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Pathological investigation into outbreak of inclusion body hepatitis from poultry flocks

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Abstract
Present study is planned, In order to identify the agent of a suggestive clinical case of Inclusion body hepatitis that affected 1-10 week old broiler and layer flock poultry in and around college region in Satara and Pune district of western Maharashtra in the year 2016. Total 192 samples showing gross lesions, which were selected for the study. Selected birds had hepatomegaly with marked degenerative and necrotic changes on surface. Congested with soft and friable hepatic parenchyma. Petechial hemorrhages were noted on the liver surface with diffuse involvement of both lobes. Histological analysis revealed reflection of gross lesions. Hepatitis with presence of intranuclear inclusion bodies and marked necrotic and inflammatory changes of hepatic parenchyma. Polymerase chain reaction revealed the amplican of 0.897 kb in agarose gel eletrophorosis. It was concluded that polymerase chain reaction has emerged as the method of choice for detection of inclusion body hepatitis birds.

Keywords: Grosspathology, histopathology, IBH, PCR and poultry

Introduction
Inclusion body hepatitis (IBH) was first described in 1963 in USA. The disease has been reported in many other countries in the world. In recent years, reemergence of IBH has increased the importance of adenoviruses infection in the poultry industries. The disease was first described in young broilers in Angara Goth, Pakistan, during the year 1987. Thereafter, it spread throughout Pakistan [1] as well as neighboring countries like India [2]. IBH was recorded subsequently in Iraq, Mexico, Ecuador, Peru, Chile, South as well as Central America, Slovakia, Russia and Japan [3]. It was earlier believed that the inclusion body hepatitis (IBH) could only be caused by adenovirus if the bird's immune system was first weakened by exposure to immunosuppressive agents such as infectious bursal disease, chicken infectious anemia or mycotoxicosis [2, 4]. The disease is widely prevalent in India and Pakistan [5-7]. Fowl adenoviruses (FAdVs), belonging to the Avi-adenovirus genus of the family *Adenoviridae*, have been classified into five species (A to E) based upon their restriction enzyme fragment patterns, phylogenetic relationships, pathogenicity, cross-neutralization and recombinant potential [8] and further divided into 12 serotypes [9-12]. Icosahedral viruses are mainly responsible for naturally acquired outbreaks of inclusion body hepatitis (IBH). Gizzard erosions that have high economic impacts [13-18]. Generally all the AAVs belong to three groups. Group I comprises of five Aviadenovirus species (A–E) with 12 serotypes isolated from poultry, with largely fowl adenovirus (FAdV) (aviadenovirus C) strains causing hydropericardium syndrome (HPS). Group II includes turkey haemorrhagic enteritis virus (HEV), virus causing marble spleen disease in pheasants and the splenomegaly adenoviruses of chickens. Group III consist of EDS–76 virus, which causes egg drop syndrome (EDS) in domestic poultry birds. Virulent strains have emerged which alone can produce the severe disease with mortality ranging from 10-30 per cent[2] which may reach up to 80 per cent [4] in presence of other immunosuppressive factors. The major predilection sites for FAdV are lymphoid organs *viz*., spleen, thymus, bursa of Fabricius and caecal tonsil which results in immunosuppression [19]. There are no reports of IBH in the birds from western region of Maharashtra. Therefore authours has done efforts to put same on the record.

Materials and methods
In the present investigation of 192 birds from 5 flocks which referred to the Department of Veterinary Pathology, KNPVC, Shirwal were studied.

Systemic post mortem examination of affected birds was done. Gross lesions, in various tissues were recorded and collected in 10 per cent neutral buffered formalin for histopathological examination. The formalin fixed tissues were subjected for histological and Liver samples were collected on ice and stored in deep freeze for PCR. Tissue processing using alcohol-xylene protocol in automated tissue processor and tissue section were cut on automated microtome. The tissue sections were stained by routine Hematoxylin and Eosin following standard protocol. The microscopic examination of tissues was undertaken and noticed lesions were recorded and photographed. Polymerase chain reaction (PCR) was used for detection of viral DNA in homogenates of infected Liver. All the amplicons were confirmed for specific size by using gel electrophoresis. Total DNA was extracted from the liver using Phenol-Chloroform reagent. Extracted DNA and specific primers targeting the conserved region in the IBH virus genome were used for the amplification of viral fragment which yielded about 0.897 kb size amplicons in the 1.5 per cent agarose gel after electrophoresis.

The primer sequence as per reported earlier were used. The FAdV hexon gene sequence of the viral DNA forward-5'CAARTTCAGRCAGACGGT3' and Reverse 5'TAGTGATGMCGSGACATCAT3' were used in the PCR[20]. DNA amplification was carried out in a total volume of 25 µl containing 10 ng total DNA, 20 pmol of each forward and reverse primer, 200 lMdNTPs mix, 1.5 mM $MgCl_2$ and 2.5U Taq DNA polymerase. The reaction was carried out in a thermal cycler with initial denaturation at 94° C for 2 min followed by 35 cycles of denaturation at 94° C for 1 min, annealing at 60° C for 1 min and extension at 72°C for 90 Sec with a step of final extension at 72° C for 2 min. The amplicon were run on 1.5 per cent agarose gel made in TrisBorate EDTA (TBE) buffer (containing 5 µg/ml of Ethidium bromide) at 80V for 1 hr. Mass Ruler DNA ladder with size 100 base pair also run as a stranded marker. The gels were visualized for appropriate band size under a UV transilluminator and photographed and analyzed by Gel Documentation system (G-Box- Syngene).

Results

In the present study 5 flocks suspected for involvement of IBH were included. It showed mortality ranging from 0.67 to 8.46 per cent (Table 1). Birds from affected flocks showed clinical sings including acute and increased mortality pattern from day 10 onwards with varying clinical signs like depression, congested comb, swollen heads and congested eye, reduced feed intake, reduced water intake, poor weight gain and ruffled feathers, wet litter, respiratory, disease lameness. Occasional diarrhea with greenish yellowish discoloration was also recorded in few of the birds.

Table 1: Details of Poultry Flocks examined

Flock Number	Layer / Broiler	Flock Size	Age of birds (days) during study period	Number of birds died (Mortality)	Mortality (%)
1.	Layer	150	10 weeks	10	6.67
2.	Broiler	100	8 days	3	3.0
3.	Broiler	2000	10 days	120	6.0
4.	Broiler	600	14 days	4	0.67
5.	Broiler	650	18 days	55	8.46

The detailed necropsy of 192 birds revealed mild to moderate pathological lesions including enlarged liver (Fig 1) with marked degenerative and necrotic changes on surface. Congested hepatic parenchyma and petechial hemorrhage (Fig 2) were noted on the liver surface with diffuse involvement of both lobes. Bursa of fabricious showed mild enlargement cheesy exudate and hemorrhages in bursal follicles. Kidneys revealed pale and swollen appearance. Spleen showed congested, swollen and hemorrhagic changes on surface. Heart revealed rupture of auricles with rounded heart, marked dilation of ventricles with right ventricular flabbiness and hydropericardium.

The microscopic examination of liver section appeared congestion of hepatic parenchyma. Marked degeneration of hepatocytes with cellular swelling, karyomegaly, vacuolar changes in cytoplasm and disruption of hepatic cords were noted. Perivascular inflammatory cellular infiltration comprised of heterophils and lymphocytes was noted with occasional large aggregates of lymphocytes as microgranuloma formation (Fig 3). Diffuse dystrophic changes with complete loss of nucleus and cellular border of hepatocytes were observed. Diffuse inflammatory changes and hemorrhages in hepatic parenchyma. Presence of intranuclear large globular inclusion bodies of basophilic (Fig 4) and eosinophilic nature with enlarged size of hepatocytes. The inclusion bodies were of variable size with small globular to large size occupying the complete cell (hepatocyte) showing scanty of cytoplasm. Bursa was observed with occasional degenerative changes and heamorrhage in follicles. Kidney section showed marked necrotic and degenerative features of renal parenchyma. Glomerular swelling was observed with accumulation of eosinophilic content in glomerular space. The renal tubules showed cellular swelling of tubular epithelium, diffuse nephrosis and necrosis. Few sections showed extensive hemorrhagic foci with glomerulonephritis. Interstitial edematous and focal infiltration of mononuclear cells between the renal tubules. Spleen revealed multifocal depletion of lymphocytes in white pulps. There was disruption of white and red pulp with presence of dark colored foci of congestion and hemorrhagic changes. Diffuse areas of subcapsular hemorrhages of were noted. Heart showed presence of diffuse cardiac dilation and derangement of length of cardiac fibers throughout the section. There was presence of edematous exudate on the pericardium with fibrinous deposits. Multiple foci with broken cardiac fibers were also noted. Focal to diffuse myocarditis with accumulation of mononuclear inflammatory cellular population was observed. Liver tissue samples used for PCR assay and the PCR amplicons was confirmed by agarose gel electrophoresis yielding 897 bp sized amplicons (Plate 1).

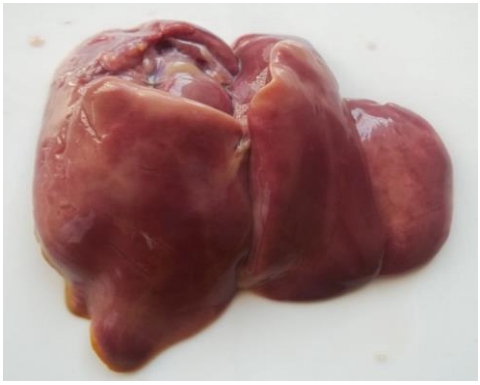


Fig 1: Enlargement of liver

Fig 2: Pin point hemorrhages

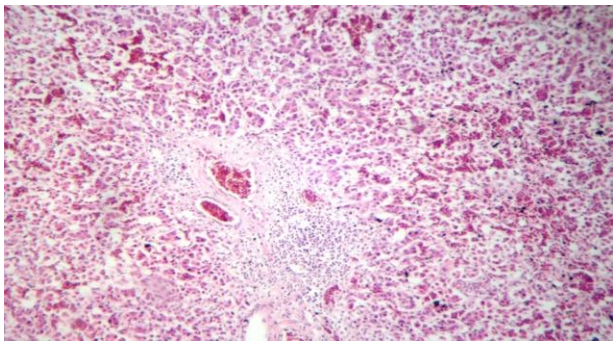


Fig 3: Inflammatory changes in hepatic parenchyma with perivascular aggregates of mononuclear cells. H&E, x100.

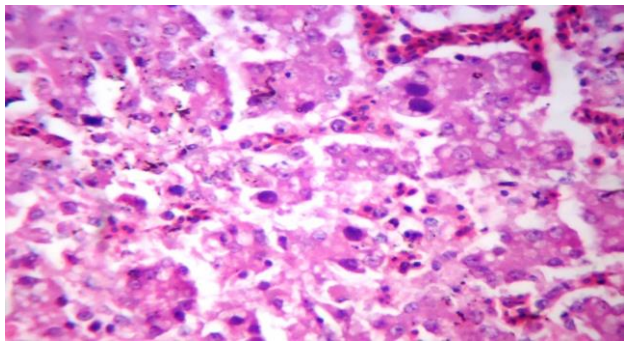
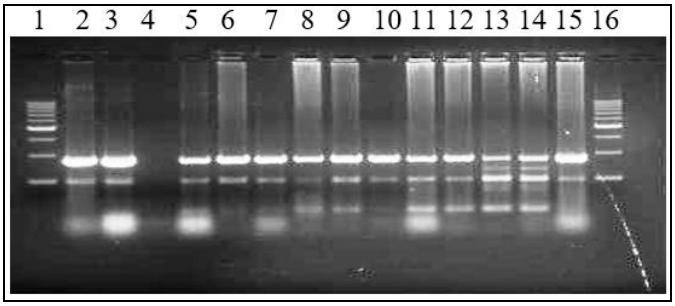


Fig 4: Basophilic intra-nuclear inclusion bodies in the hepatocyte H&E, x200



Lane 1 & 16- DNA ladder
Lane 2 & 3- Positive control
Lane 4- Negative control
Lane 5 to 15 - Positive field samples for IBHV

Plate 1: Gel electrophoresis photograph depicting 897bp size amplicons of IBHV (Adenovirus)

Discussion

Inclusion body hepatitis–hydropericardium syndrome (IBH–HPS) associated with FAdV has been reported to occur in broilers as well as layers throughout the world [21-22]. In domestic poultry, the disease usually affects birds at the age of 2 to 6 weeks [22]. The onset of disease occurs in hot and humid weather [23-24]. High (60-100 per cent) mortality and statistically significant depression of body weights due to IBHv [25]. There is scanty information on the FAdV and its interaction with the immune system of broiler chickens. The growth of both primary and secondary lymphoid organs is depressed significantly in case of HPS. The major predilection sites for FAdV are lymphoid organs *viz*., spleen, thymus, bursa of Fabricius and caecal tonsil which results in immunosuppression [19]. Primarily inflammatory and metabolic related changes in liver, kidney, spleen and heart were due to infection by adenovirus. The affinity of IBHv (all strains of Adenovirus) towards hepatocytes for replication and further causing cellular swelling and inflammatory changes in hepatic parenchyma are considered to be the cause for petechial hemorrhages and friable nature of liver due to diffuse damage of hepatocytes with vacuolar and fatty change. Histological confirmation for presence of intranuclear inclusion bodies with post mortem lesion of liver suggested that the etiological agent as IBH virus (FAdv) [12, 24, 26]. The presence of intranuclear inclusion bodies of basophilic and eosinophilic type in hepatocytes with marked necrotic and inflammatory changes due to primary infection by the IBH virus (FAdv). Multiple foci with broken cardiac fibers due to metabolic stress. Degeneration of bursal follicles observed indicated that IBH virus may be involved in suppression of humoral immunity. The PCR amplicons from the positive sample and positive control was confirmed by agarose gel electrophoresis yielding 897 bp sized amplicons suggestive of etiology of Fowl Adenovirus [27].

Conclusion

The adenovirus infections in poultry birds can occur either as primary or secondary infection after immunosuppression process due to other previous viral infections like Gumboro disease or infectious anaemia, playing a role of secondary pathogen in these cases. Excess of mycotoxin is also reported to be one of cause for liver damage and further occurrence of adenovirus infection with marked damage to liver. The pathogenesity of the infection due to fowl adenovirus is enhanced by infectious bursal disease virus (IBDV) and chicken anaemia virus (CAV); as well as mycotoxins [28-30]. But it is still not clear whether the mortality observed in broilers could be a direct result of infection with FAdV affecting liver parenchyma or whether the infection results in an immune dysfunction, which may then lead to enhanced secondary infections with concurrent bacterial, viral and fungal agents resulting in death. Fowl adenoviruses are reported in increased outbreaks with prominent pathological

lesions of liver and heart. IBHV has a high economic importance because the high mortality rate, contagiousness, the least use of the vaccines and the absence of an effective treatment against this disease. Further studies are also required to determine whether the commercial vaccines would be able to accord protection against FAdV serotypes. Such studies can help in minimizing economic losses to poultry farmers as both diseases can cause considerable mortality. Clinical diagnosis is based on the characteristic hydropericardium in association with hepatomegaly and hepatic necrosis. Recently, molecular detection using PCR has emerged as the method of choice for detection of IBH in birds. Findings of present study add to epidemiological data of disease in the country and virus isolates obtained can be used for further molecular characterization, phylogenetic analysis, immunological and virological studies.

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