Original Research Article

Epidemiological Observations on Some Natural Outbreaks of Inclusion Body Hepatitis-Hydropericardium Syndrome (IBH-HPS) in Domestic Chicken

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A B S T R A C T

Fowl adenoviruses (FAdVs) are economically significant pathogens of domestic poultry. Fowl adenoviruses are associated with many disease conditions in domestic fowl such as IBH, IBH-HPS, Egg drop syndrome, Quail bronchitis, and gizzard erosions etc. The present study deals with epidemiological investigation of forty natural outbreaks of IBH/IBH-HPS in broiler farms of Uttar Pradesh and Uttarakhand during 2011-2016. Involvement of fowl adenovirus was suspected on the basis of post mortem lesions, which was confirmed by AGID, histopathology, virus isolation and hexon gene (L1 loop) PCR. Disease was mainly present in 3 to 6 week age broiler birds; however, birds less than 3 weeks of age were also affected. Mortality was in the range of 0.5% to 20% and disease incidence was found to be more in August and September months of the year. In AGID with known antiserum, viral antigen was indicated by presence of single precipitin line for each isolate. Histopathology of liver revealed presence of basophilic intra nuclear inclusion bodies. Virus isolates were successfully propagated in CEL cell culture and a band of ~900bp was observed in PCR. In histology, intra nuclear inclusion body was found in hepatocyte.

Keywords: Fowl adenovirus, Epidemiology, IBH-HPS, chicken

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Introduction

Fowl adenoviruses (FAdVs) are classified under family *A.denoviridae* and genus *Aviadenovirus* according to the 9th ICTV report (Kings et al., 2011). There is huge diversity among FAdVs and they are classified into five genotypes, A to E based on polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) (Benko, et al., 2005; Raue and Hess., 1998; Meulemans et al., 2001) and into 12 serotypes (1-8a, 8b and 9-11) based on serum neutralization profiles (Grimes and King, 1977). In 2011, the ICTV has accepted the previously classified genotypes (A to E) as the...
five species of FAdVs (Kings et al., 2011). FAdVs are economically significant pathogens of domestic poultry and have been associated with a number of disease conditions including inclusion body hepatitis (IBH), hydropericardium syndrome (HPS), respiratory disease, tenosynovitis, impaired growth, reduced egg production, aplastic anemia, atrophy of bursa and thymus enteritis and conjunctivitis in chickens and other birds (Ahmad et al., 2001; Meulemans et al., 2001; Kumar et al., 2003a; Kumar et al., 2003b; McFerran and Adair, 2003; Kumar et al., 2010). FAdVs are stable in environment as they are resistant to several disinfectants, heat and pH changes and are easily transmitted both horizontally and vertically (McFerran and Adair, 2003; Grgic et al., 2006). Horizontal transmission occurs by oral-fecal route and also by mechanical means and by contamination with infected feces. Vertical transmission is considered to be an efficient means to spread virus from parent to progeny (Hafez, 2011; Rahimi and Minoosh Siavosh Haghighi, 2015). The IBH is caused by several serotypes of fowl adenovirus but HPS is caused by some strains of serotype 4 (Kumar et al., 2013; Asthana et al., 2013). IBH causes high morbidity among broiler birds leading to production losses although average mortality is low (5–10%) but 30% mortality has been reported from Australia (McFerran and Smyth, 2000), however, IBH accompanied by HPS may cause heavy mortality upto 80% (Asthana et al., 2013). IBH normally occurs in broiler chickens at 3 to 7 weeks of age, but it has been reported in birds as young as 7 day-old and as old as 20 weeks (Rahimi and Minoosh Siavosh Haghighi 2015). Clinically, IBH affected birds show lethargy, huddling, ruffled feathers, and in appenence (Hafez, 2011). Gross lesions of IBH include an enlarged pale and friable liver sometimes with necrotic foci. Ecchymotic hemorrhages may be also seen in the liver and less consistently in leg and breast muscles. In most cases, the main lesions are in the liver (Howell et al., 1970; McFerran and Adair, 1977). In HPS accumulation of straw/amber coloured fluid/gel is observed in addition to lesions described for IBH (Asthana et al., 2013; Kataria et al., 2013). The laboratory diagnosis of FAdV infections, including IBH-HPS is in most cases based on histological investigations, detection of intra-nuclear inclusion bodies in hepatocytes, detection of the antigen using serological test, electron microscopy, virus isolation or by molecular methods(Kumar et al., 2003a, b, 2004; Hafez, 2011; Meulemans et al., 2001). Present study describes investigation and epidemiological observations of fourty natural outbreaks of IBH-HPS in different broiler farms of Uttar Pradesh and Uttarakhand.

Materials and Methods

Collection of epidemiological data

Epidemiological data related to suspected cases of Inclusion body hepatitis (IBH) and Inclusion body hepatitis-hydropericardium syndrome (IBH-HPS) was recorded from different poultry farms of Uttar Pradesh and Uttarakhand. Data was collected from year 2011 to 2016 from total 62 suspected outbreaks. Tissue samples like liver, spleen and kidney were collected and pooled in 50% glycerol saline for virus isolation and in 10% formalin for histopathology, aseptically from dead or moribund birds from different poultry farms of tarai region of Uttarakhand and Uttar Pradesh.

Detection of IBH-HPS virus in tissue samples

Primary screening was done on the basis of clinical signs and post mortem findings followed by serological detection of viral
antigens by agar gel immunodiffusion (AGID) test. Samples found positive in AGID were further confirmed by histopathology of liver, virus isolation and PCR amplification of L1 loop of hexon gene of the IBH-HPS virus.

**Preparation of virus inoculum**

Pooled tissues samples were rinsed 3 times in PBS (pH 7.2) and triturated in pestle & mortar using sterile neutral sand as abrasive to make in 20% (w/v) suspension in HBSS (pH 7.2). Tissue homogenates were frozen and thawed thrice and centrifuged at 5000 rpm for 15min at 4°C and supernatant collected aseptically. After addition of streptomycin @100µg/ml and penicillin @100IU/ml, the suspension was kept at room temperature for 30min, then filtered through the 0.22µm syringe filter and stored at -20°C until used for AGID and virus isolation.

**Agar gel immunodiffusion (AGID) test**

AGID was performed according to the method described previously (Kumar et al., 2003) briefly. Agar gel was prepared by dissolving 1 gm of noble agar in 100 ml of 9% sodium chloride solution in boiling water bath. The ten ml molten agar was poured in a Petri plate and allowed to solidify and kept at 4°C for 30 minutes. Five wells of 3mm diameter at a distance of 3mm were punched and sealed with molten agar. The central well was charged with hyper-immune serum raised against IBH-HPS virus and three peripheral wells were filled with test antigen and another with known positive antigen. The Petri plate was then incubated at 37°C for 24 hours in a humid chamber.

**Virus isolation**

AGID positive samples were inoculated in chicken embryo liver (CEL) culture for virus isolation and passaged upto 4th passage. Primary chicken embryo liver cell culture was prepared from 14 days old embryonated chicken egg (Kumar et al., 2003). The livers were removed aseptically and taken out in a petridish with Hank’s Balanced Salt Solution (HBSS, pH 7.2). These were minced into small pieces and washed with HBSS. The tissue was trypsinized in 0.125% trypsin solution. The cells were filtered through sterilized muslin cloth and centrifuged at 3000 rpm for 10 minutes at 4°C. The cells were washed twice in HBSS and finally in Medium-199. The final cells were diluted 1:200 (v/v) in Medium-199 containing 15% newborn calf serum and 1% tryptose phosphate broth (10% w/v) and dispensed in tissue culture bottles (25 cm²) @ 10 ml.

The bottles were incubated at 37°C for 48-72 hrs until a uniform monolayer of the chicken embryo liver cells was formed. After the proper confluent layer was made, 1 ml of inoculums was added into each bottle. The bottles were incubated at 37°C for 1 hr with frequent dispersion for adsorption of the virus. After adsorption, maintenance medium (containing 2% NBCS) was added to the bottles and incubated at 37°C. Each monolayer was examined twice daily upto 96 hours for the appearance of cytopathic effects (CPE).

**Histopathology of liver samples**

Liver samples were processed for histopathological examination as per standard protocol, fixed in 10% formal saline, washed in running tap water overnight and then dehydrated for one hour in different concentrations of ethanol, 50%, 60%, 70%, 80%, 90% and absolute alcohol for dehydration of tissues in same order. Then the tissues were cleared in xylene and embedded in paraffin wax. Sections of 4–5 µ thickness were cut and stained with Haematoxylin and Eosin (H & E) staining procedure as described by Kumar et al., (2003a).
PCR amplification of L1 loop of hexon gene

Genomic DNA was also extracted from infected CEL cells at 4th passage level with the help of DNeasy kit (Qiagen, Germany) and quantified. Amplification of the L1 loop of hexon gene of all the serologically positive isolates was done as per the method of Meulemans et al., (2001) with minor modifications. The reaction was performed in volumes of 25 µl each containing 10pm each of forward and reverse primer, 1x concentration of PCR buffer, 2.5mm each of dNTPs, 1unit of DNA polymerase enzyme and 30ng of DNA template. Reaction condition was set as initial denaturation (95ºC x 4min), Denaturation (94ºC x1min), Annealing (59ºC x1min), Extension (68ºC x3.30min) and Final Extension (68ºC x10min). The PCR reaction was done upto 35 cycles.

Results and Discussion

Epidemiological and post-mortem observations

Epidemiological data is presented in table-1. The affected birds are of mainly 3 to 6 weeks of age; however, birds less than 3 weeks of age are also affected. The IBH-HPS is an emerging and immunosuppressive disease of 3–6 week old broilers and mainly occurs in 1 to 5 week old broiler chickens (Dhama et al., 2002; Balamurugan and Kataria, 2004). It has also been reported as early as 2 to 4 day old broiler chickens (broiler breeders and layers) of varying ages. Our observations are in agreement with previous reports (Schachner, et al., 2018; Niczyprouk et al., 2016; Kumar et al., 2003a). The mortality rates at different farms were in the range of 0.5% to 20%.Choi et al., (2012) suggested that mortality in IBH is varies from as low as 1% to as high as 30%, but often remains between 5 to 10%. But in HPS-IBH, chickens less than 6 weeks of age, the mortality usually varies from 2–40 per cent. Under certain conditions however mortality up to 80 per cent has been recorded on the basis of the pathogenicity of the virus (Asthana et al., 2013). Disease occurrence was present round the year and found concurrently with other immunosuppressive disease like IBD and chicken infectious anaemia in some cases. Disease incidence was found to be more in August and September months of the year, which may be attributed to hot and humid conditions prevailing in these months (Kumar et al., 2013; Shah et al., 2011).

Liver and kidney lesions were most frequent post mortem finding. Accumulation of amber/straw coloured fluid in pericardial sac was also observed in three out of forty outbreaks investigated (Fig. 1). Lesions in bursa, spleen and skeletal muscles were also recorded in few outbreaks. Respiratory involvement indicated by involvement of trachea and lung was also evidenced. Liver is the principal target organ in fowl adenovirus infections. Involvement of kidney, spleen, muscles and accumulation of fluid in pericardial sac leading to development of hydropericardium syndrome are consistent findings reported by several workers (Kumar et al., 2013; Kumar et al., 2003a; Shah et al., 2011; Kataria et al., 2013).

Virus isolation

The CEL cell culture inoculated with virus inoculum resulted in the isolation of IBH-HPS virus. The cytopathic effects characterized by rounding and degeneration of cells were evident from the first passage itself and appeared as early as 36 hr PI. By 72 hr PI, micro plaques were evident (Fig. 2). At second passage level and onwards, CPE was evident at 24 hr PI and by 48 hr PI; 50-70% of cells were involved. By 72 hr PI, almost 40-60% of monolayer was detached. Severity of CPE varied in most of the isolates.
Table 1: Epidemiological observations in natural outbreaks IBH-HPS in broiler farms of U.P. and Uttarakhand

| S. No. | Isolate (sample) | Location       | Flock Strength | Age (days) | Mortality | P. M. Lesion and Clinical symptoms |
|--------|------------------|----------------|----------------|------------|-----------|-----------------------------------|
| 1.     | Pantnagar/KA-11/R-8 | Kamry         | 3500           | 30         | 7%        | Enlarged liver, swollen kidney     |
| 2.     | Pantnagar/SH-11/R-10 | Shantipuri    | 1000           | 28         | 5%        | Pale liver with necrotic foci on liver and kidney |
| 3.     | Pantnagar/SI-14/R-20 | Sitarganj    | 1500           | 35         | 3.5%      | Hydropericardium, large, congested liver |
| 4.     | Pantnagar/HA-14/R-21 | Halduchaur    | 6500           | 36         | 5%        | Hydropericardium, large, congested liver with necrotic foci on the surface |
| 5.     | Pantnagar/NA-14/R-22 | Nanakmatta    | 1000           | 35         | 3.5%      | Pale liver with necrotic foci     |
| 6.     | Pantnagar/PU-14/R-23 | Pulbhatta     | 5000           | 25         | 5%        | Pale liver with necrotic foci on surface |
| 7.     | Pantnagar/KH-14/R-24 | Khatima       | 3000           | 35         | 5.5%      | Swollen liver and kidney, fluid was present in peritoneal cavity |
| 8.     | Pantnagar/KH-14/R-25 | Khatima       | 1800           | 35         | 0.5%      | Swollen liver and kidney       |
| 9.     | Pantnagar/KH-14/R-27 | Khatima       | 3000           | 28         | 2.5%      | Swollen liver and kidney, enlarged bursa |
| 10.    | Pantnagar/Ha/R-28  | Halduchaur    | 700            | 28         | 20%       | Swollen liver and kidney, enlarged bursa |
| 11.    | Pantnagar/NA-15/R-30 | Nanakmatta    | 1000           | 28         | 10%       | Enlarged kidney and liver       |
| 12.    | Pantnagar/KH-15/R-31 | Khatima       | 2500           | 40         | 3%        | Enlarged liver and bursa, congestion in thymus |
| 13.    | Pantnagar/HA-15/R-32 | Halduchaur    | 10000          | 28         | 10%       | Hydropericardium, pale liver having necrotic foci congested kidneys |
| 14.    | Pantnagar/HA-15/R-33 | Haldwani      | 15000          | 30         | 2%        | Pale necrotic foci present on liver |
| 15.    | Pantnagar/BI-15/R-34 | Bindukhatta   | 2000           | 35         | 7.5%      | Pale liver having necrotic foci |
| 16.    | Pantnagar/BI-15/R-35 | Bilaspur, Rampur | 15000         | 30         | 8%        | Pale liver having necrotic foci, swollen kidney |
| 17.    | Pantnagar/BI-15/R-36 | Bilaspur, Rampur | 10000        | 30         | 10%       | Pale liver having necrotic foci, swollen kidney |
| 18.    | Pantnagar/HA-15/R-37 | Haldwani      | 3500           | 21         | 14%       | Pale liver having necrotic foci, swollen kidney and anaemia in birds |
| 19.    | Pantnagar/SH-15/R-38 | Shantipuri    | 2500           | 30         | 20%       | Pale liver having necrotic foci, swollen kidney, hydropericardium, haemorrhage in breast and thigh muscle |
| 20.    | Pantnagar/HA-15/R-39 | Halduchour    | 2500           | 21         | 5%        | Liver and kidney have necrotic foci, fluid was present in abdominal cavity |
| 21.    | Pantnagar/HA-16/R-40 | Haldwani      | 5000           | 18         | 4%        | Liver and kidney have necrotic foci, |
| No. | Location/Code    | Place          | Pool No. | Mortality | Lesion Description                                                                 |
|-----|------------------|----------------|----------|-----------|-----------------------------------------------------------------------------------|
| 22  | Panthagar/SH-16/R-41 | Shantipuri     | 15000    | 30        | Pale and swollen liver, haemorrhage was present on kidney                          |
| 23  | Panthagar/KH-16/R-42 | Khatima        | 1500     | 24        | Haemorrhages on bursa and skeletal muscles, Swollen and haemorrhagic kidney, pale liver, haemorrhagic foci on spleen |
| 24  | Panthagar/BA-16/R-43 | Khatima        | 5000     | 42        | Swollen liver and kidney, congested trachea and lungs                             |
| 25  | Panthagar/BI-16/R-44 | Bindukhatta    | 2000     | 35        | Pale and swollen liver, swollen kidney                                               |
| 26  | Panthagar/HA-16/R-45 | Haldwani       | 15000    | 21        | Pale and swollen liver, swollen kidney                                               |
| 27  | Panthagar/BI-16/R-46 | Bilaspur, Rampur | 20000  | 28        | Liver was pale and swollen, haemorrhage was present on kidney                      |
| 28  | Panthagar/SH-16/R-47 | Shantipuri     | 2000     | 30        | Swollen and pale liver, lung and trachea have pin point haemorrhages               |
| 29  | Panthagar/HA-16/R-48 | Halduchour     | 2500     | 23        | Pale and enlarged liver                                                            |
| 30  | Panthagar/BI-16/R-49 | Bindukhatta    | 1500     | 28        | Swollen liver and kidney with haemorrhagic spots on the surface                     |
| 31  | Panthagar/HA-16/R-50 | Halduchour     | 2000     | 25        | Liver was pale and swollen                                                          |
| 32  | Panthagar/BA-16/R-51 | Bajipur        | 3000     | 28        | Swollen and pale liver and kidney, haemorrhages on bursa and thigh muscles         |
| 33  | Panthagar/SH-16/R-52 | Shantipuri     | 1500     | 30        | Swollen liver, pale foci was present, respiratory problem was also present, congestion on trachea |
| 34  | Panthagar/SH-16/R-53 | Shantipuri     | 2000     | 22        | Pale and enlarged liver, haemorrhagic kidney, congestion in trachea and lungs       |
| 35  | Panthagar/KI-16/R-54 | Kichha         | 3000     | 30        | Pale liver, swollen kidney, pin point haemorrhages on trachea, congestion in lungs |
| 36  | Panthagar/SH-16/R-55 | Shantipuri     | 2000     | 40        | Pale and enlarged liver, haemorrhagic kidney, congestion in trachea and lungs       |
| 37  | Panthagar/HA-16/R-56 | Haldwani       | 5500     | 28        | Liver and kidney were pale and swollen, haemorrhage was present on lung and trachea |
| 38  | Panthagar/BI-16/R-57 | Bilaspur       | 2000     | 21        | Liver and kidney were pale and swollen                                              |
| 39  | Panthagar/SH-16/R-58 | Shantipuri     | 1500     | 28        | Liver and kidney were pale and swollen                                              |
| 40  | Panthagar/HA-16/R-59 | Halduchour     | 2000     | 21        | Liver and kidney were pale and swollen                                              |
**Fig. 1** Post-mortem lesions in birds died in natural outbreaks of IBH-HPS a. Congested and swollen kidney b. Pale liver c. Hydropericardium with necrotic foci on liver

![Post-mortem lesions](image1)

**Fig. 2** Photograph of CEL culture, liver cells showing micro-plaque formation after 96 hr of infection (Unstained X 200)

![Photograph of CEL culture](image2)

**Fig. 3** Photomicrograph showing precipitin lines in AGID

![Photomicrograph](image3)

1- Known hyper-immune serum, Peripheral wells- 4-Known antigen, 2, 3, 5- Test antigens
Uninfected monolayers did not exhibit any change. Use of CEL cells is considered to be best for virus isolation and has been used by several investigators for the purpose. CPE observed are in agreement with earlier findings (Kumar et al., 2003a, b; Oberoi et al., 1996; Asthana et al., 2013).

Detection of IBH-HPS virus in tissue samples

After 24 hrs of incubation, tissue samples positive for IBH-HPS viral antigens showed clear precipitin lines with known hyper immune serum in AGID (Fig. 3). Serological tests including AGID have been successfully implied for detection of IBH-HPS virus antigens by several investigators (McFerran and Smyth, 2000; Kumar et al., 2003a, b; Choi et al., 2012; Asthana et al., 2013). Histopathology of liver samples revealed necrosis of hepatocytes, vacuolar degeneration and infiltration of mononuclear cells. Many hepatocytes revealed large basophilic intranuclear inclusion bodies, which were round and compact and occupied almost entire nucleus (Fig. 4). These findings are in accordance with those observed by

Fig. 4 Photomicrograph of liver showing basophilic intranuclear inclusion bodies in hepatocytes (H&E X 400)

Fig. 5 Agarose gel analysis of amplicons of L1 loop of hexon gene

(M-1Kb ladder, 1 to 10– test samples)
earlier workers (Kaur et al., 2003; Kim et al., 2008). The PCR was carried out on DNA extracted from CEL cell culture with HexonLA and HexonLB primers (Meulemans et al., 2001). The amplified products of all the serologically positive isolates showed a single DNA band of ~ 900bp size (Fig. 5). Polymerase chain reaction (PCR) is used to detect FAdV for confirming the infection status (Rahul et al., 2004). The PCR is usually targeted against the variable region of hexon gene flanked by conserved primer sites (Meulemans et al., 2001; Hess, 2000; Thakor et al., 2012). Jiang et al., (1999) and Hess et al., (2000) suggested the PCR for hexon gene is suitable diagnostic tool for fowl adenovirus infections.

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