

STUDIES ON ISOLATION, IDENTIFICATION
AND MOLECULAR CHARACTERIZATION OF
HYDROPERICARDIUM SYNDROME VIRUS

M.V. Sc. THESIS

by

ABHISHEK SHARMA

DEPARTMENT OF VETERINARY MICROBIOLOGY
COLLEGE OF VETERINARY SCIENCE
AND ANIMAL HUSBANDRY
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MOLECULAR CHARACTERIZATION OF
HYDROPERICARDIUM SYNDROME VIRUS**

THESIS

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**By
ABHISHEK SHARMA**

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Roll No.: 9960

ID No. : UG/VETY/DURG/2002/1

VITA

*The author of this thesis, **ABHISHEK SHARMA** was born on February 18th, 1986 at Bah, Agra (U.P.). he passed his Higher Secondary examination in the year 2002 with first division. In July 2002, he started his carrier with Veterinary profession for his B. V. Sc. & A. H. degree, from, College of Veterinary science and A. H., Anjora, Durg, Indira Gandhi Krishi Vishwavidyalaya, Raipur, (Chhattisgarh), and completed it in December 2007 in first division with distinction with OGP of 7.5/10.00. Due to his keen interest for specialization in animal diseases, he joined for his Master degree in the Department of Veterinary Microbiology, in January, 2008. he completed his course work of M.V.Sc. degree successfully with O.G.P.A. of 8.87 /10.00 with distinction. he is the member of Veterinary Council of India and veterinary council of U.P.*

Address:

Dr. Abhishek Sharma

RZ D-20, West Sagarpur,

(Near Gandhi Market),

New Delhi-110046

Phone no. – 09229436921, 09810523513, 011-25394513

Email – drabhishek_86@yahoo.com

CERTIFICATE-I

This is to certify that the thesis entitled “**Studies on isolation, identification and molecular characterization of hydropericardium syndrome virus**” submitted in partial fulfillment of the requirements for the degree of “**Master of Veterinary Science**” of the Indira Gandhi Krishi Vishwa Vidyalaya, Raipur, is a record of the bonafide research work carried out by **Abhishek Sharma** under my guidance and supervision. The subject of the thesis has been approved by the Student’s Advisory Committee and the Director of Instructions.

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

Date: April 8, 2010

Dr. Sanjay Shakya
Chairman, Advisory Committee

THESIS APPROVED BY THE STUDENT’S ADVISORY COMMITTEE

Chairman	(Dr. S. Shakya)	:	
Member	(Dr. S.P. Ingole)	:	
Member	(Dr. S.L.Ali)	:	
Member	(Dr. G.K. Dutta)	:	

CERTIFICATE-II

This is to certify that the thesis entitled **“Studies on isolation, identification and molecular characterization of hydropericardium syndrome virus”** submitted by **Abhishek Sharma** to the Indira Gandhi Krishi Vishwa Vidyalaya, Raipur, in partial fulfillment of the requirements for the degree of **“Master of Veterinary Science”** in the Department of Veterinary Microbiology has been approved by the Student’s Advisory Committee after oral examination in collaboration with the external examiner.

External Examiner

Major Advisor

Head of the Department

Dean

Director of Instructions

Date

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ABBREVIATIONS

Abbreviations	Full Form
AGPT	Agar gel precipitation test
AAV	Avian adenovirus
Amp	Ampicillin
bp	Base pair
CEL	Chicken embryo liver cell culture
Cm	Centimeter
⁰ C	Degree celcius
DNA	Deoxyribonucleic Acid
dNTPS	Deoxynucleoside Triphosphates
EDS	Egg drop syndrome
ELISA	Enzyme linked immunosorbent assay
Etbr	Ethidium bromide
<i>et al.</i>	et alia (and other)
etc.	Etcetera
FAV	Fowl adeno virus
Fig.	Figure
Gm	Gram
HHS	Hepatic hydropericardium syndrome
hr(s)	Hour(s)
HPS	Hydropericardium syndrome
HPSV	Hydropericardium syndrome virus
i.e.	id est (that is)
IBH	Inclusion body hepatitis
ICTV	International commette on the virus

INCB	Intranuclear inclusion body
IU	International unit
I/M	Intra muscular
Kb	Kilobase
Kda	Kilo Dalton
M	Molar
Mm	Milli molar
µg	Microgram
µl	Microliter
Mg	Milli gram
ml	Milli liter
Mm	Milli meter
Min	Minute
NFDW	Nuclease free water
Ng	Nano gram
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
Ppm	Parts per million
/	Per
%	Percent
PBS	Phosphate buffer saline
PI	Post inoculation
Pm	Picomoles
RE	Restriction enzymes
RFLP	Restriction fragment length polymorphism
Rpm	Revolution per minute

SPF	Specific pathogen free
TAE	Tris acetic EDTA
TE	Tris EDTA
S/C	Sub cutaneous
U	Unit
UV	Ultra violet
V	Volt
viz.	Vide licet (Namely)
v/v	Volume by volume
w/v	Weight by volume

CHAPTER-I

INTRODUCTION

Poultry rearing in India has been transformed itself from being a backyard enterprise to full fledged industry. Currently India rank fourth and ninth in egg and broiler production respectively with an annual growth rate of 8 % for egg and 15 % for broilers, thereby contributing much towards employment generation and food security. Higher demand for broiler meat, earlier market age and rapid returns over the invested capital has increased the popularity of broiler farming but it has always encountered with stumbling block in the form of various diseases. These diseases, caused by viruses, bacteria, fungi and parasites, led to major economic losses in the form of mortality, loss of production and posing a serious threat to the broiler industry (Biggs, 1982). Reduction in the net profit has observed when mortality level was increased from 2.5 to 10 % (Kitsopanidis and Ma, 1991).

Among avian viral diseases, Fowl Adenovirus (FAV) infections are widespread in most of the poultry raising countries of the world and become a serious threat to the poultry industry. Different manifestations due to FAV infection *viz* egg drop syndrome, respiratory infections, inclusion body hepatitis, hydropericardium syndrome (HPS) *etc.* have been reported. HPS is caused by fowl adenovirus (FAV) serotype 4 belonging to family of *Adenoviridae* (Jadhao *et al.*, 1997). The disease is observed in three to six week old growing broilers. The virus occasionally affects layer and breeder pullets aged more than 20 weeks (Akhtar *et al.*, 1992; Shukla *et al.*, 1997).

The syndrome is characterized by accumulation of clear straw coloured fluid in pericardial sac, discoloured liver and enlarged kidneys (Cheema *et al.*, 1989). The liver of the infected birds shows necrotic foci and basophilic intranuclear inclusion bodies in the hepatocytes (Abdul-Aziz and Hasan, 1995). The typical heart lesions simulate with de-shelled litchi fruit hence also called Litchi disease.

About 10 to 60 percent mortality has been reported in young broilers (Ahmed *et al.*, 1989; Abdul-Aziz and Al-Attar, 1991). In India Ravi Kumar *et al.* (1997) reported 30 to 80 percent mortality in broilers. The sudden onset of the disease and mortality as high as 75% in 3 to 6 weeks old group led to severe losses to the poultry producers in India and Pakistan (Gowda and Satyanarayana, 1994)

The affected birds do not exhibit any clinical sign other than sudden death with heavy mortality (Kumar *et al.*, 1997) however, in the last stages the birds become dull and depressed and show a characteristic posture with their chest and beak resting on ground with closed eyelids. Birds inoculated experimentally by the subcutaneous route become off feed, show ruffled feather, reluctant to move and die within 60 h PI (Muneer *et al.*, 1989).

In immunosuppressed birds the mortality and severity of lesion may be higher. The agent fowl adenovirus-4 causes immunosuppression by damaging lymphoid tissue. The virus replicates in erythroid and lymphoid progenitor cells causing inapparent, subclinical infections that lead to depletion of these cells with consequent immunosuppressive effects. The presence of infectious bursal disease (IBD) and chicken anemia virus (CIA) may predispose for HPS or HPS may predispose for other viral infections (Balamurugan and Kataria 2006).

The hydropericardium syndrome was first reported in Angara goth near Karachi, Pakistan during 1987 hence named Angara disease (Khawaja *et al.*, 1988). Thereafter disease has been reported from Iraq (Abdul Aziz and Al-Attar, 1991), Japan (Abe *et al.* 1998), Russia (Borisov *et al.*, 1997) South and Central America (Shane, 1996) Slovakia (Jantosovic *et al.*, 1991) and many other countries. In India the disease was first reported in some parts of Jammu and Kashmir, Punjab and Delhi during April–July 1994 (Gowda and Satyanarayan, 1994) and later from M.P., Orissa and Maharashtra (Survashe *et al.*, 1996). Rare outbreaks of HPS in older broilers reported rarely (Asrani *et al.*, 1997), Besides affecting the poultry this virus also causes

disease in pigeons (Naeem and Akram, 1995) and it can be presumed that this disease may affect the other species of the birds, which are used at par to the broilers.

The epidemiological factors associated with the spread of HPS are unclear (Akhtar *et al.*, 1992). Proper disinfection of premises and equipment, restricted entry of visitors and vaccination crews and ventilation and proper lightening in the poultry house play a significant role in prevention of disease (Abdul-Aziz and Hasan, 1995).

The HPS virus is highly pathogenic and spreads readily from flock-to-flock and farm-to-farm (Cowen 1992). Vertical as well as horizontal transmission play a role in the spread of disease. Most outbreaks are initiated by transmission of virus through embryonated eggs and hens exposed during production typically shed virus to their progeny for three to six weeks until development of immunity occurs. Horizontal spread occurs primarily by contact with infected feces. This seems to occur quite frequently and most cases are not diagnosed because they do not become a clinical problem.

Clinical diagnosis of the disease before the occurrence of the mortality is difficult since the birds do not show clinical signs. Various methods have been described for the diagnosis of fowl adenovirus infection including virus isolation in cell culture (Cowen *et al.*, 1978) indirect immunofluorescent assay (Adair *et al.*, 1980), ELISA (Calnek *et al.*, 1982) and double diffusion (Cowen *et al.*, 1978). Diagnosis of HPS can also be done on the basis of gross lesions, electron microscopy, histopathological lesions *etc.*

Although these methods are useful for detection of fowl adenovirus, but most of them are laborious and time consuming, moreover they have low sensitivity or specificity. The main problem with any serological test used for the diagnosis of fowl adenoviruses is the interpretation of results, because birds may be frequently infected with number of serotypes therefore in order to overcome such problem, *in vitro* amplification of target gene sequence by Polymerase Chain Reaction has been applied

as a rapid diagnostic tool for the detection of fowl adenovirus in recent years (Raue and Hess, 1998; Ganesh *et al.*, 2001). Even it is possible to detect the mixed infection with more than one fowl adenovirus serotypes (Singh *et al.*, 2002). This method is rapid, more sensitive and specific over other diagnostic procedures. The fowl adenoviruses specific polymerase chain reaction combined with restriction enzyme analysis was found to be very useful in investigating the epidemiological situation in the fields (Meulemans *et al.*, 2001). Hexon is the major surface protein of the virus on which, type, group and subgroup specific antigenic determinants are located (Norrby, 1969) hence molecular characterization of hexon gene is of prime importance.

Thus considering these facts the proposed work was undertaken with following objectives:-

1. To isolate the HPSV from field samples
2. To identify the isolate as HPSV by Polymerase Chain Reaction

CHAPTER- II

REVIEW OF LITERATURE

2.1 History and Incidence:

Hydropericardium Syndrome, a disease primarily of broiler birds, has emerged as a severe hazard to poultry producers, particularly in India and Pakistan, has led to closure of many farms. The disease causes high mortality in fast growing broiler chicks and was observed for the first time in August 1987 from Angara Goth of Karachi in Pakistan. The disease is commonly known as Angara disease (Afzal *et al.*, 1991) because of its first appearance in Angara Goth, Karachi. In India, the farmers called this condition Leechi disease because the heart, surrounded by hydropericardium, appear similar to a peeled Indian Leechi fruits. The disease had also reported from Faisalabad in 1988. A large number of outbreaks were reported from various parts of Pakistan (Anjum, 1988; Jaffery, 1988; Khan *et al.*, 1988; Khawaja *et al.*, 1988 and Qureshi, 1988) and from Kuwait (Hasan, 1989). The disease has also been reported from Iraq (Abdul- Aziz and Al-Attar, 1991), Slovakia (Jantosovic *et al.*, 1991), Mexico, Peru, Chile (Shane, 1996), Russia (Borisov *et al.*, 1997), Equador (Mazaheri *et al.*, 1998) and Japan (Nakamura *et al.*, 1999).

In India, the disease was first reported in Jammu and Kashmir, Punjab and New Delhi during the year 1994 (Gowda, 1994) and afterwards outbreaks of disease has been reported from different parts of the country (Bhowmik, 1996; Nighot *et al.*, 1996; Mahajan *et al.*, 1996 and Asrani *et al.*, 1997).

The incidence of disease has also been reported from Madhya Pradesh (Survashe *et al.*, 1996) Orissa, Maharashtra, Karnatka (Kumar *et al.*, 1997), West Bengal (Chakraborty *et al.*, 2000) and Haryana (Dahiya *et al.*, 2002).

2.2 Epidemiology:

The first epidemic of hydropericardium syndrome in broiler chicks was reported from Angara Goth, Karachi, Pakistan in autumn of 1987 (Khawaja *et al.*, 1988; Cheema *et al.*, 1989). Since then the disease has emerged throughout the world. Hydropericardium syndrome predominantly affects commercial broiler chicks of either sex in 3 to 5 weeks age group (Abdul- Aziz and Al-Attar, 1991; Gowda and Satyanarayana, 1994 and Kumar *et al.*, 1997), but it has also been reported sporadically in layers and breeder pullets aged between 2 and 20 weeks (Cheema *et al.*, 1989; Asrani *et al.*, 1997; Shukla *et al.*, 1997). All broiler strains of chicken are equally susceptible under field conditions (Akhtar and Cheema, 1990).

Gowda (1994) investigated the broiler birds in HPSV infection in Jammu and Kashmir and reported 10-80% mortality in 3 – 5 weeks old broilers during the course of the disease.

Khan *et al.* (1995) reported that Hubbard strain is more susceptible to HPSV than Indian River or Lohmann strains of broilers.

Naeem and Akram (1995) reported the outbreak of HPS in a pigeon flock with continuous heavy mortality for three weeks. They observed typical lesions of HPS in affected birds on post-mortem examination.

Abdul- Aziz and Hasan (1995) studied the disease in experimental infection of HPSV. They observed 100 per cent mortality by intramuscular and 30 per cent mortality by oral route of inoculation of liver homogenate from HPS affected cases.

Kumar *et al.* (1997) described the HPSV infections with 30 – 80% mortality in broilers.

Dalal (2000) experimentally produced HPS in turkey poults and reported that they are highly susceptible to the disease.

Roy *et al.* (2004) experimentally studied hydropericardium syndrome (HPS) in two different synthetic lines of chickens, white (B1) and coloured (B2) plumage broilers in their 3rd and 4th weeks of age. B1 broilers were found more susceptible and mortality ranged from 92.3% to 97%. B2 broilers were less susceptible and mortality ranged from 15.7% to 23.3%.

2.3 Etiology:

Hydropericardium Syndrome in poultry was initially considered to be caused by poisonous food additives, mycotoxins or phycotoxins, chemical or drug poisoning or infectious agents (Qureshi, 1988). The Rancid fat, rancid fishmeal, imbalances in vitamins and minerals in the feed were also assumed to be responsible for the disease (Jaffery, 1988; Qureshi, 1989).

Khawaja *et al.* (1988) tentatively suggested the etiology of HPS as an adenovirus after isolation of agent from liver, heart and kidneys

Qureshi (1988) reported that HPS was observed in chicks fed on all types of diet. He reported that the disease was progressed despite of implementing the control measures by addition or elimination of different ingredients in diet.

Ahmad *et al.* (1989); Anjum *et al.* (1989) and Muneer *et al.* (1989) reported the role of feed additives and toxic compounds in causation of HPS

Hasan (1989) reported that HPS was probably caused by avian adenovirus among the infectious agents.

Anjum *et al.* (1989) investigated on 40 HPS affected commercial broiler farms to determine the possible role of chicks, management, feed, salts and water, vaccines and vaccination schedule, antibiotics, growth promoters and coccidiostats in the emergence of the disease. They proposed that there was no correlation with these factors in the occurrence of disease.

Afzal *et al.* (1991) reported that co-infection with other infectious agents along with adenovirus may be required for the production of the clinical signs of the disease syndrome.

Gowda and Satyanarayana (1994) experimentally reproduced HPS by inoculation of liver homogenate from infected birds. They observed intranuclear inclusions in the hepatocytes of the infected birds and described the role of adenovirus in etiology of this disease.

Naeem *et al.* (1995) isolated the agent of HPS, propagated it in chicken embryo liver cell culture, adapted it in chicken embryos and reproduced the disease in susceptible birds. They proposed that the disease was associated with fowl adenovirus serotype 4.

Chandra *et al.* (1997) demonstrated the intranuclear adenovirus particles measuring 80-90 nm in diameter, in infected hepatocytes by transmission electron microscopy.

Jadhao *et al.* (1997) isolated the virus in chicken embryo liver cell culture and grouped as fowl adenovirus type 4.

2.4 Fowl Adenoviruses :

The family Adenoviridae is divided in to four genera Mastadenoviridae, Aviadenoviridae, Atadenoviridae and Siadenoviridae. Fowl adenoviruses belong to Aviadenovirus and they share a common group specific antigen (Kawamura *et al.*,1964). There are atleast 12 fowl adenoviruses which are grouped on the basis of neutralization test (Cowen and Naqi, 1982).

Egg Drop Syndrome virus which was earlier classified under genus Aviadenovirus has now been shifted to new genus Ataadenovirus. This virus share antigen cross-reactivity with fowl adenoviruses (Mcferran, 1991). The virus is resistant to lipid solvent, acidic PH and is heat stable at 56 °C (Ytes and Fry, 1957).The virus is resistant to trypsin, 2% phenol, 50% ethyl alcohol (Kawamura *et al.*, 1964).

Adenovirus named after the original source of the tissue, adenoid in which prototype virus strain was discovered (Benko and Harrach, 2003), are non enveloped virus particles containing a linear double stranded DNA genome. Avian adenovirus are very diverse group of pathogen causing a variety of problem to poultry production, routinely avian adenovirus classified in three groups mainly-Egg Drop syndrome 76 in hens (group III), Haemorrhagic enteritis in turkeys (group II), and Marble spleen disease of pheasants (group I). Fowl adenoviruses are widely distributed in poultry flocks (Monreal, 1992).

2.5 Characteristics of Fowl Adenovirus:

2.5.1 Morphology :

Adenovirus are non-enveloped double stranded DNA virus with internal protein organized in a core and surrounded by icosahedral capsid which is composed of 252 capsomere, 240 are hexon and 12 are penton at vertices of icosahedral. The penton base carry projection known as fibers (Ginesber *et al.*, 1966). The fiber is the structure protein which plays an important role in adenovirus infection by attaching the virus to the host cell by recognizing primary cellular receptors. All the fowl adenoviruses have 2 fibers of same length on each vertex penton except fowl adenovirus serotype 1 in which two fibers are of different length while mammalian adenoviruses have only a single fibers per penton base (Gelderblom and miachle-luppe, 1982).

The hexon composed of the 60S mass of virion (Monreal, 1992) and carry specific antigen which were designated as alpha (Allison *et al.*, 1960; Pereira, 1960) similarly, antigen present on the penton was

designated as Beta and antigen on fiber is designated as Gamma. Fiber antigen Gamma, present on the head is a type specific (Norrby, 1969), responsible for haemagglutination activities. Fiber antigen has been reported to induce type specific neutralizing antibodies.

Hexon antigen induces a group and type specific neutralizing epitopes on adenovirus. Hexon has been partially mapped to the loop 1 (L1) of the native protein (Toogood *et al.*, 1992).

2.5.2 Viral Genome :

Fowl adenovirus genome consist of a linear double stranded DNA of molecular weight of 30,000,000 dalton with inverted terminal repetition of 20 – 200 bp. G+C contents of 54-55 % and a virus coded 55 KDa protein attached to the 5' end of each DNA strand (Alestron *et al.*, 1982).

Zsak and Kisary (1984) reported that the fowl adenovirus genome is 43 – 45 kb in size which is significantly larger than its human equivalent (

Sheppard *et al.* (1995) identified the gene for the major capsid protein (Hexon) of fowl adenovirus serotype 10. The hexon genes of fowl adenovirus serotype 10 was found to have an ORF of 1808 bp, coding for 936 amino acids with molecular mass of 105.5 KDa.

Chiocca *et al.* (1996) sequenced CELO genome and found G+C content of 54.3 %.

Barua (2002) sequenced full length hexon (2916 bp) of Indian isolates of fowl adenovirus serotype 4. The average genome size of fowl adenovirus serotype 4 was found to be 45.99 Kb.

2.5.3 Viral Proteins:

Valentine and Pereira (1965) reported that the major structural proteins are the hexon and fibers which is noncovalently linked to a structure named as penton.

Norrby (1969) reported that the hexon protein is the major capsid protein of the enveloped icosahedral virion on which type, groups and sub groups specific determinants are located.

Roberts *et al.* (1986) reported that hexon protein consist of trimer. polypeptide II and central core VI, VII and IX are minor polypeptide also associated with the hexon and thought to be involved in stabilization and or assembly of particles. The crystallographic investigation of protein led to the establishment of it's three dimension structure .

Roberts *et al.* (1986); Toogood and hay (1988) and Toogood *et al.* (1989) reported that the major amino acid changes whether there be deletion, insertion or mutation are concentrated more frequently in the region forming three triangular projection that form the exposed surface of the capsid. The triangular projection or tower consists of three intertwined loops (L1 and L2 and L3) that are not conserved therefore allowing the various serotypes to present different surface while maintaining the necessary conservation to all the hexon-hexon contacts that are necessary for the capsid formation. The C3 region is used to stabilize the interface between P1 and P2, thus is not the part of the exposed hexon surface. It has a higher degree of amino acids sequence conservation than is found in the other loop region.

Toogood and Hay (1988) and Toogood *et al.* (1989) demonstrated the model of hexon protein. According to this model hexon are homotrimer structure that give rise to pseudo-hexagonal shape with a triangular top superimposed on the base. The pseudo-hexagonal base has been shown to composed of three copies of each of the two more conserved pedestal regions P1 and P2.

Hess *et al.* (1995) reported that penton are more complex consists of a pentamer of polypeptide III, besides five molecules of III a are also associated with the pentone base. The penton protein has a toxin like activity. Purified penton cause cytopathic effect in the absence of any other virus

components. From each of 12 vertices a trimeric fiber protein extend and is responsible for the recognition and binding to the cellular receptors .

2.5.4 Physicochemical properties:

Khawaja *et al.* (1988); Ravi Kumar *et al.*, (1997) reported that the causative agent of HPS is filterable through seitz filter and membrane filter of 0.1 µm average pore diameter.

Khawaja *et al.* (1988) reported that the virus has also been reported to agglutinate red blood cells similar to adenovirus serotype 1 but this property has not been confirmed by other investigators.

Afzal *et al.* (1991) noticed that the agent withstands heating at 60°C for 30 min or 50°C for 1 h. However, heating at 60°C for 1 h, 80°C for 10 min or 100°C for 5 min inactivated the virus. They also found that Treatment with either 5 percent chloroform or 10 percent ether appears to be partially detrimental to the causal agent and the sensitivity of the agent is variable from pH 3 and pH 10 .

2.5.5 Clinical Disease:

Fowl Adenovirus are known to cause many disease condition viz respiratory infection, hepatitis, problem in egg production etc. in chicken of all groups (Fadly *et al.*, 1973; Rosenberger *et al.*, 1974; Mcferran *et al.*, 1976). Among these manifestation Egg drop syndrome in layers and inclusion body hepatitis in young broiler chickens are frequently encountered (Rosenberger *et al.*, 1974; Macferran *et al.*, 1976). Even though various serotypes of fowl adenovirus produces inclusion body hepatitis in broiler chickens but inclusion body hepatitis along with hydropericardium syndrome producing virus is popularly called as “leechi heart disease” in broilers.

Shafique and Shakoori (1994) studied on the transmission of HPS in broiler and layer chicks kept on the infected litter and reported that the mortality was higher in broilers than in layers, which may be due to factors associated with the increased growth rate of broilers.

Avian adenovirus was first reported by Yates *et al.* (1954) from chicken embryo and inclusion body hepatitis by Helmboldt and frazier (1963) in USA, since then the disease has emerged in various countries viz Mexico, Peru, Chile, Iraq, Russia, Japan. Hydropericardium was first reported from Angara Goth at Karachi, Pakistan in 1987 (Khawaja *et al.*, 1988) and showed that Hydropericardium syndrome is more recent disease than inclusion body hepatitis. In India disease was first reported in Jammu and Kashmir, Punjab and Delhi in 1994 (Gowda and styanarayana, 1994) and thereafter throughout India (Asrani *et al.*, 1997).

The etiological agent for inclusion body hepatitis hydropericardium was diagnosed to be an adenovirus as it could be propagated via yolk sac route in chicken embryos and in chicken kidney cell, producing inclusion bodies in kidney cells and agglutinating RBCs of rats (Khwaja *et al.*, 1988). The causative agent of hydropericardium hepatitis syndrome has been identified as fowl adenovirus serotype 4 (Jadhao *et al.*, 1997).

2.5.6 DIAGNOSIS:

The disease can be diagnosed on the basis of clinical signs, gross lesions and histopathological lesions (Gowda and satyanarayana, 1994).

Adair *et al.* (1980); Grimes and king (1977) and Monreal *et al.* (1980) reported that Group specificity with immunoflouresence assay could determine the exact serotype.

Oberoi *et al.* (1996) and Saifuddin and Wilks, (1991) performed diagnosis of HPS disease by employing serological test such as gel diffusion, indirect haemagglutination, immunoperoxidase and enzyme linked immunosorbent assay which are considered to be specific and reliable.

Ganesh *et al.* (2001) demonstrated adenovirus particles in the nucleus of infected cells by transmission electron microscopy and it is helpful in confirmation of disease.

Molecular biology techniques such as polymerase chain reaction, nucleic acid hybridization, restriction enzyme analysis and DNA sequencing can also be used for early and quick diagnosis.

2.6 Isolation of HPSV:

Abe *et al.* (1998) isolated Group I adenovirus (serotype 4) from liver samples of adult broiler breeders and broiler chicks with HPS.

Toro *et al.* (1999) isolated three (341, 344, and 215) fowl adenovirus (FAV) obtained during 1996-97 from field outbreaks of inclusion body hepatitis/ hydropericardium syndrome (IBH/HPS) affecting broilers and broiler breeders in Chile and these isolates were characterized by virus neutralization tests (VNTs) and restriction enzyme analysis of a DNA fragment.

Ganesh *et al.* (2002) isolated fowl adenovirus serotype 4 from infected liver tissues from field outbreaks and reproduced disease experimentally in broiler chicks.

Dahiya *et al.* (2002) isolated fowl adenovirus (FAV) from 3-to-6-wk-old broilers in 45 different flocks of Haryana, India, with mortality ranging between 10% and 30%. Fowl adenovirus (FAV) was isolated from one of the affected flocks by chicken embryo liver cell culture.

Kumar *et al.* (2003) isolated hydropericardium syndrome virus in chicken embryo liver cell culture and performed serological characterization of HPSV.

Kim *et al.* (2008) isolated the several adenoviruses from the outbreaks of hydropericardium syndrome (HPS), caused by fowl adenovirus serotype 4 (FAdV-4), occurred in Korea and caused severe economic loss due to mortality and weight loss. From these outbreaks, several adenoviruses were isolated and identified in samples from broilers, layers, breeders, and native Korean fowl.

2.7 Revival of HPSV in experimentally infected broilers:

To find out the etiology of HPSV, it has been reproduced by inoculation of bacteria free liver homogenate from affected birds (Ahmad *et al.*, 1989; Anjum *et al.*, 1989 and Chisti *et al.*, 1989).

Khawaja *et al.* (1988) and Cheema *et al.* (1989) reproduced the disease by inoculation of a bacteria free liver homogenate to the susceptible chicks and demonstrated its infectious nature.

Gowda and Satyanarayana (1994) experimentally reproduced HPS by inoculation of liver homogenate from infected birds. They demonstrated intranuclear inclusion in the hepatocytes of the infected birds and described the infectious nature of the disease in general and the role of adenovirus in particular.

Naeem *et al.* (1995) purified the HPSV, propagated it in chicken embryo liver cells, passaged it in chicken embryos and reproduced the disease in susceptible birds. They observed symptoms and lesions in experimentally infected birds characteristic to fowl adenovirus serotype 4 as the causative agent of HPS.

Dubey *et al.* (2009) experimentally reproduced HPS in 3 week old broiler chicks by inoculation of bacterial free liver homogenate from infected birds and reported the similar incubation period.

2.8 Identification of disease:

2.8.1 Clinical signs:

In natural outbreaks of HPS, the affected birds do not exhibit any clinical sign (Jaffery, 1988) other than sudden heavy mortality (Ravi Kumar *et al.*, 1997). However, in the terminal stages, the birds become dull and depressed and showed a characteristic posture with their chest and beak resting on the ground with closed eyelids (Asrani *et al.*, 1997). Birds inoculated experimentally by the subcutaneous route declined eating and drinking, become debilitated, showed ruffled feathers, reluctant to move and die 60 h PI (Muneer *et al.*, 1989; Ravi Kumar *et al.*, 1997).

Dubey (2003) reported that incubation period of the disease ranged from 48-120 hrs in experimentally induced HPS cases via subcutaneous route of infection.

Shafiq *et al.* (1993) reported higher mortality and severity of disease in colichine-treated chicks.

Shukla *et al.* (1997) demonstrated the role of infectious bursal disease agent in precipitating HPS in layer flocks.

2.8.2 Gross pathology:

Khan *et al.* (1988); Anjum *et al.* 1989 and Cheema *et al.* (1989) found that the heart appears flabby and its cone is found floating in the pericardial sac.

Qureshi (1988) observed deposition of urates in the kidney tubules and uerters.

Anjum *et al.* (1989); Sreenivas Gowda and Satyanarayana (1994) and Mazaheri *et al.* (1998) reported that the most predominant and consistent lesions from the field cases of HPS included accumulation of fluid in the pericardial sac.

Cheema *et al.* (1989); Sreenivas Gowda and Satyanarayana (1994) and Ravi Kumar *et al.* (1997) reported that The pericardial fluid may be clear, watery/jelly like, straw/amber or green coloured, ranging from 3 – 20 ml in quantity with a pH of 7.0.

Anjum *et al.* (1989); Abdul- Aziz and Hasan (1995) and Ravi Kumar *et al.* (1997) reported that HPSV infected liver is pale yellow, swollen, friable and mottled with large areas of focal necrotic patches.

Anjum *et al.* (1989); Gowda and Satyanarayana (1994); Asrani *et al.* (1997) and Ravi Kumar *et al.* (1997) observed HPSV infected birds showing edematous and congested lungs and pale yellow, swollen and friable kidneys.

Anjum *et al.* (1989) reported yellowish discolouration of subcutis and abdominal fat in 20-30 percent of affected birds.

Anjum (1990) reported enlarged bursa of fabricious after experimental infection of broiler birds.

Gowda and Satyanarayana (1994) observed the congested intestinal blood vessels.

Asrani *et al.* (1997) found that HPSV infected pale liver containing petecheal and echymotic haemorrhages may also be observed in some birds and also noticed a consistent bursal and thymic atrophy in 10 percent of the total birds examined.

Asrani *et al.* (1997) and Dimri (1997) found that pericardial fat may exhibit yellowish discolouration with petecheal haemorrhages.

Dimri (1997) and Ravi Kumar *et al.* (1997) observed some enlarged bursa of Fabricious in some of the birds.

Nakamura *et al.* (2002) also reported pin point white foci in the pancreas and ventricular erosions.

Lakra (2008) reported prominent gross lesion in the heart and appear cone like with pointed tips along with accumulation of clear watery fluid in the pericardial sac. The pericardial fluid ranged from 10-15 ml. The multifocal haemorrhage was observed on the epicardium. The liver was pale yellow, swollen and enlarged and contains ecchymotic as well as pin point hemorrhages.

2.8.3 Histopathology:

Anjum *et al.* (1989); Cheema *et al.* (1989); Sreenivas Gowda and Satyanarayana (1994); Ravi Kumar *et al.* (1997); Basher *et al.* (1999) and Nakamura *et al.* (1999) performed histopathology of HPSV infected liver and observed focal areas of necrosis and mononuclear cellular infiltration in HPSV infected liver .

Cheema *et al.* (1989); Sreenivas Gowda and Satyanarayana (1994); Abdul- Aziz and Hasan (1995); Asrani *et al.* (1997) and Ravi Kumar *et al.* (1997) observed that the hepatocytes were shrunken in some areas and had pyknotic nuclei. Scattered hepatocytes always contain basophilic intranuclear inclusion bodies, surrounded by a clear halo or filling the entire enlarged nucleus.

Anjum *et al.* (1989); Ravikumar *et al.* (1998); Basher *et al.* (1999) and Thakur and Grewal (1999) reported that the affected heart exhibit infiltration of mononuclear cells heterophils and massive haemorrhage

Cheema *et al.* (1989) reported severe vascular changes such as calcification and presence of proteinaceous material between fibers.

Cheema *et al.* (1989); Asrani *et al.* (1997) and Ravi kumar *et al.* (1998) observed congestion and inflammatory cell infiltration in lungs with haemorrhagic exudates in the bronchi and alveoli.

Abdul- Aziz and Hasan (1995) observed centrilobular and diffuse degeneration and necrosis of hepatocytes. The degenerative changes consisted of swelling of hepatocytes with partial clearing of the cytoplasm or rupture of the cell membrane.

Abdul- Aziz and Hasan (1995) however, in experimental HPS infection did not observe any changes in the heart and lungs.

Dimri (1997) observed that Massive edema leads to disruption and separation of muscle bundles and fibers.

Asrani *et al.* (1997); Sreenivas Gowda and Satyanarayana (1994) and Surveshe *et al.* (1996) observed myocardial arteries and revealed vacuolar degeneration in the intimal layer and tunica adventitia and endothelial proliferation of blood vessels and also observed Zenker's degeneration of heart muscles with eosinophilic and vacuolated cytoplasm and interstitial edema as prominent lesion.

Abe *et al.* (1998); Thakur and Grewal (1999) and Nakamura *et al.* (2000) noticed that intranuclear eosinophilic inclusion bodies were also seen with halo but these were fewer in number than basophilic type. These inclusion bodies were frequently noticed around the necrotic foci of hepatocytes.

Abe *et al.* (1998) noticed that the interlobular interconnective tissue show edematous loosening and lobule have a parenchymatous appearance caused by marked increase of macrophages in the air and blood capillary areas.

Nakamura *et al.* (1999) observed that intranuclear inclusion bodies were also reported from the glandular epithelium cells of gizzard, the epithelium of glandular lobules of proventriculus, the epithelial cells of duodenum and caecum and kidney of one day old chicks inoculated with 10 percent liver homogenate from broiler chicks or broiler breeder hens affected naturally with HPS. However, neither

inclusions nor lesions were reported from these organs in older chicken and also noticed mild serous exudation in the submesothelial tissues of epicardium of the chicken.

Thakur and Grewal (1999) observed intranuclear basophilic inclusions in the alveolar and the bronchial epithelial cells of the secondary bronchi.

Rani *et al.* (2000) reported interstitial edema, catarrhal inflammation of parabronchi and goblet cells hyperplasia.

Nakamura *et al.* (2000) have also reported basophilic intranuclear inclusion bodies without halo.

Dubey *et al.* (2009) performed histopathology of HPSV infected tissues and reported degeneration and necrosis of hepatocytes as well as the presence of basophilic intra nuclear inclusion bodies surrounded by a clear halo. In kidneys congestion and haemorrhages, which ranged from mild to moderate degree were observed.

2.8.4 Detection of HPSV by AGPT:

Cowen (1987) reported that the sensitivity of AGPT can be increased by using antigen pooled from different Fowl adenovirus serotypes.

McFerran and Smyth (2000) reported that the AGPT has a satisfactory sensitivity when performed on serum from commercial flocks that are often exposed to multiple adenovirus serotypes, but it is less sensitive in detecting adenovirus infections in specific-pathogen-free (SPF) flocks.

Chandra *et al.* (2000) reported that fowl adenovirus serotype 4 can be detected by serological tests, such as agar gel diffusion, counter immunoelectrophoresis, indirect haemagglutination and ELISA.

McFerran and Adair (2003) reported that the agar gel precipitation test is a widely used serological test for the detection of Fowl adenovirus antibodies, since it is fast and economical. They observed lower sensitivity of this test for detection of Fowl adenovirus antibodies.

Dubey (2003) performed AGPT by double immunodiffusion technique, the hyperimmune serum raised against liver homogenate extract showed two precipitin lines against infected liver homogenate (20% w/v) after 24 hrs of incubation with no increase in number of lines on prolonged incubation at 37°C. however sharpness of precipitin lines got increased when the plates/slides were kept overnight at 4°C.

Lakra (2008) detected HPSV by AGPT using hyper immune sera raised in the birds and 20% of liver homogenate extract of infected birds as antigen. Single precipitation line was developed after 24 hrs of incubation at 37°C which confirmed the presence of HPSV in liver sample. She advocated the use of AGPT for confirmation of the virus involved in HPS.

2.8.5 Transmission Electron Microscopy:

Carl *et al.* (1956) performed Electron microscopy of HeLa cells infected with adenoviruses (types 1–4) and found Clusters of virus-like particles were found within the nuclei of infected cultures but not in those of uninfected controls. The particles were often arranged in rows as if in crystalline formation. Maximal diameter of particles was approximately 65 nm, and internal bodies were demonstrated.

Chandra *et al.* (1997) demonstrated adenoviral particles in the nucleus of infected cells by transmission electron microscopy.

Abe. *et al.* (1998). performed electron microscopy in different age groups of broilers and demonstrated numerous viral particles (65-70 nm in diameter) were demonstrated in the intranuclear

inclusions of hepatocytes. Group I adenovirus (serotype 4) was isolated from liver samples of adult broiler breeders and broiler chicks with HPS.

Ganesh *et al.* (2002) conducted electron microscopy in Hydropericardium hepatitis syndrome (HHS) infected birds. The virus from infected livers was purified and confirmed by electron microscopy and by experimental infection.

Kumar *et al.* (2004) performed electron microscopy in poultry birds during outbreaks of hydropericardium syndrome in a private poultry farm in Haldwani area of Uttaranchal and observed that intranuclear adenovirus like particles were distributed in paracrystalline arrangement demonstrated in hepatocytes.

Kumar *et al.* (2006). Performed electron microscopy in experimental infection of hydropericardium syndrome and Ultrastructurally numerous large vacuoles, fragmented endoplasmic reticulum and swollen mitochondria in the cytoplasm of hepatocytes and myocytes at 36 hours post inoculation (PI) in electron microscopy. The infected nuclei were irregularly outlined and contained large intranuclear masses. Virus particles were found attached with these masses. At higher magnification, virus particles appeared isometric and roughly spherical between 75-80 nm in diameter.

Kim *et al.* (2008) carried out the electron microscopy in Korean fowl during outbreaks of hydropericardium syndrome in Korea and observed adenovirus particles in the nucleus of hepatocytes.

2.9 Extraction of viral DNA:

Shinagawa *et al.* (1983); Clavijo *et al.* (1996) and Mazaheri *et al.* (1998) developed a sensitive method for extraction of adenoviral DNA from infected cells using protein bound nature of viral DNA from the purified virus preparation or cell culture for use in restriction enzyme analysis.

Capua *et al.* (1995) demonstrated extraction of DNA from viral suspension initially treated with SDS and proteinase k and subsequent extraction with phenol and chloroform.

Jadhao *et al.* (2000) demonstrated a method of DNA isolation from initial small volume of fowl adenovirus infected cultures or allantoic fluid .The method yielded DNA pure enough for restriction enzyme digestion beside providing satisfactory yield of DNA from a small volume of adenovirus infected culture.

2.10 Molecular Charecterization of HPSV:

Polymerase Chain Reaction And Restriction Fragment Length Polymorphism :

Zsak and Kisary (1984) analyzed fowl adenovirus DNA using *Bam* III and *Hind* III enzymes and placed recognized serotypes of fowl adenovirus in to five groups designated A to E.

Erny *et al.* (1995) reported about the genome of fowl adenovirus serotype 4 and 10 using nine different restriction enzymes *Hind* III, *Bgl* II, *Dra*I, *Nae*I, *Xba*I, *Not*I, *Sfi*I and *Sma*I and found that although these serotypes were similar on cross neutralization and cross protection restriction enzyme analyses but also revealed few differences.

Capua *et al.* (1995) studies restriction profile of avian adenoviruses isolated from Psittacine birds using *Bam* III and *Hind* III restriction enzymes and concluded that isolates were similar to fowl adenovirus serotype 3 in chicken.

Clavijo *et al.* (1996) mapped the genome of fowl adenovirus serotype 8 using *Not* I ,*Apa* I, *stu* I, *Xba*I, *Eco*RI, *Kpn* I and *Hind* III restriction enzymes and found differences in physical map of fowl adenovirus serotype 8 and fowl adenovirus serotype 1 and fowl adenovirus serotype 10.

Rue and Hess (1998) reported the implementation of the fowl adenovirus polymerase chain reactions and combined them with restriction enzyme analysis for detection and differentiation of all 12 fowl adenovirus serotypes and Egg Drop Syndrome virus. By using two sets of primers (H1/H2, H3/H4) hybridizing in three conserved regions of hexon gene, they concluded that polymerase chain reaction and restriction enzyme analysis were suitable to detect all avian adenoviruses infecting chickens and to distinguish all 12 fowl adenoviruses reference strains and to differentiate fowl adenoviruses from Egg Drop Syndrome viruses. Egg Drop Syndrome viral DNA was found to be negative with these primer pairs. Restriction enzyme analysis of amplified fragment with *Hae* II and *Hpa* II differentiate most of the fowl adenoviruses. H5/H6 primer combination only amplified fragments from Egg Drop Syndrome virus DNA and do not detect any of the fowl adenovirus serotype.

Ganesh (1998) amplified HHS virus specific 0.7 kb DNA from the purified viral DNA and extracted DNA directly from the HHS positive liver samples using the FAVH (L) and FAVH (R) primers (Table 1) from the consensus region of the fowl adenovirus to hexon gene and reported about the 728 bp polymerase chain reaction amplified DNA, labeled and used as a probe for the detection of virus by dot hybridization of viral genome.

Hess *et al.* (1999) reported that fowl adenoviruses could be detected and typed using polymerase chain reaction combined with restriction enzyme analysis. The value of this combination has been demonstrated useful in detection of several fowl adenovirus isolated from clinical cases of infectious hydropericardium.

Xie *et al.* (1999) developed an avian adenovirus specific polymerase chain reaction. The oligonucleotide primers MK 89 and MK 90 designed from the sequence data of fowl adenovirus serotype 1 and amplified an avian adenovirus specific 421 bp DNA product.

Jiang *et al.* (1999) reported a polymerase chain reaction for the detection of a number of different fowl adenoviruses isolates. The primers FAd-1 and FAd-2 were designed to amplify the fragment of viral DNA in both FAV-8 and CELO virus genome. These primers amplified a 1406 bp polymerase chain reaction product for FAV-8 and 1273 bp for CELO virus and concluded that polymerase chain reaction has high specificity and sensitivity for the detection of FAV-8 and CELO virus.

Jadhao *et al.* (2000) purified and distinguished avian adenovirus serotype 1 and avian adenovirus serotype 4 and Egg drop syndrome 76 virus genome with Hind III restriction enzyme.

Dhaiya *et al.* (2002) isolated fowl adeno virus from affected flocks in chicken embryo liver cell culture and confirmed isolated virus as fowl adenovirus serotype 4 by serum neutralization test and Polymerase chain reaction assay coupled with restriction enzyme analysis.

Ganesh *et al.* (2002) reported the isolated DNA either from infected liver or purified virus when subjected to polymerase chain reaction using hexon gene specific primer amplified at 700 bp section of the variable region of viral DNA. This PCR amplified DNA when labeled with a digoxigenin (*DIG II, DUPT*) can 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 adeno virus isolates from suspected cases of 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 resulting into fragments approximately 1219 and 1319 bp respectively. *Hae* II digestion of the H1/H2 polymerase chain reaction products and *Hpa* II digestion of H3/H4 PCR products characterized all the isolates in fowl adenovirus groups.

Rahul *et al.* (2004) analyzed eight fields isolates of IBH-HPS by amplification of hypervariable regions of hexone gene of adenovirus.

2.11 PREVENTION AND CONTROL:

Khawaja *et al.* (1988) reported that vaccination of birds against infectious bursal disease did not affect the outcome of disease, as vaccinated birds were equally susceptible to the adenovirus isolated from HPS infected birds as non-vaccinated chicks.

Abdul-Aziz and Al-Attar (1991) reported that the addition of 2.5% iodophor disinfectant solution in drinking water @ 0.07-0.1% has been found to be very effective in reducing the severity and course of HPS in broiler chicks.

Akhtar *et al.* (1992) reported that epidemiological studies on the factor associated with the development and spread of HPS in Pakistan indicated that flocks visited frequently by vaccination crews were 15 times more likely to be affected than flocks not visited at all. The premises where chicken flocks were raised regularly were less likely to be affected than those premises where flocks were raised for the first time. This is probably due to the fact that experienced farmers are more cautious and disinfect their premises regularly. The use of electricity as a source of light and heat entailed much lower risk than the use of kerosene oil. Hence, proper disinfection of premises and equipments, restricted entry of visitors and vaccination crews and proper lighting and ventilation in the poultry houses play a significant role in preventing the disease.

Table 1. Description of primers :

FORWARD PRIMERS	REVERSE PRIMERS	SEQUENCE(5'-3')	PCR PRODUCT (bp)	ADENO VIRUS GENE	AUTHORS
MK 89		CCCTCCCACCGCTTACCA	421	FAV-1	Xie <i>et al</i> (1998)
	MK 90	CACGTTGCCCTTATCTTGC		hexon	
FAD 1		TACGATTACGGGGCTATGGA	1406	Pentone	Jiang <i>et al</i> (1999)
	FAD 2	TGTTCCGTCCACCGTAGG		based	
H1		TGGGACATGGGGCGACCTA	1219	FAV-1	Rue and Hess (1998)
	H2	AAGGATTGACGTTGTCCA		hexon	
H3		AACGTCAACCCCTTCAACCACC	1319	FAV-1	Rue and Hess (1998)
	H4	UGCCTGTGGCGAAAGGCG		HEXON	
H5		TTCTGTCACCGATAAAGGT	1901	EDS virus	Rue and Hess (1998)
	H6	AGTTATTCCAAATGGGCAT		hexon	
FAVH (L)		GACATGGGGTCGACCTATTTGACAT	728	FAV-10	Ganesh <i>et al</i> (2001)
	FAVH (R)	AGTGATGACGGGACATCAT		hexon	

CHAPTER-III

MATERIALS AND METHODS

Present study was conducted in the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anjora, Durg.

3.1 Materials:

3.1.1 Chemicals, Reagents and buffers:

Standard molecular biology grade chemicals were procured from reputed national and international firms viz Bangalore genei, Sigma chemicals (USA), Himedia laboratory (India), Qiagen (USA) and Sdfine chemical (India).

3.1.2 Glass wares:

Glass wares were thoroughly washed and sterilized as per the protocol recommended by manufactures. Glass wares were procured from Borosil (India).

3.1.3 Plastic wares:

Various types of plastic wares were used viz. Eppendorf tubes, PCR tubes, micropipette tips, centrifuge tubes etc. These plastic wares were procured from Tarsons and Himedia (India).

3.1.4 Experimental chicks:

Apparently healthy two weeks old broiler chicks were procured from Indian Broiler Farm, Rajnandgaon and reared in isolation under cage system. They were reared under optimum conditions of management and nutrition up to 3 weeks of age.

3.1.5 Hydropericardium syndrome virus:

The known Hydropericardium syndrome virus, in the form of infected liver samples stored in 50% glycerol saline, available at the Department of Veterinary Microbiology, College of Veterinary Science & A. H., Anjora, Durg was used in present study.

3.2 Epidemiological study:

Epidemiological features of the disease were recorded between November 2008 to February 2009 in Durg and Rajnandgaon areas of Chhattisgarh. A total of 7500 broiler birds up to five weeks of age were screened for the presence of disease (Table 2).

Table 2. Description of virus isolates :

Poultry farms	Isolates	Year of isolation	Flock size	Age and types of birds
Lokesh poultry farm, Thanod (Durg)	Durg 1	2008	2300	Broiler 3-5 weeks
Devangan poultry farm, Chandkhuri (Durg)	Durg 2	2008	1500	Broiler 3-5 weeks
Yadav poultry farm (Rajnandgaon)	Rajn 1	2009	1200	Broiler 4-5 weeks
Verma poultry farm(Rajnandgaon)	Rajn 2	2009	2500	Broiler 3-5 weeks

3.3 Collection of samples:

Liver samples were collected from broiler birds, showing the symptoms of hydropericardium syndrome *viz* depression, dullness and tucked in appearance.

The samples were collected in 50% glycerin saline and immediately transferred to the laboratory and stored at 4⁰C for isolation of fowl adenovirus serotype 4. Part of tissue samples were also stored at - 20⁰C for further use.

3.4 Preparation of samples:

The liver samples, stored in 50% glycerol saline, were rinsed three times in PBS (pH 7.2) and then triturated in sterilized pestle and mortar using sterilized neutral sand as abrasive to make a 20% (w/v) suspension. The tissue homogenate was frozen and thawed thrice and then centrifuged at 5,000 rpm for 15 min and supernatant was collected aseptically. After adding penicillin and streptomycin @ 1,000 IU / ml and 1,000 µg / ml, respectively, the suspension was incubated at room temperature for 30 min and then filtered through 0.22 µm sterile Millipore filter. The filtrate thus obtained was stored at 0°C. It was used as inoculums for experimental infection in three weeks old healthy broilers.

3.5 Revival of HPSV in experimentally infected broilers:

Filtered liver tissue suspension of known isolate of HPS infected broilers was inoculated at the rate of 0.5 ml in three week old healthy broilers. The experiment was carried out in 3 groups of 10 birds each inoculated by different routes *viz.* subcutaneous (S/C) route, oral route and intra ocular route as mentioned in table 3. Similarly groups of 5 broiler chicks each, were inoculated with sterilized phosphate buffer saline by S/C, oral, ocular route and considered as control. The birds were daily observed for the development of clinical signs like depression, dullness and tucked in appearance.

Table 3. Experimental inoculation of broiler chicks by different routes :

S.No	Route of inoculation	No. of birds inoculated	Dose
1	Subcutaneous	10	0.5 ml
2	Oral	10	0.5 ml
3	Intra ocular	10	0.5 ml

3.6 Preparation of hyperimmune sera:

The hyperimmune serum against HPSV was raised in 3-week-old broiler birds as per the procedure described by Lakra (2008). The inactivated inclusion body hepatitis (IBH) / hydropericardium syndrome (HPS) vaccine was used as source of antigen and hyperimmune serum was raised by inoculating inactivated vaccine.

The first dose of antigen was administered at zero day, whereas, second dose was administered at 7th day. The rest of doses were given as mentioned in Table 4. Final bleeding was done on 14th day of last injection and the serum was collected and tested for the presence of antibodies by AGPT. Sample was stored at 0°C for further use.

Table 4. Schedule for raising hyperimmune serum against HPSV

No. of Injections	Days	Dose	Route
I	0 day	0.5 ml	S/C
II	7 day	1.0 ml	S/C
III	14 day	1.0 ml	S/C
IV	21 day	2.0 ml	S/C
V	28 day	2.0 ml	S/C

3.7 Identification of disease :

3.7.1 Agar gel precipitation test (AGPT):

Agar gel precipitation test was performed according to the method of Cullen and Wyeth (1975) with slight modifications. Agar gel was prepared by dissolving 1 gm of noble agar in 100ml of normal saline containing 8% sodium chloride kept in a boiling water bath. About 4-5 ml of molten gel was poured on clean scratch free glass slides and 8 ml on small petriplates. The molten gel was allowed to solidify at room temperature and then kept at 4⁰C for 30 min. There after, 3 wells arranged in triangular fashion were punched in the petriplates at a distance of 3 mm and bottom of the well were sealed with molten agar gel.

The central well was filled with hyperimmune serum and peripheral wells were filled with infected and uninfected liver homogenate (20% w/v). The petriplates were kept in moist chamber, and

incubated at 37°C for 24 hrs and examined for the precipitin lines. After 24 hrs, the petriplates were transferred to refrigerator at 4°C for further precipitation of lines.

3.7.2 Histopathology of tissue sections:

Three week old broiler birds (n=5) were inoculated with HPSV infected liver tissue homogenate. After development of disease birds were sacrificed and various tissues like kidneys, heart and liver were collected for the demonstration of histopathological changes after routine Haematoxylin and Eosin (H and E) staining of paraffin embedded tissue sections, following the method of Lillie (1965) with slight modifications. The tissues fixed in 10% buffered formalin saline were washed overnight in running tap water and then dehydrated for 1 hr each in 50%, 70%, 80%, 90%, 95% and absolute ethyl alcohol. The tissues were then cleared in xylene and embedded in paraffin wax. Thereafter, sections of 4-6 µm thickness were cut and subjected to H and E staining as described in the flowchart –1. After staining, the slides were mounted with DPX and examined under light microscope.

3.7.3 Electron Microscopy :

Processing and staining of tissues for Transmission Electron Microscopy was done at All India Institute of Medical Sciences New Delhi. Livers samples of HPSV infected birds were collected and used for the demonstration of HPS Virus *i.e* FAV- 4 by electron microscope. Liver samples were trimmed to 1-2 mm cube and preserved in 2.5% gluteraldehyde and 2 % formaldehyde in 0.1 M phosphate buffer saline (pH 7.3) for 12 hrs at 4°C. After washing in buffer, the samples were postfixated in 1% OsO₄ for 2 hrs at 4°C. The samples were dehydrated in an ascending grade of acetone, infiltrated and embedded in araldite CY 212 (TAAB, UK). Thick Sections (1 µm) were cut with an ultramicrotome, mounted on to glass slides, stained with

aqueous toluidine blue and observed under a light microscope for gross observation of the area and quality of the tissue fixation. For electron microscope examination, thin sections of grey-silver colour interference (70-80 nm) were cut and mounted onto 300 mesh- copper grids. Sections were stained with alcoholic uranyl acetate and alkaline lead citrate, washed gently with distilled water and observed under a Morgagni 268D transmission electron microscope (Fei Company, Netherlands) at an operating voltage 80 kV. Images were digitally acquired by using a CCD camera (Megaview III, Fei Company) attached to the microscope.

3.7.4 Polymerase Chain Reaction :

Polymerase chain reaction was performed in the Eppendorf thermal cycler (Germany) and amplification of hexon gene was accomplished as per the method of Meulemans *et al.* (2001) with minor modifications.

3.7.4.1 Extraction of DNA from liver tissue:

Extraction of DNA from the infected liver tissue was carried out by using DNeasy blood and tissue kit (Qiagen) following manufacturer's recommendations.

3.7.4.2 Qualitative and quantitative assessment of extracted DNA at different time intervals suitable for PCR :

DNA was extracted from the HPS infected liver tissues at different time intervals using DNeasy blood and tissue kit (Qiagen). Liver tissues from infected birds were collected and preserved at -20°C for the extraction of DNA. Extraction of DNA was performed at 0 day (fresh tissue), 15, 30, 45 and 60 days. Two elutions of DNA of each tissue were taken and extracted DNA were stored at -20°C. The samples were collectively run in agarose gel electrophoresis and results were demonstrated by using gel documentation system.

3.7.4.3 Quantitation and quality assessment of extracted DNA :

Quality and purity of DNA was checked by Submarine Agarose Gel Electrophoresis using 0.8 % agarose in 1 X TAE (pH 8) buffer (Sambrook *et al.*, 1989). Ethidium bromide was added to final concentration of 0.5 µg / ml and mixed thoroughly.

The wells were charged with 5 µl of DNA (1:10 diluted) preparations mixed with 1 µl of 6 X gel loading dye solution. Electrophoresis was carried out at 5 v/cm (50 V) for 90 minutes at room temperature and DNA was visualized under U.V transilluminator.

Quality of DNA was calculated by Spectrophotometric method. O.D at 260 and 280 nm was taken in UV spectrophotometer with distilled water as reference.

Purity of DNA was checked on the basis of OD ratio of 260:280 nm. The samples with acceptable purity (1.7 – 2.0) were quantified. The DNA having ratio of 1.8 was considered as having good purity and used as template for the PCR. The concentration of DNA was estimated using the following formula.

$$\text{Concentration of DNA (mg/ml)} = \text{OD}_{260} \times 50 \times \text{dilution factor}$$

Where 50 is the concentration of ds DNA expressed in µg/ml at OD of 1.

3.7.4.4 Primers :

The oligonucleotide primers in hexon gene region was used in the study which was commercially synthesized by Bangalore genei (India). Primer pair used was designed based on the available sequence information of FAV- 4 (Meulemans *et al.*, 2001). The sequence of primer used in PCR is as described in table 5.

Table 5. Primer sequence :

Primer	Sequence (5' to 3')	Position	PCR Product (bp)	Reference
Forward primer	CAARTTCAGRCAGACGGT	144- 161	897	Meulemans (2001)
Reverse primers	TAGTGATGMC GSGACATCAT	1041- 1021		

3.7.4.5 Master mix composition:-

Polymerase chain reaction was carried out in a final reaction volume of 50 µl using 0.2 ml thin wall PCR tube. A master mix was prepared and aliquoted in 48 µl quantities in each PCR tube. Two µl sample of DNA was added in each tube to make the final volume of 50 µl as mentioned in Table 6.

Table 6. Composition of master mix for PCR :

S. No.	Components	Quantity	Final concentration
1	Nuclease free water	34 μ l	-
2	10 X Taq buffer	5.0 μ l	1X
3	MgCl ₂ (25 mM)	3.0 μ l	1.5 mM
4	dNTPs (10 mM each)	3.0 μ l	2.5 mM each
5	DNA Template	2.0 μ l	30 ng
6	Foreward Primer (125pmole/ μ l)	1.0 μ l	125 pm
7	Reverse Primer (125pmole/ μ l)	1.0 μ l	125 pm
8	TaqDNA polymerase	1.0 μ l	1 unit
	Total	50 μ l	

3.7.4.6 Standardization of cycle steps of PCR :

Standardization of PCR was based on the procedures described by Meulemans *et al.* (2001). Various components of the reaction mixture viz. MgCl₂, each primer, Taq polymerase and each nucleotide were optimized and DNA amplification was performed in an automatic thermal cycler in 50 μ l reaction mixture volumes in 0.2 ml thin walled PCR tubes. The denaturation (Td), annealing (Ta) and

extension temperature (Te) and their time period were tested over range to standardize the cycle steps as mentioned in Table 7.

Table 7. cycling conditions :

Cycling conditions				
Initial				Final Extension
denaturation	Denaturation	Annealing	Extension	
94°C X 5'	94°C X 5'	45°C X 2'	72°C X 1.30'	72°C X 2'
Repeated for 35 times				

3.7.4.7 Confirmation of PCR products :

The PCR products generated were confirmed for their expected size (897 bp) in 1 % (w/v) agarose gel in 1X TAE buffer as per the method of Sambrook *et al.* (1989) using horizontal submarine electrophoresis apparatus (Biometra, USA).

3.8 Restriction Endonuclease (RE) analysis :

The PCR products of 897 bp, generated by using genomic DNA from field isolates of HPSV were subjected to restriction endonuclease enzyme digestion using different restriction enzymes viz. *Bgl* I and *Mlu* I. Digestion of each RE enzymes was performed as per the manufacturer's protocol using specific assay conditions and the buffers supplied. The component used and its volume used for digestion is

given in Table No 8. Digestion was carried out with calculated DNA concentration and enzyme at 37°C for one hr.

Table 8. Component for RE analysis:-

Sr. No.	Component	Volume
1	Restriction enzyme (10 U / μ l)	1 μ l
2	PCR Product	3 μ l
3	10 X Compatible enzyme buffer	2 μ l
4	Nuclease free water	14 μ l
5	Total volume	20 μ l

The reaction was stopped either by adding loading dye or stored at – 20 °C until it was subjected to agarose gel electrophoresis. Digested products along with 100 bp DNA ladder marker were electrophoresed in 2 % (w/v) agarose gel in 1 X TAE buffer at 80 V for 3 hr using horizontal submarine electrophoresis apparatus. After completing the electrophoresis, the gel were examined on UV transilluminator and photographed by Gel Documentation System.

The molecular weights of various fragments of digested viral DNA were determined using Image analysis Software and expressed in base pairs (bp).

CHAPTER- IV

RESULTS AND DISCUSSION

HPS associated problems continued to be reported from all over the world. Due to widespread nature of the disease, the development of appropriate diagnostic techniques is going on to relieve the poultry industry from such devastating disease. Presumptive diagnosis of disease may be made on the basis of clinical signs, gross lesions *etc.* However, definitive diagnosis required isolation, identification and serotyping of the causative HPSV. Therefore present study was designed considering the above mentioned facts.

4.1 Epidemiological investigation:

The epidemiological features of the disease were recorded between November 2008 to February 2009 in Durg and Rajnandgaon areas of Chhattisgarh. A total of 7500 broiler birds, up to five weeks of age from four flocks were screened for the presence of disease. Affected birds showed depression, dullness and tucked in appearance followed by sudden death. The overall mortality up to 28 % was recorded in broiler flocks (Table 9). Dahiya *et al.*(2002) also reported mortality upto 10 to 30 % in young broilers of 3 to 5 weeks of age which is in agreement of present observation. Higher mortality in birds was also reported in immunosuppressed birds by Balamurgan and Kataria (2006). HPS predominantly affected commercial broiler chicks of either sex in 3 to 5 weeks age group (Abdul-Aziz and Al-Attar, 1991; Gowda and Satyanarayana, 1994 and Kumar *et al.*, 1997) but it has also been reported sporadically in layers and breeder pullets aged 2 to 20 weeks (Cheema *et al.*, 1989; Asrani *et al.*, 1997; Shukla *et al.*, 1997). During present investigation affected birds did not exhibit any clinical sign other than sudden death with heavy mortality, however in the last stages of disease the birds became dull and depressed and

showed a characteristic posture with their chest and beak resting on ground with closed eyelids. Similar clinical signs were also reported by Kumar *et al.* (1997). The postmortem examination revealed accumulation of clear straw coloured fluid in pericardial sac, discoloured liver and enlarged kidneys. Aforementioned clinico-pathological observations during the present study indicated involvement of hydropericardium syndrome virus. The occurrence of similar clinical features has also been reported by many other researchers (Cheema *et al.*, 1989; Ahmed *et al.*, 1989). The involvement of hydropericardium syndrome virus in disease has been reported from different parts of India (Bhowmik, 1996; Nighot *et al.*, 1996; Mahajan *et al.*, 1996 Asrani *et al.*, 1997; Ravikumar *et al.*, 1998; Chakravarti *et al.*, 2000; Dahiya *et al.*, 2002) and reports of incidence of disease from neighboring Madhya Pradesh state (Survashe *et al.*, 1996) further supporting the involvement of HPSV in present study.

Table 9: Mortality rates in broiler birds due to HPSV infection

Poultry flocks	Age and types of birds	Flock strength	Condition of disease	Percentage of mortality
Durg 1	Broiler 3-5 weeks	2300	IBH-HPS	19.80
Durg 2	Broiler 3-5 weeks	1500	HPS	25.13
Rajn 1	Broiler 4-5 weeks	1200	HPS	26.66
Rajn 2	Broiler 3-5 weeks	2500	HPS	28.40

4.2 Isolation of Hydropericardium syndrome virus:

Hydropericardium syndrome virus was isolated from infected liver of dead and moribund broilers from different regions of Durg and Rajanandgaon. Liver suspension (20% w/v) was inoculated to reproduce disease experimentally in 3 weeks old broilers birds. Purification of the virus was undertaken directly from the infected liver to avoid additional steps of adapting the virus to an indicator system. The presence of fowl adenovirus serotype 4 in the sample collected and nature of purified virus was confirmed by agarose gel precipitation test. Ganesh *et al.* (2002) also adopted similar procedure for isolation of fowl adenovirus from liver and reproduce the disease in young broilers. Adenovirus serotype 4 from liver samples in field outbreaks of inclusion body hepatitis/ hydropericardium syndrome (IBH/HPS) affecting broilers and broiler breeders has been isolated by several workers (Abe *et al.* 1998; Toro *et al.* 1999 and Kim *et al.* 2008) corroborate the finding of present study. However virus strain from the affected flocks has also been isolated in chicken embryo liver cell culture (Dahiya *et al.*, 2002; Kumar *et al.*, 2003).

4.3 Revival of HPSV in experimentally infected broilers:

Hydropericardium syndrome was experimentally reproduced in 3 weeks old broiler birds by inoculation of HPSV infected liver homogenate extract. Incubation period varied between 48 hrs and 60 hrs and by third day, 70-100% of the infected birds died (Table 10). The maximum mortality (100 %) was observed in birds inoculated *via* S/C route, whereas minimum mortality of 30 % was reported in intraocularly inoculated birds. The control birds did not exhibit any clinical signs. The mortality was seen after 48 hrs P.I. Dubey *et al.* (2009) also experimentally reproduced disease in 3 week old broiler chicks after inoculation of homogenate of liver collected from infected birds with similar incubation period. The infectious nature of HPSV infected liver suspension has been demonstrated by several workers (Khawaja *et al.* 1988; Cheema *et al.* 1989 and Gowda and Satyanarayana 1994) and

experimentally reproduced the disease in similar way (Ahmad *et al.*, 1989; Anjum *et al.*, 1989 and Chisti *et al.*, 1989). Clinical signs and lesions observed in present study were similar to those recorded in natural outbreaks. Anjum (1990), Afzal *et al.*, (1991) and Gowda and Satyanarayana (1994) reported that the clinical signs and death due to hydropericardium hepatitis syndrome were observed 2- 5 days after infection, further support the findings of present study. The results clearly indicated that S/C routes is better than other for experimental infection of broiler birds.

Table 10. Mortality pattern in experimentally infected broiler chicks with HPSV infected liver tissue homogenate extract (20 % W/V)

S.No	Route of inoculation	No. of birds inoculated	Dose	Mortality after post inoculation (in hrs)					Total mortality (%)
				36	48	60	72	84	
1	Subcutaneous	10	0.5 ml	0	2	6	2	0	10 (100)
2	Oral	10	0.5 ml	0	0	3	4	0	7 (70)
3	Intra ocular	10	0.5 ml	0	0	1	2	0	3 (30)

4.3 Identification of disease:

4.3.1 Clinical signs:

Thirty healthy broiler birds were procured for propagation of virus. The infected birds and their respective controls remained healthy without manifestation of any clinical signs up to 24 hrs PI. It was observed that all infected birds declined feeding and refused to drink water after 24 hrs PI. The birds became dull, depressed and showed characteristic posture with their chest and beak resting on the ground and with closed eyes (Fig 1). They became weak and reluctant to move and showed mortality after 48 hrs PI. Similar clinical signs were observed by several workers (Muneer *et al.*, 1989; Asrani *et al.*, 1997; Gowda and Satyanarayana, 1994). During present investigation mortality was found ranging from 9.8 % to 21.4%. Dahiya *et al.* (2002) also reported similar findings *i.e* 10-30% mortality in broiler birds whereas other workers reported the higher mortality rates ranging from 20% to 75% in various outbreaks in Pakistan (Khawja *et al.*, 1988; Anjum *et al.*, 1989; Cheema *et al.*, 1989). Transmission of hydropericardium syndrome virus in broilers and layers chicks kept on infected litter revealed that mortality occurred in broilers and layers chicks (Shafique and Shakoori, 1994). These observation are in agreement to the finding of present study. The present findings corroborate the observation made by Anjum (1990) and Ravikumar *et al.* (1997). They prepared liver homogenate extract from infected birds and inoculated by parental route in broiler birds. The birds developed sign of disease 48 hrs PI and showed mortality after 60 hrs PI and incubation period following oral inoculation was ranged from 7 to 15 days (Anjum *et al.*, 1989). Dubey (2003) also reported the incubation period of the disease ranged from 48-120 hrs in experimentally induced HPS cases *via* subcutaneous route of infection with mortality ranging from 60 to 72 hrs PI.

4.3.2 Gross Pathology:

Postmortem examination of experimentally infected birds showed most prominent gross lesion in the heart and cone like appearance along with accumulation of clear watery fluid in the pericardial sac as shown in fig. 2. The pericardial fluid ranged from 10-15 ml and the pH was about neutral. The multifocal haemorrhages were observed on the epicardium. The liver was pale yellow, swollen and enlarged and contains ecchymotic as well as pin point hemorrhages, lungs were oedematous and congested. Kidneys were pale yellow, swollen, friable contained urate deposits in the tubules and ureters with haemorrhagic foci. The bursa and thymus were atrophied. The intestinal blood vessels were congested. These observation are in agreement to the finding of Cheema *et al.* (1989) and Anjum *et al.* (1989) who observed hydropericardium in more than 90% of the affected birds with the flabby heart floating in the pericardial sac. Several workers found that pericardial fluid ranges from 3 to 20 ml in quantity (Gowda and Satyanarayana, 1994; Abdul-Aziz and Hasan, 1995; Asarani *et al.*, 1997; Ravi kumar *et al.*, 1997). Gowda and Satyanarayana, (1994) also reported the pH 7.0 of pericardial fluid and the friable/mottled, pale yellowish discoloration of liver with large areas of focal necrotic patches, petechial and ecchymotic hemorrhage, finding similar to the present study have also been reported by several workers (Anjum *et al.*, 1989; Cheema *et al.*, 1989; Gowda and Satyanarayana, 1994; Abdul-Aziz and Hasan, 1995). Anjum *et al.* (1989) and Cheema *et al.* (1989) reported oedematous and congested lungs which is in agreement of finding of present study. Abdul-Aziz and Hasan, (1995), Asrani *et al.* (1997), Anjum *et al.* (1989) and Cheema *et al.* (1989) reported pale yellow swollen, friable and haemorrhagic kidneys. These findings are in accordance to the present study. Asrani *et al.* (1997) also observed bursal and thymic atrophy in 10 % of birds. Gowda and Satyanarayana, (1994) observed congested intestinal blood vessels. Nakamura *et al.* (2002) reported pin point white foci in the pancreas and ventricular erosions. Dubey (2003) and Lakra (2008) also observed similar lesions in various organs.

4.4.3 Histopathology:

The histopathological features in the H and E stained sections from different organs of HPS infected birds showed the typical changes of HPS. In liver the characteristic histopathological changes included degeneration and necrosis of hepatocytes as well as the presence of basophilic intra nuclear inclusion bodies surrounded by a clear halo (Fig 3). Similar histopathological changes have been reported by several workers (Anjum *et al.*, 1989; Sreenivas Gowda and Satyanarayana, 1994; Abdul-Aziz and Hasan, 1995; Asrani *et al.*, 1997). The morphology of inclusion bodies was similar to those described by Hasan (1989); Abdul-Aziz and Al-Attar (1991), Ravikumar *et al.* (1998). Dubey *et al.* (2009) also reported degeneration and necrosis of hepatocytes as well as the presence of basophilic intra nuclear inclusion bodies surrounded by a clear halo. However few workers have also reported the presence of basophilic intra nuclear inclusion bodies without clear halo, instead filling the entire enlarged nucleus (Nakamura *et al.*, 2000). The degenerative changes encountered were fatty and cystic hepatocytic degeneration. In general all these findings are confirmative with the findings of several other workers (Khawaja *et al.*, 1988 and Afzal *et al.*, 1991). The changes in the kidney consisted of congestion and haemorrhages, which ranged from mild to moderate (Fig 4 and 5) degree only. The above findings clearly suggested the ability of the HPS virus to reproduce the disease in experimentally infected birds and also that the virus was able to produce the typical lesions as reported by earlier workers (Gowda and Satyanayarana, 1994; Asrani *et al.*, 1997 and Dubey *et al.*, 2009). The histopathological lesions in the heart were characterized by edema and degenerative changes. Loss of cross striations and fragmentation of cardiac fibers were other notable changes (Fig 6). The intensity of the lesions progressed from mild to severe. The present findings are in agreement to those of Hasan, 1989; Ravikumar *et al.* (1988); Abdul-Aziz and Al-Attar (1991) and Gowda and Satyanarayana (1994). The

findings further suggested that the pathognomonic lesions were encountered mostly in only two organs – heart and liver. Where in the hydropericardium was the pathognomonic lesion in heart and the extensive degenerative changes in the liver along with demonstration of basophilic intranuclear inclusion bodies in the hepatic cells constituted another notable lesions to be considered for the confirmation of diagnosis.

4.3.4 Detection of HPSV by Agar gel precipitation test:

The AGPT was performed for detection of HPSV using hyperimmune serum against HPSV isolate. Hyperimmune serum was raised by inoculating inactivated inclusion body hepatitis (IBH) / hydropericardium syndrome (HPS) vaccine. A thin band of single precipitation line was developed after 24 hrs of incubation at 37°C which confirmed the presence of HPSV in liver samples. The thickness of precipitin band got increased with further incubation. It may be due to the slow diffusion of the precipitating antigens as well as antibodies. AGPT may be advocated for confirmation of the virus involved in HPS. Lakra (2008) and Dubey (2003) also performed AGPT by raising hyperimmune sera in the broiler birds by inoculating 20% of liver homogenate extract of HPSV infected birds as antigen. They observed a precipitation band after 24 hrs of incubation at 37°C which is in agreement with the findings of the present study. Several workers also reported application of AGPT for detection of fowl adenovirus serotype 4. (Cheema *et al.*, 1989; Khera *et al.*, 1995; Chandra *et al.*, 2000; Roy *et al.*, 2004; Balmurgan *et al.*, 2006). Ravi kumar *et al.* (1997) also found precipitating line in AGPT after 30 hrs of incubation. Some workers found AGPT has a satisfactory sensitivity when performed on serum from commercial flocks that are often exposed to multiple adenovirus serotypes (Cheema *et al.*, 1989), however McFerran and Smyth (2000) reported that AGPT is less sensitive in detecting adenovirus infections in specific-pathogen-free (SPF) flocks.

4.3.5 Transmission electron microscopy:

Fresh small pieces of livers from HPSV infected birds were used for the transmission electron microscopy. A large region of electron dense mass was seen in the nucleus of infected liver at 500 nm bar magnification (Fig 7) resembling the presence of HPSV. An intranuclear crystalline array of virion was observed in the thin section of infected liver tissues. The intranuclear virus particles were aggregated in the form of sheets of particles arranged in honeycomb pattern, occupying the entire nucleus. At 100 nm bar magnification (Fig 8,9 and 10), rows of roughly spherical, ovoidal, viral particles were scattered in the nucleus as clumps. Non-enveloped, icosahedral, adenovirus like particles were observed. The morphological features of the virus in liver tissue section under transmission electron microscopy were those of a typical adenovirus with characteristic hexagonal shape.

Ganesh *et al.* (2002) also performed electron microscopy in liver tissue of Hydropericardium hepatitis syndrome (HHS) infected birds. They observed non enveloped icosahedral adenovirus like particles with a diameter of 75 nm at X 44000 magnification and in thin section of liver tissues an intranuclear crystalline array of adeno like virus particles were observed. These findings corroborate the results reported in present study. Kumar *et al.* (2004) also performed electron microscopy in poultry birds during outbreaks of hydropericardium syndrome in a private poultry farm in Haldwani area of Uttaranchal. They observed that intranuclear adenovirus like particles distributed in paracrystalline arrangement in hepatocytes. Kumar *et al.* (2006) also performed electron microscopy in experimental infection of hydropericardium syndrome. They observed ultrastructurally numerous large vacuoles, fragmented endoplasmic reticulum and swollen mitochondria were found at 36 hours PI in the cytoplasm of hepatocytes and myocytes. The swollen mitochondria and fragmented endoplasmic reticulum reported by Kumar *et al.*, (2006) were not found in present study. This could be attributed to the lower magnification in electron microscopy used in the present study. The infected nuclei were

irregularly outlined and contained large intranuclear masses. Virus particles were found attached with these masses. At higher magnification, virus particles appeared isometric and roughly spherical between 75-80 nm in diameter. Kim *et al.* (2008) also carried out the electron microscopy in Korean fowl during outbreaks of hydropericardium syndrome in Korea. Specific adenovirus particles were also observed in the nucleus of hepatocytes, which is in agreement to the findings of present study.

4.3.6 Polymerase chain reaction and Restriction enzyme analysis:

In present study all the four virus isolates recovered from different locations within Durg and Rajnandgaon district of Chhattisgarh were taken. Extraction of DNA was done from all four infected liver tissues isolates (Fig 11) and at different time interval DNA was extracted by using DNeasy blood and tissue kit (Qiagen). DNA extracted from fresh tissue at 0 day yielded high quality of DNA however, second elution of same isolates gave less amount of total DNA. DNA extracted after 15, 30, 45 days gave relatively less amount of whole DNA than fresh liver tissue. Extraction after 60 days did not yield genomic DNA. Thus after 45 days DNA extracted from liver tissue was not suitable for the PCR. Considering the above findings, whole genomic DNA from all the four isolates were extracted separately from fresh infected liver tissue by using DNeasy blood and tissue kit (Qiagen). Genomic DNA was analyzed in 0.8 % agarose by agarose gel electrophoresis. Viral DNA was found to be intact as indicated by a single compact band. The optical density in UV spectrophotometer (OD₂₆₀ / OD₂₈₀) were in the range from 1.6 to 1.7 for all the four isolates indicating adequate purity of DNA. The findings of the present study are in agreement to those of Ganesh *et al.* (2002) who reported isolation of DNA using kit with purity between 1.6 and 1.9. In the present study hypervariable regions of hexon genes was amplified by polymerase chain reaction. Several workers have reported use of polymerase chain reaction in amplification of target hexon gene sequence and used as a rapid diagnostic tool for the detection of fowl adenovirus (Rue and Hess 1998; Xie *et al.*, 1999; Ganesh *et al.*, 2001). In majority of

avian adenovirus polymerase chain reaction, the hexon gene was used for the primer designing. The hexon consist of (Pedestals P1 and P2) which are located more inside the virion and the variable loops (L1 to L2) which protrude from the surface. The loop contains the type specific neutralizing epitopes (Toogood *et al.*, 1992). Due to its exposure to the immune system the sequence identity between loop regions of different serotypes of fowl adenoviruses is rather low (Sheppard *et al.*, 1995). Thus the pedestals and loop fulfill the criteria for selecting primers for establishing a polymerase chain reaction. Raue and Hess (1998) designed two primers H1/H2 and H3/H4 from conserved regions of fowl adenovirus-1 hexon gene and successfully amplified the hexon gene of fowl adenovirus-4. The polymerase chain reaction product expected at the variable loop regions L1 and L2 (1219 bp) and L3 and L4 (1319 bp) respectively. Ganesh *et al.* (2002) also reported a 728 bp fowl adenovirus-4 hexon gene sequence from Indian fowl adenovirus isolates associated with hydropericardium syndrome. The primers pair used were located in the L1 variable region of the fowl adenovirus-4 hexon gene. Barua *et al.* (2002) also developed a polymerase chain reaction for the full length amplification of fowl adenovirus-4 yielded a 2916 bp product with an Indian isolates of fowl adenovirus-4 that was further cloned and sequenced.

In the present study the published primer set hexon A and hexon B were used which were designed from the conserved regions of hexon gene sequence of fowl adenovirus and the polymerase chain reaction yielded 897 bp product (Fig 12) these finding are in agreement with the findings of Meulemans *et al.* (2001). The optimized polymerase chain reaction yielded expected size of complete hexon gene from all four fowl adenovirus isolates. Rahul *et al.* (2004) also analyzed eight field isolates of IBH-HPS by amplification of hypervariable regions of hexone gene of adenovirus. Kim *et al.* (2008) also performed Polymerase chain reaction (PCR) analysis of the hexon gene and identified all of the isolates as FAdV-4. Restriction enzyme analysis was performed and PCR product was split in to 193 bp and 692 bp by *mlu* I restriction enzyme and restriction enzyme *bgl* I failed to split the product which confirm the

isolate as HPS serotype 4. However, all isolates did not give similar results, this may be due to lower quality of restriction enzymes which need further validation.

The study revealed that polymerase chain reaction can be used for the confirmatory diagnosis of the hydropericardium syndrome disease.

CHAPTER-V

SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH WORK

Hydropericardium syndrome (HPS) is an emerging disease of poultry, primarily of broiler chickens. The disease is caused by *Fowl adenovirus* serotype 4 belonging to family *Adenoviridae*. The disease primarily affects the young broiler birds (3 to 5 week).

A total of 7500 broiler birds, up to five weeks of age from four flocks were screened for the presence of disease. The infected birds exhibit depression, dullness and showed characteristic posture with their chest and beak resting on the ground and with closed eyes (tucked in appearance) followed by sudden death. The overall mortality up to 28 % was recorded in broiler flocks. The postmortem examination revealed accumulation of clear watery fluid in the pericardial sac. The pericardial fluid ranged from 10-15 ml and the pH was about neutral. The liver was pale yellow, swollen and enlarged and contains ecchymotic as well as pin point hemorrhages, Lungs were oedematous and congested. Kidneys were pale yellow, swollen, friable contained urate deposits in the tubules and ureters with haemorrhagic foci. Bursa and thymus were atrophied. The HPSV was isolated from infected liver of dead and moribund broilers.

HPS was experimentally reproduced in 3 week old broiler birds by subcutaneous inoculation of bacteria free virus infected liver homogenate extract (20% w/v). The infected birds and their respective controls remained healthy without manifestation of any clinical signs up to 48 hrs PI. After 48-72 hrs PI, the infected birds showed the clinical manifestations characteristic to the disease viz. off feed, reluctance to move, ruffled feathers, tucked in appearance with closed eyelids for a brief period just

before death. On post mortem, the infected birds showed characteristic accumulation of clear, straw-coloured fluid in the pericardial sac with discoloured liver

The presence of fowl adenovirus serotype 4 in the samples collected and the nature of purified virus was confirmed by agarose gel precipitation test. Hyperimmune serum was raised by inoculating inactivated inclusion body hepatitis (IBH) / hydropericardium syndrome (HPS) vaccine. A thin band of single precipitation line was developed after 24 hrs of incubation at 37°C which confirmed the presence of HPSV in liver sample. The thickness of precipitin band got increased with further incubation.

The histopathology of different organs *viz* Liver, heart and kidney was performed and the sections were stained by H & E stains. In liver typical characteristic histopathological changes included degeneration and necrosis of hepatocytes as well as the presence of basophilic intra nuclear inclusion bodies surrounded by a clear halo. The changes in the kidney consisted of congestion and haemorrhages, which ranged from mild to moderate degree only. The histopathological lesions in the heart were characterized by edema and degenerative changes. Loss of cross striations and fragmentation of cardiac fibers were other notable changes.

Transmission electron microscopy of HPSV infected liver revealed large region of electron dense mass in the nucleus of infected liver at 500 nm bar magnification resembling the presence of HPSV. An intranuclear crystalline array of virion was observed in the thin section of infected liver tissues. The intranuclear virus particles were aggregated in the form of sheets of particles arranged in honeycomb pattern, occupying entire nucleus. At 100 nm bar magnification, rows of roughly spherical, ovoidal, viral particles were scattered in the nucleus as clumps. Non-enveloped, icosahedral, adenovirus like particles were observed. The morphological features of the virus in liver tissue section under transmission electron microscopy were those of a typical adenovirus with characteristic hexagonal shape.

Extraction of DNA was done from all the four virus isolates recovered from different locations within Durg and Rajnandgaon district of Chhattisgarh. DNA was extracted from Infected liver tissues collected at different time interval by using DNeasy blood and tissue kit (Qiagen). Fresh tissue or DNA extracted at 0 day yielded high quality of DNA however, second elution of same isolate gave less amount of total DNA. Extracted DNA after 15, 30, 45 days gave relatively less amount of whole DNA than fresh liver tissue. Extraction after 45 days did not yield genomic DNA.

Genomic DNA was analyzed in 0.8 % agarose by agarose gel electrophoresis. Viral DNA was found to be intact as indicated by a single compact band. The optical density in UV spectrophotometer (OD₂₆₀ / OD₂₈₀) was between 1.6 to 1.7 for all the four isolates indicating adequate purity of DNA.

Polymerase chain reaction was carried out using the published primer set hexon A and hexon B, which were designed from the conserved regions of hexon gene sequence of fowl adenovirus. The polymerase chain reaction yielded 897 bp product. The optimized polymerase chain reaction yielded expected size of hexon gene from all the four fowl adenovirus isolates.

Restriction enzyme analysis was performed and PCR product was split in to 193 bp and 692 bp by *mlu* I restriction enzyme and restriction enzyme *bg* I failed to split the product which confirm the isolate as HPS serotype 4. However, all the isolates did not give similar results, this may be due to lower quality of restriction enzymes which need further validation.

CONCLUSIONS:

Based on the results obtained from present investigations, following conclusions could be drawn.

1. HPSV infection is present in the different regions of chhattisgarh which is clearly evident by characteristic clinical signs and lesions in post mortem
2. Etiological agent of HPS is able to reproduce the disease in susceptible birds.
3. HPS can be reproduced experimentally in growing broiler birds with better adaptability and recovery of good titre of HPSV for further studies.
4. Positive findings of AGPT with good specificity and sensitivity showed the occurrence of HPSV.
5. Clinical, gross, as well as histopathological findings may be used as supportive evidence along with other diagnostic tests.
6. Transmission electron microscopy may be used for tentative diagnosis of HPS disease
7. Polymerase chain reaction can be used for confirmatory diagnosis of hydropericardium syndrome disease.

SUGGESTIONS FOR FUTURE RESEARCH WORK:

1. Penton based primers can be used for polymerase chain reaction.
2. Cloning and gene sequencing of PCR products can be done.

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APPENDIX

I Reagents for AGPT

1) 1% Agar	
Noble agar	1.0 g
Normal saline solution (8%)	100 ml

II Reagents for Histopathological work

1) Harris Haematoxylin

Haematoxylin	2.5 gm
Absolute alcohol	25 cm ³
Potassium alum	50 gm
TGDW	500 ml
Mercuric iodide	1.25 gm
Glacial acetic acid	20 cm ³

2) Eosin Y

Eosin	1 gm
TGDW	100ml

3) Glycerol albumin

Fresh egg albumin	50 cm ³
Glycerol	50 cm ³
Sodium salicylate	1 cm ³

Dilute the fluid to 1:20 with distilled floated water and sections floated on fluid.

III Reagents for Electronmicroscopy

1) 2.5 % Gluteraldehyde

25 % Glutetaldehyde	100 ml
Distilled water	400 ml
Phosphate buffered saline	500 ml

IV Reagents for polymerase chain reaction

1) 0.5 EDTA (Disodium Ethylene Diamine Tetra-Acetate, pH 8.0) 500mM solution

Na ₂ EDTA·2H ₂ O	181.6 gm
Distilled water	800 ml

While stirring with a magnetic stirrer, adjust to pH 8.0 with pellets of NaOH (about 20 g NaOH)

2) 50 X Tris Acetate EDTA (TAE)

Tris base	242 gm
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
Deionized water to make	1000ml

The pH of this buffer is not adjusted and should be about 8.5.

Working concentration: 1X (0.04M Tris-Acetate, 0.001M EDTA)

3) 6X Gel loading buffer

Bromophenol blue	.25 gm
Xylene cyanol	.25 gm
Sucrose or glycerol	4 ml
TAE (1X) to make	10 ml

(Filter sterilize and store at 4°C)

THESIS ABSTRACT

Title of Thesis:

Studies On Isolation, Identification And Molecular Characterization Of Hydropericardium Syndrome Virus

Name of Student:

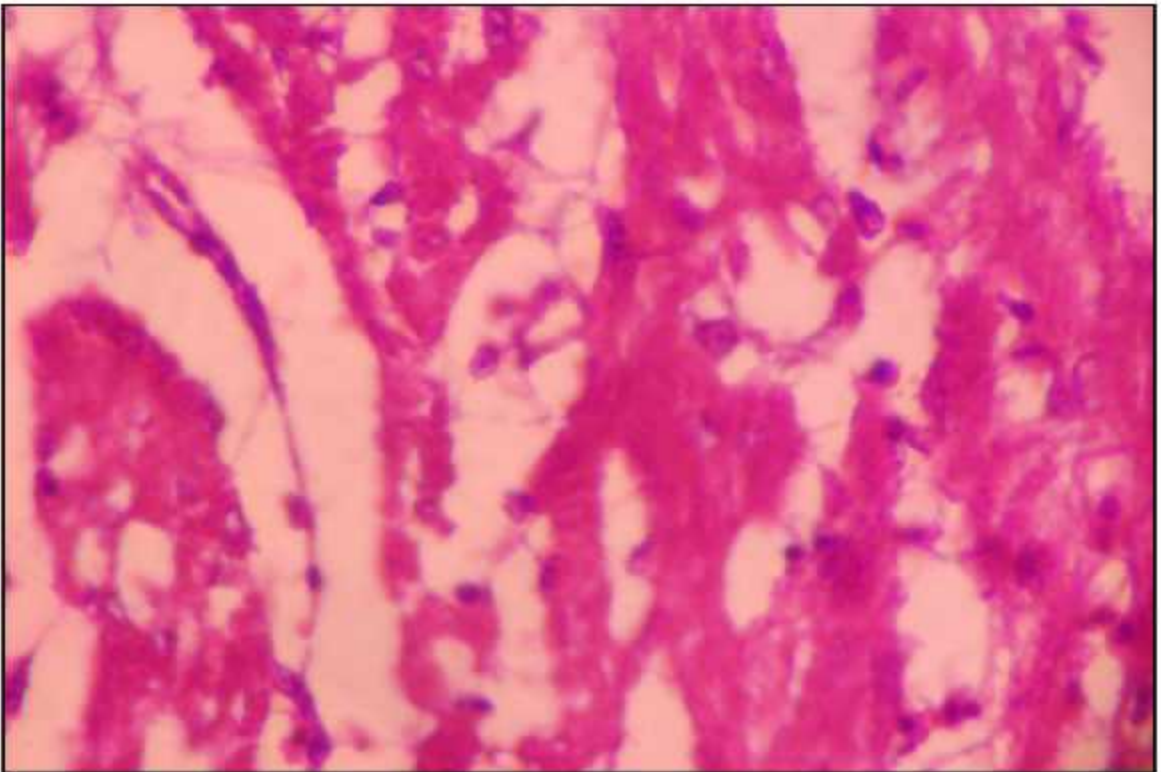
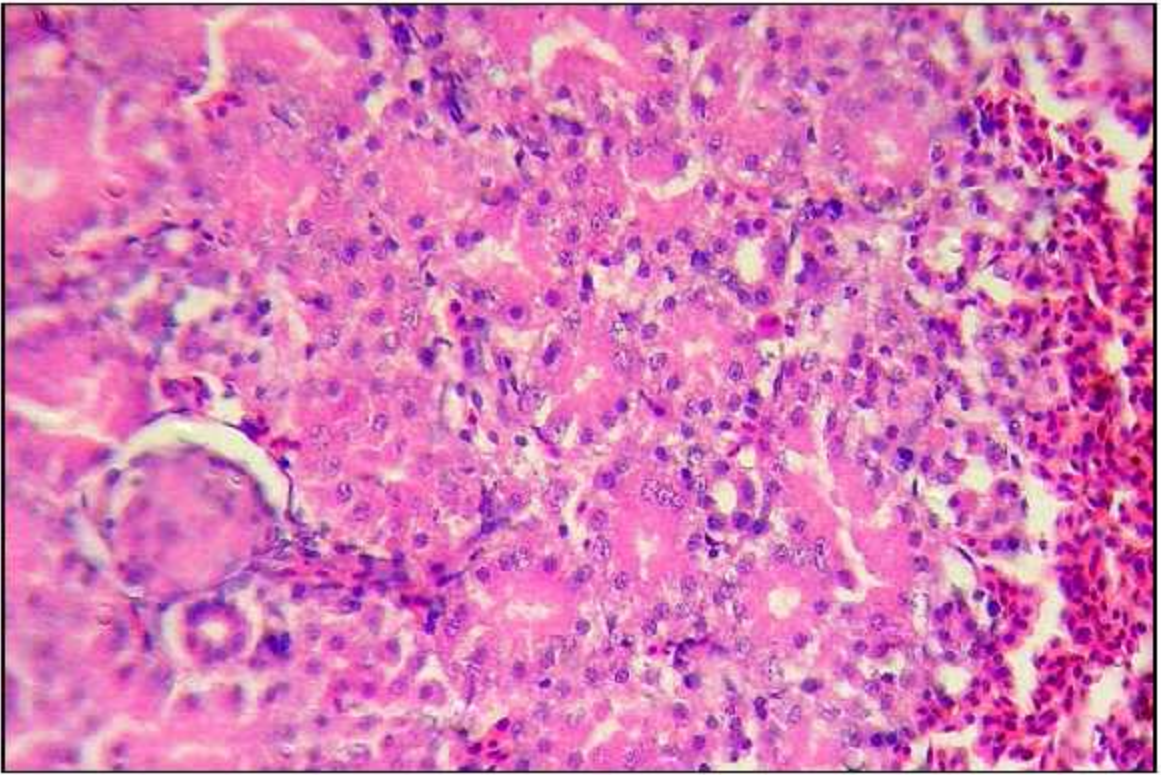
Abhishek Sharma (Roll No. 9960, ID No.UG/VETY/DURG/2002/1)

Hydropericardium syndrome (HPS), is an emerging viral disease of broiler chickens caused by Fowl adenovirus serotype 4 belonging to family *Adenoviridae*. The present study was undertaken by isolation and identification of HPSV from broiler flocks (3-5 weeks age) showing mortality up to 28% (average 24.99%). HPSV was experimentally reproduced in young broiler birds. The liver from dead and moribund broiler birds were aseptically collected and 20% (w/v) tissue homogenate was prepared. The filtered liver tissue homogenate was inoculated in subcutaneously 3 week old broilers.. The infected birds became dull, depressed and showed characteristic posture with their chest and beak resting on the ground and with closed eyes. On postmortem the infected birds showed accumulation of straw coloured fluid in the pericardial sac with discoloured liver. Agar gel precipitation test (AGPT) performed using HPSV infected liver tissue homogenate (20%, w/v) and hyperimmune serum against inclusion body hepatitis (IBH) / hydropericardium syndrome (HPS), produced thin band of single precipitation line after 24 hrs of incubation at 37°C which confirmed the presence of HPSV in liver samples. . To confirm the field isolate as HPSV, histopathology of different organs viz. Liver, heart and kidney was performed and the sections were stained by H and E stains. In liver typical characteristic histopathological changes included degeneration and necrosis of hepatocytes as well as the presence of basophilic intra nuclear inclusion bodies surrounded by a clear halo was observed. The changes in the kidney consisted of congestion and haemorrhages, which

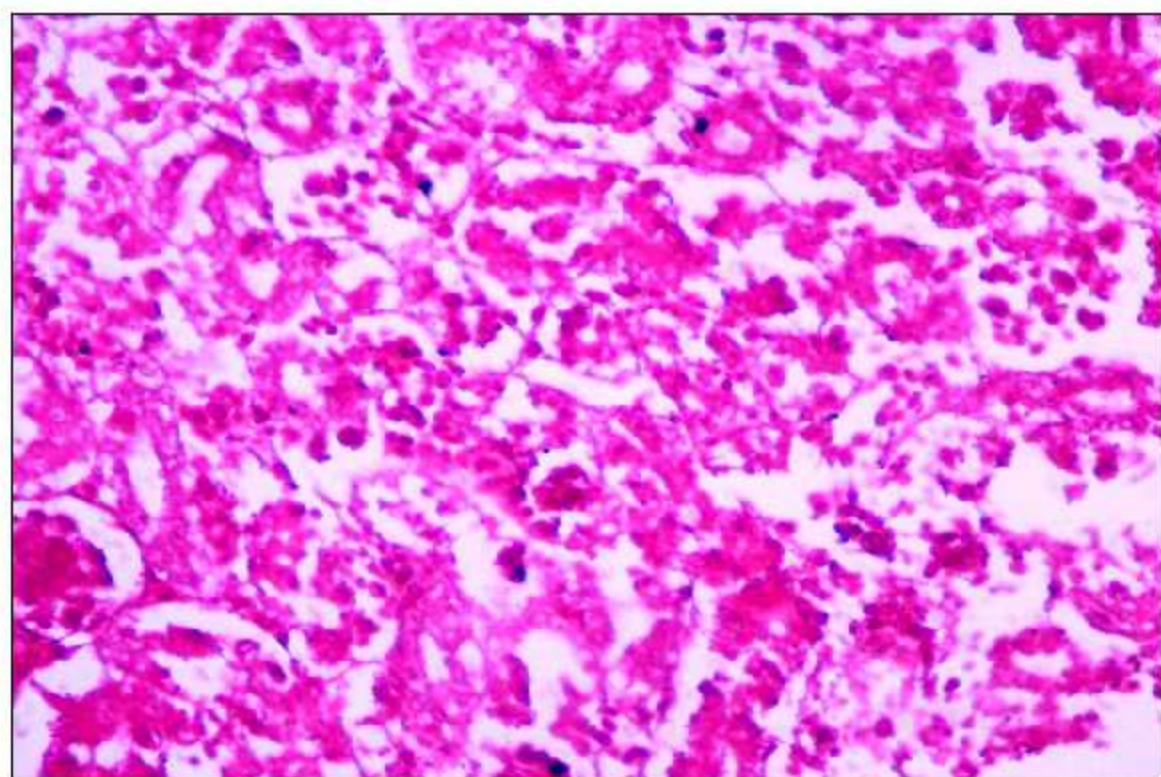
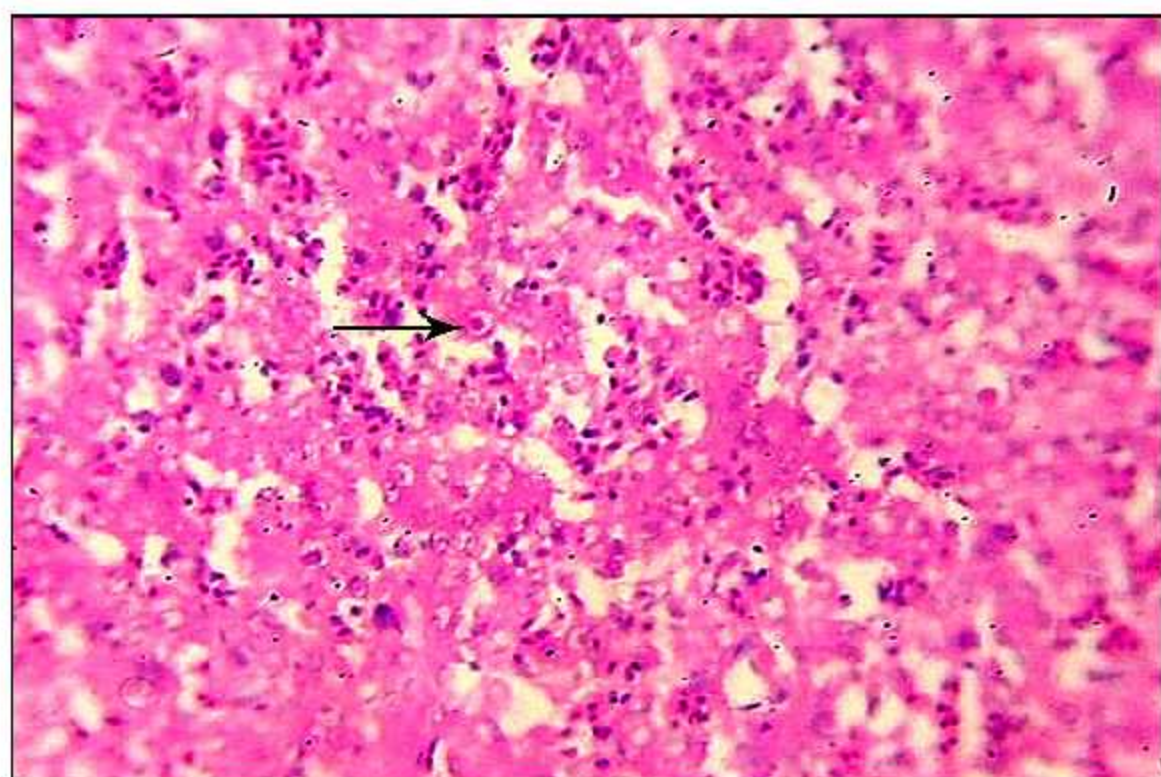
ranged from mild to moderate degree only. The histopathological lesions in the heart were characterized by edema, loss of cross striations and fragmentation of cardiac fibers. Further, transmission electron microscopy (TEM) of HPSV infected liver was performed. A large region of electron dense mass was seen in the nucleus of infected liver at 500 nm bar magnification resembling the presence of HPSV. An intranuclear crystalline array of virion was observed in the thin section of infected liver tissues. The intranuclear virus particles were aggregated in the form of sheets of particles arranged in honeycomb pattern, occupying the entire nucleus. Extraction of DNA from infected liver tissue was done at 0, 15, 30, 45 and 60 days revealed that extraction after 45 days did not yield genomic DNA, thus not suitable for polymerase chain reaction (PCR). Polymerase chain reaction was standardized for rapid detection of HPSV in field samples. The published primer set hexon A and hexon B based on conserved regions in hexon gene sequence of fowl adenovirus used in polymerase chain reaction yielded 897 bp product. Restriction enzyme analysis was performed and PCR product was split in to 193 bp and 692 bp by *mlu* I restriction enzyme and restriction enzyme *bgl* I failed to split the product which confirm the isolate as HPS serotype 4.

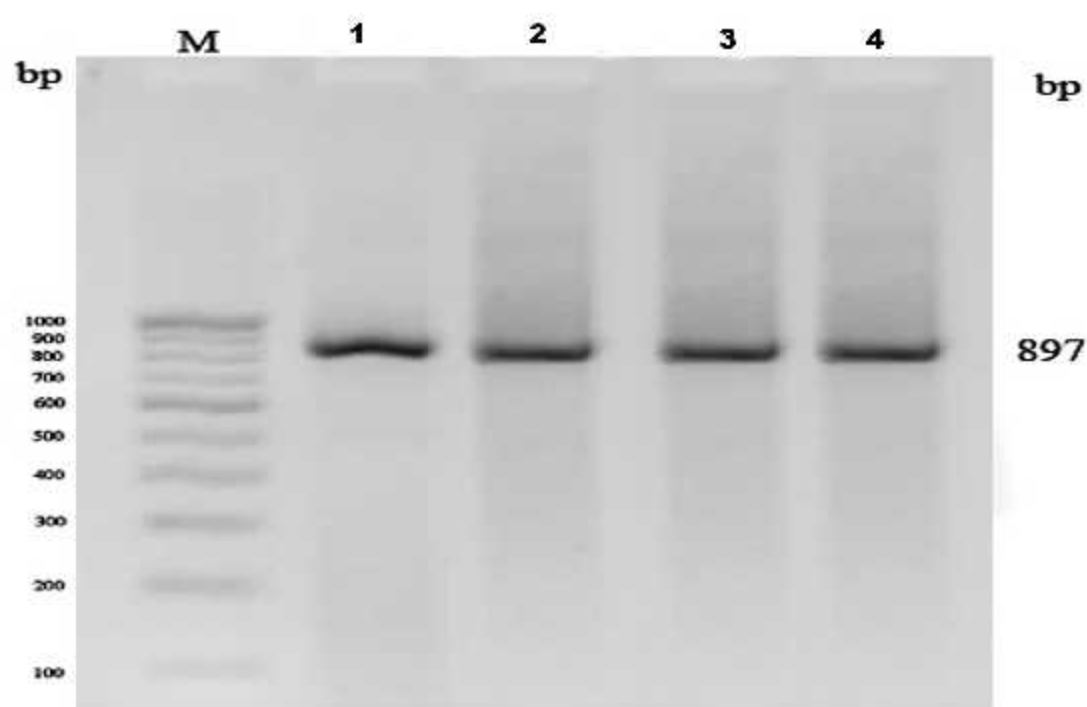
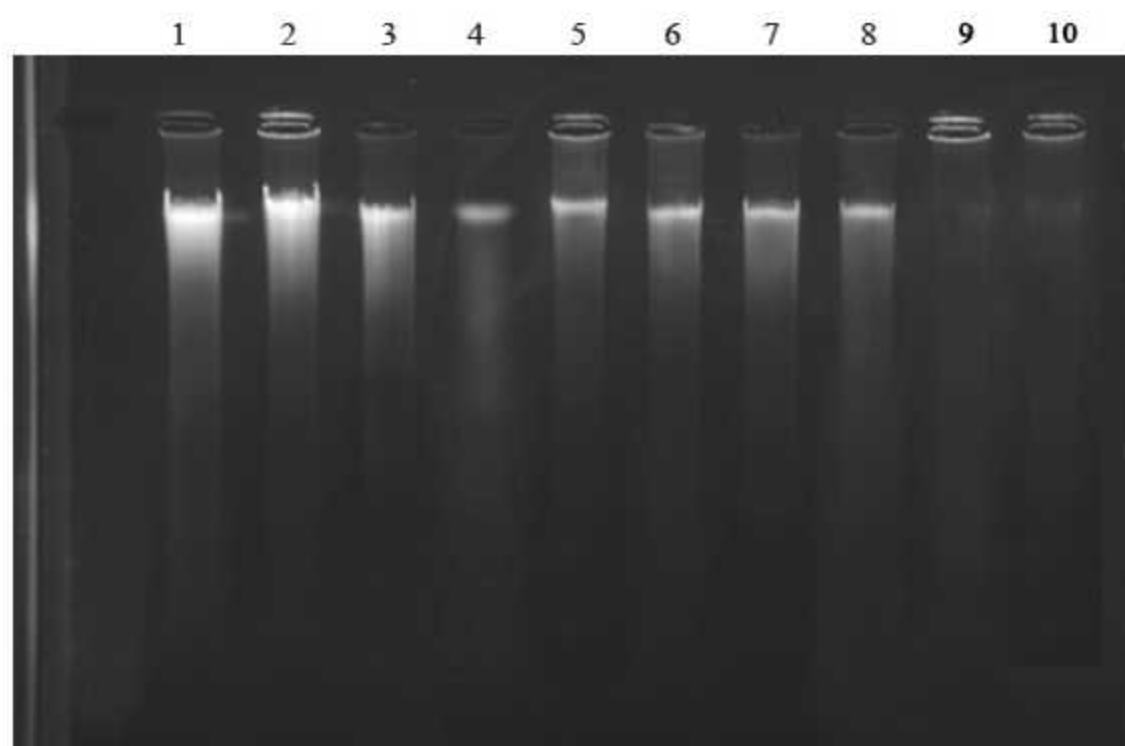
HPSV infection is prevalent in Chhattishgarh with moderate mortality and can be diagnosed on the basis of clinical signs, lesions, histopathological changes and electron microscopy. The molecular characterization of isolated viruses using hexon gene primer in PCR further validated the presence of HPSV

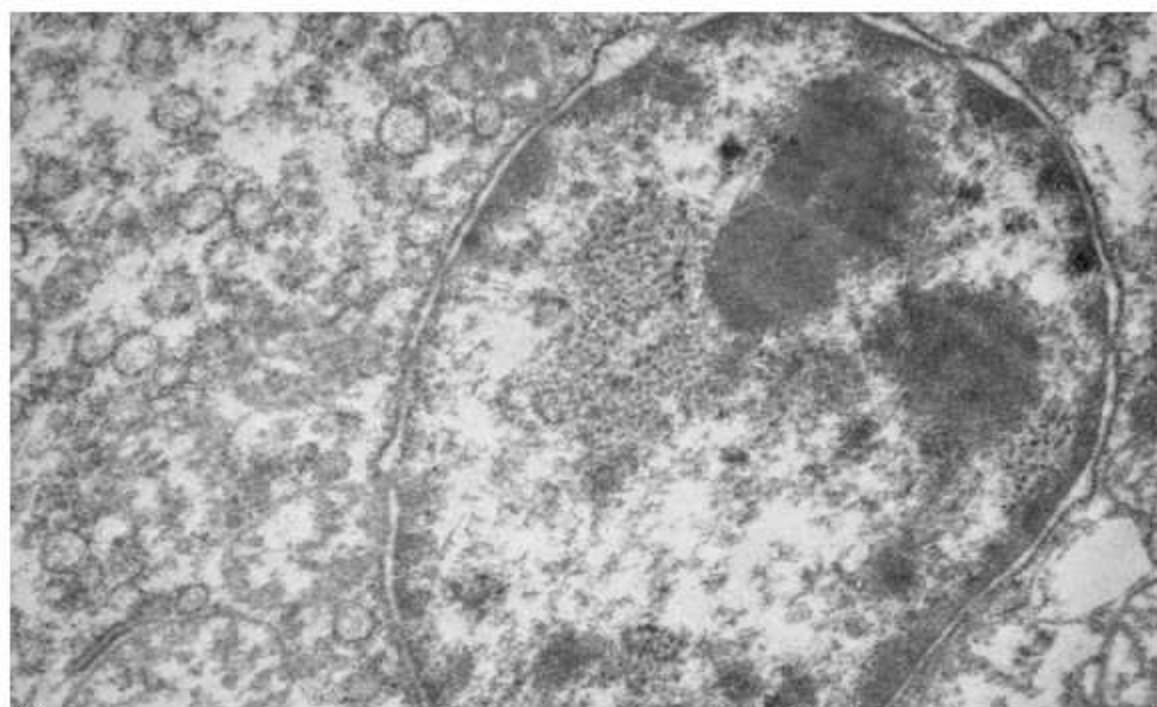
Sanjay Shakya
Major Advisor





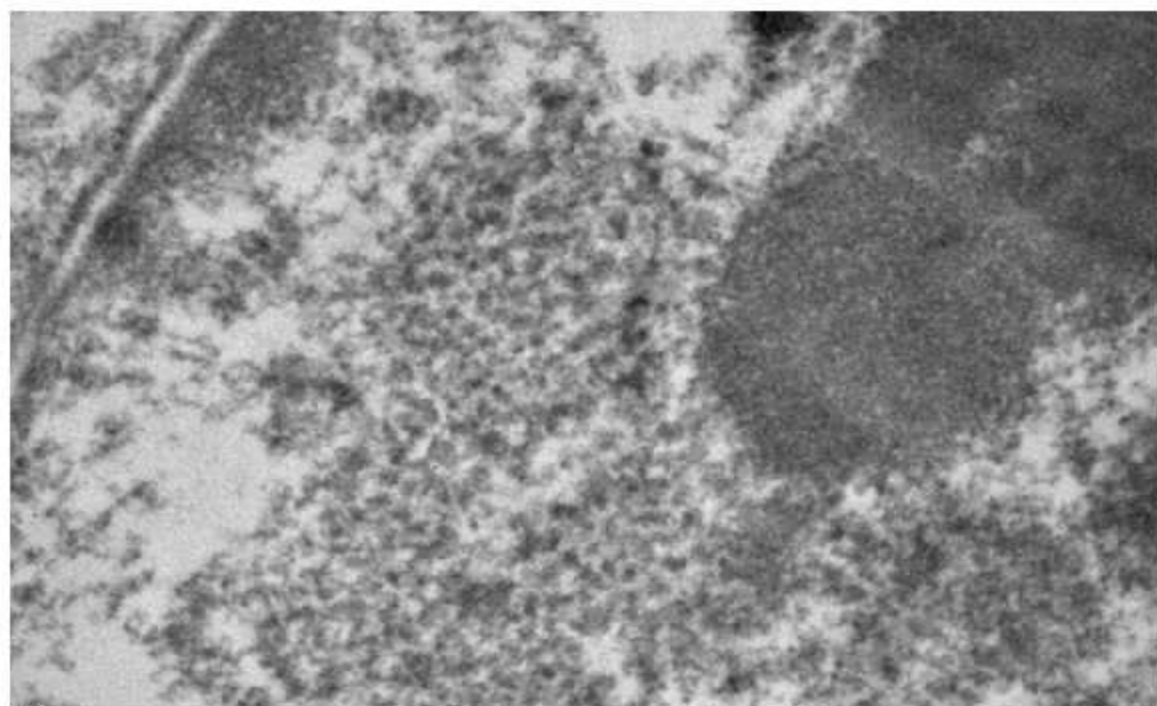






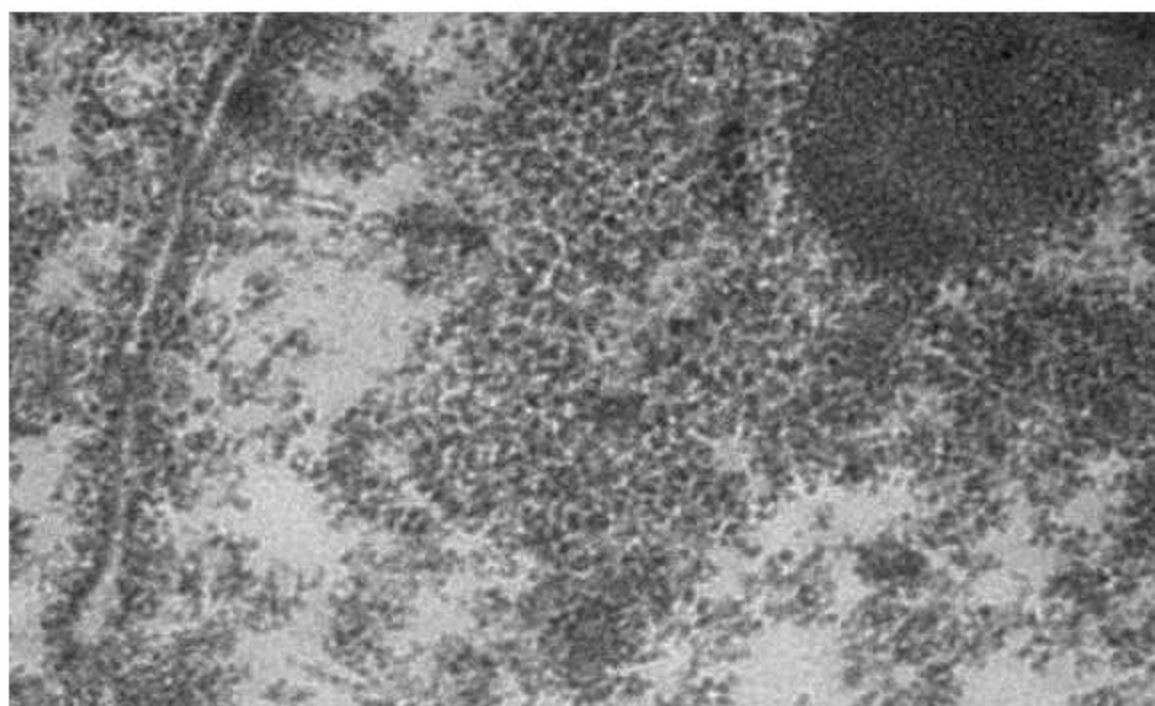
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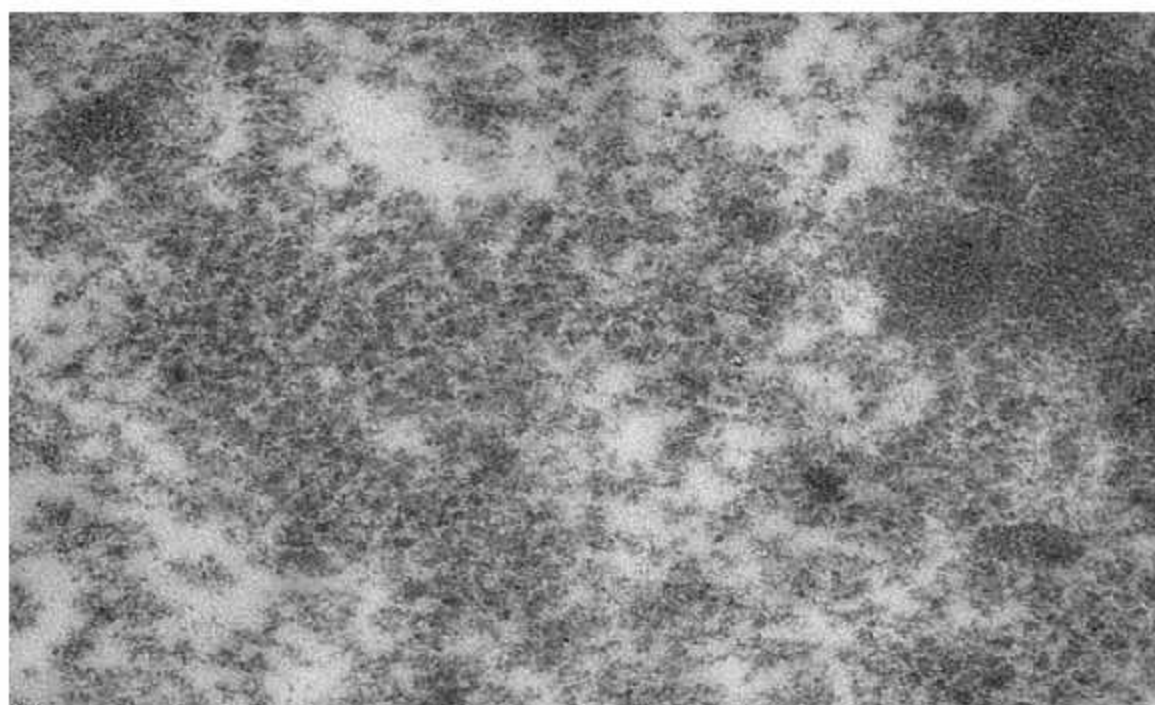
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