SEROPREVALENCE OF HYDROPERICARDIUM SYNDROME IN BROILER CHICKENS

By
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THESIS

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DECLARATION

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

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CERTIFICATE

Certified that this thesis, entitled **"SEROPREVALENCE OF HYDROPERICARDIUM SYNDROME IN BROILER CHICKENS "** is a record of research work done independently by Mr. Binu T.V. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to him.

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INTRODUCTION

1. INTRODUCTION

Agriculture is heralded as the backbone of Indian economy. Livestock and poultry have contributed a lion's share to this agriculture-based economy. Poultry scenario has changed from an age-old backyard farming system to a fast growing industry with the full backing of science and technology.

Poultry industry has played a major role in raising the economic status of the marginal farmers and the under privileged rural masses, besides it's contribution in boosting the nutritive status of common man.

In the poultry sector, the broiler production has achieved tremendous growth rate over the last three decades and no other sector in agriculture has reported such a remarkable progress as the broiler sector. A mere four million broiler population in 1971 was raised to 235 millions in 1993 and was projected to be 330 million in 1995 (Anonymous, 1994). By 2000, a broiler population of 622.5 million was expected (Ahmad, 1998). During the last decade the annual

growth rate in broiler production was recorded to be 20 per cent (Kotaiah, 1996).

Per capita consumption of meat in India was 220 g per annum in 1971 and 521 g per annum in 1997. It is estimated to be 1500 g per annum in 2000 (Shibu, 1999). ICMR recommends 10.8 kg per annum at the rate of 30 g of meat per day.

With the exceptional growth potential and the intensive system of raising of the modern units, new disease problems also emerge. Large scale movement of poultry stock and poultry products without adequate control measures and restrictions across the world, migratory birds, import and export of pet birds etc., play a major role in the spreading of many diseases. Prevention and control of disease have become a significant aspect of any successful management strategy of broiler industry.

Ironically, when we succeed in containing existing disease problems, new diseases and syndromes emerge due to various reasons.

Hydropericardium syndrome (HPS) is such an emerging threat that rocked the broiler industry in the later half of '80s and early '90s. It still continues as a serious problem leading to closure of many a broiler unit. In India this syndrome has been reported from Jammu and Kashmir, Delhi, Maharashtra, TamilNadu, Andhra Pradesh and Karnataka.

In Kerala no systematic study has so far been undertaken to estimate the magnitude of the syndrome though many outbreaks are reported from different parts of the state by field veterinarians.

So the present study was undertaken with the following objectives

1. To study the seroprevalence of hydropericardium syndrome in broilers in Kerala.

2. To compare different serological tests like agar gel precipitation test, counter immunoelectrophoresis and indirect enzyme linked immunosorbent assay for serological diagnosis of HPS.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Hydropericardium syndrome (HPS) is an emerging syndrome of chickens. This condition was first reported from Angara Goth, Karachi, Pakistan in 1987 (Anonymous, 1988). The syndrome mainly affects commercial broiler chickens of three to six weeks of age and causes heavy mortality (30-60 per cent).

The characteristic lesions are typical hepatitis and severe hydropericardium and hence named as hydropericardium syndrome.It is well known among farmers as "Leechi disease" because the hydro-pericardium and heart of affected birds resemble decorticated Leechi fruit.

So many synonyms are used to describe this condition such as Hydropericarditis syndrome (Anjum, *et al.*, 1989), Angara disease (Akhtar,1994), Infectious hydropericardium (Abdul-Aziz and Hassan, 1995), Hydropericardium-hepatitis syndrome(Shane, 1996; Ganesh, 1998), Hydropericardium-hepatopathy syndrome (Arsani *et al.*, 1997) and Inclusion body hepatitis-hydropericardium syndrome(Jadhao *et al.*, 1997;

Balamurugan *et al.*, 1999). This syndrome is of high economic importance as it causes devastating losses to the broiler industry.

2.1 Distribution

The first report of this condition was from broiler chickens from Angara Goth, Karachi, Pakistan (Anonymous, 1988). Subsequently Anjum *et al.* (1989) reported HPS with high mortality in broiler chickens from Karachi and from Faisalabad.

Hydropericardium syndrome (HPS) was also reported from Iraq (Abdul-Aziz and Al-Attar, 1991); South and Central America (Shane, 1996); Mexico, Equador and Peru (Voss *et al.*, 1996); and from Russia (Borisov *et al.*, 1997).

In India Gowda (1994) reported hydropericardium syndrome from various parts of Srinagar, Jammu and Punjab, describing it as a mysterious, emerging disease of poultry.

Gowda and Satyanarayana (1994) observed that the disease was slowly spreading to other parts of the country.

An outbreak of Leechi disease at Palladam poultry belt, Coimbatore in Tamil Nadu, affecting popular broiler breeds like Vencobb, Hubchix, Hubbavel, Ross and Anak was reported by Karunamoorthy *et al.*(1996).

Arsani *et al.* (1997) recorded that this new syndrome had wrecked the north Indian poultry industry and was rapidly spreading to other parts of the country.

2.2. Etiology

Several factors were implicated as the cause of HPS including nutritional disorders (Jaffery, 1988; Qureshi, 1989), rancid fat, rancid fish meal, vitamin and mineral imbalance in the feed (Qureshi, 1988) and toxic compounds (Ahmed *et al.*, 1989; Qureshi, 1989).

In a survey conducted by Anjum *et al.* (1989), they noted that there was no correlation between the cause of HPS and source of chicks, management, feed, salts in water, vaccines, vaccination schedules and drugs. An infectious nature of this syndrome was put forth through successful reproduction of the syndrome by inoculation of a bacteria free liver homogenate by Cheema *et al.* (1989) and Khawaja *et al.* (1988). They

detected adenoviruses in the liver homogenate. Hasan (1989) suggested that the disease was inclusion body hepatitis caused by the Tipton strain.

Central Veterinary Laboratory, Weybridge, U.K., isolated an adenovirus from the lyophilised homogenate of liver, taken from an HPS infected bird and later on a German laboratory confirmed it as a new isolate of adenovirus designated as K31/89 (Voss, 1989).

Anjum (1990) reproduced the disease by inoculation of the HPS infected liver homogenate to susceptible chicks.

In a study to investigate the etiology of HPS, Afzal *et al.* (1991) indicated that an unknown agent was involved in causing the disease, which required co-infection by an adenovirus for the production of this syndrome. Akhtar *et al.* (1992) also supported this observation.

The view that this syndrome was caused by an unknown virus which became activated only when there was co-infection with an adenovirus was argued upon by Akhtar (1994).

By demonstrating intranuclear inclusion bodies in hepatocytes, Gowda and Satyanarayana (1994) suggested adenovirus as the possible causative organism of HPS.

From the experimental studies conducted at Poultry Diagnostic and Research Centre, Pune, Survashe *et al.* (1996) evidenced a multifactorial etiology, which included adenovirus similar to type I and II and immunosuppression caused by IBD virus or by feed toxicity such as that due to clostridial toxin, aflatoxin etc.

Arsani *et al.* (1997) supported the hypothesis that another infectious agent in addition to an adenovirus was involved in the etiology of HPS. They suggested a possible involvement of chicken anaemia agent, based on clinical signs and lesions.

Jadhao *et al.* (1997) typed three Indian isolates of fowl adenoviruses recovered from inclusion body hepatitis-hydropericardium syndrome (IBH-HPS) of poultry, from different geographical areas of the country, as Fowl Adeno Virus (FAV) serotype 4.

Group I adenovirus (serotype 4) was isolated from liver samples of adult broiler breeders and broiler chicks with HPS by Abe *et al.* (1998). They suggested that HPS might be caused by Group I adenovirus.

Ganesh (1998) by serological studies and electron microscopy suggested fowl adenovirus serotype 4 as the sole causative agent of HPS.

With the support of restriction enzyme studies, Mazaheri *et al.* (1998) opined that some specific strains of FAV serotype 4 caused HPS.

Murphy *et al.* (1999) classified HPS agent as Aviadenovirus serotype-4.

2.3. Epidemiology

The disease typically occurred in three to six-week-old growing broiler chicks and resulted in upto sixty per cent mortality (Jaffery 1988; Akhtar *et al.* 1992).

Anjum *et al.* (1989) reported this syndrome in medium weight layer strains and broiler strains of chickens.

Significant association was noticed between the incidence in a flock and number of visits by poultry crew, the number of flocks raised and the source of light and heat. Flocks that had one or more visits by a poultry crew, were 15 times more likely to be affected by the syndrome than flocks that had no such visits. Premises where one flock was raised were nearly three times more likely to be affected than premises where two flocks were raised. The use of electricity as a source of light and heat, had a much lower risk than kerosene oil (Akhtar *et al.*, 1992).

Akhtar (1994) reported that HPS affected three to five weeks old chicks, causing 60 to 70 percent mortality.

Gowda (1994) observed that the occurrence of this syndrome in broiler chickens was between three to five weeks of age, with its peak at fourth week and a mortality rate ranging from 10 to 80 per cent.

Gowda and Satyanarayana (1994) recorded that the syndrome was characterised by sudden high mortality among broilers aged three to five weeks.

In an outbreak of Leechi disease in Tamil Nadu, Karunamoorthy *et al.* (1996) recorded a mortality rate ranging from 10 to 95 per cent, affecting four to seven-week-old broilers. They also observed that in many of the cases, HPS occurred following infectious bursal disease.

Arsani *et al.* (1997) observed that the syndrome was evidenced by sudden high mortality, mostly in two to six-week-old broilers. Broiler breeder flocks (32 weeks old) and commercial layer flocks (17 weeks old) were rarely affected with about five to eight per cent mortality. Mortality, which ranged from 15 to 60 per cent, reached its peak within three to four days, followed by an almost constant rate for five to seven days and then declined.

Ravikumar (1998) in his study recorded an overall occurrence of HPS in broilers as 6.5 per cent, with a seasonal variation. He observed that the incidence was low during January to July and high from August to December, i.e. low occurrence in hot and cold seasons and high during Monsoon season.

2.4. Clinical Signs

According to Anjum *et al.* (1989) there was sudden mortality in HPS with no morbidity and the birds remained active until just before death.

In an experimental study using HPS infected liver homogenate as inoculum, Anjum (1990) noted sudden mortality without any premonitory signs.

Gowda and Satyanarayana (1994) observed depression, ruffled feathers with tucked in appearance, off feed and greenish diarrhoea in affected flocks.

In an outbreak of HPS, Karunamoorthy *et al.* (1996) recorded that the birds were alert and taking feed and water normally but mortality occurred suddenly.

Ruffled feathers, drooping of wings and severe anaemia or icterus leading to death were reported as clinical signs by Survashe *et al.* (1996).

Arsani *et al.* (1997) observed that the birds assumed a characteristic posture with their chest and beak resting on the ground about 36 hours before death. The affected birds were dull and depressed with their

eye lids completely closed in the later stages of the disease. The mortality reached its peak within three to four days.

The birds affected with HPS were off feed, dull and depressed with ruffled feathers. The dullness progressed to listlessness and coma, the head was hung down and eyes were closed, followed by prostration and death within two to four hours (Kharole and Mishra, 1998).

2.5. Clinical pathology

Haematological values have been compared between birds affected with HPS and control birds. A noticeable decrease ($p<0.01$) in total leukocyte count (TLC), erythrocyte count (TRBC), haemoglobin (Hb), erythrocyte sedimentation rate (ESR) and lymphocyte values and an increase in heterophil and mean corpuscular volume (MCV) were recorded in affected birds (Bhatti *et al.* 1988; Papsalmontos, 1988; Niazi *et al.*, 1989).

Significant increase in the mean levels of serum enzymes including lactic dehydrogenase, creatine phosphokinase, alkaline phosphatase and alanine transaminase were recorded in birds affected with HPS,

compared with normal birds. A slight decrease in the level of aspartate transaminase was also observed (Zaman and Khan, 1991).

Akhtar (1994) observed a significant ($p<0.05$) decrease in all the blood values except MCV and the numbers of heterophils and eosinophils, when compared to normal chicks. A decrease in TRBC, TLC, Hb, ESR and haematocrit values was observed by Gowda (1994). Differential leukocyte count showed an increase in heterophils, eosinophils and monocytes and a decrease in lymphocytes and basophils.

A decrease in haematocrit values was also observed by Cowen *et al.*(1996) and Voss *et al.* (1996).

Arsani *et al.* (1997) reported a significant decrease in TRBC, Hb, packed cell volume (PCV) and TLC. There was an increase in heterophil count. Total serum protein and total serum cholesterol showed a decrease in their values whereas creatinine, SGOT and SGPT levels were higher than that of the controls.

In an experiment conducted by Mujeeb *et al.* (1998), they observed a decrease in protein content

both in the blood serum and the pericardial fluid. There were increases in the potassium ions and lactate dehydrogenase (LDH) activity in blood serum as well as in the pericardial fluid.

2.6. Gross pathology

The predominant lesion in HPS was hydropericardium with varying amounts (ranging from 5 to 20 ml) of clear, straw coloured, watery or jelly like fluid, surrounding a flabby and misshapen heart (Khan *et al.* 1988; Anjum *et al.* 1989; Cheema *et al.* 1989). Other lesions in heart included necrotic foci on myocardium (Muneer *et al.*, 1989), glistening white patches with or without pin point haemorrhage (Gowda, 1994; Gowda and Satyanarayana, 1994), petechial or ecchymotic (Abdul-Aziz and Hassan, 1995) and streaks of haemorrhages on the myocardium (Survashe *et al.*, 1996).

Liver showed enlargement (Abdul-Aziz and Al-Attar 1991), pale or dark to yellow discolouration (Afzal *et al.* 1991), mottling and varying degrees of haemorrhages (Gowda and Satyanarayana, 1994). In many instances liver was swollen and friable (Abdul-Aziz and Hassan, 1995; Oberoi *et al.* 1996; Arsani *et al.*, 1997).

Karunamoorthy *et al.* (1996) recorded subcapsular haemorrhage and focal necrosis of liver in HPS cases.

Spleenomegaly was cited as a consistent lesion in HPS by many workers (Cheema *et al.*, 1988; Muneer *et al.*, 1989), whereas some others recorded mottling (Gowda and Satyanarayana, 1994), congestion (Karunamoorthy, *et al.*, 1996) and atrophy or enlargement (Oberoi, *et al.*, 1996; Survashe, *et al.*,1996;Arsani *et al.*, 1997) as prominent lesions of HPS in spleen.

Bursa of Fabricius showed regression or atrophy in some cases (Gowda, 1994; Karunamoorthy *et al.*, 1996) and in some other cases hypertrophy (Muneer *et al.*, 1989) with mucus accumulation (Survashe *et al.*, 1996).

Enlargement of kidneys with extended tubules was recorded as a major lesion of HPS by Afzal *et al.* (1991) and Abdul-Aziz and Hassan (1995). Kidneys were pale or congested and swollen in some cases (Oberoi *et al.*, 1996; Arsani *et al.*, 1997). Survashe *et al.* (1996) recorded that kidneys had a cooked meat appearance.

Major lesions of lungs included congestion and oedema with frothy fluid oozing out upon pressure (Gowda and Satyanarayana, 1994; Abdul-Aziz and Hassan, 1995). Karunamoorthy et *al.*(1996) described the lung as reddish and spongy.

Other lesions observed in HPS included engorgement of intestinal mesentery with blood, mucoid enteritis, pale and anaemic bone marrow and dull, gray abdominal fat with occasional yellow shining, giving a jaundiced look (Gowda and Satyanarayana, 1994). Thymus, heart and thigh muscles showed streaks of haemorrhage (Survashe *et al.*, 1996).

2.7. Histopathology

In HPS, myocardial fibres were oedematous with mononuclear cell infiltration between them (Anjum *et al.*, 1989). Multiple areas of necrosis on the ventricular wall, particularly in the papillary muscles, shrunken, fragmented, eosinophilic and calcified muscle fibres and deposition of proteinaceous material and extravasation of erythrocytes in between the muscle bundles were described by Cheema *et al.*(1989). Gowda (1994) reported massive interstitial oedema, haemorrhage, endothelial proliferation of blood

vessels and degenerative changes of heart muscles. Zenker's degeneration of heart muscles with eosinophilic, vacuolated cytoplasm and interstitial oedema were noticed by Survashe *et al.* (1996). The haemorrhages and endothelial proliferation in the blood vessels in the heart muscles were also seen. Arsani et *al.* (1997) observed that heart showed disruption, separation of muscle bundles and fibres due to oedema and there was vacuolar degeneration in the intimal layer and tunica adventitia of myocardial arteries. Pericarditis and myocarditis were reported by Ravikumar *et al.* (1998).

In the histopathology of HPS, liver showed marked changes. There were multi focal areas of coagulative necrosis with mononuclear cell infiltration and large, round basophilic intra nuclear inclusion bodies could be demonstrated in hepatocytes (Khan *et al.*, 1988; Anjum *et al.*, 1989; Cheema *et al.*,1989; Muneer *et al.*,1989; Survashe *et al.*, 1996). Gowda (1994) recorded varying degrees of haemorrhages, cellular degeneration, large hallowed basophilic or eosinophilic intranuclear inclusions, infiltration of mononuclear cells around portal triad and kupffer cell hyperpalcia of varying degrees. Fatty changes in liver (Karunamoorthy et

al.,1996) and focal areas of lymphocyte infiltration were also reported (Arsani *et al.*, 1997). Abe *et al.*(1998) observed that basophilic type of inclusion bodies was more common than eosinophilic type. They reported that the inclusions were frequently seen around the necrotic foci of hepatocytes and also that they showed positive staining by Fuelgen reaction and immunoperoxidase staining. Congestion or haemorrhages, fatty changes, areas of coagulative necrosis and presence of intranuclear inclusions in the hepatocytes were described by Ravikumar *et al.*(1998).

There were lymphocytosis and atrophy of the follicles in the spleen (Gowda , 1994). Degenerative and necrotic changes in germinal centres of spleen and moderate depletion of lymphocytes in the follicles were observed by Survashe *et al.*(1996). Arsani *et al.* (1997) reported lymphocyte depletion from peri arteriolar lymphoid sheath, reticulo endothelial cell hyperplasia and vacuolar degeneration of the tunica adventitia of spleenic arteries as the predominant histological lesions in the spleen. Abe *et al.* (1998) observed erythrophagocytosis in spleenic sinus.

Bursa of Fabricius in HPS cases revealed degenerative follicles, varying degrees of follicular necrosis and vacuolation in the epithelium in some cases (Gowda, 1994). Abdul-Aziz and Hassan (1995), Oberoi *et al.* (1996) and Arsani *et al.* (1997) described degenerative changes and lymphocytolysis in Bursa of Fabricius.

In HPS, degeneration of tubular epithelium of kidneys was reported by many researchers(Khan *et al.*,1988; Anjum *et al.*,1989; Cheema *et al.*,1989 and Muneer *et al.*, 1989). Gowda (1994) noticed inter-cellular haemorrhages and degeneration of tubular epithelium. Nephrosis (Karunamoorthy *et al.*, 1996), degenerative necrosis in the epithelial lining of kidney tubules, presence of hyaline casts in the lumen and haemorrhages in the interstitial tissues of kidney parenchyma were also documented (Survashe et *al.* 1996). Arsani *et al.* (1997) reported proliferative glomerulitis.

Lungs showed oedema, congestion, infiltration of mononuclear cells and heterophils in the alveolar walls (Khan *et al.*, 1988; Cheema *et al.*,1989; Muneer *et al.*,1989; Gowda, 1994 and Arsani *et al.*, 1997). Gowda

(1994) observed catarrhal inflammation of parabronchiolar epithelium and presence of "cell nests" or cartilagenous masses. Abe *et al.* (1998) reported the presence of macrophages in the air capillary and blood capillary areas of lungs.

2.8. Seroprevalence

In a study conducted by Ravikumar (1998), he observed the overall occurrence of HPS in broilers in Bangalore, to be 6.5 per cent, using AGPT as the diagnostic test.

2.9. Diagnosis

2.9.1. Clinical signs

It is very difficult to diagnose HPS based on clinical signs. In natural outbreaks, birds die suddenly without any premonitory signs. The mortality due to HPS started in the third week of flock age (Jaffery, 1988; Khan *et al.*, 1988).

2.9.2. Post-mortem lesions

On post-mortem examination, hydropericardium is one of the predominant pathognomonic lesions of the syndrome, both in the field cases and following experimental inoculation (Anonymous, 1988).

The characteristic post-mortem lesion is hydropericardium with the pericardial sac filled with five to twenty ml of clear straw coloured fluid and the presence of large basophilic intranuclear inclusion bodies in the hepatocytes (Khan *et al.* 1988; Anjum *et al.*,1989; Cheema *et al.* 1989 and Muneer *et al.*, 1989).

Typical deshelled "Leechi fruit" appearance of heart in HPS is also considered as a pathognomonic lesion (Gowda, 1994; Survashe *et al.* 1996; Ravikumar *et al.*, 1998).

2.9.3. Electron microscopy

Using negative staining electron microscopy, Cheema *et al.* (1989) demonstrated adenovirus particles in the infected liver homogenate.

Adenovirus was detected by electron microscopy in the liver homogenate and in the pellet from ultracentrifugation at 40,000 rpm, but failed to detect in the supernatant, even after immune electron microscopy (Afzal *et al.*, 1991).

Chandra *et al.* (1997), in their study demonstrated the presence of virus particles with similar morphological features of adenovirus, both in the pellet after ultracentrifugation and in hepatocytes of infected liver and indicated the involvement of adenovirus in HPS cases.

2.9.4. Virus isolation

An adenovirus from HPS infected bird was isolated by Khawaja *et al.*(1988) and they could successfully reproduce the disease.

Adenovirus was isolated from liver of affected birds where it produced intranuclear inclusion bodies in the hepatocytes (Cheema *et al.*, 1989; Ahamad *et al.*,1990).

The Central Veterinary Laboratory, Weybridge, U.K. isolated an adenovirus from lyophilised homogenate of liver taken from affected birds and later confirmed it as a new isolate of adenovirus, designated as K31/89 by a laboratory in Germany (Voss, 1989).

An isolate LA/C; serotype 4 was recovered from birds showing classical lesions of HPS in Chile (Cowen

et al., 1996) and they could recover fowl adenovirus from rectal swabs of affected birds.

Isolates from HPS cases produced typical cytopathic effects (CPE) in chick embryo liver cell culture and these isolates produced high mortality in chicks with characteristic lesions of hydropericardium and hepatopathy (Rabbani and Naeem, 1996).

Hess *et al.* (1997) isolated fowl adenovirus from HPS cases in Equador and Pakistan. They reproduced the disease by oral inoculation in day-old SPF chicks and reisolated the virus. According to them serotype 4 of adenovirus caused the syndrome.

Abe *et al.* (1998) isolated fowl adenovirus from liver samples by growing in chicken kidney (CK) cells and confirmed the isolate as Serotype 4 of group I adenovirus using neutralization test.

In India, Oberoi *et al.* (1996) isolated avian adenovirus from outbreaks of inclusion body hepatitis - hydropericardium syndrome. They also used chicken embryo liver cell culture and confirmed the agent using

counter immuno electrophoresis, DOT-ELISA and double antibody sandwich ELISA.

Three Indian isolates of fowl adeno virus, recovered from inclusion body hepatitis-hydropericardium syndrome of poultry from different parts of India were typed as FAV serotype 4 using *in vitro* cross neutralisation studies (Jadhao *et al.*, 1997).

2.10. Serological tests

2.10.1. Indirect haemagglutination test

Several workers made an attempt to standardise indirect haemagglutination test in the diagnosis of HPS (Rahman *et al.*, 1989; Hassan *et al.*, 1993).

The indirect haemagglutination test has been used both with human 'O' group and sheep RBC for the detection of HPS antibody (Qureshi, 1990).

According to Akhtar (1994), indirect haemagglutination test gave fewer non-specific reactions and its sensitivity was greater when compared to agar gel precipitation test.

2.10.2. Virus neutralisation test

Jadhao *et al.* (1997) used micro neutralisation and cross- neutralisation tests, in serotyping three isolates of fowl adenovirus recovered from IBH-HPS cases.

By neutralisation test, Abe *et al.* (1998) confirmed the identity of an adenovirus isolated from HPS cases as group I adenovirus, serotype 4, using antiserum against KR5 strain, the representative strain of serotype 4 of group I adenovirus.

2.10.3. Agar gel precipitation test

Kataria *et al.* (1997) used Agar gel precipitation test(AGPT) in their study to assess the immunosuppressive effect of fowl adenovirus serotype 4 isolated from IBH-HPS cases to Ranikhet disease-F strain. They used antigen prepared from chicken embryo liver cell culture propagated virus and obtained satisfactory results.

Ravikumar (1998) used AGPT as a diagnostic test of HPS while studying the pathology and epidemiology of this syndrome.

2.10.4. Counter immunoelectrophoresis

Standardisation of counter immunoelectrophoresis (CIE) was done by Oberoi *et al.* (1990) for the study of egg drop syndrome in poultry and they recommended that it could be used for the quick detection of avian adenovirus in field samples.

Khehra *et al.* (1994) compared AGPT, CIE and ELISA for the detection of avian adenoviruses in chicken tissues and they got a better result for CIE over AGPT.

Oberoi *et al.* (1996), while isolating avian adenovirus from three, out of four outbreaks of inclusion body hepatitis-hydropericardium syndrome used CIE, DOT-ELISA and double antibody sandwich ELISA for identification of the virus. They recorded that all the three samples gave positive reaction in CIE, DOT-ELISA and double antibody sandwich ELISA with aviadeno-virus-1(AAV-1) antiserum.

2.10.5. Enzyme linked immunosorbent assay (ELISA)

A sensitive and simple method for the quantitative determination of antibodies was reported by Engvall and Perlmann (1972). They opined that antibodies in unknown

sera could be quantified by comparison with a standard antiserum.

Dawson *et al.* (1979) applied ELISA for the detection of antibodies to avian adenovirus and avian adeno associated virus. They stated that ELISA was comparable to virus neutralisation test and that it was more sensitive than immunodiffusion.

An indirect ELISA for the detection of antibody to a haemagglutinating duck adenovirus was described by Piela and Yates (1983) and they claimed ELISA as a sensitive and reliable method for detecting antibodies, but conceded that positive titres were not always in agreement with haemagglutination inhibition (HI) and immunodiffusion (ID) results.

Malkinson *et al.* (1983) and Ianconescu *et al.* (1984) confirmed the sensitivity of ELISA for the detection of antibodies to avian adenovirus and the latter remarked that ELISA was more sensitive than virus neutralisation (VN) test.

Enzyme linked immunosorbent assay (ELISA) was more sensitive than the immunodiffusion and

haemagglutination tests. It was specific, rapid, inexpensive and easy to perform (Nicholas and Thornton, 1986).

Nagal *et al.* (1990) used DOT-ELISA and neutralisation test for antigenic characterisation of virus isolates obtained from inclusion body hepatitis cases from broilers.

Enzyme linked immunosorbent assay was applied to detect antibodies against fowl adenovirus type I by Lal *et al.* (1992). They opined that ELISA could be readily used to screen the sera for antibodies against FAV with higher specificity and sensitivity. They also obtained higher titres for ELISA when compared with virus neutralisation test.

Hassan *et al.* (1993) employed enzyme linked immunosorbent assay in the diagnosis of HPS using infected liver homogenate as crude antigen.

Virus isolates from IBH - HPS were identified as avian adenovirus by DOT-ELISA and double antibody sandwich ELISA by Oberoi *et al.* (1996).

Balamurugan *et al.* (1999) developed an indirect DOT-ELISA for the detection of fowl adenovirus group I antigen, in various tissues of chicks, experimentally infected with FAV 4. They could detect antigen as low as 40 ng in spleen, bursa, thymus, kidney and liver of bird affected with IBH-HPS.

2.10.5a. Interpretation of optical density (OD) values

Different workers have interpreted the OD values differently. Marquardt *et al.* (1981); Mockett and Darbyshire (1981) and Lana *et al.* (1983) took the base line for positivity as the mean of all the readings obtained from the negative control sera plus two or three times the standard deviation. The titre of any test serum was then calculated where its absorbance curve crossed the base line.

Nandapalan *et al.* (1982) took the lowest score of the test sera above the highest score of the control negative as the end point.

Garret *et al.* (1984) took an arbitrary cut off point as twice the mean absorbance of negative serum.

Nicholas *et al.* (1985) showed that positive titres had a much wider spread than negatives and suggested the retesting of sera with values in the doubtful area.

2.11. Prevention and control

Various attempts were made to control hydropericardium syndrome in poultry by many workers to curtail the incidence and reduce economic loss.

Autogenous formalin inactivated vaccine prepared from infected liver homogenate was found to be very effective in protecting birds from the syndrome (Cheema *et al.*, 1989; Chishti *et al.*, 1989; Afzal and Ahmad 1990; Ahmad *et al.*, 1990).

Anjum (1990) compared two liver homogenate preparations, one inactivated by 0.1 per cent formalin for 24 hours and the other inactivated by 0.5 per cent formalin for 72 hours, by injecting into broilers at 10 to 15 days of age. He found that the latter preparation gave better protection under field conditions.

Mashkoor *et al.* (1984); Shane (1996) and Kumar *et al.* (1997) also attempted vaccination using formalin inactivated liver homogenate.

A cell culture vaccine was experimentally prepared by growing the virus in chicken embryo liver cells by Naeem *et al.* (1995).

Kataria *et al.* (1997) developed an oil emulsified vaccine of inactivated Indian isolates of HPS grown in chick embryo liver cell cultures.

Akhtar and Cheema (1990) and Akhtar *et al.* (1992) advised for strict biosecurity measures, optimal hygiene and proper storage of feed to control HPS effectively.

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MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Glass wares and reagents

Borosil brand of glass wares and Tarson brand of plastic wares were used in this study. Analytical grades of chemicals only were used.

3.1.2. Chicken eggs

Nine to eleven-day-old embryonated chicken eggs obtained from University Poultry Farm, Mannuthy were used for the revival of HPS virus.

3.1.3. Chicks

Three-week-old unvaccinated healthy broiler chicks procured from University Poultry Farm, Mannuthy were used for the reproduction of HPS and collection of infected liver for further processing.

3.1.4. Chicken

One-month-old healthy chickens procured from University Poultry Farm, Mannuthy were used for the production of antiserum against HPS.

3.1.5. Freund's Complete adjuvant(FCA)

Adjuvant obtained from Difco Laboratories, Michigan, USA was used in the study.

3.1.6. Antibiotic solution

Penicillin	-	10,00,000 IU
Streptomycin	-	1 g
Sterile distilled water	-	40 ml

This was added to the allantoic fluid / liver homogenate so as to get 250 IU of penicillin and 250 µg of streptomycin per ml of the preparation.

3.1.7. Phosphate buffered saline

Sodium chloride	-	8 g
Potassium chloride	-	0.2 g
Disodium hydrogen phosphate	-	1.608 g
Potassium dihydrogen phosphate	-	0.2 g
Distilled water	-	1000 ml

pH adjusted to 7.4 with 1N HCl

Sterilised by autoclaving at 121°C and 15 lbs pressure for 15 min.

3.1.8. Passive Haemagglutination Assay (PHA)

3.1.8.1. Alsever's solution

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

Sterilised by autoclaving at 115°C, 10 lbs pressure for 10 min.

3.1.8.2.Physiological saline

Sodium chloride	-	0.85 g
Distilled water	-	100 ml

Sterilised by autoclaving at 121°C and 15 lbs pressure for 15 min.

3.1.8.3. PBS

As in 3.1.7.

pH was adjusted to 7.2 using 1N HCl.

For making RBC suspension, pH was adjusted to 6.4 using 1 N HCl.

3.1.8.4. Formaldehyde (3 per cent)

Formaldehyde	-	3 ml
Physiological saline	-	97 ml

3.1.8.5. Tannic acid

3.1.8.5a. Tannic acid stock solution

Tannic acid	-	10 mg
PBS (pH 7.2)	-	10 ml

(To make 1: 1000 dilution)

3.1.8.5b. Working solution

Stock solution	-	2 ml
PBS (pH 7.2)	-	38 ml

(To make 1:20000 dilution)

3.1.8.6. Anticoagulant

Sodium citrate	-	3.8 g
Distilled water	-	100 ml

Sterilised by autoclaving at 121°C, 15 lbs pressure for 15 min and stored at 4°C.

3.1.9. Agar Gel Precipitation Test (AGPT)

3.1.9.1. 0.5 per cent agarose for coating the slides

Agarose	-	0.5 g

Distilled water - 100 ml

Agarose was dissolved by boiling in a water bath

3.1.9.2. Gel

Agarose	-	0.8 g
Sodium chloride	-	8.5 g
Sodium azide	-	0.3 g
Distilled water	-	100 ml

Agarose was melted by boiling and used.

3.1.9.3.Physiological Saline

As in 3.1.8.2

3.1.9.4. Staining solution

Amidoblack 10B	-	1.0g
Sodium chloride	-	8.5g
Distilled water	-	1000 ml

3.1.9.5. Decolouriser I

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

3.1.9.6. Decolouriser II

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

3.1.10. Counter Immunoelectrophoresis (CIE)

3.1.10.1. Agarose 0.5 per cent for coating of slides

Prepared as in 3.1.9.1.

3.1.10.2. Tris-barbitone buffer

Barbitone sodium	-	9.9 g
Tris (hydroxymethyl amino methane)	-	17.7 g
Sodium azide	-	0.3 g
Distilled water	-	2000 ml

pH adjusted to 8.6 with 1N HCl.

3.1.10.3. Gel

Agarose	-	0.8 g
Tris barbital buffer	-	100 ml

3.1.10.4. Physiological saline

As in 3.1.8.2.

3.1.10.5. Staining solution

As in 3.1.9.4.

3.1.10.6. Decolouriser I

As in 3.1.9.5.

3.1.10.7. Decolouriser II

As in 3.1.9.6.

3.1.11. Enzyme linked immunosorbent assay (ELISA)

3.1.11.1. ELISA plates

ELISA plates supplied by Tarson, with 96 flat bottom wells were used.

3.1.11.2. Antigen coating buffer

3.1.11.2a. Solution A

Sodium carbonate	-	2.12 g
Triple glass distilled (TGD) water	-	100 ml

3.1.11.2b. Solution B

Sodium bicarbonate	-	1.68 g
TGD water	-	100 ml

3.1.11.2c. Working solution

Solution A	-	7 ml
Solution B	-	17 ml
TGD water	-	76 ml

pH adjusted to 9.6 using 1N NaOH, sterilised by autoclaving at 121°C and 15 lbs pressure for 15 min and kept at 4°C.

3.1.11.3. Phosphate buffered saline (PBS)

As in 3.1.7.

3.1.11.4. PBS Tween 20 (PBST)

Tween 20	-	500 µl
PBS	-	1000 ml

3.1.11.5. Bovine serum albumin (BSA) -2 per cent

BSA	-	2 g
PBST	-	100 ml

3.1.11.6. BSA-PBST for dilution of serum and anti-chicken IgG

BSA	-	1 g
PBST	-	100 ml

3.1.11.7. Sodium citrate buffer

Sodium citrate	-	1.471 g
Distilled water	-	100 ml

pH adjusted to 4.2 using 1N HCl.

3.1.11.8. Substrate solution

ABTS (2'- 2 azino-di-ethyl Benzthiazoline-6-sulfonic acid)	-	11 mg
Sodium citrate buffer	-	50 ml
Hydrogen peroxide(30 per cent)	-	25 µl

3.1.11.9. Hydrofluoric acid

0.1 M hydrofluoric acid

3.1.12. Electron microscopy

3.1.12.1. Grids

Commercially available uncoated copper grids (Taab Laboratories Equipment Ltd., England) were used.

3.1.12.2. Formvar solution (0.25 per cent) for coating

Formvar	-	0.125 g
Chloroform	-	50 ml

Kept at 4°C for chilling.

3.1.12.3. Phosphotungstic acid

Phosphotungstic acid	-	0.2 g
Distilled water	-	10 ml

3.1.13. Biologicals

3.1.13.1.HPS virus and antiserum

HPS virus and antiserum procured from The Department of Animal Biotechnology, Madras Veterinary College, Chennai were used for the study.

3.1.13.2. Sera samples

Three hundred and fifty sera samples were collected from broiler chickens from different parts of Kerala, including Government and private farms.

3.1.13.3. Antichicken IgG

Antichicken IgG was purchased from Sigma-Aldrich, Bangalore.

3.1.13.4. Sheep RBC for PHA

Sheep RBC was prepared from the blood collected from the sheep maintained at University Goat Farm, Mannuthy.

3.1.13.5. Normal Rabbit serum (NRS) for PHA

Rabbit serum was prepared from the blood collected from rabbits procured from the Small Animal Breeding Station, Mannuthy.

Rabbit serum	-	1 ml
PBS (pH- 7.2)	-	99 ml

To make one per cent rabbit serum.

3.2.METHODS

3.2.1.Revival of antigen

The HPS virus procured from the Department of Animal Biotechnology, Madras Veterinary College, Chennai, was revived by passaging in embryonated chicken eggs.

3.2.1.1. Chicken embryo inoculation

Allantoic cavity inoculation was done as per the method of Bishai *et al.* (1974).

Nine-day-old embryonated chicken eggs were candled and position of the air cell and embryo were marked. The air cell region was disinfected with tincture iodine and a small hole was drilled on the shell 0.5 cm away from the border of the air cell and away from the head region, without breaking the outer shell membrane. Using a sterile syringe with 22gauge needle, 0.2 ml of the HPS antigen (3.1.13.1) was injected into the allantoic cavity. The embryos which were kept as controls were inoculated with 0.2 ml of sterile saline

into the allantoic cavity. The hole was sealed using melted paraffin and the eggs were incubated at 37°C in an upright position. Eggs were candled daily to check any mortality. The embryos which died 24 h after inoculation and those which did not die even after five days of incubation were transferred to a refrigerator and kept overnight for chilling. Those embryos that died within 24 h of inoculation were discarded.

3.2.1.2. Harvesting of the inoculated eggs

After chilling, the eggs were brought to room temperature. Afterwards, the air cell region of the eggs was cleaned and disinfected with 70 per cent alcohol. Using sterile forceps and scissors, air cell region was cut and removed. The inner shell membrane and chorio-allantoic membrane were peeled off. Using a sterile glass pipette, the allantoic fluid was collected into a sterile vial. The embryo was cut open and examined for any lesions. The impression smear of embryonic liver was taken on a microscopic slide to find out the presence of inclusion bodies. The allantoic fluid was clarified by low speed centrifugation at 800 x g for 10 min at 4°C. This allantoic fluid after the first passage was used as the inoculum for the second passage. The presence of virus

in the allantoic fluid was checked by AGPT using the HPS antiserum (3.1.13.1). The passaging in embryo was continued till embryo mortality could be detected. The titre of the virus in the allantoic fluid was detected using PHA. The allantoic fluid was clarified and stored at -20°C until further use.

3.2.2. Inoculation of virus into chicks

The revived virus suspension (allantoic fluid), 0.5 ml, was inoculated into three-week-old healthy broiler chicks. Five birds each were inoculated with the virus through intramuscular , subcutaneous and intraperitoneal routes. Five chicks of the same age group were housed separately as controls, which were given 0.5 ml of allantoic fluid from normal embryos subcutaneously. Before inoculation all the chicks were tested negative for HPS antibodies using AGPT. The chicks were kept at constant observation for one week. The dead chicks were autopsied. Liver was collected aseptically and stored at -20°C for future use.

3.2.3. Preparation of liver homogenate as antigen

Liver collected from affected birds was taken in a sterile mortar and cut into small pieces using sterile scissors. Aseptic precautions were taken to avoid

contamination. A small quantity of sterile silica gel was added and with a sterile pestle, the liver was thoroughly triturated in PBS to make a 20 per cent (w/v) liver homogenate. This was then subjected to low speed centrifugation at 8000 x g for 15 min at 4°C. The supernatant was collected, the protein concentration was checked by Biuret method and distributed in small aliquots with antibiotics and stored at -20°C for further use.

The liver homogenate (antigen) was further concentrated by ultracentrifugation at 80000 x g using a Beckmann type 28 rotor for three hours at 4°C. The pellet was resuspended in three millilitre of PBS. The protein concentration of the final preparation was determined by Biuret method.

3.2.4. Production of antiserum

Antiserum was prepared by following the procedure described by Polly (1977).

Five, two-months-old healthy chickens weighing about 1.5 kg procured from UPF, Mannuthy, were used for the production of antiserum. All the birds that were

found negative for HPS antibodies by AGPT alone were used.

The antigen was inactivated using 0.1 per cent formalin, keeping at 4°C overnight. One ml of this inactivated antigen was homogenised with one ml (1:1) of Freund's complete adjuvant. Each bird received 0.5 ml of this emulsion by intramuscular route. Three booster doses of this antigen, 0.5 ml each, without adjuvant were given at seven day intervals by the same route. The birds were test bled to check the presence of antibody in the serum by AGPT. The serum was checked at weekly intervals to monitor the increase in the level of antibody using indirect ELISA. When the antibody level was found satisfactory, the chickens were bled completely, 10 days after the last booster dose. The serum was separated, inactivated at 56°C for 30 min and stored at -20°C in small aliquots for further use.

3.2.5. Collection of sera samples

A total of 350 sera samples from broiler chickens were collected from different parts of Kerala.

Blood was collected from the wing vein of broilers, six to eight weeks of age, using clean dry syringe with 22gauge needle. This was immediately transferred into clean test tubes and kept at 45° angle for 15 min for separation of serum. This was then kept in a refrigerator overnight, centrifuged at 1000 x g for 15 min and the serum was collected in two millilitre storage vials, inactivated at 56°C for 30 min and stored at -20°C for further use.

3.2.6. Passive Haemagglutination Assay (PHA)

The test was done as per the technique of Boyden (1951) with slight modifications to assess the titre of antigen used for the study.

3.2.6.1. Collection of sheep blood cells

The blood was collected from healthy adult sheep in Alsever's solution (3.1.8.1) in 1:4 (v/v) dilution. This was kept at 4°C for three days to age and then subjected to low speed centrifugation to remove plasma and buffy coat. The RBCs were washed thrice in phosphate buffered saline at pH 7.2 at 800 x g for five minutes and a fourth washing in PBS for 10 min after the third washing. A 2.5 per cent RBC suspension was made in PBS.

3.2.6.2. Formalinised tanned erythrocyte (FTE) preparation

To the 2.5 per cent suspension of RBC in PBS an equal volume of three per cent formol saline was added. The mixture was incubated at 37°C for 20 h. These formalinised erythrocytes were then washed three times in PBS, pH 7.2 and stored as a 2.5 per cent suspension in PBS (pH- 7.2) at 4°C until used for tanning.

Tannic acid solution, 1: 20000, was prepared in PBS at pH 7.2. Equal proportions of this tannic acid and 2.5 per cent sheep RBC in PBS were mixed and incubated in a water bath at 37°C for 10 min.

This was then centrifuged for 5 min at 800 x g, washed with PBS, pH- 7.2 and resuspended to a 2.5 per cent suspension of FTE in PBS at pH 6.4.

3.2.6.3. Sensitisation of FTE by antigen

Different dilutions of antigen were prepared in PBS, pH- 6.4, (1: 25, 1: 50, 1: 100, 1: 200, 1: 400 and 1: 800). Sensitised the cells with each dilution of antigen by adding equal amounts of tanned cell suspension with different dilutions of antigen.

Incubated the mixtures in a water bath at 37°C for 15 min. These mixtures were then centrifuged for 5 min at 800 x g and the cells were washed twice with one per cent normal rabbit serum (NRS)(3.1.13.5).

A final cell suspension of 1.5 per cent FTE in one per cent NRS was made for each dilution of antigen.

3.2.6.4. Test proper

The antiserum prepared against the HPS antigen (liver homogenate) with a titre of 1:6400 (by indirect ELISA) was used as positive serum.

The positive serum was diluted in one per cent NRS to make a 1:10 dilution.

In a microtitre plate, 0.05 ml of the diluted serum was pipetted out to all the wells except in the last row, which was used as the diluent control.

Added 0.025 ml of 1.5 per cent sensitised cell suspension of each antigen dilution to each row, except the last two, with constant shaking of the plate. The cells were allowed to settle for three hours at room temperature.

The last two rows were used as serum control and diluent control respectively. To the serum control 0.025 ml of 1.5 per cent unsensitised tanned RBC suspension was added to each dilution of serum.

To the diluent control 0.05 ml of one per cent NRS was transferred to each well to which 0.025 ml of 1.5 per cent suspension of sensitised cells was added.

The results were read after three hours of incubation at room temperature.

3.2.7. Tests to assess the seroprevalence of HPS virus

A total of 350 sera samples from broiler chickens were collected from different parts of Kerala to screen the presence of HPS antibodies. In the initial phase, 50 sera samples were tested by AGPT, CIE and indirect ELISA. The results were compared and the remaining 300 samples were screened by the most reliable test.

3.2.7.1. Agar gel precipitation test (AGPT)

(i) AGPT was done as per the method described by William and Chase (1968) with suitable modifications.

Clean glass slides, 7.5 x 2.5 cms, were precoated with 0.5 per cent agarose and dried in an incubator. Three millilitre of the melted 0.8 per cent agarose was poured onto the glass slides, allowed to solidify, first at room temperature and then in a refrigerator at 4°C. Wells were punched in a five well pattern keeping six-millimetre distance from the central well. The central well was charged with HPS antigen and the remaining four peripheral wells were charged with two test and two control sera (both positive and negative controls were used). After charging, the slides were kept in a humid chamber at 37°C for 48h and examined against light for the presence of any precipitation line.

(ii) Staining

After 48 h, the slides were washed in normal saline. Two changes were made in normal saline for 24 h each and a third change in distilled water was done for another 24 h. A filter paper soaked in distilled water was placed on the slides and they were kept in an incubator for slow drying. Afterwards slides were stained with amidoblack for 15 min and then decolourised in decolouriser I and II for 20 min each. The slides were dried at 37°C for 1h and examined.

3.2.7.2. Counter immunoelectrophoresis (CIE)

Counter immunoelectrophoresis was done as per the method described by William and Chase (1968) with slight modifications.

Precoated slides (0.5 per cent agarose) were used for the test.

Agarose (0.8 per cent) in tris barbitone buffer (3.1.10.2) was melted and five millilitres of this was poured on to a clean precoated glass slide (7.5 x 5.5 cms) kept on a levelled surface. The agarose was allowed to solidify first at room temperature and then at 4°C in a refrigerator. Five pairs of wells were punched, one pair below the other with a distance of six millimetre between the wells. The wells at the anodal side were charged with sera and cathodal side with antigen with appropriate controls. The wells of the first row were charged with positive serum and antigen, second row with negative serum and antigen and the third, fourth and fifth rows with test sera and antigen. Electrophoretic apparatus (Amersham Pharmacia Biotech. Germany) was used for CIE. Each buffer chamber was filled with tris barbitone buffer at pH 8.6 and the electrophoresis was carried out at 12 mA for one hour

at room temperature. After electrophoresis the slides were examined immediately. The slides were washed, dried and stained with amidoblack as described earlier for AGPT.

3.2.7.3. Indirect Enzyme Linked immunosorbent assay (Indirect ELISA)

Indirect ELISA was done as per the method of Piela and Yates (1983) with slight modifications.

3.2.7.3.1. A checkerboard titration was carried out to find out the optimal dilutions of antigen and sera as per the method of Gangadhar (1993) with some modifications.

The liver homogenate antigen (3.2.3) with a protein concentration of 29.07 mg per ml and a PHA titre of 1:100 was used as antigen.

Negative antigen was prepared from the liver collected from a non-infected, disease free bird (checked by AGPT), following the same method used for the antigen (3.2.3) preparation.

Sera collected from adult layer birds with no history of HPS (checked by AGPT) were used as negative serum controls.

1. A serial two fold dilution of the antigen was made in antigen coating buffer to provide dilutions ranging from 1: 25 to 1: 800.
2. 100 µ l each of various antigen dilutions were transferred to the wells across the plate in rows. Wells in the last two rows were charged with 1:25 and 1:50 dilutions of negative antigens respectively. The plate was incubated overnight at 4°C.
3. The contents of the wells were emptied and the plate was washed with PBST and slapped the inverted microplate onto a lint free absorbent to remove all residual contents. This was repeated twice..
4. The wells were then charged with 100 µ l each of two per cent BSA solution and incubated for one hour at 37°C. This was done to block the unreacted sites of the wells.
5. After one hour of incubation, the plate was washed as described previously.

6. Two fold dilutions of known positive and negative sera samples were prepared in one per cent BSA-PBST ranging from 1: 200 to 1: 6400. 100 µl of each of the serum dilution was added to individual wells down the plate in columns and the plate was incubated at 37°C for one hour. Washing and drying were done as before.
7. 100 µl of 1: 30,000 dilution of antichicken IgG-HRP conjugate (Sigma- Aldrich) in one per cent BSA PBST was transferred to each well and incubated for one hour at 37°C. Washing and drying were done as mentioned earlier.
8. 100 µl each of freshly prepared chromogen / substrate solution (ABTS and H_2O_2) was charged to all the wells including the controls. The plate was incubated at 37°C for 30 min.
9. The reaction was stopped by adding 50 µl of 0.1M hydrofluoric acid to each well
10. The optical density (OD) values were read at 405 nm in an automated Multiscan MS ELISA reader.
11. The optimum dilutions of antigen and sera were found out from the OD values.

12. The baseline for finding out the optimum dilution was determined, as the dilution at which there was a sudden drop in the OD value.

3.2.7.3.2. Test proper

All the wells of the ELISA plate except two were coated with 100 µl each of 1:400 dilution of antigen in antigen coating buffer. This was kept for overnight incubation at 4°C.

The plates were washed thrice with PBST, drying each time onto a lint free absorbent. The uncoated sites were blocked with two per cent BSA and incubated at 37°C for one hour.

After washing the plates as before, sera samples, 100 µl each of, 1:100 dilution were added to the wells in duplicate.

The antiserum prepared against the liver homogenate antigen, at a dilution of 1:800 as obtained by checkerboard titration, was used as the positive serum control. A weak positive serum control was also kept using the same antiserum at a dilution of 1:1600. Negative serum control and conjugate control without

any serum were also kept. The plates were incubated at 37°C for one hour.

The plates were washed and dried as described earlier and each well was charged with 100 µl of 1:30,000 antichicken IgG -HRP (Sigma-Aldrich) diluted in one per cent BSA-PBST. Plates were incubated at 37°C for one hour.

Substrate/ chromogen and stopping reagents were added as described before and readings were taken using a Multiscan MS ELISA reader at 405 nm and data were interpreted.

Optical density (OD) values above twice the mean OD values of negative sera were taken as positive (Garret *et al.*, 1984).

Based on the results obtained by AGPT, CIE and indirect ELISA, the remaining 300 samples were tested by the most reliable test.

The results of the three tests were compared statistically using Z-test (Snedecor and Cochran, 1967)

to find out statistically significant difference among the three, if any.

3.2.8. Electron microscopy

Electron microscopy was carried out as per the method described by Hamvas (1974) with slight modifications.

The liver homogenate material processed as per the procedure described earlier (3.2.3) was used for electron microscopy. One drop of the suspension was placed on a formvar-coated grid. After 30 sec, the excess fluid was absorbed in a Whatman No. 1 filter paper. The grid was then stained with two per cent phosphotungstic acid for 30 sec. The excess fluid was absorbed as mentioned before. The grid was then dried at room temperature for one hour and examined in a Hitachi 500 G electron microscope at 75 kV at a magnification of 80,000.

3.2.9. Histopathology

The birds died of HPS were subjected to detailed autopsy examination. Gross lesions of different organs were recorded. Representative samples of heart, liver, lungs, spleen, proventriculus, intestine, kidneys and

bursa were collected for histopathological examination. The tissue samples were preserved in 10 per cent neutral buffered formalin and processed by routine paraffin embedding technique. Sections of four µm thickness were cut from each paraffin block and were stained with Harris Haematoxylin and Eosin as per the method of Sheehan and Hrapchak (1980). Various histopathological lesions observed were systematically documented.

RESULTS

4.RESULTS

4.1. Revival of the virus

The virus procured from the Department of Animal Biotechnology, Madras Veterinary College, Chennai, was inoculated into the allantoic cavity of nine-day-old chicken embryos. Embryo mortality occurred only in the fifth passage. In the first, second and third passages, no lesions were observed in the embryo. In the fourth passage, embryo showed congestion of liver as the only lesion. The allantoic fluid was tested for the presence of virus by AGPT using the antiserum procured from Madras Veterinary College, Chennai. A thin precipitation line was visible by AGPT. Lesions were more pronounced after the fifth passage.

The dead embryos showed congestion and haemorrhage of liver, spleenomegaly and petechial haemorrhages on the heart. Impression smear of liver showed basophilic intranuclear inclusion bodies. Hydropericardium was not seen in embryos. A sharp precipitation line was obtained between the antigen and antibody wells when tested by AGPT (Fig. 1). In the controls, no lesions were noticed in the embryos and the allantoic fluid did

not give any precipitation line when tested by AGPT. The allantoic fluid had a titre of 1:50 in PHA.

4.2. Inoculation into chicks

The allantoic fluid obtained after the fifth passage was injected into three-week-old chicks at a dose rate of 0.5 ml per bird. Five birds each were inoculated by intramuscular, subcutaneous and intraperitoneal routes. All the birds died within three to seven days post exposure. All the birds except two showed sudden mortality without any clinical signs. Two birds injected through intraperitoneal route showed droopiness with the head hung down from the fourth day onwards and died on the seventh day. The mortality started earlier when the inoculum was given intramuscularly. Two birds died on the third day and the remaining three died on the fourth day when inoculated by intramuscular route. Majority of the birds (eight birds out of fifteen birds inoculated) died on the fourth day. Incubation period was prolonged when the inoculum was given intraperitoneally, viz., one bird died on the fourth day, two birds on the fifth day and two more on the seventh day. The results are presented in table 1. Upon autopsy, the typical lesion was hydropericardium with the pericardial sac filled

with about 10 ml of straw coloured fluid (Fig. 2). There were ecchymotic haemorrhages in the epicardium. Liver showed mottling and congestion. Spleen was enlarged and bursa of Fabricius showed hypertrophy in some cases and atrophy in some other cases. Impression smear taken from the cut surface of liver showed basophilic, intranuclear inclusion bodies (Fig. 3). Infected liver was collected aseptically into sterile vials and kept at -20°C. The five birds kept as controls remained healthy throughout the observation period.

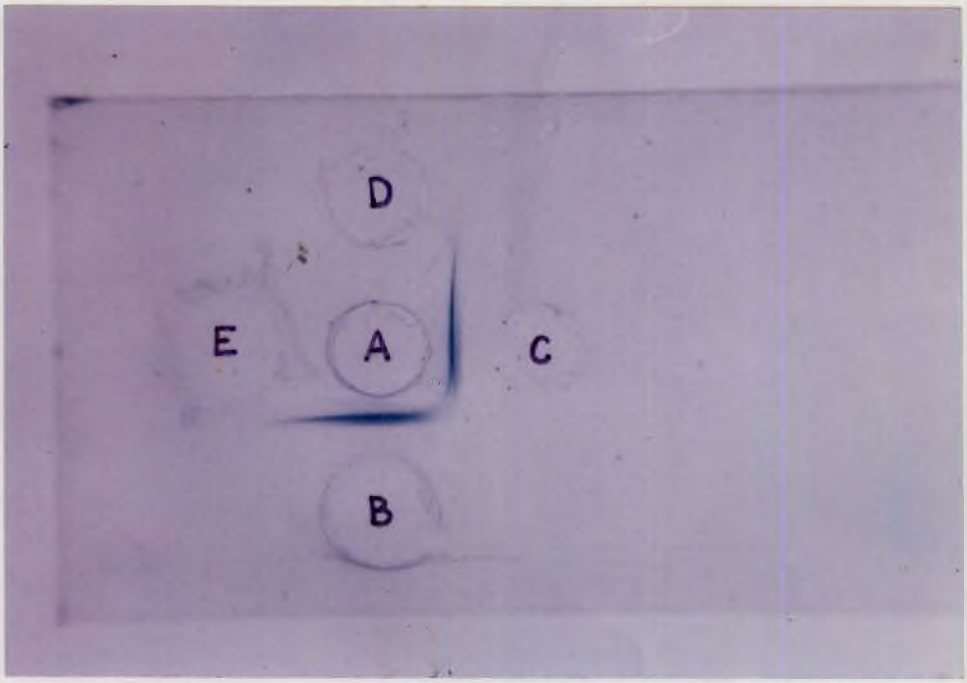
4.3. Preparation of liver homogenate as antigen

Twenty per cent liver homogenate was prepared in PBS and it was subjected to low speed centrifugation. The supernatant was collected. This homogenate had a protein concentration of 13.6 mg/ml. This liver homogenate gave positive reaction by AGPT using the antiserum procured from the Department of Animal Biotechnology, Madras Veterinary College, Chennai. This was subjected to ultracentrifugation and the pellet was dissolved in PBS. The final preparation had a protein concentration of 29.07 mg/ml. A PHA titre of 1: 100 was obtained for this preparation and it constituted the antigen used in the serological tests.

Fig.1. AGPT of allantoic fluid against the reference antiserum

A - reference antiserum
B & C-infected allantoic fluid
D & E-negative control(non-infected allantoic fluid)

Fig.2. Heart-Distended pericardium with straw coloured fluid.


D
E
A
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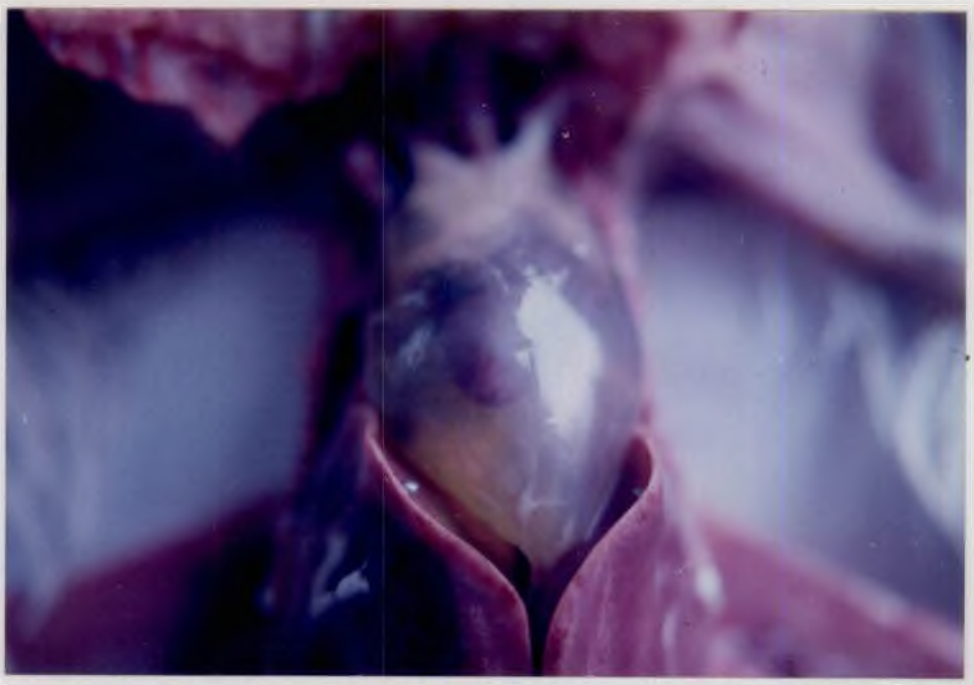
B

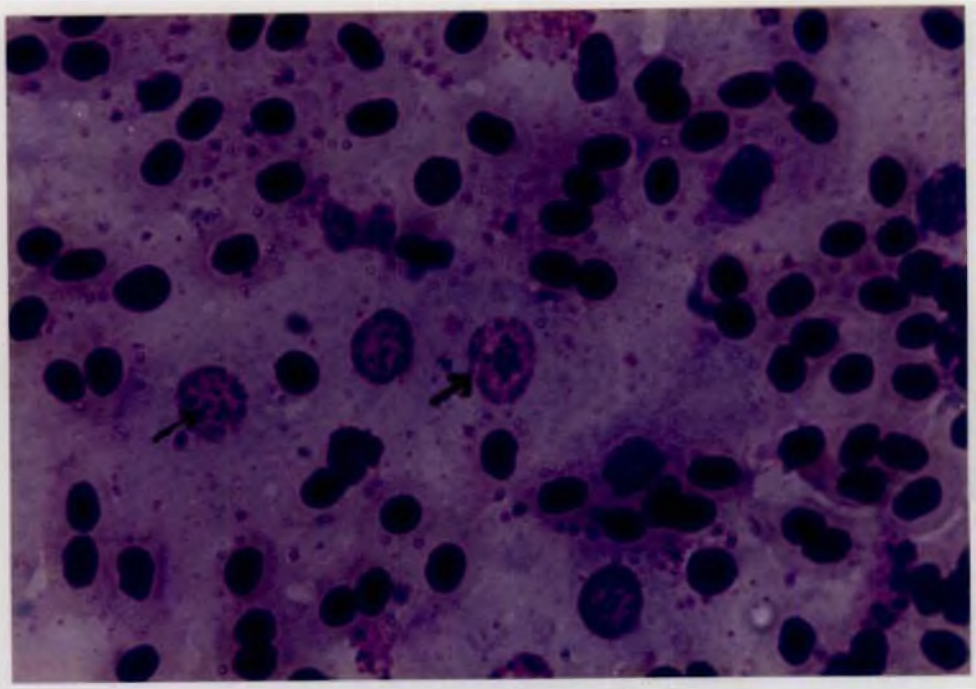


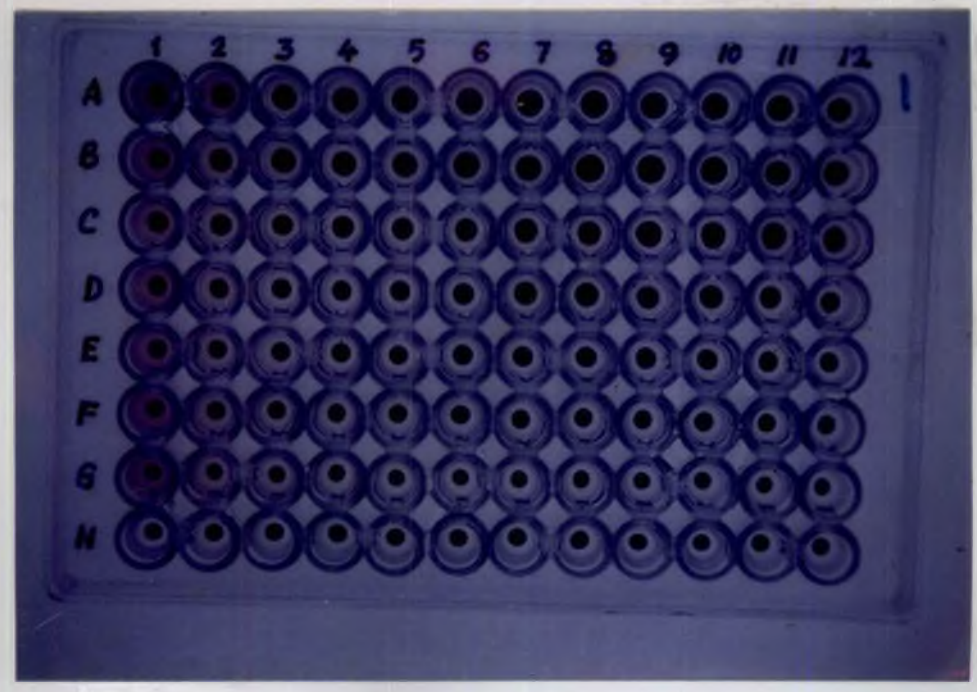
4.4. Production of antiserum

The liver homogenate preparation with a PHA titre of 1:100 and a protein concentration of 29.07 mg/ml was used as the antigen. The antiserum raised against the prepared antigen was used as the positive serum control. This serum was also counter checked using the allantoic fluid collected after the fifth passage (3.2.1.2) and the HPS virus procured from Department of Animal Biotechnology, Madras Veterinary College, Chennai, employing AGPT. In both the cases a single sharp precipitation line was observed between the antigen and antibody wells. All the five birds were bled 10days after the third booster and serum was separated. The presence of antibodies was detected using AGPT. A faint precipitation line was observed after the first booster doses. The lines gradually became thicker and sharp after the second and third booster dose. The antibody level was monitored using indirect ELISA as well. After the first booster dose, the titre was found to be 1: 200. After the second booster dose, indirect ELISA gave 1: 800 as titre and 1:3200 after the third booster dose. Ten days after the third booster, the titre was found to be 1: 6400.

Fig.3. Liver-Basophilic intranuclear inclusion bodies in the hepatocytes. Giemsa stain x 1000.

Fig.4. PHA of the liver homogenate antigen
A to F -Antigen dilutions (1:25 to 1:800)
G -Serum control
H -Diluent control




1 2 3 4 5 6 7 8 9 10 11 12
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H

4.5. Passive haemagglutination assay

Passive haemagglutination assay was done to assess the titre of the antigen preparations, viz., allantoic fluid and liver homogenate, used for serological tests. The liver homogenate was pelleted and the pellet was dissolved in three millilitres of PBS. Different dilutions of this antigen (1:25 to 1:800) and a known positive serum were used for this test. The titre was found to be 1:100 (Fig.4). The allantoic fluid showed a titre of 1: 50. The values are presented in Table 2.

4.6. Serological tests

4.6.1. Agar gel precipitation test

Out of the 350 samples collected, 50 samples were tested for the presence of HPS antibodies using AGPT. Three samples out of 50 showed precipitation lines between the central (antigen) and peripheral (serum) wells. No other samples showed any positive reaction. The precipitation line was sharp, single and confluent with the precipitation line obtained with positive control (Fig.5).

4.6.2. Counter immunoelectropshoresis

Counter immunoelectrophoresis was used to test the presence of HPS antibodies in the 50 sera samples

Table 1. Experimental inoculation of revived antigen in chicks

No of birds inoculated	Route of inoculation	Inoculum	Dose (ml)	Mortality (days)				
				3	4	5	6	7
5	Intra muscular	Infected allantoic fluid	0.5	2	3	-	-	-
5	Sub-cutaneous	,,	0.5	-	4	1	-	-
5	Intra-peritoneal	,,	0.5	-	1	2	-	2
5 (Control)	Subcutaneous	Normal allantoic fluid	0.5	-	-	-	-	-

Table 2. Comparative estimation of antigen titres with PHA and indirect ELISA

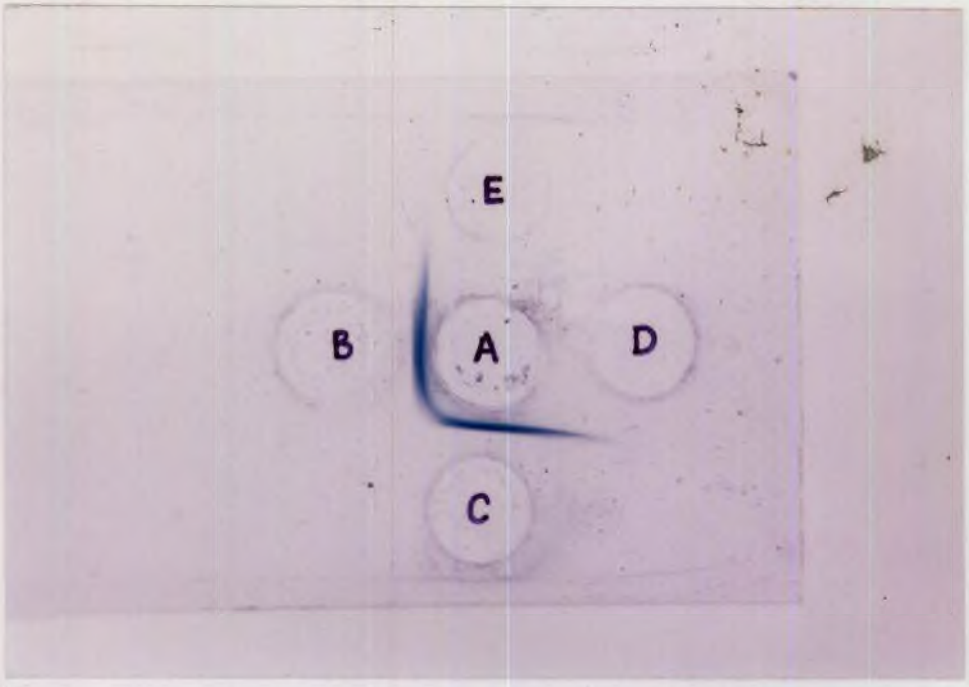
Sample	Titre	
	PHA	Indirect ELISA
Allantoic fluid	1 : 50	1 : 200
Liver homogenate	1 : 100	1 : 400

Fig.5. AGPT of test sera against liver homogenate antigen

A -Liver homogenate antigen
B -Positive serum control
C & D-Test sera
E -Negative serum control

Fig.6. CIE of test sera using liver homogenate antigen

A -Positive serum control
B -Negative serum control
C,D & E-Test sera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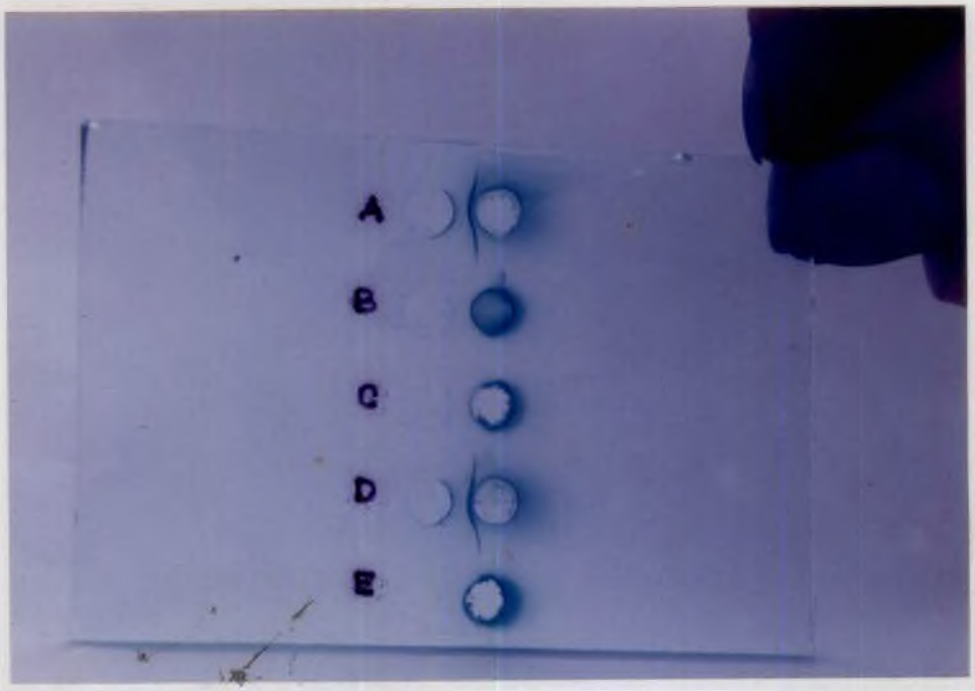
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E

collected. Three out of 50 samples showed precipitation lines between the anodal (serum) and cathodal (antigen) wells. The precipitation line was sharp and single and positioned more towards the anodal well (Fig.6). These three positive samples were the same sera samples, which gave positive results by AGPT.

4.6.3. Indirect ELISA

In checkerboard titration, the optimum dilution of antigen was found to be 1:400. The optimum dilution of the hyper immune serum used as positive control was found to be 1: 800 (Fig.7).

While standardising the test, the optimum dilution of the test sera was found to be 1:100. This dilution of serum was used for screening the field sera samples. Optical density values which were above the double of the mean optical density (OD) value of negative control were taken as positive (fig.8).

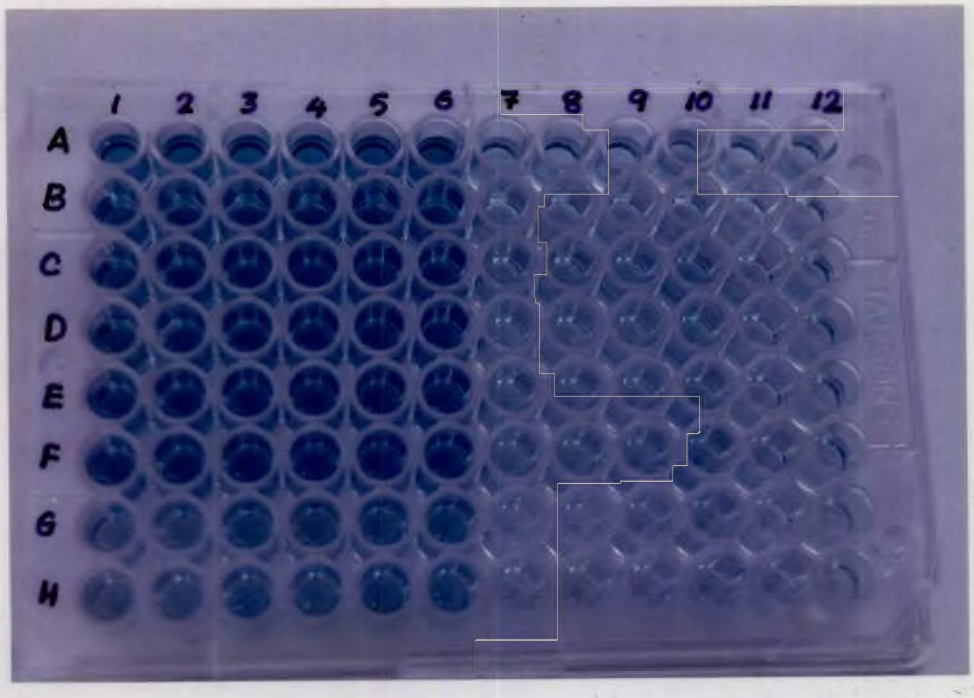
Out of the 50 sera samples screened initially seven samples were found to be positive for HPS antibodies. The titres of these seven samples are shown in Table 3. These seven samples included the three samples detected positive by AGPT and CIE. Four samples

Fig.7. ELISA checkerboard titration

A to F -liver homogenate antigen (1:25 to 1:800)
G & H -Negative antigen
1 to 6 -Positive serum
7 to 12-Negative serum

Fig.8. ELISA of test sera

A 1&2 -Blank
B 1&2 -Positive control(1:800)
C 1&2
D 1&2 -Positive control(1:1600)
E 1&2
F 1&2 -Negative control
G 1&2
H 1&2 -Conjugate control
A to H rows, 3 to 12-Test sera in duplicate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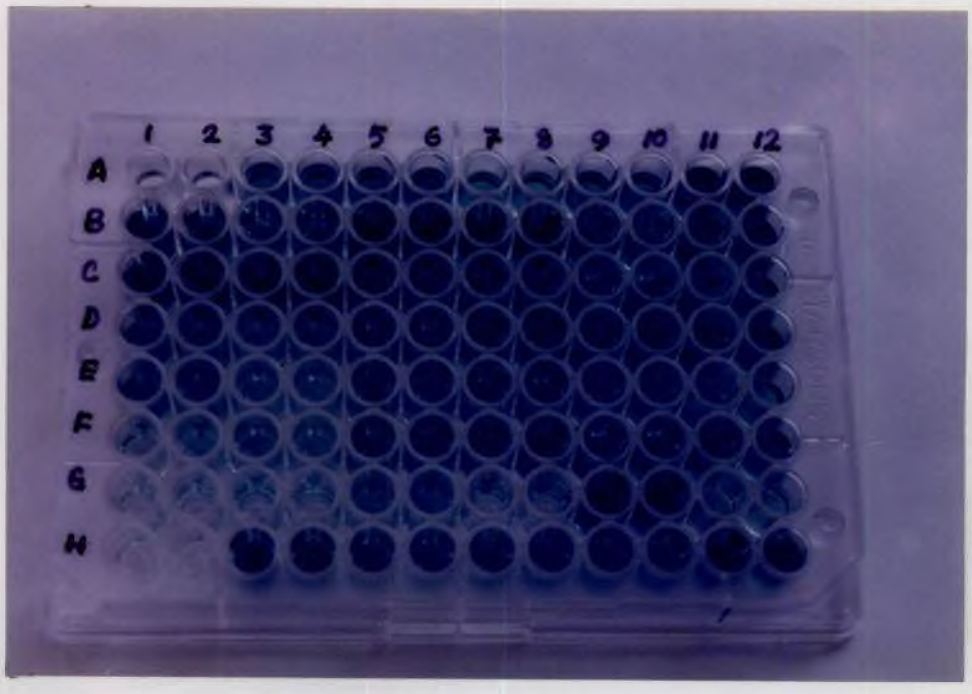
1 2 3 4 5 6 7 8 9 10 11 12
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which were found to be negative by AGPT and CIE showed positive results in indirect ELISA. The results are tabulated in Table 4.

The remaining 300 samples were screened by using indirect ELISA. Out of these 300 sera samples, seventeen samples were found to be positive for HPS antibodies.

So a total of 24 samples out of 350 samples were positive for HPS antibodies.

The seroprevalence of HPS by indirect ELISA was estimated to be 6.86 per cent.

The three serological tests, viz., AGPT, CIE and indirect ELISA used in this study showed no significant difference among them when the results were compared statistically using Z-test at five per cent level.

4.7.Electron microscopy

Virus particles could be detected by electron microscopy in the sample prepared from the infected liver homogenate pellet. The particles were hexagonal in shape and were suggestive of adenovirus. The virus

Fig.9. Electron micrograph showing hexagonal virus particles in the liver homogenate of birds affected with HPS. Negative staining. Magnification x 80000

Table 3. HPS antibody titres of field sera samples by indirect ELISA

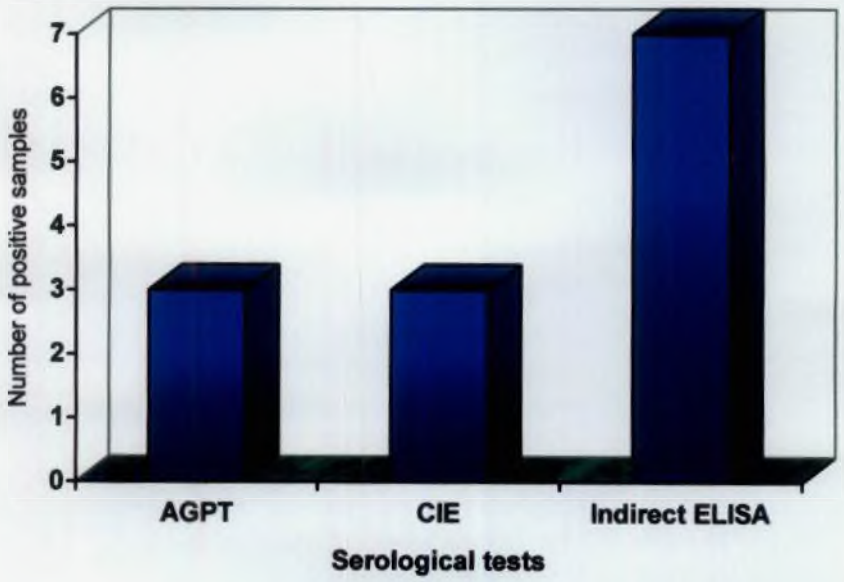
Antiserum	1: 6400
Sample 1	1 : 800*
Sample 2	1 : 3200*
Sample 3	1 : 1600*
Sample 4	1 : 400
Sample 5	1 : 400
Sample 6	1 : 800
Sample 7	1 : 200

* - positive for AGPT and CIE

Table 4. Comparison of AGPT, CIE and indirect ELISA in HPS antibody detection

Number of samples tested	Test used	No. Positive	No. Negative	Percentage positive
50	AGPT	3	47	6
50	CIE	3	47	6
50	Indirect ELISA	7	43	14

Fig. 10. Comparison of AGPT, CIE and indirect ELISA in HPS antibody detection



particles measured an average of 75 nm in diameter (Fig. 9).

4.8. Gross lesions

All the infected birds developed varying degrees of distension of the pericardial sac with moderate to large quantities of straw coloured fluid. The fluid was transparent and clear. The minimum quantity of fluid present in the sac was 10 ml.

The heart appeared shrunken. Diffuse foci of necrosis could be detected on the surface. Focal petechiae and streaks of haemorrhages could also be observed on the myocardium.

The liver was enlarged and hard in consistency. Diffuse greyish white areas of necrosis, congestion and petechiae present in the parenchyma gave the liver a mottled appearance.

Lungs were congested and oedematous and frothy fluid oozed out from the cut surface.

Kidneys appeared congested in some and pale in others, giving a cooked meat appearance. Ureters were distended with urates.

Bursa appeared either hypertrophic or atrophic. Spleenomegaly, engorgement of mesenteric vessels and catarrhal enteritis were the other gross lesions observed.

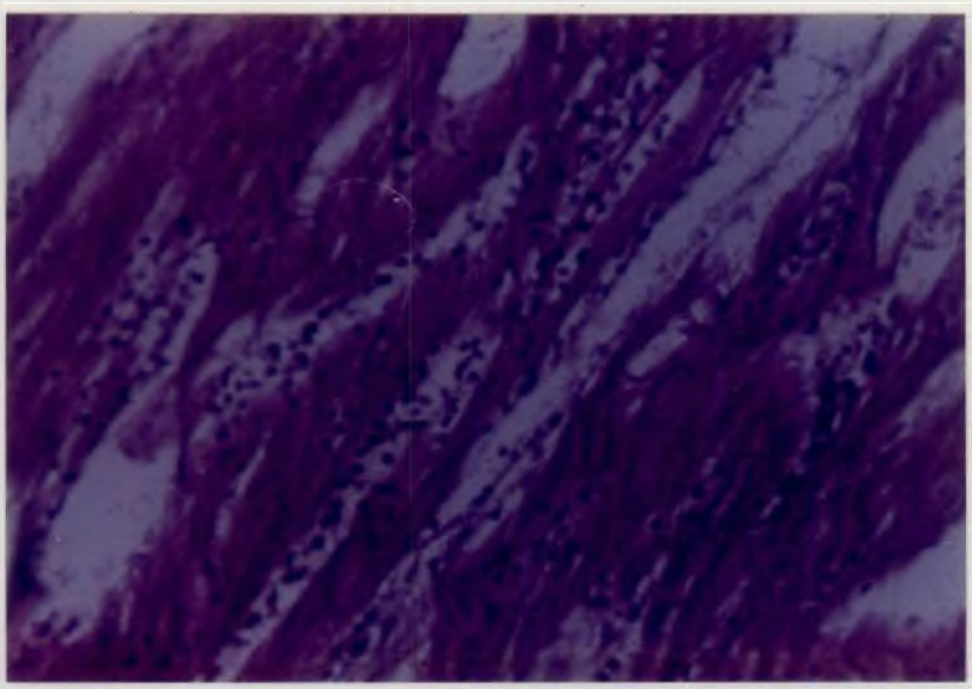
4.9. Histopathological lesions

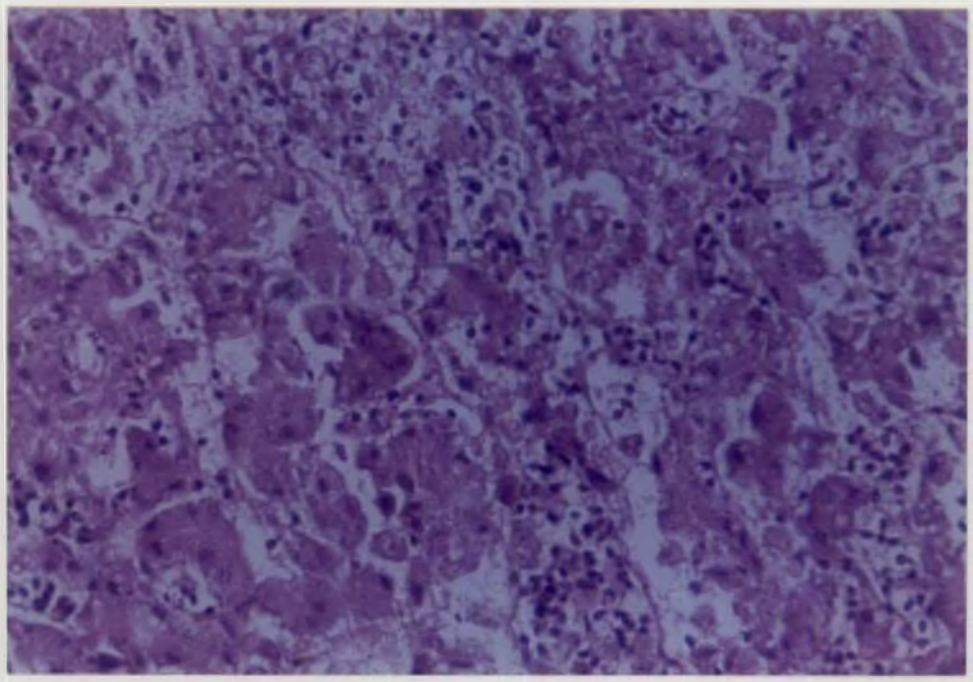
The heart on examination revealed degeneration and necrosis of muscle fibres. The muscle fibres were fragmented and more eosinophilic. Diffuse congestion, oedema, separation of fibres and moderate mononuclear cell infiltration between these were seen (Fig.11).

Liver lesions were either focal or diffuse. This included fatty changes and necrosis of hepatocytes. Small multifocal areas of coagulative necrosis and mononuclear cell infiltration were observed (Fig.12). Dilatation of central vein and sinusoids, perivascular oedema, sinusoidal accumulation of mononuclear cells and occasional Kupffer cell hyperplasia were the other lesions. Many of the hepatocytes surrounding the necrotic areas showed the presence of large round

Fig.11. Heart- Separation and fragmentation of muscle fibres, oedema and mononuclear cell infiltration. H & E x 400

Fig.12. Liver- Coagulative necrosis and mononuclear cell infiltration. H & E x 400





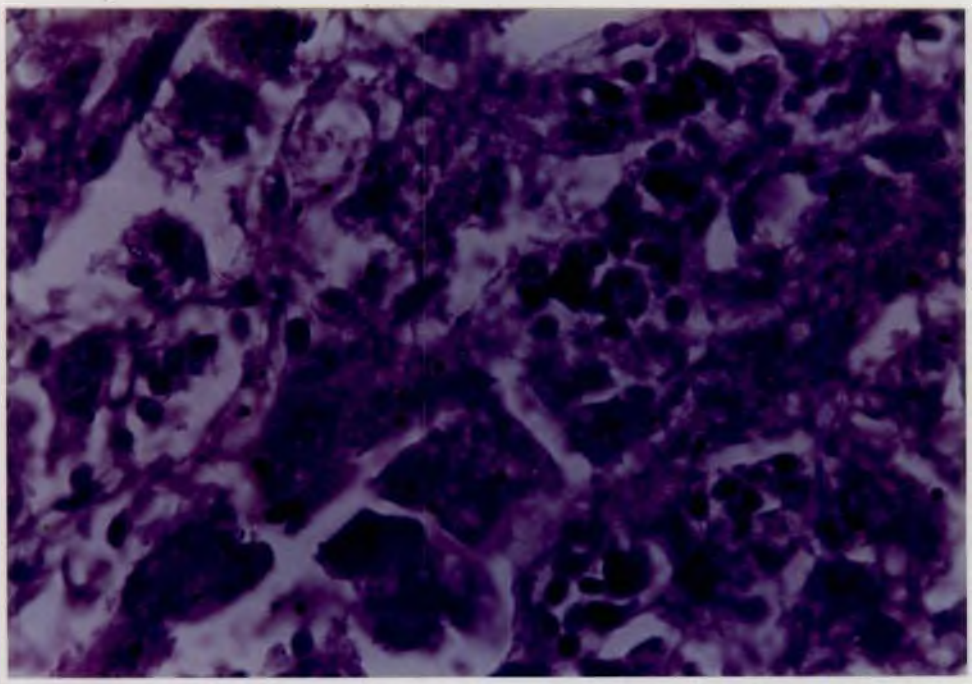
basophilic intranuclear inclusion bodies (Fig.13 & 13a).

Spleen showed moderate vascular sclerosis and diffuse lymphoid depletion and necrosis.

Vacuolar degeneration of the epithelium lining most of the tubules (Fig.14), widening of the interstitium, intertubular haemorrhage, inflammatory cell infiltration (Fig.15), tubular dilatation, peri-tubular fibrosis and presence of hyaline casts in the lumen of some of the tubules were the characteristic kidney lesions. A few of the tubules appeared highly distended and cystic, containing homogenous pink staining fluid.

The lumen of some of the cystic tubules appeared clear and the lining cells were absent (Fig.16). Glomeruli appeared less cellular. Damaged cells appearing as eosinophilic structures could be detected in some of the dilated capsules of the glomeruli. Vascular changes included thinning of the vessel wall, dilatation and rupture.

Fig.13 and 13a. Liver- Necrotic hepatocytes, mononuclear cell infiltration and presence of intranuclear inclusion bodies. H & E x1000.



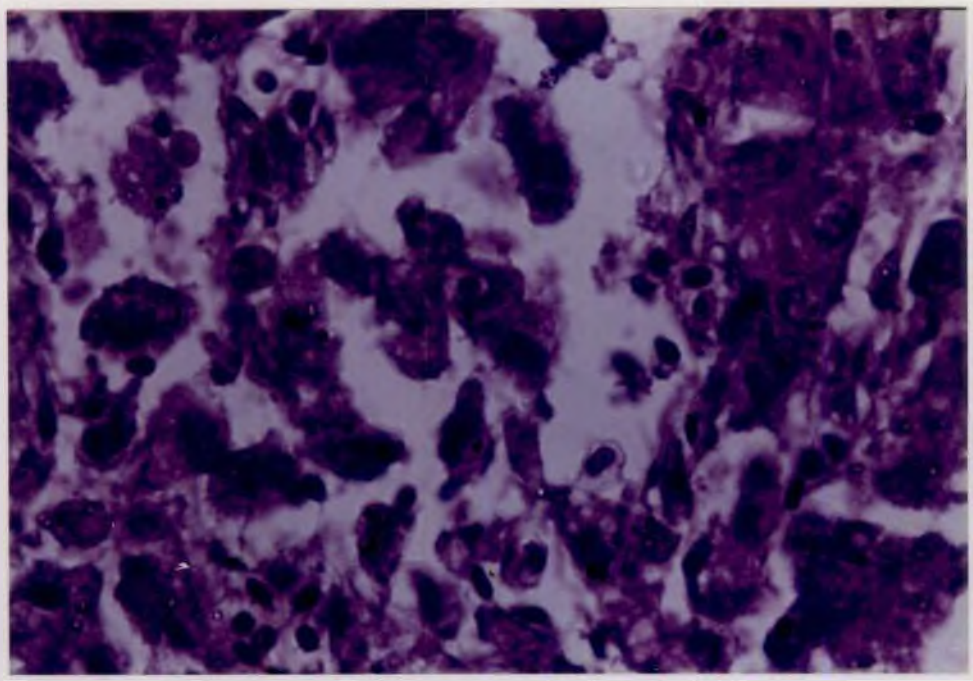
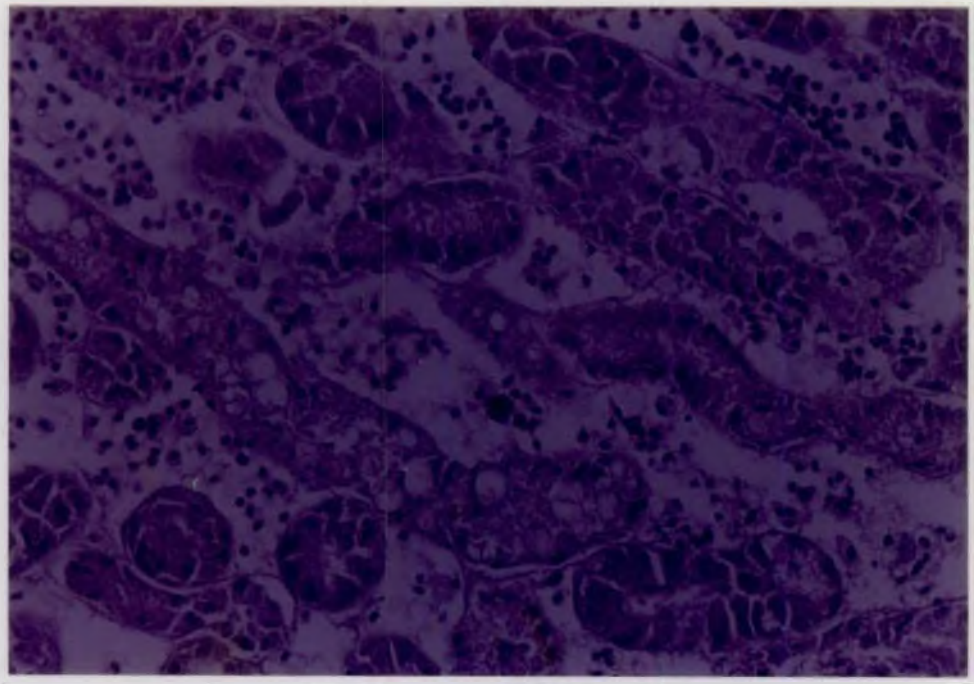


Fig.14. Kidney- Vacuolar degeneration of lining epithelium. H & E x 400.

Fig.15. Kidney- Widening of interstitium, haemorrhage and inflammatory cell infiltration. H & E x 400.



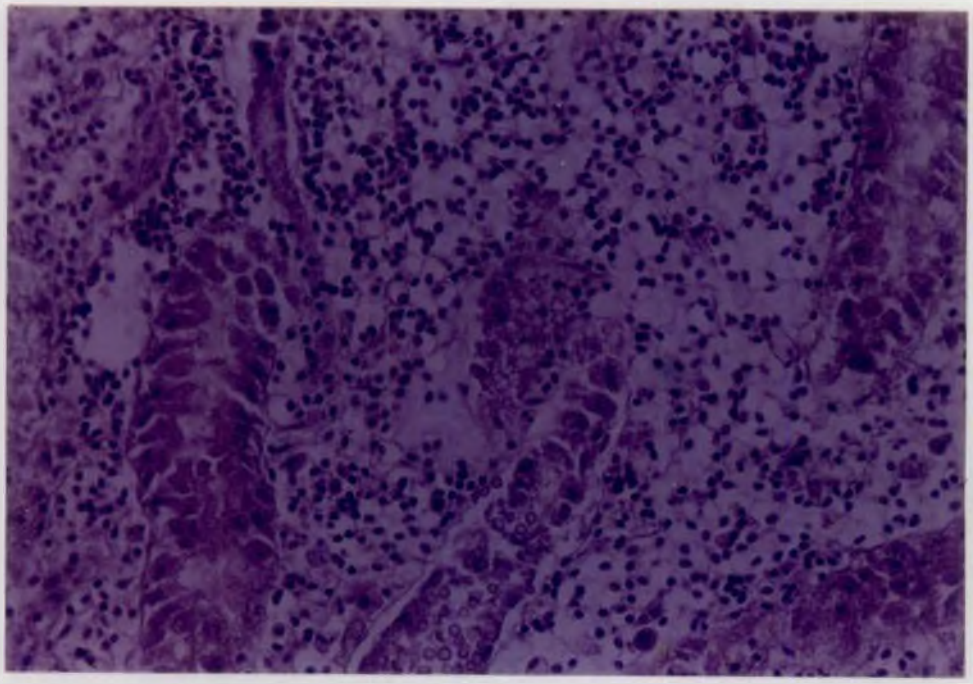
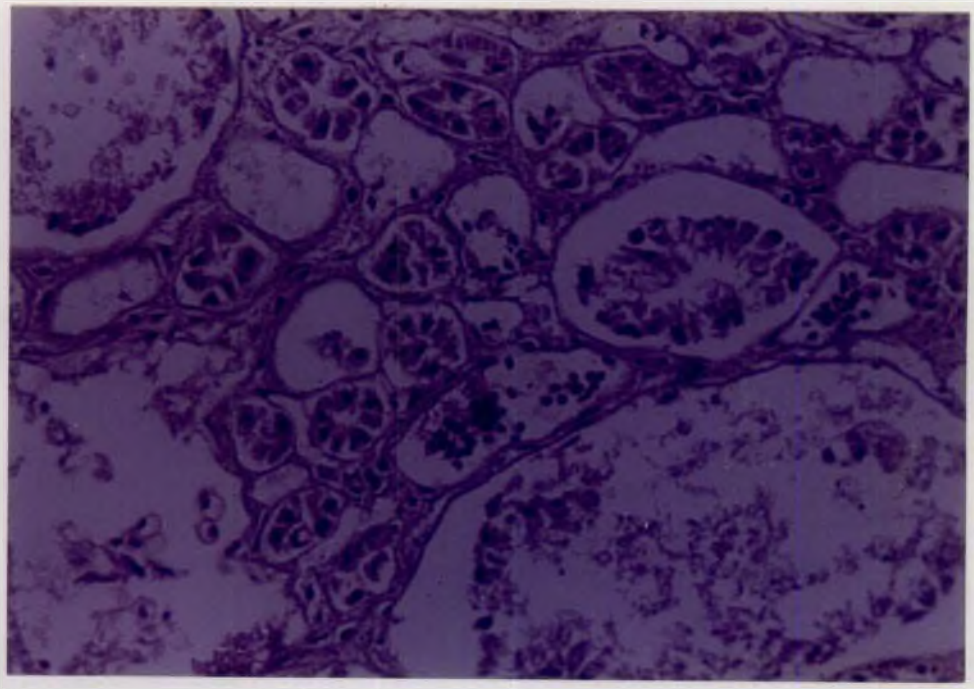


Fig.16. Kidney- Degeneration, necrosis of tubules and cystic tubules with homogenous material inside the lumen. H & E x 400.

Fig.17. Lung- Oedema. H & E x 400.



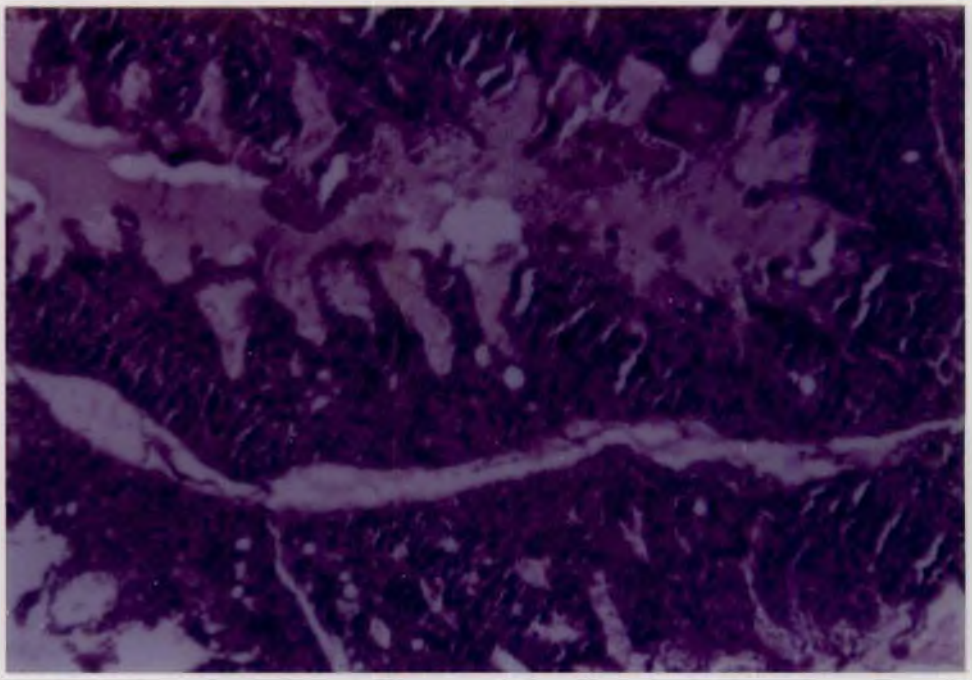
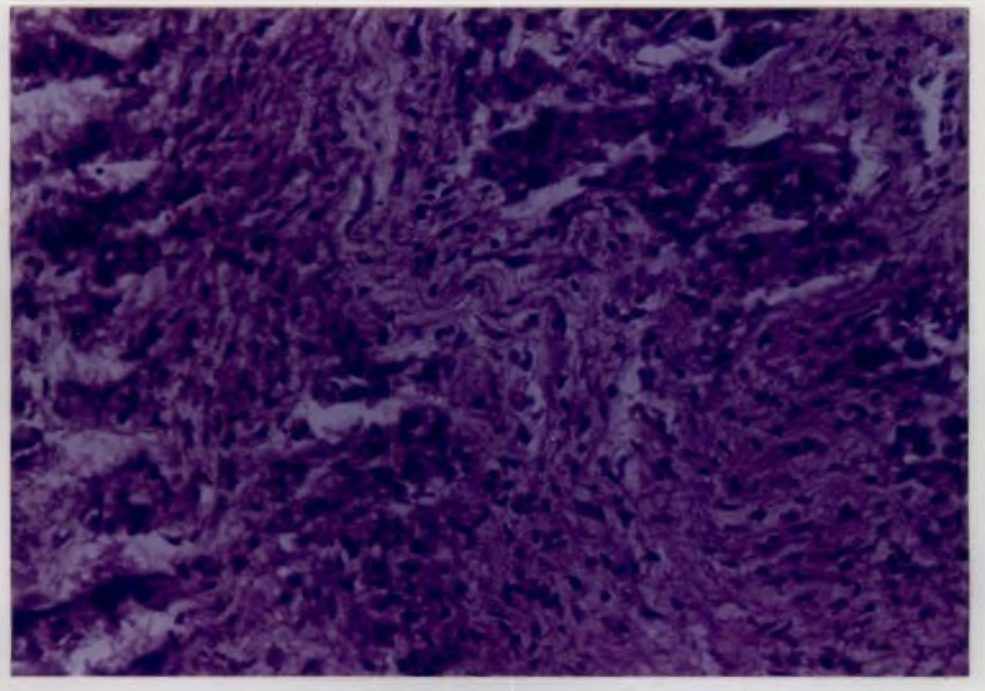
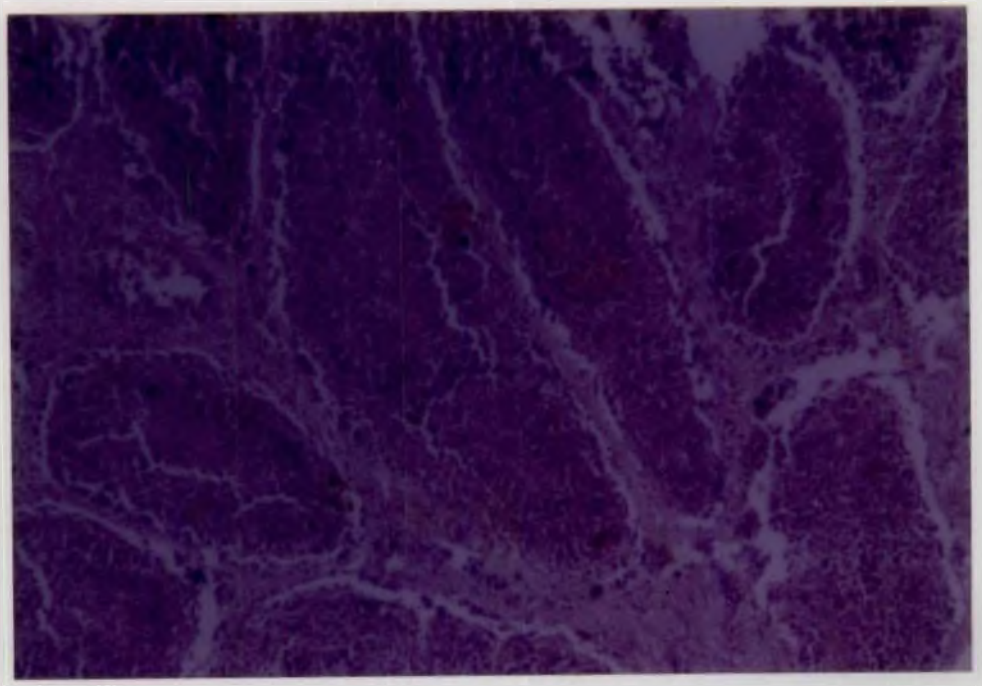


Fig.18. Proventriculus- Glandular atrophy and fibrosis. H & E x 400.

Fig.19. Bursa- Diffuse lymphoid depletion, inter follicular fibrosis and oedema. H & E x 250.





Lungs showed congestion and oedema (Fig.17). Atrophy of the glands and marked fibrosis were the lesions observed in the proventriculus (Fig.18).

The follicles of the bursa appeared loosely textured. Diffuse lymphoid depletion and lymphocytolysis were seen in these follicles. Inter and intra follicular oedema and fibrosis were predominant in few of the follicles (Fig.19). The epithelial component of the bursa remained intact.

Degeneration and necrosis of the glandular and villous epithelium were the lesions observed in the small intestine.

DISCUSSION

5. DISCUSSION

5.1. Revival of the virus

Cowen (1988) tried different routes of embryo inoculation and suggested that the yolk sac route of inoculation of the chicken embryo was a practical and efficient method for isolation and propagation of all type I adenovirus serotypes. Kharole and Mishra (1998) opined that virus concentration was maximum in nine to 14 day old embryo at six to seven passage level.

In the present study virus was revived in nine-day-old embryos by allantoic route and embryo mortality was produced at the fifth passage. There was a slow but steady increase in the virulence of the organism by each passage. This was evident from the pattern of development of lesions in the embryo. In the first three passages there were no lesions in the embryo. After the fourth passage, liver showed congestion. The lesions were more pronounced in the fifth passage. Liver showed congestion and haemorrhage. Spleen was enlarged and heart showed petechial haemorrhage. Hydropericardium, which was a characteristic feature of HPS in affected chicks, was not seen in dead embryos.

The results showed that the virus required some adaptation in embryo before it produced any mortality.

The allantoic fluid collected from the dead embryonated eggs reacted with HPS antiserum and produced precipitation line by AGPT, confirming the presence of Aviadenovirus. An increase in the virus concentration after each passage was obvious in the results obtained for AGPT. When the allantoic fluid after the third passage gave a faint precipitation line for AGPT, it gave a sharp line after the fifth passage.

The embryo mortality and the experimental reproduction of the disease by inoculation of the allantoic fluid into susceptible chicks, revealed that the chick embryo could very well be used for virus isolation and diagnosis of the disease.

5.2. Reproduction of HPS in chicks

Several researchers have successfully reproduced HPS in susceptible chicks. Khawaja *et al.*(1988) and Cheema *et al.* (1989) reproduced the disease by inoculation of a bacteria free liver homogenate. Anjum (1990) used two preparations viz., the supernatant of affected liver homogenate in PBS after centrifuging at

800 x g and an affected liver homogenate in normal saline without centrifugation and inoculated 0.25 ml each sub-cutaneously. He recorded a higher mortality with the latter preparation, though it was not statistically significant. Afzal *et al.* (1991) reproduced the disease using a 20 per cent liver homogenate in PBS after centrifuging at 2500 x g or 30 min. They inoculated 0.5 ml of the suspension sub-cutaneously. Gowda and Satyanarayana (1994) reproduced the disease using tissue samples suspension in PBS. One millilitre of the inoculum was injected intraperitoneally. The incubation period varied between two to five days.

In the present study intramuscular, subcutaneous and intra peritoneal routes of inoculation were tried. All the routes produced HPS, but more characteristic lesions were produced by the subcutaneous route of inoculation and mortality occurred within three to five days. The inoculum used was the allantoic fluid from the fifth passage, which showed the presence of aviadenovirus by AGPT.

Mortality started on the third day, when the inoculum was given intramuscularly, whereas it started

only on the fourth day when inoculated by the other routes. This indicates that the experimental reproduction of HPS is quicker in intramuscular inoculation. In the intraperitoneal route, mortality occurred between three to seven days. This may be due to the presence of intraperitoneal macrophages, which might have offered an initial resistance to the virus. This observation was in contrast to that of Gowda and Sathyanarayana (1994) where the mortality occurred between three to five days.

5.3. Preparation of liver homogenate as antigen

In the present study 20 per cent suspension was prepared and centrifuged at 8000 x g for 15 min and was used without filtration. This was in agreement with Ganesh, (1998) except in the filtration of the suspension. The liver homogenate was further concentrated using ultracentrifugation. The pellet was dissolved in PBS. This preparation with a protein concentration of 29.07 mg/ml and a PHA titre of 1:100 was used as antigen in the serological tests. It gave satisfactory results indicating, the effective concentration of HPS antigen in the preparation to successfully carry out the serological tests.

Various researchers have prepared the liver homogenate in PBS (Cheema *et al.*, 1989; Anjum, 1990; Afzal *et al.*, 1991) and in normal saline (Anjum 1990). They all made a 20 per cent suspension, though the speed of centrifugation was different and successfully used it to reproduce the syndrome experimentally. But the attempt to reproduce the syndrome experimentally using the pericardial fluid of affected birds or homogenate prepared from other organs like bursa, lungs, heart and kidneys was a failure (Anjum *et al.*, 1989). This may be due to the absence or very low level of HPS virus in these organs. These results also suggest that liver contains the maximum concentration of virus both in the natural and in the experimental infection and is a good choice for antigen preparation.

5.4. Production of antiserum

Antiserum against HPS virus was produced using the liver homogenate antigen. The slow release of the antigen was ensured by emulsifying it with Freund's complete adjuvant. Three booster doses at weekly intervals were given intramuscularly and this produced good immune response as evidenced from the indirect ELISA titres. A titre of 1:200 in the first booster dose was increased to 1:3200 in the third booster. Ten

days after the third booster a titre of 1: 6400 was observed.

This antiserum gave satisfactory results when used as positive serum control in the serological tests. It was also cross checked with the HPS antigen (3.1.13.1) and allantoic fluid (3.2.1.2). a single sharp precipitation line was observed in both the cases. This clearly indicated the involvement of aviadenovirus in HPS cases and that HPS virus produced specific antibodies in affected chickens. The presence of only a single precipitation line suggested that the antigen preparation was fairly pure and the interference of non-specific antibodies was negligible. It also suggested that the liver homogenate could be effectively used for the preparation of autogenous as well as inactivated vaccines.

5.5. Passive haemagglutination assay

Passive haemagglutination assay was done to find out the titre of the antigen preparations viz., allantoic fluid and the liver homogenate. This test has been used for the diagnosis of HPS by several researchers (Rahman *et al.*, 1989; Qureshi, 1990; Hassan

et al., 1993). A titre of 1: 50 was obtained for the allantoic fluid and 1:100 for the liver homogenate.

5.6. Agar gel precipitation test

Agar gel precipitation test produced a pattern of identity or coalescence with the positive field sera and positive control sera using HPS antigen. There was only a single precipitation line between the sera and the antigen preparation. It indicated that the HPS antigen produced specific antibodies in susceptible birds and that the syndrome was caused by a single agent, *i.e.*, aviadenovirus serotype 4. Kataria *et al.* (1997) used antigen prepared from chicken embryo liver (CEL) cell culture propagated virus and Ravikumar (1998) tested the antigen in tissue samples using AGPT.

5.7. Counter immunoelectrophoresis

Counter immunoelectrophoresis revealed a single precipitation line between the cathodal and anodal wells and the line was more towards the anodal well, indicating a higher concentration of antigen compared to the concentration of antibodies in the sera.

Oberoi *et al.* (1990) used acetate buffer (pH 5.6) while doing CIE to detect EDS-76 in samples. In the

present study tris -barbitone buffer (pH-8.6) was used and a sharp precipitation line was obtained as described by Oberoi *et al.* (1990) and Khehra *et al.* (1994).

5.8. Indirect ELISA

5.8.1. Interpretation of optical density (OD) values.

Different researchers have interpreted the results in different ways. Most workers took the base line for positivity as the mean of all the readings obtained from the negative control sera plus two or three times the standard deviation (Marquardt *et al.*, 1981; Lana *et al.*, 1983). Garret *et al.* (1984) took an arbitrary cut off point as twice the mean absorbance of negative serum. Nandapalan *et al.* (1982) took the lowest score of the test sera above the highest score of the control negative as the end point. Here there is a chance of mistaking the negative sera which show high but non-specific OD values as positive. In the present study OD values above twice the mean OD value of the negative sera were taken as positive as supported by Garret *et al.* (1984). This will eliminate the false positive values.

5.9. Comparison of AGPT, CIE and indirect ELISA

In the present study, after initial screening, AGPT and CIE gave positive results for three samples, whereas, indirect ELISA gave seven positive results. The three tests were compared statistically using Z-test. Though the test showed no significant difference between the serological tests in detecting HPS antibodies at five per cent level ($p<0.05$), the results indicated indirect ELISA as a more reliable test for detection of HPS antibodies.

5.10. Seroprevalence of HPS in broiler chickens

No systematic study has been undertaken so far to assess the magnitude of HPS in Kerala. In the present study, 6.86 percentage of occurrence of HPS was estimated. This finding indicates that the disease deserves attention and has to be controlled and prevented for economic broiler production. A detailed investigation is warranted to isolate and characterise the HPS virus as well as a systematic approach is to be initiated to evolve a successful vaccination and control programme.

5.11. Electron microscopy

The adenovirus has a naked icosahedral capsid and it measures 70 to 90 nm in diameter. The virus has 252 capsomeres, which are arranged in an icosahedron. There are 20 triangular faces and 12 vertices with a fibre projecting from each vertex (Murphy *et al.*, 1999).

In the present study, typical hexagonal virus particles were demonstrated in the pelleted liver homogenate prepared from infected birds. The observations were similar to that of Cheema *et al.* (1989) and Chandra *et al.* (1997). The virus particles measured an average of 75 nm in diameter. This particular preparation was also tested by AGPT using antiserum against HPS and found positive. These observations strongly establish the involvement of aviadenovirus in cases of HPS.

5.12. Gross and Histopathology

Gross and histopathological lesions observed were comparable to the observations made by Anjum *et al.* (1989) and Gowda (1994). In most of the cases the heart appeared shrunken, which indicated the pressure effect of the accumulating fluid on the heart and Histologically, it was characterised by breakage and

shrinkage of cardiac muscle fibres. Extravasation of erythrocytes in between the muscle bundles was reported as a characteristic feature of HPS by Cheema *et al.* (1989). Though diffuse congestion was seen in the affected muscles, haemorrhages were not a feature. Haemorrhages can be expected when there is extensive damage to the vascular endothelium.

The depletion of lymphocytes from the bursal follicles and focal lymphocytolysis indicated the immuno suppressive nature of the disease, which will perforce be subjected to other infections. Atrophy of the glandular structures of the proventriculus and extensive fibrosis observed here has not been reported by other workers and it appears only as an incidental finding or probably an indication of exhaustion due to infection. Basophilic intranuclear inclusion bodies seen in the hepatocytes were the products of virus cell interaction characteristic of adenovirus infection and the presence of these both in the impression smears and sections are of much diagnostic value.

Anjum *et al.* (1989), Cheema *et al.* (1989), Gowda (1994) and Arsani *et al.* (1997) also observed

intranuclear basophilic or eosinophilic inclusions in most of the hepatocytes of infected birds.

Changes in the spleen were not prominent, except moderate vascular sclerosis and diffused lymphoid cell depletion. Gowda (1994) observed atrophy of follicles in spleen and lymphocytolysis. Moderate depletion of lymphocytes and necrotic changes in germinal centres were reported by Survashe *et al.* (1996).

The extensive histological lesions seen in the kidney indicated the effect of the virus on this organ, which resulted in the accumulation of products of excretion. This might have added to the damage already produced in the vital organs like heart and liver, contributing to exudation of fluid. Vascular damages like thinning of the vessel wall, dilatation and rupture prove the toxic injury. Histological changes of the kidney supported fully the findings of Khan *et al.* (1988), Cheema *et al.* (1989) and Muneer *et al.* (1989).

Lungs showed congestion and oedema as described by Khan *et al.*(1988), Cheema *et al.* (1988) and Arsani *et al.* (1997). They showed infiltration of mononuclear cells and heterophils in the alveoli. Gowda (1994)

reported catarrhal inflammation in parabronchiolar epithelium and presence of "cell nests" or cartilagenous masses, apart from congestion and oedema.

SUMMARY

6. SUMMARY

Hydropericardium syndrome (HPS) is an emerging disease of chickens, mainly affecting three to six-week-old broilers. It produces high mortality and heavy economic loss. Even though many outbreaks of HPS in Kerala were reported by field veterinarians, a detailed study was not attempted. In the present study the seroprevalence of HPS in broiler chickens in Kerala was evaluated.

The HPS virus procured from the Department of Animal Biotechnology, Madras Veterinary College, Chennai was revived in chicken embryo by allantoic route of inoculation. Embryo mortality occurred only in the fifth passage, indicating the requirement of adaptation in chicken embryo. The allantoic fluid after fifth passage gave a virus titre of 1:50 in passive haemagglutination assay (PHA).

The revived virus was inoculated into three-week-old broiler chicks and the disease was reproduced. Various routes of inoculation viz., intramuscular, subcutaneous and intraperitoneal routes were tried and all these routes produced mortality. More

characteristic lesions of HPS were obtained in birds inoculated by subcutaneous route. The impression smears taken from the cut surface of liver of affected chicks revealed basophilic intranuclear inclusion bodies in the hepatocytes.

A 20 per cent (w/v) liver homogenate was prepared from the liver of affected birds after experimental inoculation of virus. This was subjected to low speed centrifugation and had a protein concentration of 13.6 mg/ml. this was pelleted by ultracentrifugation and dissolved in PBS. The preparation with a protein concentration of 29.07 mg/ml was used for the production of antiserum. Three booster doses were given before collecting the serum. This serum was used as positive control in the serological tests.

Passive haemagglutination assay was done to find out the titre of the antigen preparation used for indirect ELISA. The antigen preparation with a protein concentration of 29.07 mg/ml gave a titre of 1: 100 in PHA. A total of 350 sera samples were collected from different parts of Kerala and tested for HPS antibodies. In the first phase, 50 sera samples were screened using agar gel precipitation test (AGPT),

counter immunoelectrophoresis (CIE) and indirect enzyme linked immunosorbent assay (ELISA). AGPT and CIE showed precipitation lines in three samples. In AGPT a single sharp precipitation line was produced between the antigen and test serum wells, which was confluent with the line produced for the positive control. In CIE, a single precipitation line was produced between the cathodal and anodal wells. The line was placed more towards the anodal well showing a higher concentration of antigen. The indirect ELISA of 50 sera samples gave positive results for seven samples. Three out of these seven samples were the same which gave positive results by AGPT and CIE and the remaining four samples were negative by AGPT and CIE. The results were compared statistically by Z-test. Though there was no statistically significant difference between these tests, the results clearly indicated indirect ELISA as a more reliable test in detecting HPS antibodies.

The remaining 300 samples were tested by indirect ELISA and 17 samples were found positive for HPS antibodies. So a total of 24 sera samples were found to be positive out of the 350 samples tested by indirect ELISA. The seroprevalence of HPS in broiler chickens in Kerala was evaluated to be 6.86 per cent. This

indicates the strong presence of HPS among broiler chickens in Kerala and invites further detailed investigation to isolate and characterise the local strain. Also an effective vaccination programme should be charted out to curtail the incidence and reduce economic loss.

The liver homogenate of infected birds under electron microscopy showed hexagonal virus particles suggestive of adenovirus and they measured about 75 nm in size.

Autopsy of the experimentally infected birds showed hydropericardium with accumulation of straw coloured fluid. In most cases heart appeared shrunken. This could be attributed to the effect of pressure of the accumulating fluid on the heart. Histologically there were breakage and shrinkage of myocardial fibres. Haemorrhage was not a consistent feature. Bursa showed depletion of lymphocytes from the follicles and focal lymphocytolysis, indicating the immuno suppressive nature of the syndrome. Presence of basophilic intranuclear inclusion bodies in the hepatocytes was a consistent feature. Necrosis of hepatocytes and focal areas of coagulation in liver were also seen. Spleen

showed moderate vascular sclerosis and diffuse lymphoid cell depletion. Vacuolar degeneration of kidney tubules, congestion and oedema of lungs and infiltration of mononuclear cells and heterophils in the alveoli were also observed.

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SEROPREVALENCE OF HYDROPERICARDIUM SYNDROME IN BROILER CHICKENS

By
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ABSTRACT OF THE THESIS

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ABSTRACT

In the present study, seroprevalence of hydropericardium syndrome (HPS) in broiler chickens in Kerala was evaluated. The results of agar gel precipitation test(AGPT), counter immunoelectrophoresis (CIE) and indirect enzyme linked immunosorbent assay (ELISA) were compared in detecting HPS antibodies.

A total of 350 sera samples were collected from different parts of Kerala. In the first phase, 50 sera samples were screened by AGPT, CIE and indirect ELISA. Three samples were found positive by AGPT and CIE. Seven samples were found to be positive by indirect ELISA. Based on the comparison, another 300 samples were screened using indirect ELISA and 17 samples were found to be positive.

A total of 24 samples were found positive for HPS antibodies out of 350 samples screened by indirect ELISA. The seroprevalence of 6.86 percentage was evaluated in broiler chickens in Kerala using indirect ELISA.

The syndrome was experimentally reproduced in three-week-old broiler chicks. Various routes of inoculation viz., intramuscular, subcutaneous and intraperitoneal routes were tried. The lesions were more pronounced when the birds were inoculated by subcutaneous route. Chick mortality was high on the fourth day.

Electron microscopy of the infected liver homogenate revealed hexagonal virus particles of about 75 nm in size. These are suggestive of adenovirus.

Histopathology of necropsied birds showed degeneration and necrosis of myocardial fibres, necrosis of hepatocytes and focal areas of coagulation in liver. Basophilic intranuclear inclusion bodies were demonstrated in hepatocytes. Vacuolar degeneration of kidney tubules, lymphoid depletion of bursa, vascular sclerosis of spleen and congestion and oedema of lungs were also seen.

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