CULTURAL, SEROLOGICAL AND MOLECULAR STUDIES ON FOWL ADENOVIRUS SEROTYPE - 4 CAUSING HYDROPERICARDIUM SYNDROME AMONG POULTRY IN HIMACHAL PRADESH

THESIS

BY

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Submitted to

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IN

Partial fulfillment of the requirements for the degree

OF

DOCTOR OF PHILOSOPHY IN VETERINARY SCIENCE
(VETERINARY MICROBIOLOGY AND IMMUNOLOGY)
(2009)

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CERTIFICATE-I

This is to certify that the thesis entitled "**Cultural, serological and molecular studies on fowl adenovirus serotype-4 causing hydropericardium syndrome among poultry in Himachal Pradesh**" submitted in partial fulfillment of the requirement for the award of the degree of **Doctor of Philosophy in Veterinary Science** in the subject of **Veterinary Microbiology and Immunology** of CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Prasenjit Dhar,** son of **Mr. P.K. Dhar** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

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

(Mandeep Sharma)
Chairman,
Advisory Committee

Place: Palampur
Dated: 12.07.2009

CERTIFICATE-II

This is to certify that the thesis entitled **"Cultural, serological and molecular studies on fowl adenovirus serotype-4 causing hydropericardium syndrome among poultry in Himachal Pradesh"**, submitted by **Prasenjit Dhar,** son of **Mr. P.K. Dhar** to the CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Veterinary Science** in the subject of **Veterinary Microbiology and Immunology**, has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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DEDICATED TO MY

LITTLE KIDS

(TANISHTHA & TIJIL)

AND

THE NUMEROUS INNOCENT BIRDS THAT DIED DURING MY EXPERIMENT

Acknowledgement

At the beginning, I would like to thank "***The Almighty***" for the benevolence and wisdom bestowed upon me during this research work and all through my life. Thank you God for each and everything.

The first person, I would like to thank is Dr. Mandeep Sharma, Head and Major Advisor, Department of Veterinary Microbiology, DGCN CSKHPKV, Palampur. His keenness, august, guidance, support and integral view on research has helped me shape this thesis. His eager interest and ever available help throughout the tenure of this investigation will stay with me for ever.

Dr. R.C. Katoch, Former Dean, DGCN COVAS and my former guide deserves my gratitude for helping me through out my post graduate academic period. His ardor, wealth of knowledge and simple persona has made a deep lasting mark in my heart.

Dr. Rajesh Chahota, Assistant Professor, Department of Veterinary Microbiology deserves my unqualified gratitude for the interest he took in my research programme. He is a erudite man with an eye on minute research matters. I would always remember his contribution to my investigation with his creativity. He deserves my unqualified thanks for the help he extended all through my research period

I would also like to express thanks to the other members of my advisory committee Dr. V.K. Gupta, Head, Department of Veterinary Pathology, and Dr. Dr.Anjan Kalia, Professor and Head, Department of Agricultural Engineering and R.K. Agnihotri, Head, Department of Veterinary Parasitology for taking keen interest in my research and helping me out in the midst of their busy schedule. I am also highly thankful to Dr. S. Mitra who monitored my work, gave moral support and took personal interest in my research work. I am thankful to all of them.

My special thanks is set aside for Dr. Vipin Katoch, Assistant Professor, Department of Veterinary Microbiology, for his direction, ideas, and guidance from the very early phase of this research as well as giving me amazing experiences all the way through out my research work. His prescience, resourcefulness, imagination and helping hand would be cherished by me for all times to come. He was always available whenever I needed him and for that I must say a very big "Thank You" to him from the core of my heart.

I am deeply indebted to Dr. R.K. Asrani, Department of Veterinary Pathology, who gave me the idea to start on a new and intriguing journey during my doctoral study. I am grateful to him for his help in the form of both information and materials to start this research programme.

Many thanks go to Dr. Subhash Verma, Assistant Professor, Department of Veterinary Microbiology for his help and brotherly advice during the making of this thesis.

It is my proud privilege to articulate my heart felt gratitude and thanks to Dr. A.A. Zaidi, a virologist of repute. My research work would have been incomplete without his help and guidance. That a man of his stature can be so modest is a thing worth emulating right through my life.

I would really like to treasure my association with Dr. Vipin Hallan, a man of knowledge and professional competence who guided me into new areas of molecular biology with his ingenious thinking and vast knowledge of the subject. It was really a delight to be associated with a man of his standing.

Words would not be sufficient to express the contribution of Dr. Raja Ram Verma, whose precious suggestions, ever ready helping outlook and support during my research work helped me in the execution of this daunting task. I acknowledge his help and contribution with sincere gratitude.

Words are not enough to acknowledge the help of my friends at IHBT campus. I would like to say a very very special Thank you to Amit Singh, Birender, Kaushik, Anuradha, Tanuja, Vanita, Yogesh, Salik, Renu, Yashika, , Prachi, Puja, Sunny, Rahul, Arpana, Nagar and Tombisana who not only helped me in each and every activity in the department but also gladly and smilingly acceded to my numerous requests of helping me out even during odd hours. I am particularly amazed at the zeal and enthusiasm shown by them for completing their research work and heartily recognize their contribution in the successful achievement of my research work. I would also like to thank the ministerial and technical staff particularly Ranjit Bhaiya and Jagdeep for helping me out in the Department. Special thanks are also due to Vivek, Dr. Ramesh Kasana and Dr. Srinivasulu.

Pabitra, Rimali and Sanjay Chanda deserve my warm and sincere gratitude for helping me whenever I needed them. Their company provided me with the much needed succor in terms of professional distress.

Special thanks are also due to the Director, IHBT for allowing me to do research in the institution.

I would be failing in my duty if I do not record the help rendered by Dr. O.P. Sharma, Dr. N.P Kurade, Dr. J. Bhat and Dr. Jyoti Babu of IVRI Regional station, Palampur.

I gratefully acknowledge the help of Dr. T. G. Prabhakar, who provided me with standard isolates to help me in my research. I am also indebted to Dr. Ramneek Verma, who not only provided me isolates for my research work but also helped me each time I needed his help. My thanks are also due to Dr. Dwivedi, Dr. Narender, Dr. Arora, Dr.T.S.Rai, Dr.

Mudit Chandra, Dr. Gurpreet Kaur, Dr. Paviter Kaur and Dr. Harimohan Saxena who made my stay in Ludhiana comfortable with their friendly behavior.

A very special thanks are due to Deepti didi for helping me all through my research. I did not have to think twice in seeking her help and that made my journey a lot easier. I gratefully acknowledge the help rendered by Dr. Kuldeep Gupta, Dr. Patil, Dr. Arthnarieshwaran, Dr. Kulkarni, Siddhartha, Kiran, Prabhdeep, Rajneesh, Hitesh, Ripil and Ashwani.

I acknowledge with thanks the suggestions rendered to me by Dr. Alok, Dr. Deshraj and Dr. K.B. Nagal.

I am enormously grateful for the help extended by Department of Surgery and Radiology. I owe my genuine thanks to Dr. Adarsh for his advices, moral support, counsel and brotherly love extended right through my tenure. Dr. S.P. Tyagi deserves my sincere and unqualified thanks for the help, motivation and moral support extended to me during my research work. His help with the camera and timely advice during my research programme will forever make me ever grateful to him. Dr. Arvind Sharma deserves a big thank you for the advice and moral support extended to me.

Dr.Pankaj Sood, Dr. Anupam Dr. Rajput, Dr. Ravi Thakur, Dr. Nanda, Dr.Satish Kapoor and Dr. Shivani deserve a big thank you for all the help, motivation and advice rendered to me.

I acknowledge with thanks the help, advice and moral support extended by my dear friends Drs. Arun Sharma, Virender Pathak, Siddtharth Dev Thakur, Devina and Bandu Mane.

My special thanks is set aside for my dear friends Drs. Surinder Chauhan and Dorin, who were always available throughout my good and bad times. The contribution of both of them can be summarized in few words. I would just like to repeat the age old saying: "A friend in need is a friend indeed".

Dr. Madan, Dr. Sarvesh, and Dr. R.D. Patil, deserve genuine thanks from my side for helping me out with my research and thesis writing. Their help, encouragement and company helped me to tide over this academic ocean with ease.

I wish to thank and gratefully acknowledge the help rendered to me by Dr. Pooja Kanwar , Dr. Durairajan and Dr. Sandeepa Bhan during my research work.

Thanks are also due to Drs. K.K.Sharma, Prabhat, Vikas, Vinay Kant, Gaurav, Kapil, Amit, Deepti, Ankita, Lovit, Harshit, Kailash, Deepa, Neeraj, Rajesh Kumar and Vishal,

I would also acknowledge Abhishek, Aneesh, Durairajan, Vikas, Gaurav, Umeshwari, Nistara, Ramswaroop, Prabhat, Rita, Rajneesh, Shivali, Manish, Pankaj, Prabhdeep, Amit, Kailash, Kapil, Krishnender Dinesh, Rajesh, Isha, Shashwati, Priyanka, Madhu, Sonia, Shalmali, and Manisha for their help and company which were much needed during my research.

Special thanks are also due to my classmates: Hamender, Karkra, Hakikat, Mehta, Rajiv, Manchali, Monika, Harsha, Sahu, Vikas, Joginder, Sandeep, Negi, Bhatti and Amitabh Thakur who gave me moral support during this period.

Special thanks are also due to Pratapji, Rameshji, Kamaljeet, Shruti, Vinod and. Rameshji , Rangiluji ,Bihariji and Ramesh for their help from time to time.

Rama, Anilji, Dograji, Jamwalji, S.R.Thakurji, R.N.Sharmaji, Chaudharyji and Rajinder deserve my unreserved thanks for helping me out with my official responsibilities

I also gratefully acknowledge the help rendered by Pantji, Sandeep Khimta, Pawan, and Karam Chand Negi of IVRI, Izatnagar.

Esteemed thanks are also extended to Dr. Pradeep K. Sharma, Dean, PGS, for providing necessary facilities to carry out this work.

I am also highly thankful to Dr. Chakraborty, Didi and Rohan, Dr.Mukherjee, Mukherjee Baudi, Mitra Baudi, David Aunty, Neerajji, Vijay, Anshu, Mitra da, Buna Baudi, Chachra Uncle, Aunty, Bhowmick da and Baudi, Hajra da and Baudi, Goswami da and Top for giving me good company, advice and help and keeping up with my non availability during many social functions.

I also like to remember Ravikantji, Chauhan, Sheetalji, Bhabiji, Auntyji, Dr. and Madam Chadha, Dr. Sukhdev Paliyal and family, Dr. Manoj Gupta and Dr. Manmahon Singh in this occasion.

I acknowledge the help rendered to me by Research Aid, Palampur, Deep Distributors, Maranda and Bioserve, Genome valley for the help rendered to me.

I also thank all my teachers and present senior and junior faculty members who helped me directly or indirectly and gave me company during this time. I also thank all my undergraduate and post graduate students without whose help and wishes, this would have just remained a dream.

In this hour of academic achievement my deepest gratitude goes to my parents and my parents in law for their sacrifices and prayers. This achievement would not have been possible without their blessings. Special thanks are also due to my brother Bishnu, his wife

Kajari and my nephew, Vinayak. I also gratefully acknowledge the moral support extended by my brother in law Dipanjan, his wife Pampa and love by my niece, Tanussha.

My heart weeps on this day when I remember my dear Kaku. His absence today, takes a lot of sheen out of this professional achievement. Kaku, I miss you.

I would like to thank Dipali for her help during my writing and also remember fondly the numerous cups of tea made by her.

My deepest gratitude goes to my family for their indefatigable love and support throughout my life. This research was simply impossible without them. I would like to wholeheartedly thank my wife Chandrani, my kids Tanishtha and Tijil for their unconditional support, love and sacrifices throughout my academic pursuit. In particular, the patience and understanding shown by my wife during the research time is greatly appreciated.

All may not have been mentioned but none is forgotten.

Place: Palampur

Prasenjit Dhar

Date: 13/7/2009

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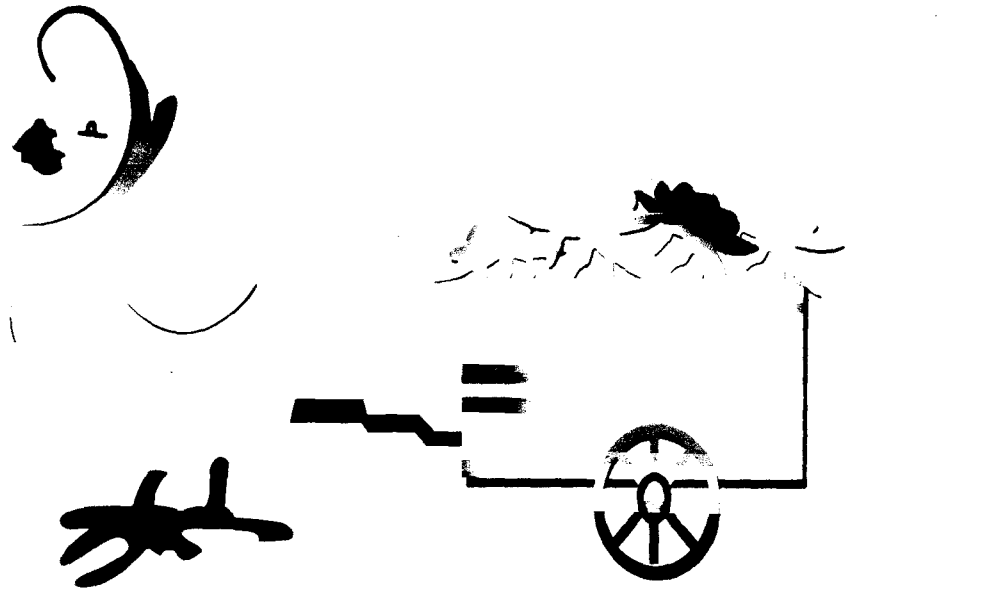
ABBREVIATIONS

Abbreviations / Symbols	Meaning
<	Less than
>	More than
□	Less than equal to
□	More than equal to
AAV	Avian adenovirus
AGPT	Agar Gel precipitation test
AGID	Agar Gel immunodiffusion
bp	Base pair
°C	Degree Celsius
CEF	Chicken embryo fibroblast
CEFC	Chicken embryo fibroblast cells
CEL	Chicken embryo liver
CELC	Chicken embryo liver cells
CEK	Chicken embryo kidney
CEK	Chicken embryo kidney cells
CIE	Counter immunoelectrophoresis
CKC	Chicken kidney cells
CPE	Cytopathic effects
Da	Dalton
DAB	Diaminobenzidine
DEPC	Diethly Pyrocarbonate
DIA	Dot immuno assay
DNA	Deoxyribonucleic acid
DPI	Days post infection
EDTA	Ethylene Diamine Tetra acetic acid
EIA	Enzyme Immuno assay
ELISA	Enzyme linked Immuno sorbent assay
e.g.	example gratia (for example
et al.	et alii (and others)
FAT	Flourescent antibody technique

FAV	Fowl adenovirus
Fig.	Figure
FITC	Flourescent isothiocyanate
g	RCF (relative centrifugal force)
gm	Gram
H &E	Hematoxylin and Eosin
hrs.	Hours (s)
HRPO	Horse radish Peroxidase
i .e.	idest (that is)
IU	International Unit
kDa	Kilodalton
kg	kilogram
L	Litre
µg	microgram
M	Molar
MDCC	Madin Derby Cell culture
mA	milli ampere
mg	milligram
min.	minute (s)
ml	milliliter
MW	Molecular weight
NCM	Nitrocellulose membrane
ng	Nanogram
No.	Number
OPD	Ortho phenylene diamine
p.	page
p.a.	per annum
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymérase chain reaction
pg	Picogram
pp.	pages

ppb	Parts per billion
ppm	Parts per million
psi	Pounds per square inch
RNA	Ribo Nucleic acid
rpm	revolutions per minute
S	Svedberg unit
secs.	Second(s)
SDS	Sodium dodecyl sulphate
SPF	Specific embryonated eggs
Sr. No.	Serial Number
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
Taq	*Thermus aquaticus*
viz.	Videlicet (namely)
UV	Ultraviolet
V	Volts
wt.	Weight
µ	micro
µl	Microlitre
Yr.	year

Introduction



Chapter I

INTRODUCTION

In India, about 65 to 70 per cent of the human population is dependent on agriculture for their livelihood while agriculture and allied sectors contribute nearly 22 per cent of Gross Domestic Product (GDP) of India (GOI, 2009).

Poultry rearing has always been an integral component of livestock production system in India. The concept of composite farming production system with crop, livestock, fish and poultry production has been practiced for centuries in India. However, poultry production in India has taken a quantum leap during the last four decades, emerging from an entirely unorganized and unscientific farming practice to commercial production system with state-of-the-art technological interventions. India, with poultry population of 489 million and estimated more than 47 billion eggs production per year, ranks among the top three countries in egg production in the world. The broiler production is growing at the rate of nearly 8 to 10 per cent every year and the country produces about 2.0 million metric tonnes of chicken meat annually, at present. The annual per capita availability of eggs and chicken meat has also increased from a mere 10 eggs and 146 grams in 1970's to more than 41 eggs and 1.6 Kgs respectively presently. India's share of the world trade in poultry and poultry products is very small. However, the nation has come a long way during the last decade increasing its value of exports from nearly Rs. 11.00 crores in 1993-94 to around Rs. 316 crores during 2005-06 (GOI, 2009).

Poultry sector, besides providing direct or indirect employment to nearly 3 million people is a potent tool for subsidiary income generation for many landless and marginal farmers. The sector also provides nutritional security especially to the rural poor. Further,

landless labourers derive more than 50 per cent of their income from livestock especially from poultry (GOI, 2009).

Backyard poultry undoubtedly has become the source of livelihood and improved nutrition for these farmers. Poultry rearing is also a viable commercial activity. The country is positioned 17th in world poultry production in the overall market for poultry products. The Indian Poultry industry is expected to record an annual growth rate of 2.7 per cent per annum with an estimated total output of 3.24 million metric tonnes (Poultry International, 2009). During the past three decades, poultry keeping, particularly broiler rearing has become a profit making venture especially considering the small time of turnover and relatively low input costs by the farmers with respect to infrastructure, feed or stock. However, the short turnover time and the economic viability require the raising of a large number of birds in close proximity under an intensive system of husbandry. Undoubtedly, intensive rearing of birds results in increased disease risks resulting in decline in the output of the finished product since poultry farms are frequently attacked by infectious and non infectious diseases and other frequent managemental problems.

In Himachal Pradesh, where land holdings are small and unemployment is on the rise, the prospects of earning one's livelihood through poultry farming is undoubtedly gaining ground these days.

Periodic outbreaks of potentially catastrophic diseases continue to impede the development of poultry. They can be ascribed to poor quality input such as genetically poor stock, feed, drugs and vaccines. On occasions, managemental lapses especially high stocking density, poor in house hygiene and continuous medication in sub lethal dosages lead to the breakdown of the bird's immunity resulting in immediate morbidity and

mortality in flocks. Not commonly realized is the fact that disease that kills even 10 per cent of birds cuts off all profits (Mouahid *et al.*, 1989).

Adenoviruses are common infectious agents in poultry. Most of the viruses replicate in healthy birds with little or no apparent signs of infection, although they can quickly take on the role of opportunistic pathogens when additional adverse factors, particularly other concurrent microbial infections, affect the health of the avian host. The subgroup I avian adenoviruses comprise the Genus *Aviadenovirus* within the *Adenovirus* family.

Hydropericardium syndrome (HPS) is a recent and mysterious disease affecting chickens. It mainly affects commercial broiler flocks aged 3 to 8 weeks and is characterized by a swollen pericardial sac filled with straw colored clear fluid. It is an emerging disease occurring in many areas of world where broilers are reared under intensive condition (Asrani *et al.*, 1997).

The disease was first observed in Angara Goth, Karachi, Pakistan (Khawaja *et al.*, 1988a; Cheema *et al.*, 1989) and gradually made its appearance in areas of Karachi, hence, also called "Angara disease". Thereafter, the disease traveled to India and other countries like Mexico, Ecuador, Peru, Chile (Cowen *et al.*, 1996), South and Central America (Shane, 1996), Russia (Borisov *et al.*, 1997) and Japan (Abe *et al.*, 1998). More recently, outbreaks of HPS have been reported in Korea that caused severe economic losses due to mortality and weight loss (Kim, 2008).

In India, the disease was first noticed in the poultry belt of Jammu in 1994. Later in the same year albeit at different time intervals the disease was recorded in some poultry farms in Gurdaspur, Amritsar and Batala districts of Punjab. The disease later on emerged

in to Haryana, Delhi and then to Uttar Pradesh where it was recorded for the first time in Haldwani region of Nainital district in November, 1994 (Gowda and Satyanaryana, 1994). This disease was also reported from Himachal Pradesh by Asrani *et al.* (1997), which inflicted huge mortality in organized poultry farms.

The disease is also commonly named as "Litchi disease" since the heart of the diseased birds resembles the shelled out litchi fruit. In birds, HPS is generally characterized by sudden occurrence with high morbidity and high mortality of up to 80 per cent in 3 to 7 weeks old broilers with low mortality *i.e.* below 10 per cent in layers (Anjum *et al.*, 1989; Shane, 1996). All broiler strains irrespective of breed and sex are equally susceptible under field conditions.

The disease is caused by a Fowl adenovirus (FAV serotype-4), a non enveloped, and icosahedral virus with double stranded DNA belonging to Adenovirus genus of *Adenoviridae* family. The disease is characterized by hydropericardium and hepatitis with characteristic intranuclear inclusion bodies in hepatocytes (Ahmad *et al.*, 1989; Anjum *et al.*, 1989). Histopathologically, basophilic intranuclear inclusion bodies are invariably seen in the hepatocytes of liver (Abdul-Aziz and Hasan, 1995). The disease is contagious by nature, hence, transmission from one flock to another occurs horizontally, either by contact or mechanically by vaccinators as well as other contaminated materials. The disease could be induced by the inoculation of bacteria free liver homogenate from diseased bird (Khawaja *et al.*, 1988a; Chishti *et al.*, 1989). The syndrome is manifested by the accumulation of a clear, straw colored fluid in the pericardium, a swollen discoloured and friable liver and pale enlarged kidneys with distended renal tubules (Anjum *et al.*, 1989).

Chand (2006) studied the pathology of hydropericardium syndrome in the state and could reproduce the disease in caged broilers, japanese quails and pigeons. Similarly, Kanwar (2008) adapted the virus in cell cultures and also characterized the virus at various ranges of physiochemical parameters.

The diagnosis of HPS still poses a considerable challenge since the infected birds die before they exhibit typical clinical signs. Current procedures for identification involve clinical signs, post mortem alterations, typical histopathological observations and electron microscopy. Other methods of diagnosis involve isolation of the virus in cell cultures and performing a battery of serological tests.

The conventional methods of virus isolation in cell cultures and routine serological tests again have their own merits and demerits. Many times these methods are labour intensive and time consuming, in addition, some of them have low specificity and/or sensitivity. The main problem in many cases of serological tests for adenoviruses are the interpretation of the serological results because birds may be frequently infected with a number of serotypes. Rapid and quick diagnosis of the disease is based on isolation of the agent in morbid chicken embryo liver and chicken embryo kidneys though with varying reports about the rapidity of the effect. Little is known, however, about the suitability of various other conventionally used cell cultures or commercially available cell lines that can be potentially used for the rapid isolation of this pathogen apart from the conventional ones.

Polymerase chain reaction (PCR) for in vitro amplification of target gene sequences has been applied as a rapid diagnostic tool for the detection of the fowl adenovirus in recent years (Raue and Hess, 1998; Ganesh *et al.*, 2002). This method is not only rapid but

also more sensitive and specific than the other diagnostic procedures. Although some work was initiated with regard to the isolation of adenovirus agent from outbreaks in Himachal Pradesh by Asrani *et al.* (1997), Chand (2006) and Kanwar (2008), yet many questions still remained unanswered.

Keeping in view, the implications of this problem among poultry enterprise in the state of Himachal Pradesh and to better understand the various characters of this virus for future diagnosis and control of this pathogen, this investigation envisaged to find out the suitability of different standard animal cell cultures for isolation of the HPS causing agent in vitro. This investigation also planned to assess the utility of few routine serological test(s) frequently used in the laboratory for rapid identification of the agent and also to adjudge the best serological tool applicable for diagnosing HPS. This study also planned to characterize the locally involved HPS isolates at the molecular level to see the likely source of origin of this virus so that better surveillance measures could be initiated in future. Thus, the study was undertaken with the following objectives:

1. To observe the suitability of different animal cell cultures for the propagation of the virus.
2. To compare the efficacy of routine serological test(s) used to detect the virus.
3. To characterize the virus at molecular level.

Review of Literature

Chapter II

REVIEW OF LITERATURE

2.1 Adenovirus

According to Madigan *et al.* (2000), Adenoviruses were first isolated from the tonsils and adenoid glands of humans, thus the term "adeno" derived from a Latin word meaning "gland".

So far, four genera of the *Adenoviridae* family have been accepted by the International Committee on Taxonomy of Viruses (ICTV) and these are the *Mastadenovirus* (infecting mammals), the *Aviadenovirus* (infecting birds), *Atadenovirus*, formerly designated group III *Aviadenovirus* which came about when some bias of the genomes towards high A+T contents was observed and *Siadenovirus*, formerly designated group II (infecting a variety of species). There is also a fifth genus that has been proposed to the already existing *Adenovirus* genera. This fifth genus has been an implication due to a partial genome sequence of the only confirmed fish adenovirus (Davison *et al.*, 2003).

Adenoviruses are common infectious agents in poultry. Most of the viruses replicate in healthy birds with little or no apparent appearance of symptoms, although they can quickly act as opportunistic pathogens as and when additional factors, particularly concurrent infections, adversely alter the health of the avian host.

The subgroup I avian adenoviruses comprise the Genus *Aviadenovirus* within the family *Adenoviridae*. In contrast with the clear association of subgroup II and subgroup III adenoviruses with affliction, the role of most subgroup I avian adenoviruses as pathogens is not well defined. (Hess,2000). Notable exceptions include the FAV-1 strains that causes "Quail Bronchitis" and also the FAV-4 strains, which play a major role in the etiology of hydropericardium syndrome. Fowl adenoviruses belong to genus *Aviadenovirus* that

includes conventional avian adenoviruses. They share a common group specific antigen (Kawamura *et al.*1964). There are at least 12 fowl adenoviruses (FAV 1 to 12) those are grouped on the basis of the neutralization test (Cowen and Naqi, 1982).

The International Committee on Taxonomy of Viruses (ICTV) has grouped the 12 FAV serotypes in five serotypes (FAV-A to FAV-E) on the basis of restriction fragment length polymorphism (RFLP) profiles and the sequencing data. (Table 2.1). This classification brings the *Aviadenovirus*es in line with the mammalian adenoviruses, which are subdivided into species using similar criteria. Viruses within each species are further subdivided into serotypes based mainly on the results of cross neutralization tests (Kawamura *et al.*, 1964; Khanna, 1964; Cox, 1966; Mcferran and Connor, 1977; Mcferran *et al.*, 1971; Calnek and Cowen, 1975; Cowen *et al.*, 1977; Grimes *et al.*, 1977 and Kefford *et al.*, 1980).

Table 2.1. ICTV classification of Group I avian adenoviruses

Genus *Aviadenovirus* (Group I avian adenoviruses)

Groups:	Serotypes:	Type strains
Fowl adenovirus A	Fowl adenovirus 1 (FAV-1)	CELO, 112, QBV, Ote, H1
Fowl adenovirus B	Fowl adenovirus 5 (FAV-5)	Tipton, M2, 340, TR-22
Fowl adenovirus C	Fowl adenovirus 4 (FAV-4)	KR5, J2, H2, 506, K31
	Fowl adenovirus 10 (FAV-10)	C-2B, M11, SA2, CFA20
Fowl adenovirus D	Fowl adenovirus 2 (FAV-2)	GAL-1, 685, SR-48, H3,P7
	Fowl adenovirus 3 (FAV-3)	SR-49,75, H5
	Fowl adenovirus 9 (FAV-9)	90, A2, CFA19
	Fowl adenovirus 11(FAV-11)	UF71, 380
Fowl adenovirus E	Fowl adenovirus 6 (FAV-6)	CR119, 168
	Fowl adenovirus 7 (FAV-7)	YR36, X-11, 122
	Fowl adenovirus 8a (FAV-8a)	58, TR-59, T-8, CFA40
	Fowl adenovirus 8b (FAV-8b)	764, B3, VRI-33

* Based on Benko *et al.* (2000).

2.1.1 Morphology

2.1.1.1 Adenovirus structure and symmetry

Adenoviruses are non enveloped, double stranded DNA virus with icosahedral structure and measure 70 to 90 nm in diameter (Mcferran, 1981). Densities between 1.32 and 1.37 g/ml in cesium chloride (CsCl) have been estimated for *Aviadenovirus*es. The virion is composed of 252 capsomeres, surrounding a core of 60 to 65 nm in diameter. Capsomeres are arranged in triangular faces with six capsomeres along each edge. There are 240 non vertex capsomeres (hexons) of 8 to 9.5 nm diameter and 12 vertex capsomeres (penton bases). Vertex capsomeres carry projections known as fibers (Russel, 2000). Mammalian adenoviruses have one fiber on each penton base, and the *Aviadenovirus*es appear to have 2 (Gelderblom and Maichle- Laupper, 1982; Chiocca *et al.*, 1996). In most cases, both fibers are of similar length and antigenic properties, because serotypes which are related in cross neutralization tests have fibers of similar length except FAV-1 which has two fibers of different length (Gelderblom and Maichle- Laupper, 1982). The fiber is the structural protein that plays a pertinent role in the adenovirus infection by attaching the virus to the host cells by recognizing primary cellular receptors.

2.1.1.2 Strain classification

The hexons comprise and carry the group specific antigens which was designated as alpha (Allison *et al.* 1960., Pereira, 1960). Similarly antigen present on penton was designated as beta and that on fiber gamma. The fiber antigen present on the head is type specific (Norrby, 1969) responsible for hemagglutinating activities. Fiber antigen has been reported to induce type

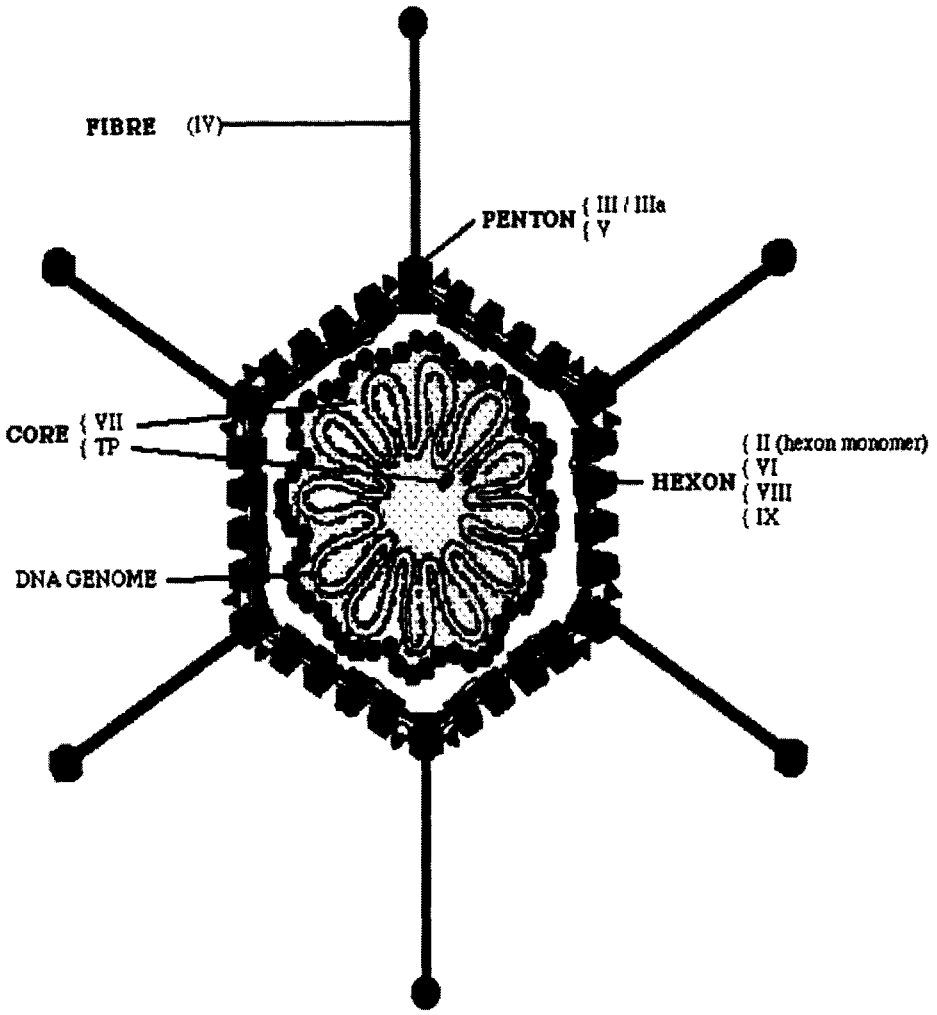


Fig 2.1. Structure of an adenovirus (Courtesy of C. Büchen-Osmond)

specific neutralizing antibodies. Hexon antigen induces a group and type specific neutralizing antisera (Kjellen, 1968).The adenovirus hexon is the major capsid protein and contains type, group and subgroup specific antigenic determinants (Mcferran and Adair, 1977; Mcferran,1981) and comprising about 60 per cent of the mass of the virion (Monreal, 1992). Birds infected with *Aviadenovirus*es, therefore, produce type-specific, group-specific, and subgroup-specific antibodies. The group specific determinants are shared by all subgroup I viruses, but are not present in subgroup II or subgroup III

adenoviruses. Neutralizing epitopes on adenovirus hexon has been partially mapped to the loop 1 (L1) of the native protein (Toogood *et al.,* 1992).

2.1.1.3 Genomic structure of fowl adenoviruses

They are linear, non-segmented, d/s DNA, 30 to 38 kbp (size varies from group to group) that has the theoretical capacity to encode 30 to 40 genes. Genome structure (cross-hybridization, restriction map) is one of the characters used to assign viruses to groups (70 to 95 per cent homology within groups, 5 to 20 per cent homology between groups).

The restriction pattern of Egg drop syndrome (EDS) and Chicken embryo lethal orphan (CELO) virus was determined by Zsak and Kisary (1981) by employing seven different restriction endonucleases. No similarity could be detected in the restriction patterns observed between the two. The calculated molecular weight of CELO virus was 43.7 kbp while that for EDS virus was about 34.2 kbp. Complete sequencing of the gene of major capsid proteins (hexon) for FAV-10 was done by Sheppard *et al.* (1995). The hexon gene of FAV-10 was found to have an ORF of 2808 bp, coding for 936 amino acids with a molecular mass of 105.5 kDa. Chiocca *et al.* (1996) sequenced CELO genome and found it to be 43,804 bp in length with G+C content of 54.3 per cent. The length was approximately 8 kb longer than in those of human subgenus C of adenoviruses. The two fiber proteins and core proteins are important in condensing the longer CELO viral genome. The structural proteins of the virus and virus encoded enzymatic function of CELO virus were homologous with human adenovirus at genomic level, whereas genes encoding proteins that interact with host cells such as E2, E3 and E4 were not conserved. The average genome size of a FAV-4 isolate was found to be 45.99 kbp by Barua (2002) who sequenced a full length hexon (2916 bp) sequence of an Indian isolate of FAV-4.

2.1.1.4 Viral proteins

The major structural proteins are the hexon and the fiber non covalently linked to a structure named as penton (Valentine and Pereira, 1965).

2.1.1.4.1 The Hexon protein

The hexon protein is a complex protein of greater than 900 residues and each hexon capsomere of the protein is a homotrimer (Crawford-Miksza and Schnurr, 1996). This complex protein has a molecular mass of 109,077 Da which includes its acetylated N terminus.

According to Rux and Burnett (2004) the hexon protein was the first animal virus protein to be crystallized and the first adenovirus protein to have its X-ray crystal structure determined. The outer surface of the virion is formed by three loops, L1, L2 and L4 and the tower region projects away from this surface, while the L3 region combines conserved pedestral (P) regions P1 and P2 forming the inner surface . The tower regions of the other two copies of the protein in the trimer interact with the L2 and L4 loops on either side by coiling of the loops. The longest and most complex loop is the L1 loop as it folds back on itself several times and thus projects the furthest into the solvent, providing maximal interaction with the environment (Miksza and Schnurr, 1996). This loop has a length of more than 130 amino acids (a.a) and shows a 42.5 per cent homology between FAV-1 and FAV-10 (Raue *et al.*, 2005).

The extreme structural stability of the hexon trimer thus comes from the adjacent pedestral interactions combined with the intertwining of the loops. In fact, disentangling one of the subunits could result in the disruption of both the tertiary and quaternary molecular structure of the hexon trimer. Due to the unusual configuration of the hexon trimer, it is said to be highly resistant to proteolysis and stable enough to retain its physical

and immunological characteristics even after exposure to 8 M urea (Rux and Burnett, 2004).

2.1.1.4.2 The Penton base

This protein together with the fiber forms the penton complex which seals the capsid at each of the 12 virion vertices. The penton base is responsible for the internalization of the virus to the cell. This protein interacts with the host cell surface alpha intergrin molecules to trigger membrane permeabilization and virus internalization during entry. The penton base also undergoes structural rearrangement on fiber binding (Rux and Burnett, 2004).

Rux and Burnett (2004) suggested that the penton base causes the intergrins to aggregate upon interaction with the cell surface. They hypothesized that this aggregation signals to activate the intergrin-mediated signaling pathway that induces virus endocytosis. This hypothesis is supported by the fact that the penton base activates a 72 kDa tyrosine kinase and promotes B-lymphoblastoid cell adhesion, whereas conserved Arg-Gly-Asp (RGD) peptides derived from the penton base sequence have no effect.

2.1.1.4.3 The Fibre

The fibre protein is responsible for virus attachment on to the host cell. The structure of the protein has 3 domains: a N-terminal tail that attaches to the penton base, a central shaft with repeating motifs of ~ 15 residues and a C-terminal globular "knob" domain that functions as the cellular attachment site. This protein became the second adenovirus structural protein to be crystallized (Rux and Burnett, 2004), but could not allow for structure determination due to insufficient crystal ordering.

Li *et al.* (1984b) reported that CELO (FAV-1) contains at least 14 structural proteins with polypeptide molecular weights ranging from 6 kDa to about 100 kDa. Peptide mapping suggested that the long fiber and short fiber were not related in their

primary sequences and are therefore, probably encoded by separate genes. The time course of synthesis of the CELO virion polypeptides indicated that they are synthesized after viral DNA replication. The hexons comprises of a trimer of polypeptides II with a central core, VI, VIII and IX are minor polypeptides also associated with the hexon and thought to be involved in stabilization and/or assembly of the particle. The pentons are more complex, the base consists of a pentamer of peptide III, besides 5 molecules of IIIa are also associated with the penton base. The pentons have a toxin like activity. Purified pentons cause cytopathic effects (CPE) in the absence of any other virus components. A trimeric fiber protein extends from each of the 12 vertices and is responsible for recognition and binding of cellular receptors. The hexon and penton fibers are responsible for type specific neutralization (Norrby 1971). Ruigrok *et al.* (1990) examined isolated fiber of adenovirus 2 by electron microscopy and observed that N- terminal structure of fiber causes heterogenecity in the length.

2.2 Hydropericardium syndrome

2.2.1 Work done Abroad

Avian adenoviruses are a very diverse group of pathogens causing a variety of problems for poultry production. Among the various emerging diseases in general, and avian adenoviruses in particular, they have been incriminated as the etiological agents for various clinical conditions in poultry (Fadly and Winterfield, 1973; Rosenberger *et al.*, 1974). Even though various serotypes of fowl adenovirus (FAV) produce inclusion body hepatitis (IBH) in broiler chicks, IBH along with the hydropericardium syndrome (IBH-HPS), popularly called "Litchi heart disease", has recently been reported to be particularly important in some countries of Asia and America (Jaffery, 1988; Shane, 1996 and Abe *et al.*, 1998). HPS, a disease primarily of broiler birds, emerged as a severe hazard to poultry

producers, particularly in India and Pakistan, and has led to closure of many farms. It causes high morbidity and variable mortality (Shane, 1996).This disease has caused huge economic losses to the poultry enterprise in Pakistan since 1987, when it was first reported at Angara Goth, an extensive broiler producing area near Karachi, hence it was given the name Angara disease (Jaffery, 1988; Akhtar, 1994). It is caused by FAV-4, a non enveloped, icosahedral virus belonging to the *Adenovirus* C species of the *Adenovirus* genus of the *Adenoviridae* family, and is characterized by hydropericardium and hepatitis, with intra nuclear inclusion bodies (INIBs) in hepatocytes (Ahmad *et al.*, 1989., Anjum *et al.*, 1989).

2.2.2 Work done in India

HPS, a disease primarily of broilers, has emerged as a severe economic hazard to poultry producers particularly, in the broiler industry in India and Pakistan (Gowda and Satyanarayana, 1994). The disease caused heavy mortality of 20 to 75 per cent in Pakistan and 30 to 80 per cent in India, with no apparent prior signs of disease (Kumar *et al.*, 1997). The disease is also called "leechy disease" or "litchi disease" in India after the peculiar appearance of the heart floating in pericardial fluid, which appears similar to that of a deshelled leechy (lichee) fruit (Gowda and Satyanarayana, 1994; Gowda,1994) or inclusion body hepatitis hydropericardium syndrome (IBH-HPS) (Jadhao *et al.*, 1997; Balamurugan *et al.*, 2001, 2002) and hydropericardium hepatopathy syndrome (Asrani *et al.*, 1997) or the hydropericardium hepatitis syndrome (HHS) (Ganesh *et al.*, 2001a).

Gowda (1994) reported HPS in 3 to 5 weeks old broiler chicks in northern parts of India. The mortality in affected flock was as high as 80 per cent. The pathological changes included hepatitis with large basophilic intranuclear inclusions and hyper chromatic hepatocytes, atrophied bursa, degenerative changes in kidneys and alterations in blood

vessels of lung. Oberoi *et al. (*1996) isolated the avian adenovirus of group I from liver of IBH-HPS affected birds. Shukla *et al.* (1997a) reported the association of IBD with hydropericardium syndrome in layer flock.

Asrani *et al. (*1997) reported the disease in caged broiler birds of an organized poultry farm in the state of Himachal Pradesh

2.3. Etiology

2.3.1. Work done Abroad

Initially, while conducting studies on the etiology of HPS, it was hypothesized that this disease was caused by toxicity or a nutritional deficiency. The possible causative factors investigated were mycotoxins, toxic fat agents, sodium chloride, polychlorinated biphenyls, chlordane and phytotoxins. Rancid fat, fish meal or vitamin and mineral imbalances in the feed were thought to be causative factors. All of these factors were associated with hydropericardium (Jaffery, 1988; Qureshi, 1988, 1989). However, all attempts failed to reproduce the disease experimentally with feed samples from farms having natural outbreaks of HPS or feed containing 100 ppm mycotoxins or both (Anjum 1988, 1990). The search of an infectious agent was initiated by several diagnostic laboratories seeking to isolate a possible causative pathogen. The successful transmission of disease with subcutaneous inoculation of the infected liver homogenate had indicated the infectious nature of the disease (Khawaja *et al.,* 1988a; Ahmad *et al.*, 1989 and Anjum, 1990). Subsequent studies confirmed that an adenovirus was associated with the condition, this being incriminated by the demonstration of either basophilic or eosinophilic inclusion bodies in the hepatocytes from the diseased birds (Niazi *et al.*, 1989; Abdul-Aziz and Al-Attar, 1991; Afzal *et al.* 1991) and by the demonstration of discrete icosahedral virions in purified liver extracts by negative staining electron microscopy (Cheema *et al.*, 1989).

However, Afzal *et al.* (1991) suggested the possibility that another agent was involved that requires co-infection by an adenovirus to produce the classical signs of this syndrome.

In another study, with a Mexican liver homogenate, an RNA virus was implicated as an additional agent, on the basis of lack of inhibition by 5-bromodeoxyuridine (Shane and Jaffery, 1997). As FAVs had been isolated from both healthy and diseases chickens, associating FAVs with this specific disease had been difficult.

In Pakistan and in several South American countries, inclusion body hepatitis (IBH-HPS) virus, in many cases associated with hydropericardium, was predominant, and the condition was caused by FAV-4 (Voss *et al.*, 1996). An adenovirus (K31/89), isolated from field cases of HPS in Pakistan (Voss *et al.*, 1996), was identified as adenovirus serotype 4 and as the causative agent of HPS (Voss *et al.*, 1996). European serotype 5 (FAV-8), itself associated in the reproduction of the HPS with IBH in Mexico, had also been reported to be involved (Shane and Jaffery, 1997). Toro *et al.* (1999) conducted virus neutralization tests with isolates from HPS outbreaks in Chile and reported that their isolates were FAV-4. They added that the Chilean FAV strains might require an association with other agents (immunosuppressive agents) to induce IBH/HPS outbreaks in the field. The liver damage observed in HPS was also noticed in IBH, but hydropericardium in 3 weeks old chicks, due to an adenovirus infection, was not a feature until recently. FAV isolated from liver samples from cases of HPS was neutralized by antisera against the KR 5 strain (FAV-4 reference strain) and the failure of attempts to isolate other viral agents from these liver samples in chicken kidney, chicken embryo and MDCC-MSB-1 cells confirmed the causative agent (Abe *et al.*, 1998). Hess *et al.* (1998) classified 12 fowl adenovirus (FAV) isolated from clinical cases of infectious hydropericardium from field outbreaks in 7 countries in Asia and America through application of serological tools, restriction enzyme analysis and polymerase chain

reactions. It was concluded that all adenoviruses isolated from various field cases of infectious hydropericardium (Angara disease) in several countries were FAV-4. Nakamura *et al.* (1999) recovered adenovirus from liver of SPF chickens affected with hydropericardium. The intranuclear inclusion bodies of various organs revealed positive reactions against group I avian adenovirus.

Naeem *et al.* (1995a) purified the HPS agent, propagated it in chicken embryo liver cells, passaged it in chicken embryos and further reproduced the disease in susceptible birds. Their observations supported the view in favor of FAV-4 as the causative agent of HPS. The isolate was designated as PARC-1.

The HPS was reproduced in SPF chicks using isolated and purified virus from field cases of HPS (Cowen *et al.*, 1996., Mazaheri *et al.*, 1998), thus proving the association of FAV-4 with HPS as the sole agent responsible for causing the disease.

Kim *et al.* (2008) could isolate and identify several adenoviruses from samples of broilers, layers, breeders, and native Korean fowl in an explosive outbreak of hydropericardium syndrome.

2.3.2 Work done in India

In India, Gowda (1994) and Gowda and Satyanarayana (1994) mentioned that an adenovirus was associated with hydropericardium syndrome, by the demonstration of either intranuclear inclusions in the hepatocytes from the affected cases of disease. In India also, intranuclear adenovirus particles measuring 80 to 90 nm in diameter were demonstrated in hepatocytes by transmission electron microscopy by Chandra *et al.* (1997) and the virus was isolated in chicken embryo liver cell culture and typed as FAV-4 (Jadhao *et al.*,1997). Kataria *et al.* (1995) also isolated adenoviruses from cases of HPS from various parts of India, these reacting in an AGPT test with antiserum against FAV-1 (Verma *et al.*, 1971).

Simultaneously, Survashe *et al.* (1996) described that an adenovirus similar to type 1 and 4 was the primary causative agent of this syndrome and viral virulence was augmented after immunosuppression caused by any one or combination of infectious bursal disease exposure, aflatoxin, other mycotoxins or clostridial and other bacterial toxins. Subsequently, isolation of FAV had been reported from many of the poultry farms in India, which were experiencing HPS (Kataria *et al.*, 1996; Oberoi *et al.*, 1996; Vairamuthu *et al.*, 2002). All the isolates from field outbreaks of IBH-HPS have been serotyped as FAV-4 using standard antisera to the 12 different serotypes of FAV (Jadhao *et al.*, 1997) and identified as HPS virus (Vairamuthu *et al.*, 2002). Dahiya *et al.* (2002) revealed that FAV-4 was associated with outbreaks of infectious hydropericardium in broiler in Haryana state. Asrani (1997) and Chand (2006) could identify the etiological agent in the outbreak of HPS in the state of Himachal Pradesh as FAV-4 by PCR and AGPT with reference hyperimmune serum against FAV-4.

2.4 Prevalence of the disease

2.4.1 Work done abroad

The first epidemic of HPS in broiler chicks was reported from Angara Goth near Karachi, Pakistan, in late 1987 (Jaffery,1988; Khawaja *et al.*, 1988a; Cheema *et al.*, 1989; Hasan,1989), although sporadic cases were recorded as early as 1985 (Cheema *et al.*, 1989). It has been subsequently recorded in Iraq (Abdul-Aziz and Al-Attar, 1991), Slovakia (Jantosovic *et al.*, 1991), Mexico, Ecuador, Peru, Chile (cited by Voss *et al.*, 1996; Cowen *et al.*, 1996), South and Central America (Shane, 1996), Russia (Borisov *et al.*, 1997) and Japan (Abe *et al.*, 1998).

2.4.2 Work done in India

In India, the disease was first recorded in some parts of Jammu and Kashmir, Punjab and Delhi during April to July 1994 (Gowda and Satyanarayana, 1994) though some cases had been noticed prior to 1994 (Singh *et al.*, 1996). It spread to Uttar Pradesh in November 1994 (Kumar *et al.*, 1997) and this was followed by distribution of disease throughout the country.

Indian states that have recorded HPS included Andhra Pradesh, Tamil Nadu, Himachal Pradesh, Karnataka, Maharastra and Haryana (Gowda and Satyanarayana, 1994; Bhowmik, 1996; Kataria *et al.*, 1996; Nighot *et al.*, 1996; Asrani *et al.*, 1997; Shukla *et al.*, 1997 b). During 1996, a study group on poultry diseases reported the mortality rate due to HPS as ranging from 10 per cent to 80 per cent (Anonymous, 1996) and identified HPS as an emerging problem of the broiler industry.

2.5 Epidemiology

2.5.1 Work done abroad

HPS has been observed in broiler chickens (Agsar-Hasan, 1989; Cheema, 1989), aged 3 to 6 weeks (Khawaja *et al.*,1988a; Anjum *et al.*, 1989; Niazi *et al.*,1989; Abdul-Aziz and Al-Attar, 1991; Javed *et al.*, 1994) or over 5 weeks of age (Ahmad *et al.*, 1989; Muneer *et al.*, 1989; Akhtar and Cheema, 1990) and occasionally in layers and breeder pullets aged from 8 to 20 weeks (Jaffery,1988; Ahmad *et al.*, 1989; Cheema *et al.*, 1989; Akhtar, 1992; Javeed *et al.*,1994). Rare outbreaks of HPS in other species of poultry including pigeons (Naeem and Akram, 1995) had been recorded. Most of the researchers had reported that different strains of broilers are equally susceptible in the field (Anjum *et al.*, 1989; Akhtar and Cheema, 1990) and in experimental cases (Afzal and Hussain, 1993). However, Khan *et al.* (1995) found the Hubbard strain of broilers to be relatively most susceptible, followed in order by the Indian River and Lohmann strains.

The course of the disease under natural conditions or following oral inoculation ranged from 7 to 15 days (Anjum *et al.*, 1989; Cheema *et al.*, 1989; Abdul-Aziz and Al-Attar, 1991; Akhtar, 1995). However, a liver homogenate prepared from infected birds and inoculated by the parenteral route caused disease within 2 to 5 days (Anjum, 1990). IBH is also seen in young broilers, but high rates of mortality of 60 to 70 per cent in Pakistan, 10 to 30 per cent in Iraq and 10 to 60 per cent in India are only characteristic of HPS (Abe *et al.*, 1998).

Studies on epidemiological features in Pakistan indicated that flocks that were visited frequently by vaccination crews were 15 times more likely to be affected by the syndrome than flocks that had no visits (Akhtar *et al.*, 1992) and also that the use of electricity as the source of light and heat entailed a much lower risk of HPS than when kerosene oil was used for these purposes. A survey of HPS carried out in 131 flocks in 105 broiler production units in Pakistan from July 1989 to August 1990 revealed a prevalence of 46.6 per cent (Akhtar *et al.*, 1992).

In Russia, HPS was recorded in chickens aged between 2 and 13 weeks, with mortality ranging from 3.5 per cent to 30 per cent in broilers and from 2.6 per cent to 15.29 per cent in layers (Borisov *et al.*, 1997). It was also recorded in a number of poultry farms in Russia as an acute disease with hydropericardium in 3 to5 week old broilers (Aliev *et al.*, 1997).

2.5.2 Work done in India

In India, the disease was observed more often in broilers of either sex aged 3 to 6 weeks. Occasionally, the disease was reported in layers and breeder pullets aged up to 20 weeks by Asrani *et al.*, 1997; Shukla *et al.*, 1997a). Rare outbreaks of HPS in older birds/broilers (Asrani *et al.*, 1997) and in other species of poultry and quails

(Karunamoorthy and Manickam, 1998) have been reported. Fast growing broilers were the most affected, mortality peaking on the third or fourth day, followed by an almost constant death rate for 5 to 7 days, before it declined, giving and average mortality of 15 to 60 per cent (Asrani *et al.*, 1997). The presence of aflatoxins in the feed at higher concentrations than 20 ppb was commonly associated with a large number of outbreaks of IBH, causing heavy mortality among 3 to 5 week old broiler chicks, which displayed typical lesions of IBH in addition to hydropericardium (Singh *et al.*, 1996). The mortality rate in various outbreaks in broiler farms in India ranged from 30 per cent to 80 per cent with an average of 61.62 per cent (Kumar *et al.*, 1997; Singh *et al.*, 1997). Occasional outbreaks in older broilers (32 weeks old) and commercial layers (17 weeks old) had also been recorded, with 5 to 8 per cent mortality (Asrani *et al.*, 1997). The role of infectious bursal disease in precipitating HPS in layer flocks had been well documented by Shukla *et al.* (1997a). Although immunosuppression due to chicken anemia virus has not been conclusively proved in HPS outbreaks, this might also hold true under field conditions.

2.6 Transmission

2.6.1 Work done abroad

The origin of the virus is often questioned during a disease outbreak investigation and vertical transmission has frequently been blamed. Avian Adenoviruses have been found to be vertically and horizontally transmitted (Grgi´ *et al.*, 2006).

Since fowl adenoviruses (FAVs) are present in feces, tracheal, nasal mucosa and kidney they are readily transmitted horizontally (Grgi´ *et al.*, 2006). The hepatocytes and enterocytes of infected birds commonly show adenoviral particles (Ritchie and Carter, 1995). Juvenile and adult patterns of excretions have been observed in birds. In adult birds, lower peak titers of fecal virus have been demonstrated exhibiting the adult pattern were

excretion of the virus is shorter than in newly hatched chicks adapting the juvenile pattern. Direct fecal contact seemed to be the main mode for horizontal spread with aerial contact over short distances being the other. Contrary to the normal excretion pattern seen in commercial flocks, experimentally infected and adventitiously infected SPF flocks have also shown this excretion pattern (McFerran, 1997). Other important contributors to the spread of the virus are fomites, personnel and transport (Grgi´ *et al.*, 2006).

Adenoviruses are vertically transmitted through embryonated eggs and in cell cultures prepared from embryos of infected flocks this transmission is often unmasked (Grgi´ *et al.*, 2006; McFerran, 1997). From week 3 onwards *Adenoviruses* are normally excreted although they can be isolated from day 1 onwards. Peak excretion in broilers occurs between 4 to 6 weeks of age and in layer replacements at 5 to 9 weeks. Around the egg production period, *Adenoviruses* are often present and the virus is presumably reactivated due to stress and high levels of sex hormones ensuring maximum egg transmission to the next generation (McFerran, 1997). There have been a limited number of publications reporting on vertical transmission in field outbreaks although some researchers reported that this transmission infrequently occurs or not at all (Grgi´ *et al.*, 2006).

The disease HPS was normally transmitted by mechanical means horizontally among broilers (Akhtar *et al.*, 1992) and by contamination with infected feces (Shafique and Shakoori, 1994) and was classified as highly contagious (Abdul-Aziz and Hasan, 1995). The virus was highly pathogenic (Khawaja *et al.*, 1988a), spreading rapidly from flock to flock and farm to farm (Cowen, 1992). The bird to bird transmission of the virus in a flock occurs by the oral fecal route (Abdul-Aziz and Hasan, 1995) as well as horizontally (Akhtar, 1995). Anjum (1990) failed to reproduce the disease by oral inoculation or by

contact with naturally diseased birds. However, Cowen *et al.* (1996) mentioned that the oral fecal route is a possible mechanism for the spread of the disease under field conditions. The disease could be reproduced in broiler chicks by inoculation a liver extract from naturally infected pigeons, but the role of wild birds in spreading the disease under natural conditions needs confirmation (Naeem and Akram, 1995), which might help in identifying a specific reservoir for the infection. A study on the transmission of HPS in broiler and layer chicks kept on infected litter revealed that the mortality was higher in the broilers than in the layers (Shafique and Shakoori, 1994),that might be due to factors associated with the increased growth rate of broilers. However, Voss *et al.* (1996) recorded mortality from 6 to 17 DPI among SPF chicks, following intramuscular inoculation of a liver homogenate. In a similar study, Aliev *et al.* (1997) recorded the incubation period as 48 to 72 hrs. in experimental infection, and the duration of disease was 7 days.

2.6.2 Work done in India

Even though the causative agent of HPS was not ascertained initially, still the disease could be reproduced in broilers by inoculation of bacteria free liver homogenate from cases of dead naturally infected birds (Gowda and Satyanarayana, 1994; Asrani *et al.*, 1997; Chandra *et al.*, 1997). Mortality was generally observed 3 to 5 days after experimental infection. Generally, broilers dying less than 60 hrs. after infection did not show any clinical symptoms (Kumar *et al.*, 1997).

The presence of adenovirus particles in the liver homogenate and in the hepatocytes of broilers experimentally infected with the HPS agent could be demonstrated by electron microscopy by Chandra *et al.* (1997). They could also reproduce the disease by inoculation of birds with the infected liver homogenate recovered from naturally occurring cases of hydropericardium. Kataria *et al.* (1997a), Deepak (1998) and Balamurugan *et al.* (2001)

could also experimentally induce IBH-HPS in broiler chicks aged 1 to 3 weeks by inoculating FAV-4 propagated in liver cell cultures.

The condition was experimentally created in broilers by injecting liver homogenate from infected birds by Gowda and Satyanarayana (1994). The disease was manifested by hydropericardium with the accumulation of about 12 ml of straw colored fluid, a pale and enlarged liver, edematous and congested lung and pale and enlarged kidneys. Dahiya *et al.* (2002) could also reproduce the disease experimentally in broilers aged around 28 days by subcutaneous or oral inoculation of isolated FAV-4, with signs of typical hydropericardium and intranuclear inclusion bodies in hepatocytes observed at 4 DPI. Chand (2006) and Kanwar (2008) could reproduce the disease in broiler chicks that died with typical signs of hydropericardium and enlarged and hemorrhagic livers.

2.7 Clinical signs

2.7.1 Work done abroad

The natural outbreaks of the disease were normally incriminated with high mortality with no outward signs as recorded by Jaffery (1988). The disease had been observed by many in well nourished growing healthy broilers at 3 to 5 weeks of age with sudden mortality of 20 to 70 per cent without any morbidity. It had been observed that the birds remained active just before death. Hydropericardium is a prominent sign of HHS and had never been noted in many reports of field and experimental cases of HPS (Gallina *et al.*, 1973).Various authors like Anjum *et al.* (1989) and Muneer *et al.* (1989) had observed that the mortality started at about 3 weeks of age and reached its peak in 4 to 5 weeks of age with the manifestation of no outwards clinical signs. The mortality rate in various outbreaks in broiler farms in Pakistan ranged from 20 per cent to 75 per cent (Khawaja *et al.*, 1988a; Anjum *et al.*, 1989.; Cheema *et al.*, 1989).

In layers and breeder pullets, average mortalities of 8.7 per cent and 10.7 per cent, respectively, had been recorded (Cheema *et al.*, 1989; Javeed *et al.*, 1994). In Iraq, the disease was also observed in 3 to 5 week-old broilers, causing 10 to 30 per cent mortality (Abdul Aziz and Al-Attar, 1991). A range of incubation period from 5 to 18 days, with a mean of 10 days had been reported in controlled experiments (Akhtar, 1992).However, this depended upon the route and dose of infection, on the type of exposure and on the age of the birds. In another study (Akhtar, 1995) reported an incubation period of 9.5 to 14.5 days, with the duration of the disease being 10 to 15 days. The reasons for these variations in the incubation period had not been satisfactorily explained by any of the workers.

2.7.2 Work done in India

In natural outbreaks of HPS, the diseased birds might not exhibit gross clinical signs other than sudden heavy mortality (Kumar *et al.*, 1997). However, in the terminal stages, the birds become dull, depressed and show a characteristic posture, with their chest and beak resting on the ground and with closed eyelids (Asrani *et al.*, 1997). Birds inoculated experimentally by the subcutaneous route stopped eating and drinking, became debilitated, showed ruffled feathers, were reluctant to move and died around 60 hrs. PI (Kumar *et al.*, 1997). Field observations at six poultry farms in the Haldwani area of Uttar Pradesh during November-December 1994, and in other parts of northern India, revealed sudden mortality in broilers, ranging from 30 per cent to 80 per cent with an average of 61.62 per cent (Kumar *et al.*, 1997; Singh *et al.*, 1997). The disease was continuously recorded up to March 1996, with an average mortality of 34.6 per cent in broilers (Shukla *et al.*, 1997 b). In Punjab, the average mortality in affected broiler flocks ranged from 15 per cent to 60 per cent. Occasional outbreaks in older broilers (32 weeks old) and

commercial layers (17 weeks old) were also recorded, with 5 to 8 per cent mortality (Asrani *et al.*, 1997).

Chand (2006) recorded clinical signs of dullness, depression, anorexia, ruffled feathers and resting of chest and beak on the cage floor with eyes completely closed in broiler chicks while clinical signs of dullness, depression and ruffled feathers were shown by Japanese quail infected with HPS virus. Similar observations were also recorded by Kanwar (2008) who found depression, ruffled feathers, anorexia and greenish diarrhea in broiler chicks infected with the virus of HPS in her study.

2.8 Gross lesions

2.8.1. Work done abroad

The most common and predominant gross lesion seen at necropsy of a hydropericardium affected bird is hydropericardium. This lesion occurred in almost 90 per cent of the affected birds (Anjum *et al.*, 1989; Cheema *et al.*, 1989; Qureshi, 1988, 1989). This is characterized by the accumulation of clear watery/jelly-like (Cheema *et al.*, 1989), straw/amber or green colored fluid in the pericardial sac (Cheema *et al.*, 1989) with a pH of 7.0. Anjum *et al.* (1989) had observed lesions in heart, liver, kidneys and lungs. They had observed hydropericardium in which the pericardial sac had a balloon like appearance having up to 20 ml of the clear straw colored fluid. Apart from the hydropericardium, some other changes had been observed that included swollen, friable, congested and dark to yellow coloured livers with large areas of focal necrosis and petechial hemorrhages (Anjum *et al.*, 1989; Cheema *et al.*, 1989; Muneer *et al.*, 1989). Pale yellow, swollen and friable kidneys, containing deposits of urates in the tubules and ureters (Cheema *et al.*, 1989; Abdul -Aziz and Hasan, 1995; Nakamura *et al.*, 1999) and edematous lungs had also

been observed (Anjum *et al.,* 1989). Shafique *et al.* (1994) had documented that mortality and severity of the lesions might be greater in severely immuno compromised chickens.

Similar gross lesions had also been described in various organs from experimentally infected broilers (Anjum, 1990). In addition to the typical hydropericardium, Nakamura *et al.* (2002) observed pinpoint white foci in the pancreas and ventricular erosions in broilers. The mortality and the severity of the lesions might be greater in immunosuppressed birds, as was evident in colchicines treated birds (Shafique *et al.*, 1993). Nakamura *et al.* (2000) indicated that IBH strains of adenovirus can also reproduced the lesions of HPS and mortality in day old specific pathogen free (SPF) chicks and that IBH and HPS strains might had similar pathogenicities, except for the difference in their virulence against older chickens.

2.8.2 Work done in India

The predominant lesions noticed at necropsy *i.e.* hydropericardium had also been recorded by authors from India like Gowda and Satyanarayana (1994) and Kumar *et al.,* (1997) which was usually manifested by the accumulation of straw/amber or green coloured fluid in the pericardial sac ranging from 3 to 20 ml in quantity (Gowda and Satyanarayana, 1994; Asrani *et al.*, 1997; Kumar *et al.*, 1997) and with a pH of 7.0 (Gowda and Satyanarayana, 1994). The heart appeared flabby and its cone was found floating in the pericardial sac (Kumar *et al.*, 1997). The pericardial fat may exhibit yellowish discoloration and petechial hemorrhages (Asrani *et al.*, 1997). The liver was pale yellow, swollen, friable/mottled and contained large areas of focal necrotic patches that might also contain petechial and ecchymotic hemorrhages (Gowda and Satyanarayana, 1994; Asrani *et al.*, 1994; Kumar *et al.*, 1997). A yellowish discoloration of the subcutis and abdominal fat was also recorded in a few birds, along with bursal and thymic atrophy in 10 per cent of the birds examined (Asrani *et al.*, 1997). They also reported that the pericardial fat might

exhibit yellowish discoloration and petechial hemorrhages. The intestinal blood vessels were congested (Gowda and Satyanarayana, 1994), while the bursa of Fabricus of some affected birds was enlarged (Kumar *et al.*, 1997). Soni (1999) observed enlargement of liver with petechial hemorrhages and edema in lungs in experimentally affected broiler chicks with HPS while Rani *et al.* (2000) observed birds for sequential gross and histopathological changes after experimentally inducing disease in 3 to 4 weeks old birds. The gross changes recorded were hydropericardium, enlarged and tan yellow colored liver. Ravishankar and Balasubramaniam (2002) reported that grossly the liver was pale, enlarged and friable and heart revealed hydropericardium with accumulation of straw coloured fluid in the pericardial sac. However, inclusions in kidneys, heart, spleen and pancreas at various stages of infection were reported by Singh *et al.* (2004). They also documented necrotic hepatitis and intranuclear inclusion bodies in hepatocytes.

Chand (2006) and Kanwar (2008) found hydropericardium as the main characterizing feature of HPS in their study. They also recorded congestion, hemorrhages and enlargement in the liver. Other lesions seen were congested and edematous lungs, congested and enlarged spleens and swollen kidneys.

2.9 Histopathology

2.9.1 Work done Abroad

Histopathological examination of the heart revealed mononuclear cell infiltration (Anjum *et al.*, 1989), severe vascular changes (Cheema *et al.*, 1989), massive edema leading to disruption of muscle bundles. Microscopic findings in the heart also included necrotic areas in the myocardium of reticular walls. Swelling and vacoulation of intimal cells of cardiac arteries along with mild leucocytic infiltration in the tunica media and adventitia of the blood vessels of HPS affected chicks were recorded by Cowen (1992).

The liver being the primary organ manifested focal areas of necrosis and mononuclear cell infiltration (Anjum *et al.*, 1989; Cheema *et al.*, 1989). Centrilobular or diffuse degeneration and necrosis of hepatocytes, swelling of the hepatocytes with partial clearing (absence of granularity) of the cytoplasm or rupture of cell membrane were also noticed in some birds. The hepatocytes were shrunken in some areas and had pyknotic nuclei (Abdul- Aziz and Hasan, 1995). Abe *et al.* (1998) observed the necrosis of hepatocytes accompanied with intranuclear basophilic inclusions in hepatocytes and hemorrhages, activation of macrophages in the splenic sinus and ellipsoids and erythrophagocytosis in the splenic sinus of spleen and interlobular interstitium of the lung in the broiler breeder and broiler chicks suffering from HPS.

The most prominent changes observed in the lungs included congestion, edema in the alveolar walls and a moderate diffuse infiltration of macrophages into the pulmonary parenchyma (Nakamura *et al.*, 1999).The kidneys had marked swelling of the tubular epithelium, necrosis and extensive hemorrhages (Abdul-Aziz and Hasan, 1995). Macrophages, containing erythrocytes and prominent yellow pigment in the red pulp, were also recorded in the spleen (Nakamura *et al.*, 1999).

Other changes observed were the depletion of lymphocytes in the medullae of the follicles in the burse of Fabricus (Abdul- ziz and Hasan, 1995). Qureshi (1990) observed mild depletion of lymphocytic cells in bursa of Fabricus and spleen in HPS affected chicks. Nakamura *et al.* (1999) observed the presence of intranuclear inclusion bodies (INIBs) in the cells of the gizzard, pancreas, proventriculus, duodenum, cecum, kidneys and lungs of chicks experimentally inoculated at one day of age. In another experimental infection, Nakamura *et al.* (2002) observed that infected chickens had multifocal hepatic necrosis, with INIBs in the hepatocytes, a marked increase in macrophages in the spleen and lung,

mild epicardial edema, multifocal necrosis of pancreatic acinar cells, with intranuclear inclusions, focal necrosis of the ventricular kaolin layer and degeneration of the ventricular glandular epithelium, with intranuclear inclusions.

2.9.2 Work done in India

In India too, the liver was identified as the major affected organ by various authors (Gowda and Satyanarayana, 1994; Asrani *et al.*, 1997 and Kumar *et al.*, 1997) revealing histological changes, such as small multifocal areas of coagulative necrosis, mononuclear cell infiltration and the presence of basophilic INIBs. These changes were confirmed by transmission electron microscopic observation of the hepatocytes (Chandra *et al.*, 1997). Asrani *et al.* (1997) noticed fatty changes and focal areas of lymphocytic infiltration in liver with intranuclear inclusion bodies in the hepatocytes of HPS affected chicks. They also observed that there were also edema and vacuolar degeneration in the intimal layer and tunica adventitia of myocardial arteries and diffuse congestion, hemorrhages and perivascular edema with mononuclear cells infiltration in the lungs.

Histopathological examination of the heart revealed mononuclear cell infiltration, massive edema, hemorrhages (Asrani *et al.*, 1997; Kumar *et al.*, 1997) and degenerative alterations (Gowda and Satyanarayana, 1994). Trivedi *et al.* (1996) revealed profuse interstitial edema, hemorrhages of varying degree, endothelial proliferation of blood vessels and mononuclear cells infiltration in the myocardium along with intranuclear basophilic inclusion bodies in the hepatocytes of HPS affected chicks. Singh *et al.* (1997) mentioned a catarrhal inflammation in the parabronchiolar epithelium with hypertrophy of parabronchiolar smooth muscles due to HPS in chicks. The lungs showed congestion, edema and infiltration by inflammatory cells and there was hemorrhagic exudate in the bronchi and alveoli (Asrani, 1997; Kumar, 1997).

Histologic features in kidneys mainly depicted by marked swelling of the tubular epithelium, necrosis and extensive hemorrhages were recorded by Asrani *et al.*, (1997). The gastrointestinal tract had catarrhal inflammation of the mucosa, particularly of the villi of the intestine (Kumar *et al.*, 1997).Other changes noticed were lymphocytolysis and cyst formation in the bursa of Fabricus, thymus and spleen (Gowda and Satyanarayana, 1994; Asrani *et al.*, 1997) and led to depletion of lymphocytes in the medullae of the follicles in the bursa of Fabricus (Kumar *et al.*, 1997). Survashe *et al.* (1996) observed findings like degeneration and necrotic changes in the germinal centres of spleen and moderate depletion of lymphocytes and haemorrhages in the lobule of thymus and bursa of Fabricus of HPS affected chicks. Deepak (1998) also observed parallel histological alterations in various organs.

Thakur and Grewal (1999) recorded detailed gross and histopathological changes upon inducing HPS in 21 days old chicks from fresh liver samples of natural outbreaks of HPS. After fourth day post-inoculation, pericardium was seen to be distended with excess fluid with swollen fatty liver. Histopathologically, liver showed hyperchromatic nucleus and large basophilic intranuclear inclusions with clear halo after third day post inoculation. Rani *et al.* (2000) experimentally induced the disease in 3 to 4 weeks old birds with liver homogenate obtained from natural cases of HPS and observed birds for sequential gross and histopathological changes at various time intervals in different organs.

The histopathological lesions recorded were fatty and cystic hepatocytic degeneration with periportal and centrilobular coagulative necrosis and intranuclear basophilic viral inclusion bodies. Kumar and Grewal (2002) also experimentally inoculated 21 days old chicks with infective liver homogenate derived from natural cases of HPS. They discerned that liver had multifocal hepatic necrosis and hemorrhages with

intranuclear inclusion bodies within hepatocytes. Ravishankar and Balasubramaniam (2002) studied spontaneous cases of HPS in broilers. Histopathologically, in liver, hepatocytes had vacuolar degeneration with focal mononuclear cell infiltration and contained large round basophilic or eosinophilic intranuclear inclusion bodies with halo.

Rajkhowa (2002) investigated an outbreak of hydropericardium-hepatitis syndrome (HHS) in Mizoram. They observed that liver revealed multifocal coagulative necrosis with mononuclear cell infiltration, hemorrhage and basophilic karyorrhexis.

Chand (2006) found histopathological changes in heart of broiler chicks that included thickening of pericardium due to edema, congestion and hemorrhages in myocardium. Changes in liver included congestion, vacoulation and intranuclear basophilic inclusion bodies.

2.10 Cultivation of virus

2.10.1 Work done Abroad

2.10.1.1 *Embryonated eggs*

Afzal *et al.* (1990) reported the cultivation of virus in embryonated hen's egg through different routes. They recorded the death of embryos inoculated via the chorioallantoic membrane, chorioallantoic sac and yolk sac between 4 to 9 days post inoculation. In some other studies on isolation of virus in embryonated eggs, stunted growth, hemorrhages and death of embryos had also been reported (Cheema *et al.*, 1989; Shafique *et al.*, 1993; Naeem *et al.*, 1995a).

Mahmood and Hussain (1995) attempted to propagate the HPS virus in duck embryonated eggs via the yolk sac and chorioallantoic sac route. They observed hemorrhages, stunted embryos and death of the embryo via both routes with some variation

in the severity. Moreover, intranuclear inclusion bodies were also been detected in hepatocytes of inoculated embryos by these researchers. Inoculation of filtered liver homogenate resulted in embryonic death within a period of four to seven days. The pathogenic agent produced small syncytia on both kidney and liver monolayers (Shane, 1996).

Embryo age and route of inoculation are other factors to be considered before propagation of *Aviadenoviruses* (Cowen, 1988). The yolk sac route is believed to be the most sensitive route although the chorioallantoic and allantoic fluid route have previously been used (Cowen, 1988; Cotten *et al.*, 1993; Hess, 2000). Replication or infectivity of AAV in chicken embryos can be seen as embryo deaths and/or gross microscopic lesions observed in hepatocytes. Stunting and curling of the embryo, hemorrhage of body parts and enlargement of the liver and spleen are also signs observed in AAV infections in specific pathogen free (SPF) eggs (Cowen, 1988).

2.10.1.2 *Cell culture system*

The HPS agent could be isolated or propagated in primary cell cultures of chicken kidney (Khawaja *et al.*, 1988a). They observed specific cytopathic effects in the cell cultures like degeneration and detachment of cells from surface and presence of basophilic intranuclear inclusion bodies.

Khawaja *et al.*, (1988a) isolated the virus from the liver, heart and kidneys of chickens affected by HPS by inoculating embryonated chicken eggs via the yolk sac route and also in chick embryo kidneys (CEK) cell cultures. The virus induced production of INIBs in CEK cell cultures and also agglutinated the red blood cells of rats. The cytopathic effects produced in the cell cultures were rounding of cells, degeneration within three to

four days, detachment of the cells from the surface and the presence of INIBs in the infected cells (Khawaja *et al.*, 1988a).

Mazaheri *et al.* (1998) prepared chicken embryo liver cells from 11 days old SPF embryos that were supplemented with 5 per cent newborn bovine calf serum for cell propagation. Raue and Hess (1998) propagated adenovirus in chicken embryo liver cells from 11 days old specific pathogen free embryos. For cell propagation, Medium M199 containing Earle's salts supplemented with 10 per cent new born calf serum was used. Mazaheri *et al.* (1998) also used similar methods for cell propagation except mediuM199 supplemented with 5 per cent new born calf serum. Hess *et al.* (1998) demonstrated the cytopathic effects at 6^{th} day post inoculation of chicken embryo liver cell culture. A few ballooned and detached cells were observed and the cytopathic effects (CPE) were intensified after 2^{nd} passage. For the multiplication of FAVs, both chicken kidney cell culture and chicken liver cell culture were equally efficient as reported by Toro *et al.* (1999). Chicken kidney cells (CKCs) were produced from one day old SPF chickens for virus replication and chicken embryo liver cell culture were prepared from 18 days old embryonated SPF eggs for both virus titration and virus neutralization tests.

For propagation of *Aviadenovirus*es (AAV), primary chicken embryo kidney (CEK) or chicken embryo liver (CEL) cell cultures are believed to be more sensitive than embryonated chicken eggs. The CEL cells have been reported to be more sensitive than CEK cell cultures for primary isolation of certain strains from pigeons as lower virus titers are obtained in CEK cells after serial passage of the virus when compared to CEL cells (Hess *et al.*, 1998).

CEF cell cultures have been reported to be less sensitive although homologous fibroblast cells can be used for certain AAV like the *Duck Adenovirus* (DAV) and *Goose*

Adenovirus (GAV) (Hess, 2000). In a recent report by Lüschow *et al.* (2007) the successful isolation of an Adenovirus naturally occurring in psittacine birds was demonstrated by using homologous cell cultures of psittacine embryo fibroblasts (PsEFs). Unfortunately psittacine embryonated eggs are very expensive and are not readily available as only a few psittacine eggs are laid yearly compared to chicken embryonated eggs which are frequently available (Lüschow *et al.*, 2007).

2.10.2 Work done in India

2.10.2.1 *Embryonated eggs*

Due to limited resources in certain diagnostic laboratories chicken embryos are more suitable for the isolation and/or propagation of these viruses. An essential point is that the eggs should be free of antibodies against group I AAV.

The HPS virus could also be passaged or isolated in embryonated chicken eggs, in which it induced stunted growth, haemorrhages and death of embryos (Jadhao, 1998). Madhumati *et al.* (2004) experimentally induced HPS (FAV-4) in nine days old chick embryos via yolk sac route and in 11 days old embryos via allantoic route infection with type I *Aviadenovirus* (AAV). The infected embryos were sequentially harvested from two days post infection (DPI) onwards up to six DPI and various gross and histopathological lesions were systemically recorded. The gross lesions in the infected embryos from both the routes of inoculation, revealed dwarfing, curling of toes, congestion, petechiae all over the body, pale/green livers or necrotic/dark livers, hepatomegaly, pale/parboiled heart, hydropericardium, splenomegaly and urate deposition. Kanwar (2008) could also record mortality in embryos characterized by dwarfing, curling of toes, hydropericardium, enlarged, dark and necrotic livers though the hydropericardium was not a very regular feature in embryos.

2.10.2.2 *Cell culture system*

Oberoi *et al.* (1996) isolated avian adenovirus from three out of four outbreaks of IBH-HPS of poultry in CEL cell culture. The virus isolates were identified by counterimmunoelectrophoresis (CIE), Dot ELISA and double antibody sandwich ELISA. The CPE produced in cell cultures were characterized by Balamurugan (1999) and Balamurugan *et al.* (2001, 2002) they reported round cell degeneration within three to four days, detachment of cells from the surface and presence of intranuclear inclusion bodies in the infected cells. The cultivation of causative virus of HPS in VERO cell lines after fourth blind passages which after 96 hrs. produced CPE characteristic of an adenovirus was documented by Roy *et al.* (2001). They used immunoperoxidase test to confirm their findings. Dahiya *et al.* (2002) prepared CEL cell culture with 12 to 14 days old embryonated eggs in medium M199 supplemented with 10 per cent fetal calf serum, 1000 IU/ml benzyl penicillin G and 1mg/ml streptomycin sulphate at 37°C for 48 hrs. The CEL monolayer was inoculated with liver homogenate suspension and incubated at 37°C in M199 containing 3 per cent fetal calf serum for five days and observed daily for cytopathic effects.

Kaur *et al.* (2003) noticed cytopathic effects typical of avian adenoviruses (AAV) in CEL cell culture with 10 isolates obtained from cases of IBH-HPS. The pathology was characterized as rounding, swelling and clustering of cells, intracytoplasmic vacoulation and detachment of cells from glass surface at 24 to 48 hrs. post inoculation. Kumar *et al.* (2003) isolated the virus that caused HPS in broilers in CEL cell culture. The CPE characterized by rounding and degeneration of cells were observed 36 hours post infection. May-Grunwald and Giemsa stained monolayer discerned typical basophilic intranuclear inclusion bodies.

Barua and Rai (2003) propagated the FAV-4 in CEL cell culture and reported that the virus produced CPE in CEL cell culture from third passage onwards. The CPE in infected CEL cell culture appeared within 48 hrs., ultimately characterized by swelling and rounding of infected cells. By 72 hrs. post infection the cells became refractile and started detaching from the surface. By 96 hrs. post infection, the monolayers completely detached from the surface. Kanwar (2008) also could successfully grow the HPS virus in CEL cell cultures. The characteristic CPE were rounding and aggregation and detachment of cells. The effects were visible from the first passage level onwards.

2.11 Pathogenesis

2.11.1 Work done Abroad

The HPS virus has predilection for hepatic and endothelial cells. The incubation period in cases of natural outbreaks varied from 5 to18 days (Akhtar, 1992). There were reports showing the simultaneous presence of infectious bursal disease (IBD) and chicken infectious anemia (CIA) viruses in areas where HHS occurs (Shane and Jaffery, 1997). IBD and CIA are known for their immunosuppressive effects and FAVs required impairment of the immune response to induce their pathogenic potential (Monreal, 1996). These viruses could be the other speculated agents required in addition to the adenovirus for the production of typical signs of the IBH-HPS syndrome (Afzal *et al.*, 1991). The HPS agent also had predilection for lymphoid tissues, and can result in immunosuppression (Naeem *et al.*, 1995b). Therefore, the presence of IBD or CIA viruses might predispose for HPS, or HPS might predispose for these other viral infections.

2.11.2 Work done in India

Deepak (1998) mentioned that in addition to hepatic and other cells the HPS virus also had special predilection for lymphoid tissues which can result in immunosuppression.

The role of IBD in precipitating HPS in layer flocks had been well documented by Shukla *et al.* (1997a). Studies on the pathogenesis of FAV isolates had also suggested a synergism with CIA or other viruses, or that prior immunosuppression is necessary to produce IBH-HPS in chickens (Deepak, 1998) and the susceptibility of chickens to oral infection with FAV, resulting in IBH-HPS varied throughout the course of CIA infection. Balamurugan and Kataria (2006) described that the agent, FAV-4, causes immunosuppression by damaging lymphoid tissues; the presence of IBD and CIA viruses may predispose for HPS or HPS may predispose for other viral infections. Synergism with CIA or other virus infections or prior immunosuppression is necessary to produce IBH-HPS in chickens and the susceptibility of chickens infected with FAV varies throughout the course of CIA infection.

2.12 Clinical Pathology

2.12.1 Work done Abroad

HPS affected birds had severe anemia (Niazi *et al.*, 1989; Asrani *et al.*, 1997), with significant reductions in all hematological parameters except the mean corpuscular volume and the percentages of heterophils and eosinophils (Niazi *et al.*, 1989). A loss of fluid from the blood and its accumulation in the pericardial sac and other organs, and the resultant hemoconcentration, might be responsible for increased hematological values. The lymphopenia might be due to lymphocytolysis, as reported by Abdul-Aziz and Hasan (1995). These contrary reports on blood values appear to be due to variations in the time of blood collection and the presence of other concurrent infections in the commercial birds.

The activities of the AST, ALT and CPK serum enzymes were lowest in normal birds, intermediate in vaccinated birds and highest in HPS-affected birds, while the AP and LDH activities were also parallel in normal and vaccinated birds but raised in HPS

diseased birds (Iqbal *et al.*, 1994). The serum protein profile revealed a decrease in albumin with an increase in beta-globulins and no change in □1- and □ 2-globulins (Mahmood *et al.*, 1995). These changes were owing to involvement of the liver, kidney and heart in HPS. The decrease in blood glucose, proteins and cholesterol and the increase in uric acid and triglycerides were primarily due to liver damage. AST, ALT, AP and LDH enzymes were present in higher concentrations in the liver, kidney and heart, whereas CPK was present in muscles. Any damage to these organs resulted in release of these enzymes into the circulation (Benjamin, 1978).

Alexander *et al.* (1962) recorded a drastic reduction in total serum protein in birds affected with hydro pericardium. Any damage to the liver results in reduced synthesis of albumin, which in turn reduced the colloidal plasma osmotic pressure and allowed leakage of fluid into the pericardial sac. The elevation of AST activity might be attributed to liver damage and damage to the cardiac muscle that resulted in heart failure. Heart failure might also be one of the factors for the increased urea nitrogen values in HPS-affected birds as and when this occurred, renal blood flow and glomerular filtration pressure were reduced and the excretion of urea nitrogen was impaired (Benjamin, 1978).

2.12.2 Work done in India

HPS affected birds revealed severe anemia, with significant reduction in all the hematological values except the mean corpuscular volume (MCV) and the percentages of heterophils and eosinophils (Asrani *et al.*, 1997). The decrease in hematological values and severe anemia might be attributed to low cell production or might be a result of concurrent infection, such as with chicken anemia virus. Contrary to these findings, Bhatti *et al.* (1989) observed leukocytosis, erythrocytosis and an increased hemoglobin concentration. Among the leukocytes, heterophils revealed a marked increase, while lymphocytes, monocytes and eosinophils decreased and basophils were altogether absent (Bhatti *et al.*,

1989.; Asrani *et al.*, 1997). There was a decrease in the concentration of blood glucose and plasma protein, whereas the uric acid, potassium, calcium and triglycerides concentrations were significantly raised in HPS-affected birds (Bhatti *et al.*, 1989). Decreased total proteins and cholesterol, and increases in creatinine, urea nitrogen and in the activity of ALT and AST were documented by Asrani *et al.* (1997). There was also an enhancement in the potassium and calcium concentrations in the serum (Bhatti *et al.*, 1989), that appeared to be due to the accumulation of fluid in the pericardial sac and other organs.

2.13 Molecular characterization

2.13.1 Work done Abroad

The isolation and characterization of FAVs were successful from several cases of HPS in Ecuador and Pakistan. FAV-4 isolates recovered from different outbreaks of HPS in Pakistan were found to differ in their pathological characteristics. The studies conducted by Rabbani and Naeem (1996) revealed the association of FAV-4 with the outbreaks of HPS. No differences were noticed between the protein profiles of different isolates of avian adenovirus recovered from various field outbreaks (Rabbani and Naeem, 1996). The polypeptides of HPS virus had also been separated by sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) and identified by known protein markers (Izhar-ul-Haq *et al.*, 1997). The extracted nucleic acid was identified as DNA, with a molecular weight of 23 kDa through agar gel electrophoresis. The results of protein profile analysis of the purified HPS agent from Pakistan by SDS-PAGE had eight polypeptides, ranging in molecular weight from 15.7 to 119 KDa. On the other hand, Rabbani *et al.*, 1998a, separated seven bands of polypeptides ranging in molecular weight from 24 to 120 kDa. However, variation existed in the findings of both of these researchers. The differences in the concentrations of stacking and resolving gels applied by these workers had been attributed to the observed discrepancy between their findings.

Other studies showed that antibodies against serotypes 4 and 10 (FAV strains KR5 and 2B) neutralized new isolates of FAV characterized from several cases of HPS (Mazaheri *et al.*, 1998).

However, cross neutralization tests and restriction enzyme (RE) analysis strengthened their grouping under sero group 4. It had been shown by immunological and molecular analytical methods that FAV-4 and FAV-10 were very closely related (Erny *et al.*, 1995). The classification of HPS field isolates as FAV-4 strains was confirmed by RE digestion with *Bam* HI, *Dra* I and *Eco* RI, generating similar restriction fragment patterns between KR5 and some filed isolates, *Pst* I digestion differentiated field isolates from KR5. This study perceived that only some specific strains of FAV-4 caused the disease (Mazaheri *et al.*, 1998). Three field isolates of FAV from IBH-HPS cases in chicks were identified by virus neutralization tests and RE analysis of a DNA fragment and also identified as FAV-4 resembling the KR5 strain (Toro *et al.*, 1999).

The comparison of a predicted KR 95 strain hexon sequence and eight mammalian and avian adenovirus hexon sequences revealed the highest homology between KR 95 and FAV-10 and FAV-1 (91.1 per cent and 80.1 per cent respectively, (Lobanov *et al.*, 2000).

2.13.2 Work done in India

When Balamurugan *et al.* (2002) subjected three field isolates of FAV-4 from HPS from India to SDS-PAGE that showed eight similar polypeptides, with molecular weights ranging from 20 to 107 kDa but differing from FAV-1, particularly in their 24.2 kDa protein. Immunoblot analysis of HPS virus with other FAV serotypes, with type specific or polyclonal serum antibodies directed against proteins of FAVs, reflected the presence of common bands, that revealed serological relatedness among the FAV serotypes, with a close relationship between serotypes 4 and 9 in group I of FAV (Balamurugan *et al.*, 2002).

The typing of Indian isolates from field outbreaks of IBH-HPS in poultry at different geographical locations as FAV-4 were carried out by Jadhao *et al.* (1997) and the agent was characterized by RE analysis of its genomic DNA by the same authors. Ganesh *et al.* (2001 b) amplified a variable region of hexon gene, encoding 728 bp of L1 and part of P1, and both the amplified nucleotide and the derived amino acid sequences were compared with FAV-1, FAV-8 and FAV-10. There was variability of 28.1 per cent, 40.3 per cent and 8.2 per cent in the nucleotide sequence from FAV-1, FAV-8 and FAV-10 respectively. However, the derived amino acid sequences showed percentage variability as high as 28.8 per cent, 38 per cent and 45.1 per cent with FAV-10, FAV-1 and FAV-8, respectively. Although the nucleotide sequence had showed only an 8.2 per cent difference between HHS and FAV-10, yet the amino acid sequences differed by 28.8 per cent. Such a high degree of variability had been attributed to be due to the shift in the reading frame caused by deletions, indicating that the FAV-4 associated with HHS was unique and also different from FAV-10 (Ganesh *et al.*, 2001b).

2.14 Polymerase Chain Reaction (PCR) and Restriction Fragment length polymorphism (RFLP)

2.14.1 Work done Abroad

Polymerase chain reaction (PCR) has also been used as a diagnostic tool for investigating microorganisms due to the sensitivity and specificity of the assay. Viruses are among those microorganisms, especially those belonging to a widely distributed group in which a link between the infection and a specific disease has not yet been established (Raue *et al.*, 2002). The important thing to remember when using PCR for diagnosing viruses, in this case the *Aviadenovirus* family is to be able to understand their epidemiological behavior. The reason being is that some avian adenoviruses, like the FAV are non- species specific and some are restricted to their hosts e.g. EDS virus (Hess, 2000).

Differentiation of all the *Aviadenoviruses* (AAVs) infecting an appropriate host may consequently be achieved by PCR. The detection of only specific AAVs, possibly a single FAV serotype or the detection of all the AAVs is to be considered before proceeding with PCR.

Diagnosis based on PCR may take a general or specific approach. The general approach entails detection of as many as possible of the known and unknown strains of a particular pathogen. The primers designed for that pathogen must be able to hybridize the most highly conserved region of its genome. Although the primers designed may be complementary to the conserved region, they may not be universally applicatory in practice to the given pathogen. This is true for viruses with high mutation frequencies e.g. RNA viruses relative to DNA viruses. The general PCR product is usually further analyzed by restriction fragment length polymorphism (RFLP) or nucleotide sequencing.

The specific approach explains itself, where the primers designed hybridize to only the subset of strains of a pathogen. Further analysis is done when epidemiological studies are pursued and this involves sequencing, but usually no further analysis is required. Genotype, serotype and pathotype may be identified with this PCR approach (Cavanagh, 2001).

To date, several reports dealing with nucleic acid technology for detection and differentiation of avian adenoviruses existed. At the beginning, RE was applied to differentiate isolates and strains. In comparison with serological methods, many more differences on the genomes could be detected, not only those located on the structural proteins. Major structural proteins of relevance for serology represent only 15 per cent of the adenovirus genome. The REA depended on the amount and type of the selected restriction enzymes, which meant the nucleotide sequence at the cutting site.

Regarding the number of strains combined into 12 serotypes, the REA was very sensitive for the differentiation of FAVs. The grouping of fowl adenoviruses was conducted by Zsak and Kisary (1984) on the basis of restriction patterns of DNA generated by *Bam* HI and *Hind* III. Seventeen fowl adenovirus strains representing 11 serotypes were classified into five groups.

RE analysis demonstrated limited sequence differences when Erny *et al.* (1995) undertook a detailed characterization of representatives of fowl adenovirus serotypes in order to determine if they should be grouped together or retained as distinct serotypes. Digestion of two FAV serotypes was undertaken by Tiemessen and Nel (1996) they used the primers designed from the long fiber gene to detect and type subgroup F adenovirus. Hess *et al.* (1998) collected nine homogenized livers to isolate the causative agent of adenovirus type I and type II infections in pigeons. Only six type II adenoviruses were isolated but none of the three type I infections. Serologically, the isolated adenoviruses were classified as FAV-4. Restriction enzyme analysis of two isolates in comparison with FAV-4 reference strain KR-5 confirmed the serological results and classification of the pigeon isolates as FAV-4 strains. Direct detection of adenoviral DNA in tissue samples by *in situ* hybridization had also been reported. The probes applied in these investigations were based on the nucleic acid sequence of the FAV-10 penton base and the virus associated RNA of FAV1 (Ramis *et al.*, 1994; Goodwin *et al.*, 1996; Latimer *et al.*, 1997).

Mazaheri *et al.* (1998) isolated and identified fowl adenoviruses from several cases of hydropericardium syndrome in Ecuador and Pakistan. Cross neutralization test and restriction enzyme analysis confirmed it as serotype 4 strains. The restriction endonucleases included permitted the differentiation among field isolates and reference strains. All field isolates tested induced high embryo mortality. Hess (1999b) conducted epidemiological studies on fowl adenoviruses isolated from cases of infectious

hydropericardium from field outbreaks in seven countries in Asia and America. All isolates belonged to FAV-4. Minor differences were perceived in the *Bam* HI restriction profiles and more variability was recorded with *Sam* I, *Bgl* I and *Pst* I restriction profiles. More than 80 per cent of the fragments were identical in size in the five *Pst* I profiles, indicated the close genomic relationship between the isolates.

Several PCRs for detecting avian adenoviruses were reported. Most of the published PCRs were established to detect primarily FAVs (Raue and Hess, 1998; Jiang *et al.*, 1999). Recently, a PCR suitable for the amplification of a DNA fragment from all three groups of avian adenoviruses was identified and established (Xie *et al.*, 1999).

The majority of avian adenovirus PCRs published to date, took the hexon gene for primer design. The crystallographic investigations of the protein were fundamental to establish the three dimensional structure of this major capsid protein (Roberts *et al.*, 1986; Athappilly *et al.*, 1994).

According to this model, the hexon consists of conserved regions (pedestal, P1 and P2), which are located more inside the virion, and the variable loops (L1 to L4), that protrudes from the surface. Subsequently, it was shown that these loops contain the type specific neutralizing epitopes (Toogood *et al.*, 1992; Adam *et al.,* 1998). The predominant role of FAV4 as the etiological agent of Angara disease of infectious hydropericardium was also reported by Abe *et al.*, 1998; Mazaheri *et al.*, 1998). The isolation of FAV-4 strains from clinical cases of the same disease in various countries of two continents is a novelty in the field of FAV pathogenecity. The published PCRs combined with REA were applied to detect FAV-4 strains from several countries.

All isolates were detected using the H1/H2 or H3/H4 primer pair. Digestion of the H3/H4 PCR products with *Hpa* II, according to Raue and Hess (1998), offered some variation between the isolates from India and Pakistan, and all others. However, it could be

shown that together with the identical restriction profiles of the H1/H2 PCR products, all isolates were typed as FAV-4 strains (Hess *et al.*, 1999b; Toro *et al.*, 1999).

2.14.2 Work done in India

The variable region of hexon gene, encoding 728 bp of L1 and part of P1, was amplified and both the nucleotide and the derived amino acid sequences were compared with FAV-1, FAV-8 and FAV-10 by Ganesh *et al.* (2001b). Variability percentage to the tune of 8.2 per cent, 28.1 per cent and 40.3 per cent in the nucleotide sequence from FAV-10, FAV-1 and FAV-8 was reported by the authors. Dahiya *et al* (2002) confirmed the isolated virus to belong to FAV-4 after isolation of the FAV from the affected flocks on chicken embryo liver cell culture. Serum neutralization test coupled with PCR assay and RE analysis confirmed their findings.

Ganesh *et al* (2001b) documented that DNA isolated either from infected liver or purified virus when subjected to PCR using hexon gene specific primers amplified a 700 bp section of the variable region of viral DNA. The PCR amplified DNA labeled with digoxigenin (DIG-II DUPT) could be used as a probe for the detection of virus by dot blot hybridization of the viral genome. Singh *et al.* (2002) characterized 10 fowl adenovirus isolates from suspected cases of inclusion body hepatitis (IBH) in quails and broilers, by hexon based PCR combined with restriction enzyme analysis. Isolates were detected by using H1 /H2 and H3/H4 primer sets. Amplification of DNA with H1/H2 and H3/H4 primer sets resulted in fragments approximately 1219 bp and 1319 bp, respectively. *Hae* II digestion of the H1/H2 PCR products. *Hpa* II digestion of the H3/H4 PCR products characterized all the isolates in FAV groups. Kaur (2004) applied PCR technique to detect fowl adenovirus in tissue inoculums from suspected cases of HPS. The PCR and RE revealed FAV-4 that was associated with HPS outbreak.

2.15 Molecular cloning of viral DNA

2.15.1 Work done Abroad

Only sparse literature is available on cloning of fowl adenoviruses. Albiges-Rizo and Chroboczek (1990) reported the expression of the adenovirus serotype 3 (AD3) fibre in *Escherichia coli* as an insoluble protein. They included gene expression system based on bacteriophage T7 polymerase. The recombinant protein was purified with urea extraction. These researchers described that on expression and purification, recombinant protein acquired a folded structure. The gene for the major capsid protein (hexon) of FAV-10 was identified by Sheppard *et al.* (1995) by the use of expression vector pGEX. They mapped, cloned and sequenced the hexon gene for FAV-10 from a Sau3A genomic library of FAV-10. Similarly, Clavijo *et al.* (1996) mapped the FAV-8 genome by cloning selected RE fragments of the viral DNA into unique sites of the plasmid vectors pBluscript-11 (Invitrogen) or pGEM-32 (Promega). The 4.8 kb of *Eco*R1 E fragment of FAV-1(CELO) DNA located between 21 and 34 map units was gel purified and cloned into pBluescript - 11, in order to make a digoxigenin labeled probe to orient the left end of the FAV 8 DNA. According to the restriction enzyme fragments, the size of the FAV-8 genome was calculated to be 44.7 kb. Sub cloning of viral DNA fragments and hybridization studies with selected viral DNA fragments facilitated construction of the physical map of FAV-8 DNA, which would be useful for further characterization of the DNA and development as a vector virus.

2.15.2 Work done in India

In India, Barua (2002) amplified FAV-4 hexon gene into a mammalian expression vector and sequenced the clones to reveal an ORF of 2814 bp coding for a 937 amino acid long polypeptide of molecular mass 106.04 kDa and a G+C content of 58.39 per cent.

2.16 Sequencing of Viral DNA

2.16.1 Work done Abroad

Nucleotide sequencing has been extensively applied by many researchers for the molecular characterization of adenoviruses.

The hexon of murine adenovirus type 1 (MAV-1) was sequenced by Weber *et al* (1994). The sequence predicts a 908 residue of hexon protein that is flanked by a portion of the upstream pVI gene and downstream endopeptidase gene. Sheppard *et al.* (1995) sequenced the complete hexon gene for FAV 10, the first mammalian hexon to be identified. The sequence analysis recognised an open reading frame (ORF) of 2808 bp coding for a putative polypeptide 936 amino acids long with a molecular mass of 105.5 kDa. The location of the hexon gene in the FAV genome was from 46.85 to 52.881 map units, that is to the left of the hexon gene in the genomes of both bovine and human adenoviruses (52.4 to 60.5 map units). They further compared the amino acid sequence of the FAV 10 hexon with the bovine, human and murine hexons and found the highest levels of identity in the regions corresponding to the pedestals that formed the base region of the hexon, while the lowest identity in the regions corresponding to the loops that were exposed to the external environment. Chiocca *et al.* (1996) documented the complete DNA sequence of the fowl adenovirus CELO virus (FAV 1). The genome was analyzed as 43,804 kb in length, approximately 8 kb longer than those of the human subgenus C adenoviruses (Ad2 and Ad5).

The complete sequence of CELO virus revealed a larger number of striking differences between Ad2 and CELO. Cao *et al.*(1998) sequenced 7.5 kb of the left and 17 kb of the right ends of FAV-8 to compare FAV type 1, CELO strain with another FAV. FAV 8 genome sequence indicated that as for CELO virus, the left and right terminal

regions were unique to FAV in comparison to mastadenoviruses. Sheppard *et al.* (1998) analyzed the complete nucleotide sequence and map location of the short fiber gene of FAV-10 strain CFA 20. The coding sequence of the short fiber gene was adjudged as 1383 bp, encoding a putative polypeptide of 461 amino acids.

The location of gene was between 69.2 to 71.7 map units on the FAV-10 genome. The sequence of FAV-8 genome was deduced by Ojkic and Nagy (2000) revealed it to be 45063 nucleotides in length, the longest adenovirus genome. No regions homologous to early regions E1, E3 and E4 of mastadenoviruses were recognized. Gene homologous for early region 2 (E2) proteins, intermediate protein *Iv*a 2 and late proteins were grouped by their similarities to protein sequences from other adenoviruses. However, sequences homologous to intermediated protein IX and late protein V could not be identified. Two regions of repeated sequences were noticed on the FAV-8 genome. Lobanov *et al.* (2000) sequenced the complete hexon gene of KR95 strain of FAV-4 and the nucleotide sequence revealed a continuous ORF of 2814 bp coding for a 937 residue protein. They compared the predicted KR95 hexon sequence with 8 mammalian and avian hexon sequences, and observed the highest homology between KR95 strain and avian adenoviruses FAV-10 and FAV-1 being 91.1 per cent and 80.1 per cent respectively. Meulemans *et al.* (2004) compared the sequence of L1 loop of hexon protein of FAV strains from Europe and America. The study highlighted lack of consensus in the numbering of the individual serotypes between American and European classification. Phylogenetic analysis depicted six clusters, three of them were independent groups A, B and C whereas three others we re clustered in a single subgroup denominated D. Japanese strain TR 22 formed a seventh cluster.

2.16.2 Work done in India

Suresh *et al.* (1995) identified the penton base gene of hemorrhagic enteritis virus (HEV), a type II avian adenovirus, in a 2477 base pair (bp)- *Eco R1* fragment of the viral DNA by sequence analysis. The 1344 bp penton base gene of HEV encoded a 448 amino acid polypeptide of molecular weight of 50,843 Da. The penton base lacked the RGD motif, present in most human adenoviruses suggesting that HEV might not use □V integrins to gain entry into host cells. However, it had LDV motif similar to human adenoviruses. Barua (2002) cloned and sequenced full length hexon gene of Indian FAV-4 isolate. He found the sequence to be larger than the hexon coding region of FAV-10. The splice acceptor sequence was located 12 bp upstream of the hexon initiation site of the translation codon, that indicated, FAV-4 hexon could be a late gene product. They recorded a high degree of amino acid variation (9.6 per cent) between FAV-4 and FAV-10 as compared to 1.5 per cent variation between the Indian isolate of FAV-4 and the reference strain KR 95. The L1 loop region revealed the maximum sequence variation among FAV serotypes. They suggested that FAV4 might had been evolved by recombination of FAV-9 and FAV-10. The nucleotide sequence of adenoviruses (Ad) type 1 and 6 fiber genes was determined by Adhikary *et al.*(2004) to clarify the molecular basis of the distinct haemagglutination properties of subgenus C adenoviruses and their phylogenetic relations.

2.17 Diagnosis

2.17.1 Work done abroad

For the detection and identification of *Aviadenoviruses* a certain number of technologies like transmission electron microscopy (TEM), agar gel precipitin (AGP),

enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and hemagglutination (HA) may be utilized (Hess,2000).

The diagnosis of HPS before the occurrence of mortality is difficult as the birds do not show specific clinical signs. In spontaneous outbreaks, the clinical diagnosis of HPS is hardly if ever possible because of the acute nature of the disease. The disease might be suspected on the basis of a sudden occurrence of high mortality among broiler chicks with hydropericardium as the predominant lesion (Anjum *et al.*, 1989; Cheema *et al.*, 1989; Abdul-Aziz and Al-Attar, 1991) and the demonstration of intranuclear inclusion bodies in hepatocytes which were basophilic in nature were pathognomonic (Akhtar,1994). The diagnosis of IBH-HPS infection had been carried out on the basis of gross lesions, histopathological lesions and demonstration of adenovirus particles in the nuclei of infected cells by transmission electron microscopy (Cheema *et al.*, 1989). The confirmation of the isolate had been done by neutralization tests (Rabbani and Naeem, 1996). Since its etiology had been confirmed, immunodiagnosis was possible through serological tests such as gel diffusion (Noor-ul-Hassan *et al.*, 1994). Akhtar (1994) diagnosed HPS from liver homogenate by AGPT by using liver homogenate as a crude antigen.

Takase *et al.*(1995) compared the agar gel precipitation responses obtained for serologically different strains of FAV in tests employing antigens prepared from FAV infected chorioallantoic membranes (CAM) antigen and chicken kidney cell cultures (CKC) antigen. The findings explained that both types of antigens exhibited less sensitivity to heterologous than to homologous antisera and quantitative differences in sensitivity were apparently present between serotypes. CAM antigens were more sensitive than CKC antigens to heterologous antisera. Likewise, Abe *et al.* (1998) performed AGPT and CIE using hyper immune serum. The indirect hemagglutination test was shown to be highly

efficacious tool to diagnose the agent of HPS by (Rahman *et al.*, 1989 and Hassan *et al.*, 1993). The dot immunobinding assay was analyzed to be specific and reliable for diagnosis of the agent (Naeem *et al.*, 1995a; Rabbani *et al.*, 1998b). The polymerase chain reaction (PCR) had also been developed for the quick diagnosis of HPS (Toro *et al.*, 1999).

2.17.2 Work done in India

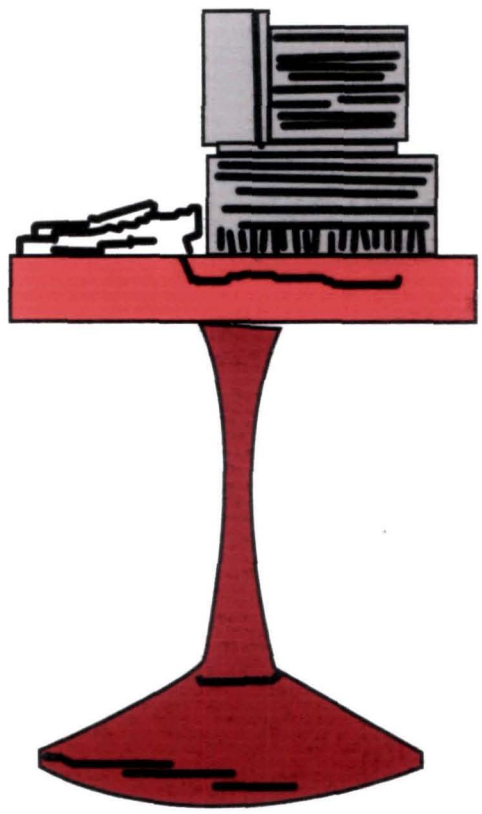
The sudden occurrence of high mortality among broiler chicks aged between 3-6 weeks with hydropericardium is one of the fool proof ways of diagnosing of hydropericardium syndrome (Kumar *et al.*, 1997). Diagnosis of IBH-HPS infection had also been carried out on the basis of gross lesions, histopathological lesions, particularly of intranuclear inclusion bodies in hepatocytes (Gowda and Satyanarayana, 1994) demonstration of adenovirus particles in the nuclei of infected liver cells by transmission electron microscopy (Chandra *et al.*, 1997; Ganesh *et al.*, 2001b) or isolation of the virus, either in cell culture or in embryonated eggs (Kataria *et al.*, 1996, 1997a). CIE remained as one of the major diagnostic tools to confirm the presence of FAVs (Oberoi *et al.*, 1996).

Kumar *et al.* (2003) applied various serological tests, *i.e.* AGPT, CIE, micro serum neutralization test and ELISA to confirm the isolation of etiological agent of HPS in CEL cell culture and to diagnose the disease in poultry. The fluorescent antibody technique (FAT) was successfully applied for diagnosis by Deepak (1998) and Balamurugan (1999) and various modifications of ELISA were tested by Oberoi *et al.* (1996) and Balamurugan *et al.* (1999, 2001). Chand (2006) also used AGPT to confirm and characterize the virus used in his study as FAV-4 after positive reaction with hyperimmune reference serum of FAV-4. Deepak (1998), while studying the pathogenesis of HPS in 2 weeks old chickens, detected an immunoflourescent FAV antigen in the thymus, spleen and bursa from 3 to 10 DPI, in the liver from 3 to 14 DPI and in the heart up to 5 DPI. In an attempt to develop a

laboratory method for diagnosing HPS in chickens, Balamurugan *et al.* (2001) detected viral antigen in various tissues *viz* .liver, kidney, bursa of Fabricus, spleen and thymus from experimentally infected chickens by antigen capture (sandwich) ELISA, using guinea pig and chicken hyper immune sera for trapping and tracing the viral antigen, respectively.

Dahiya *et al.* (2002) and Ganesh *et al.* (2002) performed PCR to diagnose HPS in India. The DNA isolated from either infected liver tissue or purified virus was subjected to PCR with hexon gene specific primers that amplified a 700 bp section of the variable regions of HPS viral DNA (Ganesh *et al.*, 2002). The PCR amplified DNA was labeled with digoxigenin (DIG-11-DUTP) and applied as a probe for the detection of virus by dot blot hybridization of the viral genome (Ganesh *et al.*, 2002).

Materials and Methods



Chapter III

MATERIALS AND METHODS

3.1 Source of materials

3.1.1 Source of the virus

In the present study, the following isolates of fowl adenovirus (FAV) were taken for further studies.

1) Two FAV isolates which were recovered from different outbreaks of HPS in broilers in the state of Himachal Pradesh and one characterized as FAV-4 by the Department of Veterinary Pathology (Chand, 2006), Dr. G. C. Negi College of Veterinary and Sciences, Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya (DGCN, COVAS, CSKHPKV), Palampur. These isolates were in the form of samples of infected liver homogenate suspension and infected liver pieces.

2) One standard isolate of FAV-4 along with hyperimmune serum was kindly provided by the Department of Veterinary Microbiology (*courtesy* of Dr. T.G. Prabhakar), Tamil Nadu University of Veterinary and Animal Sciences (TANUVAS), Chennai. This isolate was also used as source of positive viral DNA.

The description of isolates is as under:

Virus isolate(s)	**Place of Origin**
a) Palampur-I (P-I)	Himachal Pradesh
b) Palampur Himachal-II (PHII)	Himachal Pradesh
c) Palampur Chennai-III (PC-III)	Chennai

3.1.2 Antigens, antisera and conjugates

The standard fowl adenovirus serotype-1 (FAV-1), chicken embryo lethal orphan (CELO) virus in the form of infected allantoic fluid and corresponding hyperimmune sera raised in rabbit were received from the Department of Veterinary Microbiology (*courtesy* of Dr. Ramneek Verma), College of Veterinary and Animal Sciences, Guru Angad Dev Veterinary and Animal Science University (GADVASU), Ludhiana, Punjab. Vaccine strain (Lasota) of Newcastle disease was procured from Indian Veterinary Research Institute (IVRI), Izatnagar, U.P. The antispecies Horseradish Peroxidase (HRPO) and Fluorescein isothiocyanate (FITC) conjugated immunoglobulins were procured in the department from Sigma Immunochemicals, St. Louis, USA.

3.1.3 Embryonated eggs

Day old embryonated eggs were continuously procured from the poultry unit, Department of Animal Breeding, Genetics and Biostatistics, DGCN COVAS, CSKHPKV, Palampur, H.P. Eggs were thoroughly cleaned with lukewarm water, rubbed with 70 per cent alcohol and incubated at 37°C with 80 per cent relative humidity in an egg incubator (Remi Egg Incubator). Further, these were inoculated at an appropriate age for the propagation of the CELO virus and the virus isolates and also for the preparation of primary chicken embryo fibroblast, chicken embryo liver and chicken embryo kidney cell cultures.

3.1.4 Calf serum

Heat inactivated fetal calf serum (HyClone laboratory, USA) available in the department was used after filtration through membrane filter of pore size 0.22 µm. Aliquots of 50 ml were made and stored at -20°C for further use. One small aliquot of 2 ml was kept at 37°C for a week to check for any contamination.

3.1.5 Plastic wares

All plastic wares including tissue culture flasks, plates, disposable pipettes and membrane filter assembly used were obtained from Nunc-Denmark and Tarsons-India.

3.1.6 Glasswares

All the glass wares used in this study were available in the department and were procured from Borosil (India) and Schott Duran (Germany).

3.1.7 Chemicals and culture media

All the standard reagent grade chemicals were procured from Sigma chemicals (USA), SRL (India), Merck (India), Hi Media (India) etc.

3.1.8 Solutions and buffers

Composition of solutions, buffers and media used in this study are given in the Appendix or at appropriate places in the text.

3.1.9 Washing and sterilization of glass wares for cell culture work

3.1.9 (a) *New glasswares*

All the new glasswares were immersed in 0.1 per cent sodium carbonate solution for 24 hrs. These were then washed thoroughly and rinsed 10 to 15 times in tap water and later on rinsed 10 times in double distilled water, dried upright, plugged with cotton plug, covered with aluminum foil and paper and sterilized in hot air oven at 160^{o}C for 2 hrs.

3.1.9 (b) *Used glasswares*

After use, all the used glass wares were autoclaved at 121^{o}C at 15 lbs pressure for at least 15 min. and further rinsed in tap water to remove all the soiled material.

Glass wares were then put in hot water with mild detergent and washed thoroughly with tap water for 10 to 15 times to remove all the detergent. These were finally rinsed

with double distilled water at least 6 times, then dried upright, plugged with a cotton plug, covered with aluminum foil and paper and sterilized in hot air oven at 160°C for 2 hrs.

3.1.9 (c) *Pipettes*

All the glass pipettes were put in pipette washer under running water for 16 to 24 hrs. Pipettes were then rinsed with double distilled water at least six times. After washing, all the pipettes were kept in hot air oven for drying. Pipettes were then plugged with cotton and placed in canisters and then sterilized at 160°C for 2 hrs.

3.1.10 Experimental animals and birds

Adult healthy rabbits (4 to 5 months of age) were obtained from Rabbit Breeding farm, Kandbari, Department of Animal Husbandry, Himachal Pradesh. Healthy chickens around a week old, were procured from poultry unit of Department of Animal Husbandry, Himachal Pradesh. These were reared for 3 to 4 months in the Department of Veterinary Microbiology and were used for raising antisera against fowl adenovirus. Some of these chickens at 2 to 3 weeks of age were also used for testing the viability of the isolates.

Day old unvaccinated broiler chicks of either sex were also procured from the above source that had no earlier history of occurrence of HPS in the parent flock for experimental infection for the detection of virus and for histopathological studies.

These chicks were kept in well ventilated rooms of the departmental animal house under strict hygienic conditions. Prior to procurement of chicks, the rooms of the birds were thoroughly sterilized by fumigation. The cages were disinfected using 5 per cent phenol solution, and were thoroughly flamed using a blow lamp while the utensils were autoclaved and then they were kept in a fumigated room (fumigated using a mixture of 35 ml of concentrated formalin and 17.5 gm potassium permanganate per cubic feet area). All the birds were given *ad lib* autoclaved standard chick feed procured from the Department

of Animal Nutrition, DGCN COVAS, CSKHPKV, Palampur (*Appendix II*) and provided *ad lib* clean autoclaved drinking water throughout the experiment.

3.2 Virus preparation

3.2.1 Preparation of the virus inoculum

All the samples (infected liver homogenate suspension, infected liver tissue samples and lyophilized culture fluid) were tested for the virus viability before starting the proposed study. The infected liver samples were weighed and 30 per cent liver homogenate suspension (w/v) suspension was prepared using sterile phosphate buffered saline (PBS) (0.01 M, pH 7.4) in a sterile pestle and mortar containing sterile sand. The supernatants were filtered through 0.22 μm pore size filter to eliminate any bacterial contamination.

The samples of liver homogenate suspension and lyophilized culture fluid (after reconstitution in sterile PBS) were also filtered, before the supernatant was used. All the samples were tested for the virus viability by inoculating into six birds aged 2 to 3 weeks old @ 0.5 ml / bird by subcutaneous route (two birds per isolate) and were observed for a period of one week. Three birds inoculated with equal amount of sterile PBS were kept as control.

Following the death of the inoculated birds, post mortem were carried out. The control chicks were also sacrificed to match the findings *i.e.* gross lesions. Liver samples so obtained from these dead birds were collected in sterile PBS (pH 7.4) for the preparation of virus inoculum. These were cut into small pieces, weighed, triturated in sterile pestle and mortar with sterile sand and then a 20 per cent liver homogenate (w/v) suspension was prepared in sterile PBS (0.01 M, pH 7.4). The supernatants were collected and two cycles of freezing and thawing at -20°C and at 37°C for 30 min., respectively were given. The suspensions were then centrifuged at 10,000 rpm for 30 min. at 4°C. Antibiotics (penicillin

1000 IU/ml and streptomycin 1 mg/ml) were added to the supernatant and then incubated for 2 hrs. at 37°C. These were then filtered through a 0.22 μm pore size Millipore filter.

The entire procedure was carried out under sterile conditions in a laminar air flow. The suspension so obtained was checked for sterility by inoculating on blood agar plates and thioglycollate broth tubes by incubating them for 48 hrs. and seven days, respectively. After confirmation of sterility, these suspensions were then preserved as small aliquots in sterile vials at -20°C till further use. One representative aliquot each from the aliquots of the three samples were then tested with the reference anti FAV-4 hyper immune serum by Agar gel precipitation test (AGPT) and then further classified as the isolates (P-I, PH-II and PC-III) used in our study.

For determination of single approximate infective dose to be used as inoculums in chicks, 10 chicks (two chicks per dose) were inoculated with different doses of inoculums via subcutaneous route as done by Thakur (1998). It was observed that 0.5 ml dose of viral inoculum caused 50 per cent morality in birds with characteristic gross lesions at necropsy. Additionally, 0.1 ml dose was found to cause 50 per cent mortality in embryonated eggs.

3.2.2 Propagation of standard fowl adenovirus -1 (FAV-1)

The standard FAV-1 isolate (CELO virus) obtained in lyophilized form, after reconstitution in PBS (pH 7.2) was filtered and inoculated into 11 days old embryonated eggs through allantoic sac route (0.1 ml/chicken embryo) and incubated at 37°C in an egg incubator. The embryos were candled twice daily. The embryos that died after 48 hrs. of inoculation were chilled and allantoic fluid was harvested. The harvested allantoic fluid was stored at -20°C for further use.

3.2.3 Titration of virus in embryonated eggs for determining infectivity titre

All the three isolates along with CELO virus were titrated in 6 to 8 day old chicken embryos via yolk sac route. Serial ten fold dilutions of each isolate were made logarithmically (10^{-1} to 10^{-10}) in sterile PBS (pH 7.4). Each dilution was inoculated into five embryos in a quantity of 0.1 ml per embryo. Control eggs were inoculated with equal quantity of sterile PBS. The eggs were then incubated at 37°C for a week. Death of embryos within first 48 hrs. of inoculation were considered to be nonspecific. The titer of virus was calculated in terms of 50 per cent embryo infective dose ($\log_{10}$ $EID_{50/}$ ml) by the method of Reed and Muench (1938).

3. 3 Cell cultures

3. 3.1 Primary cell cultures

The following primary cell cultures *viz.* chicken embryo fibroblast, chicken embryo liver and chicken embryo kidney cell cultures were used in the study for the adaptation of the virus.

3.3.1.1 Chicken embryo fibroblast cell culture

Primary chicken embryo fibroblast (CEF) cell cultures were prepared by warm trypsinization method (Cole and Paul, 1966; Freshney, 2000 and Schat and Purchage, 1989).

Two eight to ten days old embryonated eggs were candled for ensuring their viability and the air spaces were marked. The shells of each egg were cleaned with 70 per cent alcohol and painted with Lugol's iodine. The top of the egg was punctured carefully with the help of sterile scissors and the embryos were pulled out from the egg with the help of a curved forceps, and the underlying membranes cut with sterile scissors. The embryos were

collected in a petri dish containing sterile PBS (pH 7.4). The head, appendages, eyes and viscera were removed and the embryos were washed thrice with PBS. The embryos were minced into fine pieces and transferred to a 500 ml capacity Erlenmeyer flask. The minced tissues were further washed with PBS in a flask by stirring with a sterilized teflon coated magnetic bar on a magnetic stirrer for about five min. and the supernatant was discarded.

Then, 0.25 per cent prewarmed trypsin versene (TV) solution was added to the embryos @ 20 ml per embryo. The tissues were gently agitated on a magnetic stirrer at room temperature. After 10 to 15 min., the supernatants were decanted into a sterilized flask containing 2 per cent fetal calf serum in minimum essential medium (MEM) (Hi Media limited). Thereafter, the flask was kept on ice to neutralize the trypsin activity and fresh TV solution was added followed by stirring for additional 10 to 15 min. The trypsinization was continued till the time there were only fibrous tissues left behind. Each time the supernatants were decanted into the flask containing 2 per cent fetal calf serum. The cell suspensions so obtained, were then filtered through sterile double layered muslin cloth tied to a beaker to remove the larger tissue fragments. The cellular suspensions were then spun in a centrifuge at 2500 rpm for five min. The supernatants were discarded and the cell pellet suspended in PBS and centrifuged again. One more washing was given to the cell pellet likewise.

Finally, the cells were suspended in growth medium (MEM with 5 per cent heat inactivated fetal calf serum) to give a final concentration of 1×10^5 cells per ml. The cell suspension was seeded into tissue culture flasks, Roux flasks and Leighton tubes with cover slips @ 10 ml, 100 ml and 1 ml, respectively. The flasks and tubes were incubated at 37°C with 5 per cent CO_2 tension in a CO_2 incubator (Thermo Electron Corporation) for the monolayer to form. For maintaining the CEF cell cultures, the cells were washed thrice with PBS and supplemented with MEM containing one per cent fetal calf serum.

3.3.1.2 Chicken embryo liver (CEL) cell culture

Chicken embryo liver cell cultures (CEL) were prepared as described by Adair *et al.* (1979). Chicken embryos of 14 to 16 days old were used and the cells were prepared in M 199 medium supplemented with 20 per cent fetal calf serum, while, 5 per cent fetal calf serum was added in the maintenance media.

Eight to ten, 14 to 16 days old embryonated chicken eggs were candled to ensure their viability and the air sac was marked with a pencil. Eggs were then placed in a sterilized egg tray with blunt end up and swabbed liberally with 70 per cent alcohol and then painted with lugol's iodine. The top of the egg was punctured carefully with the help of sterile scissors thus exposing the underlying membrane. With the help of a curved forceps, the embryo was pulled out from the egg and the underlying membranes cut with a sterile scissors. Embryos were carefully removed and put in a sterile petri dish containing PBS (pH 7.4). Livers from the embryos were removed aseptically and washed twice with PBS (pH 7.4). The livers were then finely minced with curved scissors. The minced pieces were trypsinized gently using 0.25 per cent TV solution. The trypsinized liver suspension was then filtered through a muslin cloth into a beaker containing 2 per cent serum in M199 medium. The collected filtrates were centrifuged at 2500 rpm for 10 min. and the cell pellet obtained was washed twice with growth medium M199 supplemented with 15 per cent (v/v) fetal calf serum to remove residual trypsin.

The final cell pellet was resuspended in M199 containing 20 per cent fetal calf serum. The live or dead cell count was done by then counted by Trypan blue exclusion and the final cell concentration were then adjusted to contain 5×10^6 live cells/ml. before seeding. The amount of cell suspension that were seeded into Leighton tubes, plastic tissue culture flasks 25 cm^2, 75 cm^2 and roux flasks were @1 ml, 10 ml, 30 ml and 100 ml respectively. The flasks were incubated at 37°C under 5 per cent CO_2 tension in a CO_2

incubator (Thermo Electron Corporation). After 5 to 6 days of incubation, when 70 to 80 per cent monolayer was formed, it was further used to propagate the virus. The monolayers were examined using an inverted phase contrast microscope (Nikon TMS inverted microscope).

3.3.1.3 Chicken embryo kidney (CEK) cell culture

Monolayers of primary CEK cells were prepared by the method of Cunningham and Spring (1965) from the kidneys of six to eight, 18 days-old chicken embryos with minor modifications.

Six to eight 18 days and above old embryonated chicken eggs were candled to ensure their viability and the air sac was marked with a pencil. Eggs were then placed in a sterilized egg tray with blunt end up and swabbed liberally with 70 per cent alcohol thereafter painted with lugol's iodine. The top of the egg was punctured carefully with the help of sterile scissors thus exposing the underlying membrane. With the help of a curved forceps, the embryo was pulled out from the egg and the underlying membranes cut with a sterile scissor. Embryos were carefully removed and put in a sterile petri dish containing PBS (pH 7.4). The abdomen was cut open and all the internal organs were dissected out with the help of a curved scissor and forceps. The kidneys that lied at the posterior part of the body attached to the backbone were then gently cut with sterile scissors and placed in sterile PBS and washed thoroughly with three to four changes of sterile PBS (pH 7.4). The kidneys were then finely minced with curved scissors.

The minced pieces of kidneys were trypsinized gently using 0.25 per cent TV. The trypsinized kidney suspension was then filtered through a muslin cloth in a beaker. The collected filtrate was centrifuged at 2500 rpm for 10 min. and the cell pellet obtained was washed twice with PBS. Finally, one washing was given with medium M199 and the cells

resuspended in medium M199 containing 15 per cent fetal calf serum. The amount of cell suspension that were seeded into plastic tissue culture flasks 25 cm^2, 75 cm^2 and Leighton tubes were @10 ml, 30 ml and 1 ml, respectively. The flasks were incubated at 37°C under 5 per cent CO_2 tension in a CO_2 incubator (Thermo Electron Corporation). The monolayers were examined daily under an inverted phase contrast microscope for the formation of a complete monolayer. When 70 to 80 per cent monolayer was formed, it was further used to propagate the virus. For maintaining the CEL and CEK cell cultures, the monolayer were washed lightly with PBS thrice and supplemented with M 199 containing 5 per cent fetal calf serum.

3.3.2 Cell lines

3.3.2.1 Types of cell lines

The following cell lines were procured from the National Center for Cell Sciences (NCCS), Ganeshkhind, Pune, Maharashtra. These cells were Vero cell line (African green monkey kidney cell line), McCoy cell line (Mouse cells / Human fibroblastic cells), HeLa cell line (Human cervical cancer cell line) and BHK-21 cell line (Baby hamster kidney). The cells were received as a monolayer cultures in 25 cm^2 tissue culture flask.

3.3.2.2 Propagation of cell lines

All the cell lines were grown to confluence with growth medium containing MEM with 10 per cent fetal calf serum. The cells were grown at 37°C under 5 per cent CO_2 atmosphere conditions. The split ratio and medium change for each cell lines were done strictly according to the instructions given by NCCS. For maintaining the cell lines, medium MEM with 1 per cent fetal calf serum was used. The cell lines were grown to confluence in plastic tissue culture flasks.

After complete formation of monolayer, the medium was removed and three washings were given with sterile PBS to remove serum. Afterward, one ml of Trypsin Versene Glucose (TVG) (*Appendix I*) was added to the flasks. The flask was kept at room temperature with occasional tilting until the cells detached. Then fresh culture medium with serum @ 10 per cent was aspirated and dispensed into new culture flasks along with the cells.

3.3.2.3 Infection of cell cultures / continuous cell lines

When the monolayers were 80 to 90 per cent confluent, the growth medium was decanted and 0.1 ml of inoculum was added to a 25 cm^2 tissue culture flask and 1 ml to 75 cm^2 or Roux flask. Cover slip cultures were also prepared for infection and controls. Simultaneous inoculations of similar flasks with equal amount of sterile PBS served as uninoculated monolayer controls. The inoculum was allowed to adsorb at 37°C for one hour. After one hour, the inoculum was pipetted out and the monolayer was washed with PBS. Finally, maintenance medium (MEM/M199) were added to each monolayer including the control and incubated at 37°C. Each monolayer was examined daily for the appearance of cytopathic effects (CPE).

3.3.2.4 Harvesting of virus

The cultures were observed for CPE for about 5 to 7 days after inoculation. The tubes and flasks were thoroughly shaken and repeatedly pipetted in order to detach the cells from surface of tubes and flasks. The virus was released from the cells by three alternative cycles of freezing (-20°C) and thawing (37°C) of 30 min. duration, each. The contents were then centrifuged at 4000 rpm for 15 min. at 4°C and filtered through 0.22 µm pore size millipore filters for use as inoculum for further passage.

For blind passages, the cells were harvested by freezing and thawing thrice. The clear supernatant obtained after centrifugation at 4000 rpm for 15 min. at 4°C were filtered as described above before using the inoculum for blind passages.

All the three isolates used in the investigation were passaged ten times in CEF cell cultures, eight times in each cell line and five times in CEL and CEK cultures. All the cell cultures were tested for the presence of virus by observing CPE and by serological and molecular tests.

3.3.2.5 Staining of the monolayer

The maintenance medium was decanted and the cover slips were washed 2 to 3 times with PBS (pH 7.4) and fixed in absolute methanol for 10 min. The fixed monolayer on the cover slips was stained with May-Grunwald stain (Merck India limited) diluted 1:2 in buffered distilled water for 15 min. Thereafter, counterstaining was done with Giemsa stain (Giemsa stock freshly diluted 1:10 in buffered distilled water (*Annexure I*) for 30 min. After washing one or two times with buffered distilled water for differentiation, the cover slips were mounted upside down on slides with DPX and observed under suitable magnification and photographed.

3.3.2.6 Titration of virus harvested after passage from cell culture

Harvested materials from cell cultures that exhibited CPE *viz.* CEF, CEL, CEK and Vero cell culture were again titrated in chicken embryos to find out the change in virus infectivity titer after cell passage. The infectivity titer of virus after passage was calculated in terms of $\log_{10} EID_{50}$ / ml by the method of Reed and Muench (1938).

3.3.2.7 Re infection in chicks with virus harvested from cell cultures

The virus harvested from different cell cultures was inoculated into groups of three, day old chicks to check whether the virus retained the ability to infect chickens after passage in cell culture. The harvested cell culture supernatants from four infected cell cultures *viz.* CEF (tenth passage level), CEL (fifth passage level), CEK (fifth passage level) and Vero cell line (eighth passage level) were used for inoculating the chicks.

Eighth passage level culture supernatants from HeLa, McCoy and BHK-21 cells were also used for re infective studies even though cytopathic changes could not be detected in these three cell cultures.

The infective viral inoculums harvested from cell cultures were frozen and thawed thrice. Thereafter, these were centrifuged at 4000 rpm for 15 min. at 4°C and the supernatants were collected. The supernatants were filtered through 0.22 µm pore size filter and 0.5 ml of these inoculated into three chicks by subcutaneous route. Two chicks per group were kept as control and inoculated with an equal amount of sterile PBS.

The chicks were observed for one week and the mortalities were recorded. The dead chicks were opened and different organs were collected in sterile PBS for detection of the virus by different serological tests as well as for DNA extraction from the tissues for further detection by PCR. The pooled liver homogenates per cell culture infected group were again collected, processed by triturating, then freeze thawed, centrifuged and filtered through 0.22 µm size filter before inoculating one set of respective cell cultures for confirmation of growth.

3.3.2.8 Studies in embryonated eggs

To observe the severity of effects of fowl adenoviruses on chicken embryos on each subsequent passage, the three isolates were passaged thrice in five (6 to 8 days old)

chick embryos @ 0.1 ml of each isolate via yolk sac route as described by Bansal (1996) along with suitable controls. The embryonated eggs were incubated at 37°C for 7 days and candled twice daily. The death of embryo within first 48 hrs. of inoculation was considered to be non specific. The control embryos were simultaneously inoculated with an equal volume of sterile PBS. Following the death of the embryo, the inoculated eggs were chilled at 4°C for at least 2 hrs. before harvesting. The pathogenicity of the causal agent was observed by recording the growth rate, mortality and gross lesions of embryos. The growth rate was observed by recording the length, width and weight of embryos.

The infected livers that were obtained from the dead embryos were processed to make a 20 per cent suspension that was used to inoculate eggs further. The materials like livers, yolk as well as amnioallantoic fluid were also collected from eggs. Antibiotics such as penicillin 1000 I.U and streptomycin @ 1 mg per ml of the collected materials were added and the mixture was incubated at 37°C for 2 hrs. and preserved in small aliquots at -20°C until further used. Detection of virus by both serological and molecular methods was done from these infected eggs.

3.4 Detection of virus

The detection of the virus was done both by serological as well as by molecular tests from different tissues and cell cultures.

3.4.1 Serological tests for the detection of the virus

Various serological tests like AGPT, Counter immunoelectrophoresis (CIE), Dot Enzyme linked immunosorbent assay (Dot ELISA) and Fluorescent antibody technique (FAT) were used to detect the presence of the virus in different samples. These tests were standardized before use.

3.4.1.1 Standardization

3.4.1.1.1 Production of hyper immune sera

The hyper immune serum to the fowl adenovirus group antigen was prepared for use in serological tests.

3.4.1.1.2 Bulk production of fowl adenovirus for production of hyper immune serum

The CEL cell monolayer prepared in roux flasks and in 75 cm^2 flasks were washed twice with sterile PBS and infected with 3 ml and 1 ml of P-I virus inoculum, respectively. The cultures were incubated at 37° C for one hour with occasional tilting for adsorption. Following adsorption, the inoculum was pipetted out and maintenance medium containing 5 per cent fetal calf serum was added to each flask. The flasks were incubated at 37° C for 3 to 4 days and cultures were harvested when it exhibited 80 to 90 per cent CPE and further frozen at -20° C.

3.4.1.1.3 Partial purification of fowl adenovirus for raising hyper immune serum

The FAV (P-I isolate) was concentrated and partially purified for raising hyper immune serum following the method of Capua *et al.* (1995). CEL culture propagated virus was frozen and thawed three times. The supernatant was homogenized with about half the amount of reagent grade chloroform by mixing the supernatant very vigorously. The homogenized virus preparation was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was collected and centrifuged at 1,00,000×g for 3 hrs. at 4°C in ultracentrifuge (Fixed angle rotor A-641, Sorvall Ultra Dupoint OTD 65 B). The resultant virus pellet was suspended in 1.0 ml of TE buffer (Tris HCl 100 mM, EDTA 10 mM, pH 8) per tube and kept overnight at 4°C. The virus suspension was then layered over 5 to 7 ml of 30 per cent (w/v) sucrose cushion and centrifuged at 1,00,000×g for 2 hrs. at 4°C in swinging out rotor (Swing out rotor AH-629). Finally, the virus pellet was suspended in 1.0 ml of TE buffer

per tube and kept overnight at 4°C. The virus pellet was collected in sterile micro centrifuge tubes and stored at -20°C until further use.

3.4.1.1.4 Raising of hyper immune sera against FAV in chicken and rabbits

The hyperimmune serum was raised against P-I isolate in rabbits and chickens as described by Saifuddin and Wilks (1990). The pellet of semi purified isolate of P-I was resuspended in about 2 ml of PBS (pH 7.2). Two healthy chickens of about 3 to 4 months of age and free from fowl adenovirus antibody (tested negative by AGPT with FAV isolate) and two healthy adult rabbits of approximately 1.5 kg body weight were used. Serum collected from the animals prior to injection of the antigen served as negative control serum. Viral antigen with protein concentration of 470µg/ml (measured spectrophotometrically) was mixed with an equal volume of Freund's Complete Adjuvant (FCA) (Sigma Immunochemicals, USA). Each chicken and rabbit was injected intradermally at different sites with 1 ml of mixture. After 3 weeks, a second injection was prepared in Freund's Incomplete Adjuvant (FIA) (Sigma Immunochemicals, USA) and injected @ 1 ml to each chicken and rabbit intramuscularly in thigh muscle. Two weeks later, a second booster was injected @ 1 ml intramuscularly in thigh muscles to each animal. A week after the last injection, maximum blood was collected from each animal. The serum was then separated, heat inactivated at 56°C for 30 min., mixed with sodium azide @ 0.01 per cent for preservation and stored in small aliquots at -20°C until further use.

3.4.1.2 Removal of cross reacting antibodies from hyper immune sera

Liver tissue powder prepared from healthy day old chicks was used to remove antibodies from hyper immune sera that normally react with healthy chicken liver cells.

3.4.1.2.1 Preparation of healthy chicken liver tissue powder

The chicken liver tissue powder was prepared as per the method of Sambrook *et al.* (1989). The livers were aseptically removed from healthy unvaccinated one day old chicks after sacrificing them. The liver tissues were minced with scissors and washed thrice with 0.1 M ice cold NaCl solution. The serum of chicks was checked for fowl adenovirus antibodies by AGPT to declare it as negative.

After homogenization of the minced tissue, the cell suspension was filtered through three layers of sterile muslin cloth and the filtrate was again washed with ice cold sterile normal saline. The filtrate was centrifuged and the cell pellet was resuspended in ice cold saline @ of 2 ml per gm of cells. To the resuspended cells, 4 volumes of chilled acetone at -20° C was added, mixed vigorously and kept on ice for about 1 hr.

The cell suspension was then centrifuged at 10,000 rpm for 10 min. at 4°C in a cooling centrifuge (Remi High Speed Centrifuge). After discarding the supernatant, the pellet was resuspended in 4 volumes of chilled acetone and kept on ice for 10 min. After incubation, cells were recentrifuged, supernatant was discarded and acetone extracted cell pellet was transferred to a piece of filter paper and mixed from time to time to evaporate the acetone. The completely dried powder was then triturated, filtered through fine sieves and finally transferred into screw capped vial and stored at -20°C.

For removal of the cross reacting antibodies from chicken and rabbit hyper immune sera, chicken liver cell powder was adsorbed with sera at the final concentration of 1 per cent (w/v) and stored on ice for 15 min. The adsorbed sera were centrifuged at 10,000 rpm for 10 min. at 4°C. Cross reactivity of chicken and rabbit hyper immune sera (absorbed and unabsorbed) with healthy CEL cells was also checked by AGPT. For this, the rabbit and chicken raised hyper immune sera were checked against healthy CEL culture fluid and the results recorded.

Various serological tests were standardized for the detection of viral antigens in different tissues using the hyper immune sera raised in rabbits and chickens. The sera collected both from rabbits and chickens were checked by AGPT against the three isolates used in the study and were found to react and all the isolates were found to be identical.

3.4.1.3 Experimental infection in chicks

For standardization of serological tests, six healthy 2 to 3 week birds, free of FAV antibodies were infected with virus @ 0.5 ml / chick, subcutaneously and observed daily for any mortality. Various tissues *viz.* liver, heart, bursa, thymus and kidney were collected from the dead chicks showing gross lesions. 50 per cent tissue homogenate suspensions in PBS (w/v) prepared from the dead chicks were used as positive control and stored at -20°C until used.

3.4.1.3.1 Design of experiment

For detection of FAV antigen in the infected tissue materials, thirty, day old healthy chicks (10 chicks per isolate with suitable control chicks) free of FAV antibodies as checked by AGPT were infected with 20 per cent liver homogenate of the three isolates (P-I, PH-II and PC-III) @ 0.5 ml / chick, orally and observed daily for any mortality. The control chicks were inoculated with an equal amount of sterile PBS and kept in a separate enclosure. Following the inoculations, the birds were observed for any mortality for up to 7 days post inoculation and the results recorded. Various tissues like liver, heart, bursa, thymus and kidney were collected at various time intervals. The control chicks were also sacrificed at the end of the experiment. Different measurements were taken from both the infected and control groups.

Various tissues *viz.* liver, heart, bursa, thymus and kidney were collected from the dead chicks showing gross lesions. Virus/ viral antigen in different organs *viz.* liver, heart, bursa, thymus and kidney at various post inoculation intervals was detected by AGPT and it was matched with other serological techniques like CIE, Dot ELISA and IFAT. Tissues from control birds were used as negative controls.

The tissues were also collected in 10 per cent buffered formalin and histopathological studies were carried out in the Department of Veterinary Pathology, DGCN COVAS, CSKHPKV, Palampur, H.P. Simultaneously, DNA was also extracted from the tissues and subjected to viral detection by PCR.

3.4.1.4 Serological tests

The following tests *viz.* AGPT, CIE, Dot ELISA and FAT were employed to serologically detect the presence of virus in different samples like organs of experimental chicks, cell culture supernatants, parts of infected embryonated eggs and organs from chicks given re infection with cell culture supernatants.

3.4.1.4.1 Agar gel precipitation test and staining of slides

AGPT was performed as per standard procedure (Woernele, 1966). For this, one per cent agarose prepared in 8 per cent NaCl (pH 7.4) was poured on microscopic slide precoated with 0.3 per cent agar in 8 per cent NaCl. Precoating was done to prevent the slipping of gel from the slide during subsequent staining procedures. After solidification of agar, one central and six peripheral wells (3 mm diameter) at a distance of 5 mm from the centre of central well were punched out.

AGPT was used to detect the virus in different forms of samples. The AGPT was standardized using both positive and negative samples. The centrifuged supernatant of known positive liver homogenate at various dilutions (10 to 50% w/v in PBS) was tested

against various dilutions (1:2 to 1:32 v/v) of hyperimmune serum against FAV-4 raised in chicken. It was found that 20 per cent liver homogenate suspension gave a single distinct precipitation line against 1:4 dilution of hyperimmune serum and thus was used as positive control while 20 per cent uninfected liver homogenate was used as negative control.

The different tissues from experimentally infected birds *viz.* liver, heart, thymus, bursa and kidneys were collected from experimentally infected and control chicks in sterile PBS. The tissue samples obtained from chicks at various time intervals were triturated in sterilized pestle and mortar in PBS (pH 7.4) and a 20 per cent (w/v) suspension of each sample was prepared. The tissue suspensions were centrifuged at 4000 rpm for 15 min. The clear supernatants were collected and used for testing by AGPT and CIE.

For detection of viral antigen from various tissues, the central well was filled with anti FAV-4 hyper immune serum raised in chicken and peripheral wells were filled with the supernatants obtained from tissue homogenates. The slides were kept at room temperature for 48 hrs. in a humid chamber and observed for appearance of precipitation lines.

The preinfection sera from day old chicks used in the experimental study were screened for the antibodies against FAV-4 by AGPT. The yolk, amnioallantoic fluid and liver from infected embryonated eggs, tissue samples obtained from chicks infected with virus harvested from cell cultures along with infected cell culture supernatants were also screened for presence of the virus by AGPT.

The slides showing precipitation lines after incubation of 24 to 48 hrs. were kept overnight in PBS to remove excess proteins. Thereafter, the slides were dried in an incubator at 37°C for 2 to 4 hrs by placing a wet filter paper over it. After incubation, the filter paper was soaked with a little water and the paper peeled off. The slides were then immersed in Coommasie brilliant blue staining solution (*Appendix I*) for 15 to 30 min.

Thereafter, the stained slides were destained for 30 min. each with repeated changes of the destaining solution (*Appendix I*) for removing the excess dye.

3.4.1.4.2 Counterimmunoelectrophoresis (CIE) and staining of gels

The CIE test was carried out as described by Oberoi *et al.* (1990). The tissue samples used in AGPT were used as antigen in CIE also. A 0.9 per cent agarose gel was prepared in 0.05 M acetate buffer, pH 5.6 (*Appendix I*). Approximately, 4.5 ml of molten agarose was poured on a 75 mm x 25 mm slide and allowed to solidify. After solidification, opposing rows of wells 5 mm in diameter and at a distance of 5 mm, were punched out. The wells were filled with 30 µl antibody in appropriate wells, known positive and negative were also included in the test. The well containing antigen (positive unknown) was kept towards cathode and serum well towards anode. Electrophoresis was conducted in the presence of 0.05 M acetate buffer, pH 5.6 using 6 mA current per slide for 1 hour at room temperature. The gel slides were examined immediately and after 1 hr. storage at 4°C and results were recorded.

The staining procedure used for staining positive CIE slides was same as used for slides that were positive in AGPT. All samples tested by AGPT were also tested by CIE and the findings were recorded.

3.4.1.4.3 Dot ELISA / Dot immuno assay (DIA)

The Dot ELISA was carried out as per the method of Oberoi *et al.* (1993) with slight modifications. Nitrocellulose membrane (NCM) sheet of 0.45 nm pore size was cut into strips of 5 x 0.5 cm and marked with lead pencil for the orientation of the antigen dots.

Chloroform treated known positive liver homogenate (50% w/v suspension) was used as positive control and chloroform treated uninfected liver homogenate (50% w/v suspension) was used as negative control. Similarly, the supernatant fluids from the tissue

homogenates and infected cell culture supernatant treated with equal volume of chloroform and centrifuged at 4000 rpm for 15 min was used as an antigen in Dot ELISA.

Two microlitres of the antigen in PBS–Tween-20 (PBS-T) along with positive and negative control antigens were dotted on strip at 1 cm apart and were allowed to dry at room temperature for about 20 to 30 min. The antigen free sites on NCM strips were blocked by immersing in blocking buffer (1 per cent casein in PBS-T) at 37°C for 30 min. The NCM strips were washed 3 times in PBS-Tween 20. The strips were then incubated with anti FAV-4 hyper immune serum raised in rabbits diluted 1:80 dilution in PBS-T and incubated at 37°C for 30 min. The optimal dilution of 1:80 was determined earlier by titration on positive samples with 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320 dilutions of hyperimmune serum. Following further 3 washings to remove unbound antibodies, the nitrocellulose membrane strips were incubated at 37°C for 30 min. with goat raised-anti rabbit IgG HRPO conjugate (Sigma Immunochemicals, St. Louis, USA) diluted to 1:1000 in PBS-Tween. The optimal dilution of 1:1000 was determined earlier by titration with 1:500, 1:1000, 1:2000, 1: 2500, 1:5000 and 1:10,000 dilutions on positive samples.

The strips were washed again in the manner described above and were dipped in substrate solution (*Appendix I*). The reagents were allowed to react for 30 secs. The enzymatic reaction was stopped by washing the NCM in tap water and air dried before visual interpretation of the results. The appearance of brown spot at the site of antigen coating was considered as a positive reaction. The following controls were also run in Dot ELISA.

i) Chloroform treated positive liver homogenate (50% w/v suspension) incubated with rabbit hyperimmune serum and then incubated with goat anti-rabbit HRPO conjugated immunoglobulin and finally dipped in substrate solution.

ii) Chloroform treated uninfected liver homogenate (50% w/v suspension) treated with rabbit hyper immune serum and then incubated with goat anti-rabbit HRPO conjugated immunoglobulin and dipped in substrate solution.

The Dot ELISA was used for detecting the viral antigens in samples tested by AGPT and the findings were compared.

3.4.1.4.4 Fluorescent antibody technique

The indirect fluorescent antibody test (IFAT) was performed for the detection of virus antigen in the infected cells /smears from tissue supernatants. Anti FAV-4 hyperimmune serum against FAV-4 raised in chickens was used as the primary antibody. Rabbit anti chicken Fluorescein isothiocyanate (FITC) conjugated globulins against chicken immunoglobulins (Sigma Immunochemicals, St. Louis, USA) were used as conjugated secondary antibody for indirect FAT.

Smears of infected cells/impression smears of tissues were prepared on clean, dry microscopic slides. The smears were fixed in chilled acetone for 10 min. and air dried. The smears were then washed thrice in PBS (pH 7.4) for 5 min. each. Thereafter, anti FAV-4 hyper immune serum raised in chickens diluted to 1:16 dilution with PBS was poured over the slides @ 20 µl and kept at 37°C for 1 hour in a petri dish containing moistened paper at the bottom. The optimal dilution of 1:16 was determined earlier by titration on positive liver homogenates (50 % w/v suspension) with 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 dilutions of hyperimmune serum. The slides were subsequently washed with PBS-T thrice for 5 min. each. The slides were then incubated with rabbit anti-chicken FITC conjugated immunoglobulin (Sigma Immunochemicals) diluted to 1:80 in PBS and reincubated for 1 hour at 37°C in a moist chamber. The optimal 1:80 of the conjugate was determined earlier using 1:10, 1:20, 1:40, 1:80 and 1:160 dilutions on positive samples. The smears were thoroughly washed in PBS (pH 7.4) and counterstained with Evan's blue

(1:10,000) for 5 min. before mounting in 50 per cent glycerin-PBS. The slides were then examined for the presence of specific fluorescence under fluorescent microscope (Nikon, UV microscope). The following controls were also run simultaneously for immunoflourescent test:

i) Positive liver homogenate (50% w/v suspension) treated with normal chicken serum that was shown to be negative for FAV-4 antibodies by AGPT and then stained with rabbit anti-chicken FITC conjugated immunoglobulin.

ii) Uninfected liver homogenate (50% w/v suspension) treated with anti FAV-4 hyper immune serum and then stained with rabbit anti-chicken FITC conjugated immunoglobulin.

iii) Positive liver homogenate (50% w/v suspension) stained directly with rabbit anti-chicken FITC conjugated immunoglobulin.

The IFAT was used for detecting viral antigens in samples and the findings were recorded.

3.4.2 Additional tests performed for virus detection

3.4.2.1 Hemagglutination test (HA)

HA was done by standard procedure (Hitchner *et al.*, 1975). All the three isolates along with CELO virus were screened for their haemagglutinating properties against the RBC's of eight species *viz.* avian (chicken), bovine (cattle and buffalo), anatid (geese), rodent (rat), porcine (swine), canine (dog) and piscine (fish). The blood samples from different species were collected in Alsever's solution or Dextrose gelatin veronal (DGV) (1:1 v/v) (*Appendix I*) and stored under refrigeration. The RBCs from each species were washed thrice in sterile PBS in a table top centrifuge at 1000 rpm for 5 min. each and suspended @ 0.3 per cent and 0.5 per cent in PBS (pH7.2). Serial two fold dilution of virus was made in plastic U bottom microtitre plates. For this, 50 µl of diluent (PBS) was

added in all the wells of the microtitre plate. 50 µl of virus was added to the first well of the column and after thorough mixing, 50 µl was transferred to next well of the column. The process was repeated from column 1 to column 10 so that dilution from 1:2 (first well) to 1: 1024 (tenth well) were made. For RBCs of eight species, eight rows of a microtitre plate were used. 0.3 per cent of RBC suspension from each species *viz.*, chicken, cattle, buffalo, geese, rat, swine, dog and fish were added to each row @ 50 µl with one species per row in the first plate. For each virus isolates two plates were used. In the second plate, 0.5 per cent RBCs suspension was added in a similar fashion.

The plates were mixed well by gently tapping against the palm of hand and the plates were then incubated for 2 hrs. at room temperature and the results recorded. RBCs suspension (0.3 per cent and 0.5 per cent) from each species were also added in two wells (Well number 11) with PBS (50 µl/ well) as negative controls while Lasota virus at 1:64 dilution (4 HA units) were added in well no. 12 along with chicken RBCs as positive control.

3.4.2.2 Virus neutralization test

The neutralization test was carried out using the diluted virus and constant serum method known as alpha-method (α- method) as described by Page and Cunningham (1962) for neutralization and serotyping of the FAV isolates. For doing the neutralization tests, 10 fold serial dilutions of each of the three isolates and CELO virus were made in PBS. To each dilution, equal amount of 1:5 dilution of standard heat inactivated rabbit anti FAV/CELO hyperimmune was added. The serum virus mixtures were incubated at 37°C for 2 hrs. in an incubator with intermittent shaking. After incubation, 0.1 ml of virus serum mixture was inoculated via yolk sac route into five 6 to 8 days old chicken embryos for each dilution. The embryonated eggs were examined daily up to 7 days .The control

embryos (two embryos each for virus control, uninoculated control and serum control) were also included in the study. Death of embryos within 48 hrs. of inoculation were taken to be nonspecific. The observations were recorded and the titer was calculated by standard formula (Reed and Muench, 1938) to find out the neutralization index.

3.4.2.3 Immuno peroxidase test (IPT)

Immuno peroxidase test was carried out as described by Roy *et al.* (2001) with slight modifications. The IPT was used to detect the presence of virus in suspensions of infected cell culture and impression smears of liver and kidney samples from infected birds.

Suspensions from infected cell cultures and impression smears of liver and kidney from infected birds made on microscopic slides were air dried and then fixed in chilled acetone for 24 hrs. Thereafter, the slides were washed thrice in PBS.

To destroy the endogenous peroxidase activity normally associated with cells, the smears on the slides were left as such for 30 min. at room temperature with freshly prepared endogenous peroxidase inhibitor solution (methanol–hydrogen peroxide, 97µl : 3 µl). The slides were then incubated in various dilutions (1:10, 1:20, 1:40, 1:80, 1:160 and 1:320) dilution of rabbit raised anti FAV-4 hyper immune serum for 2 hrs. at room temperature in a humid chamber (Petri dish with a soaked filter paper in it). The optimal dilution of hyperimmune serum as determined by titration on positive samples was found to be 1:80. This dilution was used for subsequent studies. Following incubation, the slides were again washed thrice with PBS.

The slides were again rewashed with PBS thrice and incubated with various dilutions

(1:100, 1:200, 1:300, 1:400,1:500, 1:600, 1:800 and 1:1000) of goat raised anti-rabbit IgG peroxidase conjugate (Sigma Immunochemicals, St. Louis, USA) for one hour at room temperature. The optimal dilution of peroxidase conjugated antibody was found to be 1:500 that was employed in the present study. Following incubation, the slides were again washed thrice with PBS.

After washing with PBS, the slides were treated with freshly prepared 3-3'Diaminobenzidine (DAB) (Sigma Immunochemicals, St. Louis, USA) substrate solution (*Appendix I*) for half an hour. The slides were washed once with PBS for 10 min. and then once with distilled water, then dried and viewed under microscope. Following controls were also run for immunoperoxidase test:

i) Impression smears from positive liver samples treated with rabbit hyperimmune serum and then incubated with goat anti-rabbit HRPO conjugated immunoglobulin and dipped in substrate solution (Positive control)

ii) Impression smears from uninfected liver samples treated with rabbit hyperimmune serum that then incubated with goat anti-rabbit HRPO conjugated immunoglobulin and dipped in substrate solution (Negative control).

3.4.2.4 Histopathological studies

Pieces of liver, heart, bursa, thymus and kidney collected from birds given experimental infection with FAV were collected in 10 per cent neutral buffered formalin and subjected to histopathological studies using standard techniques. The fixed tissues were washed in running water overnight, dehydrated in ascending grades of alcohols, cleared in acetone and benzene and embedded in paraffin wax. Paraffin sections cut at 5 to 6 micron thickness were stained by routine hematoxylin and eosin (H and E) technique (Luna, 1968).

3.4.3 Molecular detection of fowl adenovirus

The detection of the fowl adenoviruses from different samples was done by Polymerase Chain Reaction (PCR) and Dot blot hybridization.

3.4.3.1 Detection of virus by amplification of DNA by polymerase chain reaction

3.4.3.1 A Processing of samples for PCR

3.4.3.1. A.1 Preparation of tissue samples for detection of fowl adenovirus by PCR

PCR was standardized and used for the detection of fowl adenoviruses from different samples in the study. Various tissues *viz.* liver, heart, thymus, bursa and kidney obtained from experimentally infected and control chicks were collected in sterile PBS. These tissue samples at various time intervals were triturated in sterilized pestle and mortar in PBS (pH 7.4) and a 20 per cent (w/v) suspension of each sample was prepared. The tissue suspensions were centrifuged at 4000 rpm for 15 min. The clear supernatants were collected and then used for detection of the virus by PCR.

3.4.3.1. A.2 Extraction of DNA

The conventional Phenol extraction method of DNA extraction was followed for isolating the DNA from the samples as per Sambrook *et al.* (1989). 500 µl of the sample (infected cell culture fluid, homogenates from different organs, infected allantoic fluid etc.) in duplicate were taken in a sterile 2 ml micro centrifuge tube. To this samples, 50 µl each of 10 per cent sodium dodecyl sulphate (SDS) and Proteinase K (20 µg/ml) was added, mixed properly and incubated overnight at 37°C in a shaking incubator set at 100 rpm/min to completely dissolve the sample. To this mixture, an equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) were added, mixed thoroughly for about 15 min. and centrifuged in a micro centrifuge (Sigma 1-15K Micro centrifuge) at 13,000 rpm for 15 min.

The aqueous supernatant layers were taken in a new sterile 2.0 ml micro centrifuge tube. To this, an equal amount of Chloroform: Isoamyl alcohol (24:1) were added, mixed thoroughly for 15 min. and recentrifuged at 13,000 rpm for 15 min. The aqueous supernatant layer was again taken in a new sterile micro centrifuge tube and the DNA was precipitated from aqueous phase by addition of 2.5 volumes of chilled ethanol and $1/10^{th}$ volume of 3 M sodium acetate, pH 5.2. The mixture was kept at -20°C overnight.

After overnight precipitation, the samples were centrifuged at 13,000 rpm for 15 min. at 4°C. to settle the precipitate and the supernatant was discarded.

The pellet was then washed twice with 70 per cent chilled ethanol. The material was spun at 8000 rpm for 15 min., ethanol discarded and the precipitate air dried in room temperature. The pellet was resuspended in 50 µl of sterile DNAase free water for reconstituting the DNA which was then kept at -20°C till further use.

3.4.3.1. A. 3 Estimation of DNA concentration

The DNA concentration was measured in a Nano Drop ND 1000 Spectrophotometer. Milli Q water was used as a blank control. Approximately 100 ng of DNA was used for each PCR reaction.

3.4.3.1. B Avian adenoviruses (AAV) group specific PCR

The purified DNA was initially amplified by PCR using primer pairs H1/H2 and H3/H4 specific for fowl adenoviruses initially at Department of Veterinary Microbiology, COVAS, GADVASU, Ludhiana, Punjab and later on in our department.

Primer sequence

Oligonucleotide primers corresponding to the relatively conserved region of gene as per Raue and Hess (1998) were used in the present study. The sequence of the primer pair is shown in Table 3.1.

Table 3.1. Sequences of H1/H2 and H3/H4 primers

Primers		**Sequences**	**PCR product**
Forward	*Reverse*		
H1		5'-TGGGACATGGGGGCGACCTA-3'	1219 bp
	H2	5'-AAGGGATTGACGTTGTCCA-3'	
H3		5'-AACGTCAACCCCTTCAACCACC-3'	1319 bp
	H4	5'-TTGCCTGTGGCGAAAGGCG-3'	

Amplification

1) A 25 µl of PCR reaction was set up with 5 µl of extracted DNA sample.

To this PCR mix (20 µl) was incorporated as given below:

Composition of PCR mix

Composition	Volume / Reaction
10 X PCR buffer (containing 1.5mM $MgCl_2$)	2.5 µl
Forward primer (25 p mol)	1.0 µl
Reverse primer (25 p mol)	1.0µl
dNTP's	0.5 µl
Taq polymerase (5 units/ µl)	0.5 µl
Distilled water	14.5 µl

Bulk PCR mix was prepared according to the number of samples.

2) PCR tubes were placed in DNA thermocyclers (Hybaid U.K) and (Gene Amp PCR system 9700) and subjected to following conditions of amplification.

Thermo cycling parameters for amplifications of DNA

Step	Time	Temperature	Cycles
Initial Denaturation	2 min.	94°C	1
Denaturation	2 min.	94° C	30
Annealing	1 minute	60°C (H1/H2) 50° C (H3/H4)	
Extension	1 ½ min.	72°C	
Final extension	2 min.	72°C	1

3.4.3.1. C Hexon gene specific PCR for detection of FAV

Amplification of the hypervariable region of the hexon gene of FAV isolates was accomplished as per Ganesh *et al.* (2001).

Primer sequence

Oligonucleotide primers corresponding to the hypervariable regions of hexon gene region of FAV used in study were as reported by Ganesh *et al.* (2001). All the isolates were subjected to PCR by using FAV hexon gene fragment specific primers the sequences of which are shown in Table 3.2.

Table 3.2 Sequences of primers for amplification of FAV hexon gene fragment (Ganesh *et al.*, 2001)

Primers		**Sequence**	**PCR product**
Forward	*Reverse*		
FAVHL		5'-GACATGGGGTCGACCTATTTCGACAT-3'	~700 bp
	FAVHR	5'-AGTGATGACGGGACATCAT-3'	

Amplification

1) A 25 µl of PCR reaction was set up with 5 µl of extracted DNA sample.

To this PCR mix (20 µl) was added as given below:

Composition of PCR mix

Composition	**Volume / Reaction**
10 X PCR buffer (containing 1.5mM $MgCl_2$)	2.5 µl
Forward primer (25 p mol)	1.0 µl
Reverse primer (25 p mol)	1.0 µl
dNTP's	0.5 µl
Taq polymerase (5 units/ µl)	0.5 µl
Distilled water	14.5 µl

2) PCR tubes were placed in DNA thermo cycler (Gene Amp PCR System 9700) and

subjected to following conditions of amplification.

Thermo cycling parameters for amplifications of DNA for hexon gene fragment

Step	Time	Temperature	Cycles
Initial Denaturation	10 min	95° C	1
Denaturation	1 min	94° C	35
Annealing	1 min	57° C	
Extension	2 min	72° C	
Final extension	5 min	72°C	1

3.4.3.1. D Fibre gene specific PCRs

3.4.3.1. D.1 Fibre gene specific PCRs for detection of FAV

The isolates were subjected to PCR by using FAV-4 fiber gene specific primers.

Primer sequence

Oligonucleotide primers corresponding to the gene for FAV-4 whole fibre were used in the study as reported by (Singh, 2000). The primer sequences are shown in Table 3.3.

Table 3.3. Sequences of primers for amplification of FAV-4 whole fiber (Singh, 2000)

Primers		Sequence	PCR product
Forward	*Reverse*		
FAV4 Fifo		5'-AAGATCTTCATGCTCCGAGCCCTAA-3'	~1400 bp
	FAV4 FiRe	5'-GAAGATCTTCTTCGGGAGGGAGCCCG-3'	

The primer sequences were got commercially synthesized from Lifetech technologies, Bangalore.

Amplification

1) A 25 µl of PCR reaction was set up with 5 µl of extracted DNA sample. To this PCR mix (20 µl) was added as given below:

Composition of PCR mix

The PCR mix was made as follows and 5µl of DNA was added to make the final volume to 25 µl.

Composition	Volume / Reaction
10X PCR buffer (containing 1.5mM $MgCl_2$)	2.5 µl
Forward primer (25 p mol)	1.0 µl
Reverse primer (25 pmol)	1.0 µl
dNTP's	0.5 µl
Taq polymerase (5 units/ µl)	0.5 µl
Distilled water	14.5 µl

2) PCR tubes were placed in DNA thermocycler (Gene Amp PCR System 9700) and subjected to following conditions of amplification.

Thermo cycling parameters for amplifications of DNA

Step	Time	Temperature	Cycles
Initial denaturation	2 min	94° C	1
Denaturation	1 min	94° C	30
Annealing	1 min	55° C	
Extension	1 ½ min	72° C	
Final extension	2 min	72° C	1

3.4.3.1. D.2 Fiber gene specific PCRs for detection of FAV-1

Amplification using FAV-1 fiber gene specific primers were done to check the three isolates used in the study for any contamination with FAV-1. The standard CELO virus was also subjected to amplification as a positive control.

Primer sequence

Oligonucleotide primers corresponding to the gene for FAV-1 whole fibre were used in the study as reported by (Singh, 2000). The primer sequences are shown in Table 3.4.

Table 3.4. Sequence of primers for amplification of FAV 1 fibre gene (Singh, 2000).

Primers		**Sequence**	**PCR product**
Forward	*Reverse*		
FAV Fi_for		5'-GGTGGCGTGGTCAGCGGGACC-3'	~350 bp
	FAV Fi_rev	5'-GAGTTCTCCGCTCGCGAGCTGC-3'	

Amplification

1) A 25 µl of PCR reaction was set up with 5 µl of extracted DNA sample.

To this PCR mix (20 µl) was added as given below:

Composition of PCR mix

Composition	Volume / Reaction
10 X PCR buffer (containing 1.5mM $MgCl_2$)	2.5 µl
Forward primer (25 p mol)	1.0 µl
Reverse primer (25 p mol)	1.0µl
dNTP's	0.5 µl
Taq polymerase (5 units/ µl)	0.5 µl
Distilled water	14.5 µl

Bulk PCR mix were prepared according to the number of samples.

2) PCR tubes were kept in DNA thermocycler (Gene Amp PCR System 9700) and subjected to following conditions of amplification.

Thermo cycling parameters for amplifications of DNA

Step	Time	Temperature	Cycles
Initial Denaturation	2 min	94° C	1
Denaturation	1 min	94° C	35
Annealing	1 min	65° C	
Extension	1 min	72° C	
Final extension	10 min	72°C	1

3.4.3.1. E Preparation of solutions / buffers

The compositions of stock solutions of TAE (10X) and working solution (1X) are given in *Appendix I.* The PCR products were analyzed in a gel electrophoresis system.

The gel electrophoresis was conducted using a submarine horizontal agarose slab gel as described by Sambrook *et al.* (1989). During gel preparation, ethidium bromide

(0.5µg/ml) was added to the lukewarm gel for staining. The PCR product was analyzed on 1 per cent agarose gel made in 1× TAE buffer at 80V for an hour. Along with the test sample Gene Ruler DNA ladder plus 100 bp (MBI, Fermentas) was also run.

After electrophoresis, the gel was visualized using UV Trans-illuminator and photographed using Gel Documentation system (Alphamager 2200 Documentation and Analysis System, Alpha Innotech Corporation, USA).

3.4.3.2 Detection of FAVs by Dot blot hybridization

Dot blot hybridization is an efficient tool for detecting viruses based on DNA-DNA hybridization. To detect fowl adenoviruses in tissue samples infected with the virus, a gel purified 700 bp product specific for FAV-4 was used as a biotinylated labeled probe. The procedure followed is being described below.

3.4.3.2.1 Preparation of the Dot blot

Various tissues (~1 g) from birds that had died after been infected with the three isolates were triturated in a pestle and mortar containing 1.5 ml Tris NaCl EDTA (TNE) buffer (10mM Tris, 100mM NaCl, 1mM EDTA, pH 8.0) and mixed well. Then the mixture was transferred to a 1.5 ml eppendorf tube. The tubes were centrifuged for 1 min at 13,000 rpm. The supernatant (100µl) was taken in a new eppendorf tube and diluted with 300µl of millipore water. Then the mixture was denatured at 65°C for 10 min and immediately placed on ice before loading on to the Dot blot apparatus containing the nylon membrane. Additionally, DNA extracted from these tissues that were used in PCR was also used in preparation of the Dot blot.

Nylon membrane was cut according to the size of the Dot blot apparatus and a small portion of one corner was cut for demarcation. Blots were prepared from different

infected organ supernatants infected with all the three isolates using nylon membrane. The membrane was dipped in deionised water for 5 min. The membrane was then dipped in 6 per cent SSC solution (*Appendix I*) for 5 min. followed by a dip in 10 per cent SSC solution for 5 min. and thereafter adjusted on the Dot blot apparatus. Drying of the membrane was done at 40 V During this time, a table was prepared where the sample numbers were recorded according to the location of each dot for further easily identification of location of samples which will show positive. Thereafter different samples were loaded on the Dot blot manifold and it was again vacuumed. After drying of the membrane, 10X SSC was used. The membrane was cross linked by giving UV exposure in a UV transilluminator (spotted side down) for 2 min. post drying to fix DNA on the membrane and then stored at 4°C. A positive control probe for FAV was also included while for negative control only TNE buffer was used.

3.4.3.2.2 DNA probe preparation (*DNA labeling with Biotin -11-dUTP*)

The DNA probe was labeled with Biotin-11-dUTP in a eppendorf. The reaction mixture contains 10 µl eluted DNA Template (100 ng to 1 µg), 10 µl Decanucleotide in 5X reaction buffer, 44 µl nuclease free water. The tube was vortexed and spun down in a micro centrifuge for 3 to 5 secs. The tube was then incubated in a boiling water bath for 5 to 10 min., cooled on ice and spun down quickly. Thereafter, 5µl Biotin labeling mix and 1µl Klenow fragment, exo (5u)- were added to the tube. The tube was shaken and then the contents were again spun down in an eppendorf for 3 to 5 secs. The tube was incubated for 1 hour at 37°C. The reaction was stopped by the addition of 1µl 0.5M EDTA, pH 8.0.

3.4.3.2.3 Control labeling reaction

The reaction control was prepared in same way, 25 µl of *Hind* III fragment (10 ng/µl) was incorporated instead of eluted DNA. 10 µl of Decanucleotide in 5 X Reaction buffer and 9 µl of deionised water was added to the *Hind III* fragments in a eppendorf tube. The other protocols used in the control labeling reaction were parallel to the DNA probe preparation. The control labeled DNA was stored at -20°C for further studies.

3.4.3.2.4 Pre hybridization

For pre hybridization, a pre hybridization solution was added into a bottle at 42°C for 2 to 4 hrs. The biotin labeled probe was denatured at 100°C for 5 min and subsequently the denatured probe was added to the pre hybridization solution. The pre hybridization solution along with probe, were then incubated at 42°C overnight with constant shaking (Hybridization oven/shaker, Stuart Scientific SI 20 H). The membrane was washed twice with wash buffer I at 42°C for 15 min. and subsequently washed twice with wash buffer II at 25°C for 15 min. Thereafter, the membrane was dried on filter paper after washing twice with wash buffer III at 65°C for 15 min.

3.4.3.2.5 Detection procedure

After hybridization, the membrane was washed with 30 ml of blocking /washing buffer for 5 min. at room temperature on a platform shaker with constant shaking. The membrane was then blocked with 30 ml of blocking buffer for 30 min. at room temperature on platform shaker with constant shaking. The membrane was incubated in 20 ml of diluted Streptavidin-AP conjugate for 30 min at room temperature with constant shaking. Afterwards, the membrane was incubated with 60 ml of blocking /washing buffer for 15 min. After discarding the solution, the membrane was again incubated in 60 ml of blocking

/washing buffer for 15 min. after which the solution was again discarded. The membrane was again incubated with 20 ml of detection buffer for 10 min. thereafter the membrane was incubated in 10 ml of freshly prepared substrate solution in dark at room temperature. The presence of blue purple precipitate is considered as a positive result that often becomes visible after 15 to 30 min. of incubation. The reaction was stopped by the addition of 0.5 M EDTA pH 8.0.

3. 5 Molecular characterization of fowl adenovirus

The characterization of fowl adenovirus was done both by protein profiling of the isolates as well as by molecular methods gene sequencing.

3. 5. 1 Protein profile of fowl adenovirus

The protein make up of the fowl adenoviruses used in our study were resolved by SDS-PAGE.

3.5.1.1 Purification of fowl adenoviruses

The purification of FAVs for SDS-PAGE was carried out by the method of Winters and Russell (1971).

Infected cells were pelleted by centrifugation at 1000 rpm for 10 min. The pelleted cells were redispersed in 2 ml of sodium phosphate buffer. The cell suspension was homogenized by shaking vigorously for about 10 min. with an equal volume of *Genetron* 113 (1, 1, 2,-Trichlor-trifluoroethane) (HiMedia Limited) and the total mixture was immersed in an ice bath. This procedure disrupted many cells and denatured most of the cellular proteins while retaining the virus integrity. The extract was centrifuged at 1000 rpm for 10 min. and the aqueous phase was retained. The *Genetron* phase and the cellular material at the interphase was re- extracted with an equal volume of fresh buffer and the

aqueous extracts was pooled. Healthy uninfected CEL cell culture fluid was treated in the same manner for control.

The pellet was carefully layered over the top of two layers of CsCl (Sigma, USA) solution (0.8 ml of density 1.45g / ml and 1.5 ml of density 1.33g / ml in 5mM Tris- HCl, 1 mM EDTA pH 8) in 5 ml centrifuge tubes and centrifuged at 90,000×g for 90 min in Swing out rotor (TH 641 rotor Sorvall RC 90). A virus band as an opalescent layer at the interface of higher and lower density solution was collected by using Pasteur pipette. Virus preparation thus obtained was dissolved in TE buffer overnight and pelleted at 1,00,000 × g for 1 hr at 4°C. The virus pellet was dissolved in 700 µl of TE buffer and kept at -20°C.

3.5.1.2 Purification of CELO virus

The purification of virus was done as per the method described by Maiti and Sarkar (1997).

3.5.1.2.1 Virus concentration

Infected allantoic fluid collected after 48 hrs of inoculation was mixed with equal volume of chloroform. Following vigorous shaking or homogenization, the material was centrifuged at 4000 rpm for 30 min. at 4°C (Remi High speed centrifuge).The supernatant was saved and subjected to precipitation of virus with 55 per cent saturated ammonium sulphate by slowly adding grinded salt to the fluid. The mixture was allowed to stand overnight at 4°C. The pellet was dissolved in about $1/50^{th}$ of the original volume of PBS (pH 7.2). The sulphate ions were removed by dialyzing the virus against 4 changes of PBS at 4°C.

3.5.1.2.2 Virus purification

The concentrated virus material was pelleted at 1,00,000×g for 60 min. in ultracentrifuge (TH 641 rotor, Sorvall RC 90).The pelleted virus was suspended in a small volume of 10 mM Tris- HCl buffer, pH 8 and kept overnight at 4°C.

The virus suspension was then layered on two layers of 20 to 45 per cent cesium chloride gradient (1.5 ml of 20 per cent CsCl on top of 1 ml of 45 per cent CsCl) in 5 ml centrifuge tubes then centrifuged at 1,10,000x g for 3 hrs in a swinging out rotor (TH 641 swing out rotor). Following centrifugation the virus band formed at the interface was visualized by indirect light and collected with a Pasteur pipette.

The virus suspension was dissolved in 10mM Tris –HCl buffer. The CsCl was removed from the virus preparation by dialysis at 4°C for several hrs. against 10mM Tris buffer. Finally, the virus was pelleted at 1, 00,000 × g for 1 hr at 4°C and dissolved in minimal volume (500 µl) of TE buffer. The virus was then checked in AGID for confirmation.

For determining the optical density of virus, the absorbance values of the purified preparation were taken at 260 nm and 280 nm with the help of UV spectrophotometer (Nanodrop ND 1000 Spectrophotometer). The approximate protein content in the samples was determined by using the formula

Protein (mg/ml)= 1. 55 × O.D. at 280 nm - 0.77 × O.D. at 260nm

Ratio of absorbance values at 260/280 nm was calculated to check the purity of virus sample. A virus preparation giving a value of 1.2 was taken as purified.

3.5.1.3 SDS-PAGE of virus proteins

The purified virus was subjected to SDS-PAGE in order to study the protein profile of the virus. All the three viral isolates and one standard reference CELO virus were tested by this procedure.

The method of Laemmli (1970) was followed, molecular weight (M.W.) of different proteins and purity of virus samples were checked by SDS-PAGE in Minigel electrophoresis unit (Amersham Biosciences). Ten per cent polyacrylamide resolving gel and 4.5 per cent stacking gel were used. Purified virus preparation was mixed with equal volume of sample buffer and the mixture was boiled for 5 min. The MW marker (ranging from 14.3 to 97.4 kDa Genei, Bangalore) was prepared similarly.

Prepared virus samples and MW markers were loaded in wells and the gel was run at 10 mA of constant current until the tracking dye bromophenol blue) entered the separating gel. When the tracking dye entered the separating gel, the current was increased to 15 mA till the dye reached the bottom. Healthy CEL cell culture treated in the same manner was used as control.

After the completion of electrophoresis the gel was stained with Coomassie brilliant blue (CBB) to detect the protein bands. Gel was kept in CBB for 6 hrs. and destained in destaining solution (*Appendix*) with frequent changes and stored in 5 per cent acetic acid.

3.5.1.4 Western Blot

The western blot was attempted in the study. The polypeptides of viruses separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membrane according to the method of Burnette (1981) for detection of immunogenic proteins. For these, nitrocellulose membrane (Immuno–PVDF membrane) (Bio Rad) were cut according to the size of the gel and equilibrated in transfer buffer / Towbin buffer (*Appendix*) for 30

min. Additionally, strips of whatman filter paper no. 3 (three pieces) of equal size were also cut according to the size of the gel and kept on the flat plate (graphite plate) of the western blot apparatus (TE 70 semidry transfer unit, Amersham Biosciences) and equilibrated in transfer buffer for few min.

Thereafter, the marked nylon gel was placed over the filter paper taking care to avoid air bubble formation between various layers of the sandwich. The gel was then placed over the membrane and covered with another 3 pieces of filter papers. A current of 2 mA/sq.cm was passed for 90 min. for transferring the proteins.

After transfer of proteins, the membrane was blocked with blocking solution (*Appendix I*) overnight at 4°C. The membrane was washed thrice with washing buffer (*Appendix I*) by repeated agitation for 5 min. in orbitory shaker (Innova 4230 refrigerated incubator shaker). The membrane strip was then kept in rabbit anti FAV-4 hyperimmune serum/CELO antiserum (1:100) for 1 hour and washed thrice as before.

The strip was then dipped in anti-rabbit HRPO conjugate (1:1000) dilution for 1 hr. at 37°C. The unbound conjugate was removed by washing as described earlier. Thereafter the membrane was transferred into substrate solution (*Appendix I*) and incubated in dark till colour developed. As the bands appeared, the membrane strip was washed in excess of distilled water and result recorded.

3.5.2 Molecular profile of fowl adenovirus by gene sequencing

3.5.2.1 Cloning of fowl adenovirus hexon protein gene

The cloning of fowl adenovirus hexon protein gene was done for finding out the sequencing data.

3.5.2.1.1 Purification of PCR products

Extraction, elution of DNA and purification of DNA from agarose gel was done by HiPurA™ gel extraction kit (HiMedia Limited).

3.5.2.1.2 Procedure of Elution

The gel slice containing the PCR amplified product was excised with a clean, sharp scalpel under UV transilluminator. The gel slice was weighed and placed in to a sterile 1.5 ml eppendorf tube. Then gel bind buffer (HG) thrice the volume of the gel was added into the gel slice and kept in water bath at 60°C for 10 min. until the gel slice totally dissolved in the gel bind buffer. For preparation of binding column, the HiElute Miniprep Spin Column was placed into a 2 ml collection tube.

The solubilized gel sample was added to the HiElute Miniprep Spin Column and centrifuged for 1 min at 13,000 rpm. The spin column was removed and the flow through was vortexed in the 2 ml collection tube for 5 secs. and again recentrifuged. The flow through liquid was discarded and the HiElute Miniprep spin column was placed in a new 2.0 ml collection tube. To this, 500 µl of Gel Wash Buffer (BIW) was added and the column was washed by centrifuging for 1 min. at 13,000 rpm. The flow through was again discarded. The column was again one time centrifuged for 1 min. at 13,000 rpm. The HiElute Miniprep spin column was carefully transferred to a clean 2.0 ml collection tube provided and about 15 µl of RNase free water was added on to the centre of the HiElute Miniprep spin column membrane and incubated for 1 min at room temperature. After letting the column stand for 1 to 2 min., it was centrifuged for 1 to 2 min. to elute DNA. Again 15 µl of RNAse free water was added on to the centre of the membrane and the above step repeated. The eluted DNA was stored at -20°C till further use.

3.5.2.1.3 Checking of eluted DNA

Agarose gel (1 per cent) was prepared in 1X TAE buffer. DNA (5 µl) were loaded with 1X DNA loading dye (2 µl) and electrophoresed at 80 V for 1 to 2 hr. During gel preparation, gel was stained by adding ethidium bromide (0.5 mg/ml). After electrophoresis, the gel was visualized in U.V. transilluminator.

3.5.2.1.4 Ligation of eluted DNA in to the pGEM®-T easy (Promega, USA) vector

The eluted DNA was ligated into the pGEM®-T easy Vector System that are convenient systems for the cloning of PCR products.

3.5.2.1.5 TA cloning vector ligation

The 700 bp PCR product (hexon gene) was ligated into PGEM- T easy vector (3.0 kb). The ligation was carried out at an insert vector ratio ranging from 1:1 to 3:1. The ligation reaction was set up as follows:

3.5.2.1.5.1 Ligation reaction

Contents	Volume
2x rapid ligation buffer	5 µl
pGEM-T Easy vector (50ng)	0.5 µl
Eluted DNA (Insert)	4 µl
T4 DNA ligase (3 Weiss units)	0.5 µl
Total	10 µl

3.5.2.1.5.2 Procedure

1) The eluted DNA was vortexed briefly along with the pGEM®-T Easy Vector by spinning at 5000 rpm for 30 sec.

2) The reaction mixture was mixed by pipetting and incubated overnight at 4°C in a refrigerator and stored at -20°C for next transformation step.

3.5.2.2 Preparation of competent cells

Competent cells can be prepared either freshly for each use or can be stored at – 70^0C for future use. For the transformation experiments either DH5α or XL1-Blue (tet ^{R}) strains of *E. coli* were used. Single colony of the appropriate strain of the bacteria was streaked on to Luria Bertani (LB) agar plate with appropriate antibiotics. This was incubated at 37°C for 12 to 16 hrs. These freshly grown colonies of *E. coli* cells were used for preparation of competent cells.

3.5.2.2.1 Procedure

One ml of overnight culture of *E. coli* was inoculated in 200 ml SOB medium (*Appendix I*) at 22°C (preferable temperature is 18°C, but growth is slower) and grown for 24 to 28 hrs. with continuous shaking. The culture containing cells was split into four 50 ml tubes and then the tubes were placed on ice for 10 min. The culture was spun for 15 min at 4000 rpm. The supernatant was discarded and the culture resuspended in 64 ml HTB (*Appendix*) (16 ml for each tube) that were finally pooled into one tube. The dimethyl sulphoxide (DMSO) (filter sterilized) was gradually added to it while gently swirling aliquot immediately into eppendorf tubes. The cells were stored at -70°C until further use.

3.5.2.2.3 Transformation of Competent cells

The competent cells which are stored at -80°C (vol. 200 µl) were thawed on ice for 15 min. The ligation mixture (10 µl) was simultaneously thawed on ice for 15 min and centrifuged briefly and added to the thawed competent cells in laminar air flow condition.

The two components were mixed by gentle flicking and incubated on ice for 30 min. Then heat shock was given to the cells for 90 secs. in a water bath at 42°C (Heat shock

converts the bacterial cell membrane in to gel state from solid state. This lead to formation of gaps in membrane which are sufficient for the entry of ligated plasmid cells in to bacteria. The cells were cooled immediately on ice for 5 mins. (Due to cooling the membrane gets repaired and the gaps were closed after entry of ligated plasmid cells in to bacteria). 800 µl of Luria broth (previously autoclaved) was added to the tubes containing transformed cells in laminar air flow condition and incubated the tubes at 37°C for 1 hr with moderate shaking (~150 rpm).

During the mean time LA plates were prepared containing ampicillin (100 µl amp / 100 ml LA).The incubated culture was centrifuged and cells were pelleted down. 850 µl broth was discarded. Pellet was resuspended in remaining broth, and then plated on LA-amp plate. And the plates were sealed tightly with parafilm. The above plates were incubated overnight at 37°C for development of transformed colonies. Next day after 12 to 16 hrs, the plates were observed for checking of development of transformed colonies.

3.5.2.2.4 Checking of transformed colonies

About 10 colonies, growing on the LB agar plates were picked up, cultured separately on fresh LB agar plates as well as in tubes in laminar air flow condition and incubated at 37° C overnight to recheck the development of transformed colonies.

3.5.2.3 Plasmid isolation

3.5.2.3.1 Plasmid isolation from transformed cells

Transformed colony was picked up and inoculated on the LA-amp plates with the help of sterile toothpick and same was used for the incubation of LB-amp tubes. These plates and tubes were grown overnight at 37°C in a shaking incubator.

3.5.2.3.2 Boiling Miniprep method of Plasmid isolation

The boiling miniprep method of (Holmes and Quingley, 1981; Sambrook *et al.*, 1989) was followed for plasmid isolation. Approximately 5 ml of the overnight grown LB-amp culture was pelleted down in the centrifuge for 1 min at 13,000 rpm. The supernatant was discarded and the pellet was resuspended in 110µl of STET Buffer (*Appendix I*). Then lysozyme (10mg/ml stock) was made in 10 mM Tris HCl, pH 8 and from this stock, 10 µl of lysozyme was added to the resuspended cells. The tubes were gently mixed 3 to 4 times and incubated at room temperature for 5 min. Then heat shock was given for 40 secs. in boiling water bath. The suspension was centrifuged at full speed for 20 min. at room temperature.

The pellet containing the cell debris was removed with the help of a sterile toothpick. The DNA in the supernatant was precipitated by adding one volume of isopropanol (*i.e.* 120 µl).The precipitated DNA was collected immediately by centrifugation for 25 min. at 13,000 rpm. Then complete removal of the supernatant was done and the pellet was dried at 37°C for 15 min. The pellet was resuspended in 50µl nuclease free water and left for 15 to 20 min at room temperature to dissolve. The plasmid DNA was stored in -20°C.

3.5.2.3.3 Agarose gel electrophoresis of isolated plasmid

The isolated plasmid (3 µl) mixed with 2 □l of gel loading dye was loaded on 1 per cent agarose gel and electrophoresed in TAE buffer at 80 V for 1 to 2 hrs. During gel preparation, gel was stained by adding ethidium bromide (0.5 mg/ml). After electrophoresis, the gel was visualized in U.V. transilluminator and photographed in Alpha Digidoc™ (Alpha Innotech Corporation).

3.5.2.3.4 Checking of recombinant clones

The recombinant clones were checked for the presence of FAV hexon protein gene through restriction digestion of plasmid DNA.

3.5.2.3.5 Digestion of the plasmid DNA using *Eco* RI

pGEMT® Easy Vectors contain multiple restriction sites within the multiple cloning sites. These restriction sites allow for the release of the insert by restriction digestion with a single restriction enzyme. pGEMT® Easy Vector multiple cloning sites is flanked by recognition sites for the restriction enzymes *Eco* RI, *Bst* ZI, *Not* 1, *Pvu* II. The single enzyme can, therefore, be utilized for release of insert that is further proved by running the digested product on gel electrophoresis. Before digesting the isolated plasmid, RNase treatment was given to degrade RNA. (2 µl of ribonuclease A was taken and diluted to 50 times by adding 48 µl of double distilled water. This 2 µl RNase was used for entire Plasmid DNA and incubated at 37°C for 1 hr in water bath).

3.5.2.3.5.1 Digestion mixture

Contents	**Quantity**
Plasmid DNA	17.0 µl
10x Buffer 'O' (*MBI Fermentas*)	5.0 µl (2x final concentration)
EcoRI Enzyme	1 µl
Water	27 µl
Total	50.0 µl

3.5.2.3.5.2 Procedure

The digestion mixtures were thoroughly mixed and centrifuged for a short run. The digestion mixtures were incubated at 37°C for 2.5 to3 hrs. 2.5 volumes of cold ethanol was added in each mixture and incubated at -70°C for 1 hr. After that the mixtures were centrifuged at maximum speed for 30 min. at 4°C. The supernatant was removed and the pellet was dried at room temperature and resuspended in 12µl RNase free water. The resuspended solution was mixed with 3µl of dye and loaded on 1 per cent agarose gel along with marker to view the digestion pattern.

3.5.2.3.6 Purification of plasmid DNA for sequencing

One of the colonies containing the gene of interest ligated with vector was chosen for further downstream reaction. Plasmid DNA was isolated and purified for sequencing as the procedure given below using AuPreP spin^{m} plasmid isolation minikit (Life technologies India Pvt. Ltd.).

Transformed *E.coli* grown overnight LB-amp culture (0.5 to4 ml) was subjected to centrifugation at 13,000 rpm for 1 min. The supernatant was discarded and all medium residues were removed by pipetting. Then 250 µl of MX1 Buffer was incorporated into the pellet and resuspended the cells completely by vortexing or pipetting. 50 µl of MX2 Buffer was added and gently mixed to lyse the cells until the lysate became clear. Incubation was done at room temperature for 1 to 5 mins.

About 350 µl of MX3 Buffer was subsequently added to the mixture (to neutralize the lysate) immediately and the solution gently mixed following which a white precipitate was formed. Then centrifugation done for 5 to 10 min, meanwhile a spin column was placed onto a collection tube. The supernatant was cautiously transferred clearly into the column and centrifuge done for 1 min and the flow through discarded. The

column was washed once with 0.5 ml WF Buffer by centrifuging for 30 to 60 secs. and again the flow through was discarded. The column was centrifuged for another 3 min. to remove residual ethanol and was placed onto a new 1.5 ml centrifuge tube. 20 µl of RNase free water was then added onto the centre of the column membrane. The plasmid obtained was then stored at -20°C.

3.5.2.3.7 Quantification of purified DNA

The quantification of purified DNA was carried out in Nanodrop 1000 spectrophotometer and absorbance of DNA was recorded in UV range from 260/280 nm. Values of A_{max}, A_{min} (A_{260}/A_{280}) were calculated to know the appropriate concentration.

3.5.2.4 Direct sequencing of fibre gene

The ~1400 bp of fibre gene from P-I isolate was eluted out from the gel with gel extraction kit, purified and sequenced directly without cloning.

3.5.2.5 Sequencing of FAV hexon protein gene

The transformants containing putative recombinant vector plasmids (P-I, PH-II and the DNA from non viable FAV isolate described earlier) were purified by AuPreP spin^{m} plasmid isolation minikit, quantified and analyzed to contain in excess of 100 ng/ µl per sample. Approximately 20 µl of plasmid containing a total concentration of 1 to 2 µg of DNA were lyophilized (DNA 120 Speed vac, ThermoSavant) and sent by courier to Bioserve, Genome Valley, and Hyderabad for commercial sequencing.

3.5.2.6 Sequence analysis

The sequence was aligned with corresponding sequences of other established FAV sequences from the database using Basic local alignment search tool (BLAST) from the website http://www.ncbi.nlm.nih.gov/blast (Altschul *et al.*, 1997). Based on the BLAST

results of BLAST server, sequences were further compared with nucleotide sequences of other members of FAV available in the Gen Bank database. The program BLASTP was used to search the amino acid sequence database. Pair wise comparisons were performed by the ALIGN-2 program utilizing the DOTHELEX algorithm (Tatusova and Maiden, 1999). Multiple alignments were generated by the MULTALIN program from the web site: http://prodes.toulouse.inra.fr/multialin/multialin.html (Corpet, 1988). Phylogenetic tree was constructed with the help of Clustal W from the website http://www2.ebi.ac.uk/clustalw/ (Higgins *et al.*, 1994) and from the website www. ddbj.nig.ac.jp. Tree was subjected to bootstrap (using 1000 replicates) and viewed with the help of Tree View from website http://taxonomy.zoology.gla.ac.uk/rod/treeview.html (Page, 1996).

Results

Chapter IV

RESULTS

HPS or hydropericardium syndrome, a recently introduced lethal disease of three to five weeks old broiler chicks causes significant economic losses to the poultry farmers. The disease is characterized by hydropericardium as well as enlarged, friable and pale liver along with significant mortality.

In the present study, the samples of infected liver along with liver homogenates were obtained from the Department of Veterinary Pathology, DGCN COVAS, CSKHPKV, Palampur, (H. P). for further studies.

The present investigation was thus undertaken to do cultural, serological and molecular studies on the locally available isolates of HPS. Further, the study also intended to observe the growth of HPS agent in primary cell cultures of avian origin as well as established cell lines and to find out the suitability of various cell cultures of avian and non avian origin for the propagation of agent *in vitro*.

Both serological as well as molecular tests were carried out to know the most effective test(s) available to diagnose the virus in the laboratory. The availability of viral isolates recovered from natural outbreaks of the disease in the State in the present study provided an opportunity to ascertain the molecular characters of the virus and compare these with already reported strains of this virus in other states of India.

4.1 Virus preparation

4.1.1 Effects on birds after viral inoculation

All the isolates used in this study were found to be viable. Infection led to the development of clinical signs in the inoculated birds which were: lethargy, closed eyes and ruffled feathers before death (Plate 4.1). The birds typically sat on their hunches, stopped

feeding, passed greenish feces and eventually died. The control birds, however, did not show any symptom of the disease (Plate 4.2). On post mortem, typical symptoms of hydropericardium along with enlarged and hemorrhagic livers were observed (Plate 4.3, 4.5 and 4.6). In kidneys, hemorrhages and enlargement was noticed (Plate 4.4). The mortality pattern of the birds at different time intervals is shown in Table 4.1, while the pathological lesions observed in the birds are depicted in Table 4.2.

Table 4.1. Mortality pattern in birds inoculated with all the three isolates

Days post inoculation	**No. of birds inoculated**	**Number of birds dead**			
		P-I	**PH-II**	**PC-III**	**Control birds(s)**
1	2	1 / 2 (50%)	1 / 2 (50%)	1 / 2 (50%)	1 / 3 (33 %)
2	2	-	-	1 / 2 (50%)	1/ 3 (33 %)
3	2	1 / 2 (50%)	1 / 2 (50%)	-	1/ 3 (33%)

S: Sacrificed

4.1.2 Determination of infectivity titres of the viruses

All the three viruses along with the standard CELO virus were titrated in embryonated chicken eggs at 6 to 8 days of age by yolk sac route. The EID_{50}/ml was recorded employing standard procedure (Reed and Meunch, 1938) for CELO virus along with the three test isolates.

Table 4.2. Gross pathological lesions in birds inoculated with three viral isolates

Days PI	No. of birds infected	Gross pathological lesions in birds			
		P-I	**PH-II**	**PC-III**	**Control birds**
1	2	Enlarged liver with slight hydropericardium	Enlarged and hemorrhagic liver	Enlarged and pale liver	No gross change
2	2	-	-	Hydro pericardium with pale and enlarged livers and kidneys	No gross change
3	2	Typical hydropericardium with enlarged livers and kidneys	Hydropericardium with enlarged livers		No gross change

The infectivity titre measured for CELO virus was found to be $log10^{5.45}$ EID_{50}/ml. The titre measured for the three virus isolates namely: P-I, PH-II and PC-III used in the study were found to be $log10^{5.67}$ EID_{50}/ml, log $10^{6.3}$ EID_{50}/ml and log $10^{6.2}$ EID_{50}/ml, respectively.

4.2 Infectivity studies of fowl adenovirus in cell cultures

4.2.1 Primary cell cultures

4.2.1.1 Chicken embryo fibroblast cell culture

4.2.1.1.1 Establishment of chicken embryo fibroblast cell culture

The first indication of attachment of the cells to the substrate was evident after six hrs. of incubation. By 18 hrs, the cells were dividing and had started to proliferate. By 24

Plate 4.1 : Bird infected with P-I virus isolate showing typical depression and ruffled feathers,(48 hrs p.i) with P-I isolate

Plate 4.2: Healthy bird(left) with infected bird(right), 48 hrs. p.i with P-I virus isolate

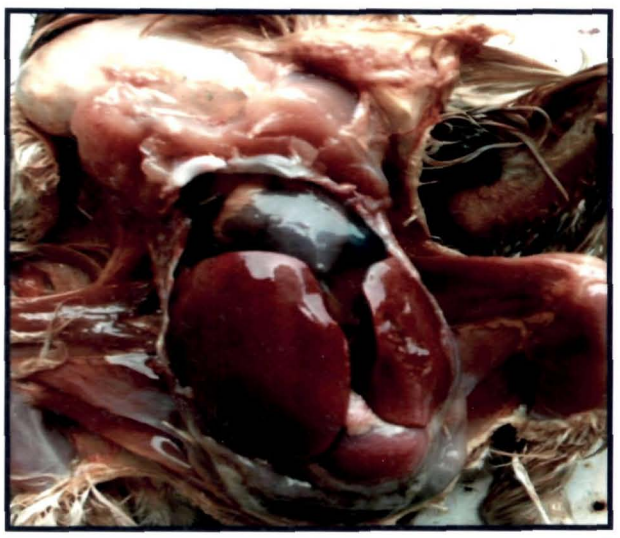


Plate 4.3: Typical hydropericardium, enlarged and hemorrhagic liver,72 hrs p.i with P-I isolate

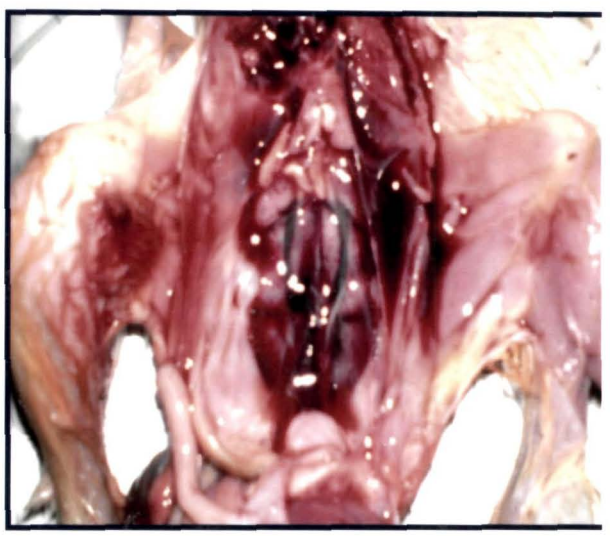


Plate 4.4: Enlarged kidneys with slight hemorrhages, 72 hrs p.i with P-I isolate

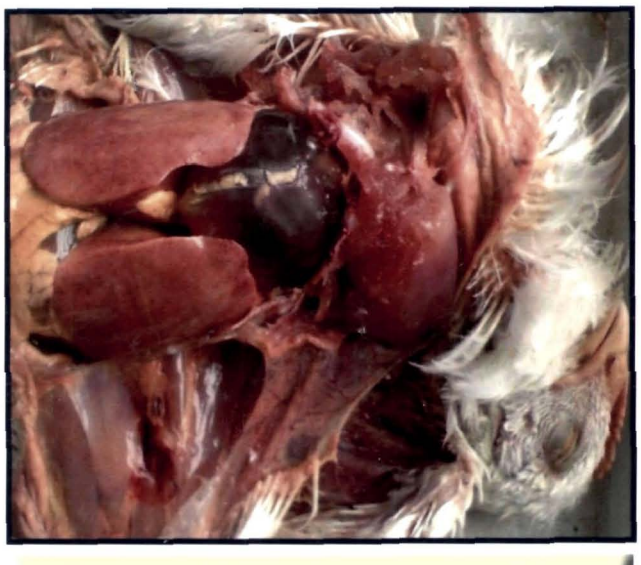


Plate 4.5: Hydropericardium, enlarged and hemorrhagic liver 72 hrs p.i with PH-II isolate

Plate 4.6: Close up of the same bird showing typical hydropericardium, enlarged and hemorrhagic liver,72 hrs p.i with PH-II isolate

hrs., 60 to 70 per cent monolayer had formed and by 48 hrs., more than 90 per cent monolayer had formed. (Plate 4.7 to 4.12).

4.2.1.1.2 Infection of chicken embryo fibroblast cell cultures

Four blind passages were required in CEF monolayer cultures before evidence of adaptation of the virus to the fibroblast culture was noticed. At the fifth passage level, after 48 hrs. of inoculation, non specific changes like slight rounding of fibroblast cells was seen.

In the sixth passage level, at 48 hrs. post inoculation (p.i.), the cell monolayer infected with P-I viral isolate showed rounding that progressed sequentially to clumping, mild detachment leading to extensive detachment of the monolayer at 120 hrs. p.i. (Plate 4.14 to 4.16). No cytopathic changes (CPE) however were observed in the control monolayer (Plate 4.13). CPE also started appearing in the monolayers inoculated with PH-II and PC-III isolates at the sixth passage level but at different time intervals.

All the isolates were further passaged up to the tenth passage level. However, successive passages of the P-I isolate from the eighth passage level onwards showed onset of early and enhanced CPE with effects ranging from significant rounding, aggregation and clumping of cells starting from 24 hrs. p.i. with complete detachment of the monolayer at 96 hrs. p.i. By the ninth passage level, P-I infected monolayer showed increased cell damage manifested by destruction of the monolayer at 72 hrs. p.i.

The monolayer was damaged very rapidly in the 10^{th} passage level with widespread and complete detachment of the monolayer at 72 hrs. p.i. The stained monolayers showing CPE along with control CEF monolayer is shown (Plates 4.17 and

4.18). Similar results were obtained at 10^{th} passage level after infection with PH-II isolate with destruction of the complete monolayer by 96 hrs. p.i.

The combined passage wise onset of CPE shown by the three isolates is depicted in Table no. 4.3. while the hour wise changes seen in the CEF cell culture inoculated with each of the three isolates are elaborated in Table 4.4.

Although the CPE shown by both PH-II and PC-III isolates were more or less similar in appearance to those shown by P-I isolate, however when titration of all of these isolates was done after the 10^{th} passage level, the virus titer revealed few changes.

The viral infectivity titer (EID_{50}/ml) recorded for P-I after the 10^{th} passage level was log $10^{7.3}$ EID $_{50}$/ml, which was different from the initial level of log $10^{5.67}$. The viral titers measured for PH-II and PC-III after 10^{th} passage level were found to be log $10^{7.7}$ and log $10^{7.5}$ respectively, that were slightly different than initial titres of log $10^{6.3}$ and log $10^{6.2}$ EID $_{50}$/ml.

4.2.1.2 Chicken embryo liver cell culture

4.2.1.2.1 Establishment of chicken embryo liver cell cultures

Attachment of the hepatocytes to the substrate of the flask started after 24 hrs. and the complete monolayer got established after 144 hrs. post seeding (Plates 4.19 to 4.24). The medium had to be changed for removal of dead and non attached cells and replenished after 48 hrs. by the addition of 5 per cent fetal calf serum.

4.2.1.2.2 Infection of primary chicken embryo liver cell cultures

The isolates were given a total of five passages in CEL monolayer cell cultures. No CPE were seen in the first passage level after an incubation period of 5 days. Effects were seen from the second passage level. In P-I infected cells, the effects started as

shrinkage of cells followed by moderate to extensive detachment of the monolayer by 96 hrs. p.i. The CPE were characterized on an average by shortening of onset time and evidence of cell detachment in the whole monolayer by 48 to 96 hrs. p.i.

Table 4.3. Passage wise CPE observed in CEF cell culture after inoculation with viral isolates

Passage level	P-I	PH-II	PC-III
Ist	-	-	-
IInd	-	-	-
IIIrd	-	-	-
IVth	-	-	-
Vth	-	-	-
VIth	Slight rounding, shortening and clumping with detachment of cells from the monolayer	Rounding and clumping with slight detachment of cells from the monolayer	Rounding ,shrinkage to moderate detachment of cells
VIIth	Rounding, clumping and shrinkage of cells with extensive detachment of cells from the monolayer	Rounding and clumping with moderate detachment of cells	Rounding, clumping of cells leading to extensive detachment of cells from the monolayer
VIIIth	Clump formation along with moderate detachment leading to complete damage to monolayer	Rounding , shrinkage along with moderate to extensive damage to monolayer	Rounding, clumping to extensive damage to monolayer by 96 hrs.
IXth	Marked clumping leading to complete sloughing of monolayer	Rounding, clumping, with moderate to extensive damage to monolayer	Rounding ,mild clumping with moderate to extensive damage to monolayer
Xth	Marked clumping leading to complete sloughing of cell sheet by 72 hrs.	Shrinkage, moderate detachment progressing to complete destruction of monolayer sheet by 96 hrs.	Shrinkage leading to extensive destruction followed by complete detachment of cells of the monolayer by 96 hrs.

-: No cytopathic effects seen

Table 4.4. Hour wise CPE produced in CEF by the viral isolates

Passage level	Isolates														
	P-I					PH-II					PC-III				
	Hours post inoculation					Hours post inoculation					Hours post inoculation				
	24	48	72	96	120	24	48	72	96	120	24	48	72	96	120
Ist	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IInd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IIIrd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IVth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VIth	-	+	++	+++	++++	-	-	-	+	++	-	-	+	+++	++++
VIIth	-	++	++	+++	++++	-	+	+	++	+++	-	+	++	+++	++++
VIIIth	+	++	+++	++++-		+	++	+++	++++		-	+	++	++++	
IXth	++	+++	++++ +			+	++	+++	++++		+	++	+++	++++	
Xth	++	++++	++++ +			+	++	+++	+++++		+	++	++++	+++++	

- : No CPE
+: Slight rounding and shortening of cells
+ +: Shrinkage of cells from monolayer & formation of clumps / slight detachment with vacoulation
+ + +: Moderate detachment of monolayer at few places
+ + ++: Extensive degeneration of monolayer in many places
++ +++: Complete sloughing/ peeling of cell sheet

P-I isolate infected CEL monolayer cultures at the third passage level started showing rounding of the cells at 24 hrs., followed by shrinkage and more rounding at 48 hrs. The monolayers started detaching by 72 hrs. p.i. (Plate 4.26) while the control monolayer remained unchanged (Plate 4.25). Complete sloughing and detachment of cells of the monolayer was seen at 96 hrs p.i. By the fifth passage level, infection of the CEL monolayer with P-I isolate led to moderate detachment at 24 hrs. p.i. subsequently leading to complete peeling of the monolayer by 48 hrs. p.i.

The effects produced by PH-II isolate on CEL culture started 72 hrs. p. i. from the 2^{nd} passage onwards. However, the effects got enhanced in the 3^{rd} passage with some initial rounding of the cells seen at 24 hrs. interval followed by moderate to complete degeneration and peeling of the monolayer by 72 hrs. p.i. Enhanced CPE manifested by degeneration of the monolayer by 24 hrs. and destruction of the complete monolayer was seen by 48 hrs. p.i. in the fifth passage.

Monolayers of CEL cell cultures when infected with PC-III isolate initially started showing CPE from the 2^{nd} passage level with complete destruction of the monolayer by 96 hrs. p.i. However, in the third and fourth passage level, the initiation of CPE started 24 hrs. onwards, while, extensive CPE leading to the destruction of the complete monolayer at 72 hrs. p.i. was seen in the fifth passage level (Plate 4.27 to 4.29). The control monolayer remained unchanged (Plate 4.30).

The effects seen in monolayer cultures of CEL were more extensive with rapid onset and involved whole of the monolayer in less time as compared to CEF cell cultures.

The combined passage wise onset of cytopathic changes is depicted in Table no. 4.5. while hour. wise changes seen in the liver cell culture inoculated with the three isolates are depicted in Table 4.6.

The virus infectivity titers (EID_{50} / ml) calculated for each of the three isolates viz. P-I, PH-II and PC-III after the 5^{th} passage level were $10^{7.3}$/ ml, $10^{7.82}$/ ml and $10^{8.5}$/ ml, respectively.

4.2.1.3 Chicken embryo kidney cell culture

4.2.1.3.1 Establishment of chicken embryo kidney cell cultures

The CEK cells got established as a monolayer after 96 hrs. of seeding.

4.2.1.3.2 Infection of primary chicken embryo kidney cell cultures:

No CPE were observed in the first passage. However, in the second passage level, CEK monolayer infected with P-I isolate started showing CPE 72 hrs. p.i. onwards. These CPE were characterized by retraction and loss of cells consequently leading to extensive destruction of the monolayer by 120 hrs. p.i. (Plate 4.32 to 4.34). The control monolayer (Plate 4.31) did not show any changes. Additional passages of the isolate decreased the time interval in initiating CPE from 72 to 24 hrs. along with evidence of complete detachment by 72 hrs. p.i.

At the start of the fifth passage however the retraction of cells from the monolayer was marked. This followed simultaneously by detachment and complete degeneration of the monolayer by 72 hrs. p.i.

Table 4.5. Passage wise changes in CEL cell culture after inoculation with viral isolates

Passage level	P-I	PH-II	PC-III
Ist	-	-	-
IInd	Shrinkage, rounding, moderate to complete detachment of the monolayer.	Rounding and cell shrinkage followed by moderate to extensive detachment of monolayer	Shrinkage, clumping of cells followed by detachment of cells from monolayer
IIIrd	Rounding, clumping along with moderate to complete detachment of cells	Rounding of cells leading to complete detachment of monolayer	Rounding ,aggregate formation with extensive detachment of monolayer
IVth	Rounding leading to moderate to complete sloughing of monolayer	Moderate to complete sloughing of monolayer	Rounding ,shrinkage to moderate to complete sloughing of monolayer
Vth	Moderate to complete sloughing of monolayer by 48 hrs.	Moderate cell detachment seen at 24 hrs. leading to complete sloughing of monolayer by 48 hrs.	Moderate to extensive detachment leading to complete peeling of monolayer by 72 hrs.

-: No cytopathic effects seen

Table 4.6. Hour wise appearance of CPE in CEL culture at different passage levels

Passage level	Virus isolates														
	P-I					PH-II					PC-III				
	Hours post inoculation					Hours post inoculation					Hours post inoculation				
	24	48	72	96	120	24	48	72	96	120	24	48	72	96	120
I[st]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
II[nd]	-	++	+++	++++		-	-	++	+++	++++	-	++	+++	++++	
III[rd]	+	++	+++	+++++		+	+++	+++++			+	++	+++	+++++	
IV[th]	+	+++	+++++			+++	+++++				+	++	++++	+++++	
V[th]	+++	+++++				+++	+++++				+++	++++	+++++		

-: No CPE
+: Slight rounding of cell
+ +: Rounding, clumping and aggregate formation
+ + +: Moderate detachment of monolayer at few places
+ + + +: Extensive detachment of monolayer at many areas
+ + + + +: Complete sloughing/peeling off from surface of cell sheet

The monolayer infected with PH-II isolate exhibited similar pattern with initial CPE becoming obvious in the second passage level from 72 hrs. and extensive degeneration of the monolayer by 120 hrs. p.i. The time required for the initiation of CPE shortened from 72 to 48 hrs. in third and fourth passage levels with complete degeneration of the monolayer by 72 hrs. p.i.

At fifth passage level, the time interval was further shortened with peeling of the complete monolayer by 48 hrs. itself (Plate 4.35 to 4.36).

Monolayers infected with PC-III isolate roughly showed similar patterns with decreasing time interval for initiating cell changes with each subsequent passage. By the fifth passage level, the cells became completely detached from the monolayer after 72 hrs. p.i.

The virus infectivity titers calculated for the three isolates after the 5^{th} passage level were log $10^{7.6}$, log $10^{7.5}$ and log $10^{6.5}$ respectively, that were higher than the initial level.

The combined passage wise effects of the three isolates on CEK monolayer cultures at each passage level are shown in Table 4.7 and the hour wise changes induced in CEK monolayers by the isolates are depicted in Table 4.8.

4.2.2 Cell lines

4.2.2.1 Vero cell lines

4.2.2.1.1 Establishment of Vero cell lines

Vero cell lines were easy to grow and maintain and could be established relatively quickly leading to faster study time between subcultures. The Vero cell line monolayers were confluent by 48 hrs. (Plates 4.37 to 4.38).

Table 4.7. Passage wise changes in CEK cell cultures after inoculation with viral isolates

Passage level	P-I	PH-II	PC-III
Ist	-	-	-
IInd	Shrinkage, clumping followed by moderate to extensive detachment of the monolayer.	Cell clumping, retraction followed by extensive detachment of monolayer	Shrinkage, clumping of cells followed by detachment of cells from monolayer
IIIrd	Clumping of cells with aggregate formation and moderate degeneration of monolayer	Clumping of cells with extensive detachment of monolayer	Rounding ,retraction of cells with extensive detachment of monolayer
IVth	Rounding, moderate degeneration leading to complete sloughing of monolayer	Moderate detachment leading to complete sloughing of monolayer	Severe aggregation, leading to complete sloughing of monolayer
Vth	Retraction, detachment, degeneration and total peeling of monolayer sheet by 72 hrs.	Detachment and peeling of the cell sheet by 48 hrs.	Shrinkage and moderate detachment followed by complete detachment of cell sheet by 72 hrs.

-: No cytopathic effects seen

Table 4.8. Hour wise appearance of CPE in CEK culture at different passage level

Passage level	Virus isolates														
	P-I					PH-II					PC-III				
	Hours post inoculation					Hours post inoculation					Hours post inoculation				
	24	48	72	96	120	24	48	72	96	120	24	48	72	96	120
Ist	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IInd	-	-	++	+++	++++	-	-	++	+++	++++	-	-	++	+++	++++
IIIrd	-	++	+++	+++		-	++	+++++			-	+	+++	+++++	
IVth	+	+++	+++++			-	+++	+++++			-	++	+++	+++++	
Vth	+++	++++	+++++			+++	+++++				++	+++	+++++		

-: No visible change
+: Rounding of cell
++: Clumping, aggregate formation and shrinkage of cells
+++: Moderate degeneration/retraction of monolayer at some places
++++: Extensive detachment of monolayer at many places
+++++: Complete sloughing/peeling of monolayer sheet

4.2.2.1.2 Infection of Vero cells

The isolates were given eight passages in Vero cells. No characteristic CPE were seen in Vero cell lines during the first five blind passages. Evidence of CPE was seen in Vero monolayer cultures infected with P-I isolate from 72 hrs. p.i. onwards at the sixth passage level. Initially, there were shrinkage and rounding of cells at a few places that progressed to clumping followed by extensive detachment of the monolayer 120 hrs. p.i.

At the eighth passage level, cytopathic changes seen with P-I isolate were rounding and shortening of Vero cell line at 48 hrs. p.i. That was followed at 72 hrs. p.i by moderate detachment leading to complete detachment of the monolayer by 96 hrs. p.i (Plates 4.39 to 4.41). The control monolayer (Plate 4.42) remained unchanged.

At the eighth passage level, the Vero monolayer infected with PH-II virus isolate exhibited CPE in sequential progression like rounding and shortening of cells by 24 hrs. p.i, rounding of cells along with moderate detachment at 48 hrs. p.i followed by complete destruction of the monolayer by 96 hrs. p.i. PC-III induced changes in the Vero monolayer included shrinkage & clumping of cells starting at 48 hrs. leading to extensive loss of cells from the monolayer that led to complete destruction of the monolayer by 96 hrs. The passage wise changes in Vero monolayer is shown in Table 4.9, while hour wise changes induced by the three isolates are detailed in Table 4.10.

The virus infectivity titers of each isolate was measured after 8^{th} passage. The virus titers after eight passage were log $10^{8.3}$, log $10^{8.8}$ and log $10^{8.2}$ EID_{50}/ml, respectively.

Table 4.9. Passage wise changes in Vero cell cultures after inoculation with viral isolates

Passage level	P-I	PH-II	PC-III
Ist	-	-	-
IInd	-	-	-
IIIrd	-	-	-
IVth	-	-	-
Vth	-	-	-
VIth	Shrinkage and clumping of cells followed by extensive detachment of the monolayer.	Rounding of cells, cell shrinkage followed by moderate to extensive detachment of monolayer	Shrinkage, clumping of cells followed by detachment of cells from monolayer
VIIth	Rounding, shrinkage and and clumping of cells with extensive detachment of monolayer	Cells shrinkage leading to extensive detachment of monolayer	Rounding ,clumping of cells along with moderate to extensive detachment of monolayer
VIIIth	Severe aggregation, rounding , shortening and clumping of cells leading to complete sloughing of monolayer by 96 hrs.	Rounding, shortening, shrinkage of cells leading to moderate to complete sloughing of monolayer by 96 hrs.	Shrinkage, clumping of cells followed by extensive to complete sloughing of monolayer by 96 hrs.

-: No cytopathic effects seen

Table 4.10. Hour wise appearance of CPE in Vero cell culture at different passage level

Passage level	Virus isolates														
	P-I					PH-II					PC-III				
	Hours post inoculation					Hours post inoculation					Hours post inoculation				
	24	48	72	96	120	24	48	72	96	120	24	48	72	96	120
Ist	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IInd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IIIrd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IVth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vth	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VIth	-	-	++	+++	++++	-	-	+	+++	++++	-	-	++	+++	++++
VIIth	+	++	+++	++++		-	++	+++	++++		-	++	+++	++++	+++++
VIIIth	-	++	+++	+++++		++	+++	++++	+++++		-	++	++++	+++++	

- : No cytopathic changes
+: Rounding of cell
++: Clumping and shrinkage of cells in monolayer
+++: Moderate detachment /loss of monolayer at few places
++++: Extensive detachment involving many cells of monolayer
+ + + + +: Complete sloughing/peeling of monolayer sheet

4.2.2.2 HeLa cell lines

4.2.2.2.1 Establishment of HeLa cell line

The HeLa monolayer became confluent by 72 hrs. post seeding (Plates 4.43 to 4.44).

4.2.2.2.2 Infection of HeLa cell line

All three isolates were given eight blind passages for adaptation of the virus to these cells. An incubation of 5 to 7 days was given to check appearance of cytopathic changes. The virus could not be adapted in HeLa cell lines even after eight serial blind passages.

4.2.2.3 McCoy cell lines

4.2.2.3.1 Establishment of McCoy cell line

The McCoy cell lines formed a complete monolayer after 96 hrs. of incubation. MGG stained cells are shown in Plates 4.45 to 4.46.

4.2.2.3.2 Infection of McCoy cells

The McCoy cells were infected with all the isolates. The attempts made at adaptation of virus in McCoy cell line failed and no cytopathic changes were observed after eight blind passages.

4.2.2.4 Baby Hamster kidney-21 cell lines (BHK-21)

4.2.2.4.1 Establishment of BHK-21 cell lines

The BHK-21 cells were split in our laboratory according to the split ratio suggested by NCCS, Pune. A complete BHK-21 monolayer usually formed within 72 hrs. (Plate 4.47 to 4.48).

4.2.2.4.2 Infection of BHK-21 cells

Eight blind passages were given in BHK cells to check adaptation of the virus to these cells without success.

The comparative passage wise changes observed in the different cell cultures used in the study are presented in Table 4.11. The passage wise comparison of CPE in cell cultures/ lines is shown in Table 4.12.

4.2.3 Re infection studies in chicks

FAV adapted in cell cultures (CEK, CEL, CEF) and Vero cell line that exhibited CPE were used to infect healthy chicks for determining the virulence of the isolate in natural host following adaptation in cell culture. Three chicks were used per cell culture with two control chicks in each group. Death of the chicks (Plate 4.49) was recorded from 24 hrs. to 120 hrs. p.i. (Table 4.13).

The chicks that were infected with virus propagated in CEL and Vero cell line showed quicker death time as compared to CEF and CEK cell cultures adapted virus. The pathological changes seen in the chicks following infection are depicted in Table 4.14. Typical hydropericardium was seen in all chicks that died at 72 hrs. p.i.(Plates 4.51 and 4.53) whereas, hydropericardium was not observed in chicks that had died early. Other pathological lesions included pale, enlarged and hemorrhagic livers (Plates 4.50, 4.52, 4.54 and 4.55).

The control chicks did not exhibit any changes and two chicks were sacrificed each day upto one week to compare the findings. The corresponding cell cultures when re-infected with the pooled liver homogenates from each respective group could record cytopathic changes

Table 4.11. Comparative passage wise description of adaptation of fowl adenovirus isolate in four cell lines

Passage level	P-I				PH-II				PH-III			
	Adaptation in cell lines				Adaptation in cell lines				Adaptation in cell lines			
	Vero	HeLa	McCoy	BHK-21	Vero	HeLa	McCoy	BHK-21	Vero	HeLa	McCoy	BHK-21
1^{st}	-	-	-	-	-	-	-	-	-	-	-	-
II^{nd}	-	-	-	-	-	-	-	-	-	-	-	-
III^{rd}	-	-	-	-	-	-	-	-	-	-	-	-
IV^{th}	-	-	-	-	-	-	-	-	-	-	-	-
V^{th}	-	-	-	-	-	-	-	-	-	-	-	-
VI^{th}	+	-	-	-	+	-	-	-	+	-	-	-
VII^{th}	+	-	-	-	+	-	-	-	+	-	-	-
$VIII^{th}$	+	-	-	-	+	-	-	-	+	-	-	-

-: No cytopathic effects seen

+: Cytopathic effects visible

Table 4.12. Passage wise comparison of cytopathic changes in cell cultures/lines

Passage level	Isolates											
	P-I				PH-II				PC-III			
	Type of cytopathic changes				Type of cytopathic changes				Type of cytopathic changes			
	CEF	CEL	CEK	Vero	CEF	CEL	CEK	Vero	CEF	CEL	CEK	Vero
1st	-	-	-	-	-	-	-	-	-	-	-	-
IInd	-	++	++	-	-	++	++	-	-	++	++	-
IIIrd	-	+++	+++	-	-	+++	+++	-	-	+++	+++	-
IVth	-	++++	++++•	-	-	++++	++++	-	-	++++	++++	-
Vth	-	+++++	+++++	-	-	++++	+++++	-	-	+++++	+++++	-
VIth	++	*	*	+++	++	*	*	++	++	*	*	++
VIIth	+++	*	*	+++	+ ++	*	*	+++	+++	*	*	+++
VIIIth	+++	*	*	++++	+++	*	*	++++	+++	*	*	++++
IXth	++++	*	*	*	++++	*	*	*	++++	*	*	*
Xth	+++++	*	*	*	++++	*	*	*	++++	*	*	*

-: No cytopathic changes
*: Not passaged further
++: Shrinkage, aggregation, rounding of cells, degeneration followed by detachment of the monolayer
+++: Extensive rounding / clumping of cells with shrinkage/ peeling off/ complete detachment of monolayer
++++: Severe aggregation, rounding and clumping of cells leading to complete sloughing/extensive degeneration of monolayer
+++++: Total detachment, degeneration and peeling of monolayer sheet

in the cell cultures. The body weights of the infected chicks along with the control chicks are shown in Table 4.15.

Table 4.13. Mortality pattern in chicks re- infected with CEF, CEL, CEK and Vero cell adapted virus

Cell cultures/ cell line	Passage level	No. of chicks	Dead (Hrs. post inoculation)						
			24	48	72	96	120	144	168
CEL	5^{th}	3	1	1	1	-	-	-	-
CEK	5^{th}	3	-	1	1	1	-	-	-
CEF	10^{th}	3	-	1	1	-	1	-	-
Vero cell line	8^{th}	3	1	1	1	-	-	-	-
HeLa	8^{th}	3	-	-	-	-	-	-	3 (S)
McCoy	8^{th}	3	-	-	-	-	-	-	3 (S)
BHK-21	8^{th}	3	-	-	-	-	-	-	3 (S)
Control chicks	S (Sacrificed)	14	2(S)	2(S)	2(S)	2(S)	2(S)	2(S)	2(S)

*** No chicks died in these groups and all chicks were sacrificed after 7 days p.i.**

Table 4.14. Pathological changes observed in chicks infected with cell culture adapted virus

Cell cultures/ cell line	No. of chicks	Dead (Hrs. post inoculation)				
		24	48	72	96	120
CEL	3	No gross changes	Hemorrhages along with enlarged livers	Hydropericardium	-	-
CEK	3	-	Pale & enlarged liver	Hydropericardium	Slight congestion on liver	-
CEF	3	-	Pale liver	Pale & enlarged liver	-	Hemorrhagic & enlarged liver
Vero cell	3	No gross changes	Severe hemorrhages, congestion & enlarged liver	Pale liver	-	-
HeLa	3	-	-	-	-	-
McCoy	3	-	-	-	-	-
BHK-21	3	-	-	-	-	-
Control chicks	14 (S)	No change	No change	No change	No change	No change

Table 4.15. Measurement of weight of chicks

Cell cultures	No. of chicks	Dead (Hrs. post inoculation						
		24	**48**	**72**	**96**	**120**	**144**	**168**
CEL	3	28.6 gm	31.2 gm	34.5 gm	-	-	-	-
CEK	3	-	30.8 gm	34.0 gm	39.8 gm	-	-	-
CEF	3	-	31.6 gm	35.2 gm	-	41.6 gm	-	-
Vero	3	28.8 gm	30.0 gm	35.4 gm	-	-	-	-
HeLa	3	-	-	-	-		-	49.0 gm
McCoy	3	-	-	-	-	-	-	49.2 gm
BHK-21	3	-	-	-	-	-	-	50.5 gm
Control chicks	14 (S)	28.5 gm	29.6 gm	35.9 gm	39.8 gm	44.5 gm	48.6 gm	50.0 gm

The table above reveals no appreciable difference between the weights of infected and control group birds.

4.2.4 Pathogenicity study in embryonated chicken eggs

When isolates were passaged in embryonated eggs via yolk sac route, the embryos were killed and gross pathological lesions were seen within 3 to 6 days of inoculation. During 1st passage level, only petechial hemorrhages were seen. At 2nd and 3rd passage level, the embryos died and extensive hemorrhages were seen on the body (Plates 4.56 to 4.57). All the isolates produced almost identical pathology in embryonated eggs (Table 4.16).

Maximum mortality was recorded by all the isolates at 4 DPI *i.e.* at 96 hrs. p.i. A total of 20 embryos died spanning all three passage levels. The mortalities produced by all the isolates passage wise were recorded in Table 4.17.

4.2.4.1 Growth rate

The growth rate was observed by recording the length, width and weight of inoculated embryos and comparing the parameters with embryos of control group at first, second and third passage levels (Table 4.18 to 4.20).

4.2.4.1.1 Length

The mean length ± S.E of embryos recorded in all infected groups of first, second and third passage levels were 2.662 ± 0.020 cms that were found to be lower when compared with corresponding values in the control group. In control group, mean length values were 3.123 ± 0.067 cms respectively.

4.2.4.1.2 Width

The mean width ± S.E values of embryos recorded in all infected groups of first, second and third passage levels were 0.827 ± 0.159 cms and these were found to be lower when compared to mean width values of control group. In control group, mean width values were 1.144 ±0.047 cms, respectively.

4.2.4.1.3 Weight

The mean weight of embryos in all infected groups at first, second and third passage levels were 1.0052 ± 0.0220 gms., whereas, the mean weight in control group were 1.5514 ± 0.062 gms, respectively. The values of infected group were found to be lower than the control group.

Additionally, the comparison of mean ± S.E value measurements of length, width and weight of infected group embryos at different time intervals to that of the control embryos at different time intervals is shown in Table 4.21.

The comparison of mean ± S.E values of length, width and weight of inoculated embryos at different time intervals to that of control group embryos at different time intervals, showed that the values of infected group embryos at different time intervals were numerically lower than the corresponding values of control group embryos.

Table 4.16. Changes in embryonated eggs after inoculation with viral isolates

Passage level	P-I	PH-II	PC-III
1st	Embryo died with slight petechial hemorrhages on head and leg region	Embryo died with slight petechial hemorrhages	Embryo died with petechial hemorrhages on head and leg region
2nd	Embryo died with hemorrhages on body	Embryo died	Embryo died with hemorrhages on body
3rd	Dead embryo with severe congestion, hemorrhages and stunting	Dead embryo with severe hemorrhages and stunting	Dead embryo with congestion

Table 4.17. Details of morality pattern of inoculated embryos

Hrs. PI	Isolates											
	P-I			PH-II			PC-III			Control chicks sacrificed		
	Passage level			Passage level			Passage level			Passage level		
	I st	II nd	III rd	I st	II nd	III rd	I st	II nd	III rd	I st	II nd	III rd
24	-	-	-	-	-	-	-	-	-	-	-	-
48	-	-	-	-	-	-	-	-	-	-	-	-
72	1/5 (20%)	1/5 (20%)	2/5 (40%)	1/5 (20%)	1/5 (20%)	2/5 (40%)	2/5 (40%)	1/5 (20%)	0/5 (0%)	2/5 (40%)	1/5 (20%)	1/5 (20%)
96	2/5 (40%)	2/5 (40%)	2/5 (40%)	2/5 (40%)	3/5 (60%)	2/5 (40%)	2/5 (40%)	2/5 (40%)	3/5 (60%)	2/5 (40%)	2/5 (40%)	2/5 (40%)
120	2/5 (40%)	1/5 (20%)	1/5 (20%)	2/5 (40%)	1/5 (20%)	1/5 (20%)	1/5 (20%)	2/5 (40%)	1/5 (20%)	1/5 (20%)	1/5 (20%)	1/5 (20%)
144	-	1/5 (20%)	-	-	-	-	-	-	1/5 (20%)	-	1/5 (20%)	1/5 (40%)

Table 4.18. Measurements of length, breadth and weight of infected and control embryos at first passage level

Hrs. PI		P-I				PH-II				PC-III				Control chicks sacrificed		
	Mortality (M)	L (cms)	B (cms)	W (gms)	M	L (cms)	B (cms)	W (gms)	M	L (cms)	B (cms)	W (gms)	S	L (cms)	B (cms)	W (gms)
72	1	2.553	0.730	0.853	1	2.520	0.693	0.813	2	2.396	0.765	0.918	2	2.905	0.923	1.305
96	2	2.532	0.756	0.919	2	2.627	0.697	0.889	2	2.491	0.809	0.968	2	2.998	0.984	1.326
120	2	2.673	0.888	1.12	2	2.717	0.767	1.162	1	2.630	0.930	1.234	1	3.339	1.263	1.793

Table 4.19. Measurements of length, breadth and weight of infected and control embryos at second passage level

Hrs. PI		P-I				PH-II				PC-III				Control chicks sacrificed		
	Mortality (M)	L (cms)	B (cms)	W (gm)	M	L (cms)	B (cms)	W (gms)	M	L (cms)	B (cms)	W (gms)	S	L (cms)	B (cms)	W (gms)
72	1	2.516	0.723	0.862	1	2.462	0.745	0.855	1	2.693	0.737	0.925	1	2.752	0.932	1.351
96	2	2.677	0.827	0.937	3	2.630	0.741	0.934	2	2.721	0.729	0.962	2	2.959	1.033	1.453
120	1	2.828	0.876	1.020	1	2.721	0.820	1.115	2	2.843	0.937	1.275	1	3.105	1.321	1.729
144	1	2.815	0.853	1.302	-	-	-	-	-	-	-	-	1	3.562	1.323	2.012

Table 4.20. Measurements of length, breadth and weight of infected and control embryos at third passage level

Hrs. PI		P-I				PC-II				PC-III				Control chicks sacrificed		
	Mortality (M)	L (cms)	B (cms)	W (gms)	M	L (cms)	B (cms)	W (gms)	M	L (cms)	B (cms)	W (gms)	S	L (cms)	B (cms)	W (gms)
72	2	2.566	0.743	0.876	2	2.622	0.805	0.887	-	-	-	-	1	2.898	0.95	1.306
96	2	2.774	0.841	0.954	2	2.722	0.975	0.952	3	2.701	0.867	0.980	2	3.035	1.164	1.367
120	1	2.816	0.926	1.106	1	2.715	1.052	1.334	1	2.952	1.113	1.112	1	3.292	1.372	1.742
144	-	-	-	-	-	-	-	-	1	2.914	1.124	1.318	1	3.722	1.456	2.004

S= Control chicks sacrificed

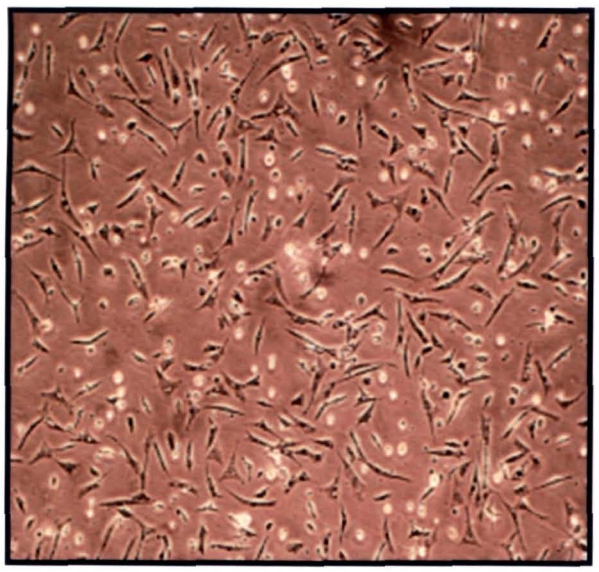


Plate 4.7; CEF culture, 18 hrs culture (100X)

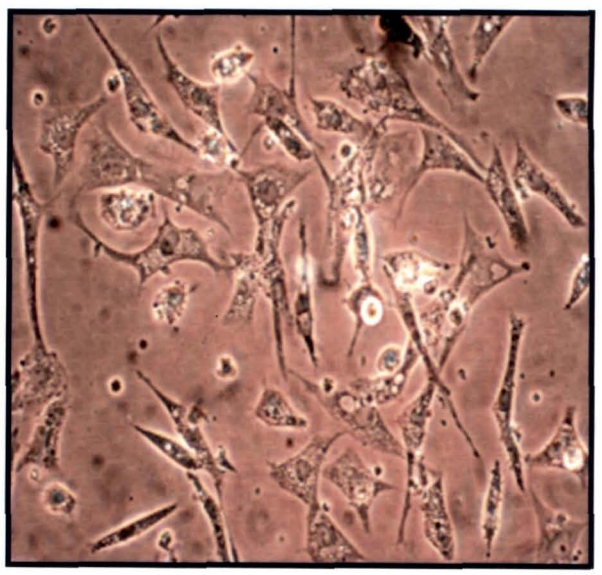


Plate 4.8: CEF culture, 18 hrs culture (400X)

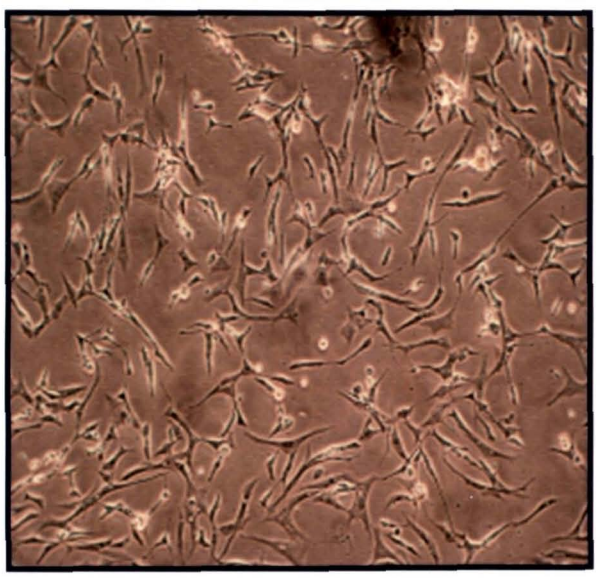


Plate 4.9: CEF culture, 24 hrs culture (100X)

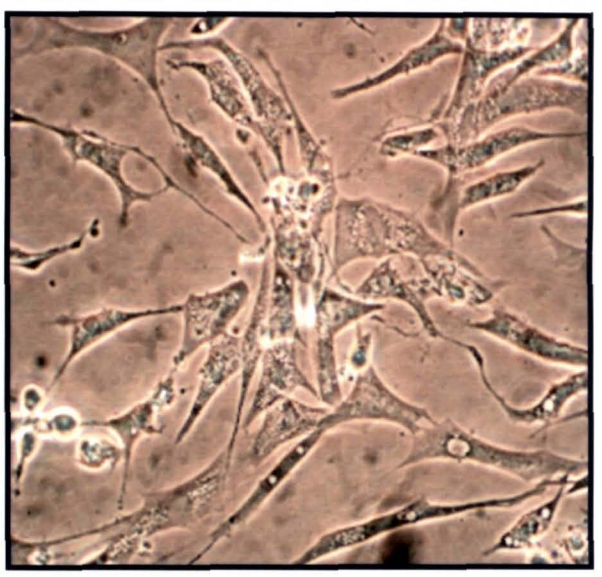


Plate 4.10: CEF,24 hrs culture (400X)

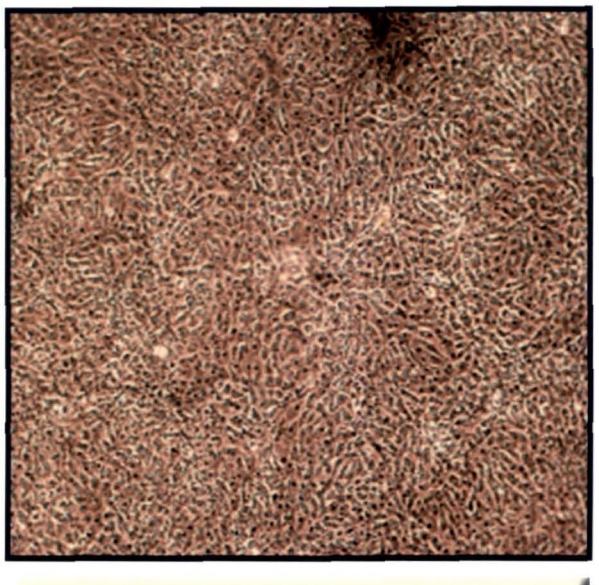


Plate 4.11: CEF complete monolayer 48 hrs culture.(100 X)

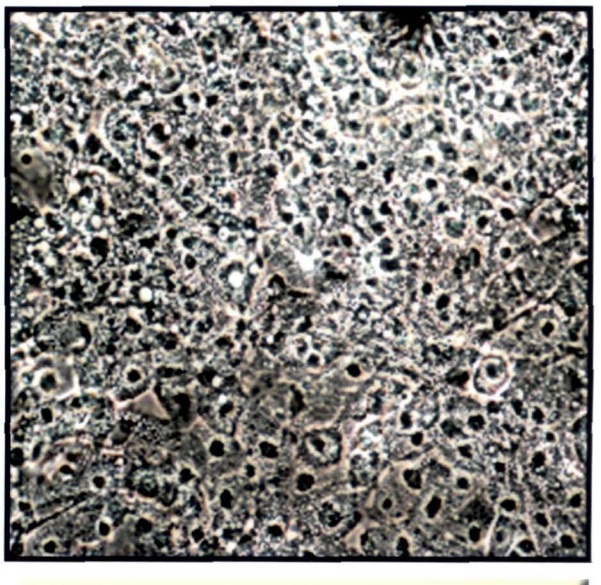


Plate 4.12: CEF complete monolayer 48 hrs culture (400X)

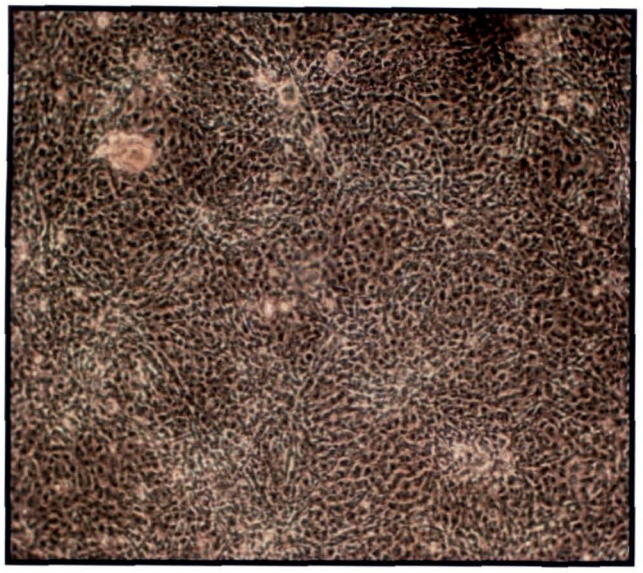


Plate 4.13 : CEF cells, Control monolayer culture (100 X)

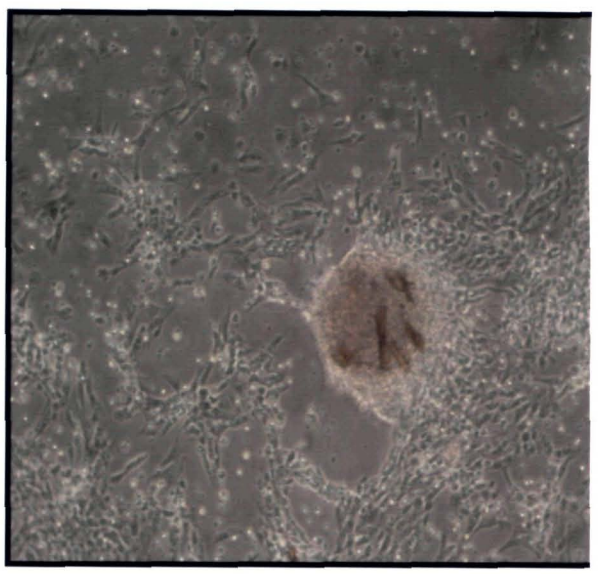


Plate 4.14: Shrinkage of cells from the monolayer, 72 hrs p.i , VIth passage with P-I

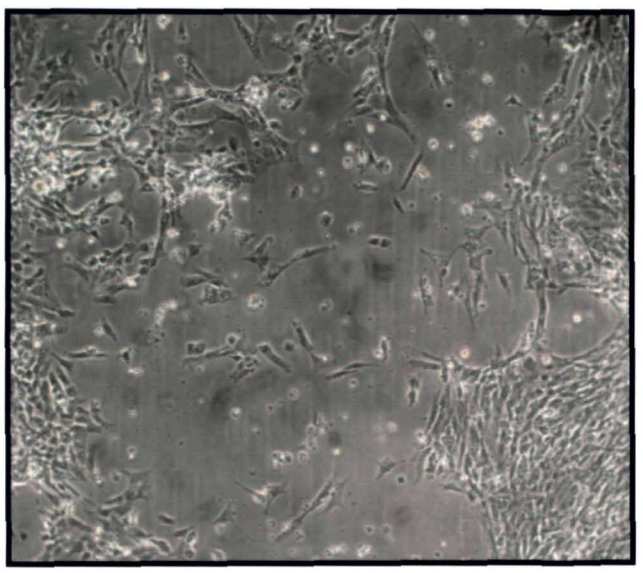


Plate 4.15 : Detachment of cells from monolayer, 96 hrs p. VIth passage (X 100)

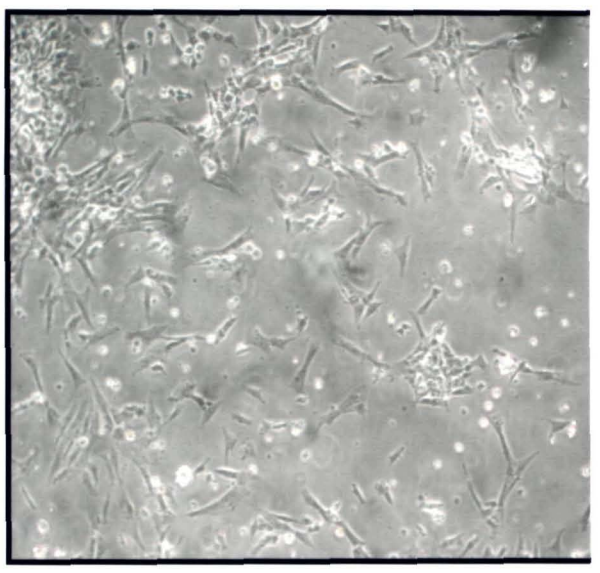


Plate 4.16 : Extensive detachment of monolayer, 120 hrs p.i , VIth passage

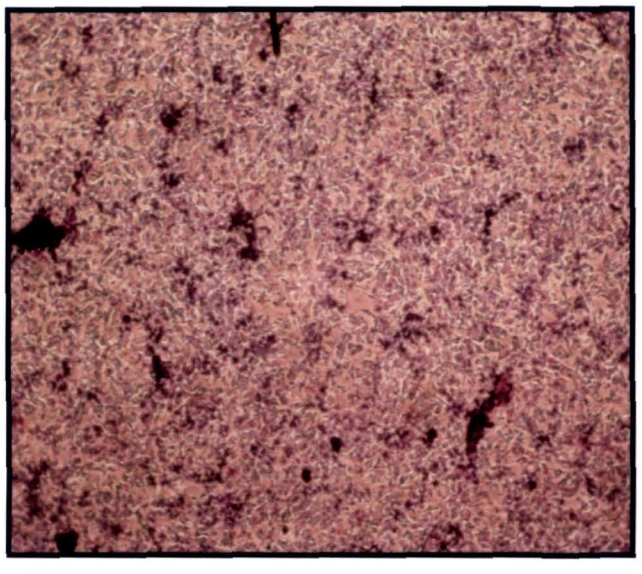


Plate 4.17: CEF cells, control monolayer MGG stain (100 X)

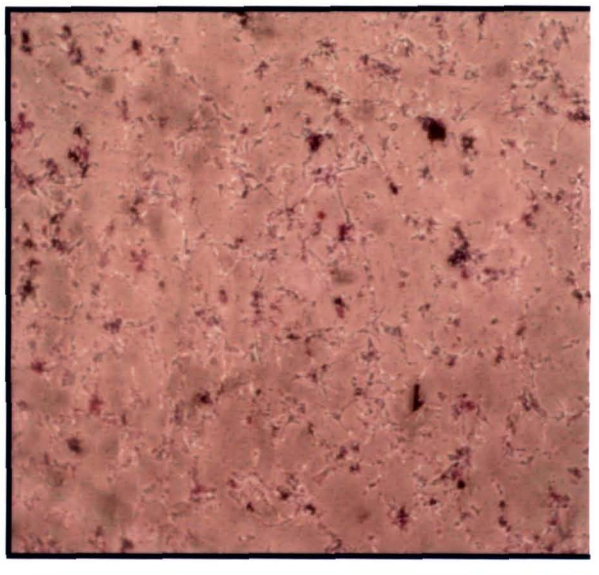


Plate 4.18: Destruction of the monolaye 72 hrs p.i , Xth passage level, MGG stair (100 X)

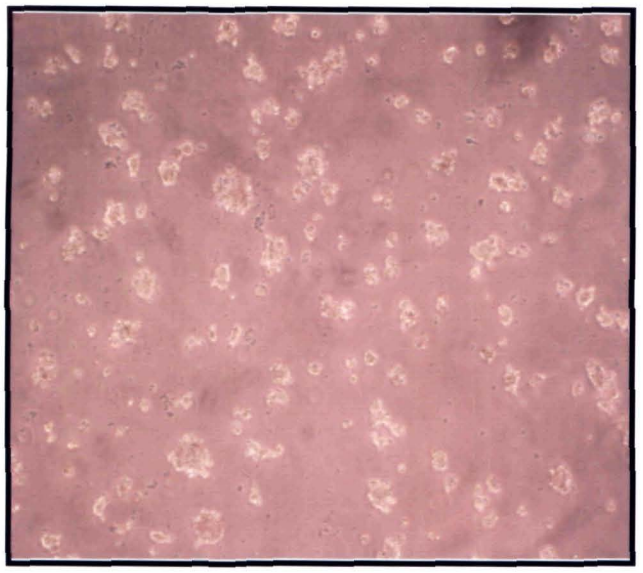


Plate 4.19: CEL cells, 24 hrs culture (100 X)

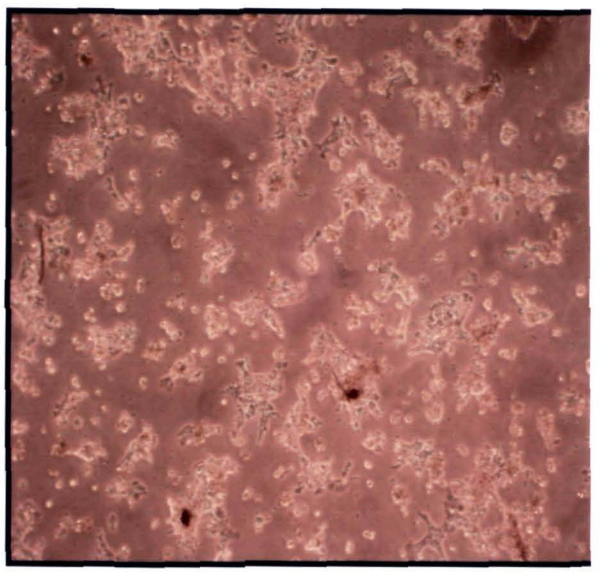


Plate 4.20 : CEL cells, 48 hrs culture (100X)

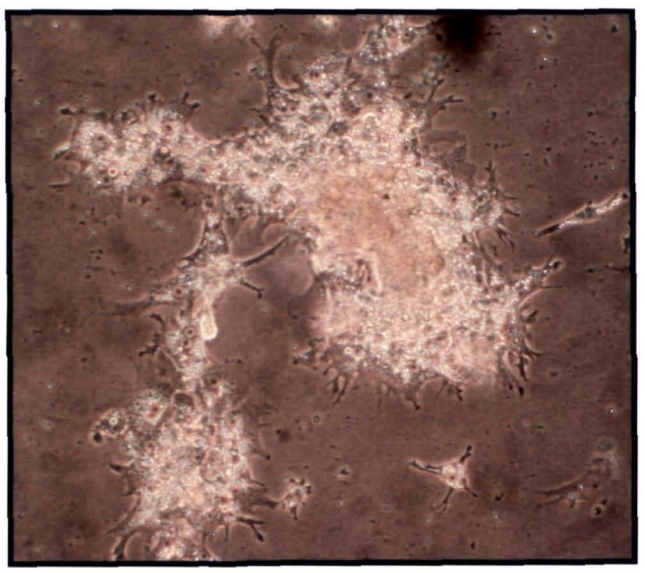


Plate 4.21 : CEL cells, 72 hrs culture (100 X)

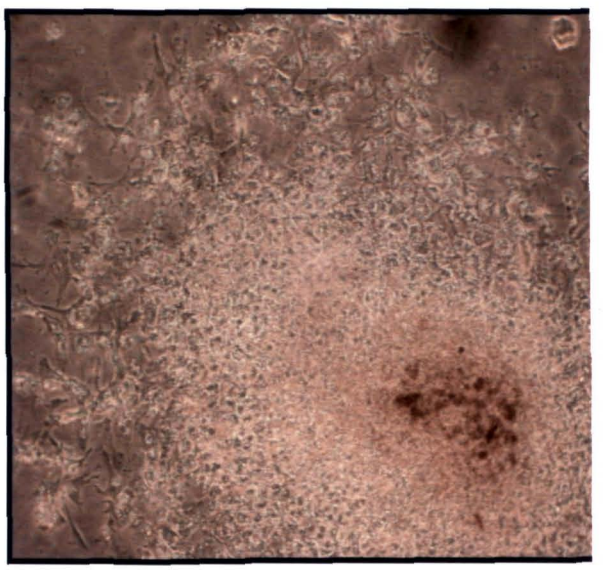


Plate 4.22 : CEL cells, 96 hrs culture (100 X)

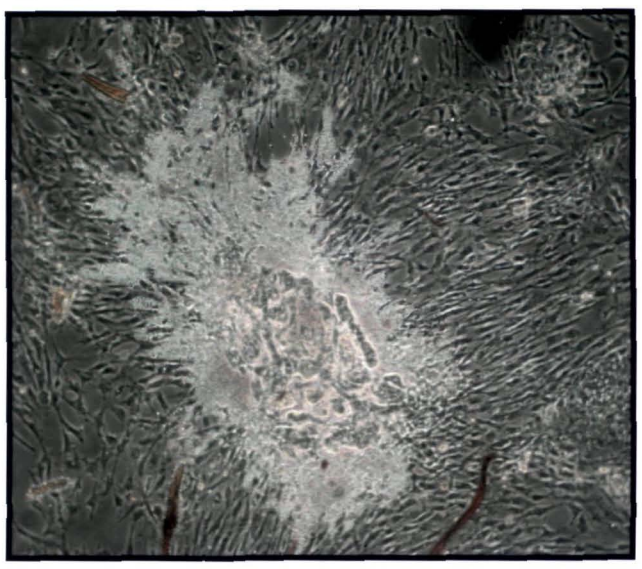


Plate 4.23 : CEL cells, 120 hrs culture (100 X)

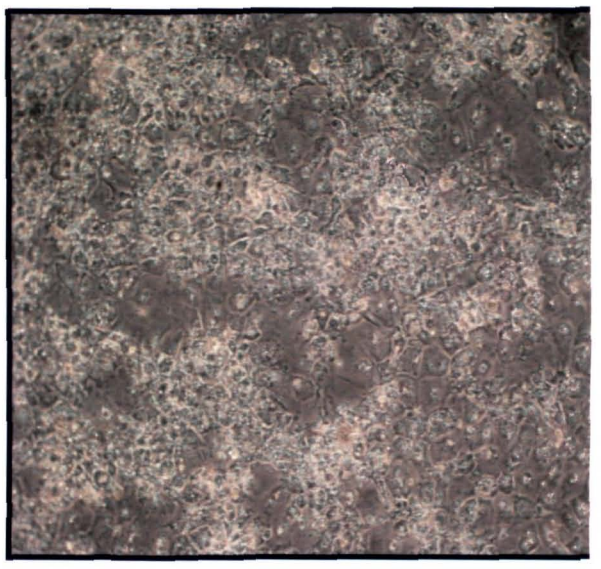


Plate 4.24: CEL cells 144 hrs culture (100 X)

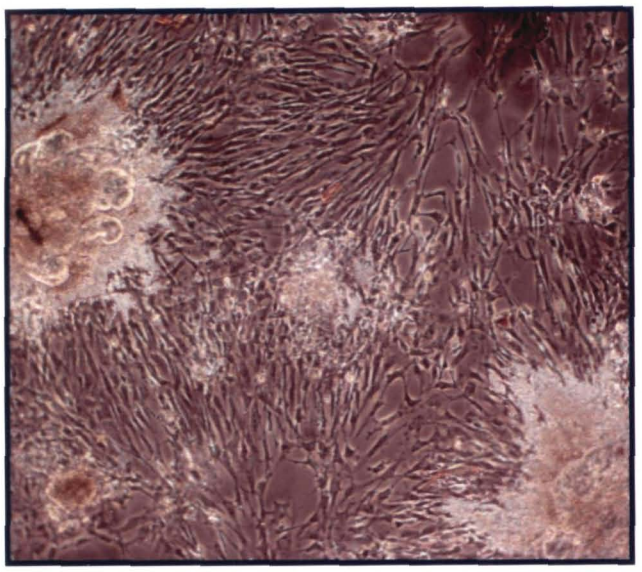


Plate 4.25: CEL cells, control monolayer (100 X)

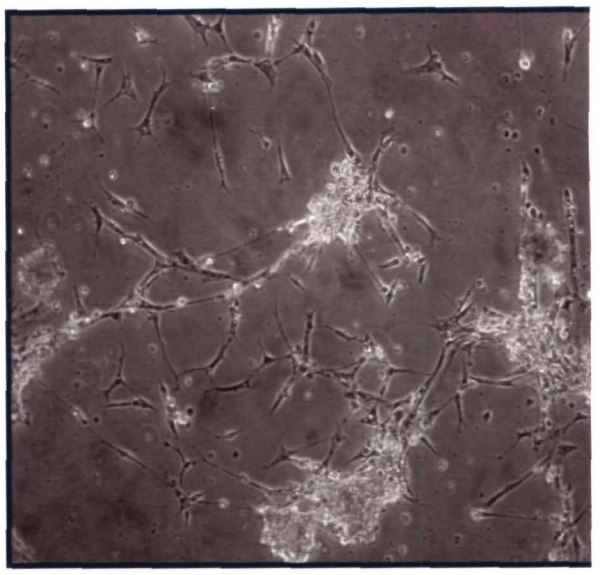


Plate 4.26: Detachment of monolayer 72 hrs p.i, III[rd] passage with P-I isolate (100 X)

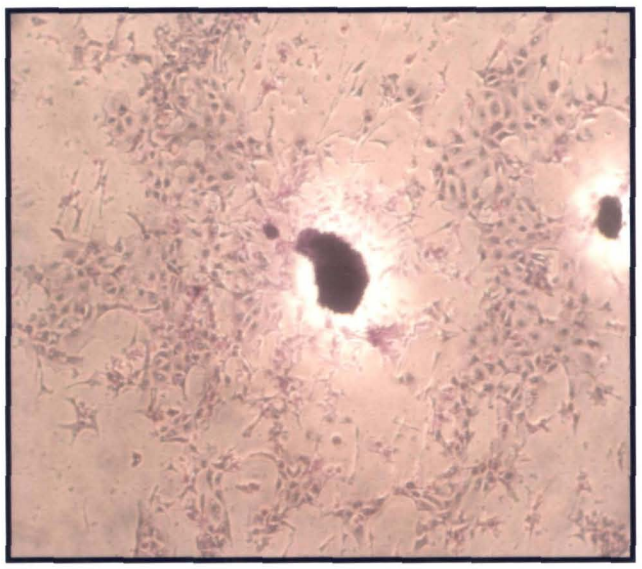


Plate 4.27: Moderate degeneration of monolayer at few places, 24 hrs p.i , V[th] passage, MGG (100 X)

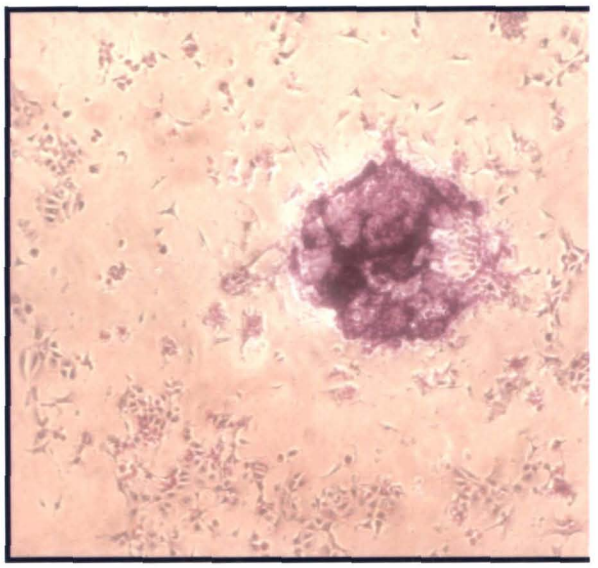


Plate 4.28: Extensive degeneration of monolayer, 48 hrs p.i, V[th] passage, MGG (100 X)

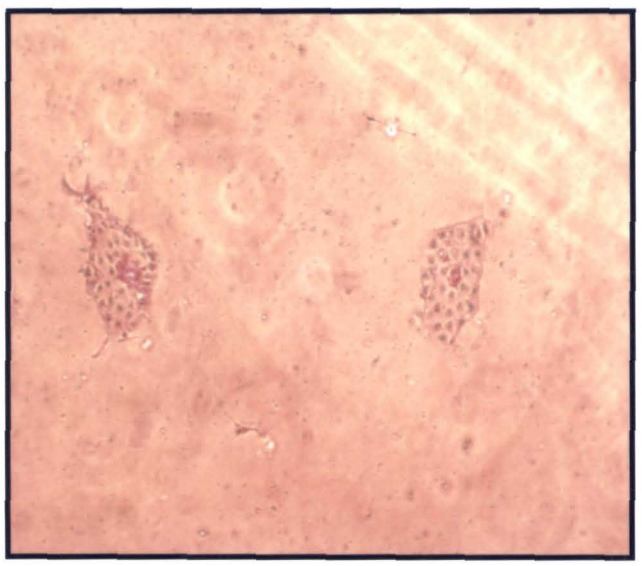


Plate 4.29: Complete loss of monolayer, 72 hrs p.i , V[th] passage, MGG (100 X)

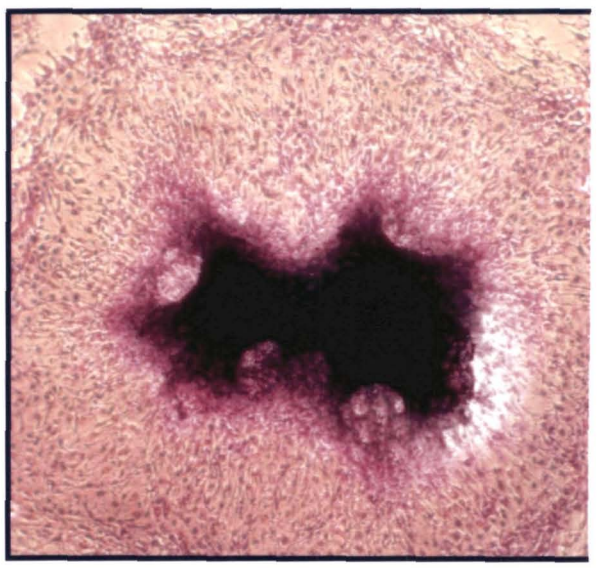


Plate 4.30: CEL control monolayer, MGG stain (100 X)

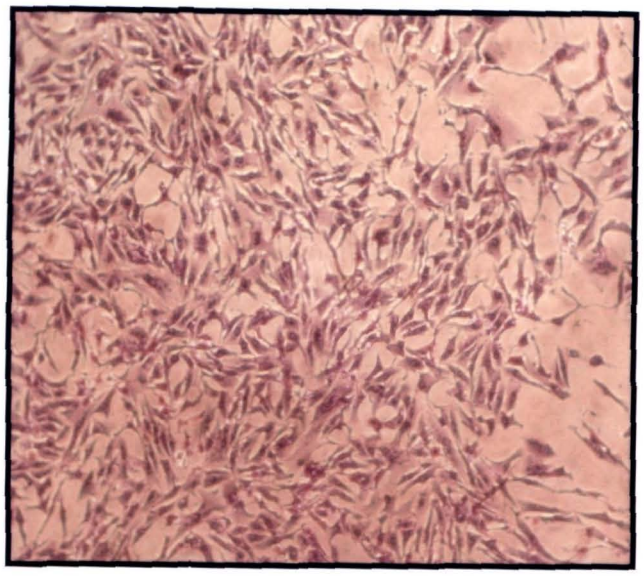


Plate 4.37 : Vero cells, 24 hrs. Culture, MGG stain (100 X)

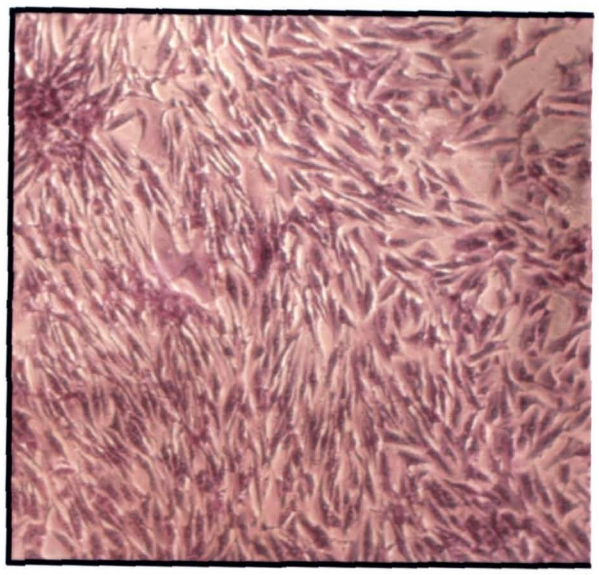


Plate 4.38 : Vero cells, 48 hrs. Culture, MGG stain (100 X)

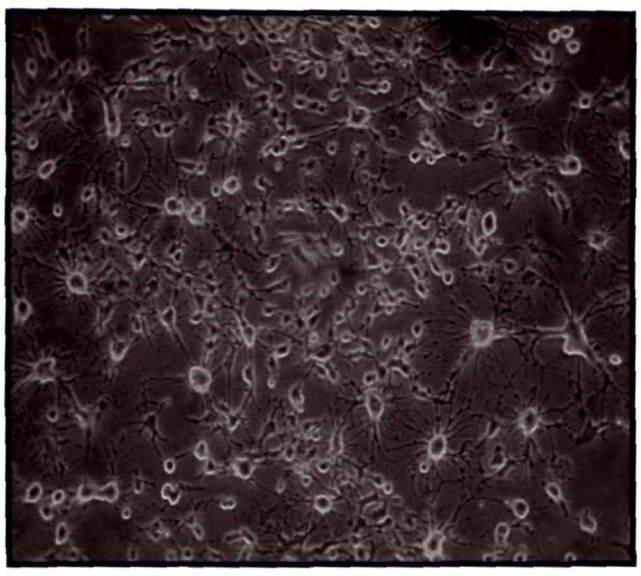


Plate 4.39: Shrinkage of cells, Vero cell culture 48 hrs p.i, VIIIth passage (100 X)

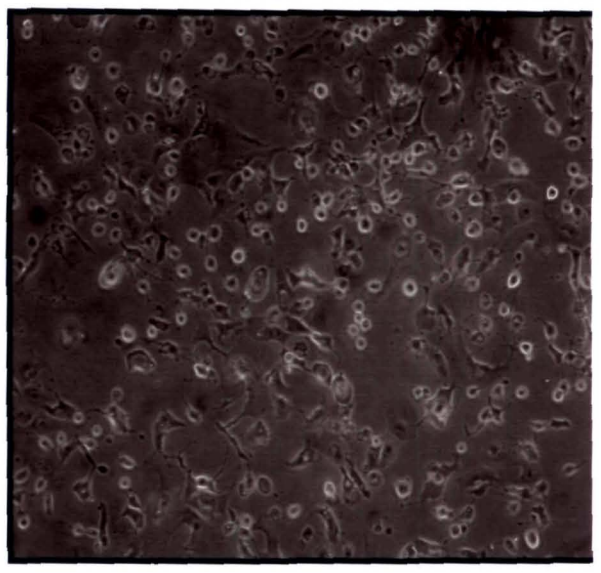


Plate 4.40: Degeneration of cells, Vero cell culture 72 hrs p.i, VIIIth passage (100 X)

Plate 4.41: Total degeneration of cells, 96 hrs p.i, VIIIth passage (100 X)

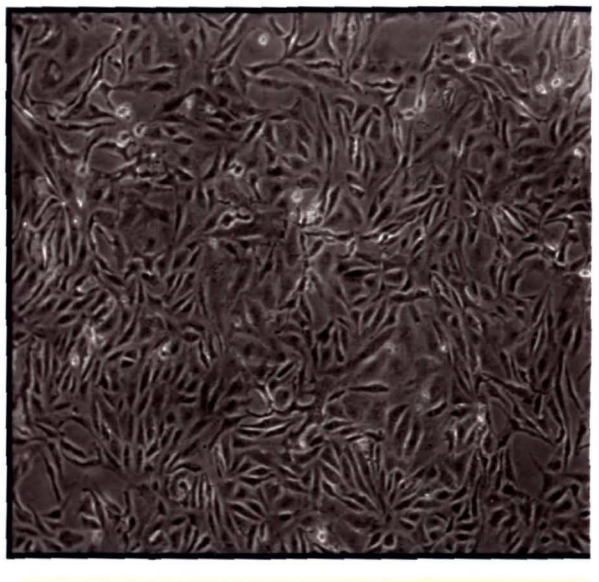


Plate 4.42: Vero cell culture , Control monolayer (100 X)

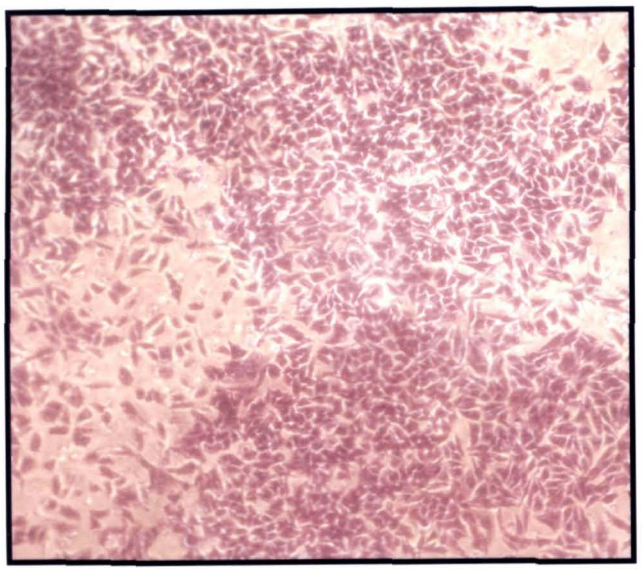


Plate 4.43: Hela cells, 48 hrs culture (100 X), MGG stain

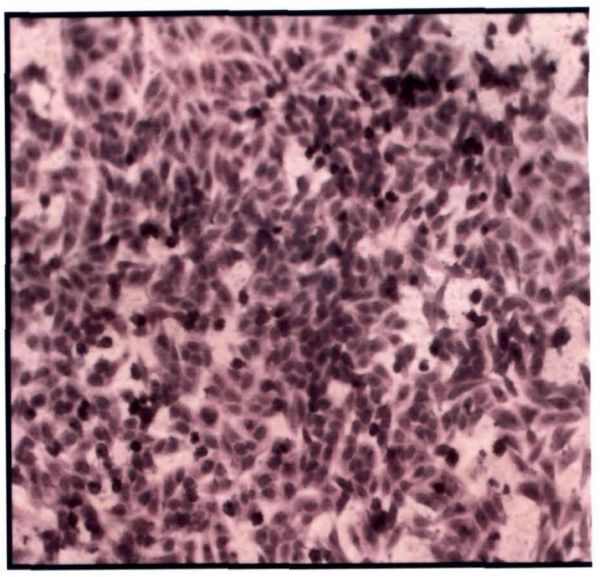


Plate 4.44: Hela cells , 72 hrs culture (100 X), MGG stain

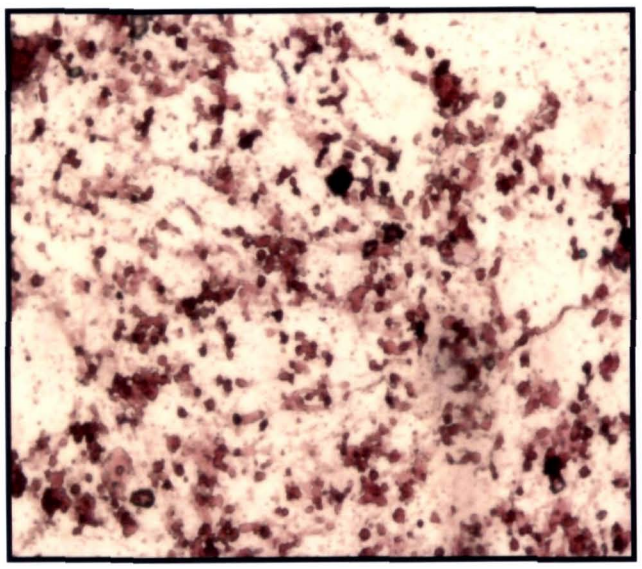


Plate 4.45: McCoy cells, 48 hrs culture (100 X)

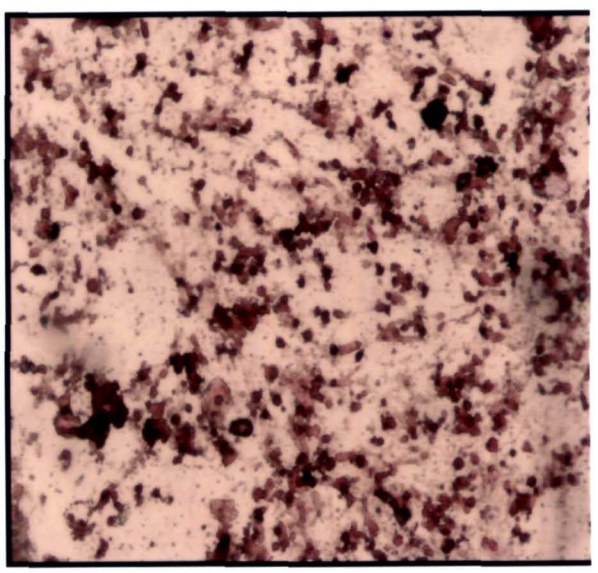


Plate 4.46: McCoy cells, 96 hrs culture MGG

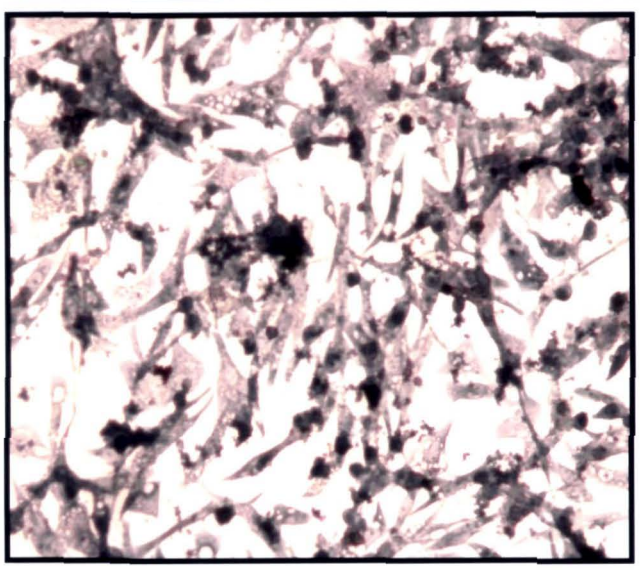


Plate 4.47 : BHK-21 cells, 48 hrs culture (100 X)

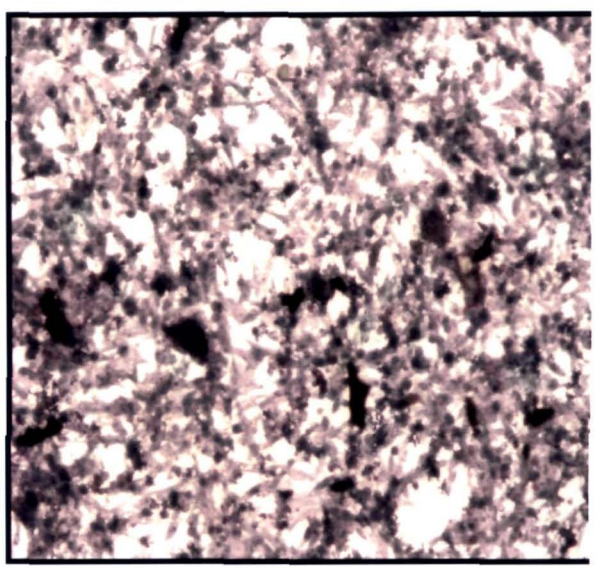


Plate 4.48 : BHK-21 cells, 72 hrs culture (400 X)

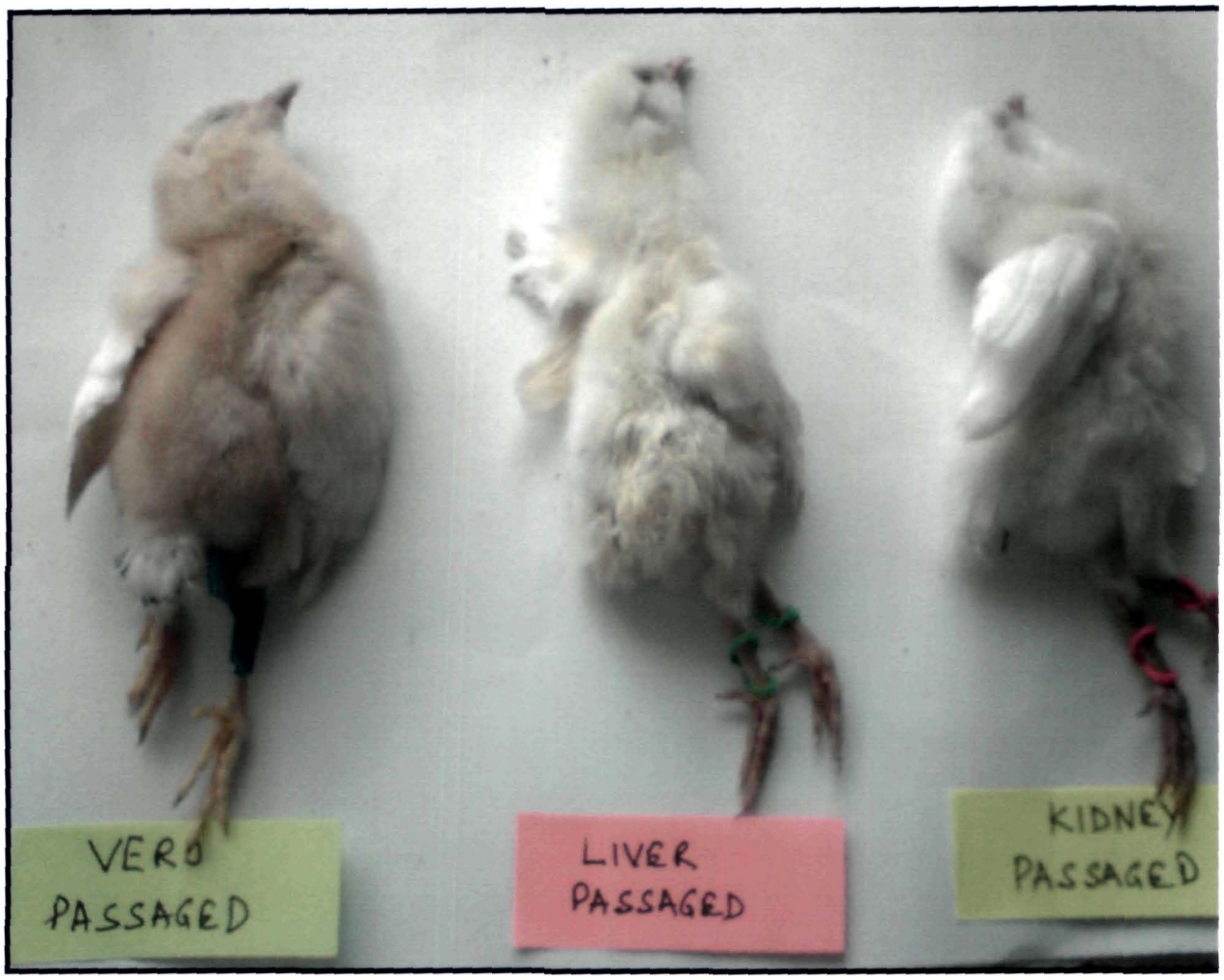


Plate 4.49 : Chicks infected with Vero, liver and kidney cell culture adapted virus

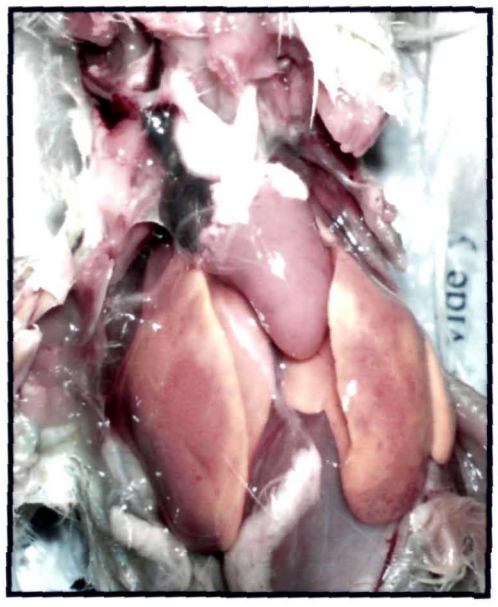


Plate 4.50 : Hemorrhages along with enlarged livers. (48 hrs p.i), CEL culture adapted virus

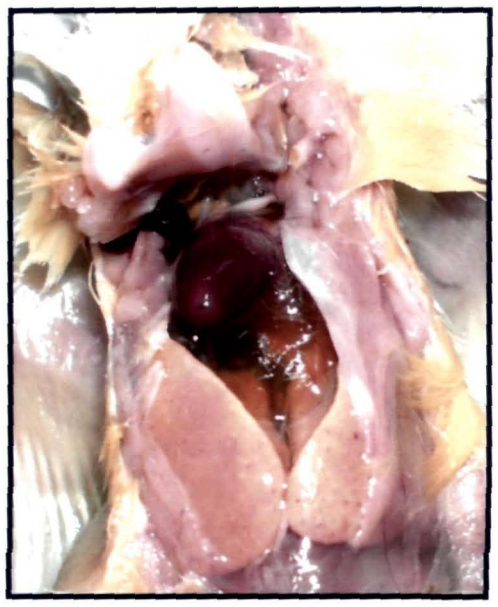


Plate 4.51: Hydropericardium along with enlarged livers. (72 hrs p.i), CEL culture adapted virus

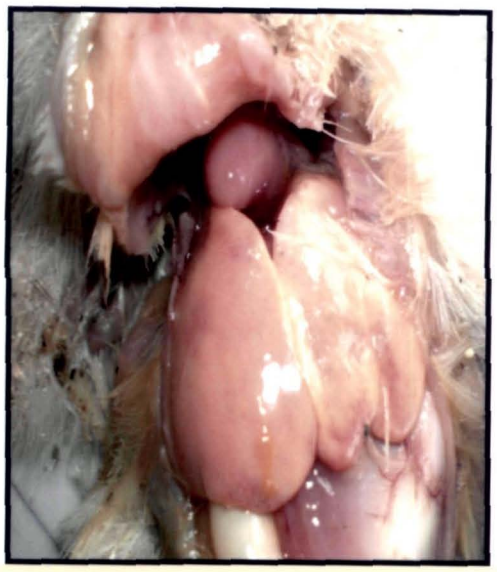


Plate 4.52 : Pale & enlarged liver (48 hrs p.i), CEK adapted virus

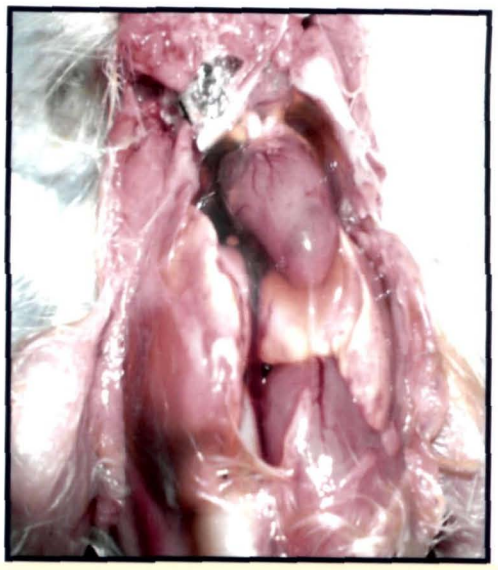


Plate 4.53 : Hydropericardium along with enlarged livers. (72 hrs P.I), CEK adapted virus

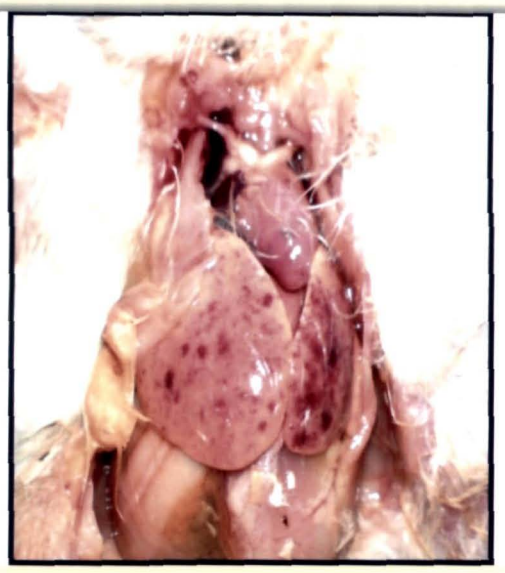


Plate 4.54: Hemorrhages, congestion on liver (48 hrs P.I), Vero passaged virus

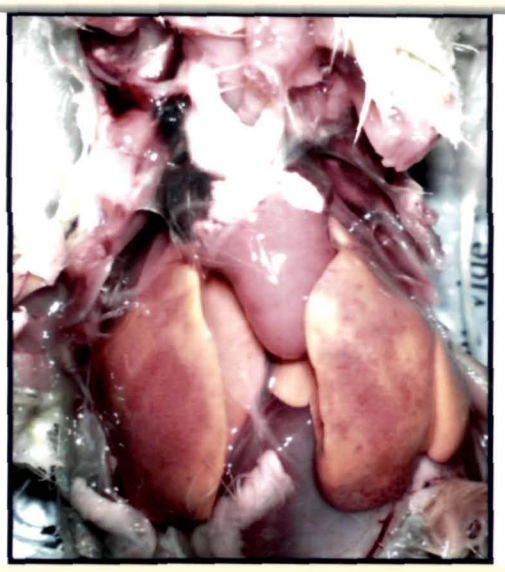


Plate 4.55: Hemorrhagic & enlarged liver (120 hrs P.I), CEF passaged virus

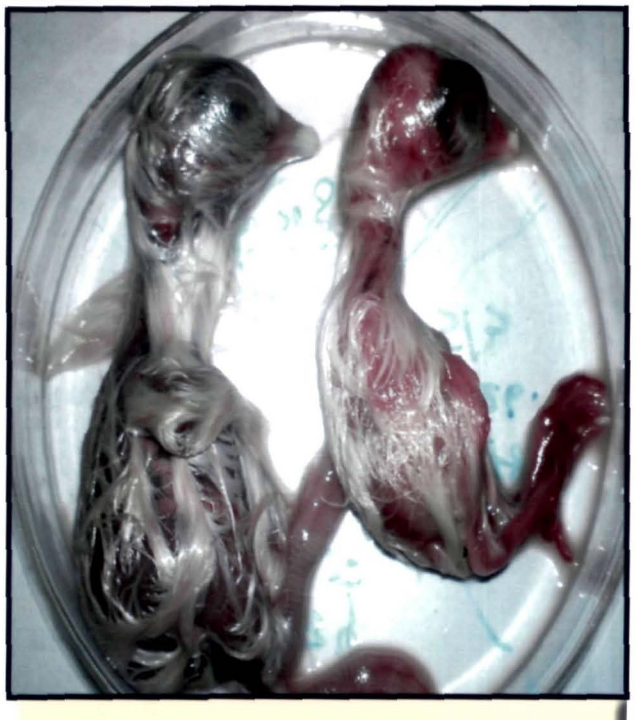


Plate 4.56 : Left (Healthy chick) , right (infected chick) after inoculation with

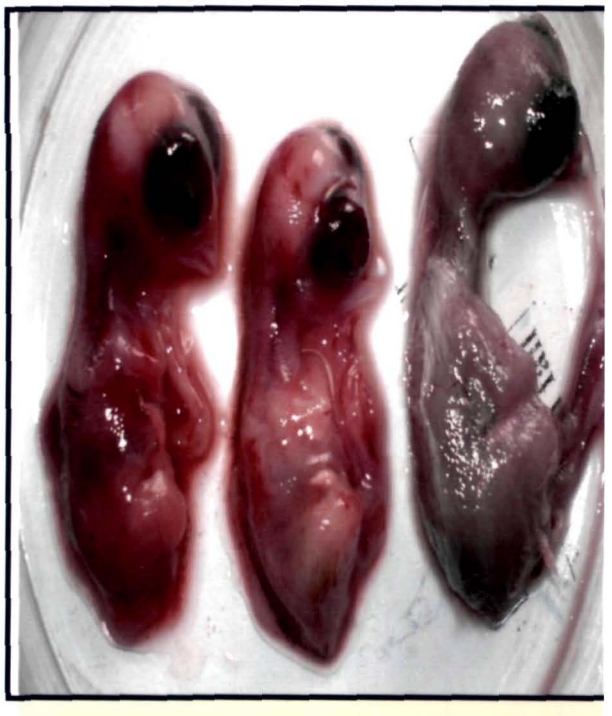


Plate 4.57 : Severe hemorrhages on body of infected chicks, (IIIrd passage), Right Control chick

Table 4.21. Mean ± S.E values of various measurements of inoculated and control group embryos.

Hrs. PI	Length (cms)		Breadth (cms)		Weight (gms)	
	Inoculated	Control	Inoculated	Control	Inoculated	Control
24	-	-	-	-	-	-
48	-	-	-	-	-	-
72	2.530± 0.031	2.866± 0.041	0.749±0.010	0.932±0.007	0.876 ± 0.009	1.316± 0.012
96	2.656± 0.023	2.997± 0.046	0.826±0.024	1.127±0.060	0.951±0.013	1.382±0.037
120	2.760± 0.027	3.255± 0.089	0.908±0.029	1.318±0.034	1.170±0.034	1.76±0.017
144	2.864± 0.049	3.642± 0.080	0.988±0.135	1.389±0.06	1.310±0.008	2.008±0.004

4.3 Detection of virus

4.3.1 Detection of virus employing serological tests

4.3.1.1 Screening of chicken for antibodies to FAV

The sera of chicks used for the experimental study were screened for the presence of FAV antibody by AGID and were found to be negative.

4.3.1.1.1 Experiments for detection of virus

The experimental infection was carried out in 30 chicks with 10 chicks per isolate with appropriate controls. The mortality pattern observed up to 7 days is given in Table no. 4.22. On day 7, the control chicks were also sacrificed.

Tissues like liver, heart, spleen, bursa, thymus and kidney were collected for histopathological studies to see the different changes in the different organs. The tissues collected were used for the detection of FAV antigen by AGID, CIE, IFAT, Dot ELISA and PCR. IPT was used for detection of the viral antigen only in the liver and kidney impression smears.

4.3.1.1.2 Following observations were made during the study

a) Body weight and organometry: Body weight of each bird was taken during the study (Table 4. 23). The average body weight of the infected group was found to be 29.506 ± 0.247 gm, while the average body weight of the control group was found to be 29.65 ± 0.29 gm. No difference in the body weight between the infected and control birds were noticed during the course of the experiment.

Table 4.24 depicts the length, breadth and weight of livers and hearts collected from infected and control birds. The length, breadth and weight of livers and hearts collected from the infected birds revealed no difference numerically when compared to length, breadth and weight of livers and hearts of control birds.

b) Clinical signs: Most of the birds affected in the study showed clinical signs of lethargy and anorexia. The other symptoms observed were depression, listlessness, reluctance to move, dropped wings, drooping head and ruffled feathers (Plate 4.58), while the control chick showed no symptoms of disease (Plate 4.59).

c) Mortality: Most of the chicks in all three groups 22/30 *i.e more* than 73 per cent of the birds died within 96 hrs. p.i. Mortality could be recorded as late as 168 days post infection.

d) Gross lesions: The characteristic gross lesion observed in most of the cases were hydropericardium characterized by accumulation of clear or slightly straw colored fluid in the pericardial sac of the infected birds (Plate 4.61). Birds that died within 24 to 48 hrs. of inoculation mostly did not exhibit hydropericardium but only paleness or slight hemorrhages along with enlargement of the liver tissue. However, birds dying 48 hrs. onwards showed the typical signs of hydropericardium, hemorrhagic kidneys and enlarged, mottled and grossly hemorrhagic liver (Plate 4.60).

Table 4.22. Mortality pattern observed in chicks after inoculation by three isolates

Mortality pattern (Days post inoculation)	**P-I** (10)	**PH-II** (10)	**PH-III** (10)	**Control group** (Sacrificed)
1 DPI	1(10%)	2 (20%)	1(10%)	2(14%)
2 DPI	2(20%)	2(20%)	2(20%)	2(14%)
3 DPI	3((30%)	1(10%)	3(30%)	2(14%)
4 DPI	1(10%)	2(20%)	2(20%)	2(14%)
5 DPI	-	2(20%)	1(10%)	2(14%)
6 DPI	2(20%)	1(10%)	-	2(14%)
7 DPI	1(10%)	-	1(10%)	2(14%)

Table 4.23. Body weight of chicks after inoculation by three isolates

Mortality pattern (Days post inoculation)	**P-I** (10)	**PH-II** (10)	**PH-III** (10)	**Control group**
1 DPI	29.6 gms	30.1 gms	29.4 gms	30.6 gms
2 DPI	29.8 gms	31.2 gms	32.1gms	32.4 gms
3 DPI	35.7 gms	32.2 gms	35.5 gms	35.3 gms
4 DPI	40.0 gms	40.5 gms	41.2 gms	39.9 gms
5 DPI	-	43.5 gms	43.6 gms	43.9 gms
6 DPI	46.2 gms	45.8 gms	-	49.1gms
7 DPI	50.6 gms	-	51.2 gms	55.0 gms

Table 4.24. Relative weight of liver and heart at various stages of mortality in experimentally infected and control chicks

DPI	(M)	P-I			M	PH-II			M	PH-III			S	Control chicks sacrificed		
		Liver				Liver				Liver				Liver		
		L (cms)	B (cms)	W (gms)		L (cms)	B (cms)	W (gms)		L (cms)	B (cms)	W (gms)		L (cms)	B (cms)	W (gms)
		2.30	1.20	2.50		2.23	1.15	2.30		2.50	1.67	2.75		2.27	1.10	2.39
1	1	Heart			2 (av)	Heart			1	Heart			2	Heart		
		L	B	W		L	B	W		L	B	W		L	B	W
		2.43	1.32	2.04		2.34	1.37	1.17		2.32	1.39	1.80		2.45	1.34	2.66
		Liver				Liver				Liver				Liver		
		L	B	W		L	B	W		L	B	W		L	B	W
		2.46	1.09	2.27		2.36	1.34	2.53		2.66	1.79	3.0		2.37	1.24	2.47
2	2	Heart			2	Heart			2	Heart			2	Heart		
		L	B	W		L	B	W		L	B	W		L	B	W
		2.53	1.47	2.04		2.36	1.49	2.28		2.37	1.48	2.17		2.49	1.40	2.74
		Liver				Liver				Liver				Liver		
		L	B	W		L	B	W		L	B	W		L	B	W
		2.73	1.35	2.63		2.43	1.38	2.61		2.52	1.87	3.03		2.41	1.26	2.51
3	3	Heart			1	Heart			3	Heart			2	Heart		
		L	B	W		L	B	W								
		2.75	1.76	2.52		2.78	1.62	2.29		2.39	1.48	2.27		2.53	1.41	2.77
		Liver				Liver				Liver				Liver		
		L	B	W		L	B	W		L	B	W		L	B	W
		3.05	1.72	2.95		2.56	1.62	2.89		2.77	1.95	3.17		2.45	1.30	2.54
4	1	Heart			2	Heart			2	Heart			2	Heart		
		L	B	W		L	B	W		L	B	W		L	B	W

		2.95	1.96	2.05		2.88	1.74	2.84		2.59	1.58	2.06		2.58	1.46	2.8
5	-	-	-	-	2	**Liver**			1	**Liver**			2	**Liver**		
						L	**B**	**W**		**L**	**B**	**W**		**L**	**B**	**W**
						3.05	1.95	3.12		2.89	1.99	4.02		2.48	1.37	2.57
						Heart				**Heart**				**Heart**		
						L	**B**	**W**		**L**	**B**	**W**		**L**	**B**	**W**
						3.12	1.98	2.20		2.63	1.73	2.20		2.62	1.49	2.81
6	2	**Liver**			1	**Liver**			-				2	**Liver**		
		L	**B**	**W**		**L**	**B**	**W**						**L**	**B**	**W**
		3.19	1.91	2.99		3.12	2.01	3.22						2.55	1.36	2.60
		Heart				**Heart**								**Heart**		
		L	**B**	**W**		**L**	**B**	**W**						**L**	**B**	**W**
		3.03	2.12	2.35		3.25	2.00	2.35						2.66	1.49	2.98
7	1	**Liver**			-				1	**Liver**			2	**Liver**		
		L	**B**	**W**						**L**	**B**	**W**		**L**	**B**	**W**
		3.24	1.96	3.05						3.02	2.08	4.12		2.65		
		Heart								**Heart**				**Heart**		
		L	**B**	**W**						**L**	**B**	**W**		**L**	**B**	**W**
		3.14	2.16	2.72						2.74	1.95	2.46		2.69	1.55	3.14

4.3.1.2 Results of different serological tests employed

4.3.1.2.1 Agar gel precipitation test (AGPT)

The AGPT was used to test pre-infected sera, yolk, amnioallantoic fluid and liver from embryonated eggs, tissue samples from chicks, re-infected with cell culture adapted virus, tissue samples from experimentally infected chicks and cell culture supernatants.

All the three isolates used in the present study gave a clear single line of precipitation with reference FAV-4 hyper immune serum. This indicated that serologically all the three isolates were FAV-4 (Plate 4.62).

a) Detection of virus from samples of embryonated eggs

Virus detection was possible employing AGPT from liver samples of infected embryonated chicks at all passage levels. However, detection of virus antigen was possible from yolk and amnioallantoic fluid only at second passage level (Table 4.25).

b) Detection of virus from cell culture supernatants

Infected supernatants of all the isolates from CEF (8^{th} to 10^{th} passage level), Vero cell line (7^{th} and 8^{th} passage level) and CEL & CEK (3^{nd} to 5^{th} passage level) showed positive reaction in AGID. No precipitation line however, could be seen from cell culture supernatants from HeLa, McCoy or BHK-21 cell lines infected with all the three isolates (Table 4.26).

c) Detection of virus from organs of chicks given re-infection with cell culture adapted virus

Pooled liver and kidney samples from chicks infected with cell culture adapted virus in re infective studies gave a single line of precipitation in AGPT. No other organ homogenate from cross infective studies gave positive reaction in AGPT. No detection was

possible with tissue homogenates from chicks infected with HeLa, McCoy and BHK-21 propagated cell culture supernatant (Table 4.27)

d) Detection of virus from organs of chicks given experimental infection

By AGPT, the liver samples collected from birds from all the groups in the experimental study that died within 4 DPI were found to be positive. Additionally, positive findings were also recorded from kidneys, and heart samples of birds up to 3 DPI, while two thymus samples were found to be positive in P-I infected group at 2^{nd} DPI, while, six bursa samples from P-I and PC-III infected groups up to 2^{nd} DPI showed positive reaction (Table 4.28).

4.3.1.2.2 Counter immunoelectrophoresis

The counterimmunoelectrophoresis (CIE) test was used to test the samples that were tested by AGPT (Plate 4.63).

a) Detection of virus from samples of embryonated eggs

Virus could be detected via CIE from livers of chicks from infected eggs in all passage levels while detection of the virus was only possible from yolk and amnioallantoic fluid of eggs from the second passage level only (Table 4.25).

b) Detection of virus from cell culture supernatants

Infected cell culture supernatants of CEF culture (8^{th} to 10^{th} passage level), Vero cell line (7^{th} to 8^{th} passage level) and CEL & CEK (3^{nd} to 5^{th} passage level) showed positive reaction in CIE. No positive reaction could however be seen from cell culture supernatants from HeLa, McCoy or BHK-21 cell lines (Table 4.26).

c) Detection of virus from organs of chicks re-infected with cell culture adapted virus

The cell culture adapted viruses also gave exactly similar results as in AGPT in CIE. Earlier results obtained with AGPT from liver and kidney samples were also positive in CIE. No detection was possible from tissue homogenates from chicks infected with HeLa, McCoy and BHK-21 propagated cell culture supernatant (Table 4.27).

d) Detection of virus from organs of chicks given experimental infection

By CIE, the liver samples collected from chicks used in experimental study from all the groups that died within 4 DPI were found to be positive. Additionally, positive reaction were recorded from kidneys of all dead birds upto 3rd DPI. Kidney samples of two birds from PH-II infected group at 4th DPI also reacted positively in CIE. Detection of viral antigen from two thymus samples was however only possible at 2nd DPI of P-I infected group. Seven samples of bursa at 2nd DPI (except one sample from PH-II infected group) reacted positively with CIE. Heart samples from all groups showed positive results upto 3rd DPI (Table 4.28).

4.3.1.2.3 Dot ELISA/Dot immunoassay (DIA)

Dot ELISA was standardized for the detection of FAV-4 antigen with known positive and negative samples. Positive results with all the three the virus isolates used in the study was seen in Dot ELISA (Plate 4.64).

a) Detection of virus from samples of embryonated eggs

Virus could be detected in the livers, yolk and amnioallantoic fluid of chicks from infected eggs in all passage levels by Dot ELISA (Table 4.25).

b) Detection of virus from cell culture supernatants

All the infected cell culture supernatants from CEF (6^{th} to 10^{th} passage level), CEL & CEK (2^{nd} to 5^{th} passage level) and Vero (6^{th} to 8^{th} passage level) showed positive results in Dot ELISA (Plate 4.65). Negative results obtained in AGPT and CIE were also obtained in Dot ELISA, when cell culture supernatants from HeLa, McCoy or BHK-21 cell lines were tested (Table 4.26).

c) Detection of virus from organs of chicks re-infected cell culture adapted virus

By Dot ELISA, virus detection was possible from samples of liver, heart and kidneys of chicks re-infected with cell culture adapted virus. Positive detection was also possible from thymus and bursa samples of chicks dying within 72 hrs. p.i. (Table 4.27).

d) Detection of virus from organs of chicks given experimental infection

Detection of virus was possible from liver, kidney and heart samples collected from all the experimentally infected birds (Plate 4.65). Positive results were also seen in bursa, thymus and kidneys samples collected from birds dying upto 6^{th} DPI (Table 4.28).

4.3.1.2.4 Fluorescent antibody technique

The IFAT could detect virus in different samples tested previously by AGPT, CIE and Dot ELISA.

a) Detection of virus from samples of embryonated eggs

IFAT could detect virus in the livers, yolk and amnioallantoic fluid of chicks from infected eggs in all passage levels (Table 4.25).

b) Detection of virus from cell culture supernatants

Virus detection by IFAT was possible from all infected cell culture supernatants from CEF (6^{th} to 10^{th} passage), CEL& CEK (2^{nd} to 5^{th} passage) and Vero (6^{th} to 8^{th} passage).These samples were also found to give positive results in Dot ELISA. Virus detection was not possible from from HeLa, McCoy and BHK-21 cell lines (Table 4.26).

c) Detection of virus from organs of chicks re infected with cell culture adapted virus

Organs from chicks that were infected by cell culture adapted virus when tested at different intervals by IFAT showed similar patterns as in Dot ELISA. IFAT could detect virus in all the samples of liver, heart, kidney, thymus and bursa. IFAT was found to be equally sensitive and could detect viral antigens like Dot ELISA (Table 4.27).

d) Detection of virus from tissue samples of chicks given experimental infection

IFAT could detect viral antigens in liver, heart, bursa, thymus and kidney samples upto 6^{th} DPI (Plate 4.66). One kidney sample from P-I infected group did not however show fluorescence at 6^{th} DPI. (Table 4.28).

The findings suggest that for detecting fowl adenoviral antigens in tissues, both Dot ELISA and IFAT were equally sensitive.

4.3.1.3 Additional tests for detection of virus

4.3.1.3.1 Hemagglutination test

The hemagglutination test of all the three isolates along with CELO virus conducted against the RBCs of nine species *viz.* chicken, cattle, buffalo, geese, rat, swine, dog, rat and fish were found to be negative. The positive control containing NDV (New castle disease virus), showed hemagglutination with chicken RBCs.

Table no: 4.25. Detection of the virus from different parts of embryonated eggs by different serological tests

Pooled samples	Pooled samples	Passage levels								
		I[st]			II[nd]			III[rd]		
		P-I	PH-II	PC-III	P-I	PH-II	PC-III	P-I	PH-II	PC-III
Liver	AGPT	+	+	+	+	+	+	+	+	+
	CIE	+	+	+	+	+	+	+	+	+
	DIA	+	+	+	+	+	+	+	+	+
	IFAT	+	+	+	+	+	+	+	+	+
Yolk sac	AGPT	-	-	-	+	+	+	+	+	+
	CIE	-	-	-	+	+	+	+	+	+
	DIA	+	+	+	+	+	+	+	+	+
	IFAT	+	+	+	+	+	+	+	+	+
Amnioallantoic fluid	AGPT	-	-	-	+	+	+	+	+	+
	CIE	-	-	-	+	+	+	+	+	+
	DIA	+	+	+	+	+	+	+	+	+
	IFAT	+	+	+	+	+	+	+	+	+

DIA: Dot Immunoassay/Dot ELISA

Table 4.26. Comparative detection of virus by different serological tests from infected cell culture supernatant at different passage levels

Cell culture	Tests	Passage level									
		1	2	3	4	5	6	7	8	9	10
CEF	AGPT	-	-	-	-	-	-	-	+	+	+
	CIE	-	-	-	-	-	-	-	+	+	+
	DOT-ELISA	-	-	-	-	-	+	+	+	+	+
	IFAT	-	-	-	-	-	+	+	+	+	+
CEL	AGPT	-	-	+	+	+	NA	NA	NA	NA	NA
	CIE	-	-	+	+	+	NA	NA	NA	NA	NA
	DOT-ELISA	-	+	+	+	+	NA	NA	NA	NA	NA
	IFAT	-	+	+	+	+	NA	NA	NA	NA	NA
CEK	AGPT	-	-	+	+	+	NA	NA	NA	NA	NA
	CIE	-	-	+	+	+	NA	NA	NA	NA	NA
	DOT-ELISA	-	+	+	+	+	NA	NA	NA	NA	NA
	IFAT	-	+	+	+	+	NA	NA	NA	NA	NA
Vero	AGPT	-	-	-	-	-	-	+	+	NA	NA
	CIE	-	-	-	-	-	-	+	+	NA	NA
	DOT-ELISA	-	-	-	-	-	+	+	+	NA	NA
	IFAT	-	-	-	-	-	+	+	+	NA	NA
HeLa	AGPT	-	-	-	-	-	-	-	-	NA	NA
	CIE	-	-	-	-	-	-	-	-	NA	NA
	DOT-ELISA	-	-	-	-	-	-	-	-	NA	NA
	IFAT	-	-	-	-	-	-	-	-	NA	NA
McCoy	AGPT	-	-	-	-	-	-	-	-	NA	NA
	CIE	-	-	-	-	-	-	-	-	NA	NA
	DOT-ELISA	-	-	-	-	-	-	-	-	NA	NA
	IFAT	-	-	-	-	-	-	-	-	NA	NA
BHK-21	AGPT	-	-	-	-	-	-	-	-	NA	NA
	CIE	-	-	-	-	-	-	-	-	NA	NA
	DOT-ELISA	-	-	-	-	-	-	-	-	NA	NA
	IFAT	-	-	-	-	-	-	-	-	NA	NA

NA: Not applicable as cells were not passaged further

Table 4.27. Comparative detection of virus by different serological tests from the organs of chicks re-infected with cell culture adapted virus

Samples	Tests	CEL adapted					CEK adapted					CEF adapted					Vero adapted			
		Days post infection					Days post infection					Days post infection					Days post infection			
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4
Liver	AGPT	+	+	+	NA	NA	NA	+	+	+	NA	NA	+	+	NA	+	+	+	+	NA
	CIE	+	+	+	NA	NA	NA	+	+	+	NA	NA	+	+	NA	+	+	+	+	NA
	DIA	+	+	+	NA	NA	NA	+	+	+	NA	NA	+	+	NA	+	+	+	+	NA
	IFAT	+	+	+	NA	NA	NA	+	+	+	NA	NA	+	+	NA	+	+	+	+	NA
Heart	AGPT	-	-	-	NA	NA	NA	-	-	-	NA	NA	-	-	NA	-	-	-	-	NA
	CIE	-	-	-	NA	NA	NA	-	-	-	NA	NA	-	-	NA	-	-	-	-	NA
	DIA	+	+	+	NA	NA	NA	+	+	+	NA	NA	+	+	NA	+	+	+	+	NA
	IFAT	+	+	+	NA	NA	NA	+	+	+	NA	NA	+	+	NA	+	+	+	+	NA
Kidney	AGPT	+	+	+	NA	NA	NA	+	+	+	NA	NA	+	+	NA	+	+	+	+	NA
	CIE	+	+	+	NA	NA	NA	+	+	+	NA	NA	+	+	NA	+	+	+	+	NA
	DIA	+	+	+	NA	NA	NA	+	+	+	NA	NA	+	+	NA	+	+	+	+	NA
	IFAT	+	+	+	NA	NA	NA	+	+	+	NA	NA	+	+	NA	+	+	+	+	NA
Thymus	AGPT	-	-	-	NA	NA	NA	-	-	-	NA	NA	-	-	NA	-	-	-	-	NA
	CIE	-	-	-	NA	NA	NA	-	-	-	NA	NA	-	-	NA	-	-	-	-	NA
	DIA	+	+	+	NA	NA	NA	+	+	-	NA	NA	+	+	NA	-	+	+	+	NA
	IFAT	+	+	+	NA	NA	NA	+	+	+	NA	NA	+	+	NA	+	+	+	+	NA
Bursa	AGPT	-	-	-	NA	NA	NA	-	-	-	NA	NA	-	-	NA	-	-	-	-	NA
	CIE	-	-	-	NA	NA	NA	-	-	-	NA	NA	-	-	NA	-	-	-	-	NA
	DIA	+	+	+	NA	NA	NA	+	+	-	NA	NA	+	+	NA	-	+	+	+	NA
	IFAT	+	+	+	NA	NA	NA	+	+	+	NA	NA	+	+	NA	+	+	+	+	NA

NA: Birds did not die at this time interval

Table 4.28. Serological detection of viral antigens in different tissues of chicks experimentally infected with FAV-4.

Tissue samples	Serological tests	Viral isolates														
		P-I					PH-II					PC-III				
		Mortality days post infection					Mortality days post infection					Mortality days post infection				
		1	2	3	4	>5	1	2	3	4	>5	1	2	3	4	>5
Liver	AGPT	1/1	2/2	3/3	1/1	0/3	2/2	2/2	1/1	2/2	0/3	1/1	2/2	3/3	2/2	0/2
	CIE	1/1	2/2	3/3	1/1	0/3	2/2	2/2	1/1	2/2	0/3	1/1	2/2	3/3	2/2	0/2
	DIA	1/1	2/2	3/3	1/1	3/3	2/2	2/2	1/1	2/2	3/3	1/1	2/2	3/3	2/2	2/2
	IFAT	1/1	2/2	3/3	1/1	3/3	2/2	2/2	1/1	2/2	3/3	1/1	2/2	3/3	2/2	2/2
Kidney	AGPT	1/1	2/2	3/3	0/1	0/3	2/2	2/2	1/1	0/2	0/3	1/1	2/2	3/3	0/2	0/2
	CIE	1/1	2/2	3/3	0/1	0/3	2/2	2/2	1/1	2/2	0/3	1/1	2/2	3/3	0/2	0/2
	DIA	1/1	2/2	3/3	1/1	3/3	2/2	2/2	1/1	2/2	3/3	1/1	2/2	3/3	2/2	2/2
	IFAT	1/1	2/2	3/3	1/1	2/3	2/2	2/2	1/1	2/2	3/3	1/1	2/2	3/3	2/2	2/2
Heart	AGPT	1/1	2/2	3/3	0/1	0/3	2/2	2/2	1/1	0/2	0/3	1/1	2/2	3/3	0/2	0/2
	CIE	1/1	2/2	3/3	0/1	0/3	2/2	2/2	1/1	0/2	0/3	1/1	2/2	3/3	0/2	0/2
	DIA	1/1	2/2	3/3	1/1	3/3	2/2	2/2	1/1	2/2	3/3	1/1	2/2	3/3	2/2	2/2
	IFAT	1/1	2/2	3/3	1/1	3/3	2/2	2/2	1/1	2/2	3/3	1/1	2/2	3/3	2/2	2/2
Thymus	AGPT	0/1	2/2	0/3	0/1	0/3	0/2	0/2	0/1	0/2	0/3	0/1	0/2	0/3	0/2	0/2
	CIE	0/1	2/2	0/3	0/1	0/3	0/2	0/2	0/1	0/2	0/3	0/1	0/2	0/3	0/2	0/2
	DIA	1/1	2/2	3/3	1/1	3/3	2/2	2/2	1/1	2/2	3/3	1/1	2/2	3/3	2/2	2/2
	IFAT	1/1	2/2	3/3	1/1	3/3	2/2	2/2	1/1	2/2	3/3	1/1	2/2	3/3	2/2	2/2
Bursa	AGPT	1/1	2/2	0/3	0/1	0/3	0/2	0/2	0/1	0/2	0/3	1/1	2/2	0/3	0/2	0/2
	CIE	1/1	2/2	0/3	0/1	0/3	0/2	1/2	0/1	0/2	0/3	1/1	2/2	0/3	0/2	0/2
	DIA	1/1	2/2	3/3	1/1	3/3	2/2	2/2	1/1	2/2	3/3	1/1	2/2	3/3	2/2	2/2
	IFAT	1/1	2/2	3/3	1/1	3/3	2/2	2/2	1/1	2/2	3/3	1/1	2/2	3/3	2/2	2/2

A/B A: Number of samples found positive B: Total number of samples tested

4.3.1.3.2 Hemagglutination inhibition test

Hemagglutination inhibition test was not performed as hemagglutination test was found to be negative.

4.3.1.3.3 Virus neutralization test

The diluted virus and constant serum known as alpha-method (□- method) was used for finding out the neutralization index of the isolates.

The titer of virus and virus serum mixture in terms of embryo infective dose, 50 per cent (EID_{50}/ml) was calculated by the method of Reed and Meunch (1938). The result of titration and neutralization of the standard AAV type-1 (CELO virus) along with the three virus isolates are shown in Table 4.29. The neutralization of CELO virus was done with anti-CELO hyperimmune serum, while the neutralization of the viral isolates used in the study was done with both anti FAV-4 hyperimmune serum. The CELO virus had a neutralization titre of 4.15, while the PH-II isolate had a neutralization titre of 4.63. The P-I and the PC-III isolate were completely neutralized by the corresponding antiserum. The FAV-4 isolates were not neutralized at all by CELO antiserum. The neutralization results of CELO and PH-II isolates showed that CELO and PH-II isolates could be neutralized by 4.15 and 4.63 folds of hyperimmune serum (Table 4.30).

4.3.1.3.4 Immunoperoxidase test

The immunoperoxidase test was performed on infected cell culture supernatants as well as the impression smears of organs particularly liver and kidney samples from infected birds. It was used to detect the presence of virus particles in the specimens. Both kidney and liver samples showed positive reaction as evident by the presence of brownish deposits in the

liver impression smears (Plate 4.67), while infected CEL along with CEK cell culture also showed the presence of the virus.

Table 4.29. List of neutralization titre of various isolates against known hyperimmune serum

		Log $_{10}$ titres (EID_{50}/ml)		
Sr. No.	**Isolate no.**	**Virus titre (VT)**	**Treated titre (TT)**	**NI=VT-TT**
1.	CELO	5.45	1.30	4.15
2.	P-I	5.67	0.0	5.67
3.	PH-II	6.3	1.67	4.63
4.	PH-III	6 .2	0.0	6.20

Table 4.30. Results of neutralization titre (NI) of virus isolates

			Log $_{10}$ values			
			Treated titre (TT)		NI=VT-TT	
Sr. No.	**Isolates**	**Virus titre**	**CELO antiserum**	**FAV-4 antiserum**	**CELO antiserum**	**FAV-4 antiserum**
1.	CELO	5.45	1.30	NN	4.15	NN
2.	P-I	5.67	NN	0.0	NN	5.67
3.	PH-II	6.3	NN	1.67	NN	4.63
4.	PH-III	6 .2	NN	0.0	NN	6.20

NT= Not neutralized **0= Completely neutralized**

4.3.1.3.5 Histopathological studies

The main histopathological lesions in the FAV infected experimental chicks were as follows:

a) Heart: The myocardium showed mild to moderated congestion and foci of hemorrhages (Plate 4.68). At places, there was disruption of myocardial fibers due to edema. Fibrinous myocarditis was evident by the fibrinous deposition over the epicardium.

b) Lungs: The lungs showed generalized alveolar congestion and mild to moderate hemorrhages. (Plate 4.69). At places, there was perivascular edema and widening of interlobular septa due to edema. In some cases there was hyperplasia of bronchial epithelium.

c) Liver: The hepatocytes showed varying degrees of degeneration viz., vacuolar degeneration and fatty changes (Plate 4.70). There were randomly scattered foci of hepatic necrosis. Distinct basophilic intranuclear inclusions bodies (Plate 4.71) with clear halo around them were present in some hepatocytes. Numerous hyperchromatic nuclei were seen in some hepatocytes.

d) Kidney: Kidneys revealed congestion and hemorrhages in parenchyma. Most of the changes were encountered in tubules which comprised of vascular degeneration and desquamation of tubular epithelium. At places, there was focal aggregation of lymphoid cells in the interstitial tissue (Plate 4.72).

e) Thymus: There was marked congestion in medullary and sub scapular vessels. Mild rarefaction is observed in medullary region.

f) Bursa of Fabricius: The follicles showed mild lymphoid depletion in medulla and hyperplasia of epithelium forming papillary projection into the lumen (Plate 4.73).

Plate 4.58: Chick showing listlessness, closed eyes and dropped wings, 48 hrs after experimental

Plate 4.59: Healthy bird (left) with infected bird (right) (48 hrs P.I)

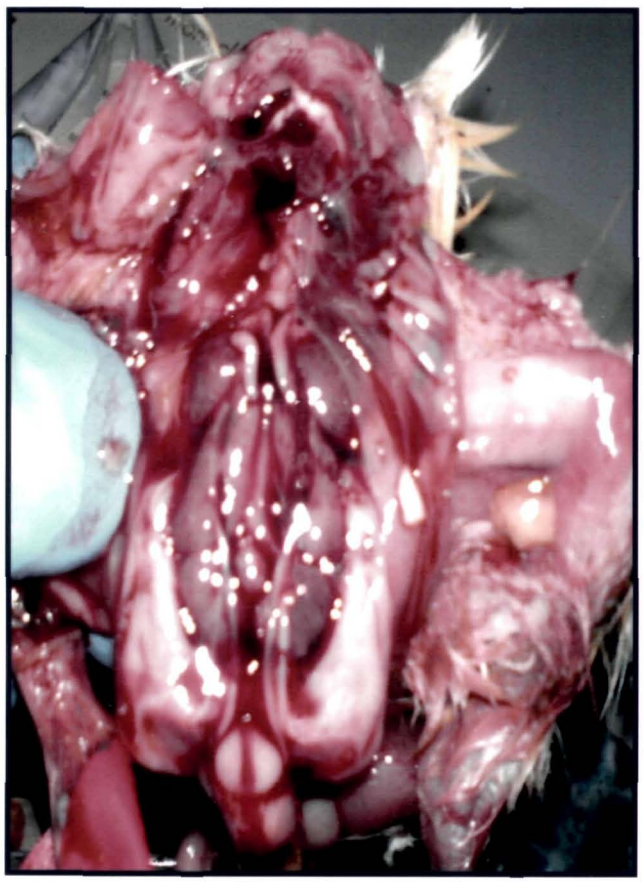


Plate 4.60 : Hemorrhagic & enlarged kidney, 72 hrs p.i

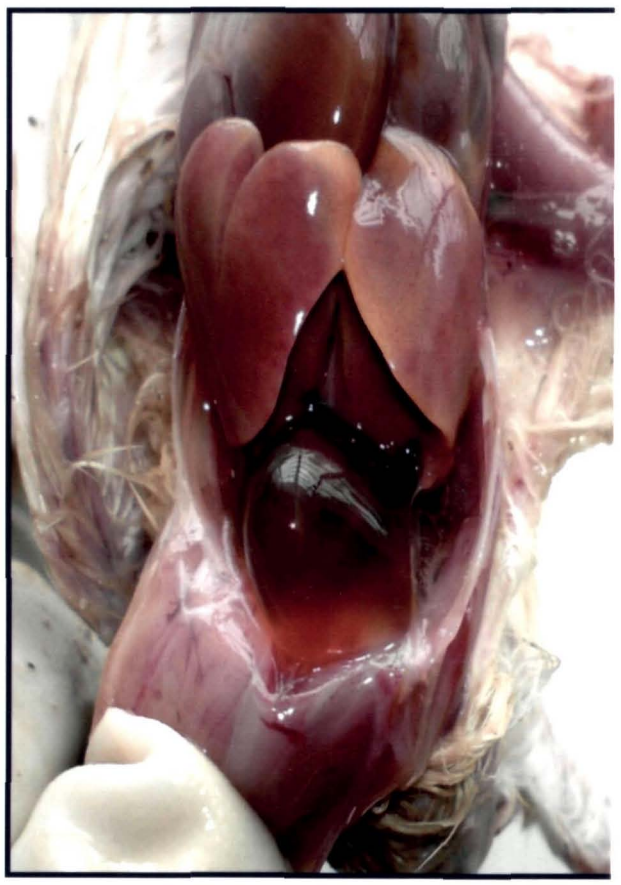


Plate 4.61 : "Litchi" heart along with hemorrhagic & enlarged liver (96 hrs p.i)

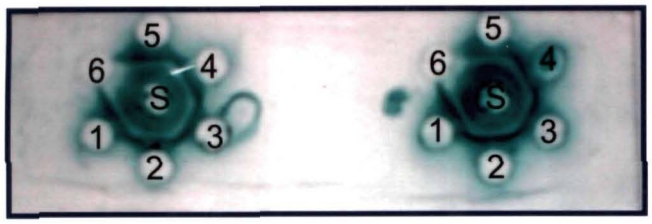


Plate 4.62: AGPT: 1: Positive control, 2: P-I, 3: PH-II, 4: PC-III, 5: Positive control 6: Negative control

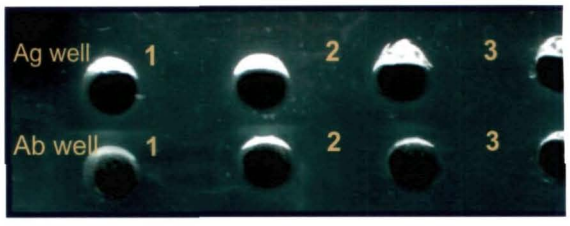


Plate 4.63: CIE: 1: P-1, 2: PH-II, 3: PC-III, 4: Negative control

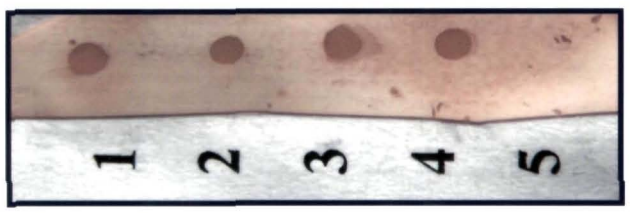


Plate 4.64: DOT-ELISA: 1: Positive control, 2: P-I, 3: PH-II, 4: PC-III, 5: Negative control

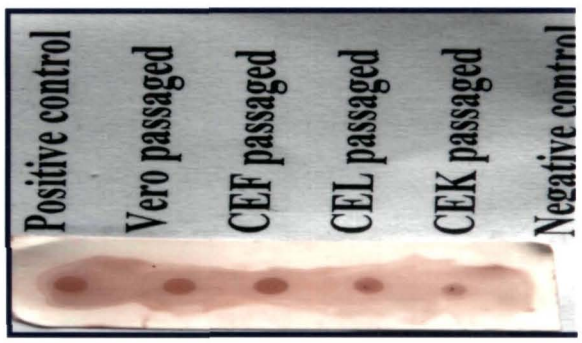


Plate 4.65: DOT-ELISA of infected cell culture supernatants

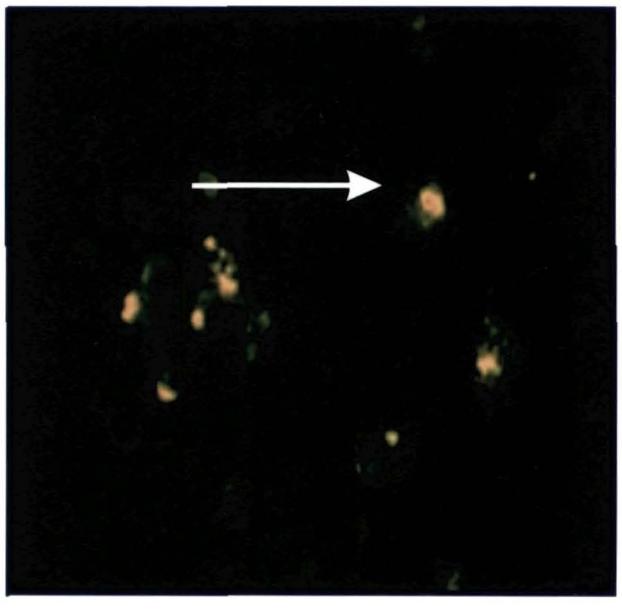


Plate 4.66: FAT showing viral antigen in infected liver samples

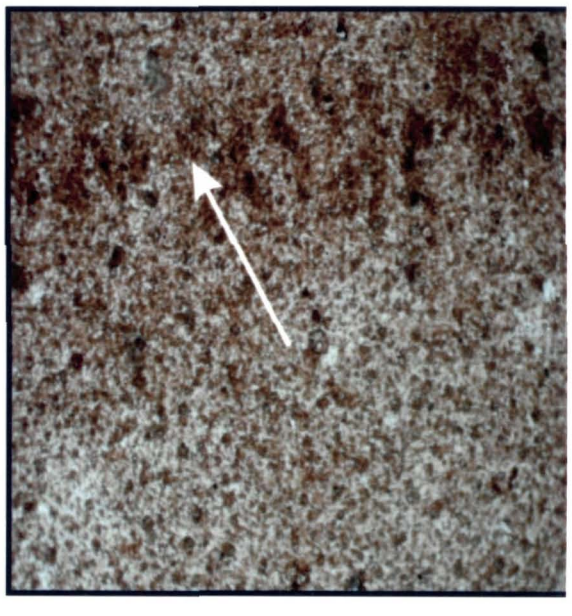


Plate 4.67 : Brown deposits depicting presence of viral antigen in liver impression smear antigen, IPT (H & E, 200 X)

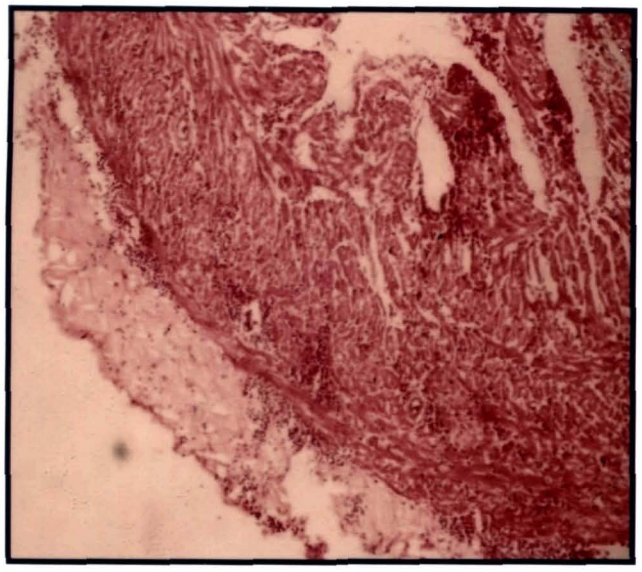


Plate 4.68: Micrograph showing moderate hemorrhages in myocardium (H & E, 200 X)

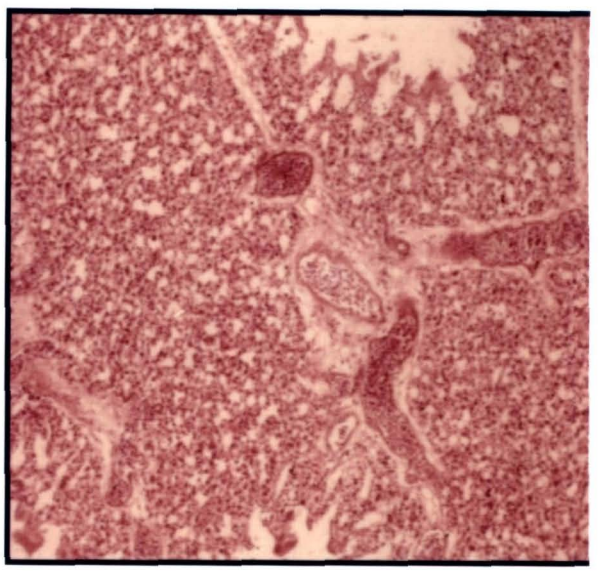


Plate 4.69: Micrograph of lung showing generalized congestion

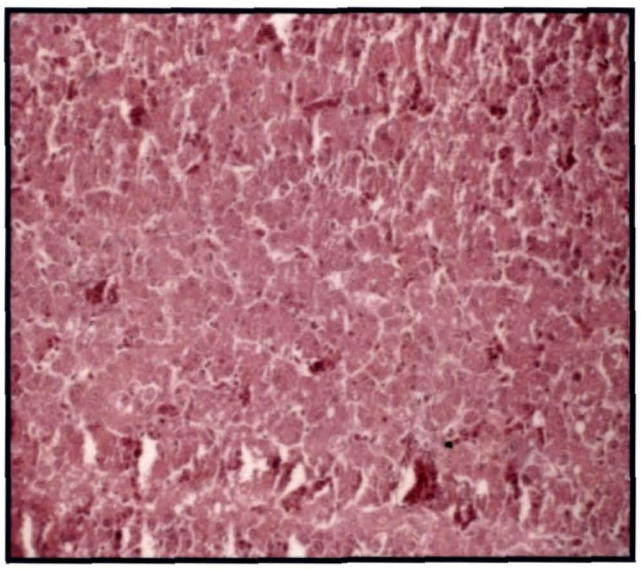


Plate 4.70: Micrograph of liver showing varying degree of degeneration and fatty changes along with hepatic necrosis.

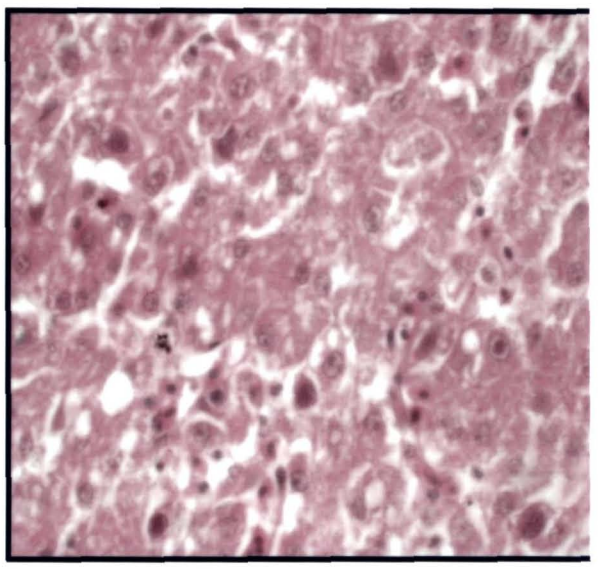


Plate 4.71: Micrograph of liver showing intranuclear basophilic inclusions in the hepatocyte (H & E)

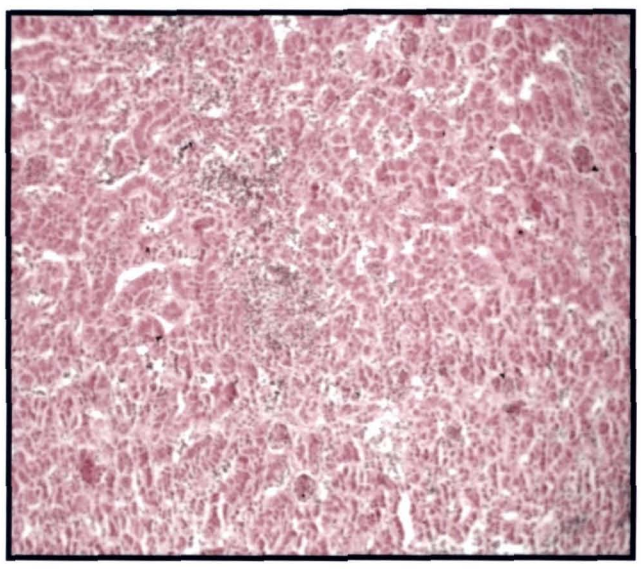


Plate 4.72: Micrograph of kidney showing MNC cell infiltration in the parenchyma (H & E, 200 X)

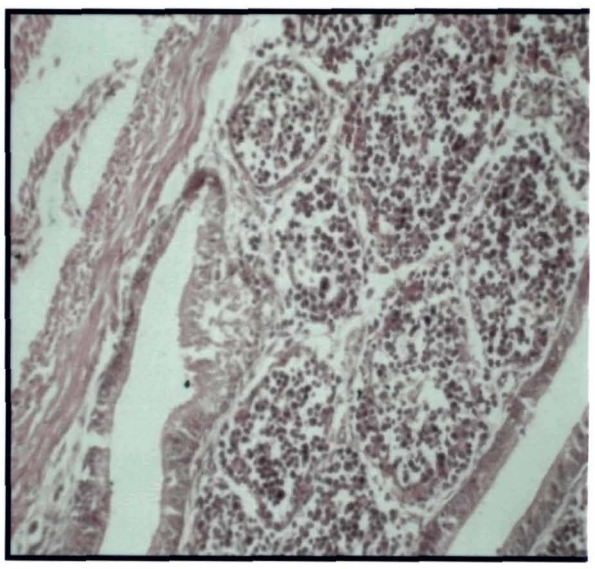


Plate 4.73: Micrograph of bursa showing leucocytic linfiltration (H & E, 200 X)

4.3.2 Molecular detection of fowl adenovirus

4.3.2.1 Detection of fowl adenovirus by PCR

4.3.2.1.1 Detection of fowl adenovirus by group specific PCR

PCR was used to detect the fowl adenovirus directly from the liver samples of birds infected with the three isolates used in the study. The DNA of all the three liver inoculum samples as well as the standard FAV-1 were amplified with the primer pairs H1/H2 and H3/ H4 that is diagnostic for avian adenoviruses. As expected, PCR products of approx. 1219 base pairs (bp) could be amplified with H1/H2 primers while by the use of H3/H4 primers, PCR products of 1319 bp were amplified that placed the isolates in the particular avian adenovirus group (Plate 4.74 and 4.75). Group specific amplification could successfully amplify the required regions of FAV from parts of infected embryonated eggs, different cell culture supernatants (Plate 4.76 and 4.77) as well as from chicks given experimental infection (Table 4.31 and 4.32).

After the group specific identification, the infected liver samples were subjected to FAV-4 species specific identification using the hexon gene primers targeting 0.7 kb fragment of FAV-4 (Plate 4.78).

Additionally, to detect the presence of FAV-4 from different sources, amplification of 0.7 kb product was also done from organs (heart / liver) and fluids (yolk and amnioallantoic fluid) of embryonated chicks infected with the three isolates. Livers, yolk and amnioallantoic fluid from embryonated chicks showed the required amplification, while hearts from the same chicks did not show any amplification.

Table 4.31. Detection of fowl adenovirus by group specific PCR

Procedure	Samples	P-I	PH-II	PC-III
Embryonated eggs	Liver	+	+	+
	Yolk	+	+	+
	Amnioallantoic fluid	+	+	+
		Passage level	Passage level	Passage level
Cell culture	CEL	+ (1^{st} - 5^{th})	+ (1^{st} - 5^{th})	+ (1^{st} - 5^{th})
	CEK	+ (1^{st}- 5^{th})	+ (1^{st}-5^{th})	+ (1^{st}-5^{th})
	CEF	+ (4^{th} - 10^{th})	+ (4^{th} - 10^{th})	+ (4^{th} - 10^{th})
	Vero	+ (4^{th} - 8^{th})	+ (4^{th} - 8^{th})	+ (4^{th} - 8^{th})
Cell lines	HeLa	-	-	-
	McCoy	-	-	-
	BHK-21	-	-	-

Table 4.32. Amplification of fowl adenovirus by group specific PCR from different samples

	Cell culture adapted supernatant from				Experimental infection to chicks		
Organs from infected chicks	CEL	CEK	CEF	Vero	P-I	PH-II	PH-III
Liver	+	-	-	+	+	+	+
Kidney	+	+	+	+	+	+	+
Heart	+	-	+	+	+	+	+
Thymus	+	+	+	+	+	+	+
Bursa	+	+	+	+	+	+	+

4.3.2.1.2 Detection of FAVs by hexon gene based PCR

The supernatants from cell cultures infected with the three isolates and showing CPE were tested for the presence of virus by using primer pairs (H1/H2) and (H3/H4) that amplified 1219 bp and 1319 bp regions of adenoviruses. Once they were found positive, the supernatants were subjected to amplification of 0.7 kb region by hexon gene specific primers and were found to be positive for the 0.7 kb fragment of FAV-4.

PCR could detect the virus in infected CEL and CEK cultures in all the five passages. However, the presence of virus was noted in fibroblast and Vero cell cultures from the 4^{th} passage level onwards.

One interesting finding in this study was that though, other cell lines viz. McCoy and BHK-21 did not show any CPE and were also found to be negative in serological tests as well as for the presence of FAV-4 DNA by PCR, HeLa cell line showed the presence of viral DNA by hexon gene PCR in both fourth and fifth cell culture passaged supernatants. Nevertheless, the supernatants were found to be negative in serological tests, by detection by H1/H2 and H3/H4 specific primers as well as the presence of CPE even after eight passages (Plate 4.79).

In chicks re infected with cell culture adapted virus, an interesting finding was noticed *i.e* in chicks infected with CEF and CEK adapted virus, the viral DNA could be detected in all organs except livers in CEF passaged birds while both liver and kidney samples were negative for the presence of the virus DNA in case of birds infected with CEK adapted virus though other organs from the same birds showed the presence of the virus. However, from birds that died after infected with Vero adapted virus, the virus could be detected by PCR from all organs tested (Plate 4.80).

Positive results were also obtained from all the organs of chicks that had been experimentally infected with the virus isolates (Plate 4.81). Interestingly, the amplification of

the 0.7 kb fragment was seen regularly from both thymus and bursa of infected birds, even though the results obtained by serological studies from these organs was not always positive.

Hexon gene specific amplification could successfully amplify the required regions of FAV from parts of infected embryonated eggs, different cell culture supernatants as well as from chicks given experimental infection (Table 4.33 and 4.34). The 0.7 kb product could regularly be amplified from the livers of experimental chicks (Plate 4.84).

4.3.2.1.3 Detection of FAVs by fibre gene based PCR

4.3.2.1.3.1 Detection of FAV-4 by amplification of fibre gene

All the samples as well as the cell culture supernatants were also subjected to amplification of the FAV- 4 fibre gene fragment by using fibre gene specific sequence targeting the 1.4 kb fragment of FAV-4. Plate 4.82 shows the amplification of the 1.4 kb region from the infected cell culture supernatants, while Plate 4.83 shows the amplification of the desired 1.4 kbp product from all the three isolates.

Fibre gene specific amplification could successfully amplify the required regions of FAV from parts of infected embryonated eggs, different cell culture supernatants as well as from chicks given experimental infection (Table 4.35 and 4.36).

The aliquots of the three isolates (as infected liver homogenates) used in the study along with the standard CELO virus were subjected to PCR for detection of the fiber gene that is specific for FAV-1 (CELO virus) to exclude any chance of contamination of the liver samples with CELO virus.

Table 4.33. Detection of FAV-4 hexon gene specific product from different samples

Procedure	Samples	P-I	PH-II	PC-III
Embryonated eggs	**Liver**	+	+	+
	Yolk	+	+	+
	Amnioallantoic fluid	+	+	+
	Heart	-	-	-
Cell culture	**CEL**	+	+	+
	CEK	+	+	+
	CEF	+	+	+
Cell lines	**Vero**	+	+	+
	HeLa	±	-	-
	McCoy	-	-	-
	BHK-21	-	-	-

+ = Amplification seen ±: Positive amplification in some and negative in others
- = Amplification absent

Table 4.34. Comparative detection of FAV-4 by amplification of hexon gene specific product

	Cell culture adapted supernatant				Experimental infection to chicks		
Organs from infected chicks	**CEL passaged**	**CEK passaged**	**CEF passaged**	**Vero passaged**	**P-I**	**PH-II**	**PH-III**
Liver	+	-	-	+	+	+	+
Kidney	+	+	+	+	+	+	+
Heart	+	-	+	+	+	+	+
Thymus	+	+	+	+	+	+	+
Bursa	+	+	+	+	+	+	+

+ = Amplification seen - = Amplification absent

4.3.2.1.3.2 Detection of FAV-1 by amplification of fibre gene

None of the three viral isolates included in the present study showed the fiber specific amplification of 350 bp that is specific for the identification of FAV-1, while, the CELO virus included in the study showed the desired amplification. (Plate 4.85).

This led to the conclusion that the isolates included in the study were not contaminated with FAV-1, that also produces similar symptoms of hepatitis in birds. Table no. 4.37 shows the comparative test results when CELO virus as well as all the three isolates included in the study were both subjected to detection by AGPT and PCR.

Additionally, when the CELO virus was propagated in embryonated eggs, different samples from fertile eggs viz., liver of chick, yolk sac fluid, allantoic fluid, CAM from embryonated eggs showed the presence of required amplification from the various samples suggesting that the virus is present in different parts of a fertile egg (Plate 4.86).

Both tissue homogenates as well as DNA samples extracted from these tissue homogenates were used for dot blotting after boiling the products for denaturing the DNA. Absolutely similar results were obtained with both tissues and DNA extracted from tissues in hybridization (Plate 4.87). The results of Dot blot hybridization is depicted in Table 4.38.

4.4 Molecular characterization of fowl adenovirus

4.4.1 Protein profile of fowl adenovirus

4.4.1.1 Purification of the virus isolates

All the three isolates of FAV-4 used in the study were purified by 1.33 to 1.45 g/ml cesium chloride density gradient.

Table 4.35. Comparative detection of FAV-4 fibre gene specific product from different samples in this study

Procedure	Samples	P-I	PH-II	PC-III
Embryonated eggs	Liver	+	+	+
	Yolk	+	+	+
	Amnioallantoic fluid	+	+	+
	Heart	-	-	-
Cell culture Cell lines	CEL	+	+	+
	CEK	+	+	+
	CEF	+	+	+
	Vero	+	+	+
	HeLa	-	-	-
	McCoy	-	-	-
	BHK-21	-	-	-

+ = Amplification seen ±: Positive amplification in some and negative in others
- = Amplification absent

Table 4.36. Comparative detection of FAV-4 by amplification of fibre gene specific product

	Cell culture adapted supernatant				Experimental infection to chicks		
Organs from infected chicks	CEL passaged	CEK passaged	CEF passaged	Vero passaged	P-I	PH-II	PC-III
Liver	+	-	-	+	+	+	+
Kidney	+	-	-	+	+	-	+
Heart	+	-	-	+	+	+	+
Thymus	+	+	+	+	+	+	+
Bursa	+	+	+	+	+	+	+

+ = Amplification seen - = Amplification absent

Table 4.37. Results obtained by different tests

		AGPT		PCR		
Sr. No.	**Isolates**	**CELO antiserum**	**FAV hyperimmune serum**	**FAVs**	**FAV-1**	**FAV-4**
1	**CELO**	+	-	+	+	-
2	**P-I**	-	+	+	-	+
3	**PH-II**	-	+	+	-	+
4	**PC-III**	-	+	+	-	+

Extinction ratio of the viruses obtained by density gradient centrifugation and their protein ratio are depicted (Table 4.39). The FAV-4 isolates contained some impurities as cellular components as evident by their extinction ratios.

4.4.1.2 Purification of the standard CELO virus

The standard CELO virus was purified by 20 to 45 per cent CsCl density gradient centrifugation. The extinction ratio calculated for CELO virus showed that it was sufficiently purified with an extinction ratio of 1.21.

4.4.1.3 FAV structural protein analysis by SDS-PAGE

The three FAV-4 isolates were analyzed by SDS-PAGE in 10 per cent gel after purification. The resultant bands that were obtained were studied after staining by Coomasie brilliant blue. The MW of the viral proteins was then compared with standard molecular weight markers used along with the proteins. The pattern of the three isolates was similar and contained seven fractions (Plate 4.88).

Table 4.38. Results of Dot blot hybridization

Sample no.	Reaction (Tissue /DNA)	Sample no.	Reaction	Sample no.	Reaction	Sample no.	Reaction
1	+	16	+	31	+	46	+
2	+	17	+	32	-	47	PC
3	+	18	+	33	+	48	NC
4	+	19	+	34	+		
5	+	20	+	35	+		
6	-	21	+	36	+		
7	-	22	+	37	+		
8	+	23	+	38	+		
9	+	24	+	39	+		
10	-	25	+	40	+		
11	+	26	+	41	+		
12	+	27	+	42	+		
13	+	28	+	43	+		
14	-	29	+	44	-		
15	+	30	+	45	+		

1:Liver of embryonated chick infected with P-I,
2:Liver of embryonated chick infected with PH-II,
3:Liver of embryonated chick infected with PC-III,
4:Heart of embryonated chick infected with P-I,
5:Heart of embryonated chick infected with PH-II,
6:McCoy 8th passage,
7:Hela 8th passage,
8:Hela 4th passage,
9:Hela 5th passage,
10:BHK-21 8th passage,
11:CEK 3rd passage,
12:CEK 3rd passage,
13:CEK 5th passage,
14:Vero 3rd passage,
15:Vero 6th passage,
16:Vero 8th passage,
17:Bursa (CEF adapted virus),
18:Thymus (CEF adapted virus,
19: Kidney (CEF adapted virus)
20:Heart (CEF adapted virus
21: Liver (CEF adapted),
22:Bursa (CEK adapted virus)
23: Thymus (CEK adapted)
24:Kidney (CEK adapted virus),
25: Heart (CEK adapted),
26;Bursa (Vero adapted virus
27: Thymus (Vero adapted),
28:Kidney(Vero adapted),
29:Liver (Vero adapted),
30:P-I infected Chick (Liver),
31: P-I inf. Chick (Heart),
32: P -I inf. Chick (spleen),
33: P-I infected chick (thymus),
34:P-I inf. Chick (bursa),
35: PH-II infected chick (Liver
36: PH-II infected chick (Heart),
37: PH-II infected chick (Kidney),
38:PH-II infected chick (Thymus),
39:PC-III infected chick (Liver),
40:PC-III inf. chick (Heart),
41: P-I
42 PH-II
43:PC-III
44:PC-III inf .chick (spleen)
45: P-I (Liver)
46:PH-II (Liver)
47:Positive control
48: Negative control

Table 4.39. Extinction ratio of purified FAV-4 isolates and CELO virus with its protein contents.

Isolate	Absorbance value at 260 nm	Absorbance value at 280 nm	Ratio 260/280nm	Protein content (mg/ml)
P-I	1.274	1.245	1.02	0.95
PH-II	1.425	1.407	1.01	1.08
PC-III	1.353	1.321	1.02	1.00
CELO	2.823	2.322	1.21	1.42

4.4.1.4 Structural protein analysis of CELO virus by SDS-PAGE

The standard CELO virus (FAV-1) was also analyzed by SDS-PAGE in 10 per cent gel after purification. The resultant bands obtained after PAGE were stained and the protein profile studied after staining with Coomasie brilliant blue.

The molecular weight of the CELO viral proteins were then compared with standard molecular weight markers used along with the test virus. A total of eight proteins could be identified on SDS-PAGE with an additional protein of 110 kDa visible in CELO virus (Table 4.40).

4.4.1.5 Western Blot

Only two proteins could be detected visible in both CELO virus and FAV (Plate 4.89). The proteins corresponded to 43 and 78 kDa proteins seen in SDS-PAGE.

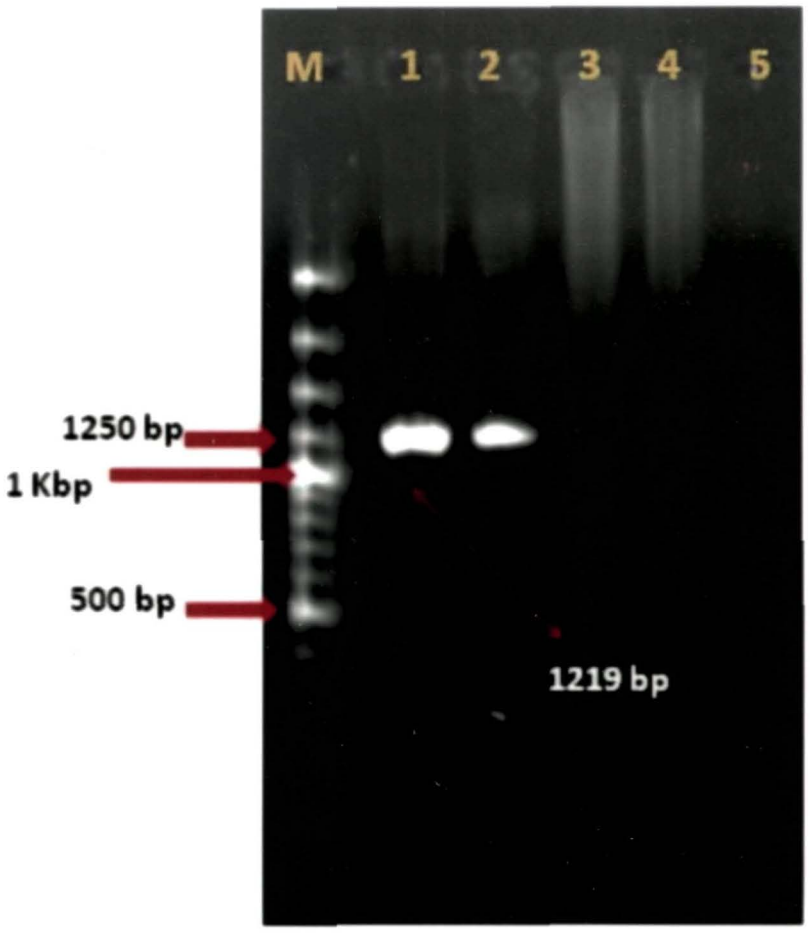


Plate 4.74: Agarose (1 %) electrophoresis of PCR products (1219 bp) of AAV M-1 Kb marker, Lane 1: CELO, Lane 2: P-III, Lane 3: P-I, Lane 4: P-II, Lane 5: Negative control

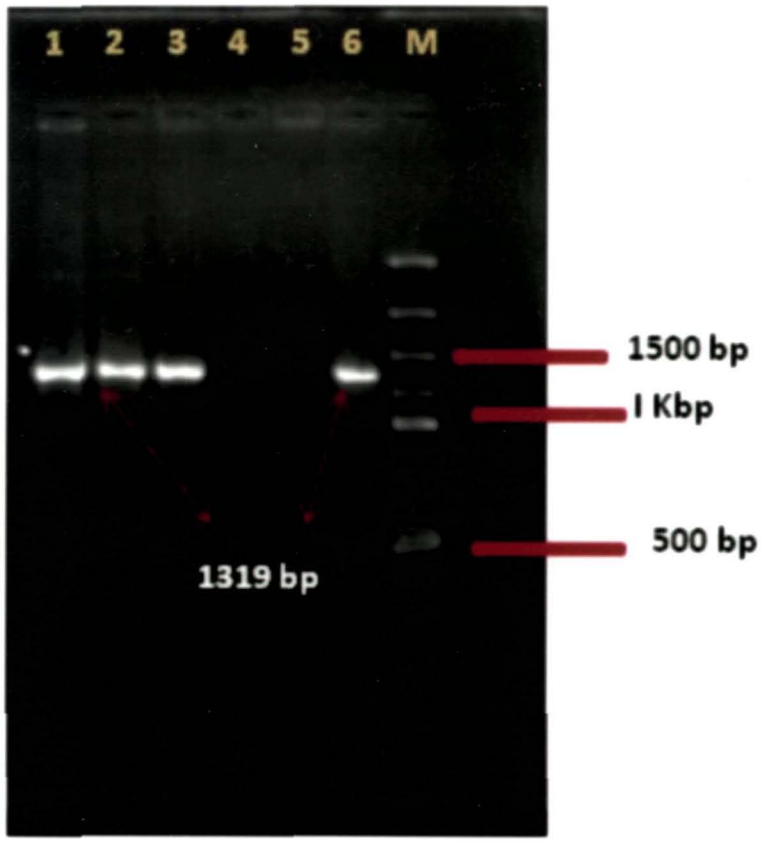


Plate 4.75 : Agarose (1 %) electrophoresis of PCR products (1319 bp) of AAV Lane 1: P-I, Lane 2: PH-II, Lane 3: PC-III, Lane 4:Blank , Lane 5: Negative control, Lane 6 : CELO, M-1 Kb marker

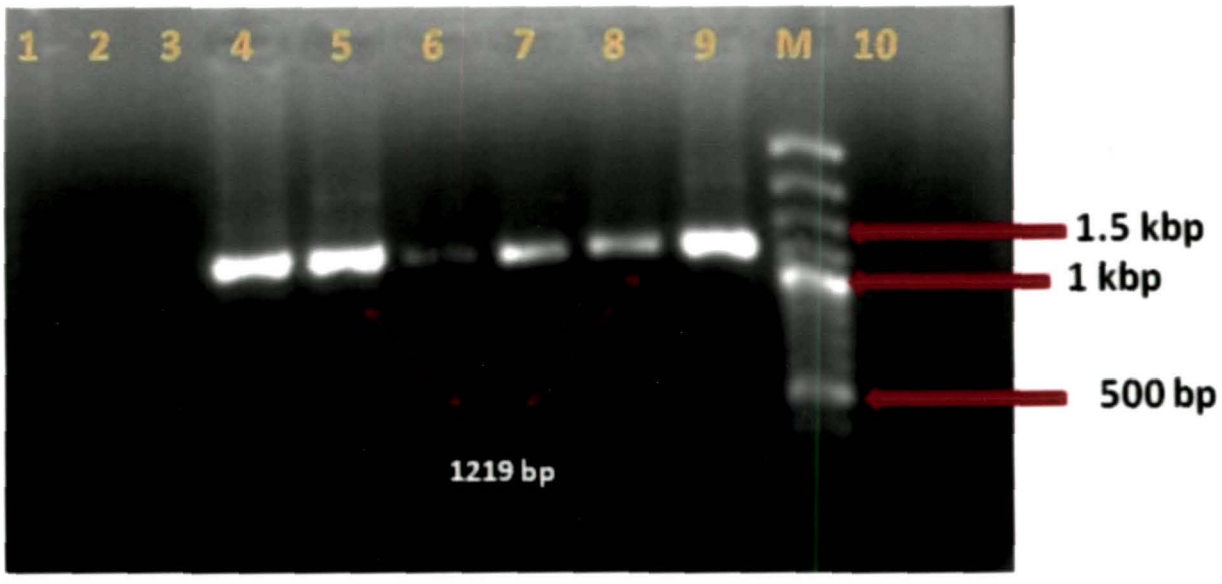


Plate 4.76 : Detection of 1219 bp product of FAV from cell culture supernatants
Lane 1: Hela 6th passage level, Lane 2: McCoy 6th passage, Lane 3: BHK-21 6th passage, Lane 4: Vero 6th passage , Lane 5: CEL 4th passage, Lane 6 :CEK 4th passage, Lane 7 :CEF 6th passage, Lane 8: CEF 10th passage, Lane 9: Positive control, M: Marker, Lane 10 : Negative control

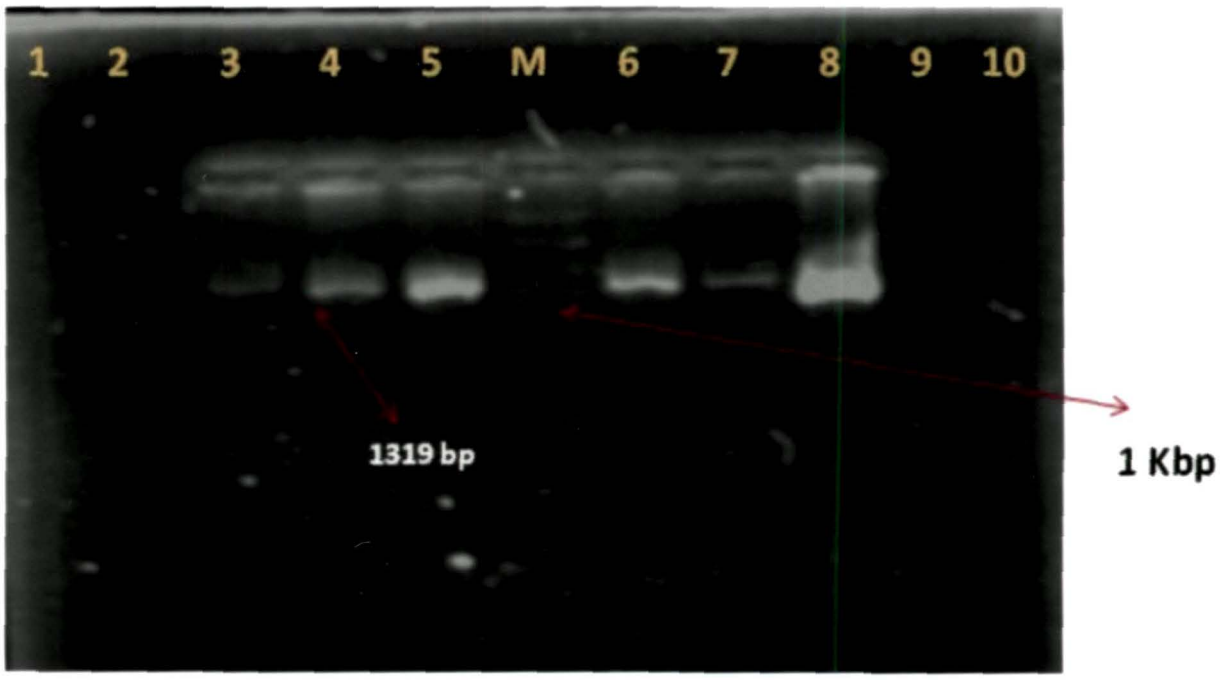


Plate 4.77: Detection of 1319 bp product of FAV from cell culture supernatants
Lane 1: Hela 6th passage level, Lane 2: McCoy 6th passage Lane 3: CEL 5th passage Lane 4: Vero 6th passage, Lane 5: Positive control, M: Marker, Lane 6 : CEK 5th passage, Lane 7: CEF 6th passage, Lane 8: CEF 10th passage, Lane 9 : BHK-21 6th passage, Lane 10 : Negative Control

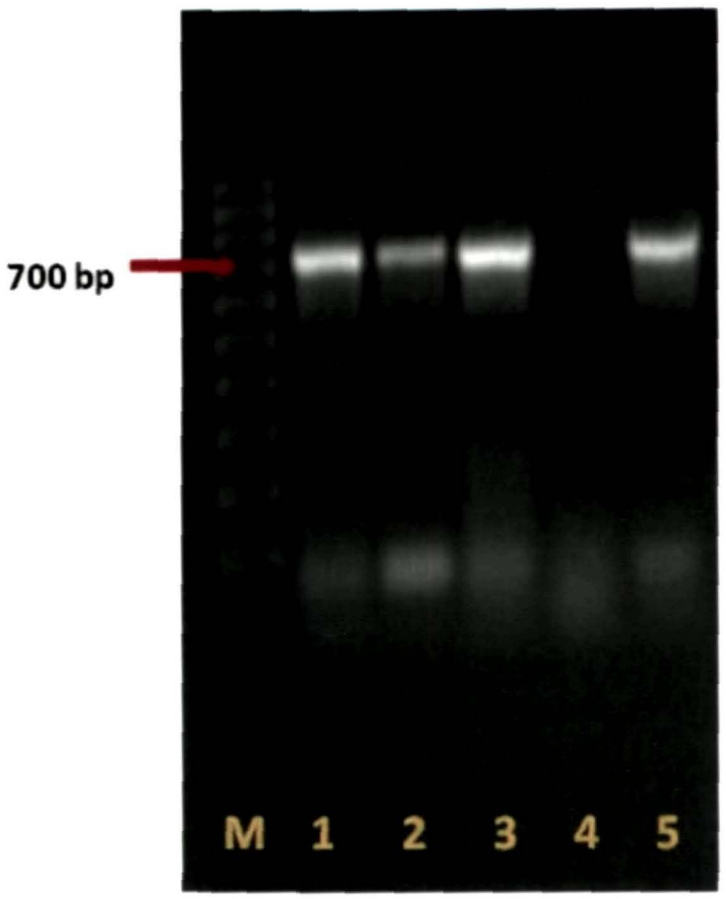


Plate 4.78: Agarose (1 %) electrophoresis of hexon gene PCR products of FAV-4
M- : 100 bp ladder , Lane 1: P-I , Lane 2: PH-II , Lane 3: PC-III ,
Lane 4: Negative control, Lane 5: Positive control

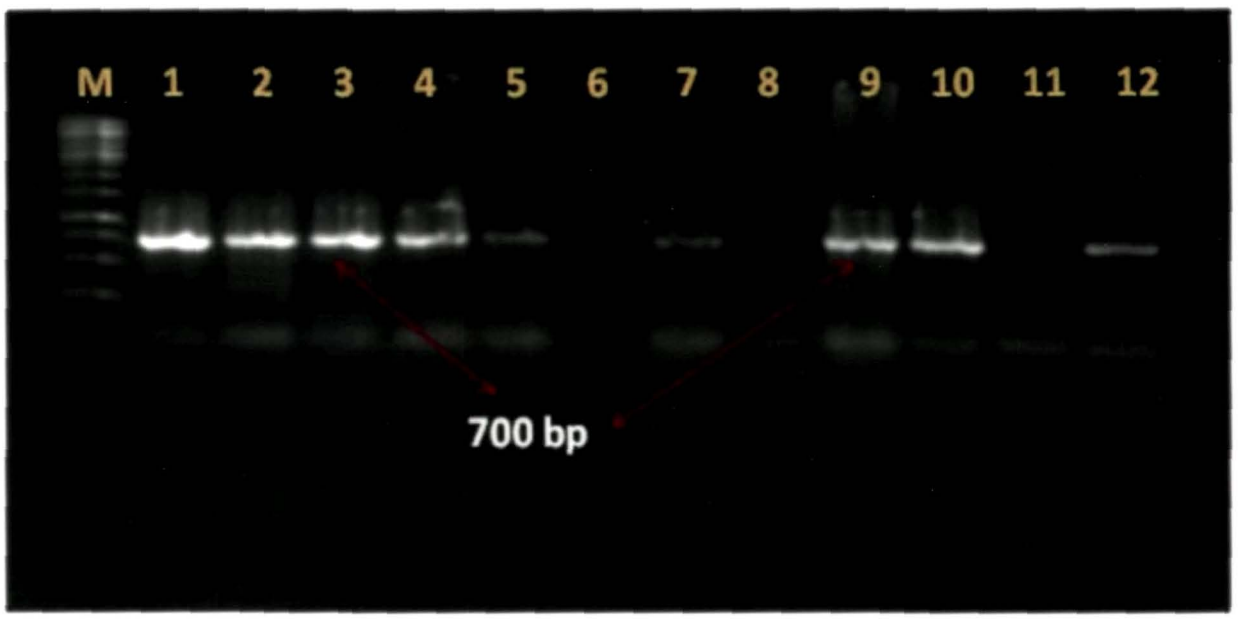


Plate 4.79 : Detection of 700 bp product of FAV-4 from infected cell culture supernatants
M: Marker, Lane 1: CEL 5th passage, Lane 2: CEK 5th passage, Lane 3: Vero 6th passage, Lane 4: Vero 8th passage, Lane 5 : Heh 4th passage, Lane 6 :McCoy 6th passage , Lane 7: Heh 5th passage Lane 8: BHK-21 6th passage, Lane 9 :CEF 8th passage level, Lane 10: CEF 10th passage, Lane 11: Negative Control, Lane 12: Positive Control

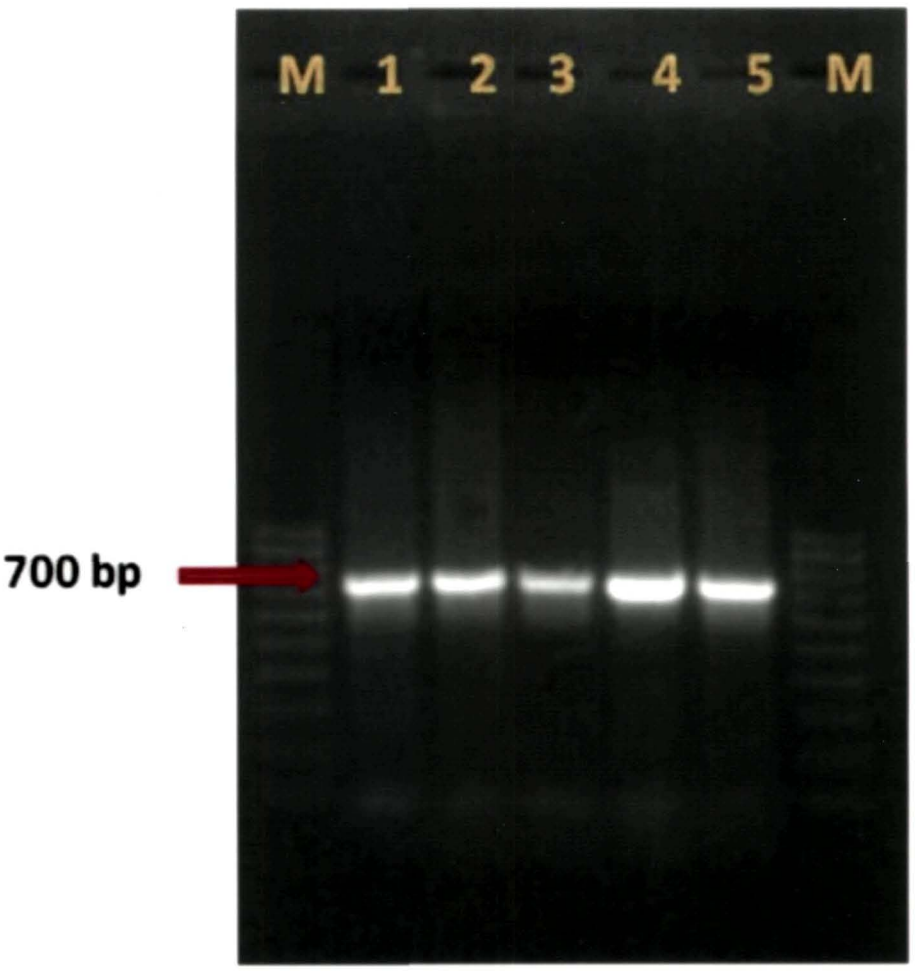


Plate 4.80: Detection of ~700 bp product FAV-4 from organs of chick infecte with Vero adapted virus
M: Marker Lane 1: Liver Lane 2: Heart, Lane 3: Kidney, Lane 4: Thymus, Lane 5 :Bursa, M: Marker

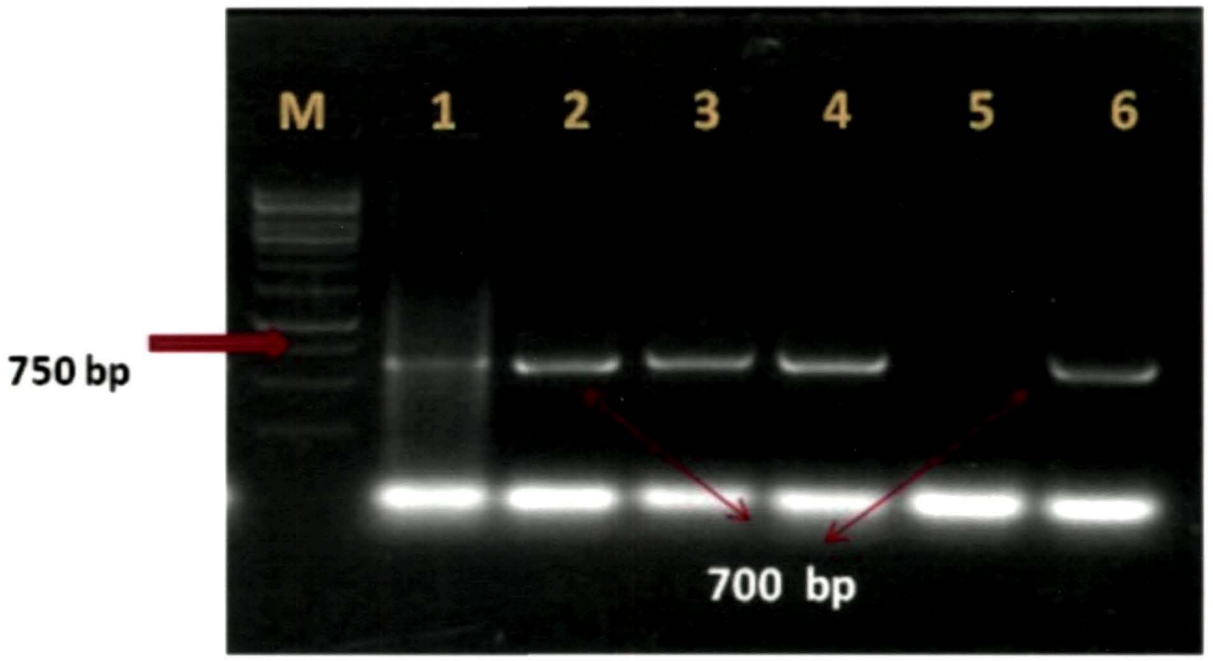


Plate 4.81: Agarose (1 %) electrophoresis of ~ 700 bp hexon gene PCR products of FAV-4 following experimental infection in chicks
M : 100 bp marker, Lane 1: Thymus of chick infected with P-I , Lane 2: Bursa of chick infected with P-I, Lane 3: Liver of chick infected with P-I , Lane 4: Heart of chick infected with P-I, Lane 5 : Spleen of chick infected with P-I, Lane 6 :Kidney of chick infected with P-I

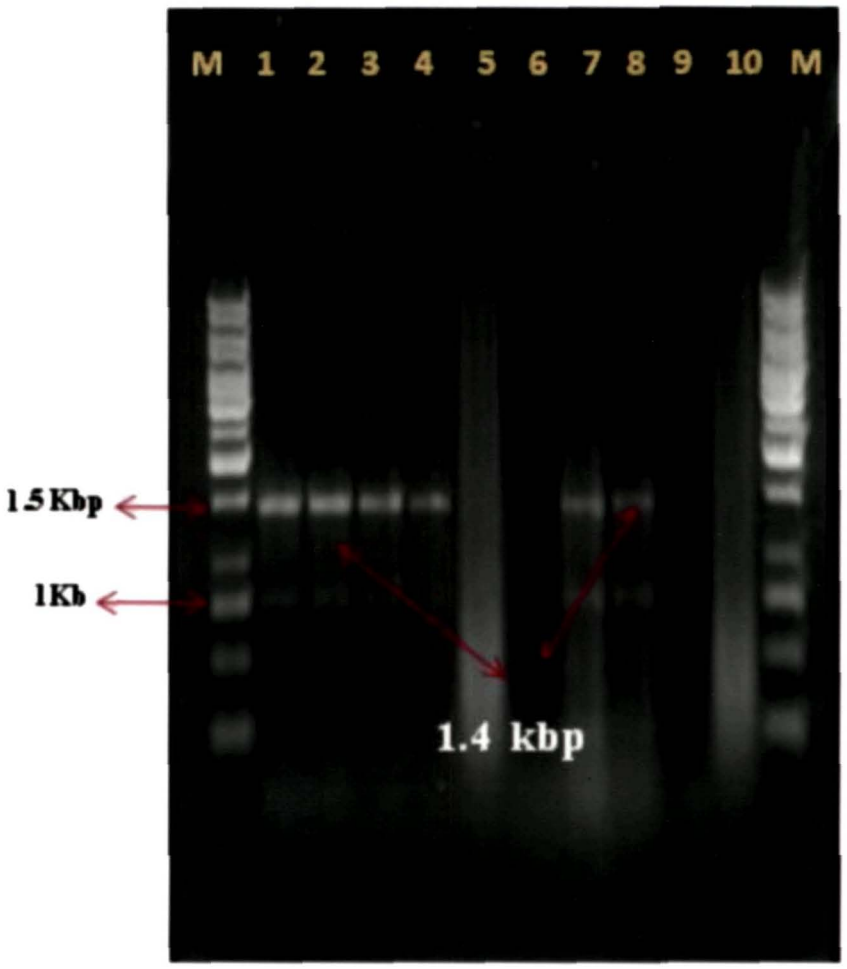


Plate 4.82: Agarose (1 %) electrophoresis of ~ 1400 bp fiber gene PCR products of FAV-4

M : Marker, Lane 1: Positive control, Lane 2:CEL 5th passage, Lane 3:CEK 5th passage,Lane 4: Vero 8th passage, Lane 5 : Hela 6th passage, Lane 6 : McCoy 6th passage, Lane 7: CEF 6th passage, Lane 8: CEF 10th passage, Lane 9:BHK-21 6th passage ,Lane 10:Negative control, M:Marker

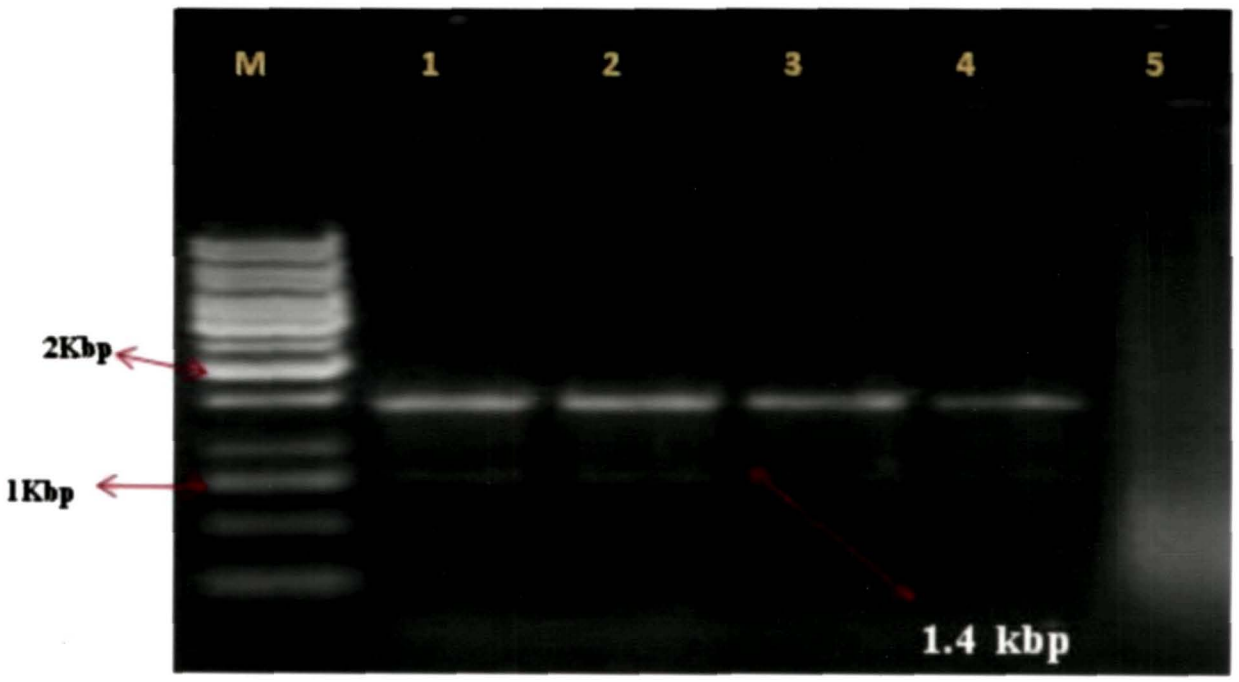


Plate 4.83: Agarose (1 %) electrophoresis of ~ 1400 bp fiber gene PCR products of FAV-4

M : Marker, Lane 1: P-I, Lane 2: PH-II, Lane 3:PC-III, Lane 4: Positive control, Lane 5: Negative control

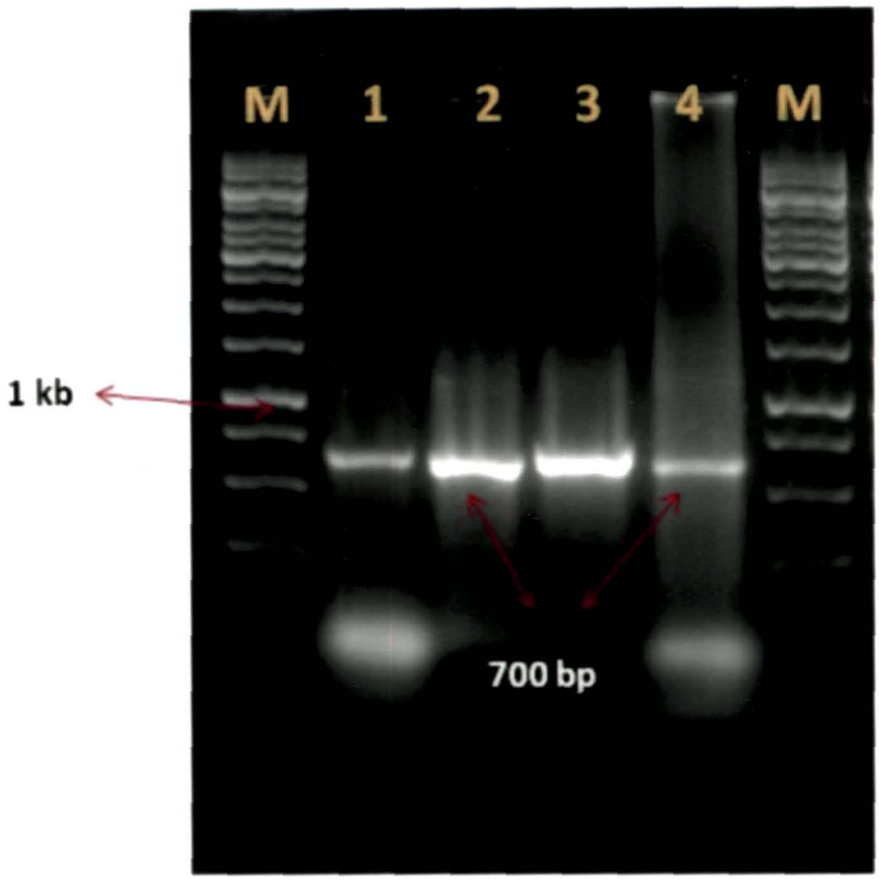


Plate 4.84: Agarose (1 %) electrophoresis of hexon gene products of FAV-4 from liver of experimentally infected chick
M: 3 Kb Marker, Lane 1: Positive control, Lane 2: P-I, Lane 3: PH-II, Lane 4: PC-III, M: 1 Kb Marker

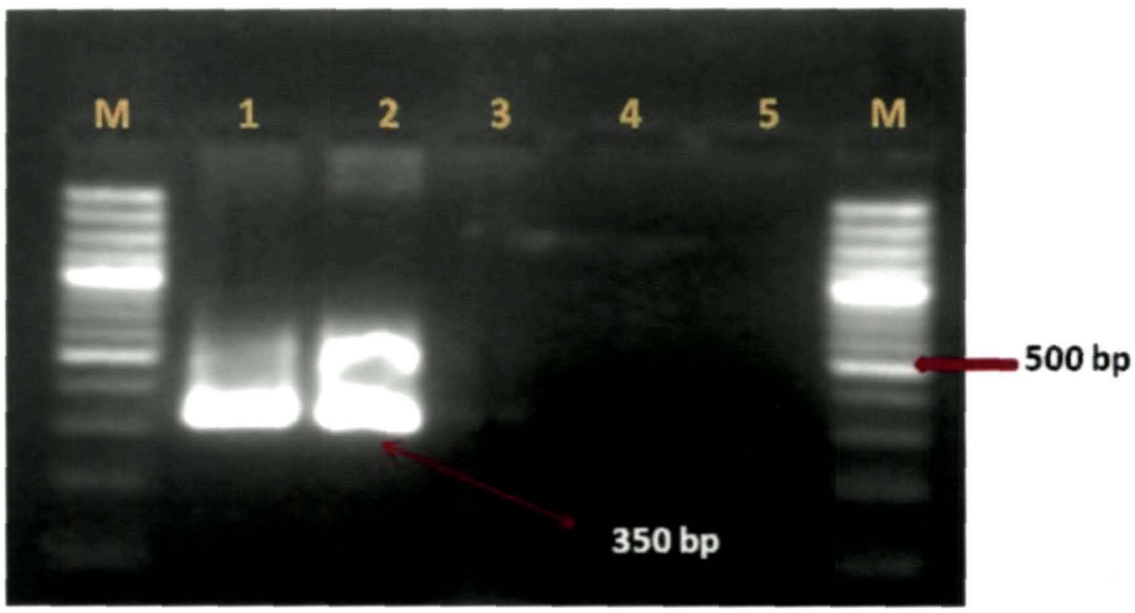


Plate 4.85: Agarose (1 %) electrophoresis of ~ 350 bp fiber gene products of FAV-1
M:100 bp Lane 1: CELO Lane 2: CELO Lane 3: P-I Lane 4: PH-II, Lane 5: PC-III

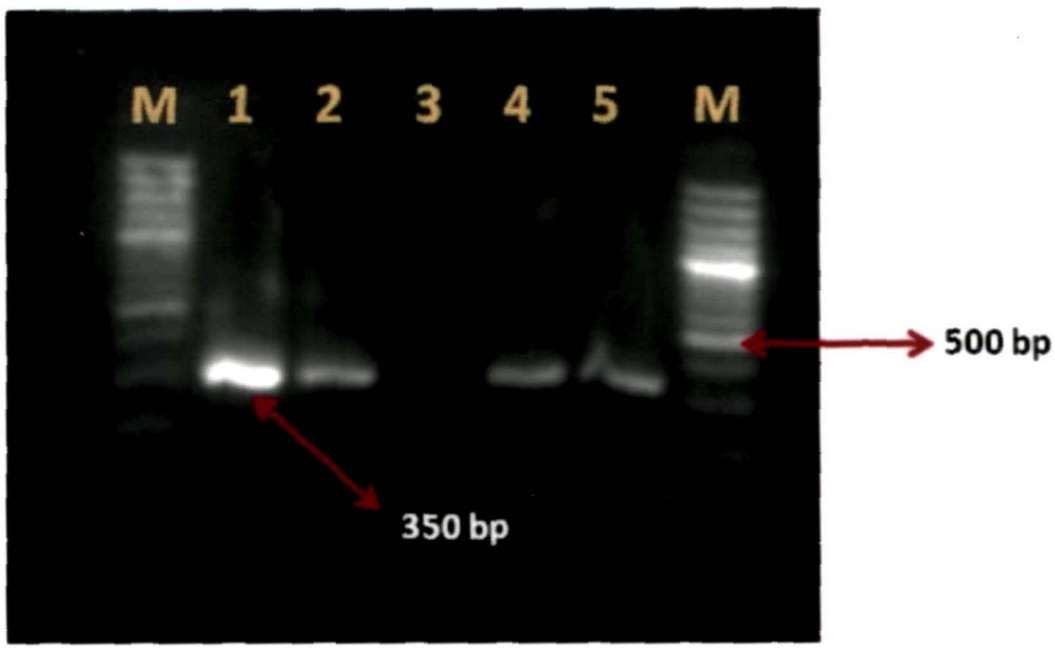


Plate 4.86: Agarose (1 %) electrophoresis of ~ 350 bp fibre gene PCR products of FAV-1 (CELO)
M: 100 bp marker, Lane 1: Liver of chick, Lane 2: CAM, Lane 3: Negative control , Lane 4: Yolk sac, Lane 5 :Allantoic fluid M:100 bp

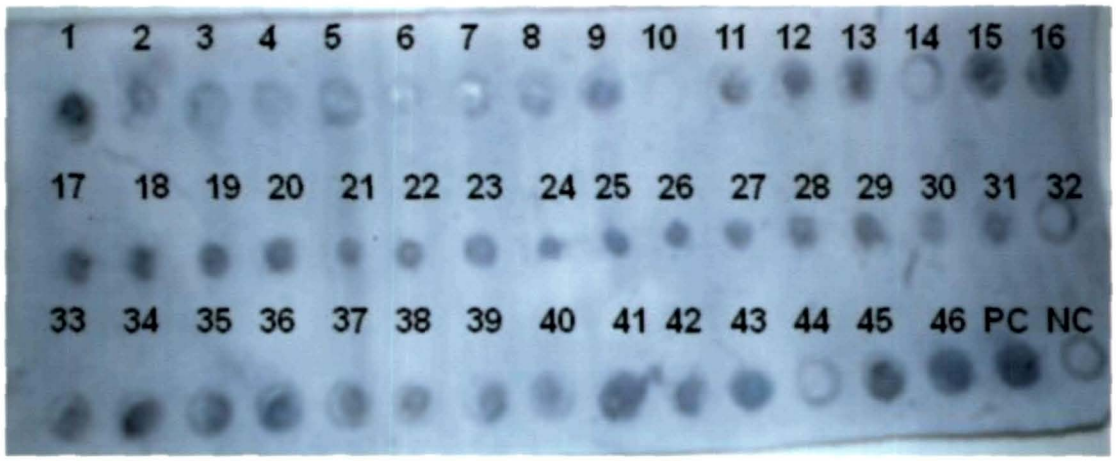


Plate 4.87: Dot Blot Hybridization
Samples 6, 7, 10, 14, 32, 44 Negative PC: Positive control, NC: Negative control

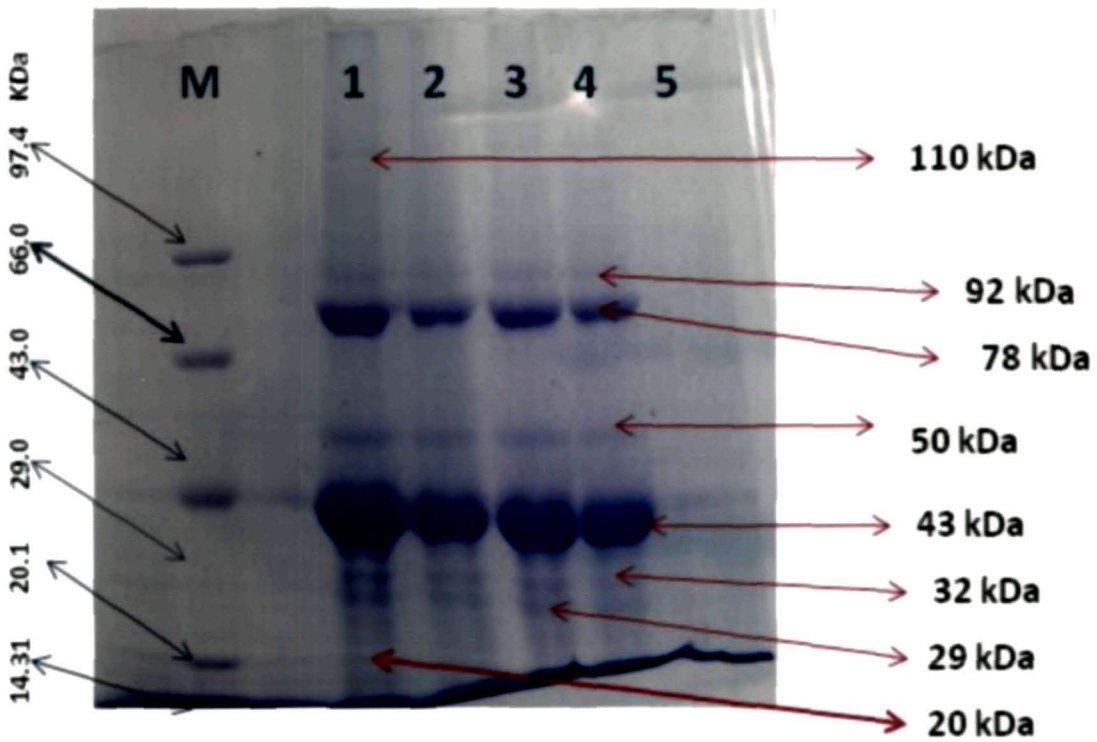


Plate 4.88: SDS-PAGE analysis of CELO virus and FAV-4 isolates
M: Protein Marker, 1 : CELO virus 2: P-I, 3 : P-II, 4 : P-III, 5: Normal CEL

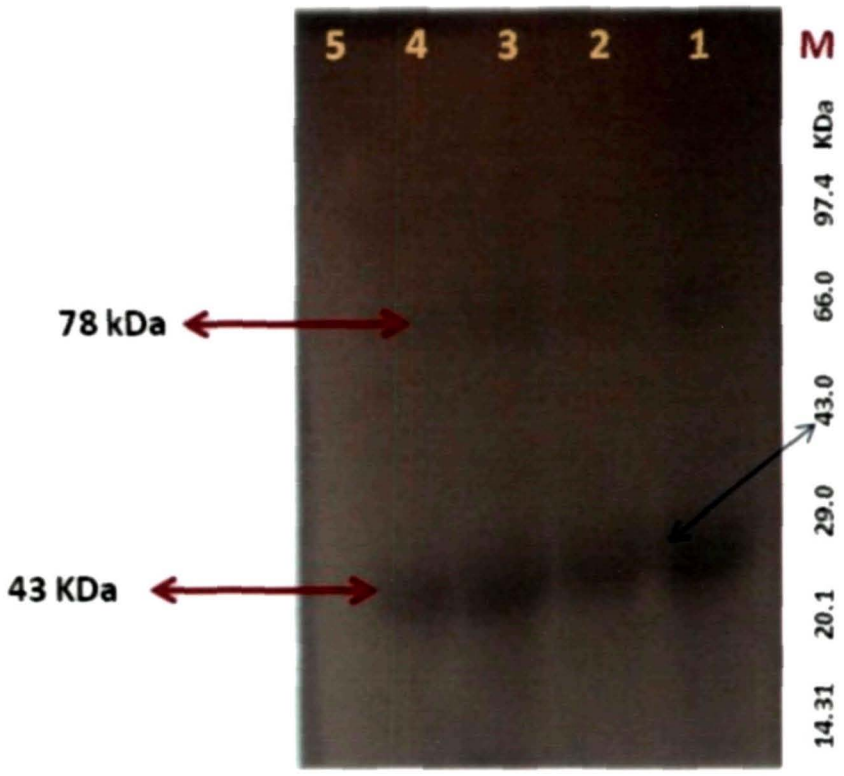


Plate 4.89: Western blot analysis of CELO virus and FAV-4 isolates
M: Protein Marker , 1 : CELO virus 2 : P-I, 3 : P-II, 4 : P-III, 5: Normal CEL

Table 4.40. Approximate MW of protein fractions obtained from FAV-4 and CELO virus.

	FAV -4 isolates			
Protein fractions	**CELO virus**	**P-I**	**PH-II**	**PC-III**
1	110	-	-	-
2	92	92	92	92
3	78	78	78	78
4	50	50	50	50
5	43	43	43	43
6	32	32	32	32
7	29	29	29	29
8	20	20	20	20

4.4.2 Genetic profile of fowl adenovirus

4.4.2.1 Gene sequencing of PCR products of fowl adenovirus

4.4.2.1.1 Direct sequencing of FAV-4 fibre gene fragment

Direct sequencing of the purified PCR product of FAV-4 fibre gene (P-I isolate) fragment showed 90 per cent identity at nucleotide level to fibre gene of Fowl adenovirus 4 isolate Punjab 1 (DQ864436), Punjab 2 (DQ864434) and short fibre gene (AY340863).

Nucleotide sequences of the fibre gene (partial sequence) of FAV-4 isolate (P-I) used in the study

i) **P-I(1.4kb)**

CGTGGCCGACCCCGTCGGAGGGTCATTCGCCTTTTTTGGGAGGCTCAGGATTCCTAG

TGGACCAGGGCGGACAGCTTACGCTCAACGCTCCGATCCCATCATATCACGTACAG

4.4.2.1.2 Sequencing of FAV-4 hexon gene fragments

The purified PCR product s of FAV -4 isolates {(P-I/FAV 4 (Palampur)}, {PH-II/FAV 4(Palampur)} were sequenced commercially from Bioserve, Genome Valley, Hyderabad. The sequences were blasted in NCBI databank and the sequences matched and aligned. The sequences were edited in "Edit Seq" programme for further analysis. The nucleotide sequence of FAV -4 hexon gene revealed a coding region of 685 bp coding for 228 amino acids.

4.4.2.1.2.1 Nucleotide sequences of the hexon gene of FAV-4 isolates used in the study

i) P-1(Isolate 1)

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
CCCGCTGGCTCCCAAGGAGTCCATGTTTAACAACTGGTCGGAGACGGCACCCGGGCA
GAACGTGTCCGCCTCCGGTCAGCTGTCCAATGTCTATACCAACACGAGCACCTCCAA
AGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
GGGACCCGGAAGAAATCCTCTGCGACGGGTAGAAAACGCCAACACCGGCGTGCTCG
GTCGCTTCGCCAAGTCTCAGTACAATTACGCTTACGGTGCCTACGTCAAGCCCGTCG
CCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGG
GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
ATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

ii) PH-II (Isolate 2)

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
CCCGCTGGCTCCCAAGGAGTCCATGTTTAACAACTGGTCGGAGACGGCACCCGGGCA
GAACGTGTCCGCCTCCGGTCAGCTGTCCAATGTCTATACCAACACGAGCACCTCCAA
AGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
GGGACCCGGAAGAAATCCTCTGCGACGGGTAGAAAACGCCAACACCGGCGTGCTCG
GTCGCTTCGCCAAGTCTCAGTACAATTACGCTTACGGTGCCTACGTCAAGCCCGTCG
CCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGG
GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
ATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

4.4.2.1.2.2 Nucleotide sequences of FAV-4 isolates used for alignment

i) AJ459805

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
CCCGCTGGCTCCCAAGGAGTCCATGTTTAACAACTGGTCGGAGACGGCACCCGGGCA
GAACGTGTCCGCCTCCGGTCAGCTGTCCAATGTCTATACCAACACGAGCACCTCCAA
AGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
GGGACCCGGAAGAAATCCTCTGCGACAGGTAGAAAACGCCAACACCGGCGTGCTCG
GTCGCTTCGCCAAGTCTCAGTACAATTACGCTTACGGTGCCTACGTCAAGCCCGTCG
CCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGG
GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
ATCACGTCTCCGGCGTGTACTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

ii) AY581274

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
CCCGCTGGCTCCCAAGGAGTCCATGTTTAACAACTGGTCGGAGACGGCACCCGGGCA
GAACGTGTCCGCCTCCGGTCAGCTGTCCAATGTCTATACCAACACGAGCACCTCCAA
AGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
GGGACCCGGAAGAAATCCTCTGCGACAGGTAGAAAACGCCAACACCGGCGTGCTCG
GTCGCTTCGCCAAGTCTCAGTACAATTACGCTTACGGTGCCTACGTCAAGCCCGTCG
CCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGG
GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
ATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

iii) AJ320166

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
CCCGCTGGCTCCCAAGGAGTCCATGTTTAACAACTGGTCGGAGACGGCACCCGGGCA
GAACGTGTCCGCCTCCGGTCAGCTGTCCAATGTCTATACCAACACGAGCACCTCCAA
AGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
GGGACCCGGAAGAAATCCTCTGCGACAGGTAGAAAACGCCAACACCGGCGTGCTCG
GTCGCTTCGCCAAGTCTCAGTACAATTACGCTTACGGTGCCTACGTCAAGCCCGTCG
CCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGG
GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
ATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

iv) AY581295

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
CCCGCTGGCTCCCAAGGAGTCCATGTTTAACAACTGGTCGGAGACGGCACCCGGGCA
GAACGTGTCCGCCTCCGGTCAGCTGTCCAATGTCTATACCAACACGAGCACCTCCAA
AGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
GGGACCCGGAAGAAATCCTCTGCGACAGGTAGAAAACGCCAACACCGGCGTGCTCG
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CCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGG
GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
ATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGC

v) AY581296

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
CCCGCTGGCTCCCAAGGAGTCCATGTTTAACAACTGGTCGGAGACGGCACCCGGGCA
GAACGTGTCCGCCTCCGGTCAGCTGTCCAATGTCTATACCAACACGAGCACCTCCAA
AGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
GGGACCCGGAAGAAATCCTCTGCGACAGGTAGAAAACGCCAACACCGGCGTGCTCG
GTCGCTTCGCCAAGTCTCAGTACAATTACGCTTACGGTGCCTACGTCAAGCCCGTCG
CCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGG
GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
ATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

vi) AY581297

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GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
ATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

vii) AY581298

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
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AGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
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CCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGG
GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
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TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

viii) AY581299

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
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CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
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ix) EU177546

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ATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

x) EU847626

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
CCCGCTGGCTCCCAAGGAGTCCATGTTTAACAACTGGTCGGAGACGGCACCCGGGCA
GAACGTGTCCGCCTCCGGTCAGCTGTCCAATGTCTATACCAACACGAGCACCTCCAA
AGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
GGGACCCGGAAGAAATCCTCTGCGACGGGTAGAAAACGCCAACACCGGCGTGCTCG
GTCGCTTCGCCAAGTCTCAGTACAATTACGCTTACGGTGCCTACGTCAAGCCCGTCG

CCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGG
GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
ATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

xi) EU931690

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
CCCGCTGGCTCCCAAGGAGTCCATGTTTAACAACTGGTCGGAGACGGCACCCGGGCA
GAACGTGTCCGCCTCCGGTCAGCTGTCCAATGTCTATACCAACACGAGCACCTCCAA
AGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
GGGACCCGGAAGAAATCCTCTGCGACAGGTAGAAAACGCCAACACCGGCGTGCTCG
GTCGCTTCGCCAAGTCTCAGTACAATTACGCTTACGGTGCCTACGTCAAGCCCGTCG
CCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGG
GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
ATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

xii) EU931691

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
CCCGCTGGCTCCCAAGGAGTCCATGTTTAACAACTGGTCGGAGACGGCACCCGGGCA
GAACGTGTCCGCCTCCGGTCAGCTGTCCAATGTCTATACCAACACGAGCACCTCCAA
AGACACGACGGCGGCGCACGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
GGGACCCGGAAGAAATCCTCTGCGACAGGTAGAAAACGCCAACACCGGCGTGCTCG
GTCGCTTCGCCAAGTCTCAGTACAATTACGCTTACGGTGCCTACGTCAAGCCCGTCG
CCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGG
GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
ATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

xiii) EU931692

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
CCCGCTGGCTCCCAAGGAGTCCATGTTTAACAACTGGTCGGAGACGGCACCCGGGCA
GAACGTGTCCGCCTCCGGTCAGCTGTCCAATGTCTATACCAACACGAGCACCTCCAA
AGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
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GTCGCTTCGCCAAGTCTCAGTACAATTACGCTTACGGTGCCTACGTCAAGCCCGTCG
CCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGG
GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCGTAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT

ATCACGACTCCGGCGTGTGCTCGGGCACCCTCACTCGGAGCGTTCGGGCATGAACGT
GGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCGA
C

xiv) EU931693

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CCCGCTGGCTCCCAAGGAGTCCATGTTTAACAACTGGTCGGAGACGGCACCCGGGCA
GAACGTGTCCGCCTCCGGTCAGCTGTCCAATGTCTATACCAACACGAGCACCTCCAA
AGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
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GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
ATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

xv) EU938324

CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAA
CCCGCTGGCTCCCAAGGAGTCCATGTTTAACAACTGGTCGGAGACGGCACCCGGGCA
GAACGTGTCCGCCTCCGGTCAGCTGCCCAATGTCTATACCAACACGAGCACCTCCAA
AGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCA
GGGACCCGGAAGAAATCCTCTGCGACAGGTAGAAAACGCCAACACCGGCGTGCTCG
GTCGCTTCGCCAAGTCTCAGTACAATTACGCTTACGGTGCCTACGTCAAGCCCGTCG
CCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGG
GCACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACC
CAGATACCATAGTCGTGCCGCCTCCCGAGGACTACGACGATTATAACATAGGCACCA
CGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTAACCTGCTGT
ATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACG
TGGTGGTCGAGCTGCCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCG
AC

4.4.2.1.2.3 Amino acid sequences of FAV-4 used for alignment

P-1 (Isolate 1)

KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDT
TAAQVTKISGVFPNPNQGPGRNPLRRVENANTGVLGRFAKSQYNYAYGAYVKPVAADG
SQSLTQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRP
NYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

PH-II (Isolate II)

KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDT
TAAQVTKISGVFPNPNQGPGRNPLRRVENANTGVLGRFAKSQYNYAYGAYVKPVAADG

SQSLTQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRP
NYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

EU177546.seq

KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDT
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SQSLTQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRP
NYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

EU847626.seq

KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDT
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SQSLTQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRP
NYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

AJ320166.seq

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TAAQVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADG
SQSLTQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRP
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AY581274.seq

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SQSLTQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRP
NYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

AY581298.seq

KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDT
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SQSLTQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRP
NYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

AJ459805.seq

KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDT
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SQSLTQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRP
NYIGFRDNFINLLYHVSGVYSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

AY581297.seq

KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDT
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SQSLTQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRP
NYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

AY581296.seq

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SQSLTQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRP
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AY581295.seq

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SQSLTQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRP
NYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

EU931693.seq

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SQSLTQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRP
NYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

EU931690.seq

KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTA
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TQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFR
DNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

AY581299.seq

KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTT
AAQVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGS
QSLTQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNY
IGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

EU931691.seq

KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTA
AHVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSL

TQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFR
DNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

EU931692.seq

KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTA
AQVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSL
TQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTVVVPPPEDYDDYNIGTTRALRPNYIGFR
DNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

EU938324.seq

KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLPNVYTNTSTSKDTTA
AQVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSL
TQTPYWIMDNTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFR
DNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD

4.4.2.1.3 Sequence alignment and phylogenetic analysis

Multialin analysis of the two FAV-4 isolates used in the study was found to be 100 per cent similar among themselves both at nucleotide and amino acid level. Multalin analysis of the isolates at the amino acid level also does not show any difference among themselves (Fig 4. 1). There were however 98 to 100 per cent homology at nucleotide level and 95 to 100 per cent at amino acid level with other FAV-4 isolates. Both the isolates in the study are together in the phylogenetic tree thus indicating that there is not much difference in these two isolates at nucleotide and amino acid level (Table 4.41). Phylogenetic analysis reveals its closeness to EU847626 isolate in the tree (Fig 4.2). Multalin of amino acids also does not show any significant change in amino acids (Fig 4.3) while phylogenetic analysis of the amino acid sequences show that the FAV-4 of Himachal cluster with EU847626 FAV-4 isolate of Haryana revealing a common origin (Fig.4.4). From this study it appeared that hexon gene sequences of FAVs are relatively conserved within serotypes.

Fig 4.1. Multiple sequence alignment of deduced nucleotide sequences of hexon gene of FAV -4

```
AJ320166.seq      1 CAAGGGAATCCTAGACCGAGGGCCGTCCTTCAAGCCCTACTGCGGCACGGCTTACAACCCGCTGGCTCCCAAGGAGTCCA  80
AJ459805.seq      1 ................................................................................  80
AY581274.seq      1 ................................................................................  80
AY581295.seq      1 ................................................................................  80
AY581296.seq      1 ................................................................................  80
AY581297.seq      1 ................................................................................  80
AY581298.seq      1 ................................................................................  80
AY581299.seq      1 ................................................................................  80
EU177546.seq      1 ................................................................................  80
EU847626.seq      1 ................................................................................  80
EU931690.seq      1 ................................................................................  80
EU931691.seq      1 ................................................................................  80
EU931692.seq      1 ................................................................................  80
EU931693.seq      1 ................................................................................  80
EU938324.seq      1 ................................................................................  80
Isolate1.seq      1 ................................................................................  80
Isolate2.seq      1 ................................................................................  80

AJ320166.seq     81 TGTTTAACAACTGGTCGGAGACGGCACCCGGGCAGAACGTGTCCGCCTCCGGTCAGCTGTCCAATGTCTATACCAACACG 160
AJ459805.seq     81 ................................................................................ 160
AY581274.seq     81 ................................................................................ 160
AY581295.seq     81 ................................................................................ 160
AY581296.seq     81 ................................................................................ 160
AY581297.seq     81 ................................................................................ 160
AY581298.seq     81 ................................................................................ 160
AY581299.seq     81 ................................................................................ 160
EU177546.seq     81 ................................................................................ 160
EU847626.seq     81 ................................................................................ 160
EU931690.seq     81 ................................................................................ 160
EU931691.seq     81 ................................................................................ 160
EU931692.seq     81 ................................................................................ 160
EU931693.seq     81 ................................................................................ 160
EU938324.seq     81 ...............................................C................................ 160
Isolate1.seq     81 ................................................................................ 160
Isolate2.seq     81 ................................................................................ 160

AJ320166.seq    161 AGCACCTCCAAAGACACGACGGCGGCGCAGGTGACGAAGATTTCCGGCGTCTTCCCCAATCCCAACCAGGGACCCGGAAG 240
AJ459805.seq    161 ................................................................................ 240
AY581274.seq    161 ................................................................................ 240
AY581295.seq    161 ................................................................................ 240
AY581296.seq    161 ................................................................................ 240
AY581297.seq    161 ................................................................................ 240
AY581298.seq    161 ................................................................................ 240
AY581299.seq    161 ................................................................................ 240
EU177546.seq    161 ................................................................................ 240
EU847626.seq    161 ................................................................................ 240
EU931690.seq    161 ................................................................................ 240
EU931691.seq    161 ..........................C..................................................... 240
EU931692.seq    161 ................................................................................ 240
EU931693.seq    161 ................................................................................ 240
EU938324.seq    161 ................................................................................ 240
Isolate1.seq    161 ................................................................................ 240
Isolate2.seq    161 ................................................................................ 240

AJ320166.seq    241 AAATCCTCTGCGACAGGTAGAAAACGCCAACACCGGCGTGCTCGGTCGCTTCGCCAAGTCTCAGTACAATTACGCTTACG 320
AJ459805.seq    241 ................................................................................ 320
AY581274.seq    241 ................................................................................ 320
AY581295.seq    241 ................................................................................ 320
AY581296.seq    241 ................................................................................ 320
AY581297.seq    241 ................................................................................ 320
AY581298.seq    241 ................................................................................ 320
AY581299.seq    241 ................................................................................ 320
EU177546.seq    241 ................................................................................ 320
EU847626.seq    241 ............G................................................................... 320
EU931690.seq    241 ................................................................................ 320
EU931691.seq    241 ................................................................................ 320
EU931692.seq    241 ................................................................................ 320
EU931693.seq    241 ................................................................................ 320
EU938324.seq    241 ................................................................................ 320
Isolate1.seq    241 ............G................................................................... 320
Isolate2.seq    241 ............G................................................................... 320
```

```
AJ320166.seq    321 GTGCCTACGTCAAGCCCGTCGCCGCCGACGGTTCCCAGTCCCTCACGCAGACCCCCTACTGGATCATGGATAACACGGGC 400
AJ459805.seq    321 ........................................................................................ 400
AY581274.seq    321 ........................................................................................ 400
AY581295.seq    321 ........................................................................................ 400
AY581296.seq    321 ........................................................................................ 400
AY581297.seq    321 ........................................................................................ 400
AY581298.seq    321 ........................................................................................ 400
AY581299.seq    321 ........................................................................................ 400
EU177546.seq    321 ........................................................................................ 400
EU847626.seq    321 ........................................................................................ 400
EU931690.seq    321 ........................................................................................ 400
EU931691.seq    321 ........................................................................................ 400
EU931692.seq    321 ........................................................................................ 400
EU931693.seq    321 ........................................................................................ 400
EU938324.seq    321 ........................................................................................ 400
Isolate1.seq    321 ........................................................................................ 400
Isolate2.seq    321 ........................................................................................ 400

AJ320166.seq    401 ACCAATTACCTGGGAGCGGTGGCCGTCGAGGACTACACCAACAGCCTCTCGTACCCAGATACCATAGTCGTGCCGCCTCC 480
AJ459805.seq    401 ........................................................................................ 480
AY581274.seq    401 ........................................................................................ 480
AY581295.seq    401 ........................................................................................ 480
AY581296.seq    401 ........................................................................................ 480
AY581297.seq    401 ........................................................................................ 480
AY581298.seq    401 ........................................................................................ 480
AY581299.seq    401 ........................................................................................ 480
EU177546.seq    401 ........................................................................................ 480
EU847626.seq    401 ........................................................................................ 480
EU931690.seq    401 ........................................................................................ 480
EU931691.seq    401 ........................................................................................ 480
EU931692.seq    401 ..........................................................G............................. 480
EU931693.seq    401 ........................................................................................ 480
EU938324.seq    401 ........................................................................................ 480
Isolate1.seq    401 ........................................................................................ 480
Isolate2.seq    401 ........................................................................................ 480

AJ320166.seq    481 CGAGGACTACGACGATTATAACATAGGCACCACGCGTGCGCTCAGGCCCAACTACATCGGGTTCAGGGATAACTTCATTA 560
AJ459805.seq    481 ........................................................................................ 560
AY581274.seq    481 ........................................................................................ 560
AY581295.seq    481 ........................................................................................ 560
AY581296.seq    481 ........................................................................................ 560
AY581297.seq    481 ........................................................................................ 560
AY581298.seq    481 ........................................................................................ 560
AY581299.seq    481 ........................................................................................ 560
EU177546.seq    481 ........................................................................................ 560
EU847626.seq    481 ........................................................................................ 560
EU931690.seq    481 ........................................................................................ 560
EU931691.seq    481 ........................................................................................ 560
EU931692.seq    481 ........................................................................................ 560
EU931693.seq    481 ........................................................................................ 560
EU938324.seq    481 ........................................................................................ 560
Isolate1.seq    481 ........................................................................................ 560
Isolate2.seq    481 ........................................................................................ 560

AJ320166.seq    561 ACCTGCTGTATCACGACTCCGGCGTGTGCTCGGGCACCCTCAACTCGGAGCGTTCGGGCATGAACGTGGTGGTCGAGCTG 640
AJ459805.seq    561 ..............T..........A......................................................... 640
AY581274.seq    561 ........................................................................................ 640
AY581295.seq    561 ........................................................................................ 640
AY581296.seq    561 ........................................................................................ 640
AY581297.seq    561 ........................................................................................ 640
AY581298.seq    561 ........................................................................................ 640
AY581299.seq    561 ........................................................................................ 640
EU177546.seq    561 ........................................................................................ 640
EU847626.seq    561 ........................................................................................ 640
EU931690.seq    561 ........................................................................................ 640
EU931691.seq    561 ........................................................................................ 640
EU931692.seq    561 ........................................................................................ 640
EU931693.seq    561 ........................................................................................ 640
EU938324.seq    561 ........................................................................................ 640
Isolate1.seq    561 ........................................................................................ 640
Isolate2.seq    561 ........................................................................................ 640
```

```
AJ320166.seq    641 CCCGACCGGAATACCGAGCTCAGCTACCAGTACATGCTGGCCGAC 685
AJ459805.seq    641 ................................................ 685
AY581274.seq    641 ................................................ 685
AY581295.seq    641 ................................................ 685
AY581296.seq    641 ................................................ 685
AY581297.seq    641 ................................................ 685
AY581298.seq    641 ................................................ 685
AY581299.seq    641 ................................................ 685
EU177546.seq    641 ................................................ 685
EU847626.seq    641 ................................................ 685
EU931690.seq    641 ................................................ 685
EU931691.seq    641 ................................................ 685
EU931692.seq    641 ................................................ 685
EU931693.seq    641 ................................................ 685
EU938324.seq    641 ................................................ 685
Isolate1.seq    641 ................................................ 685
Isolate2.seq    641 ................................................ 685
```

Fig 4.2. Phylogenetic analysis of various FAV-4 isolates based on nucleotide sequences of hexon gene

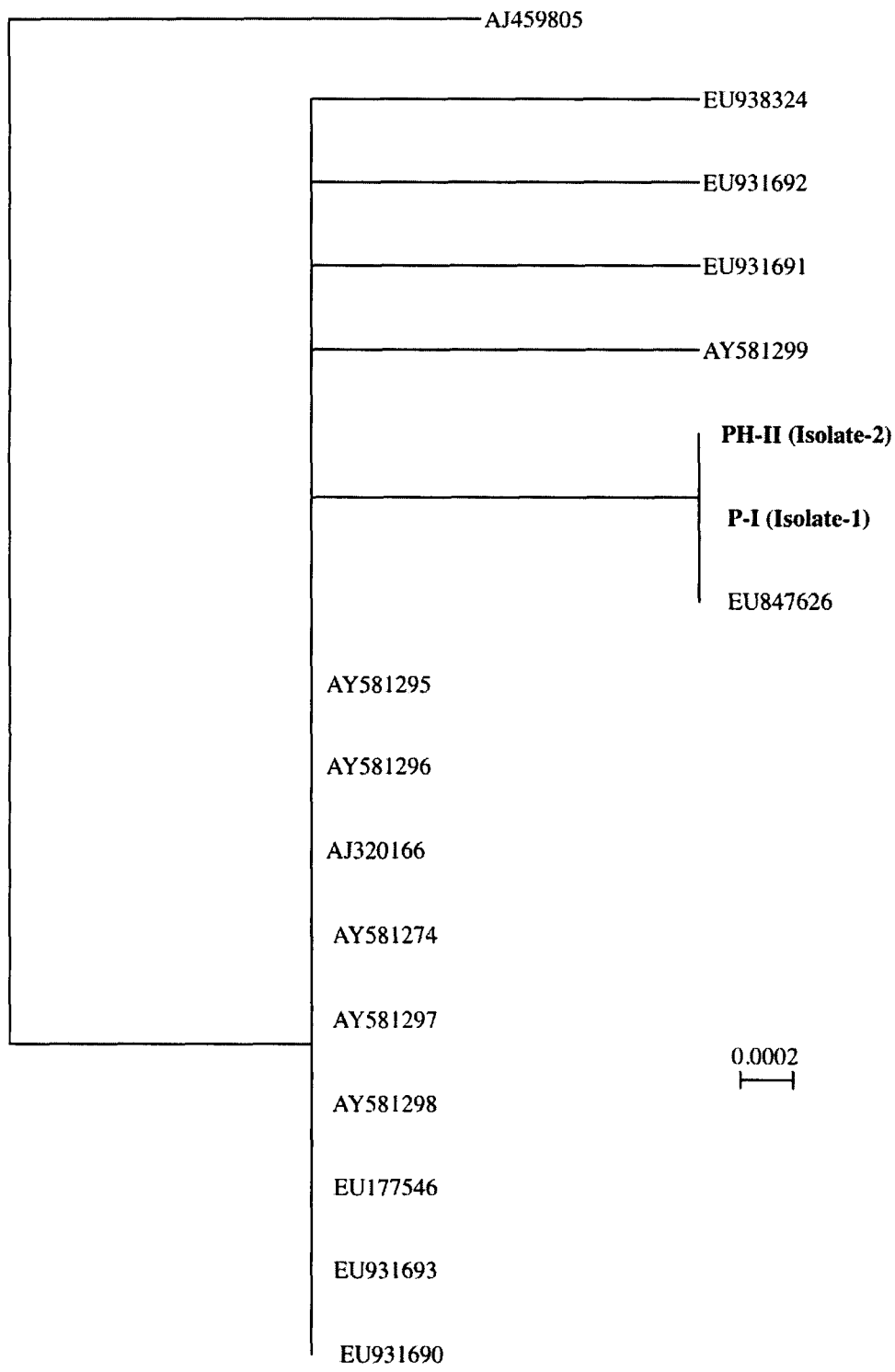

Fig 4.3. Multiple sequence alignment of deduced amino acid sequence of hexon gene of FAV-4

```
                1       10        20        30        40        50        60        70        80        90       100       110       120       130
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
          P-I   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTAAQVTKISGVFPNPNQGPGRNPLRRVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD
         PH-II   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTAAQVTKISGVFPNPNQGPGRNPLRRVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD
      EU847626   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTAAQVTKISGVFPNPNQGPGRNPLRRVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD
      EU177546   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTAAQVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD
      AJ320166   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTAAQVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD
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      AY581299   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTAAQVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD
      EU931690   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTAAQVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD
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      AY581297   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTAAQVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD
      AY581298   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTAAQVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD
      EU931692   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTAAQVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD
      EU938324   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLPNVYTNTSTSKDTTAAQVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD
      EU931691   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTAAHVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD
      AJ459805   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTAAQVTKISGVFPNPNQGPGRNPLRQVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD
    Consensus   KGILDRGPSFKPYCGTAYNPLAPKESMFNNWSETAPGQNVSASGQLSNVYTNTSTSKDTTAAQVTKISGVFPNPNQGPGRNPLRqVENANTGVLGRFAKSQYNYAYGAYVKPVAADGSQSLTQTPYWIMD

                131     140       150       160       170       180       190       200       210       220     228
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+-------|
          P-I   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
         PH-II   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      EU847626   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      EU177546   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      AJ320166   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      AY581274   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      AY581299   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      EU931690   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      EU931693   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      AY581295   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      AY581296   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      AY581297   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      AY581298   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      EU931692   NTGTNYLGAVAVEDYTNSLSYPDTVVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      EU938324   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      EU931691   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHDSGVCSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
      AJ459805   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHVSGVYSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
    Consensus   NTGTNYLGAVAVEDYTNSLSYPDTIVVPPPEDYDDYNIGTTRALRPNYIGFRDNFINLLYHdSGVcSGTLNSERSGMNVVVELPDRNTELSYQYMLAD
```
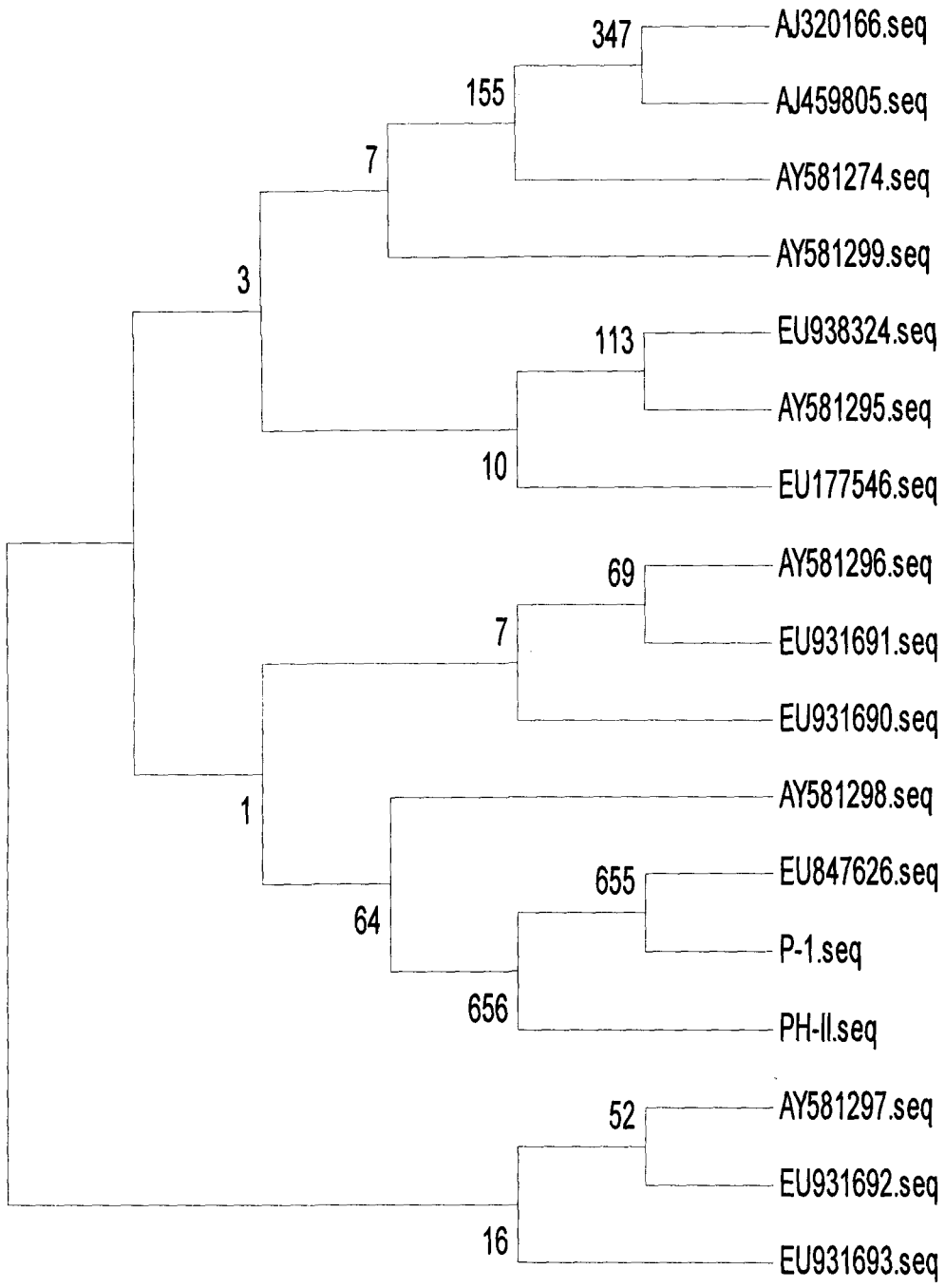

Percent divergence			Percent similarity																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
			P-I	PH-II	EU177545	EU847626	AJ320166	AY581274	AY581298	AY581297	AY581296	AY581295	EU931693	EU931690	EU177546	AY581299	EU938324	EU931692	EU931691	AJ459805
	P-I	1	X	100	98	100	99	99	99	99	99	99	99	99	99	99	99	99	99	99
	PH-II	2	100	X	98	100	99	99	99	99	99	99	99	99	99	99	99	99	99	99
	EU177545	3	96	96	X	98	98	98	98	98	98	98	98	98	98	98	98	98	98	97
	EU847626	4	96	96	99	X	99	99	99	99	99	99	99	99	99	99	99	99	99	99
	AJ320166	5	99	99	99	98	X	100	100	100	100	100	100	100	100	99	99	99	99	99
	AY581274	6	96	96	99	98	100	X	100	100	100	100	100	100	100	99	99	99	99	99
	AY581298	7	99	99	99	98	100	100	X	100	100	100	100	100	100	99	99	99	99	99
	AY581297	8	99	99	99	98	100	100	100	X	100	100	100	100	100	99	99	99	99	99
	AY581296	9	99	99	99	98	100	100	100	100	X	100	100	100	100	99	99	99	99	99
	AY581295	10	99	99	99	98	100	100	100	100	100	X	100	100	100	99	99	99	99	99
	EU931693	11	99	99	99	98	100	100	100	100	100	100	X	100	100	99	99	99	99	99
	EU931690	12	96	96	99	98	100	100	100	100	100	100	100	X	100	99	99	99	99	99
	EU177546	13	96	96	99	98	100	100	100	100	100	100	100	100	X	99	99	99	99	99
	AY581299	14	99	99	99	98	100	100	100	100	100	100	100	100	100	X	99	99	99	99
	EU938324	15	96	96	99	98	100	100	100	100	100	100	100	100	100	100	X	99	99	99
	EU931692	16	99	99	98	98	99	99	99	99	99	99	99	99	99	99	99	X	99	99
	EU931691	17	95	95	98	98	99	99	99	99	99	99	99	99	99	99	99	98	X	99
	AJ459805	18	95	95	98	97	98	98	98	98	98	98	98	98	98	98	98	98	98	X

Table 4.41. Percent homology of FAV4 hexon protein gene at nucleotide level (above diagonal) and percent amino acid similarities (below diagonal) among different FAV4 isolates using Clustal W

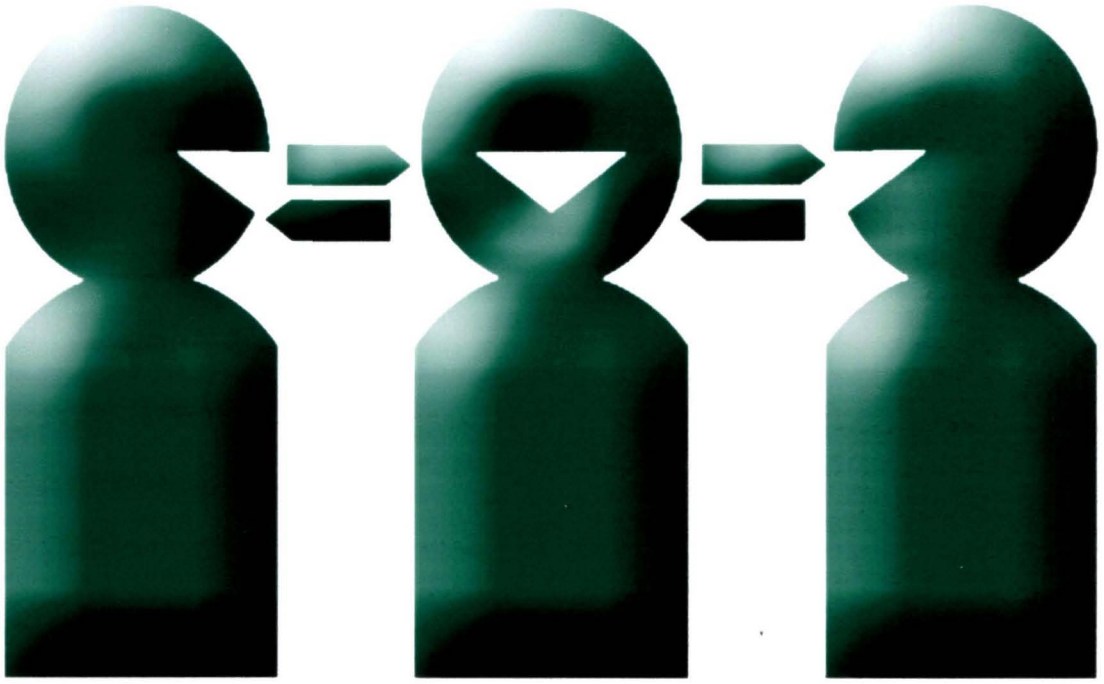
Isolates used for analysis in the study are : P-I: FAV 4 Palampur, **PH-II**: FAV 4 Palampur, **EU177545**: FAV4 Uttrakhand, **EU847626:** FAV4 Hissar, **AJ320166:** FAV4 Turkey, India, **AY581274:** FAV4 Quail, Izatnagar, **AY581298:**FAV4 Nepal, **AY581297:** FAV4 Bangalore , **AY581296: FAV4** Isolate 608, **AY581295:** FAV4 Isolate 488, **EU931693:** FAV4 Uttarakhand, **EU931690 :**FAV 4 Isolate PB-08, **EU177546:** FAV4 RC hexon, **AY581299:** FAV4 GC hexon, **EU938324:** FAV 4 PP-01 hexon, **EU931692:** FAV4 PJ-06, **EU931691:** FAV4 PB-05 , **AJ459805:** FAV 4 Hexon Indian

Fig 4.4. Phylogenetic analysis of deduced amino acid sequences of FAV-4 isolates



Phylogenetic relationship of FAV-4 infecting birds on the basis of hexon protein amino acid sequence with other FAV-4 isolates. Branch lengths are proportional to the number of inferred character state transformation. Vertical branches are arbitrary. Group designations on the right reflect major grouping identified in the analysis. Bootstrap values (% replication) are shown on the major branches

Discussion



Chapter V

DISCUSSION

Hydro pericardium syndrome (HPS) is an important disease in poultry causing heavy economic loss through high mortality (10 to 70 %) in broiler chicks of 3 to 6 weeks of age. The disease was reported for the first time from Pakistan (Khawaja *et al.,* 1988a; Jaffery, 1988). Gowda and Satyanarayana (1994) reported the disease for the first time in India with involvement of the liver that led to closure of many poultry farms in the region. The disease manifestation includes a characteristic hydropericardium, with the heart simulating a de-shelled litchi fruit (hence the name "litchi disease") and hepatitis with a basophilic intra-nuclear inclusion bodies in hepatocytes, with a mortality rate of 10 to 70 per cent. The study group on poultry diseases (Anonymous, 1996) had identified IBH-HPS as an emerging problem of the broiler industry in India.

Tentative diagnosis of the disease is based on characteristic gross lesions and histopathology. Though serological tests like gel diffusion, counter immunoelectrophoresis and enzyme linked immunosorbent assay (Oberoi *et al.*, 1996) are considered to be reliable, a confirmatory diagnosis can only be made based on isolation and identification of the virus (Chandra *et al.*, 1997).

Poultry is a fledgling business enterprise in the state and outbreaks such as those due to HPS can lead to heavy damage and subsequent closing down of many small poultry enterprises. Characterization of FAVs prevalent in a geographical area is necessary to develop any effective control strategy.

Although some preliminary work on HPS in Himachal was done by Asrani *et al.* (1997) and Chand (2006) with these isolates, the characterization of the isolate remained to be done. The typical geographical location and difficult weather conditions in Himachal Pradesh offers varied conditions for the host as well as the pathogen. Animal disease outbreaks in Himachal are not new and the presence of difficult terrain and climatic conditions along with a host of other factors makes the diagnosis of the disease even more challenging. Though considerable work has been done on fowl adenovirus in some parts of the country, yet it is not sufficient to meet the requirements to control the diseases. Characterization of FAVs prevalent in a geographical area is necessary for evolving any vaccine based control strategy factors associated with the disease in general and the pathogen in particular for better understanding of control strategies to be implemented in case of future outbreaks.

This study was particularly intended to characterize the local isolates. Antibodies against FAV have been detected even in healthy chickens (Yates *et al.*, 1976; Cook, 1970; McFerran and Adair, 1977). Therefore, detection of FAV antigens at tissue level is more relevant in the diagnosis of infection than quantification of its antibodies in serum. HPS being an acute disease hardly allows the development and detection of antibodies in serum in case of a natural outbreak. Further, the sensitivity and specificity for detection of various commonly used serological tests needs to be studied to know the suitability of different tests in case of an outbreak. Though older traditional and conventional methods have their own place in diagnosis, but the lack of sensitivity or specificity or both can hinder diagnosis in any given situation.

With the diagnostic techniques available for the detection of the adenoviral agents such as the immunodiffusion test, enzyme linked immunosorbent assay (ELISA), neutralizing test and electron microscopy, some have been reported to have low sensitivity and/or specificity and they are time consuming and laborious (Raue and Hess, 1998). Thus, diagnosis of *Aviadenoviruses* is based mainly on post mortal material and histopathological lesions on the affected organs and tissues of the affected host (Parthiban *et al.*, 2004).

Despite the serological relationship of *Aviadenoviruses* that has been used as a characteristic tool, there has been growing interest to genetically characterizing these viruses. Molecular techniques are used frequently these days and have the added advantage that even small amounts of material can be used for diagnosis. But, they have their own problems with rigorous standards and finances involved to be put on place for optimum results. So, it is always imperative to strike a balance between old and new techniques and to offer the best suitable sensitive and specific technique that is quick to perform, gives appreciable results and is easy on finances.

Most of the molecular based diagnostic progress used so far has been polymerase chain reaction (PCR) and restriction enzyme analysis (Meulemans *et al.*, 2001; Raue and Hess, 1998). Employing restriction enzyme analysis by digesting extracted DNA with *Hind* III and *Bam* HI and has resulted in differentiation between the *Aviadenoviruses* (Raue and Hess, 1998). The thrust of adenoviral research has been based mainly on the major coat proteins and it has been found that the hexon gene together with the fiber protein contains the type, group, subgroup antigenic determinants and also that the hexon gene loop regions show high variability (Raue *et al.*, 2005).

With all this information, this brought about an interest to investigate a molecular based test, particularly PCR as a diagnostic tool for detecting FAV-4.

Perusal of the available literature revealed that in India, very few attempts were made to characterize the structural and immunogenic proteins of FAV-4. The genetic makeup of the pathogen also needs to be looked into to pinpoint the origin of this virus in the state. Therefore, the present study was undertaken with the view to identify and characterize structural proteins of FAV-4 isolates recovered in the outbreak of HPS in the state and to investigate possible variation in polypeptide composition if any, and their immunogenic proteins among these virus isolates. The genetic makeup of the organism was also looked into to find some association with other FAV-4 isolates in the country.

In the present study, an attempt was therefore made to find out the suitability of some routine serological tests for detection of fowl adenovirus antigens in the infected birds so as to develop a method for rapid diagnosis of this disease in the near future. Though the CEL and CEK have been found suitable for growing the HPS agent by many workers, an attempt was made to assess the suitability of various cell lines for the isolation of HPS agent as alternative.

The isolates exhibited typical signs of hydropericardium in birds. The main pathological finding was the accumulation of a clear, straw coloured fluid in the pericardial sac along with the enlargement of the liver. The disease can be therefore called hydropericardium syndrome (HPS; Cheema *et al.*, 1989).

5.1 Cell cultures

Different systems have been put in place for isolating viruses from various sources. The systems entail the use of certain techniques which are dependent on the final achievement whether it be for diagnostic purposes or the development of vaccines.

These systems are cell cultures, embryonated eggs or intact animal. All of these have certain advantages, for instance cell cultures are considered a more sensitive medium than the other two due to the fact that they allow for primary isolation of viruses. Also, they have cells that are equivalent to the host, meaning the culture system is made up of more or less identical cells with respect to virus susceptibility and physiological conditions. Cell cultures require less space and the manifestation of CPE is an indication of virus growth, (Burleson *et al.*, 1992; Hoskins, 1967).

The purpose of this study was to adapt the fowl adenoviruses isolated from outbreaks of HPS in the state in different types of cell cultures. Though research done prior had found that FAV-4 belongs to the group I *Aviadenovirus* and can be isolated in CEL and CEK cell culture systems and embryonated eggs (Hess, 2000; Raue *et al.*, 2005), yet, it was imperative to study other additional cell cultures and other continuous cell lines to know the growth pattern of the virus.

5.1.1 Primary cell cultures

Primary cells are harvested directly from an organism and can be grown for several weeks *in vitro* in specialized cell culture medium before the cells undergo senescence. Primary cells are especially sensitive to chemicals, toxins, and viruses including influenza

virus e.g primary CEK, (Ona *et al.*,1995) and Eastern equine encephalitis virus (primary CEF) and are often used for various research and industrial applications.

Culture systems are said to have a clear advantage in the isolation of FAV-4 and so due to this information, it was decided to use cell cultures, preferentially CEK or CEL cells that have been known to support the growth of the virus and compare it with the adaptation of the virus in CEF cells. Primary cultures are quite tricky to work with as they have a limited lifespan and long-term experiments cannot be achieved with these cultures (Burleson *et al.*, 1992).

The establishment of primary cell culture systems proved to be quite interesting and challenging in our study as there is no company that supplies primary chicken embryo kidney (CEK) or chicken embryo liver (CEL) cells in India, thus the cells had to be made in-house. Primary cultures should be from a homologous species for successful virus isolation. Or at least CEL cell culture should be used in the case of group I *Aviadenovirus*es if successful cultivation of the virus is to be achieved (Hess, 2000). Hess (2000) also reported that fibroblast cell cultures gave inconclusive results when cultivating this group of viruses.

5.1.1.1 CEF cell culture

Investigators have used these cells to assay the infectivity of a strain of H5N2 (Lee, *et al.*, 2005) and test the antiviral properties of several antiviral compounds (Conti and Portincasa, 2002; Serkedjieva, 1995).

Recently successful propagation of Psittacine Adenoviruses (PsAV) in psittacine embryo fibroblasts (PsEFs) was reported (Lüschow *et al.*, 2007). Unfortunately, PsEFs are inconvenient as there is a limited supply of the psittacine embryos where only a few eggs

are laid yearly and they are very expensive. This prompted our study to see whether CEF cultures supports the growth of the HPS agent, FAV-4.

Normally, CEF cells can be grown by two methods *i.e.* the warm trypsinization method and the cold trypsinization method (Freshney, 2000). We used the warm trypsinization method as described by Freshney, 2000 for initiating the culture. The cold trypsinization method was also used and that also led to consistently good results. However, cold trypsinization requires overnight holding of the tissues in trypsin solution in a refrigerator that was somewhat time consuming, and so the warm trypsinization method was preferred because of its rapidity. An average CEF culture took 24 to 48 hrs. to establish and many culture flasks could be initiated with the initial inoculum as 1×10^4 cells were required to initiate a culture.

Hess (2000) reported that fibroblast cell cultures gave inconclusive results when cultivating this group of viruses. According to Mcferran and Adair (1977), CEF cells are less sensitive for the growth of avian adenoviruses. Miller *et al.,(*1972); Oxford and Potter, (1969); Butler *et al.*, (1972) also did not recommend CEF for the growth of adenoviruses because of their less sensitivity to adenoviruses than the CEL and CEK cell cultures.

Our study is in agreement with the above authors as virus specific CPE could only be detected at sixth passage level in CEF. However, when the virus adapted to the CEF cultures, routine and increased severity in the changes could be detected. The virus after adaptation in CEF cells was passaged up to tenth passage with increase in severity of CPE in each passage level as well as decrease in initiation time. Available literature does not record any attempt to grow the FAV-4 in CEF cells. Kraft and Tischer (1978) however, could record CPE in CEF cells after infecting them with CELO virus, a group I avian

adenovirus. Our study also records CPE with FAV-4 another group I *Aviadenovirus*. However, this investigation is in variance with the results reported by Kraft and Tischer (1978). They reported that the propagation of CELO virus (a group I *Aviadenovirus*) employing confluent monolayers of CEF yielded virus titers one to two logs lower than those from confluent chicken kidney (CK) cells. But, in our study, we could find increase in titer of the viruses by one or two log units after passage in CEF cells. Detection of the virus in the supernatant of the CEF was found out by PCR. The only advantage recorded for the propagation of this virus in CEF cells was the very less number of embryos (*i.e.* one or two) and less amount of serum required for the establishment of a monolayer. The quicker establishment of the monolayer in case of CEF cells were of some advantage and the cells could be sub cultured for at least one to two more generations making the whole process less costly and more time saving. Fibroblasts were also found to be hardy cells and divide rapidly requiring minimum amount of serum and are less fastidious in their nutrient requirements. The lack of high sensitivity of CEF cells for the virus could be explained because of the natural affinity of the avian adenoviruses for rapidly growing epithelial cells preferentially over fibroblastic cells. The adaptation of the virus in fibroblast cells shows that the virus has the potential to adapt itself to a new cell type and produce changes. The sudden change of this isolate from a comparatively innocuous form to a highly virulent form capable of causing a large epidemic with sudden death and high mortality has been traced to the presence of new proteins in the structure (Balamurugan *et al.*,1999). These finding therefore suggest that changing viral structures may allow them to adapt to fibroblastic or somatic cells.

5.1.1.2 Chicken embryo liver cell culture

Primary cultures should be from a homologous species for successful virus isolation. Or at least CEL should be used in the case of group I *Aviadenovirus* if successful cultivation of the virus is to be achieved (Hess, 2000).The cultivation of the HPS virus were attempted in chicken embryo liver cell cultures. CEL cells were made using livers from embryos of 14 days old embryonated eggs as described earlier. Once the cells were confluent (cells accumulating surface growth area) they were inoculated with FAV isolates. The virus was left to propagate and the virus should have replicated inside the nucleus of the liver cells around the third day that would be represented by a CPE. No CPE could be recorded in the first passage in both the cell cultures with any of the isolates. However, by the second passage, the virus produced characteristic cytopathic changes in chicken embryo liver cell culture.

Though characteristic CPE were seen in the CEL culture, by all the three isolates, the effects were not immediate. Hess *et al.* (1998) reported the rounding of cells and detachment of cells to be typical of *Adenoviruses* infection in cell cultures. Also, if one compares the control with the infected cells a visible detachment of some cells from the surface of the flask can be visualized when looking at the overall cell surface.

The CPE were characterized by rounding and shrinkage of cells, aggregation and degeneration of monolayer. Many authors (Kanwar, 2008; Balamurugan, 1999; Barua and Rai, 2003) have described the effects on CEL culture as characterized by rounding, detachment and ultimately total sloughing of the monolayer though the severity and time interval required to develop the lesions reported by various authors varied. Liver cells being primary target cells for the virus to multiply *in vivo* were expected to show

characteristic CPE. The virus got adapted to the *in vitro* system quickly and started to show characteristic CPE. Our study is in slight variance with the work done by Kanwar (2008) who observed cytopathic changes from the first passage itself, while Barua and Rai (2003) reported that FAV-4 virus produced CPE in infected CEL cell culture from third passage onwards. The CPE appeared after 48 hrs. and were characterized by swelling and rounding of infected cells. Complete detachment of the monolayer was observed 96 hrs. p.i.

Similar CPE in CEL were recorded in the present study. Our study is also in accordance with the CPE recorded by Kumar *et al.* (2004) who isolated and adapted FAV in CEL cell culture and observed CPE characterized by cell rounding and degenerative changes within 24 hrs. Similar changes like rounding and degenerative changes in CEL cell cultures have also been recorded by Oberoi *et al.* (1996) and Khawaja *et al.* (1988a), who however reported the changes after 3 to 4 days of infection. Naeem *et al.* (1995a) also reported that the HPS agent could be propagated in CEL cell cultures. Hess *et al.* (1998) reported slightly differently with CPE changes evident in his study only at 6^{th} day post inoculation. Chandra *et al.* (2000) reported cultivation of FAV-4 in primary cell cultures of CEL cells. Some other investigators also used primary chicken liver cell cultures for adaptation and cultivation of HPS virus (Kamal *et al.*, 2002; Dahiya *et al.*, 2002 and Gupta *et al.*, 2005). Helena *et al.* (2006) used chicken hepatoma cells of the CH-SAH cell line for isolation of fowl adenovirus and observed CPE by light microscopy.

All of these authors reported similar CPE and concluded that the virus adapts well in primary cell culture of chicken origin particularly liver cell culture.

Establishment of the CEL culture was found to be costly as it required more number of eggs and also higher concentration of serum (20 per cent) for initialization of the culture. The maintenance medium also had to be replenished with relatively higher concentration of serum (5 %). No subculture was possible as the cells were very fragile and were damaged by low amounts of trypsin. Vigorous pipetting or shaking or even slight delay reduced the chances to establish a culture to very low levels. The fastidiousness and the relatively large amounts required for establishing a monolayer makes the establishment of chicken embryo liver cell cultures a time consuming and costly venture.

The rapidity and the extent of damage seen in cell cultures in the first passage level were however not as fast as seen in an *in vivo* system. This could be explained by the presence of many hitherto unknown additional factors present inside the body of the host that aid in the rapid replication of the virus and the subsequent presence of enormous numbers of new liver cells to infect. However, in the subsequent passages, the virus produced quicker changes in the cell culture also that were similar to the lesions seen in infected birds. The virus normally produces lesions in birds 48 to 96 hrs. p.i and in some cases even death was recorded within 24 hrs. This is interesting that whereas the virus could produce death in a live animal, it failed to initiate rapid changes in the same period in the liver cells that are supposed to be site of viral replication *in vivo*.

5.1.1.3 Chicken embryo kidney cell culture

In our study, CEK cell cultures were also used for the propagation of the virus. Though no CPE were seen in the first passage, CPE similar to the changes observed in CEL culture were recorded from the second passage onwards. Toro *et al.* (1999) observed

CPE in CEK cell cultures. They observed round refractile changes in CEK cell cultures after 24 hrs. of inoculation. Though some authors like Higashihara *et al.* (1983) and Swain *et al.* (1993) have documented CEK to be characteristically less sensitive to CEL cultures for the growth of FAVs, no lack of sensitivity in CEK cultures was observed in the present investigation. CEK cultures responded well for the adaptation of fowl adenovirus and the relative ease of initiating a monolayer culture of chicken kidney cells as opposed to chicken liver cells almost outweighed the reported lack of sensitivity associated with kidney cultures.

The CEK cell cultures were established from 18 days and above aged embryos. The establishment of CEK cell cultures took relatively less time and the complete monolayer in case of CEK could be established within 96 hrs. post seeding. The signs of CPE were visible in the CEK monolayers from 2^{nd} passage onwards. Characteristic shrinkage and rounding could be seen in the monolayers. The passaged virus when inoculated into newer CEK monolayers produced CPE rapidly, thus establishing the affinity of the virus for the renal epithelial tissue and the proliferation cum adaptation of the virus to this type of epithelium *in vitro*. The CPE were characterized by rounding and clumping of cells, followed by depletion of the monolayer at many places followed sequentially by complete degeneration and destruction of the monolayer.

All the viral isolates used in the study were passaged five times in the chicken kidney monolayer culture. The average time required for destruction of the complete monolayer of CEK cells was 72 hrs. Gupta *et al.* (2005) studied a field isolate of HPSV, recovered from an outbreak in one week old broiler chicks, and adapted in CEK cell culture by successive passaging up to the 10^{th} passage. They reported a titre of 6.5 log 10

TCID50/ ml in the pooled virus harvest at 10^{th} passage. The virus titers recorded for all the isolates at the 5^{th} passage level after passage in CEK cell cultures in our study were $10^{7.6}$, $10^{7.5}$ & $10^{6.5}$ respectively, which is in accordance to the work done by the earlier authors.

Most isolations of fowl adenoviruses have been done in CEK cell cultures (Khanna, 1964; Kawamura *et al.*, 1964; McFerran *et al.,* 1972; Taylor and Calnek, 1962). Although some workers have found that CEL cells are superior to kidney or lung cells (Sharpless *et al.*, 1958), experience in our laboratory has shown little difference.

5.1.2 Cell lines

Cell lines are cancerous cells that are immortal. They have the ability to grow rapidly in culture and generations of subculture can be initiated from these cell lines indefinitely and they retain the property to grow rapidly. The propagation of FAV-4 in continuous cell lines can be advantageous because it avoids the problems associated with the use of primary cell cultures or embryonated eggs. The quality of continuous cell line is more stable than primary cell cultures and the use of a continuous cell line is less laborious and need less preparatory steps as compared to establishment of a primary cell culture.

Four cell lines were used in the present study. The cell lines were chosen keeping in view the extent and frequency of use of these cell lines in a normal virology laboratory. The additional factors leading to this choice was that these cell lines are less fastidious in their growth requirement and are a mixture of both fibroblastic and epithelial cells. These cell lines are easy to procure, easy to grow and adapt well in a virology laboratory with minimum facilities.

5.1.2.1 Vero cell lines

Vero cells are lineages of cells used in cell cultures. The Vero lineage was isolated from kidney epithelial cells extracted from an African green monkey (*Cercopithecus aethiops*). The lineage was developed by Yasumura and Kawakita (1963). The original cell line was named "Vero" after an abbreviation of "Verda Reno", which means "green. It was observed that Vero cell lines were easy to grow and maintain and could be established relatively quickly leading to faster study time between subcultures. On an average complete monolayer in case of Vero cells got established with 48 hrs. No CPE could be detected in the first five passages after infection by virus isolates. However, at the sixth passage level, evidence of CPE were seen that included shrinking and rounding of the cells. Roy *et al.* (2001) observed CPE in Vero cell lines which were characteristic of CPE produced by adenovirus. They observed CPE from fifth passage level at 96 hrs. of incubation. Kanwar (2008)too reported the detection of CPE in Vero cell lines at fifth passage level at 120 hrs. The CPE was characterized by rounding and clumping of affected cells into regular clusters resembling " bunches of grapes" while uninfected cells did not show any changes. This study is in slight variance to the study carried out by Kanwar (2008) and Roy *et al.* (2001) as we detected CPE at sixth passage level as compared to the detection of CPE by above authors from fifth passage onwards. The study is however in agreement as regards the appearance of CPE recorded by the above authors as similar CPE could be recorded in this study also.

Buxton and Frazer (1977) reported that the CPE of adenovirus may be very characteristic, with rounding and clumping of affected cells into regular clusters resembling "bunches of grapes". The results of present study are in accordance to their findings. The

viruses were passaged in Vero cell lines for two more passages *i.e.* the eight passage level. There were rapid changes leading to degeneration of the monolayer within 72 hrs. The titration of all the three isolates after the 8^{th} passage level showed an increase in virus titer.

More serial passages would be required in future for better adaptation of the virus to this cell line with consistent and regular CPE.

Attempts were also made to adapt the isolates in HeLa cell lines (human cervical cancer cell line), McCoy cell lines (human/ mouse epithelium?) and BHK-21 cell lines (Baby hamster kidney cell lines).

5.1.2.2 HeLa cell lines

A HeLa cell is an immortal cell line used in scientific research. The cell line was derived from cervical cancer cells taken from Henrietta Lacks. HeLa cells grew into a complete monolayer after 60 to 72 hrs. of incubation in our study. The split ratio recommended by NCCS, Pune was adequate in sub culturing the virus. One problem that we encountered in the subculture was the detachment of the cells from the substrate. HeLa cells took more than 15 to 20 minutes to round up and loosen from the substrate on addition of TVG and incubation in the incubator with frequent shaking. In the present study, attempts to adapt fowl adenoviruses in these cells were made. A total of eight blind passages were made in HeLa cells, but CPE could not be detected. Some authors (Winters and Russel, 1971) have grown human adenoviruses (*Mastadenoviruses*) in HeLa cells with appearance of crystalline formations.

An interesting finding that was seen in the present study was the detection of fowl adenovirus DNA from HeLa cells at 4th and 5th passage level. No gross cytopathic changes were noticed however in the cultures. The viral DNA could not also be detected in the subsequent passages. To exclude any doubt, the process was replicated twice and both times the amplification was successful. We cannot put forward a reason for the detection of fowl adenovirus DNA from HeLa cell cultures.

The non adaptability of the fowl adenoviruses to these cell lines suggests that may be other intrinsic factors play a role in attachment of viruses to certain cells.

Other than the factor of origin of the mammalian and avian adenoviruses, the finding that viral DNA could be detected in 4th and 5th passage level shows that the virus at some point of time could be detected by diagnostic tools but not macroscopically. This finding needs future investigation.

5.1.2.3 McCoy cell lines

The McCoy cell line was derived in 1955 from cells of synovial fluid obtained from the knee joint of a patient with degenerative arthritis (Pomerat *et al.*, 1957). The McCoy cell line is widely applied in medical research including culturing of microorganisms like *Chlamydia* (Black, 1997), viruses (Nogueira,1998), trichomonads (Garber *et al.*,1987) and *Helicobacter pylori* (Xia *et al.*, 1999). McCoy cells were found to be slow to grow in our laboratory and the split ratio suggested by NCCS, Pune was found to result in slower growth rates. When the split ratio was lowered than the recommended level, quicker growth rates were achieved.

Fowl adenoviruses were passaged eight times in McCoy cell lines. No evidence of CPE was seen after eight passage levels. No record exists in the literature of previously employing McCoy cell lines for the adaptation of fowl adenoviruses.

5.1.2.4 Baby hamster kidney cells (BHK-21)

BHK-21 cells established by Macpherson and Stoker (1962) are now widely used for the production of vaccine, like Foot and mouth disease vaccine since they were proved to grow in suspension culture system and to have a good property for antigen production (Capstick *et al.*, 1962; Capstick *et al.*, 1969). The BHK-21 cell lines were used in the present study for the propagation of FAV-4 and were found similar in their growth and other morphological characters to CEF cell cultures. Growth was rapid and the complete monolayer formed in 48 hrs. Split ratio suggested by the forwarding institute NCCS, Pune was adequate to get good growth rates. The BHK-21 cells grew rapidly and could be sub cultured with ease.

However, all attempts to adapt the fowl adenoviruses to BHK-21 cells met with failure. No CPE or virus isolation was possible from these cells after eight passage levels.

5.1.4 Embryonated eggs

For those diagnostic laboratories with limited resources preventing the use of cell cultures, embryonated eggs can be advantageous. According to literature, for primary isolation of Type I *Aviadenovirus*es (presently known as group I *Aviadenovirus*es) in embryonated eggs, the yolk sac route is the most sensitive although some of the other routes have been successful for certain other strains of *Aviadenovirus* (Cowen, 1988;

Cotten *et al.*, 1993; Hess, 2000). whereas in embryonated eggs, secondary tests usually have to be administered in order to confirm viral growth unless they are pock forming viruses or cause mortality of the embryo and this is applicable to only certain viruses, not all.

The virus isolates were passaged thrice in embryonated eggs to see the mortality pattern as well as the intensity of the lesions produced in embryos. It was found that the maximum mortality across three passages were recorded at 4 DPI or 96 hrs after infection. The yolk sac route inoculated chicken embryos showed haemorrhages on the body. The changes observed in developing chicken embryos with increasing mortality due to serial passaging indicated adaptation of the FAV via yolk sac route. Mazaheri *et al.* (1998) reported high embryo mortality when HPSV was inoculated via yolk sac route. Kumar *et al.* (1997) reported that the HPSV inoculated embryos showed stunted growth evident from decrease in length, width and weight. Grossly, the embryo showed haemorrhages on the body and liver. The FAV isolates adopted well in the developing chicken embryos by yolk sac route. The results of the present study confirm the findings of Kaur *et al.* (2003) who characterized FAV- 4 isolates from inclusion body hepatitis- hydropericardium syndrome and adopted it in the embryonated chicken eggs by yolk sac and allantoic cavity routes. They observed stunting of embryos along with subcutaneous haemorrhages and about 90 per cent mortality through yolk sac route as compared to 0 to 30 per cent via allantoic cavity route. The variation in susceptibility observed in FAV inoculated chicken embryos revealed variability in adaptation of the virus via different routes of inoculation. The chicken embryos were more susceptible to FAV when inoculated via yolk sac route compared to allantoic cavity route. Our study regarding the inoculation of embryos via yolk

sac route is supported by the work of earlier workers (Kawamura *et al.*, 1964 and Fadly and Winterfield, 1973). Shafiq *et al.* (1992) also reported cent per cent mortality and growth due to FAV in 10-day- old embryonated eggs. Similar findings were also recorded by Kanwar (2008) who recorded morality from 2 DPI to 5 DPI in inoculated embryos with peak at 3rd to 4th DPI. The changes seen in the embryonated chicks in our study were haemorrhages on the body increasing to severe congestion and stunting of the embryo and death of embryo. Many authors have confirmed this and exactly similar observations were recorded (Cheema *et al.*, 1989; Shafiq *et al.*, 1993; Naeem *et al.*, 1995a and Jadhao, 1998). Khawaja *et al.* (1988a) isolated a virus from the liver, heart and kidneys of chickens affected by HPS by inoculating embryonated chicken eggs via the yolk sac route and also in CEK cell cultures. The control embryos at particular time intervals recorded increased growth parameters than the infected groups.

5.1.5 Re-infection in chicks with virus harvested from cell cultures

An attempt was made to see the effect of adaptation of virus to cell cultures or its virulence *in vivo*. Typical hydropericardium were seen in all chicks that died at 72 hrs. post inoculation whereas, hydropericardium were not observed in chicks that had died early. Another finding in this study was that CEL and CEK adapted virus produced hydropericardium in infected chicks while CEF and Vero adapted virus produced only enlarged and hemorrhagic livers in chicks. The virus could be detected uniformly from all the organs of dead chicks of all groups by using appropriate test. The non appearance of hydropericardium in the chicks infected with CEF and Vero cell culture adapted virus is difficult to interpret because the sample size was too small to draw any conclusion, however, the regular recovery or detection of the virus from the organs of infected chicks

and the severe haemorrhages on liver produced by these viruses in the chick is definite indication that the virus remains pathogenic and retains its pathogenicity for its natural host even after adaptation in regular and non regular cell cultures.

5.2 Detection of virus

5.2.1 Detection of virus by serological methods

5.2.1.1 Experimental infection in chicks

The experimental infection in day old chicks could be produced by inoculation of 0.5 ml of virus isolates orally. Mortality started from 24 hrs. post inoculation and peaked at 96 hrs PI. Characteristic signs of hydro pericardium syndrome were seen in chicks. Balamurugan (1999) could produce the disease in birds by oral infection with FAV-4 while Cheema *et al.* (1989), Anjum, (1990) and Kumar *et al.* (1997) reported that the disease can be reproduced in broiler chicks by subcutaneous inoculation of 0.25 to 0.3 ml per bird of a 10 to 20 per cent suspension of liver homogenate from naturally or experimentally infected birds. The chicks appeared depressed and listless, sat on hunches, had ruffled feathers and closed eyes and were anorectic. Death usually followed 24 to 48 hrs. of showing such signs. Abdul-Aziz and Hasan, 1995 also reported 100 per cent mortality in day old broiler chicks inoculated with infected liver homogenate. Cent per cent mortality among the chicks after inoculation with infected liver homogenate in the present investigation also.

Our study on chicks is in agreement with Kanwar (2008) who also recorded maximum mortality starting from 2 DPI to 5 DPI with peak at 3rd and 4th DPI. In our study, mortality upto 7 days post inoculation were seen. Mortality was observed three to five days after experimental infection and birds dying less than 60 hrs. did not show any clinical

signs. Similar results were found in our study where approximately 73 per cent of the birds died within 96 hrs. post infection Muneer *et al.* (1989) reported that the chickens inoculated experimentally with HPS infected liver homogenate suspension showed anorexia, debility, dullness, ruffled feathers, greenish diarrhea, prostration and subsequently death. Borisov *et al.* (1997) observed ataxia, anorexia along with depression in natural as well as experimental infection among chicks in Russia.

Similar observations have been recorded by Asrani *et al.* (1997) in Himachal Pradesh. He observed that fast growing birds in flock were most affected by the HPS and died within 36 hrs. of assuming a characteristic posture with their chest and beak resting on the ground. The sick birds were dull and depressed with their eyelids completely closed in the later stages of the disease. Our study finds support from the findings of Kumar *et al.* (1997).

The gross lesions observed in most of the cases in our study were hydropericardium characterized by accumulation of clear of slightly straw colored fluid in the pericardial sac of the infected birds. Other lesions seen were enlargement of the liver tissue along with haemorrhages along with hemorrhagic kidneys and enlarged, mottled and grossly hemorrhagic liver. Gowda and Satyanarayana (1994) experimentally reproduced the condition in broilers by inoculating liver homogenate from infected birds, the disease being characterized by hydropericardium, with the accumulation of about 12 ml of straw colored fluid (giving the heart the "litchi fruit" appearance), a pale enlarged liver, edematous and congested lungs, pale and tumified kidneys and an icteric carcass. Other investigators have also described lesions of hydropericardium, splenomegaly, necrotic foci on the myocardium, hypertrophy of the bursa of Fabricus and poorly developed

musculature in induced cases of HPS. (Cheema *et al.* 1988; Muneer *et al.*, 1989; Abdul Aziz and Al-Attar, 1991). The lesions seen in our study were typical of HPS. Hydropericardium and enlargement of liver were the most consistent findings in our study. There was accumulation of straw colored fluid in the pericardial sac. The liver was discoloured, pale, enlarged in many cases with the edges swollen and friable. Additionally, small petechial to wide spread haemorrhages of the liver was seen in some of the cases. Kidneys were generally swollen and congested in majority of the dead birds. Our findings are totally in agreement with Kanwar (2008) and Cheema *et al.* (1989). These workers also reported the enlargement of liver with characteristic hemorrhagic changes in birds that died after being infected with HPS isolate. According to them, hydropericardium was a characteristic finding in most of the cases.

Anjum *et al.* (1989) recorded the distension of pericardial sac with approximately 20 ml of clear, straw coloured watery fluid in more than 90 per cent chicks that died of HPS along with flabby and misshapen heart. Abdul Aziz and Al-Attar (1991) revealed that 3 to5 weeks old HPS affected chicks had markedly distended pericardial sacs with clear yellowish fluid and hepatitis. They added that the liver was enlarged, friable and either dark brown, pale or yellowish and mottled with necrotic foci or haemorrhages or both. Cowen (1992) reported that HPS in chicks caused pulmonary edema, enlargement of spleen, atrophy of bursa of Fabricus, paleness of bone marrow, yellowish discolouration of body fat and intra muscular haemorrhages.

5.2.1.2 Serological tests

Detection of viral antigens in various tissues at different intervals following experimental infection with FAV-4 was studied by various serological tests.

5.2.1.2.1 Agar gel precipitation test

The presence and identity of virus from the infected livers were confirmed as FAV-4 using anti FAV-4 hyperimmune serum. Additionally, AGPT was used to confirm the presence of virus in infected cell culture supernatant. All samples from HeLa, McCoy and BHK-21 cells were negative in AGPT. From CEF cell cultures, samples showed positive reaction in AGPT from eight passage onwards, while from CEL and CEK cell cultures, samples from third passage level started showing positive AGPT reaction. This can be explained by the fact that for AGPT, a larger antigen concentration is usually required that is achieved only when the virus has adapted to a particular cell and has multiplied manifold.

By AGPT, the liver samples from birds in the experimental study dying within 3 DPI were found to be positive. Additionally, positive findings were also recorded from kidneys and heart samples of birds dying within 3 DPI. Thymus and bursa samples were also found to positive for viral antigen up to 2^{nd} DPI.

Viral antigens could also detected from CEF, CEL, CEK and Vero cell cultures by AGPT. Only liver and kidney samples from infected chicks dying following infection with cell culture adapted supernatant showed positive reaction in AGPT. Bhan (2006) could also detect viral antigens from CEL supernatants by AGPT in her study that is in agreement with our findings.

AGPT has been used to detect FAV antigen from tissues of birds by Hassan *et al.*, 1993 and Kumar *et al.*, 1997). Balamurugan (1999) found liver samples to be positive for the presence of FAV antigens from 3 to 10 DPI in his study. In kidney, viral antigens were

detected from 5 to 7 DPI in 2 weeks old and only at 5 DPI in 5 weeks old chicks, respectively. Fowl adenovirus antigens in other tissues, bursa, spleen and thymus could not be detected by this test. Our study is in slight variance with the study done by Balamurugan (1999) as both thymus and bursa samples were found to be positive for fowl adenovirus antigen in our study. While there is agreement with our study regarding the detection of viral antigen in liver and kidney, the time of detection of viral antigens is in contrast as we could detect viral antigens in our study within a very short time interval whereas, Balamurugan (1999) could record the presence of these antigens for a comparatively longer duration in his study. No detection of viral antigen from the heart was recorded by Balamurugan (1999) whereas heart samples recorded presence of antigens upto 3rd DPI.

5.2.1.2.2 Counter immuno electrophoresis

Detection of the viral antigens by counter immunoelectrophoresis elicited similar results to AGPT. The presence of viral antigen was detected from liver and kidney samples upto 4th DPI, in heart samples the presence of antigen was detected upto 3rd DPI. The viral antigen could be detected from thymus and bursa samples upto 2nd DPI by CIE. Oberoi *et al.* (1996) and Kumar *et al.* (1997) also recorded the efficacy of CIE in their study and described these tests as specific and reliable for the diagnosis of FAV antigen. Detection of viral antigen was possible from liver, kidneys, bursa and thymus in the study conducted by Balamurugan (1999) that is in agreement with our findings.

Comparative detection of viral antigens were also attempted from organ homogenates from chicks infected with cell culture adapted virus in cross infective

studies. Only pooled liver and kidney samples showed a positive reaction in AGPT and CIE while no other organ homogenates from these chicks reacted in AGPT.

However, contrasting results were obtained from heart, bursa and thymus in our study. The results were inconsistent as detection of viral antigens was possible by AGPT and CIE from organs of chicks given experimental infection while no detection was possible from the same organs when the chicks were infected with cell culture adapted supernatants.

5.2.1.2.3 Dot ELISA

Dot ELISA was used for testing infected tissues for presence of FAV antigens. It was found that Dot ELISA could detect presence of viral antigens in supernatants of cell cultures earlier than either AGPT or CIE. Similarly when tissues homogenates were subjected to virus detection by Dot ELISA, it was found that Dot ELISA could detect viral antigens much longer in the tissues than either AGPT or CIE.

Dot immunobinding assay (DIA) has been used successfully for the detection of various viral antibody / antigen system (Bode *et al.*1984). They stated that Nitrocellulose ELISA had 8 to 10 fold higher sensitivity than a conventional ELISA using polystyrene solid phase support for detection of adenoviral antigens. Nitrocellulose membranes have a strong protein binding capacity and has been used for immunoblotting of proteins. This property has been exploited by several researcher to develop DIA (Towbin *et al.*,1979, Heberling *et al.*, 1988; Furuya *et al.*,1984).

Naeem *et al.* (1995b) while studying immunosuppressive effect in chicken with PARC-1 isolate of HPS detected viral antigens in liver, spleen beyond 60 hrs P.I. by using DIA.

In our study, however, the relative efficacy of DIA and IFAT as compared to AGPT and CIE is comparable to the results reported by Balamurugan (1999). In our study, positive results in Dot ELISA was seen from infected cell culture supernatants as well as liver samples collected from infected birds. Viral antigen could be detected by Dot ELISA in liver, heart, bursa, thymus and kidney samples upto 6^{th} DPI from chicks given experimental infection. Positive results in Dot ELISA was also obtained from the above organs of chicks infected with cell culture adapted virus. Balamurugan (1999) could consistently detect viral antigens in spleen, bursa, thymus and kidney from 3 to 10 DPI and in liver from 3 to 15 DPI by dot immunoassay. This shows that Dot ELISA could detect low levels of viral antigens in tissues.

5.2.1.2.4 Fluorescent antibody test

FAT could detect presence of viral antigen in liver, heart, bursa, thymus and kidney samples upto 6^{th} DPI in experimental chicks. Viral antigen detection was also possible by FAT from samples of liver, heart, kidney, thymus and bursa of chicks infected with cell culture adapted supernatants. Our study is partly in agreement with the work done by Deepak (1998) who while studying the pathogenesis of HPS in 2 week old chickens, detected an immunoflourescent FAV antigen in the thymus, spleen and bursa from 3 to 10 DPI, in the liver from 3 to 14 DPI and in the heart upto 5 DPI. The presence of fluorescing FAV antigen was detected in liver upto 15 DPI by Balamurugan (1999) in 2

weeks old infected chicks whereas in spleen, thymus and bursa antigens were detected from 3 to 10 DPI. In spleen, viral antigen was also found to be detectable in one sample of 2 week old chicks at 15th DPI. Intense amount of fluorescing FAV antigens were detected in liver, spleen and bursa from 3 to 10 DPI and in thymus from 3 to 7 DPI in 5 weeks old infected groups. In kidney, the fluorescing antigens were detected from 3 to 7 DPI in 2 weeks old chicken.

Dot ELISA and IFAT could detect viral antigen in all organs of experimentally infected birds than either AGPT or CIE. These could be due to less tissue antigen in these organs which could not react in AGPT or CIE while they could be detected by a comparatively more sensitive test like DIA and IFAT. Balamurugan (1999) also reported inconsistent results with spleen, bursa and thymus in his study and indicated lesser sensitivity of AGPT and CIE than DIA and IFAT.

It is evident from these results that out of the serological tests used for its comparative evaluation for detection of FAV-group antigen in our studies, FAT and DIA were found to be equally sensitive as these tests could routinely detect viral antigens in various tissues quicker and for longer period.

5.2.2 Additional tests for the detection of virus

5.2.2.1 Hemagglutination test

For detecting the hemagglutinating property of the virus, RBCs of eight species of animals were used against virus isolates used in the study. The property of hemagglutination in fowl adenovirus is controversial as some authors have reported

hemagglutination while others have not found any evidence of hemagglutination. Khawaja *et al.*,(1988a) reported that the virus agglutinates red blood cells in a similar manner to adenovirus though this property has not been confirmed by other researchers. In our study, no hemagglutination was observed with any of the virus even after using RBC's from eight different species of animals *viz.* chicken, cattle , buffalo, geese, rabbit, swine, dog and fish ranging from ruminants, avian species, carnivores, omnivores and even aquatic animals. As no hemagglutination could be recorded with any of the isolates against RBC's of any species used in our study, hemagglutination inhibition test could not be done.

Though Mcferrran (1991) reported that CELO virus agglutinates rat RBC's, no such positive hemagglutination were seen in our studies. However, the hemagglutinating properties of a subgroup III adenovirus *i.e* EDS-76 virus is known and is frequently used for diagnosis. The results in our study indicates that hemagglutination is not a consistent property of fowl adenoviruses and could not be used for diagnosis.

The ability of human adenoviruses to agglutinate erythrocytes was first demonstrated by Rosen (1958). Later Rosen (1960) showed that the human adenoviruses could be grouped according to their haemagglutination properties. A first subgroup agglutinated rhesus monkey but not rat erythrocytes. A second subgroup agglutinated rat but not rhesus monkey erythrocytes and a third subgroup caused partial agglutination of rat but not rhesus monkey erythrocytes. A fourth subgroup comprised those viruses causing no agglutination of rat or rhesus monkey cells.

No haemagglutination or haemadsorption of fowl erythrocytes was found by Kawamura *et al.* (1964) for the 8 Japanese serotypes. Other workers have also failed to observe agglutination of fowl erythrocytes with FAV-1 (Burke *et al.*, 1968) and FAV-5

(Fadly and Winterfield, 1975; Rosenberger *et al.*, 1974). Krauss (1965) tested erythrocytes of several species including man, baboon, pigeon, mouse, rabbit, guinea pig, rat, pig and horse for agglutination by a FAV-1 isolate, without success.

However, although Burke *et al.* (1968) using FAV-1 (strains Phelps, EV89, C9103, GAL3 and GAL4) and Clemmer (1964) using FAV-1 (strains EV89, 93, GAL3 and GAL4) got no agglutination with sheep erythrocytes, Fadly and Winterfield (1975) were successful with FAV-1 (Indiana C) but not FAV-1 (Phelps).

A number of FAV-1 strains have been shown to agglutinate rat erythrocytes (Burke *et al*, 1968; Clemmer, 1964). Anderson *et al.* (1969b) demonstrated that haemagglutination by the Phelps (FAV-1) strain was associated with bands containing virus CsCl gradients.

5.2.2.2 Virus Neutralization

The etiology of the agents used in our study were further confirmed by doing the virus neutralization tests. The diluted virus and constant serum known as alpha-method was used for identification of the virus and finding out the neutralization index of the isolates. It was found that the P-I and the PC-III isolate were completely neutralized by the corresponding antiserum while CELO antiserum had a neutralization titre of 4.15 while the PH-II isolate had a neutralization titre of 4.63. The FAV-4 isolates were not neutralized at all by CELO antiserum. The neutralization results of PH-II isolates show that CELO and PH-II isolate could be neutralized by 4.15 and 4.63 folds of hyperimmune serum. Both Rabbani and Naeem, 1996 and Jadhao *et al.*,1997 could confirm the isolates used in their study as FAV-4 by neutralization tests using serotype specific sera.

5.2.2.3 Immunoperoxidase test

Liver impression smears and cell culture suspension were positive for immunoperoxidase test. Though immunoperoxidase test is usually done to identify antigen in tissues, the following samples were tried to detect the presence of the antigen in the tissues. Roy *et al.*(2001) found positive immunoperoxidase test when he detected FAV antigen in infected Vero cell lines. The presence of brown deposits were sufficiently diagnostic for the presence of fowl adenovirus antigen in the tissues.

5.2.2.4 Histopathology

Histopathological studies on the chicks revealed classical changes in different organs associated with hydropericardium syndrome.

In the present study, hydro pericardium was the hallmark of the disease. It is a pathological lesion resulting from the malfunction of hepatic, nephritic and cardiopulmonary system. Generally in animals, hydropericardium occurs in chronic heart failure and hydraemia e.g. in parasitemia with associated anaemia (Fraser *et al.*, 1991). Thus in animals, other than chickens, chronic cardiac disease may induce hydro pericardium. However, in HPS, the period to form hydropericardium is very short. So acute hydraemia and hypoproteinemia may occur and thus may cause hydro pericardium in chicken.

During the present investigation, microscopic changes revealed that liver damage was central to the pathogenesis of this condition. Dead birds showed hepatic necrosis with inclusion bodies. This hepatic insult results in decreased production of blood proteins, thus, decreasing the plasma colloidal osmotic pressure and making the bird vulnerable to edema (Slauson and Cooper, 1990). Alexander *et al.* (1962) had earlier reported a drastic

reduction in the total serum protein in birds affected with hydropericardium. Asrani *et al.* (1997) reported significant increase in SGPT, which may be attributed to hepatocellular damage with an alteration in cell membrane permeability and leakage of cytoplasmic SGPT into the blood (Benjamin, 1978).Therefore acute hepatic insufficiency may be the cause of death in chicken with HPS.

Hypoproteinemia may be caused by injury to glomeruli and to the tubules. During the current study, kidneys showed tumefaction and haemorrhages with distended tubules. Microscopically, there were proliferative glomerulitis, tubular degeneration and desquamation of the tubular epithelium into lumen. In glomerulonephritis, albumen passes more easily through the glomerular membrane resulting in fall of plasma osmotic pressure which leads to diffusion of fluid into the pericardial sac. Also it seems probable that a fall in blood volume stimulates the release of adrenal cortex hormone aldosterone which in turn causes increased adsorption of sodium, chloride and water by the renal tubules. Hypertension due to renal malfunction or failure results in increased cardiac output due to elevated sodium level and water retention (Qureshi, 1989). All this increases the capillary hydrostatic pressure and increases the chances of hydropericardium.

Pulmonary edema results due to heart failure. Because of the compromised pumping capacity of the left ventricle, the blood begins to dam up in the lungs. This increases the pulmonary capillary pressure resulting into extravasation of fluid into the lungs. In addition, it causes peripheral vasodilatation resulting in delivery of more blood to the heart, thus aggravating the condition (Guyton,1991).

Probably the increased hydrostatic pressure due to systemic and myocardial alteration is a predisposition for extravasation of fluid into the pericardial sac. When fluid

accumulates in the pericardial sac, the pressure increases and progressively compress the chambers of the heart. Because the right sided chambers have thinner walls than left sided chambers, it results into a condition referred to as cardiac tamponade (Tilley,1998). So, hydropericardium itself can depress the cardiac function and can result in death. Thus further studies on this aspect may be helpful in understanding the exact pathogenesis of HPS.

5.2.3 Molecular detection of fowl adenovirus

5.2.3.1 Detection of FAVs by polymerase chain reactions (PCR)

5.2.3.1.1 Detection of FAVs by group specific primers

Classical diagnostic methods for avian adenovirus have been described in detail(Mcferran, 1991). Recently, major progress in the field of avian adenovirus diagnosis was achieved in the direct detection of the relevant pathogens using molecular methods. As published for many other viruses, the method of choice.

PCR has also been used as a diagnostic tool for investigating microorganisms due to the sensitivity and specificity of the assay. Viruses are among those microorganisms, especially those belonging to a widely distributed group in which a link between the infection and a specific disease has not yet been established (Raue *et al.*, 2002). The important thing to remember when using PCR for diagnosing viruses, in this case the *Aviadenovirus* family is to be able to understand their epidemiological behaviour. The reason being is that some avian adenoviruses, like the Fowl Adenoviruses (FAV) are non-species specific and some are restricted to their hosts e.g. EDS virus (Hess, 2000).

Differentiation of all the *Aviadenoviruses* (AAVs) infecting an appropriate host may consequently be achieved by PCR. The detection of only specific AAVs, possibly a

single FAV serotype or the detection of all the AAVs is to be considered before proceeding with PCR.

Diagnosis based on PCR may take a general or specific approach. The general approach entails detection of as many as possible of the known and unknown strains of a particular pathogen. The primers designed for that pathogen must be able to hybridize the most highly conserved region of its genome. Although the primers designed may be complementary to the conserved region, they may not be universally applicatory in practice to the given pathogen. This is true for viruses with high mutation frequencies e.g. RNA viruses relative to DNA viruses. The general PCR product is usually further analyzed by restriction fragment length polymorphism (RFLP) or nucleotide sequencing.

The specific approach explains itself, where the primers designed hybridize to only the subset of strains of a pathogen. Further analysis is done when epidemiological studies are pursued and this involves sequencing, but usually no further analysis is required. Genotype, serotype and pathotype may be identified with this PCR approach (Cavanagh, 2001).

The majority of the avian adenovirus PCRs published till date took the hexon gene for primer design (Hess, 2000). The major viral surface protein of all Adenoviruses is the hexon protein. It contains three loop regions designated L1, L2 and L4 that are located at the outer surface of the hexon protein. The inner surface, combined by the L3 region comprises of the conserved pedestal (P1 and P2) regions and the outer surface (Raue and Hess, 1998). Amongst all these loop regions, L1 is the most variable with more than 130 amino acids (aa) in length. The adenoviral fibre is non covalently bound to the penton base and while the *Mastadenoviruses* comprises of one fiber bound per penton base, the

Aviadenovirus contain two fibres of variable length bound per penton base (Raue *et al.*, 2005).

Despite the serological relationships of *Aviadenoviruses* that has been used as a characteristic tool, there has been growing interest to genetically characterizing these viruses. Most of the molecular based diagnostic progress used so far has been PCR and restriction enzyme analysis (Meulemans *et al.*,2001; Raue and Hess, 1998). Employing restriction enzyme analysis by digesting extracted DNA with *Bam* HI and *Hind* III has been reported to result in differentiation between the *Aviadenoviruses* (Raue and Hess, 1998). The concentration of adenoviral research has been based mainly on the major coat proteins and it has been found that the hexon gene together with the fibre protein contains the type, group, subgroup antigenic determinants and also that the hexon gene loop regions show high variability (Raue *et al.*, 2005).

With all this information, we got interested to investigate a molecular based test, particularly PCR as a diagnostic tool for detecting *Aviadenovirus*.

PCR was used for the detection of FAV directly from the initial liver inoculums using the primer pairs H1/H2 and H3/H4 and also from cell culture passaged supernatants. The primer pair H1/H2 and H3/H4 used in the study successfully directed the synthesis of 1219 bp and 1319 base pair products, respectively. Amplification of the 1219 and 1319 bp products from the conserved regions of adenoviruses is diagnostic for fowl adenoviruses (Raue and Hess, 1998). Dahiya *et al.* 2002 also used H1/H2 and H3/ H4 primer set to amplify the FAV used in their study. PCR products amplified by these primer set and digested by *Hae* II and *Hpa* II restriction enzymes is normally diagnostic for confirmation of serotype of FAV. Singh *et al.*, 2002 performed PCR with the primer sets H1/H2 and

H3/H4 and advocated that the amplification of DNA with this primer set is virtually diagnostic for the fowl adenoviruses (FAVs). In our study, amplification of DNA of virus with this particular primer pair from variety of samples placed the virus in the fowl adenovirus group.

5.2.3.1.2 Detection of FAVs by hexon gene based PCR

All described adenoviruses share a common genome organization and architecture. The capsid consists of 252 capsomeres arranged in icosahedral symmetry. Hexons form 240 of the 252 virion capsomeres and contain type-, subgenus-, and genus-specific determinants. (Pring–Akerblom *et al.*, 1995; van oostrum and Burnett, 1985). The hexon genes showed three distinct segments, a variable middle segment, and 2 flanking, highly conserved regions. The outer shell of the virion (capsid) is composed of seven polypeptides. The hexon is a trimeric protein of polypeptide of polypeptide II and is referred to as a hexon polymer. The hexon is the major protein of the adenovirus capsid and has been shown to carry type-, group- and sub-group-specific antigenic determinants (Norrby, 1969). Sheppard *et al.* (1995) mapped, cloned and sequenced the hexon gene for FAV10, the first avian adenovirus for which the hexon gene sequence is available. It has been shown that regions responsible for the hexon structures (the pedestals) are more conserved, whereas the exposed regions (loops) are more variable. This allows avian adenoviruses to keep their adenovirus morphology identical while not sharing any common epitopes with the mammalian adenoviruses. As the hexon gene carries the major epitopes of FAV and the variable region of the hexon may distinguish FAV-4 and FAV-10 at the genomic level, the designing of the primers were such that they flanked the variable region of the virus. The two oligonucleotides, FAVHL and FAVHR, amplified a 0.7 kb

fragment and as expected, the product contained the highly variable region. PCR based amplification of the FAV-4 hexon gene (Ganesh *et al.*, 2002) from infected liver, has been proved a highly reliable and sensitive technique for the diagnosis of FAV infection but, the suitability of other tissues needs to be evaluated and validated.

Hence, the present study evaluated the suitability of hexon gene based PCR for the detection of FAV- 4 in various tissues *viz.* liver, heart, kidney, bursa, thymus, different parts of infected eggs and infected cell culture supernatants.

The 700 bp FAV-4 hexon gene specific product was amplified by PCR with DNA extracted from all tissues *viz* liver, kidney, heart, thymus and kidney from experimentally infected chicks. Additionally, PCR could amplify hexon gene specific product from liver and amnioallantoic fluid from infected eggs as well as from infected cell culture supernatants from CEF, CEL, CEK and Vero cell lines. Our study is in complete agreement with the work done by Rahul *et al.*, 2003, who could amplify the 728 bp FAV-4 hexon gene specific product by PCR with DNA extracted from all the tissues *viz.* liver, spleen, bursa, thymus and kidneys of SPF chicks dead with IBH-HPS. DNA extracted from tissues of uninfected controls was negative for the amplification of FAV-4 DNA by PCR.

5.2.3.1.3 Detection of FAVs by fibre gene based PCR

Laver *et al.* (1971) reported that the avian adenoviruses have general architect similar to mammalian adenoviruses with the differences in pentons as every penton in avian adenoviruses is penetrated by two fibres instead of one as in mammalian adenoviruses. CELO virus, an avian adenovirus possessed a penton base with two knobbed fibres, one long (42.5 nm) and other short (8.5 nm). Norrby (1971) reported

single fibre of 14 nm length and El Mishad *et al.* (1975) reported fibre of 17.5 nm long at the vertices of CELO virion. McFerran *et al.* (1975) found that the pentons have an antigen associated with group specificity and fibre has at least one type specific antigen.

PCR was used for the amplification of FAV- 4 from the same samples that were used for hexon gene fragment amplification. The results show that hexon gene specific primers were more successful in detecting FAV-4 DNA from a variety of samples. Fibre gene specific primers however could successfully amplify FAV- 4 DNA from a variety of samples. Bhan (2006) could amplify the whole fibre gene of FAV- 4 with the fibre based primers used in this study. They also successfully cloned and sequenced the 1400 bp fragment in their study.

To verify that the isolates used in our study were free from CELO virus (FAV-1) contamination, the virus inoculums used in our study were subjected to fibre gene product specific amplification of 350 bp that is specific for fowl adenovirus serotype -1 fibre. We used the reference CELO virus used in the study as a reference positive control. The fibre specific primers of CELO virus was not amplified from the three viral inoculums thus satisfying our requirement that the isolates used in the study were indeed FAV- 4 and not contaminated by FAV-1 which is also associated with hepatitis or inclusion body hepatitis in birds. Bhan (2006) successfully used these primer pairs to amplify the fibre gene region in their study. They also successfully cloned this ~350 bp fragment and sequenced the product.

5.2.3.2 Detection of virus DNA by dot blot hybridization

Dot blot hybridization is an efficient tool for detecting viruses based on DNA-DNA hybridization. To detect fowl adenoviruses in tissue samples infected with the virus, a gel purified 700 bp product specific for FAV- 4 was used as a biotinylated labeled probe. The dot blot hybridization was done from both raw tissue samples as well as DNA samples extracted from these samples. The samples were blotted onto nitrocellulose membrane after boiling the products for denaturing the DNA, allowed to dry and then cross linked with UV rays. The probe consisted of a 700 bp PCR product that was labeled with Biotin-11-dUTP. The DNA samples were hybridized with the DNA probe developed. The results show that dot blot hybridization could detect samples that were detected by PCR. DNA could be detected from all samples that were detected by PCR. Ganesh *et al.*, 2002, performed southern hybridization to confirm the specificity of amplified viral DNA. The PCR products after gel electrophoresis were denatured and transferred onto nylon membrane by the upward capillary transfer technique of Southern (1975). The immobilized DNA was hybridized with a digoxigenin- labeled 0.7 kb PCR product. PCR amplified DNA was labeled with digoxigenin (DIG -11-dUTP) using the DIG labelling kit. Their study is in direct agreement with our study.

5.3 Molecular characterization of fowl adenovirus

5.3.1 Protein profile of fowl adenovirus

The FAV-4 isolates and CELO virus were cultivated in CEL cell culture system and in embryonated chicken eggs, respectively for bulk production of virus. The embryo died after 48 hrs of post inoculation were chilled and allantoic fluid was harvested and

clarified at 4000 rpm for 30 minutes. The CELO virus was concentrated by saturated ammonium sulphate and purified through 20-45 per cent CsCl density gradient solution. Other workers (Li *et al.,* 1984a; Laver *et al.*,1971; Maiti and Sarkar,1997) have also used this method for purification of virus material through CsCl gradient. FAV-4 infected CEL cell cultures were harvested when it exhibited 80-90 % CPE. The infected cells were pelleted and extracted with trichlor triflourethane and supernatant aqueous phase containing virus was purified through 1.33-1.45 g/ml CsCl density gradient. Winter and Russell (1971), Rabbani *et al.* (1998a) have also used this method for purification of adenoviruses. However, McIntosh *et al.,* (1971) reported virus obtained by this method was still contaminated to some extent with cellular components and a further equilibrium density gradient centrifugation at 1,00,000 × g for 18 hrs was necessary. The virus band obtained at the interface of CsCl gradient was checked for its purity by taking OD 260/280 ratio. The extinction ratio of CELO, FAV-4 isolates P-I, P-II and PC-III was found to be 1.21,1.02,1.01 and 1.02 respectively, that indicated the purity of CELO virus and partial purity of FAV-4 isolates. Similar study has been done by Mishra (1997) and Swain *et al.*(1997) for Newcastle disease virus and Underwood and Brow (1974) for Rinderpest virus. For complete purification FAV-4 further equilibrium density gradient at 1,00,000 × g for 18 hrs. is needed.

Three FAV-4 isolates and CELO virus after purification and uninfected CEL cell culture fluid treated in similar manner were subjected to SDS-PAGE. The polypeptide profile of FAV-4 isolates showed similar proteins with minor variation in MW and protein bands to those seen in CELO virus. The approximate MW of 8 protein fractions of FAVs designed as II (hexon), III(penton), IIIa (fibre), IV, IVa, V,VI (hexon associated protein)

and VII (core protein 1) were found to be 110,92,78,50,43,32,29,20 KDa, respectively. On the other hand, uninfected CEL cell culture protein bands did not correspond with the viral polypeptides. The molecular weight of proteins by and large was found to be within the range of MW of protein of PARC-1 isolates of HPS virus and unique presence of IV (hexon associated proteins) in these isolates, has also been reported by Rabbani *et al.* (1998a). The protein bands found in our study is in slight variance to the work done by Balamurugan (1999) who found the proteins to range from 107, 87, 66, 55,45,38.5,24,2 and 20 KDa respectively. Very slight difference in the molecular weight of the proteins could be found as these may occur because of slight differences in estimating the proteins or the denaturing conditions that were used to cleave the polypeptides by SDS-PAGE.

For human adenoviruses, Patterson (1984) reported that the proteins with MW of 90-120, 70, 60, 60-65, 48, 24 and 18 kDa have been designated as II, III,IV,V,VI and VII respectively. Li *et al.*,(1984a) reported that none of the polypeptides of CELO virus except hexon, were comparable to those of human adenovirus. They suggested the possible morphological position of CELO virus polypeptides. The morphological position of several polypeptides of CELO virus in the present studies was concurrent with the findings of Yasue and Ishibashi (1977), Li *et al.* (1984a) and Maiti and Sarkar (1997). The differences in the total number of polypeptides of FAV-4 isolates and CELO virus observed during the present study varied with reports of other workers. The discrepancy in the total number of virion polypeptides lies mainly in the low molecular weight range because bands below 20 KDa could not be visualized. Yasue and Ishibashi (1977) reported three virion polypeptides with MW under 20 kDa, whereas Li *et al.* (1984a) reported six virion polypeptides in this range. This could be attributed partly to loss of small basic

polypeptides in the conventional methanol and acetic acid destaining solution (Li *et al.*, 1984a) suggested that formaldehyde fixation before Coomassie blue staining retained small and basic protein molecules better (Steck *et al.*,1980) and use of freshly grown virus which have minimized storage reduction of internal CELO virion proteins (Laver *et al.*,1971) were the probable reasons.

In Western blotting, only two protein bands (43 kDa and 78 kDa) of FAV-4 isolates resolved in SDS-PAGE, were found to be reactive with chicken hyperimmune serum. This might be due to the presence of less amount of protein or due to denaturation of protein or less antigen reactivity with heterologous serum.

Balamurugan *et al.* (1999) in his studies could detect 8 protein bands of FAV-4 isolates resolved in SDS-PAGE which were found to be reactive with chicken FAV-4 hyperimmnune serum. However, 38.5 kDa and 24.2 kDa protein bands of a isolate were not clearly visible in their study and this could be due to presence of less amount of protein that was not enough to react in western blotting. In two FAV-4 isolates in his study, 2 to 3 bands above 107 kDa was seen reacting to chicken FAV-4 hyperimmune serum. Similar findings were reported with PARC-1 isolates of HPS and 1 to 11 serotype of FAV (Rabbani *et al.*, 1998a). In the immunoblot, 7 polypeptides of CELO virus 9107, 94, 76,66, 55,32 and 20 kDa) reacted with FAV-4 serum, this indicated that these might be group specific proteins. The 76 kDa proteins (penton base) appeared distinctly on western blot which was not distinctly visible on SDS-PAGE.

The study demonstrated that all the FAV-4 isolates used were antigenically similar and had similar protein profiling. Only a single protein difference could be found with CELO virus which is at variance to the above study. The number of polypeptide bands

visible in FAV-4 isolates was seven. The comparatively less detection of the antigens of both CELO and FAV-4 isolates by heterologous serum in our study can be due to the presence of various trimers and dimers there is always a fair chance of getting more bands if different methods for polypeptide disruption are used because it is also possible that it can lead to the production of various epitopes. These epitopes do not react with antisera and might have been denatured during SDS-PAGE and these denatured proteins might have not recognized the antibodies (Burnette,1981). Based upon the observations of SDS-PAGE and western blotting, no detectable difference could be found among the three FAV-4 isolates in their proteins but they differed slightly with CELO virus.

This study demonstrated that all the FAV-4 isolates were antigenically similar and shared many common proteins with CELO virus revealing serological similarities among the FAVs. The immunoblot studies indicated that at least five major proteins are conserved in FAV-4 and FAV-1. Rabbani *et al.*(1998a) reported that five common proteins in adenoviruses reacted in immunoblot with antiserum of AAV isolate PARC-1. The number of polypeptide bands visible in FAV-4 isolates was eight. Due to the presence of various trimers and dimers there is always a fair chance of getting more bands if different methods for polypeptide disruption are used because it is also possible that some of the epitopes which did not react with antisera might have been denatured during SDS-PAGE and these denatured proteins might have not recognized the serum antibodies (Burnette, 1981). Based upon the observations of SDS-PAGE and western blotting, no difference was found among the three FAV-4 isolates in their proteins but they differed with CELO virus.

5.3.2 Gene sequencing

Comparison of direct sequencing of FAV-4 (P-I) isolate fibre gene product revealed 90 per cent identity at nucleotide level to fibre gene of FAV-4 isolate Punjab 1 (DQ864436), Punjab 2 (DQ864434) and short fibre gene (AY340863). Bhan (2006) also sequenced the FAV-4 fibre gene and could deduce that the fibre sequences of FAVs are relatively conserved within serotypes.

The purified cloned PCR product of FAV- 4 isolates (P-I and PH-II) were sequenced at commercially. The received sequences were analyzed and blasted on the NCBI GenBank Database to see their similarity with other FAV-4 isolates. They had strong similarities with the other FAV-4 available on the database. These sequences were then aligned to compare them with known FAV-4 sequences. The alignment of the nucleotide sequences revealed a very high homology amongst the sequences. The sequence similarity also confirms this high homology amongst the sequences as it shows that all these sequences share a close relationship with each other with similarity scores more than 95 per cent and thus can be assumed that these sequences may be similar isolates or may belong to the same group.

There is a full similarity in amino acid sequences that can be seen from the alignment table. An amino acid alignment was constructed to differentiate between the FAV-4 isolates and understand the similarities better. The same profile of high homology more than 95 per cent can be observed and thus it can be assumed that the isolates are similar isolates or belong to the same group. Our P-I and PH-II isolates shares quite a strong similarity with FAV-4 isolate from Haryana with Accession no. EU847626 compared to that with the reference sequence as can be observed from the alignment

differences and we can deduce that these isolates may as well belong to the FAV-4 group, but are different strains.

All of this can be clarified in the neighbor–joining phylogenetic trees that were constructed using both the nucleotide and protein sequences of our FAV-4 isolates together with the FAV-4 available with the Gen bank data base. Two major clusters are observed where the FAV-4 form their own significant cluster and the only member of *Adenovirus* (AJ 459 805) form its own cluster. We presume similarity amongst all the FAV sequences, suggesting they may belong to the same genogroup as suggested previously. There is a divergence to sub clusters where the differences between the FAV sequences are clearly seen. From the two sub clusters, the FAV-4 isolates cluster together and a high confidence level is shown suggesting that they are similar isolates, while the other two known members make their own clusters and also show a strong similarity amongst them.

This divergence into two sub clusters among the FAV may be attributed to the fact that these isolates had been isolated from different region with AJ459805 isolate and the other isolates recorded from somewhere else. It could be that all these FAV isolates have originated from the same place as suggested by the high confidence level that has been observed from the major cluster but due to the reason that poultry being moved around from one state to another for slaughtering, the virus might have been introduced in other regions. Most birds that reach different states are meant for slaughter and so less concentration is shown for the evidence of any disease. Also most poultry that are meant for meat purpose are quickly slaughtered even those birds that have been exposed to FAV-4 infection might not have shown any clinical signs of infection before they died or

slaughtered, thus no diagnosis was possible. The viral carriers appear healthy before they are slaughtered and these may have been the biggest transporters to Himachal Pradesh.

There is also high degree of homology observed between the FAV group I members represented in the tree and the local FAV-4 suggesting direct descendancy. What is also interesting is our isolate clusters with the Haryana FAV-4 isolate (Accession no. EU847626) suggesting that the isolates of FAV-4 in Himachal Pradesh have their origin in Haryana. Ganesh *et al.* (2001b) amplified the variable region of hexon gene, encoding 728 bp of L1 and part of P1, and compared both the nucleotide and the derived amino acid sequences with FAV-1, FAV-8 and FAV-10. There was variability of 8.2 % , 28.1 % and 40.3 % in the nucleotide sequence from FAV-10, FAV-1 and FAV-8, respectively. Although the nucleotide sequence showed only an 8.2 % difference between FAV-4 and FAV-10, the amino acid sequences differed by 28.8 %. Such a high degree of variability has been found to be due to change in the reading frame caused by deletions, indicating that the FAV-4 associated with HPS is unique and is different from FAV-10 (Ganesh *et al.*, 2001b).

Summary

Chapter VI

SUMMARY

The present study was undertaken to grow the locally available FAV-4 isolates from outbreaks of hydropericardium in the state and also to observe the suitability of various cell cultures and continuous cell lines for the adaptation of the virus. The study also aimed to standardize and compare the findings of different serological tests that are routinely used to detect the virus in a microbiological laboratory. Efforts were also made to characterize the isolates at the molecular level.

The locally available isolates and one standard strain of fowl adenovirus recovered from cases of HPS in Himachal Pradesh were passaged in three primary cell cultures (CEF, CEL and CEK) and four cell lines *viz.* Vero, HeLa, McCoy and BHK-21.

Ten passages were given in CEF cell cultures. CPE in CEF cell culture started only at the sixth passage level. The CPE were characterized by rounding and shrinkage of cells leading subsequently to complete detachment and degeneration of the monolayer. The effects started at 48 hrs. at sixth passage level and the complete monolayer got degenerated after 120 hrs. At higher passage levels, the rapidity and intensity of cytopathic changes increased and the complete monolayer got degenerated on an average by 72 hrs. p.i.

In case of CEL and CEK cell cultures, no CPE could be observed in both the cell cultures in the first passage level. CPE could be detected from 72 hrs. in the second passage level that were characterized by rounding of cells which increased in severity to shrinkage, detachment of cells and complete degeneration and destruction of the

monolayer by 96 hrs. A total of five passages were given in both CEL and CEK cell cultures. With each passage, the CPE increased in intensity and by the fifth passage level, the complete monolayers in case of both liver and kidney monolayer cultures got detached by 48 hrs.

The virus isolates were passaged eight times in Vero cell lines. No cytopathic changes were seen in Vero cells in the first five passage levels. However signs of cytopathic changes became visible from 72 hrs. onwards at the sixth passage level. The CPE were characterized by aggregate formation, rounding and shrinkage of cells which progressed to loss of cells from the monolayer by 120 hrs. of inoculation. Further passages of the virus isolates up to the eight passage level led to the whole monolayers getting degenerated by 96 hrs. p.i.

The virus isolates used in our study were also passaged eight times in three additional cell lines *viz.* HeLa, McCoy and BHK-21. However, virus failed to grow in any of these cell lines even after eight blind passages.

Titration of the infectivity of viruses were done before commencing the study by titrating the virus in eggs by standard procedure. Re titration of the virus isolates were done after passage in cell cultures to assess the change in the infectivity titre. It was found that the virus titre increased after passage in cell cultures.

Three passages were given in chicken embryos to find out the severity of the lesions produced at each passage level. It was observed that intensity of pathological changes increased in the last two passage levels with increased congestion and widespread haemorrhages on the body of the chicks as compared to the first passage level.

A unique attempt to study the effect of cell culture adapted virus on chicks was done. Infected cell culture supernatants from all cell cultures used in the study were used to inoculate chicks. It was found that the virus that got adapted to cell cultures retained their pathogenic potential for their traditional hosts with the appearance of hydropericardium. The mortality in chicks was recorded to peak at 96 hrs. p.i

The detection of FAV was done by both serological and molecular methods. For this, partial purification of FAV was done in order to raise hyperimmune serum to be used in our experiment. CEL cell culture adapted virus was semipurified by ultracentrifugation and was then used as an antigen. The semipurified virus elicited the production of antibodies in both rabbits and chickens following injections over a period of few weeks with both complete and incomplete adjuvants. The hyperimmune serum that was collected from the rabbits and chickens was cross reacted with liver tissue powder made from uninfected livers of chickens so as to eliminate any cross reacting antibodies developed against normal tissues. Standardization of the diagnostic tests was done by orally infecting thirty chicks with the three viral isolates and detecting the virus from tissues of the birds at different time intervals both serologically and by molecular methods.

It was found that the majority died before 4^{th} DPI. The typical signs seen in birds were hydropericardium, enlarged and pale liver and congested kidneys.

Serological tests like AGPT, CIE, Dot ELISA and IFAT were standardized and used to detect the virus from various samples and findings were compared. It was found that on an average, Dot ELISA and IFAT could detect virus antigens upto prolonged intervals in tissues as compared to AGPT and CIE. Additionally it was found that Dot

ELISA and IFAT could detect virus antigens in cell culture supernatants earlier than both AGPT and CIE.

An attempt was also made to ascertain the hemagglutinating property of the fowl adenoviruses used in this study as there were conflicting reports from many authors upon the usefulness of hemagglutination test for the diagnosis of fowl adenoviruses. RBCs from eight different species of animals were used. These species were cattle, buffalo, chicken, geese, swine, dog, rat and fish. Two dilutions of the virus were made and the standard FAV-1 isolate used in our study was also included. No detection was however possible with any of the RBC's with any of our isolates.

Efforts were also made to find out the neutralization index of the three viral isolates and the standard CELO virus used in our study. It was found that both CELO as well as the FAV-4 isolates could be neutralized by their corresponding hyperimmune serum.

The tissues processed for histopathological findings from the chicks used in the experimental study revealed mild to moderate congestion and foci of haemorrhages along with disruption of myocardial fibres due to edema in the heart. Fibrinous myocarditis was evident by the fibrinous deposition over the epicardium. Lungs showed generalized alveolar congestion and mild to moderate haemorrhages with perivascular edema and widening of interlobular septa due to edema with hyperplasia of bronchial epithelium. The hepatocytes in the liver tissue showed variable degrees of degeneration *viz.* vacuolar degeneration and fatty changes. Distinct basophilic intranuclear inclusion bodies with clear halo around them were present in some hepatocytes. Kidneys revealed congestion and haemorrhages in the parenchyma and changes like vascular degeneration and

desquamation of tubular epithelium while marked congestion in medullary and sub capsular vessels were seen in thymus. Mild lymphoid depletion in medulla of follicles of bursa and hyperplasic changes in epithelium forming papillary projection into the lumen were seen.

Immunoperoxidase test could detect viral antigens in impression smears of liver and kidney from infected birds and infected cell cultures.

PCR was standardized for the detection of the fowl adenovirus DNA from various tissues and other samples used in the study. The DNA was extracted by conventional method (Phenol: Chloroform: Isoamyl alcohol) extraction and subjected to FAV group specific PCR amplification to ascertain the group to which the viral isolates belonged. The viral isolates as expected were identified in the fowl adenovirus group. Thereafter, all the samples were subjected to hexon and fibre gene specific amplification for the identification of FAV-4 isolates. It was found that hexon gene specific amplification of ~700 bp region of FAV-4 was successful from most of the samples. It was also noted that the fibre gene specific amplification could also amplify the specific products but was not as foolhardy as hexon gene amplification.

Dot blot hybridization using DNA probe confirmed the results of the PCR for detection of viral DNA from various samples.

Molecular characterization of the FAV-4 isolates used in our study were done by protein profiling and gene sequencing. The purified FAV isolates and the standard CELO virus were subjected to SDS-PAGE in order to study the protein profile of the virus. Seven protein bands from both CELO as well as the three FAV isolates were detected, while the 110 kDa protein band could be detected in case of CELO virus. Western blot was

attempted to study the immunogenic proteins resolved by SDS-PAGE. However, only two bands corresponding to 32 and 43 kDa protein could be detected in western blot.

Purification of hexon gene fragment of P-I and PH-II isolates obtained after PCR amplification from liver tissues were done from agarose gel using gel extraction kit. The gel eluted hexon gene segments were cloned in PGEM-T easy vector and transformed into competent *E. coli* according to standard protocols. Thereafter, the cloned product was extracted out from the bacteria, purified and sequenced commercially. Fibre gene PCR product was directly sequenced to know its genetic profile.

Sequencing results showed that both isolates used in our study were identical and clustered with known Haryana FAV-4 isolate. The two isolates were found to be identical in both nucleotide and amino acid level and were also found to be 95 to 100 per cent similar in both nucleotide and amino acid profile to other FAV-4 isolates from different parts of the country. The fibre gene also showed greater than 90 per cent homology at nucleotide level and amino acid level with fibre gene of Punjab FAV-4 isolate.

Conclusions

The following conclusions were drawn from our investigation.

1. All the isolates used in the present study were characterized and confirmed to be FAV-4.
2. CEL and CEK were found to be better than CEF for adaptation of virus in primary cell culture.
3. Out of four cell lines such as Vero, HeLa, McCoy and BHK-21 tested in the present investigation, the Vero cell culture was observed to be a convenient alternative for propagation of FAV-4.

4. The pathogenicity of FAV-4 is enhanced on successive passages in chicken embryos.
5. The diagnostic methods such as Dot ELISA and FAT found to be better for the diagnosis of FAV-4.
6. HPS was further confirmed by classical gross pathology and by demonstration of characteristic basophilic intranuclear inclusion bodies in the hepatocytes of experimental birds suggestive of adenovirus infection.
7. The protein profile of FAV-4 on SDS-PAGE revealed seven polypeptides of various sizes *viz.* 92, 78, 50, 43,32,29 and 20 kDa with an eighth polypeptide of 110 kDa seen in case of CELO virus.
8. Group specific and hexon gene specific PCRs were found to be very useful and efficient for identifying the fowl adenovirus.
9. Both FAV-4 isolates used in the study were found genetically similar to Haryana FAV-4 isolate and were also to found to be 95 to 100% identical with other Indian FAV-4 isolates.

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ANNEXURE -I

A. Solutions and media for cell culture

1. Phosphate buffer saline (PBS) (Calcium and magnesium free)

Sodium chloride (NaCl)	8.00 g
Potassium chloride (KCl)	0.20 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.20 g
Disodium hydrogen phosphate ($Na_2HPO_4.7H_2O$)	1.15 g
Distilled water	to make 1000 ml

Sterilized by autoclaving at 15 p.s.i. for 20 minutes and stored at room temperature.

2. Trypsin-Versene solution (10X)

Sodium Chloride (NaCl)	85.0g
Potassium chloride (KCl)	4.0g
Glucose	10.0g
Sodium bicarbonate ($NaHCO_3$)	3.5g
Penicillin G sodium	1×10^6 units
Streptomycin sulphate	1.0 g
Trypsin (1:250) [DIFCO]	5.0 g
EDTA	2.5g
Phenol red solution (0.5%)	20ml
Distilled water	to make 1000 ml

Stirring was done for 2 to3 hours at room temperature. Trypsin-Versene was sterilized by filtration through membrane filter of pore size 0.22µm. Filtered trypsin-versene was then dispensed in 50-100 ml quantities and stored at -20°C.

3. Trypsin- Versene solution (1X)

Trypsin Versene solution (10X)	100 ml
Distilled water	900 ml

The above contents were mixed thoroughly and then filtered through membrane filter of pore size 0.22µm. It was then stored at 4°C.

4. Sodium bicarbonate solution (5 %)

Sodium bicarbonate ($NaHCO_3$)	5 g
Double distilled water	100 ml

50 ml of double distilled water was taken in a measuring cylinder and 5 g of sodium bicarbonate was added to it. It was thoroughly mixed with the help of magnetic stirrer. Then final volume was made up to 100 ml with double glass distilled water. It was filtered through membrane filter of pore size 0.22 µm and stored at 4°C.

5. D-glucose (27%)

Dextrose	108 g
Double distilled water	325 ml

Water was placed in graduated cylinder with magnetic stirring bar in motion. Dextrose was added and stirred until it was dissolved. Final volume was made to 400 ml. It was then sterilized with membrane filters of pore size 0.22μm and stored at 4°C.

6. Medium-199 (1X)

Medium -199 powder	11.0 gm
Sodium bicarbonate	0.35 gm
Tryptose phosphate broth	0.30 gm
HEPES (1 M)	2.0 ml
Benzyl Penicillin	2.5 lakhs I.U
Streptomycin	0.50 gm
Double distilled water	1000 ml

All the above contents were added in 850 ml of double distilled water in a measuring cylinder and mixed thoroughly. The pH was adjusted 0.2 – 0.3 pH units below the desired pH using 1N HCl or 1 N NaOH. Then, final volume was made to 1000 ml with double distilled water. The medium was then sterilized using membrane filter of pore size 0.22μm. The sterile medium was then dispensed in sterile containers and stored at 4°C.

7. Minimum Essential Medium (10X)

MEM (Hi media)	10.74g
Double distilled water	100 ml

10.74g of MEM was suspended in 50 ml double distilled water in a measuring cylinder and gently stirred on a magnetic stirrer until medium is completely dissolved. Then,

final volume was made 100ml with double distilled water. It was then sterilized using membrane filter of pore size 0.22µm and stored at 4°C.

8. Minimum Essential Medium (1X)

MEM (10X)	10 ml
HEPES (1M)	2 ml
Glucose	2 ml
Antibiotic solution	2.5 ml
Sodium bicarbonate solution (5%)	0.7 ml
Double distilled water	82.80 ml

All the above contents were added in 50 ml of double distilled water in a measuring cylinder and mixed thoroughly. The pH was adjusted 0.2 – 0.3 pH units below the desired pH using 1 N HCl or 1 N NaOH. Then final volume was made to 100 ml with double distilled water. Medium was then sterilized using membrane filter of pore size 0.22µm. and stored at 4°C.

9. Trypsin Versene Glucose

a. TVG consists of the following components in 1 X PBS

b. Trypsin	0.1 %
c. Versene	0.2 %
d. Glucose	0.05 %

Stock solution for TVG

a. *10 X PBS*

NaCl	80.00 gms
KCl	2.00 gms
Na_2HPO_4	14.42 gms

$KH_2 PO_4$	2.00 gms
Dist.water upto 1 liters	

To prepare 1 X PBS- added 100 ml of 10 X PBS to 900ml of distilled water

b. *2 % Trypsin*

Trypsin	2.00 gms
Distilled water upto	100 ml

Stirred the above solution on a magnetic stirrer for 4 hrs. or overnight at 4°C.sterilized by filtering through sterile membrane filter of pore size 0.22 μm. A sterility check was done before using the solution.

c.. *0.2% Versene*

EDTA	200 mg
Distilled water	100 ml

Sterilized by autoclaving at 15 lbs and 121°C for 15 minutes.

d. *10 % Glucose*

Glucose	10.00 gms
Distilled water upto	100 ml

Sterilized by autoclaving at 15 lbs and 121°C for 15 minutes.

e. *1 % Phenol Red*

Phenol Red	1.00 gms
Distilled water upto	100 ml

Preparation of working solution of TVG

Prepared 840 ml of 1 X PBS and to this added 1.0 ml of 1 % phenol red (indicator). Sterilized by autoclaving at 15 lbs and 121° C for 15 minutes. Cooled this sterile solution and then added the following sterilized solutions to it.

Trypsin 2.0 %	50 ml
Versene 0.2 %	100 ml
Glucose 10.0 %	5 ml

Did sterility test as mentioned above before using the TVG.

10. HEPES 1M solution (*Himedia*)

HEPES was added at the rate of 20mM in 1000 ml of media. It was stored at 4°C.

11. Stock antibiotic antimycotic solution (100X) [*Himedia*]

The stock antibiotic solution had 10,000 I.U. of penicillin, 10mg of streptomycin and 25µg of amphotericin B per ml. It was stored at 4°C. It was added at the rate of 2.5ml per 100ml of medium.

a) Physiological saline (0.85%)

NaCl	0.85 g
Distilled Water	100 ml

b) Trypan blue (0.4%)

Trypan blue dye	0.4 g
Physiological saline	100 ml

Paper filter before use.

B. STAINING OF AGPT SLIDES

1. *Coommasie brilliant blue staining solution*

Brilliant blue (Coomasie) R-250	0.25 g
Methanol	45 ml
Glacial acetic acid	10 ml
Double distilled water	45 ml

2. *Destaining Solution*

Methanol	45 ml
Glacial acetic acid	10 ml
Double distilled water	45 ml

C. STAINING OF CELL CULTURES

1. Giemsa stain reagents

Giemsa powder	19 gm
Glycerol	66 ml
Absolute methanol	66 ml

Giemsa powder was dissolved in glycerol at 55-60°C for about 2 hrs. Methanol is added and mixed thoroughly. The solution from above formula and the commercially available solution is mixed with buffer solution before use:

1 volume of stain to 9 volumes of buffer.

2. Buffer for Giemsa stain

A) Sodium phosphate Na_2HPO_4 (anhydrous) M/15 solution (9.47 g/litre)

OR

Sodiun phosphate $Na_2HPO_4.2H_2O$ (M/15 solution (11.87 gm/litre) = 61.1 ml

b) Potassium phosphate, KH_2PO_4 (anhydrous) M/15 solution (9.08 gm /litre) = 38.9 ml

c) Distilled water =900 ml

D. SOLUTIONS AND BUFFERS FOR VIRUS PURIFICATION

1. Phosphate buffer saline (pH-7.2)

Sodium chloride (NaCl)	8.00gm
Potassium Chloride (KCl)	0.20gm
Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$)	1.15gm
Potassium dihydrogen phosphate (KH_2PO_4)	0.20gm
Distilled water to make	1000 ml

Sterilized at 15 lb pressure (autoclave) for 20 min and stored at 4^0C

2. Tris-HCl (1M) pH 8.0

Tris	12.114gm
Distilled water	80.000ml

Stirred vigorously on a magnetic stirrer. pH was adjusted to 8.0 with NaOH and the volume was made to 100ml and sterilized by autoclaving.

3. EDTA (0.5 M) pH 8.0

EDTA.$2H_2O$	18.612gm

Distilled water	80.00ml

Stirred vigorously on a magnetic stirrer. pH was adjusted to 8.0 with NaOH and the volume was made to 100 ml and sterilized by autoclaving.

4. Sodium chloride (5M)

NaCl	29.2gm
Distilled water	100.00ml
Sterilized by autoclaving	

5. TNE Buffer (50mM Tris, 150 mM NaCl, 1mM EDTA)

1M Tris HCl	5ml
5M NaCl	3 ml
0.5 M EDTA	200µl
Distilled water to make	100ml

6. Sucrose solution 30%

Sucrose	30 gm
TNE buffer	100 ml
(Dissolved 30 gm sucrose in 70 ml TNE buffer and final volume made upto 100ml)	
Distilled water to make	100 ml

7. Sodium phosphate buffer (0.1M) pH 6.8

0.1 M Na_2HPO_4	46.3 ml
0.1 M NaH_2PO_4	53.7 ml

Sterilized at 15lb pressure (autoclave) for 20 min and stored at 4^0C.

8. Cesium chloride 1.33g/ml density solution

Cesium chloride	4.54 gm
TE buffer (5mM Tris, 1mM EDTA)	10.0 ml

9. Cesium chloride 1.45g/ml density solution

Cesium chloride	6.09 gm
TE buffer (5mM Tris, 1mM EDTA)	10.00ml

E. SOLUTIONS AND BUFFERS FOR SDS-PAGE

1. Acrylamide / Bis–acrylamide solution 30 %

Acrylamide	29.2gm
Bis-acrylamide	0.8 gm
Distilled water to make	100.0 ml

2. Lower Buffer (1.5M Tris HCl, pH 8.8)

Tris Base	18.17 gm
SDS	0.40 gm
Distilled water to make	90.0 ml

pH was adjusted with diluted HCl drop by drop and final volume made upto 100 ml

3. Upper Buffer (0.5M Tris HCl, pH 6.8)

Tris Base	6.06 gm
SDS	0.40 gm
Distilled water to make (diluted HCl added to adjust pH 6.8)	90.0 ml
Final volume adjusted to	100ml

4. Ammonium per sulphate (APS) solution (10%)

APS (dry power)	100mg
Distilled water	1ml

5. Loading Buffer/ Sample Buffer (2X)

Glycerin	2.0ml
Upper buffer	1.7ml
SDS-10%	4.5ml
2-mercaptoethanol	1.0 ml
Bromophenol blue	2.0mg
Distilled water	0.8 ml

6. Running/Resolving gel solution (10%)

Acrylamide solution	6.0ml
Lower buffer	4.5ml
Distilled water	7.5ml
APS 10%	70 µl
TEMED	10 µl

7. Stacking gel solution (4.5%)

Acrylamide solution	0.9 ml
Upper buffer	1.5ml
Distilled water	3.6 ml
APS 10%	18 µl
TEMED	10 µl

8. Electrode Buffer pH 8.3 { 0.025 M Tris, 0.192 M Glycine, 0.1 % SDS)

Tris	3.0 gm
Glycine	14.4 gm

SDS	1.0 gm
Distilled water	1000 ml

9. Coomassie Brilliant Blue (CBB) staining solution (0.25 %)

Coomassie Brilliant Blue R-250	0.25 gm
Methanol	50 ml
Glacial acetic acid	10 ml
Distilled water to make	100 ml

10. Destaining solution

Methanol	300 ml
Glacial acetic acid	100 ml
Distilled water to make	1000 ml

11. Silver staining

Solution A: 0.8 g silver nitrate in 4 ml distilled water.

Solution B: Mix 21 ml of 0.36% NaOH

Developing solution: Mix 2.5 ml of 1% citric acid with 0.25 ml of 37% formaldehyde and make the volume to 500 ml (freshly prepared).

F. SOLUTIONS AND BUFFERS FOR WESTERN BLOT

1. Transfer/Blotting/Electrode(Towbin's) buffer

(0.025 M Tris, 0.192 M Glycine, 20 % Methanol)

Tris	3.0 gm
Glycine	14.4 gm

20 % methanol	200 ml
Distilled water to make	1000 ml

2. Washing Buffer (Tris buffered saline (TBS)-Tween -20, pH 7.6)

(50 mM Tris, 150 mM NaCl, 0.05 % Tween-20)

Tris	3.025 gm
Nacl	4.380 gm
Tween-20	0.250 ml
Distilled water to make	500 ml

3. Blocking Buffer

BSA	5.0 gm
Washing buffer	100 ml

4. Development / substrate solution (50 mM Tris), pH 7.6

Tris	3.025 gm
Distilled water	500 ml

Final preparation- 10 ml Trsi (pH 7.6) + 6 mg DAB + 10 µl H_2O_2

G. SOLUTIONS AND BUFFERS FOR CIE

Acetate buffer (0.05 M) pH 5.6

1.Solution A (0.2 M)

Acetic acid	11.55 ml
Distilled water to make	1000ml

2. Solution B (0.2 M)

Sodium acetate anhydrous ($C_2H_3O_2$ Na)	16.4 gm
Distilled water to make	1000 ml

From this was prepared 0.1M acetate buffer by diluting the stock solution in the following ratio:

Solution A	48 ml
Solution B	452 ml
Distilled water to make	1000 ml

Acetate buffer of 0.05 M, pH 5.6 was prepared by diluting the 0.1M buffer solution further in distilled water.

H. DILUENT FOR RBCS

1. Alsever's solution

Dextrose	20.5 g
Sodium citrate	8.0 g
Citric acid	0.55 g
NaCl	4.20 g

QS to 1 litre with distilled water. Sterilize by autoclaving at 10 lbs. pressure for 10 minutes.

2. Dextrose-Gelatin-Veronal (DGV)

$CaCl_2$	0.02 gm
$MgSO_4.7H_2O$	0.120 gm
NaCl	8.5 gm
Gelatin	0.6 gm
Glucose	10.0 gm
Sodium barbital	0.38 gm
Barbital	0.58 gm

I. BUFFERS/ MEDIUM FOR RUNNING PCR

A. Preparation of solutions / buffers

1. Stock solution of Tris acetate EDTA buffer (TAE) (10 X)

Tris-base	48.4 g
Glacial acetic acid	10.9 g
EDTA	2.92 g
Double distilled water	To make the volume to 1ltr

2. Working buffer (1 X TAE)

10 X TAE	100 ml
Double distilled water	To make the volume to 1 liter

3. 1 per cent Agarose (Molecular biology grade) gel

It was prepared in working TAE containing ethidium bromide @ 0.5 µl/ml.

B. Agarose Gel Electrophoresis

1. 50 X TAE Buffer:

Tris base	242.1 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Adjust volume to 1 litre with ddH_2O.

2. Ethidium bromide (0.5 mg/ml)

0.5 mg of ethidium bromide in 1 ml of autoclaved ddH_2O (Prepare 100 ml of ethidium bromide solution by dissolving 50 mg of ethidium bromide in 100 ml of autoclaved

dd H_2O and stain the gel by keeping for 1 min.

3. 6X Gel loading dye

Contents	**Quantity**
Ficoll 400	15 %
Bromophenol blue	0.25 %
Xylene cyanol	0.25 %

J. BUFFERS USED FOR DOT BLOT HYBRIDIZATION

1. 20X SSC buffer: 3M NaCl and 0.3 M Trisodium citrate

2. Buffer-A: 500mM Tris HCL (pH 7.5), 500mM NaCl, 5mM EDTA and 0.5% SDS.

TNE Buffer- **Tris HCl- 20mM, NaCl- 10mM and EDTA- 10mM**

3. Prehybridization buffer

Formamide	50 % (v/v)
Na_2HPO_4 (pH-7.2)	120 mM
NaCl	250 mM
EDTA 1mM	7% (W/V) SDS

All components were prepared for 100 ml hybridization solution and kept at room temperature for further use.

4. Washing buffers

First Wash Buffer 2.0x SSC, 0.1% SDS (250C) - two washings of 15 min each

Second wash buffer 0.5x SSC, 0.1% SDS ((250C) - two washings of 15 min each

Third Wash buffer 0.1x SSC 0.1% SDS (650C) - two washings of 30 min each

K. BUFFERS/MEDIUMS USED FOR CLONING

1. 100 ml of 0.1 M $CaCl_2$, 100 ml of 0.1 M $MgCl_2$

First prepare stocks of 2.5 M of 100 ml $CaCl_2$, 2.5 M of 100 ml $MgCl_2$ respectively and filter sterilize them (not autoclaving). From the filtered stocks take4 ml of each solution to make 100 ml of 0.1 M $CaCl_2$:, 100 ml of 0.1 M $MgCl_2$ respectively.

2. 10 ml of 0.1 M $CaCl_2$ containing 10% glycerol.

2.5 M $CaCl_2$	400 µl
50% Glycerol	2 ml

Adjust the volume to 10 ml by addition of ddH_2O

3. SOB medium

Bacto tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g

Dissolve in 900 ml water, 10 ml 250 mM KCl was added to it. Adjust pH to 7 with NaOH, aliquot, autoclave, cool and 5 ml sterile 2M $MgCl_2$ was added.

4. STET buffer

Contents	**Quantity**
NaCl	0.1 M
Triton X-100	5 %
Tris HCl (pH 8)	10mM
EDTA (pH 8)	1mM

5. Lysis Buffer (1 ml)

10 % SDS	100 µl
0.5 EDTA	20 µl
10 N NaOH	10 µl
dd water	870 µl

6. 3 M Sodium acetate pH 5.2: 123.05 g of sodium acetate was dissolved in 400 ml DEPC treated water, pH was adjusted to 5.2 with glacial acetic acid and final volume was adjusted to 500 ml.

ANNEXURE-II

1. Composition of feed ingredients in the broiler ration:

Maize	50 kg
Groundnut cake	10 kg
Sunflower cake	12 kg
Soya flakes	12 kg
Fish meal	5 kg
Lime powder	1 kg
Digestible crude protein	1 kg
Molasses	5 kg
Mustard cake	4 kg

2. Feed additives

B-complex	20 kg
Vit.AB_2D_3K	25 kg
DOCTI	50 kg
Trace minerals	100 g
Biocholine	50 kg
Choline chloride	100 g