsible for induction of protective antibody

The antigenicity and immunogenicity of the EDS-76 virus polypeptides was characterized, using VN_1 virus isolate as a candidate viral isolate.

The virion polypeptide(s) responsible for protection / capable of absorbing HI antibody was isolated and characterized. To achieve this goal, the virion polypeptide elutes from the gels were tested for their ability to absorb HI antibodies

Fig. 6: Eelctroblot immunoassay of EDS-76 (VN_1 isolate) Polypeptides

Table 5 : **Relative mobility and Molecular weight determination of EDS-76 Virus immunogenic proteins by electroblot immuno assay.**

PolypeptideNumber	Distance Migrated (cm)	Molecular Weight (kDa)
1	0.6	124
2	1.7	84
3	2.9	54

from the standard EDS-76 (strain 127) virus hyperimmune serum. The results of the HI test conducted with serum absorbed with various gel elutes are shown in Fig.7. It can be seen from the figure that the 84 kDa polypeptide could absorb the HI antibody from the known hyperimmune serum.

Similarly, the virion polypeptide(s) responsible for protection / capable of absorbing neutralizing antibody was isolated and characterized by serum neutralization test (SNT) by inoculating into 9-10 day old duck embryos and testing for the presence of any residual live virus after appropriate incubation. The results of the SNT carried out on hyperimmune serum absorbed with various gel elutes showed that the polypeptide with a molecular weight of 84 kDa could absorb the neutralizing antibody from the hyperimmune serum.

b. Characterization of polypeptide responsible for induction of cell mediated immune response

The polypeptide responsible for induction of cell mediated immune response in EDS-76 viral infection in chicks experimentally infected with different viral polypeptides was studied using lymphocyte stimulation test by MTT assay method. The results of this assay are shown in Table 6. It is evident from the table that the mean changes in relative cell numbers as assayed by the MTT method ranged from 0.0449 to 1.2730. Further, the results showed that, a polypeptide viz: 84 kDa stimulated the virus sensitized lymphocytes (Fig. 8) when compared to normal

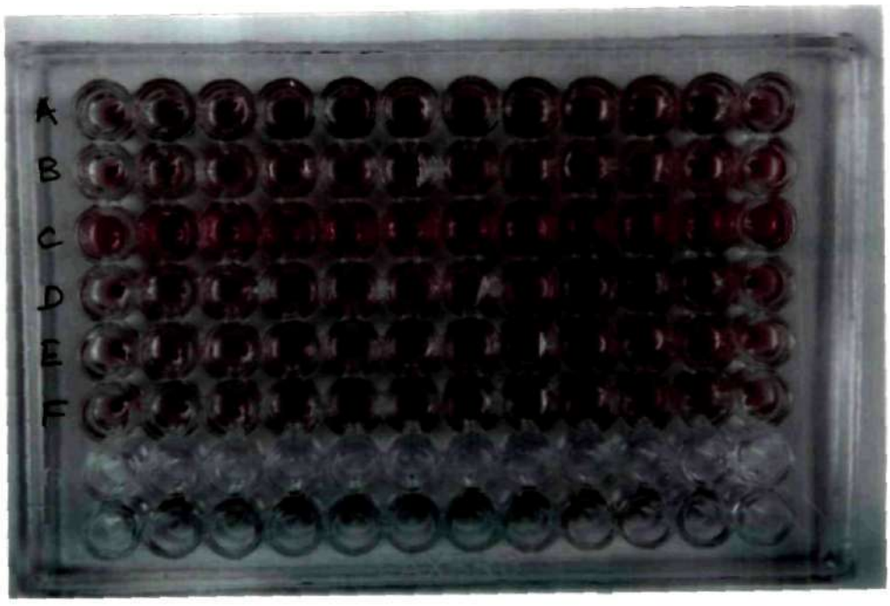


Fig. 7: Haemagglutination inhibition patterns of EDS-76 hyperimmune serum absorbed with SDS-PAGE elutes of purified virus

A. 124 kDa Polypeptide
B. 54 kDa Polypeptide
C. 84 kDa Polypeptide
D. 70 kDa Polypeptide
E. 40 kDa Polypeptide
F. 26 kDa Polypeptide

Table 6 : Comparison of the results of the MTT assay of unstimulated and stimulated cells

EDS-76 virus polypeptides used for sensitization of chicken (kDa)	Cell numbers[a]			
	Unstimulated cells (OD 630)	Stimulated cells (OD 570)	Difference[b]	SI[c]
124	1.6625	1.7842	0.1217	0.073
54	1.4102	2.6382	1.2730	0.903
84	0.6322	1.4262	0.7940	1.256
70	2.1230	2.2672	0.1442	0.068
40	0.3420	0.4126	0.0706	0.206
26	1.4364	1.4813	0.0448	0.031

Results form unstimulated and stimulated cells were significantly different

(paired t test, compared t value = 4.23, df = 5, $P < 0.01$)

a. Mean relative cell numbers (x 10^5) from four replicates

b. Stimulated - Unstimulated

c. $S.I = \dfrac{\text{Cell no. (S)} - \text{Cell no. (U)}}{\text{Cell no. (U)}}$

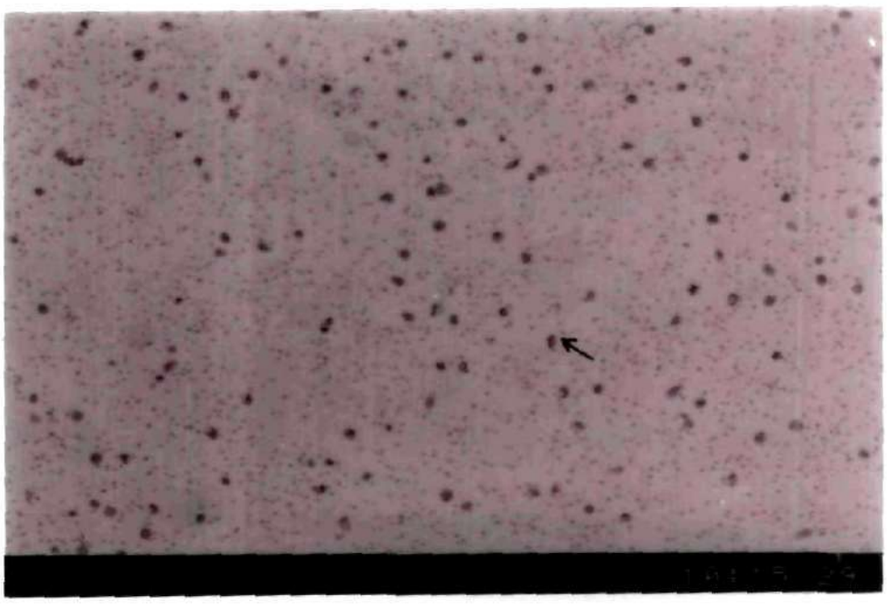


Fig. 8: Lymphocyte stimulation in chicken spleen cells sensitized with 84 kDa polypeptide (blasts)

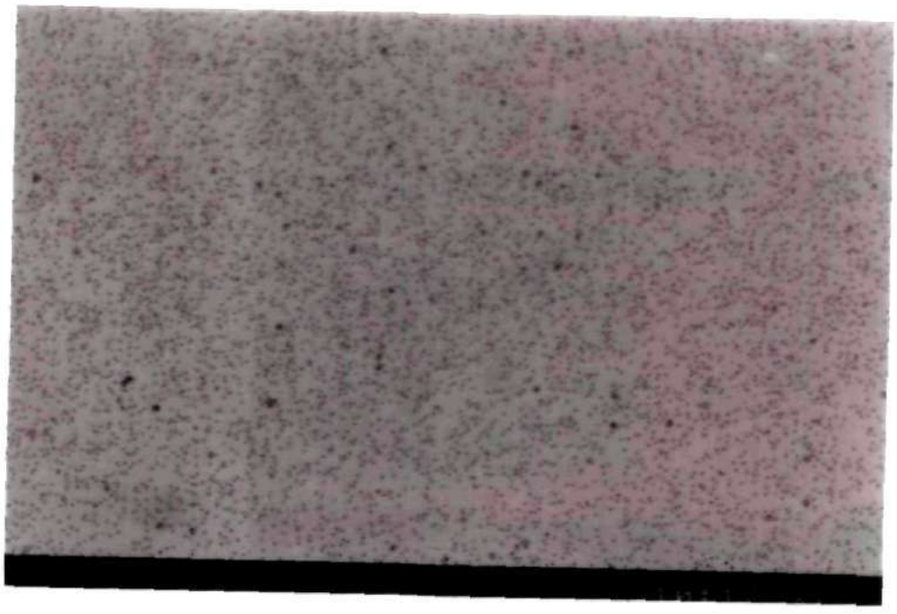


Fig. 9: Unstimulated chicken spleen cells

lymphocytes (Fig. 9). A second polypeptide with a molecular weight of 54 kDa also showed a low levels of stimulation of lymphocytes.

The response of spleen to different polypeptides of VN_I isolate of EDS-76 virus was also studied.(Fig.10). A noticeable splenomegaly was observed at 5^{th} day after injection of 84 kDa polypeptide fragment. Histological studies revealed enhanced lymphoblast formation in red pulp area of spleen (Fig.11) as compared to normal spleen (Fig.12).

Cytotoxic cell assay was used to study the CMI response induced in different groups of chickens with EDS-76 viral polypeptides (VN_I isolate) using MTT as marker and EDS-76 virus coupled chicken embryo liver cells as target cells. The results of cytotoxic effect of spleen cell extracts of different groups of polypeptide inoculated chicks as assessed by MTT uptake using coupled inactivated virus chick embryo liver cells as target cells are presented in Table 7. It can be seen from the table that the polypeptide having molecular weight 84 kDa produced more activated ' T ' lymphocytes which initiates more cytotoxic effect on virus coupled chick embryo liver cells.

The polypeptide responsible for induction of antibody forming cells (AFC) was determined using Jerne's plaque assay. The results of plaque assay conducted with immunogenic polypeptide 84 kDa was shown in Fig.13. The results of this study revealed that the 84 kDa polypeptide induced 1240 plaques/ml of spleen cells. The

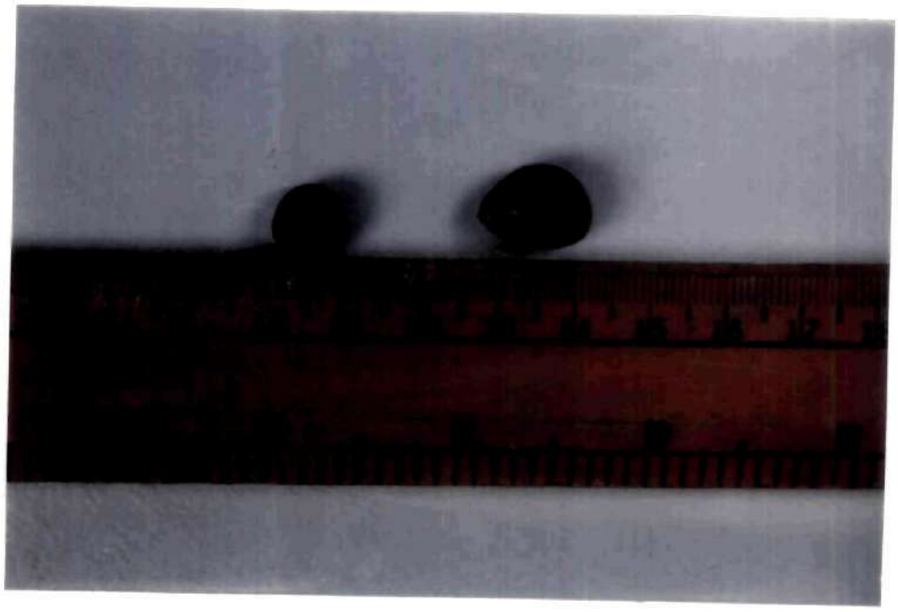


Fig. 10: Normal and Enlarged sizes of spleens collected from Control and 84 kDa injected chicken

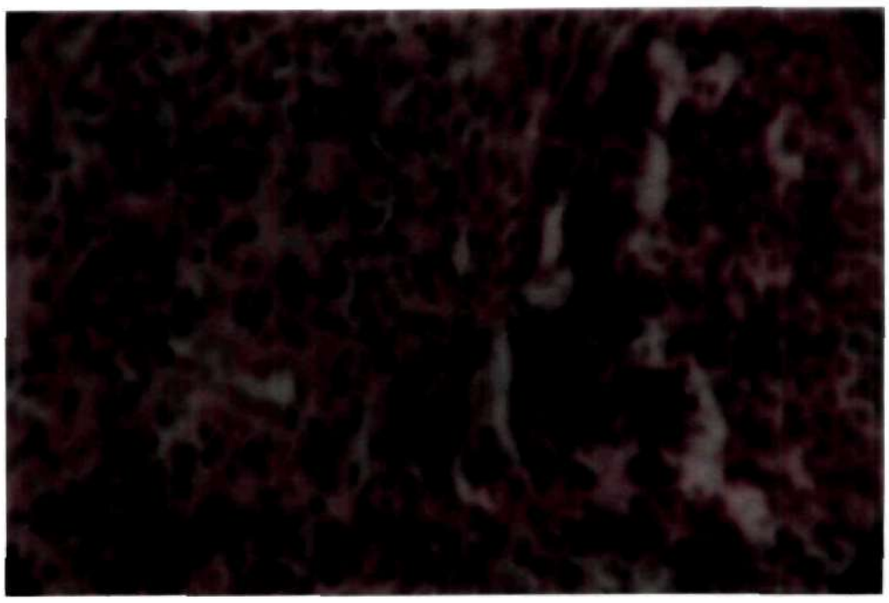


Fig. 11: A spleen section (Red pulp area) from immunogenic Polypeptide (84 kDa) infected chicken

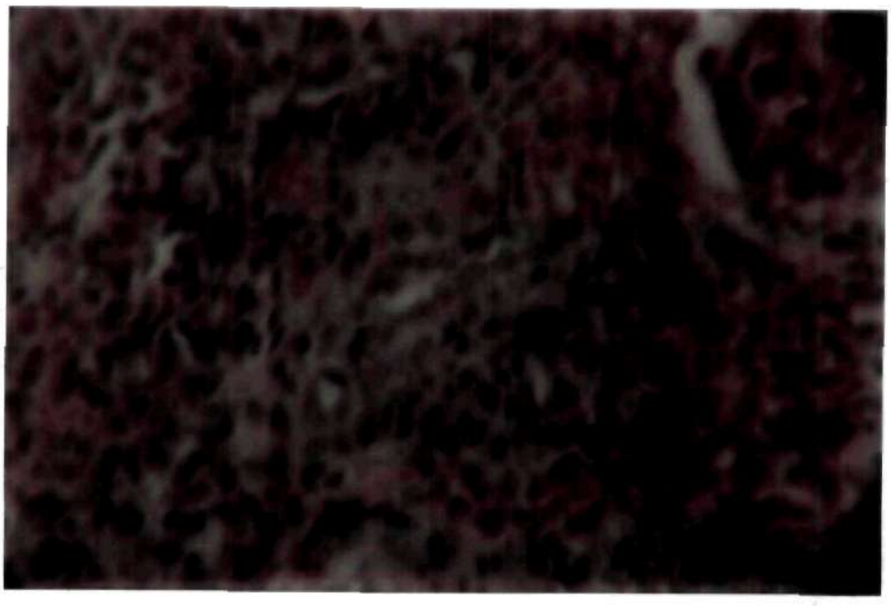


Fig. 12: A spleen section (Red pulp area) from control chicken

Table 7 : Cytotoxicity test ; MTT assay

S. No	EDS-76 virus polypeptides used for sensitization of chicken (kDa)	OD of Effector/ Target cells after incubation (570 nm)	OD of $2x\ 10^5$ Cells present in Control well (570 nm)
1	124	0.462	0.536
2	54	0.312	0.536
3	84	0.109	0.536
4	70	0.492	0.536
5	40	0.512	0.536
6	26	0.489	0.536

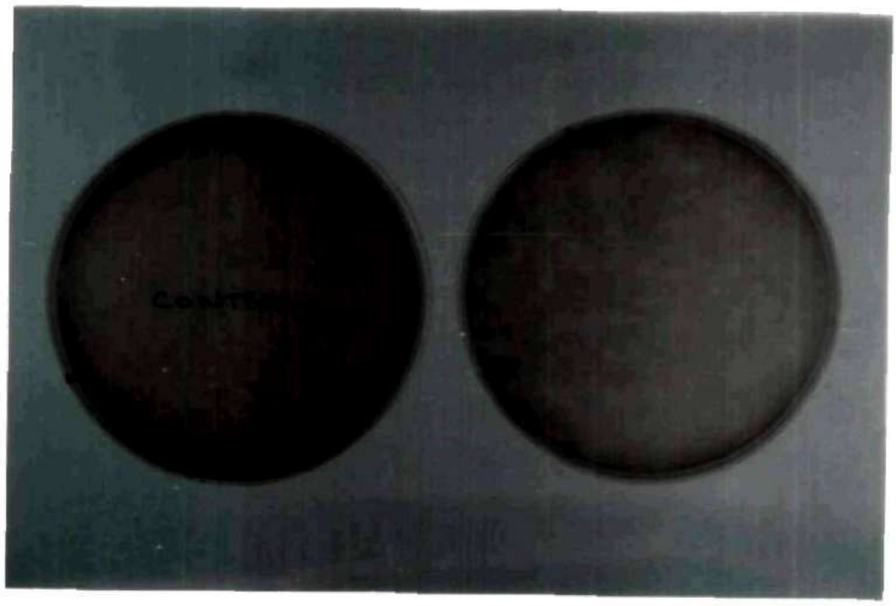


Fig. 13: Haemolytic plaque (with 84 kDa polypeptide)

polypeptides 54, 124, 26 and 70 kDa induced 676, 322, 236 and 102 plaques/ml of spleen cells respectively.

4.4 Molecular characterization EDS- 76 virus

4.4.1 Isolation of viral nucleic acid

Viral DNA isolated from purified virus by SDS-phenol-proteinase-K method. The isolated nucleic acid had A_{max} at 268nm and A_{min} at 256nm (Fig.14). It had A_{260}/A_{280} ratio of 1.80.

4.4.2 Determination of nucleic acid type

In agarose gel electrophoresis, undigested and RNase digested viral nucleic acids were resolved into one band, whereas no band was observed in DNase I digested sample (Fig.15). The viral nucleic acid gave positive reaction to diphenylamine reagent and negative to orcinol test. These tests indicated that the virus contained deoxyribonucleic acid (DNA).

4.4.3 Determination of molecular weight of nucleic acid

The DNA extracted from purified virus resolved into linear form on 1 per cent agarose gel. The molecular weight of the DNA determined using *Eco R I / Hind III* double digest as marker and was around 22.6×10^6 daltons (34.2 kb) as shown in Fig.16. The viral DNA extracted from infected allantoic fluid by disruption method (rapid isolation) was also resolved into a single band similar to that of DNA extracted from purified virus. No band was resolved from healthy allantoic fluid preparations.

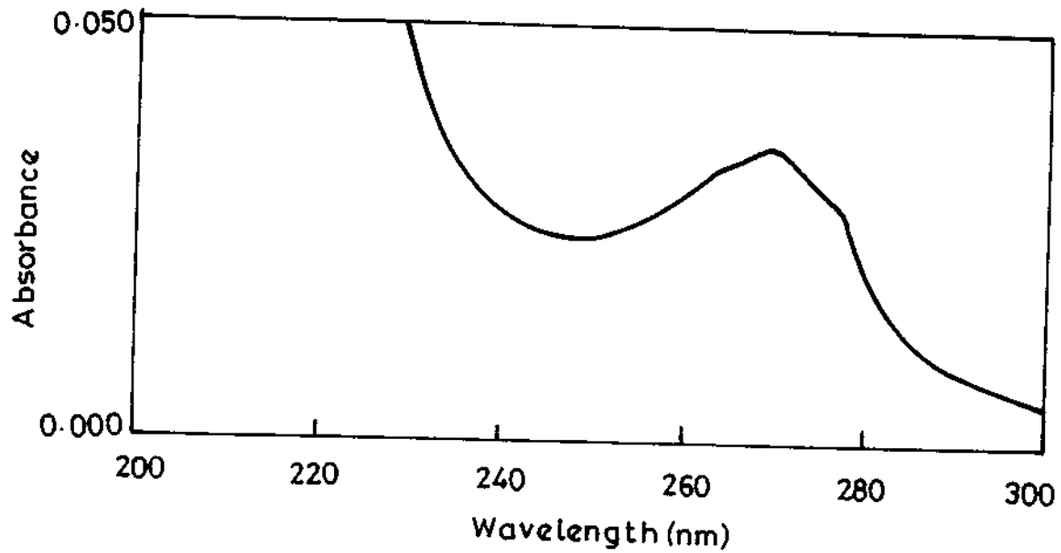


Fig. 14: U.V - visible light absorption spectrum of purified EDS-76 (VN_1 isolate) DNA

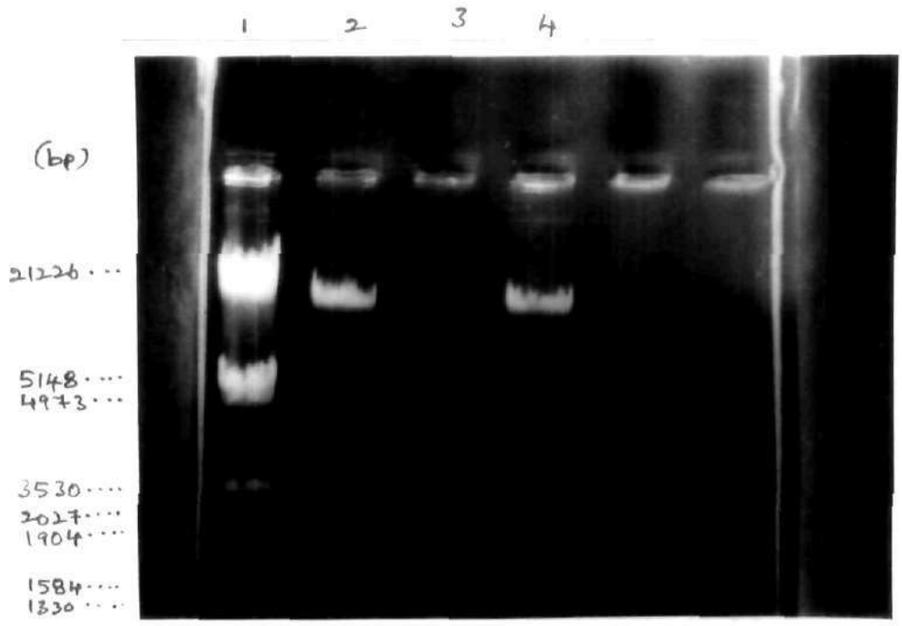


Fig. 15: Determination of nucleic acid type

Lane 1. Molecular weight marker (λDNA/*EcoRI Hind III* double digest)
Lane 2. Untreated nucleic acid (control)
Lane 3. Nucleic acid treated with DNase
Lane 4. Nucleic acid treated with RNase

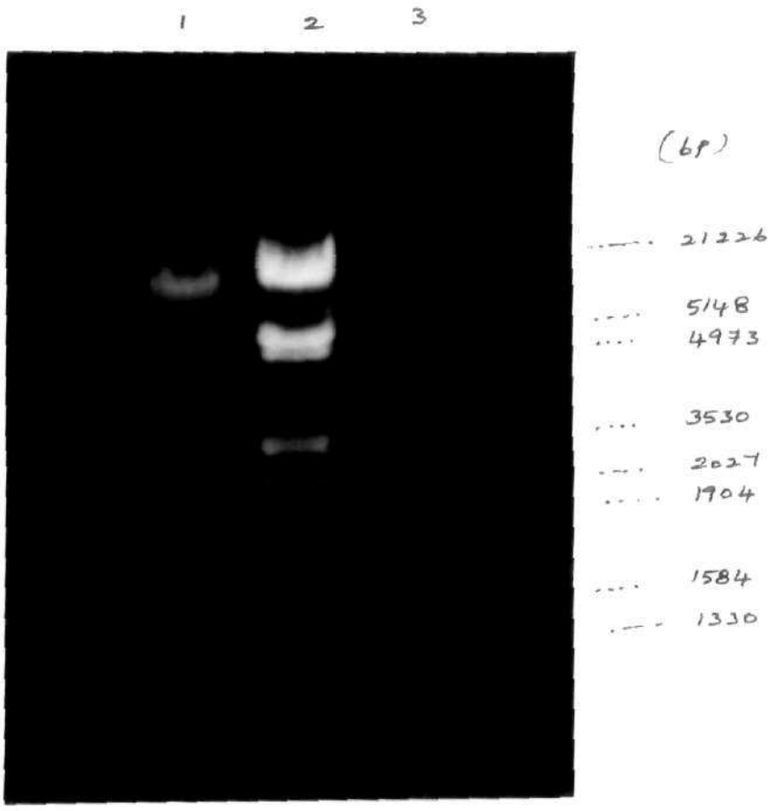


Fig. 16: Determination of Molecular weight of viral DNA

Lane 1. DNA extracted from allantoic fluid
Lane 2. Molecular weight marker (λDNA/*EcoRI Hind III* double digest)
Lane 3. DNA extracted from allantoic fluid

4.4.4 Restriction enzyme analysis of EDS-76 (VN_1 isolate) viral DNA

To confirm the double strandedness and linearity of the EDS-76 (VN-1 isolate) genome, and to obtain a reliable estimate of its size, a restriction endonuclease map has been constructed. The viral DNA had a molecular weight of 32.3 kbp when digested with *EcoRI* enzyme yielded 3 major fragments in nearly equal molar amounts with a molecular weight of 17.5, 7.5, 6.3 and one minor band resolved below, with a molecular weight of 1.0 kbp. *Pvu II* digested viral genome had a molecular weight of 32.1 kbp resolved into 9 major fragments and 3 minor fragments. *Hind III* digested DNA had a molecular weight of 30.1 kbp which resolved into seven fragments with a molecular weight of 7.1, 5.7, 5.0, 4.8, 3.0, 2.8 and 1.5 kbp. *Hae III* digested viral DNA had a molecular weight of 33 kbp and resolved into 12 major fragments with a molecular weight of 4.6, 4.2, 4.0, 3.0, 2.8, 2.6, 2.4, 2.2, 1.6, 1.4, 1.3 and 3 minor fragments with a molecular weight of 1.0, 0.9, 0.6 and 0.4 as shown in Table 8 and Fig.17 and 17a.

4.4.5 Physical mapping of EDS-76, VN_1 isolate viral DNA

The EDS virus (VN_1 isolate) restriction map for *EcoRI* was prepared. When viral DNA was digested with *EcoRI* enzyme, it was resolved into four fragments with molecular weights of 17.5, 7.5, 6.3 and one minor band resolved below, with a molecular weight of 1.0, a total of 32.3Kbp. The map was derived from the migration distance of the electrophoresed restriction fragments. Fragment C generated by *EcoR I* digestion (*EcoRI-C*) was placed at the right hand end of the genome as shown in the Fig.18.

Table 8 : **Fragment size of Restriction enzyme cleavage products of EDS-76, VN_1 DNA**

Enzymes	Fragment (kbp) number											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Eco RI	√	√	√	√								
Hind III	√	√	√	√	√	√	√					
Pvu II	√	√	√	√	√	√	√	√	√	√	√	√
Hae III	√	√	√	√	√	√	√	√	√	√	√	√

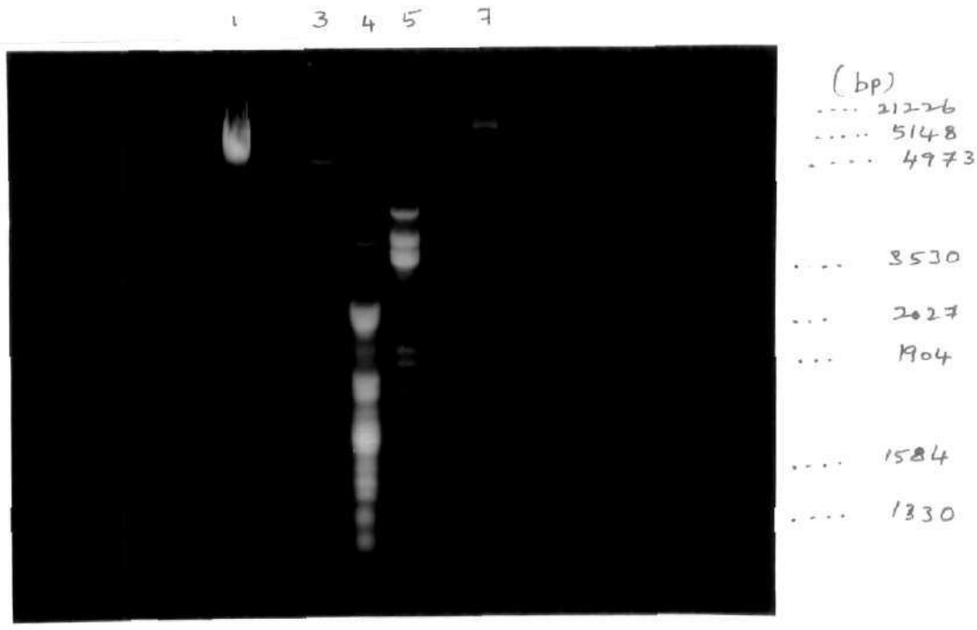


Fig. 17: Electrophoretic pattern of EDS-76 virus (VN_1 isolate) DNA treated with different restriction enzymes

Lane 1: Uncut DNA (control)
Lane 3: *EcoRI* digested sample
Lane 4: *Hae III* digested sample
Lane 5: *Pvu II* digested sample
Lane 7: Marker (λDNA/*EcoRI Hind III* double digest)

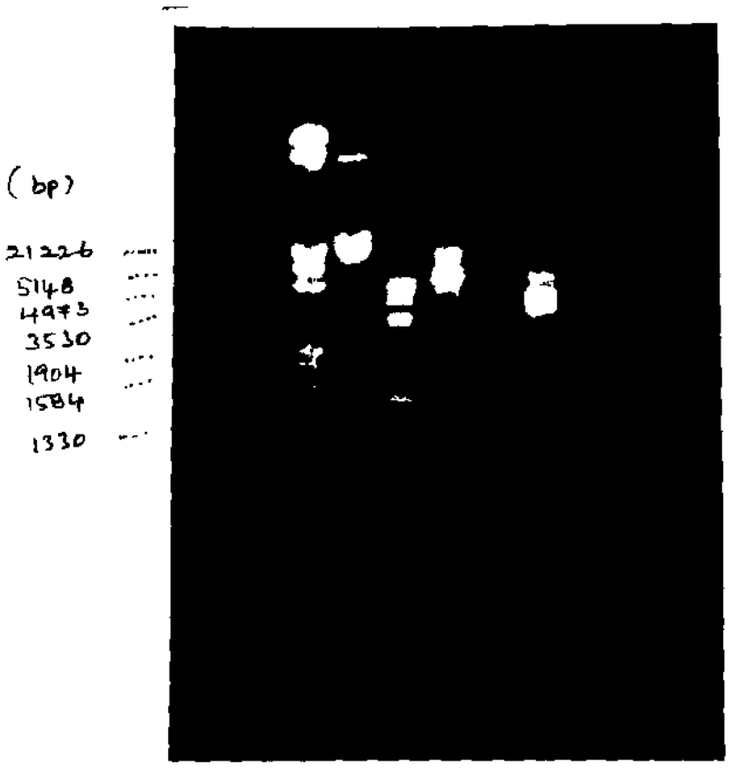


Fig. 17a: Electrophoretic pattern of EDS-76 virus (VN1 isolate) DNA treated with different restriction enzymes

Lane 1. λ DNA/Eco RI Hind III double digest marker
Lane 2. Uncut DNA
Lane 3. Hind III digested sample
Lane 4. Eco RI digested DNA
Lane 5. Hae III digested DNA
Lane 6. Pvu II digested DNA

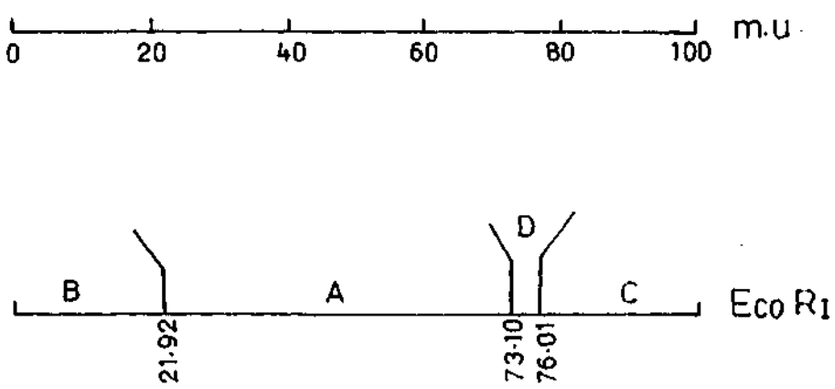


Fig. 18: Physical map of EDS-76 virus (VN_1 isolate) DNA

4.4.6 Comparison of EDS-76 viral isolates DNA by *EcoRI* restriction enzyme

The enzyme, *EcoR I* which recognize DNA sequences of six base pairs generate four restriction fragments from DNA's of the EDS-76 virus isolates (DFH, EDS-127, IVRI-A, IVRI-C). The restriction fragment patterns of 3 isolates are typical when compared with the standard isolate EDS-76 virus, strain 127 and is shown in Fig.19. The molecular weights of fragments were determined by comparing their electrophoretic mobilities with those of marker (λ DNA / *EcoR I Hind III* double digest). All isolates yielded 3 major fragments and 1 minor fragment with a total molecular weight of 32.3 kbp.

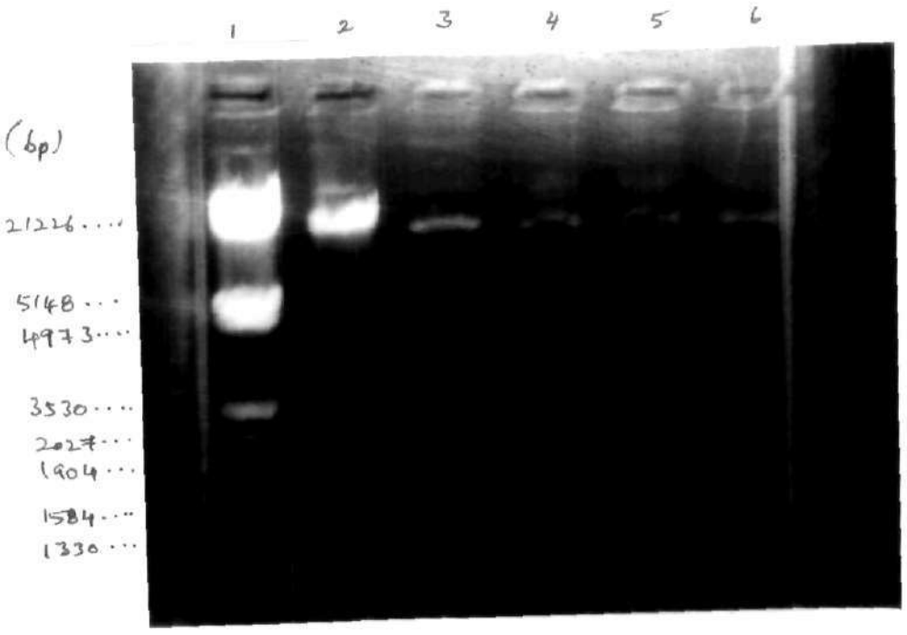


Fig. 19: Electrophoretic pattern of different EDS-76 isolates DNA treated with *EcoR I* enzyme

Lane 1. Molecular weight marker (λDNA/*EcoRI Hind III* double digest)
Lane 2. Uncut DNA (control)
Lane 3. *EcoRI* digested DFH isolate
Lane 4. *EcoRI* digested 127 isolate
Lane 5. *EcoRI* digested IVRI-A isolate
Lane 6. *EcoRI* digested IVRI-C isolate

DISCUSSION

V. DISCUSSION

The drop in egg production has been a constant problem in many poultry farms throughout India and abroad. During the recent past, EDS-76 virus has been incriminated a chief pathogen involved in this clinical syndrome at organized farms (Ramkumar, 1990). In spite of several attempts to develop vaccine against EDS-76 virus (Vanisree *et al.*, 1995), an effective commercial vaccine is not in sight in India or abroad. To develop an effective vaccine, a thorough understanding of the antigenic and molecular characteristics of the indigenous isolates is required. However, the pertinent information on the antigenic and polypeptide profiles of the EDS-76 virus isolates is limited. Similarly, the information available on the molecular characterization of EDS-76 virus is meagre. The prospect of developing a genetically engineered vaccine has rekindled considerable interest in the study of the molecular biology of this virus. Hence in the present study, the EDS-76 virus was characterized antigenically and molecular biologically.

In the present study, a total of six EDS -76 virus isolates representing different geographical regions in India were procured from different places (Table 1). The isolates were propagated in 9-10 day old duck embryos through allantoic cavity route. The virus isolates were examined for growth, infectivity, morphological and serological properties before studying their protein profiles, antigenicity and molecular characteristics in detail. The infectivity assay results revealed that the EDS-76 virus produced high titres as expected. The infectivity titres (EID_{50} / ml) of

the EDS-76 in embryonated duck eggs ranged from log_{10} 5.7 to 7.2 for different isolates (Table 3).

For studying the morphological characters of the EDS-76 virus isolates, the isolates were purified by sucrose gradient centrifugation method. Various techniques were used by different investigators for isolation of EDS-76 virus from faecal samples. The most widely used system has been the chorio allantoic cavity route inoculation in embryonated duck eggs (Adair *et al.*,1979). Similarly different methods were followed by different scientists to purify the virus (Todd and Mc Nulty,1978; Kraft *et al.*,1979). In the present study the virus isolates were purified by 10-40 per cent sucrose gradient centrifugation method. The results (Fig 1) revealed a single light scattering zone between 20 and 30 per cent sucrose gradients, indicating the purity and homogenecity of the EDS-76 virus. The zone obtained is similar to the one obtained by Swain *et al.* (1997). These workers purified EDS-76 virus by velocity gradient centrifugation in continuous density gradients of sucrose. The virus isolates were further purified clonally by terminal dilution method (Flow chart I) to obtain genetic homogenecity in virion population. These clonally purified virus isolates were used for the molecular studies of the virus.

Further the purity of the virus was studied by U.V- visible absorption spectrum. The results of the U.V- visible absorption spectrum showed A_{max} and A_{min} values of 258.9-260 and 242.3-243.4 nm, respectively (Fig.2). A_{260} / A_{280} ratio was 1.42 for the EDS-76 virus isolates. U.V- visible absorption spectra give an idea on the

nature of a bio-molecule. The absorption spectrum of the virus particle is a combination of both nucleic acid and protein. Since the nucleic acid has a much higher specific absorption at its maximum (~ 260) than does the protein, the nucleic acid spectrum dominates (Hull,1985).

In the present study, the electron microscopy of EDS-76 viral particles appeared hexagonal in shape which is a typical character of adenovirus. (Adair *et al.*, 1979 ; Firth *et al.*, 1981 ; Higashihara *et al.*, 1983). The virus particles appeared as a single particle (Zsak and Kisary, 1981). The virus particles measured about 70 nm in diameter (Fig.3) which fell with in the size range of avian adenoviruses. The morphological features of the EDS-76, VN_1 isolate confirmed to those features reported by Todd and McNulty, (1978) to the EDS-76, strain 127 virus.

The isolates were also confirmed serologically by Immunodiffusion test. The results of Immunodiffusion test indicated line of identity between the soluble antigen prepared from virus isolates and referral strain (Fig.4). This result indicates that, the isolates are identical to EDS-76 virus, strain 127.

Having confirmed the EDS-76 virus isolates by various tests as described earlier, the viral polypeptides of six isolates were analyzed and characterized. The results of analysis on 12 per cent SDS-PAGE, the virion was found to contain 13 polypeptides, whose molecular weight varied from 18 to 124 kDa ; many of which appeared as major polypeptides. The polypeptide bands in the EDS-76 isolates were

found to be similar to those in referral strain. Todd and McNulty (1978) also observed 13 polypeptide bands in EDS-76 virus, strain 127. The polypeptide band 1 obtained in the present study was found to have a molecular weight of 124 kDa. Similarly, the molecular weight determined by Todd and Mc Nulty (1978) for similar prominent band 1 was 126 kDa. Some of the minor differences that were observed between the molecular weights of the polypeptides in the present study could be due to the gradient used for purification of the virus. Discrepancies were also reported by other workers (Wilson, 1989; Zsak and Kisary, 1981; Swain *et al.*, 1992) which was ascribed to the method of virus purification and to the source of virus i.e embryonated duck eggs or chicken embryo liver cells. In the present study, we have used sucrose density gradient centrifugation for virus purification and duck embryos for growth of virus as compared to the chick embryo liver cells and cesium chloride density gradient centrifugation used by Todd and Mc Nulty (1978). However, Swain *et al.* (1992), in contrast, observed only 12 polypeptides with an indigenous EDS-76 virus isolate which varied from 10 to 126 kDa.

In this study, effects were made to determine the immunogenic polypeptide(s) among the 13 polypeptides by electroblot immunoassay (Fig.6). The results revealed that, 3 polypeptides were found to be immunogenic. The positive EDS-76 virus serum reacted with viral polypeptides bands of 124, 84 and 54 kDa. Similar observations were recorded by Kejun *et al.*, (1996) but these workers have noticed 7 polypeptides reacted with positive mouse monoclonal anti EDS virus serum. This

variation may be due to the use of monoclonal antibodies which have better resolving power and higher specificity.

In the present investigation, attempts were also made to isolate and identify the polypeptide responsible for protection / capable of absorbing haemagglutinating antibody and neutralizing antibody. The results of these experiments revealed that the polypeptide having a molecular weight of 84 kDa could absorb the neutralizing antibody from the hyperimmune serum against EDS-76 virus. Further, it was also observed that the same protein is also responsible for eliciting neutralizing antibody as well as h magglutinating antibodies when inoculated into chicken. Wilson (1989) also reported that the same protein (44 kDa) is responsible for eliciting both neutralizing and haemagglutinating antibody. However, the molecular weight differences reported by Wilson (1989) may possibly be due to the individual differences in the interpretation of the results of the gels. In contrast, Mockett *et al.*(1984) reported that the epitope responsible for production of neutralizing monoclonal antibodies was not involved in haemagglutination. The possible discrepancy in the observation may be due to the fact that in adenoviruses the neutralizing activity resides with determinant of hexon and determinant of fiber (Philipson *et al.*, 1975) whereas the haemagglutinating activity is associated with fiber only (Rosen, 1960). So, probably this monoclonal antibody of Mockett *et al.* (1984) is directed against the epitopes present on hexons.

The polypeptides responsible for producing cell mediated immunity were characterized using lymphocyte stimulation test and cytotoxicity assay by MTT assay method. The cell mediated immune response in chicks experimentally infected with EDS-76 (VN_I isolate) viral polypeptides was studied using the lymphocyte stimulation Test by MTT assay. The use of a colorimetric assay for cell growth and survival, performed in microtitre plates in combination with multichannel pipettors and an automatic scanning spectrophotometer, offers major advantages in speed, simplicity, cost and safety over conventional assays using the uptake of radio labelled compounds (Gerlier and Thomasset, 1986). This assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells that is why this method is used to measure the proliferated lymphocytes. MTT is cleaved by all living, metabolically active cells but not by dead cells. The amount of formazan generated is directly proportional to the cell number over a wide range. The results of the MTT assay of the unstimulated and stimulated spleenic cells after sensitization with different polypeptides was shown in Table 6. The results showed that the 84 kDa polypeptide sensitized spleen cells stimulated actively in more numbers when compared to spleen cells sensitized with other polypeptides. However, no comparable results are available with EDS-76 virus system.

When different polypeptides of VN_I virus isolate were inoculated, a noticeable splenomegaly was observed in the chicken sensitized with 84 kDa polypeptide (Fig.10) compared to unsensitized chicken spleen. The splenomegaly was noticed on the 5^{th} day. The results are in agreement with those of Eck and

Van (1986). The splenomegaly may be due to the fact that lymphocytes bearing antibody-antigen complexes were intercepted by macrophages in the red pulp of the spleen resulting in enhanced lymphoblast formation and by the macrophages of the ellipsoidal corona resulting in expansion of periellipsoidal lymphoid tissue.

The 84 kDa polypeptide was also found to elicit cytotoxic 'T' cell (CTL) response. The percentage of dead cells are more in the 84 kDa polypeptide introduced wells, when compared to others (Table 7). In 54 and 124 kDa polypeptide treated wells, there was a considerable percentage of CTL though not as high as in the 84 kDa polypeptide wells. These results suggest that, these polypeptides (54 and 124 kDa) also appear to have some immunogenic property. But when compared with 84 kDa polypeptide they seem to elicit less CTL as evidenced by more O.D of the effector / target cell combination, because DNA of live cells will take up more MTT. No comparable results were available with EDS virus system. However, the data reported herein confirm the previous findings of Mosmann (1983) and Green *et al.* (1984) describing the use and advantage of the MTT colorimetric assay in the study of cytotoxicity, proliferation and cell activation.

The polypeptide responsible for induction of antibody forming cells (AFC) were studied. In this study also the 84 kDa polypeptide induced more number of AFC (Fig.13) when compared to other polypeptides. This suggests that the 84 kDa polypeptide due to its immunogenic nature probably produce IgM class of antibody

that bind to complement, consequently there will be an increase in the number of plaques as reported by Jerne and Nordin,(1963).

The immunogenicity of the three polypeptides viz: 124, 84 and 54 kDa was indicated by the results of electroblot immunoassay. Further, a critical evaluation of the various EDS-76 virus polypeptides using different parameters such as, the ability of the polypeptide to induce neutralizing and haemagglutinating antibodies, antigen specific lymphocyte proliferation, induction of cytotoxic 'T' cell response and induction AFC revealed that the polypeptide with a molecular weight of 84 kDa appears to be major immunogenic polypeptide and two more polypeptides (124 and 54 kDa) appear to be minor immunogenic polypeptides as they could induce only CTL response but not antibody response. However, no comparable investigations are available using EDS-76 virus - chicken system in the available literature. It would be interesting to further evaluate 84 kDa polypeptide with respect to its potential as a non-infectious vaccine, since it is able to induce a variety of immune responses in the chicken.

In the present investigation, the molecular characterization of the EDS-76 viral isolates were studied. Viral nucleic acid was extracted from the purified virus and the purity of the nucleic acid was assessed by studying the OD ratio between A_{260} nm and A_{280} nm. The ratio between A_{260} and A_{280} provides an estimate of the purity of the nucleic acids (Sambrook *et al.*, 1989). The A_{260} / A_{280} ratio of the EDS viral nucleic acid extracted form the purified virus was 1.8 (Fig.14), which shows the

purity of the nucleic acid. The virus isolate contained DNA as its nucleic acid positively reacted with diphenylamine reagent and was completely digested by DNase, but not by RNase (Fig.15). These results are in agreement with Swain *et al.* (1995). The molecular weight of the nucleic acid was found to be 22.6 x 10^6 daltons (Fig.16) which was similar to the observations made by Todd *et al.* (1988).

In this study the isolates of EDS-76 virus could be distinguished by restriction endonuclease analysis of the virus DNA's. Restriction enzyme analysis of DNA genomes may be used for quantitative estimates of sequence divergence as great as 20 to 30 per cent (Brown *et al.*, 1978). The analysis involved the comparison of DNA's of 3 isolates with the DNA of referral strain,127 by using *EcoR I* restriction endonuclease. The *EcoR I* which recognize DNA sequences of six base pairs cleaved the EDS virus genome at a total of 4 restriction sites (Fig.19) but is of limited use for the differentiation of isolates. All the three isolates were found to have genetic similarity with referral strain 127. The restriction fragment pattern obtained with *EcoR I* for the isolates were identical to the results of Todd *et al.* (1988). In addition, the molecular weight of the 127 virus genome calculated in this study was 34.2 kb which was similar to the observation of Zsak and Kisary (1981).

The endonucleases *Hind III, Pvu II. EcoR I* which recognizes DNA sequence of six base pairs cleaved the VN_I virus genome at a total of 7,12 and 4 restriction sites respectively (Fig.17 and Table 8). The restriction enzymes like *Hae III* recognize sequences of four base pairs generates more DNA fragments of smaller

size. The ability to separate these fragments with a high degree of resolution by electrophoresis in polyacrylamide gel greatly increases the chances of detecting base sequence differences which may exist between DNA molecules. *Hae III* cleaved the viral genome at 12 restriction sites (Fig.17). The restriction fragment patterns of isolate VN_1 obtained with *Hind III, Pvu II, EcoR I* and *Hae III* were similar to the restriction enzyme profile for the previously reported variant Hungarian strain, B78/8 (Zsak and Kisary, 1981).

The physical map of EDS-76, VN_1 isolate obtained for *Eco R I* enzyme used in this experiment suggest that the genome of the EDS-76 virus is a linear duplex molecule (Fig.18). It can also be stated that one of the end regions of the DNA molecule is richer in A-T compared to the other area. These results are in coordination with the work of Zsak and Kisary (1981).

DNA finger printing is a modern technique for the present day molecular biologists to distinguish genetically different yet serologically similar strains of viruses. In general, adenovirus contains more number of inverted repeat sequences of the viral DNA. Considerable finger print variations occur since sequence 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 inter strain finger print variations occur more frequently in restriction fragments containing inverted repeat sequences of viral DNA (Zhang and Nagaraja, 1989).

Inspite of the highly sensitive molecular studies carried out using restriction length polymorphism (RFLP) analysis, no genomic divergence was observed in the EDS-76 virus isolates. Similarly, no divergence in the isolates could be observed in protein profile analysis. These indicate that the EDS-76 virus circulating in the country is genetically and antigenically homogenous and identical to the referral strain,127.

In conclusion, the present investigation on molecular and antigenic characterization of EDS-76 virus isolates helped in identifying the immunogenic polypeptide(s). These results would be critical while designing a molecularly defined non-infectious EDS-76 virus vaccine. It would be interesting to identify the genomic fragment (s) coding for the major immunogenic polypeptide (84 kDa) which will pave the way for molecularly designed vaccines.

SUMMARY

VI. SUMMARY

The present investigation was taken up with a view to understand the antigenic and molecular characteristics of the indigenous EDS virus isolates which aids in developing an effective vaccine which is very much essential now in India due to frequent outbreaks.

In this study, the virus isolates were procured from different geographical regions of India and purified by ultra centrifugation in a 10 - 40 per cent sucrose density gradients. The electron microscope observation of the purified virus preparation of VN_1 isolate revealed the presence of complete hexagonal virus particles scattering singly.

The purified viruses were subjected to electrophoresis on 12 per cent polyacrylamide gels. PAGE of the purified viruses reproducibly resulted in 13 polypeptide bands. The molecular weights of these polypeptides ranged from 124 to 18 kDa .

The polypeptide responsible for immunogenicity was identified by electroblot immunoassay. SDS-PAGE separated viral polypeptides were transferred electrophoretically on to a nitrocellulose membrane and reacted with hyperimmune rabbit serum prepared against EDS-76 virus (strain 127). Among 13 polypeptides, only 3 reacted. The molecular weights of these moieties were determined by standard curve.

The virion polypeptide (s) responsible for protection / capable of absorbing HI antibodies and neutralizing antibody was isolated and characterized. The polypeptide responsible for induction of cell mediated immunity was studied using lymphocyte stimulation test by MTT assay method. This test also Supports the results of electroblot immunoassay in that 84 kDa polypeptide sensitized chicken stimulated more lymphocytes than other polypeptide sensitized chicken. A noticeable splenomegaly was also observed on 5^{th} day after injection of 84 kDa polypeptide fragment.

The cytotoxic assay has been performed to monitor the effect of cytotoxic 'T' lymphocytes by MTT assay. This test also confirms the polypeptide having a molecular weight of 84 kDa produced more activated 'T' lymphocytes.

These results also revealed that, the 84 kDa polypeptide is responsible for induction of more number of antibody forming cells (AFC) when compared to other polypeptides.

The results provide an evidence to the fact that the isolates consist of DNA and the molecular weight of DNA was calculated as 22.6 x 10^6 daltons (34.2 kb). Restriction endonucleases *EcoRI*, *Hind III* , *Pvu II* and *Hae III* recognized 35 cleavage sites in EDS-76 ,VN_I isolate. Three isolates of EDS virus were compared with referral strain,127 by *EcoRI* restriction endonuclease analysis of the virus DNA and revealed that all the isolates were genetically same. The fragments generated from VN_I isolate DNA by *EcoRI* was physically mapped.

LITERATURE CITED

LITERATURE CITED

Adair, B.M., Mc Ferran, J.B., Cannor, T.J., Mc Nulty, M.S and Mc Killop, E.R. (1979). Biological and physical properties of a virus (strain 127) associated with the egg drop syndrome 1976. Avian Pathology, **8:** 249-264.

Agrawal, P.K and Reynolds, D.L. (1999). An evaluation of the mitogenic reactivity of intestinal intraepithelial lymphocytes of chickens. Avian Diseases, **43;** 172-181.

Anonym. (1977). Adenovirus strand nomenclature: a proposal. Journal of Virology, **22**:830-831.

Baxendale, W. (1978). Egg drop syndrome - 76. Veterinary Record, **102:** 285-286.

Bennejean,G., Gnittet, M and Picault, J.P. (1980). Fall in egg production associated with the laying of soft shelled eggs; a new infectious disease of the hen. Science Veterinaires et. de Medicine Compaya. delyon, **81:** 159-165. (Vet. Bulletin, **50**: 648).

Bounous, D.I., Raymond, P., Campagnoli and Brown, J. (1992). Comparison of MTT colorimetric assay and tritiated thymidine uptake of lymphocyte. Avian Diseases, **36:**1022-1027.

Brakke,M.K (1967). Density gradient centrifugation; Miscellaneous problems in virus purification. In: Methods in Virology (K.Maramorosch and H.Koprowski,Eds.), vol.2,pp.93-136. Academic Press: New york.

Brown, W.M., George, M and Wilson, A.C. (1978). Rapid evolution of animal mitochondrial DNA. Proceedings of the National Academy of Science USA, **76**: 1967-1971.

Burgermeister,W and Koening, R (1984). Electroblot immunoassay - a means for studying serological relationships among plant viruses. Phytopathologische Zeitschrift, **111**:15-25.

Burton,K (1956). A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of DNA. Biochemical Journal, **62**:315-322.

Cannon, M.J and Russell, P.H. (1986). Secondary in vitro stimulation of specific cytotoxic cells to Newcastle disease virus in chickens. Avian Pathology, **15:** 731-740.

Chandramohan, A. (1994). Studies on Egg drop syndrome virus. Ph.D., Thesis. Tamilnadu Veterinary and Animal Science Uinversity. Chennai.

Chandramohan, A., Shaw, A.M., Nachimuthu, K and Venkatesan, R.A. (1997). Electron and immunoelectron microscopic investigations on EDS-76 virus. Indian Journal of Animal Science, **64:** 939-940.

Chandramohan, A., Shaw, A.M., Ramadass, P., Wilson, A and Venkatesan, R.A. (1998). Protein fractionation analysis of indigenous Egg drop syndrome-76 viral isolates. Indian Veterinary Journal, **75:** 170.

Chetty, M.S. (1985). Studies on Egg drop syndrome virus (EDS-76) in chicken in A.P. Ph.D., Thesis., A.P. Agricultural University, Hyderabad.

Chubb, R.C., Huynh,V and Law, R. (1987). The detection of cytotoxic lymphocyte activity in chickens infected with infectious bronchitis virus or fowl pox virus. Avian Pathology, **16:** 395-405.

Crowle, A.J (1961). Immunodiffusion. Academic press, New york and London.

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

Das, B.C., Panda, H.K., Mohapatra, H.K and Tripathy, B.C. (1996). Isolation and identification of EDS-76 virus strains from organised poultry and duck breeding farms in Orissa. Indian Journal of Animal Health, **35:** 31-34.

Denizot, F and Lang, R. (1986). Rapid colorimetric assay for cell growth and survival - modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal of Immunological Methods, **89:** 271-277.

Eck, J.V and Van, E.J. (1986). Involvement of spleen components of mature fowl in the primary and secondary humoral antibody response following experimental EDS-76 virus infection. Veterinary Quarterly, **8:** 105-127.

Erny, K.M., Sheppard, M and Fahey, K.J (1996). Immunopathogenesis and physical maps of fowl adenovirus serotype 9. Research in Veterinary Science, **61:** 174-175

Fellowes, O.N. (1966). Hydroxylamine as an inactivating agent for foot and mouth disease virus. Journal of Immunology, **96:** 772-776.

Firth, G.A., Hall, M.H and Mc Ferran, J.B. (1981). Isolation of a haemagglutinating adenovirus related to virus 127 from Australia Poultry flock with an egg drop syndrome. Australian Veterinary Journal, **57:** 239-242.

Franklin, R.M and Wecker, E. (1959). Nature, **184:** 343. (cited by Fellowes, 1966).

Friedman, H and Goldner, H. (1970). Effect of a DNA tumor virus on antibody forming cells in neonatally infected hamsters. Nature, **225:**455-456.

Gerlier, D and Thomasset, N. (1986). Use of MTT colorimetric assay to measure cell activation. Journal of Immunological Methods, **94:** 57-63.

Gough, R.E., Collins, M.S and Spackman, D. (1982). Isolation of a haemagglutinating adenovirus from commercial ducks. Veterinary Record, **110:** 275-276.

Green, L.M., Reade, J.L and Ware, C.F. (1984). Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. Journal of Immunological Methods, **70:** 257-268.

Harrach, B., Mechan, B.M., Benko, M., Adair, B.M and Todd, D. (1997). Close phylogenetic relationship between EDS-virus, bovine adenovirus serotype-7 and ovine adenovirus strain 287. Virology, **229:** 302-306.

Hess, M., Blocker, H and Brandt, P. (1997). The complete nucleotide sequence of the Egg drop syndrome virus: an intermediate between mastadenoviruses and aviadenoviruses. Virology, **238:** 145-146.

Higashihara, M., Takai, S., Hidaka, A., Houolatsu, T., Hiruma, M., Watanbe, Y and Matumoto, M. (1983). Isolation of the virus of Egg drop syndrome -76 in a Japanese Outbreak. Japan Journal of Veterinary Science, **45:** 605-612.

Huang, G.G., Xin, C.A and Kong, D.V. (1996). Restriction endonuclease analysis of Egg drop syndrome 1976 virus DNA. Chinese Journal of Veterinary Science and Technology. **26**:11.

Hull,R (1985). Purification, Biophysical and Biochemical characterization of viruses with special reference to plant viruses. Virology- A practical approach, ed.by B.W.J.Mahy, IRL press, Oxford, Washington.

ICAR Report (1987).Final report of Adhoc Research Scheme on "Incidence, Etiopathology and control of Egg drop syndrome in poultry in Andhra Pradesh". Department of Microbiology, C.V.Sc, Tirupathi.

Jerne, N.K and Nordin, A.A. (1963). Plaque formation in agar by single antibody producing cells, Science, **140:** 405.

Kejun, Y., Deying, K., Chaoan, X., Yang, K.J., Kong, D.Y and Xin, C.A. (1996). Antigen analysis of Egg drop syndrome-76 virus by using monoclonal antibodies. Chinese Journal of Veterinary Medicine, **22:** 3-6.

Kisary, J and Zsak, L (1980). The molecular weight of the Egg drop syndrome (EDS) avian adenovirus (strain B8/78) DNA estimated by digestion with restriction endonuclease enzyme *EcoR I* - Brief Report. Archieves of Virology, **85:** 83-88.

Kraft, V., Grund., S and Monreal, G (1979). Ultrastructural characterization of isolate 127 of Egg drop syndrome 1976 virus as an adenovirus. Avian Pathology, **8:** 353-361.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature, **227:** 680-684.

Majuvani, S.P. (1986). Studies on the relationship of different indigenous viral isolates of Egg drop syndrome - 76 with respect to reference strain - 127. M.V.Sc , Thesis, Andhra Pradesh Agricultural University, Hyderabad.

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

Mc Ferran, J.B., Connor, T.J and Adair, B.M. (1978). Studies on the antigenic relationship between an isolate (127) from the Egg drop syndrome 1976 and a fowl adenovirus. Avian Pathology, **7**; 629-636.

Meulemans, G., Detegel, D., Peeters, J., Van Meirhaeghe, E and Halen, P. (1979). Isolation of an adenolike virus from laying chickens effected by Egg drop syndrome 1976. Vlams Diergeneeskd Tijdschr, **2:** 151-157 (Vet. Bulletin, **49**: 6606).

Mockett, A.P.A., Brown, T.D.K and Davis, P.J. (1984). A hybridoma secreting a neutralizing monoclonal antibodies to Egg drop syndrome virus. Avian Pathology, **13**:333-337.

Mohanthy, G.C., Verma, K.C., Pradhan, M.K and Kumar, R. (1984). Egg drop syndrome-76 (EDS-76) in India. Seroprevalence of EDS-76 virus infection in poultry flocks. Indian Journal of Poultry Science, **26:** 236-240.

Mosmann, T.J. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods. **65:** 55-63.

Mullbacher, A., Parish, C.R and Mundy, J.P. (1984). An improved colorimetric assay for T cell cytotoxicity in vitro. Journal of Immunological Methods, **68;** 205-215.

Muzhi, H., Zhang, Z., Youxin, J., Hu, M.Z., Zhang, Z.J and Jin, Y.X. (1996). Study on the analysis of nucleic acid sequence and gene amplification of egg drop syndrome-76 virus. Chinese Journal of Veterinary Science and Technology, **26:** 20-21.

Nagaraja, K.V., Emery, D.A., Patel, B.L., Pomeroy, B.S and Newman, J.A. (1982). In vitro evaluation of B-lymphocyte function in turkeys infected with hemorrhagic enteritis virus. American Journal of Veterinary Research, **43:** 502-504.

Philipson, L., Petterson, U and Lindberg, U. (1975). Molecular biology of adenoviruses. Virology Monographs, **14**: 1-115.

Ping, J., Ojkic, D., Tuboly, T., Huber, P and Nagy, E. (1999). Polymerase chain reaction for the detection of fowl adenoviruses. Canadian Journal of Veterinary Research, **63:** 124-128.

Prasad, V. 1992. Studies on experimental suppression of bursa and thymus dependent immune system in ducks. M.V.Sc., Thesis. Andhra Pradesh Agricultural University, Hyderabad.

Ramkumar, (1990). Immunopathological studies on Egg drop syndrome in poultry associated with adenovirus infection. Ph.D. Thesis., Deemed University, IVRI, Izatnagar, U.P.

Ramkumar, Kataria, 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.

Ramkumar, Mohanty, G.C. and Verma, K.C. (1992). Eptizootiological studies on Egg drop syndrome in poultry. Indian Journal of Animal Science, **62:** 497-501.

Raue, R and Hess, M (1998). Hexon based PCR's combined with restriction enzyme analysis for rapid detection and differentiation of fowl adenovirus and EDS virus. Journal of Virological methods, **73:** 211-217.

Reddy, T.V. (1984). Isolation, characterization and serology of adenovirus from Egg drop syndrome (EDS) in poultry. Ph.D.,Thesis. University of Agricultural Sciences, Bangalore.

Reed, L.J and Muench, H. (1938). A simple method of estimating of fifty percent end point. American Journal of Hygiene, **27:** 493-497.

Rosen, I. (1960). A haemagglutination inhibition technique for typing adenoviruses. American Journal of Hygiene, **71**: 120-128.

Rozhdestvenskii, I.K. (1984). A general view of the adenoviruses. Annals New York Academy Science, **101:** 466-474.

Sambrook, J., Fritsch, E.T and Maniatis, T. (1989). Molecular cloning: a laboratory manual. Cold spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schafer, W and Rott, R. (1962). Z. Hyg. Infektions K, **148:** 256 (cited by Fellowes, 1966).

Schneider, W.C (1957). Determination of nucleic acids in tissues by pentose analysis. Methods in Enzymology, **3**:680-684.

Shakya, S and Dhawedkar, R.G. (1991). Antigenic relationship among Egg drop syndrome - 1976 virus strains. Indian Veterinary Journal, **68:** 510-513.

Shapiro, A.L.,Vinnela, E and Maizel, J.V. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochemistry, Biophysics Research Communication, **28:** 815-820.

Shariff, A., Reveppa, K.L and Kesavamurthy, B.S. (1983). Abstracts on Xth Annual Poultry Symposium (15-17th Dec., 1983 held at Madras) Abst. No. A.D. SC. 4.

Swain, P., Kataria, J.M and Verma, K.C. (1993). Biological characterization of an Indian isolate of EDS-76 virus. Research in Veterinary Science, **55:** 396-397.

Swain, P., Kataria, J.M., Dharma, K and Verma, K.C. (1997). Purification of Egg drop syndrome virus by velocity density gradient centrifugation. A comparative study. Acta Virologica, **41:** 303-304.

Swain, P., Kataria, J.M., Verma, K.C and Sathish, K (1992). Characterization of a field isolate of Egg drop syndrome 76 (EDS-76) virus. Indian Journal of Virology, **8:** 8-14.

Swain, P., Verma, K.C., Kataria, J.M and Mehrotra, M.L. (1995). Purification and isolation of nucleic acid of Egg drop syndrome-76 (EDS-76) virus isolated from Japanese quail. Indian Journal of Comparative Microbiology, Immunology and infectious diseases. **16:** 141-143.

Takai, S., Higashihara, M and Matumoto, M. (1984). Purification of haemagglutinating properties of Egg drop syndrome 1976 virus. Archieves of virology, **80:** 59-67.

Thompson, D.L., Elgert, K.D., Gross,W.B and Siegel, P.B. (1980). Cell mediated immunity in Marek's disease virus - infected chickens genetically selected for high and low concentrations of plasma corticosterone. American Journal of Veterinary Research, **41:** 91-96.

Thomsson, G and Naqi, S. (1997). Cytotoxic activity of cells recovered from the respiratory tracts of chickens inoculated with Infectious Bronchitis Virus. Avian Diseases, **41:** 690-694.

Todd, D and Mc Nulty, M.S. (1978). Biochemical studies on a virus associated with Egg drop syndrome - 1976. Journal of General Virology, **41:** 63-75.

Todd, D., McNulty, M.S and Smyth, J.A. (1988). Differentiation of Egg drop syndrome virus isolates by restriction endonuclease analysis of virus DNA. Avian Pathology, **17:** 909-919.

Umesh Kumar, K., Krishnaswamy, S and Venkat Reddy, T. (1989). Cell mediated immune response to egg drop syndrome (EDS-76) virus infection in chickens. Current Science, **58:** 431-433.

Vanisree, K. (1995). Monitoring of antibody response to experimental Egg drop syndrome - 1976 (EDS-76) vaccine. M.V.Sc., Thesis, Andhra Pradesh Agricultural University. Hyderabad.

Vickery, K., Cossart, Y and Dixon, R (1999). Cellular immune response of ducks to duck hepatitis B infection. Journal of Medical Virology, **58**:19-25.

Villegas, P., Kleven, S.H., Eidson, C.S and Trampal, D. (1979). Isolation of a haemagglutinating adenovirus serologically related to adenovirus-127. Avian diseases, **23:** 507-514.

Walker, L., Piela, T.H., Yates, V.J and Gulka, C.M. (1982). Heat inactivation of a duck adenovirus serologically indistinguishable from Adenovirus 127. Avian Diseases, **26**:602-605.

Wigand, R., Bartha, A., Dreizin, R.S., Esche, H., Ginsberg, H.S., Green, M., Hierholzer, S.S., Kalter, S.S., Mc Ferran, J.B., Petterson, U., Russell, W.C and Wadell, G. (1982). Adenoviridae: Second report. Intervirology, **18:** 169-176.

Wilson (1989). Characterization of immunogen(s) of Egg drop syndrome-76 (EDS-76) virus. M.V.Sc. Thesis, Andhra Pradesh Agricultural University, Hyderabad.

Winberg, G and Hammarskjold, M.L. (1980). Isolation of DNA from agarose gels using DEAE-paper. Application to restriction site mapping of adenovirus type 16 DNA. Nucleic Acids Research, **8**: 253-264.

Xuemin, W., Zheng, M., Chen, P and Caibao, X. (1998). Analysis of EDS virus DNA by restriction endonucleases. Acta Agriculture Shanghai, **14:** 43-44. (Poultry Abstracts, **25**: 271).

Yamaguchi, S., Imada, T., Kawamura, H., Taniguchi, S., Saio, H and Shiwamoatsu, K. (1981). Outbreak of Egg drop syndrome - 1976 in Japan and its etiological agent. Avian Diseases, **25:** 528-541.

Yates, V.J., Rhee, Y.O., Fry, D.E., Mishad, E.I. and Mc Cornick, K.J. (1976). The presence of avian adenovirus and AAV in healthy chickens. Avian Diseases, **20:** 146-152.

Yishan, L., Tianxian, L., Feng, F., Lin, Z., Luo, Y.S., Li, T.X., Feng, F and Zhao, L. (1995). Study on Egg drop syndrome 1976 virus - protein characteristics of GC_2 strain. Acta - veterinaria - et - zootechnica - sinica, **26:** 534-536.

Yugou, D., Zhizhong,C., Song, G., Tiantong, G., Duan, Y.Y., Cui, Z.Z., Gao, S and Ge, T.T. (1995). Preparation and application of a DNA probe for chicken Egg drop syndrome virus. Chinese Journal of Veterinary Science, **15;** 219-223.

Yurov, G.K., Kadykov., V.A., Neugodova, G.L and Naroditsky, B.S. (1998). Analysis of virion core polypeptides of Egg drop syndrome virus. Voprosy Virusologi, **43:** 232-235. (Poultry Abstracts, **25**: 3092).

Yu You, D., Zhizhong, C., Tiantong, G., Yong Kun, W., Duan, Y.Y., Cui, Z.Z., Ge, T.T and Wang, Y.K. (1995). Restriction endonuclease analysis of viral genomic DNA of chicken Egg drop syndrome H91 virus. Journal of Jiangsu Agricultural College, **16:** 54-58.

Zakharchuk, A.N., Kruglyak, V.A., Akopian, T.A., Naroditsky, B.S and Tikhonenko, T.I. (1993). Physical mapping and homology studies of Egg drop syndrome (EDS-76) adenovirus DNA. Archieves of Virology, 128: 171-176.

Zanella, A., Donato, A.N., Nigrellia, A and Poli, G. (1980). Etiopathogenesis, Epidemiology, Immunology and control of the diseases. Clinico Vet, **103:** 549-569. (Vet. Bulletin, **51** 3219).

Zeng, L., Jin, Q., Zhang, J.G., Li, M., Li, H., Zhu, L., Yin, Z and Hou, Y. (1998). Analysis of the structure of Egg drop syndrome virus major proteins. Chinese Journal of Virology, **14:** 45-54.

Zhang, C and Nagaraja, K.V. (1989). Differentiation of avian adenovirus type-II strains by restriction endonuclease finger printing. American Journal of Veterinary Research, **50**:1466-1470.

Zhixun, X., Fadl, A.A., Girshick, C and Khan, M.I. (1999). Detection of avian adenovirus by polymerase chain reaction. Avian diseases, **43:** 98-105.

Zijun, Z., Muzhi, H and Youxin, J. (1995). Confirmation and sequencing of the J fragment of Egg drop syndrome virus DNA. Acta - Biochemica - et - Biophysica - Sinica, **27:** 598-592.

Zsak, A., Bartha, J., Mezsaros and Szurop, I. (1981). Immunization trials against the Egg drop syndrome (EDS) in laying hens. Magyar Allatrvosok Lapaka, **2:**123-127 (Vet. Bulletin, **51**: 4921).

Zsak, L and Kisary, J (1981). Some biological and physico chemical properties of Egg drop syndrome (EDS) avian adenovirus strain B8/78. Archieves of Virology, **689:** 211-219.

Zsak, L and Kisary, J. (1984). Grouping of fowl adenoviruses based upon the restriction patterns of DNA generated by *Bam HI* and *Hind III*. Intervirology, **22:** 110-114.

APPENDIX

Appendix

I. Stock solutions for polyacrylamide gel electrophoresis

1. Acrylamide : Bis acrylamide (30: 0.8)

Acrylamide	- 30.0 g
N,N'-Methylene bis acrylamide	- 0.8 g

Dissolved in 60 ml distilled water and made upto 100 ml with distilled water, filtered and stored at 4°C in an amber - coloured bottle.

2. Resolving gel buffer (1.5 M Tris-HCl, pH 8.8)

(Tris - base)	- 18.15 g
Distilled water	- 80 ml

pH adjusted to 8.8 with 1N HCl and made upto 100 ml with distilled water, filtered and stored at 4°C.

3. Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

Tris base	- 6.0 g
Distilled water	- 70 ml

pH adjusted to 6.8 with 1N HCl and made upto 100 ml with distilled water, filtered and stored at 4°C.

4. 10 per cent Sodium dodecyl sulphate (SDS)

10 g of SDS dissolved in 100 ml of distilled water with gentle stirring and stored at room temperature.

5. **Sample buffer**

0.5 M Tris HCl, pH 6.8	- 1.25 ml
Glycerol	- 1.00 ml
10% SDS	- 2.00 ml
2-Mercaptoethanol	- 0.50 ml
0.05% Bromophenol blue (w/v)	- 0.25 ml

made upto 10 ml with distilled water and stored at room temperature.

6. **Electrophoresis buffer**

Tris base	- 1.80 g
Glycine	- 8.62 g
SDS	- 0.60 g
Distilled water	- 500 ml

pH adjusted to 8.3 with 1N HCl and made upto 600 ml with distilled water and stored at room temperature.

7. **10 per cent Ammonium persulphate (APS)**

100 mg APS dissolved in 1 ml distilled water just before use.

8. **Gel staining solution**

Coomassie Brilliant Blue R - 250	- 100 mg
Methanol	- 50 ml
Dissolved then add acetic acid	- 10 ml
dH_2O	- 40 ml

Filter before use

9. Destaining solution

Methanol	- 40 ml
Acetic acid , glacial	- 14 ml
Distilled water	- 146 ml

10. Gel composition

Solution	**Resolving gel**	**Stacking gel**
	(20 ml)	**(10 ml)**
	12%	**4%**
30% Acrylamide	6.6 ml	1.3 ml
Resolving gel buffer	5.0 ml	-
Stacking gel buffer	-	2.5 ml
10% SDS	0.2 ml	0.1 ml
Distilled water	8.1 ml	6.04 ml
10% APS	0.1 ml	0.05 ml
TEMED	20 µl	10 µl

II. Stock solutions for Electroblot immunoassay

1. Immunoblot transfer buffer (pH 8.3)

0.025 M Tris	- 9.085 g
0.192 M Glycine	- 43.2 g
20% Methanol	- 600.0 ml

Made upto 3000 ml with distilled water

2. **Tris-buffered saline (TBS)**

0.02 M Tris	- 4.84 g
0.5 M NaCl	- 58.48 g

pH adjusted to 7.5 with HCl and made upto 2 L.

3. **TBS - tween (TBS-T)**

TBS containing 0.05% Tween - 20

4. **Blocking solution**

TBS with 5% (w/v) spray dried milk (SDM, "everyday" by Nestle)

5. **Antibody buffer**

TBS-T containing 5% SDM (TBS-T SDM)

6. **Goat anti-rabbit IgG conjugated with HRP** (Genei, Bangalore)

1: 5000 dilution was used

7. **Substrate buffer**

Trisodium	- 7.35 g
dH_2O	- 400 ml

pH adjusted to 5.2 and the volume made upto 500 ml

8. **Substrate solution**

Substrate buffer	- 50.00 ml
Diamino benzidine	- 20 mg
H_2O_2	- 0.5 ml
Cobaltous chloride	- 20 mg

(TBS containing 20 mM Tris-HCl, 500 mM NaCl pH 7.5)

III. Stock solution for nucleic acid isolation

1. 10 per cent SDS

Sodium dodecyl sulphate - 1 g

Distilled water - 10 ml

2. 200 mM EDTA

Ethyldiamine tetra acetic acid, disodium salt (EDTA) - 3.72 g

Dissolved in 30 ml of sterile water and the pH adjusted to 8.0 with 3 N NaOH and made upto 50 ml with sterile water, autoclaved and stored at room temperature.

3. 2.5 mM Sodium acetate

Anhydrous sodium acetate - 12.3 g

Dissolved in 30 ml sterile water, pH adjusted to 5.2 with acetic acid and made upto 50 ml with sterile water. Autoclaved and stored at room temperature.

4. Phenol

Phenol was prepared as described by Sambrook *et al.* (1989).

8-Hydroxyquinoline was added to 0.1 per cent in liquid phenol. The phenol was extracted several times with equal volumes of 1M Tris-HCl buffer, pH 8.0, until the pH of the aqueous phase was 8.0. After the phenol was equilibrated, final aqueous phase was removed and 0.1ml volume of 0.1 M Tris-HCl, pH 8.0 containing 0.2 per cent 2-mercaptoethanol was added. The phenol solution was stored in this form in an amber colored bottle at 4°C.

5. *Chloroform - Isoamyl alcohol mixture*

Chloroform and isoamyl alcohol were mixed (24 : 1, v/v) and stored in a closed amber coloured bottle at room temperature.

6. Proteinase K

Proteinase K (Sigma - p - 3090) - 20 mg/ml

Dissolved in sterile distilled water and stored at -20°C.

7. Preparation of diphenylamine reagent

One gram of diphenylamine was dissolved in 100 ml glacial acetic acid and 2.75 ml conc. H_2SO_4 and mixed well. It was stored at 4°C and warmed to room temperature before use.

8. Gel loading buffer, 1 ml

Bromophenol blue (0.25 per cent)	- 2.5 mg
Xylene cyanol FF (0.25 per cent)	- 2.5 mg
Ficoll (Type 400 Pharmacia) (15 per cent)	- 150 mg

Dissolve the above chemicals in sufficient amount of sterile distilled water to 1 ml. Store at 4°C.

IV. MTT Assay

1. Preparation of MTT dye solution

MTT (Sigma Chemical Co., St. Louis, Missouri) was dissolved in Hank's balanced salt solution to a concentration of 5 mg/ml. It was filtered to remove any amount of insoluble residue and stored in dark at -20°C.

V. Buffer Solutions

1. Alsever's slution

Sodium chloride	- 4.20 g
Trisodium citrate	- 8.00 g
Glucose	- 20.50 g

Citric acid	- 0.55 g
Distilled water	- 1000 ml

2. **Phosphate buffer saline (PBS), pH 7.2**

Sodium chloride	- 8.0 g
Disodium hydrogen phosphate	- 1.44 g
Potassium dihydrogen phosphate	- 0.2 g
Potassium chloride	- 0.2 g
Distilled water	- 1000 ml

3. **Hanks Balanced Salt Solution (HBSS) (10 X)**

Solution A

Calcium Chloride	- 1.4 g
Double distilled water	- 200 ml

Solution B

Glucose	- 10 g
Sodium Chloride	- 80 g
Magnesium sulphate	- 2 g
Pottasium dihydrogen Orthophosphate	- 0.6 g
Disodium hydrogen orthophosphate	- 1.2 g
Phenol Red (0.2 %)	- 100 ml
Double distilled water	- 800 ml

The two solutions were autoclaved separately. After cooling solution A was added to solution B slowly thoroughly. The solution was dispensed in 10 ml screw

cap tubes and stored at 4°C. From this 10 ml was added to 90 ml of sterilized distilled water to make it 1 x.

4. T.E. Buffer pH 8.0

EDTA	- 185 mg
Tris	- 605 mg
Distilled water	- 500 ml

5. TAE Buffer

Tris	- 1.4 g
Acetic acid	- 300 µl
0.04 M EDTA	- 100 mg
Distilled water	- 300 ml

VI. Sucrose gradients

Twenty grams of sucrose was dissolved in 30 ml of PBS and then made up to 50 ml to prepare 40 per cent sucrose solution. With this 40 per cent solution, dilutions were made in PBS to prepare the 30,20 and 10 per cent which were layered in order (40,30,20 and 10) in centrifuge tubes and kept overnight at 4°C for formation of linear sucrose gradients.