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Clinicopathological investigations into concurrent fowl adenovirus-induced inclusion body hepatitis and mycotoxicosis on commercial broiler farms in Kerala, South India

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ABSTRACT

Broiler farming is a significant segment of the global economy, valued at 1.5 trillion in 2020, with India among the leading producers. Disease outbreaks continue to threaten the profitability and sustenance of the industry, impacting growth and feed conversion efficiency. The present study investigated the impact of Inclusion Body Hepatitis (IBH) and mycotoxicosis in commercial broiler farms in Kerala, South India, by examining 21 outbreaks between May 2023 and November 2024, that resulted in the mortality of 2946 birds out of an affected population of 52,022. Detailed necropsy examinations were performed on 130 birds, with gross and histopathological lesions recorded, and aflatoxin B1 levels in 6 feed samples were estimated using fluorimetry. Samples subjected to hexon gene (590 bp) amplification using polymerase chain reaction (PCR) were found positive for fowl adenovirus (FAdV). Poultry feed analyses presented Aflatoxin B1 levels far above the recommended maximum levels (20 ppb). Acute mortality ranged from 0.44 to 61.9% in broiler flocks aged 6 to 37 days. The liver was the primary organ affected, followed by the kidneys, spleen and lungs. Pathognomonic histopathological lesions, such as basophilic intranuclear inclusion bodies in hepatocytes with necrosis, were observed in all the outbreaks. The present study emphasizes the association of the immunosuppressive role of aflatoxin B1 and the consequent IBH infection, and provided insights into the development of suitable biosecurity and preventive strategies. Further studies are warranted to identify major FAdV serotypes, and to study differences in the pathogenesis of IBH.

Key words: inclusion body hepatitis; Fowl adenovirus; broiler; molecular diagnosis; aflatoxin B1

Introduction

Poultry farming is one of the segments of agriculture in India which has seen a tremendous transformation from being a small-scale supplemental backyard activity to a full-fledged primary commercial activity at industrial scales, involving higher yielding breeds, research into inputs and a support infrastructure working for its continued improvements in productivity and efficiency (CHATTERJEE and RAJKUMAR, 2015; GULATI and JUNEJA, 2023). The significance of poultry farming to the larger Indian economy can be understood from the rate of growth that the sector has witnessed, posting a consistent 8% growth (KUMAR et al., 2024).The newer breeds introduced to India for their higher yield were, over a period of time,

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identified to be susceptible to diseases to which the indigenous breeds had been found to be thoroughly resistant. Many species of bacteria and viruses have been identified as having an adverse impact on the industry (KRISHNEGOWDA et al., 2020; 2022).

Fowl adenoviruses (FAdVs) are preva- lent in India, and within the poultry population most cases are reported in broiler chickens (CHA- VAN et al., 2023). FAdV is a non-enveloped, double-stranded DNA virus belonging to the genus *Aviadenovirus* and family *Adenoviridae*. FAdV is the causative agent of inclusion body hepatitis (IBH), hydropericardium hepatitis syndrome (HHS), egg drop syndrome (EDS), adenoviral gizzard erosion (AGE), and avian adenoviral splenomegaly (AAS) in chickens. Fowl adenovirus causes significant economic losses to the poultry industry in India through massive growth retardation and high mortalities due to IBH, HHS and AGE diseases (ASTHANA et al., 2013; SCHACHNER et al., 2018; EL-SHALL et al., 2022). There are 5 species of Avian adenoviruses, A to E with 12 serotypes: Fowl adenovirus A (FAdV-1), Fowl adenovirus B (FAdV-5), Fowl adenovirus C (FAdV-4 and FAdV-10), Fowl adenovirus D (FAdV-2, -3, -9, and -11), and Fowl adenovirus E (FAdV-6, -7, -8a, and -8b) (SCHACHNER et al., 2018; HESS, 2000). The FAdVs A, B and C are considered to be non-pathogenic and are ubiquitously present in healthy poultry flocks without causing any malady (ABSALÓN et al., 2017). However, Fowl adenovirus D and E (serotypes 2, 3, 6, 7, 8a, 8b, 9, and 11) are linked to IBH, characterized by the presence of intranuclear basophilic and/or eosinophilic inclusion bodies in hepatocytes (HOSSEINI et al., 2020). The disease mainly affects 3-5-week-old broilers causing mortality ranging from 2 to 40% against a range of 20 to 80% in HHS (NICZYPORUK et al., 2020). FAdV contributes significantly to the prevalence of IBH in broilers in India. FAdV- 2, FAdV-4, FAdV-8a, FAdV-8b, and FAdV-11 were commonly reported as the predominant circulating serotypes in India with a prevalence rate of 19 to 48% in commercial broiler flocks in western India (CHAVAN et al., 2023).

Aflatoxicosis is a significant economic problem in the Indian poultry industry due to the widespread contamination of feed with aflatox- ins, resulting in reduced feed efficiency and de- creased production, immunosuppression, he- patocellular tumours, increased susceptibility to infections, and mortality (DIAZ et al., 2008; YUNUS et al., 2011). Aflatoxins are a family of toxins produced by certain fungi, *Aspergil- lus flavus* and *Aspergillus parasiticus*. Aflatoxins are the most widespread and economically im- portant mycotoxins, and have carcinogenic and mutagenic potential. Aflatoxicosis is attribut- ed to feeding with grains, corn/maize, soybean, peanut, and millet contaminated with aflatoxin. Even though aflatoxicosis in poultry primarily affects the liver, it can also compromise immu- nologic, digestive, and hematopoietic functions (DALVI, 1986; OCHIENG et al., 2021). Detailed pathological studies of the association of IBH and mycotoxicosis among commercial broilers are scarce in India. Hence, the present study was conducted to investigate the unusual mortality and pathological lesions recorded during the post-mortem examination of commer- cial broilers due to the first reported concurrent occurrence of inclusion body hepatitis and myco- toxicosis in Kerala, India.

Materials and methods

Disease outbreak location. The present study was conducted on broiler birds from various commercial farms spread across different districts of Kerala State, South India, presented for postmortem examination at the Avian Disease Diagnostic Laboratory, Thiruvalla, Pathanamthitta District of Kerala, during the period from May 2023 to November 2024. The detailed history of the flocks, including the outbreak location, the age of the birds, flock strength, morbidity, clinical signs, vaccination history, mortality, and other management practices were recorded.

Postmortem examination and sample collection. Detailed necropsy examination of the dead birds was carried out, and the gross pathological findings were recorded. Representative tissue samples (liver,

Table 1. Details of disease outbreak location, age of birds, flock strength, and mortality

S. No	Outbreak location	Lab ID	Age of birds	Flock trength	Mortality	Birds presented	Clinical signs	Vaccination history	Type of rearing
1	Pothanikad village, Ernakulam district	652/23	32 days	2142	38 (1.77%)	6	Sudden onset of mortality and droopiness	NDV, IBD	Organized commercial farm
2	Cherthala, Alappuzha	653/23	34 days	1950	31 (1.59%)	5	Lethargy and decreased appetite	NDV, IBD	Organized commercial farm
3	Poonjar, Kottayam district	665/23	37 days	3650	16 (0.44%)	5	Huddling and yellow mucoid droppings	NDV, IBD	Organized commercial farm
4	Poonjar, Kottayam district	666/23	30 days	2900	39 (1.34%)	5	Decreased appetite and mortality	NDV, IBD	Organized commercial farm
5	Manimala village, Kottayam district	684/23	28 days	1350	34 (2.52%)	5	Droopiness and mortality	NDV, IBD	Organized commercial farm
6	Ayrkunnam, Kottayam district	685/23	32 days	2300	83 (3.60%)	5	Lethargy and mortality	NDV, IBD	Organized commercial farm
7	Udayanapuram village, Kottayam district	873/23	29 days	1680	92 (5.48%)	5	Greenish droppings and mortality	NDV, IBD	Organized commercial farm
8	Cherthala, Alappuzha district	875/23	33 days	1479	53 (3.58%)	5	Decreased appetite and mortality	NDV, IBD	Organized commercial farm
9	Ayrkunnam, Kottayam district	1082/23	29 days	2480	295 (11.90%)	5	Decreased appetite	NDV, IBD	Organized commercial farm
10	Vattapara village, Thiruvananthapuram district	1091/23	27 days	2000	45 (2.25%)	5	Droopiness	NDV, IBD	Organized commercial farm
11	Ayrkunnam, Kottayam district	1092/23	28 days	2800	120 (4.29%)	5	Yellowish droppings and sudden onset of mortality	NDV, IBD	Organized commercial farm
12	Edathua village, Alappuzha district	1130/23	35 days	21	13 (61.9%)	5	Droopiness	NDV, IBD	Unorganized backyard farm
13	Perumbavoor Ernakulam district	1797/23	34 days	4590	56 (1.22%)	6	Droopiness	NDV, IBD	Organized commercial farm
14	Muhamma, Alappuzha district	113/24	17 days	1700	61(3.59 %)	5	Huddling and decreased appetite	NDV	Organized commercial farm
15	Ayrkunnam, Kottayam district	1081/24	34 days	2100	124 (5.9%)	5	Droopiness	NDV, IBD	Organized commercial farm
16	Thidanad village, Kottayam district	1082/24	6 days	4386	148 (3.37%)	5	Huddling and mortality	NDV	Organized commercial farm
17	Elanthoor village, Pathanamthitta district	1256/24	25 days	500	10 (2%)	3	Decreased appetite and mortality	NDV, IBD	Organized commercial farm
18	Peruva village, Kottayam	1310/24	9 days	3976	164 (4.12%)	14	Yellowish droppings and mortality	NDV	Organized commercial farm
19	Peruva village, Kottayam district	1311/24	9 days	5118	290 (5.67%)	13	Decreased appetite and mortality	NDV	Organized commercial farm
20	Airapuram village Ernakulam	1314/24	32 days	3800	1221(32. 13%)	13	Sudden onset of mortality and droopiness	NDV, IBD	Organized commercial farm
21	Kollam	1362/24	27 days	1000	13 (1.3%)	5	Decreased appetite and dyspepsia	NDV, IBD	Organized commercial farm

Note: NDV: Newcastle disease; IBD: Infectious bursal disease

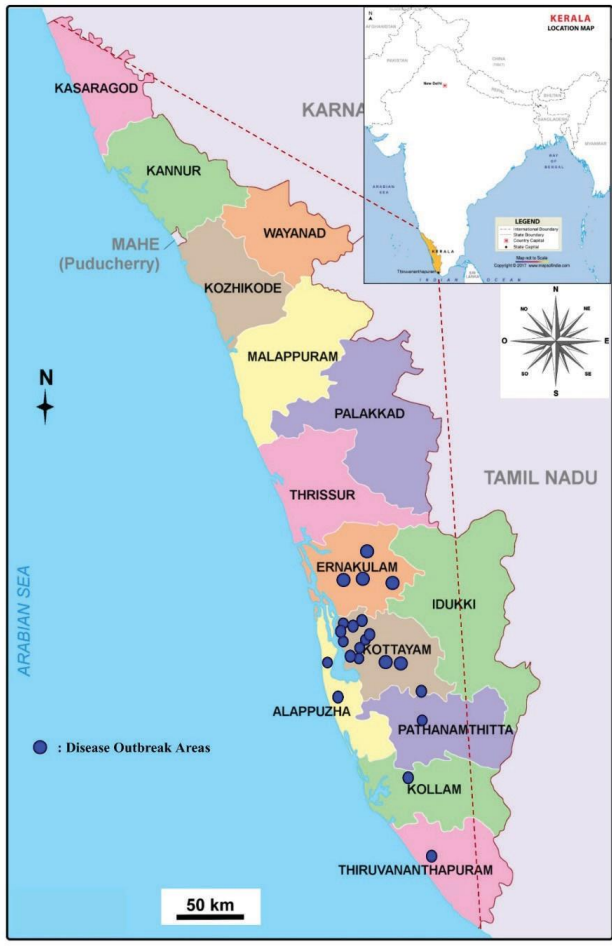

Fig. 1. Map showing the distribution of outbreaks of inclusion body hepatitis and mycotoxicosis in various commercial farms of Kerala State, South India

kidneys, lungs, heart, spleen, and brain) were collected in 10% neutral buffered formalin for histopathological studies. Various samples, such as liver, heart and spleen, were collected aseptically from the birds and transported under a cold chain for isolation and subsequent identification of the pathogen.

Blood smear and fecal examination. Blood smear examination was carried out after staining with Gram, methylene blue, and Giemsa stains to demonstrate Gram-positive/-negative, bipolar (*Pasteurella*), and hemoprotozoal organisms, respectively. Fecal examination was carried out in all birds to examine the presence of protozoan and helminth parasites.

Screening of important bacterial pathogens of poultry. Tissues, such as liver, spleen, and heart, were processed for bacterial isolation. Using a sterile loop, the tissues were penetrated and the materi- al was inoculated into four different media: tryp- tic soy agar (TSA), blood agar, MacConkey agar, and universal differential medium. After inocula- tion, the plates were incubated at 37ºC aerobically overnight. The bacterial colonies were transferred after incubation on to a clean glass slide and stained using Gram staining.

The colonies were subjected to catalase and oxidase testing in various media. The individual colonies were picked, suspended in tryptic soy broth and reinoculated onto fresh corresponding media, where from the colonies were restained to confirm uniformity, and then subjected to conventional biochemical test procedures. Further, a lawn culture was made on to phenol red agar, and the battery of various sugar-coated discs were equidistantly placed in the already lawned phenol red agar, keeping 2.5 cm between the discs. These agar plates were incubated at 37ºC aerobically overnight.

Immunochromatography tests. Representa- tive tissue samples were processed by homoge- nizing the tissues using a mortar and pestle with diluent buffer, and a few drops of the supernatant were applied to the sample hole of a rapid antigen immunochromatographic lateral flow assay (LFA) test kit (Bionote, Inc., Hwaseong-si, Korea) for screening for NDV (cloacal swab), IBD (Bursa of Fabricius), avian infectious bronchitis (IB, lungs), and type A influenza virus (AIV, cloacal swab), as per the manufacturer's instructions.

DNA extraction. DNA extraction was carried out on liver and spleen samples using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The yield and purity of the extracted DNA were measured using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA).

Polymerase chain reaction (PCR). The primers targeting the partial hexon gene of *Aviadenovirus* were: Forward: 5'-ATG GGA GCG ACC TAC TTC GAC AT-3' and Reverse: 3'-AAA TTG TCC CGA AAA CCG ATG TA-5'(ARAZI et al., 2020).

The PCR was performed in a Thermocycler (Mastercycler Personal, Eppendorf, Enfield, Connecticut, USA) using 25 µL reaction mixture containing 5 ng/µL DNA, 12.5 µL 2x Taq PCR master mix (Qiagen, Hilden, Germany), 0.5 µL of forward primer (10 pmol/µL), 0.5 µL of reverse primer (10 pmol/µL), and nuclease-free water. The cyclic conditions for primary amplification were: initial denaturation at 95°C for 15 min, followed by 35 cycles of 94°C for 45 s, 57°C for 45 s, 72°C for 45 s, and final extension at 72°C for 10 min

Agarose gel electrophoresis. The PCR products were analyzed by electrophoresis in 1.5% agarose gel in 0.5× tris-borate-EDTA (TBE) buffer in the electrophoresis apparatus (Bio-Rad, Hercules, California, USA) stained with ethidium bromide (0.5 µg/mL), and standard DNA molecular weight markers (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were run along with the PCR products. After electrophoresis, the gel was visualized and documented using the Gel Documentation system (Bio-Rad, Hercules, California, USA). The relative size of the amplified product was determined by comparison with the DNA ladder.

Histopathological examination. The formalin -fixed tissue samples were processed using a routine paraffin-embedding technique. Sections of 4-5 µm thickness were prepared, deparaffinized, and stained with hematoxylin and eosin (H&E) for detailed examination of histopathological lesions, following the standard operating procedure.

Estimation of aflatoxin B1 levels in feed using fluorimetry. Aflatoxin B1 levels in six feed samples were quantified by Fluorimetry using AflaTest® columns using a VICAM Series-4EX Fluorometer (Milford, Massachusetts, USA). Brief- ly, sample extraction was done using 50 g of the submitted feed samples, followed by dilution and filtration using a microfiber filter. The filtrate was passed through an immunoaffinity column, fol- lowed by elution with methanol. Developer was then added, and the digital readout was recorded after 60 seconds using the calibrated fluorometer.

Results

Clinical signs and mortality. The details of the clinical signs and mortality are presented in Table 1. A total of 21 outbreaks were recorded from May 2023 to November 2024. In total, 52,022 birds were affected, with 2946 mortalities reported (mortality - 5.66%). Of these, 130 birds were subjected to systematic necropsy examination. The birds, aged 6 to 37 days, exhibited clinical signs such as: sudden onset of mortality, droopiness, lethargy, decreased feed consumption, poor growth rate, huddling, ruffled feathers, pale combs and wattles, weight loss, and yellow mucoid droppings.

Gross pathological lesions. Detailed necrop- sy examination revealed significant differences in the body weights of birds from the same batch. The most characteristic and common lesions noted on the liver were severe enlargement, pale and focal or coalescing necrotic areas, friable, and streaks or patches of hemorrhages (Fig. 2a, 2c). Kidneys were enlarged and pale, with hemorrhag- ic foci and dilated ureters (Fig. 2b). In most cases, the lungs were pneumonic or congested. Frothy, catarrhal or mucoid contents were observed in the nasal sinuses. Pale streaks were noted in the breast muscle. Intestinal catarrh was observed in the ma- jority of the cases. Enlargement of the spleen (Fig. 2d) and heart was also noted.

Blood smear and fecal examination. Blood smear examination showed no presence of Gram-positive/-negative, bipolar (Pasteurella), or hemo protozoal organisms. Similarly, fecal analysis revealed no protozoan or helminth eggs or parasitic forms.

Isolation of secondary bacterial pathogens. Bacteria such as *Klebsiella*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Proteus* spp., and *Pseudomonas* spp. were isolated from some of the samples collected during necropsy. These secondary bacterial pathogens were detected in only 3 out of 21 outbreaks.

Molecular confirmation of fowl adenovirus by PCR. Tissue samples including liver and spleen, processed for molecular confirmation of inclusion body hepatitis by targeting the hexon gene of fowl adenovirus using PCR, followed by agarose gel

Table 2. Detailed gross and histopathological lesions in the individual outbreaks

S. NO.	LAB ID	GROSS PATHOLOGICAL LESIONS	HISTOPATHOLOGICAL LESIONS
1	652/23	Hepatomegaly and epicardial petechiae.	Necrotizing steatohepatitis, basophilic intranuclear inclusions occupying the entire nucleus in hepatocytes, bile duct hyperplasia, mild to moderate depletion of splenic lymphoid follicles, and degeneration and extensive desquamation of tracheal epithelium with infiltration of inflammatory cells.
2	653/23	Hepatomegaly, congestion of lungs, and hemorrhages in kidneys.	Steatohepatitis with bile duct hyperplasia and basophilic intranuclear inclusions in hepatocytes.
3	665/23	Pale and enlarged liver, and congested lungs with pneumonic patches.	Severe diffuse necrotizing pneumonia, pericarditis, bursitis, tracheitis, steatohepatitis with inclusion bodies, and nephritis.
4	666/23	Pale and enlarged liver.	Steatohepatitis with inclusions, nephritis, hyperplastic proventriculitis, pleuritis, and bursitis.
5	684/23	Pale and enlarged liver, congested lungs, and catarrhal enteritis.	Steatohepatitis with bile duct hyperplasia, inclusion bodies, nephritis, and hyperplastic proventriculitis.
6	685/23	Enlarged, pale and friable liver, enlarged and mottled spleen, and pulmonary edema.	Severe diffuse pneumonia, splenitis, necrotizing pancreatitis, and steatohepatitis.
7	873/23	Pale and enlarged liver, and enlarged heart with pale streaks and hydropericardium	Necrotizing hepatitis with basophilic inclusions and myocarditis.
8	875/23	Enlarged and pale liver, and enlarged and mottled spleen.	Necrotizing splenitis and steatohepatitis with inclusion bodies.
9	1082/23	Enlarged and pale liver, and enlarged bursa with caseous contents.	Diffuse depletion of bursal lymphoid follicles and coagulative necrosis, basophilic intranuclear inclusions in hepatocytes, and bile duct hyperplasia.
10	1091/23	Pale liver with streaks of congestion and hemorrhages, enlarged bursa, thigh muscle hemorrhage, and hemorrhagic kidneys.	Diffuse severe micro- to macrovesicular fatty degeneration, multifocal hepatic necrosis with infiltration of polymorphonuclear cells, basophilic intranuclear inclusions and multifocal bacterial colonies. In the kidneys, diffuse tubular necrosis and interstitial lymphocytic infiltration. In the bursa, severe depletion of lymphoid follicles.
11	1092/23	Enlarged pale liver.	Diffuse fatty degeneration, periportal lymphocytic infiltration mostly pleiomorphic, basophilic intranuclear inclusions, and diffuse hepatocyte degeneration.
12	1130/23	Splenomegaly, prominent proventricular glands, and enlarged liver.	Necrotizing splenitis, steatohepatitis with inclusion bodies, and hyperplastic proventriculitis.
13	1797/23	Enlarged liver with pale necrotic areas, splenomegaly, enlarged kidneys, pulmonary edema, and pericarditis.	Steatohepatitis with basophilic intranuclear inclusions in hepatocytes, myocarditis, and hyperplastic proventriculitis.
14	113/24	Enlarged pale liver, congested lungs, pulmonary edema, and pale and enlarged kidneys.	In the liver, multifocal hepatic necrosis and basophilic intranuclear inclusions. In the kidneys, diffuse tubular necrosis and interstitial lymphocytic infiltration. In the lungs, pulmonary edema and respiratory atrial infiltration of inflammatory cells.
15	1081/24	Enlarged liver with rounded ends, pale necrotic foci, enlarged spleen, and congested trachea.	Necrotizing steatohepatitis, inclusion bodies, reactive splenitis, and tracheitis.
16	1082/24	Enlarged and pale liver, enlarged spleen, pale streaks in the heart, and congested lungs.	Diffuse fatty degeneration, individualization of hepatocytes, basophilic intranuclear inclusions, necrosis of splenic lymphoid follicles, interstitial lymphocytic infiltration with fragmentation of cardiac muscle fibers, and alveolar capillary congestion.
17	1256/24	Hepatomegaly, enlarged kidneys, and splenic mottling.	Hepatitis, coagulative necrosis with basophilic intranuclear inclusions in hepatocytes, nephrosis, and splenic necrosis.
18	1310/24	Pale and enlarged liver, catarrhal enteritis, and fibrinous pneumonia.	Hepatitis with basophilic intranuclear inclusions in hepatocytes, and myocarditis. In the lungs, severe fibrous tissue proliferation, edema, and infiltration of heterophils, lymphocytes, macrophages and presence of bacterial colonies.
19	1311/24	Hepatomegaly with focal necrosis and catarrhal enteritis.	In the liver, multiple necrotic foci in parenchyma with necrotic neutrophils, bacterial colonies and basophilic intranuclear inclusions. In the intestine, severe hemorrhages on the lamina propria, desquamated epithelial cells and diffuse necrosis.
20	1314/24	Hepatomegaly, cooked appearance of breast muscle, and catarrhal enteritis.	In the liver, severe diffuse macrovesicular fatty degeneration, necrotizing hepatitis, bile duct hyperplasia, and sinusoidal congestion. In the intestine, infiltration of inflammatory cells, predominantly neutrophils and a few lymphocytes in the lamina propria.
21	1362/24	Enlarged liver and splenomegaly with mottled.	Individualization of hepatocytes, multifocal necrotizing hepatitis with basophilic intranuclear inclusions, red pulp congestion, and proliferation of splenic macrophages.

electrophoresis, produced the expected amplicon of 590 bp (Fig. 4d).

Rapid antigen-based immunochromatographic assay. Representative clinical and tissue samples tested negative for other viral etiologies such as NDV, IBD, IB, and AIV using antigen-based immunochromatographic assay.

Histopathological lesions. Histopathological examination revealed severe degenerative and necrotic changes in the liver, kidneys and spleen in most cases (Table. 2). In the liver, multifocal necrotic areas with infiltration of polymorphonuclear cells, proliferation of Kupffer cells (Fig. 3a, 3b), sinusoidal congestion, bile duct hyperplasia, the presence of basophilic intranuclear inclusions occupying the entire nucleus (Fig. 3c, 3d), and a few intracytoplasmic eosinophilic inclusions in hepatocytes were observed. Varying degrees of microvesicular to macrovesicular fatty degeneration were evident (Fig. 3e). Individualization of hepatocytes and a few bacterial colonies were observed. In the kidneys, diffuse necrosis of the tubular epithelium, cast formation, and interstitial infiltration of inflammatory cells were prominent (Fig. 3f). In the heart, multifocal infiltrations of necrotic heterophils and a few lymphocytes, and interstitial edema were observed. Infiltration of inflammatory cells, predominantly polymorphonuclear cells, and fibrous tissue proliferation, were noticed in the pericardium (Fig. 4a). In the spleen, severe edema of the reticular sheath with proliferation of splenic macrophages and the presence of necrotic heterophils were observed (Fig. 4b). Severe respiratory atrial and parabronchial congestion, interstitial edema, infiltration of heterophils and a few macrophages in the respiratory atria, pleural infiltration of inflammatory cells (pleuritis), and fibrous tissue proliferation could be observed in the lungs (Fig. 4c). In the bursa of Fabricius, bursal depletion, repopulation of follicles, and interfollicular fibrous tissue proliferation were noted. In the trachea, mild infiltration of inflammatory cells, predominantly heterophils, and a few lymphocytes were evident. Hyperplastic proventriculitis and necrotizing pancreatitis were observed in some cases. In the intestines, severe hemorrhages on the lamina propria, desquamated epithelial cells and diffuse necrosis were observed.

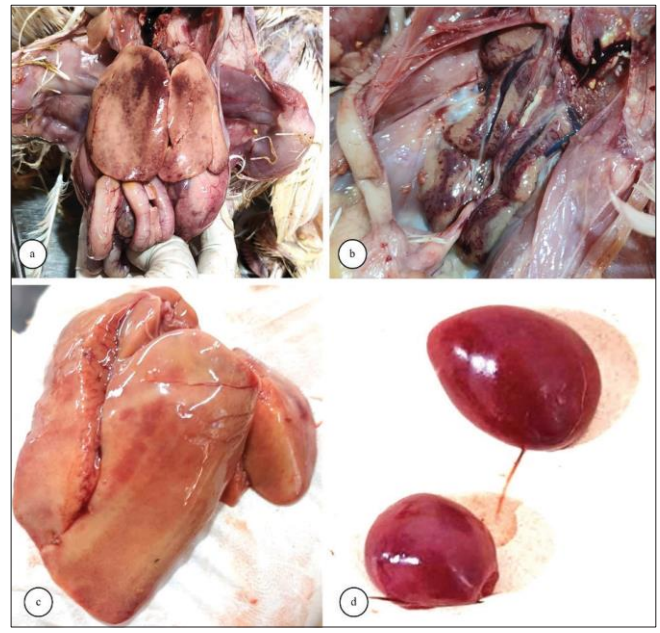

Fig. 2. Gross pathological lesions in concurrent inclusion body hepatitis and mycotoxicosis affection in commercial broilers
(a) Swollen, pale liver with necrotic and hemorrhagic patches; (b) Bilaterally swollen kidneys with pale necrotic patches and dilated ureter; (c) Enlarged and pale liver; (d) Enlarged spleen

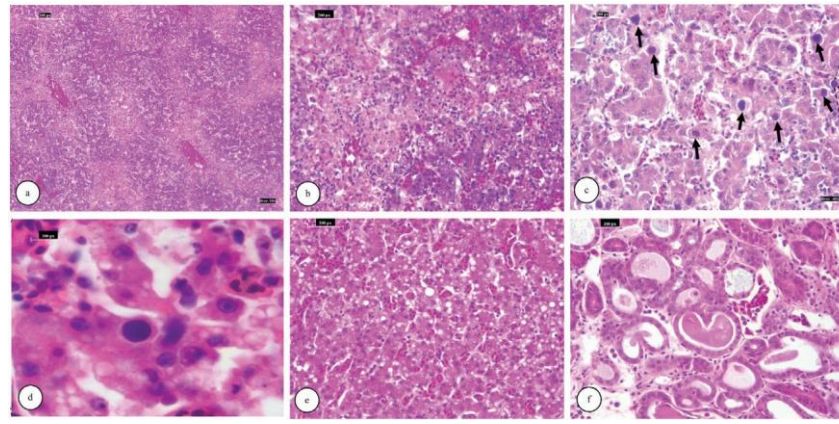

Fig. 3. Histopathological lesions in concurrent inclusion body hepatitis and mycotoxicosis affection in commercial broilers

(a & b) The liver showing multifocal diffuse hepatic necrosis and infiltration of polymorphonuclear cells. H&E x 100 (a), x400 (b). (c & d) The liver showing the presence of basophilic intranuclear inclusions occupying the entire nucleus of hepatocyte (arrows). H&E x400 (c), x1000 (d). (e) The liver showed sinusoidal congestion, diffuse micro-vesicular fatty degeneration and Kupffer cell proliferation. H&E x400. (f) The kidneys showed moderate degeneration of the tubular epithelium, cast formation, and interstitial infiltration of inflammatory cells. H&E x400.

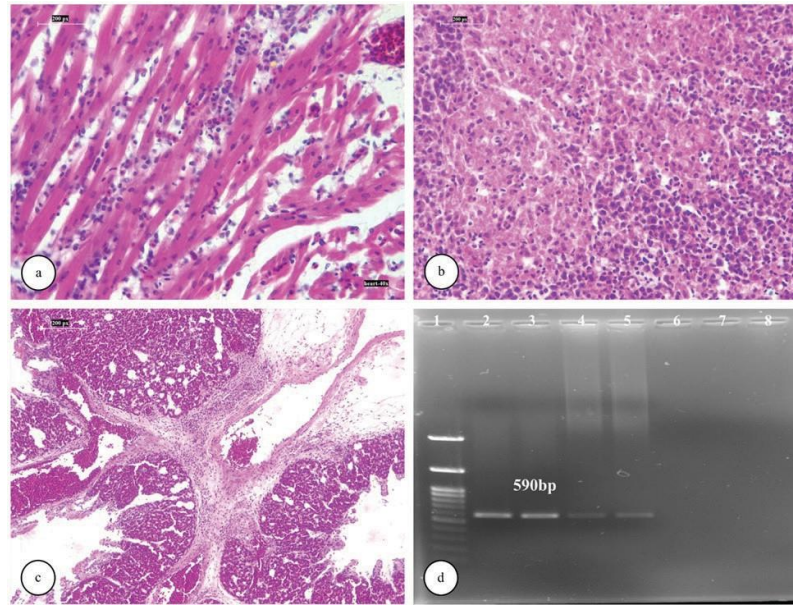

Fig. 4. Histopathological lesions in concurrent inclusion body hepatitis and mycotoxicosis affection in commercial broilers

(a) The heart showed edema, and interstitial infiltration of mononuclear cells and a few heterophils. H&E x400. (b) The spleen showed severe edema of the reticular sheath with proliferation of splenic macrophages and necrotic heterophils. H&E x400. (c) The lungs showed severe respiratory atrial and parabronchial congestion, fibroplasia, infiltration of heterophils and a few macrophages in the respiratory atria. H&E x100. (d) Agar gel electrophoresis showing 590 bp amplicon for fowl adenovirus. Lane 1: 100 bp DNA ladder; 2: Positive control; 3-5: Positive clinical samples; 6: Negative control; 7 & 8: Negative clinical samples.

Estimation of Aflatoxin B1 by fluorimetry. The levels of aflatoxin B1 in the feed samples are detailed in Table 3. All feed samples tested contained signficantl higher concentration of Aflatoxin B1 than the recommended limit of 20 parts per billion (ppb) as specified by the Bureau of Indian Standards (BIS), 2023.

Table 3. Level of aflatoksin B1 in the feed samples

S. No.	Lab ID	Type of feed	Aflatoxin B1 (ppb)
1	673/2023 (1)	Pre-starter	53
2	673/2023 (2)	Starter	100
3	673/2023 (3)	Finisher	>300
4	1312/2024 (1)	Pre-starter	56
5	1312/2024 (2)	Pre-starter	120
6	1312/2024 (3)	Finisher	36

Discussion

Inclusion body hepatitis (IBH) is a disease of high concern because of the severe losses it inflicts on the broiler industry. The Genus *Aviadenovirus* causes IBH, HHS, EDS, AGE, and AAS diseases (ASTHANA et al., 2013; SCHACHNER et al., 2018; TRIVEDI et al., 2018; EL-SHALL et al., 2022). Numerous studies have documented IBH as a major epidemic outbreak in several countries resulting in enormous economic losses (KHODAKARAM-TAFTI et al., 2016; TRIVEDI et al., 2018; MARIAPPAN et al., 2018; EL-BASREY et al., 2020; ISHAG et al., 2022). In the present study, sudden acute mortality ranging from 0.44 to 61.90% was recorded in affected broiler flocks. Mortality due to IBH has been reported to vary between 10% to as high as 50%, depending on the strain of vi- rus, host susceptibility, and other immunosuppres- sive agents (CHOI et al., 2012; EL-THOLOTH and ABOU EL-AZM, 2019). The findings of the present study corroborated previous studies documenting mortality rates as low as 0.5% to as high as 80% based on the pathogenicity of the virus (ASTHANA et al., 2013; EL-SHALL et al., 2022).

IBH can infect chickens of all ages; howev- er, young chicks are more susceptible during the first two weeks, even when immunologically in- tact (SCHACHNER et al., 2018; TRIVEDI et al., 2018; EL-SHALL et al., 2022). In the present study, the age of the infected birds varied from 6 to 37 days, where these findings were in agree- ment with previous reports of the incidence of the disease in birds as early as 2 to 4 days (SCHACHNER et al., 2018; NICZYPORUK et al., 2020), even though, the age of 2 to 6 weeks has been consid- ered as the most susceptible age in broiler chickens for IBH and/or HPS (BALAMURUGAN and KATARIA, 2006). Studies have reported the pres- ence of the virus in the very early days, due to a viru- lent strain of the virus or the fact that day-old chicks were being kept on a farm (without proper cleaning) where previous flocks suffered from HPS and/or IBH, or multiple age group flocks were kept on a farm (MITTAL et al., 2014). Vertical transmission of the virus has also been documented (EL-SHALL et al., 2022). The clinical signs observed in the affected birds were depression, dullness, decreased appetite and yellowish or greenish diarrhea, and the findings of present study are in agreement with previous reports (GRGIC et al., 2006; KUMAR and CHANDRA, 2009). Sudden mortality was reported in all the flocks which corroborated previous studies (MCFERRAN and SMYTH, 2000; KUMAR et al., 2003; CHOI et al., 2012; MARIAPPAN et al., 2018).

In the present study, gross pathological le- sions were most frequently observed in the liver and kidneys in most of the reported outbreaks. Typical pathological lesions were an enlarged and pale liver, and enlarged, hemorrhagic kidneys, which correlated with previous studies (NAKAMU- RA et al., 2011; AHMAD et al., 2016). In the pres- ent study, accumulation of amber or straw-colored fluid in the pericardial sac (hydropericardium) was not a prominent finding, being observed in only one out of the twenty-one outbreaks investigated, which was similar to the findings of RAHIMI and HAGHIGHI (2015). However, gross pathological lesions in the kidneys, spleen and breast muscle, and the accumulation of fluid in the pericardial sac leading to the development of hydropericardium syndrome, were consistent findings reported by several workers (SHAH et al., 2011; KATARIA et al., 2013; KUMAR et al., 2013). Outbreaks associated with hydropericardial syndrome had a substantially greater fatality rate than IBH. FAdV strains

associated with HHS include FAdV-4 (FAdV-C), which is highly pathogenic to chickens. Similarly, IBH is usually associated with FAdV-2-11 (species D), 8a and 8b (species E) (ISHAG et al., 2022). In the present study, many outbreaks were reported in low altitude areas, which may also be the reason for the lower incidence of hydropericardium as the partial pressure of oxygen is lower in high mountain ranges, which might contribute to the increased incidence of hydropericardium.

In the present study, the most characteristic and pathognomonic histopathological lesion observed in all the outbreaks was the presence of basophilic intranuclear inclusion bodies in hepatocytes, which was corroborated by previous studies (KUMAR et al., 2013; KHODAKARAM-TAFTI et al., 2016; MARIAPPAN et al., 2018; ISHAG et al., 2022). These intranuclear inclusions were reported as the hallmark of FAdV infection, since the mere presence of the viral genome detected by molecular tools is of less diagnostic importance due to the ubiquitous nature of the virus in healthy and sick birds (CHITRADEVI, 2025). In the present study, other histopathological lesions were sinusoidal congestion, hepatocyte degeneration, lymphocytic and heterophilic hepatitis, multifocal areas of coagulative necrosis, fatty degeneration characterized by micro- to macrovesicular changes in the hepatocytes, and bile duct hyperplasia. This is in agreement with previous findings (MARIAPPAN et al., 2018; ISHAG et al., 2022).

India has a tropical climate characterized by hot and humid (moisture) conditions, unseasonal rain, flash flooding, improper harvesting of crops during the monsoon season, and insufficient dry- ing and storage facilities, which favors fungal contamination in susceptible feed material, resulting in huge economic losses to the poultry industry as a result of mycotoxicosis. Further, improper management practices on poultry farms increases the risk of mycotoxicosis (KALITA et al., 2021). In the present study, micro- or macrovesicular fatty degenerative changes were correlated with mycotoxicosis. A denuded tubular epithelium and interstitial lympho-plasmacytic nephritis correlated with the previous study by MARIAPPAN et al. (2018), in which mycotoxicosis due to citrinin toxicity resulted in glomerulonephritis, a prominent renal pathology recorded in broiler flocks infected with IBH and complicated by citrinin mycotoxicosis. In the present study, severe pulmonary edema, congestion, hemorrhages and mononuclear cells infiltration in the lungs correlated with previous studies (MARIAPPAN et al., 2018). The liver, especially hepatocytes, is the main site for bio-activation and a major target of toxicity. The kidneys are another important organ affected by aflatoxin. Lesions frequently seen in these organs were degeneration, necrosis and inflammatory reaction. Diagnosis of mycotoxicosis is difficult as it produces clinical signs and lesions similar to other diseases. The strict control of feed quality is necessary to avoid the incidence of mycotoxicosis (KALITA et al., 2021).

The incidence of the disease and its transmission is greater in high density flocks, either due to increased contact or because of stress and overcrowding, and feed contaminated with aflatoxin results in immunosuppression, which contributes to the reactivation and shedding of the latent virus (NIU et al., 2019). Mycotoxicosis also contributes to liver damage and consequent immunosuppression. Enhanced biosecurity and decontamination of poultry houses between successive flocks are important for management of disease. The absence of hepatic tumors observed in this study could be attributed to the acute toxicity in the affected flock, likely resulting from Aflatoxin B1 concentrations far exceeding the recommended levels (BENKEROUM, 2020). Additionally, the high acute mortality caused by adenovirus infection may have prevented sufficient chronic exposure to feed-borne mycotoxins, which is typically necessary for hepatic tumor development.

Polymerase chain reaction (PCR) is an effective and sensitive technology for the diagnosis of FAdV, as opposed to other traditional diagnostic techniques (GANESH et al., 2001). In the present study, molecular diagnosis of the outbreaks was confirmed using primers targeting the hexon gene, the primary surface protein of Aviadenovirus- es which contains antigenic determinants that are type-, group-, and subgroup-specific (HESS, 2000). Studies have reported that certain immunosuppressive conditions, such as chicken infectious anemia,

IBD, and mycotoxicosis, enhance the pathogenicity of IBH (SHIVACHANDRA et al., 2003). MARIAPPAN et al. (2018) demonstrated the immunosuppressive effects and incidence of IBH due to citrinin mycotoxicosis. The aflatoxin B1 levels estimated in the present study were far above the recommended maximum levels in poultry feed according to BIS standards (20 ppb). This underlines the fact that mycotoxicosis and the consequent immunosuppression, as well as aviadenoviral infection, were likely responsible for the mortality in broiler birds.

In the present study, secondary bacterial pathogens such as *Klebsiella* spp., *Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Proteus* spp. and *Pseudomonas* spp., were isolated from various tissue samples collected during necropsy. The organisms isolated in the present study were secondary bacterial pathogens, and the gross and histopathological lesions observed in the present study were not correlated with the pres- ence of the bacteria since these organisms were commensal, either present in the gut or on the skin. Their presence in the tissues might be due to the immunosuppression induced by mycotoxicosis and FAdV infection. Further, in only 3 out of 21 outbreaks did the secondary bacteria proliferate, and the organisms were also demonstrated histopathologically. Hence, it may be concluded that in most of the outbreaks the pathological lesions observed in various organs in the present study were due to fowl adenovirus and mycotoxicosis, reported for the first-time in Kerala State, South India.

Conclusions

Fowl adenoviral infections in the poultry industry are a growing concern due to the economic losses because of mortality, reduced feed conversion efficiency, and retarded growth rates in birds. The present study reports, for the first-time, the concurrent occurrence and association of inclusion body hepatitis and mycotoxicosis in commercial broilers of Kerala State, South India. This study highlights the role of mycotoxins in the immunosuppression and incidence of fowl adenoviral infections in a subset of the examined cases. The incidence of inclusion body hepatitis as early as 6 days of age provides an insight into the need to develop suitable biosecurity and other preventive measures. The difference in pathogenesis, specifically the absence of hydropericardium in the majority of flocks warrants the need for serotyping of the virus, which is essential for studying the epidemiology, prevalence and pathogenicity of the virus to enable the adoption of suitable vaccination and control strategies.

Abbreviations

IBH: Inclusion body hepatitis; FAdV: Fowl adenovirus; PCR: Polymerase chain reaction; HHS: Hydropericardium hepatitis syndrome; EDS: Egg drop syndrome; AGE: Adenoviral gizzard erosion; AAS: Avian adenoviral splenomegaly; NDV: Newcastle disease; IBD: Infectious bursal disease; TSA: Tryptic soy agar; LFA: Lateral flow assay; IB: Infectious bronchitis; AIV: Type A influenza virus; DNA: Deoxyribonucleic acid; USA: United States of America; TBE: Tris-borate-EDTA; H&E: Hematoxylin and eosin.

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Authorship contribution statement

SV contributed to designing and drafting the work, data collection, analytical techniques, and writing of the manuscript. PP contributed to the field and laboratory studies. MM contributed to the investigations and analytical techniques. GSA contributed to the analytical techniques, labora- tory studies and data analysis. MS contributed to revising and correcting the work, data analysis, and editing of the manuscript. All the authors read and approved the final manuscript.

Declaration of competing interest

The authors declared no potential competing interest with respect to the research, authorship, financial, personal relationships, and/or publication of this article.

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Availability of data and materials

All raw datasets generated and analysed during this study were converted and arranged in table format as shown in the "Results" section of this manuscript, but are also available from the corresponding authors upon a reasonable request.

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VINEETHA, S., P. PREVIN, M. MAHESH, G. N. SANTHAMBIKA AJITHKUMAR, M. SAMINATHAN: Kliničko-patološka istraživanja istodobne pojave hepatitisa s inkluzijskim tjelešcima uzrokovanog adenovirusom peradi i mikotoksikoze na komercijalnim farmama brojlera u Kerali, južna Indija. Vet. arhiv 96, 283-297, 2026.

SAŽETAK

Uzgoj brojlera važan je segment svjetskoga gospodarstva, koji je 2020. godine procijenjen na 1,5 bilijuna dolara, a Indija je među vodećim proizvođačima brojlera. Pojava i širenje bolesti i dalje ugrožavaju profitabilnost i održivost industrije u proizvodnji brojlera, utječući na rast i učinkovitost iskorištenja hrane. Ovim je istraživanjem analiziran utjecaj hepatitisa s inkluzijskim tjelešcima (IBH) i mikotoksikoze na komercijalnim farmama brojlera u Kerali, južna Indija. Analizirano je 21 izbijanje bolesti, u razdoblju od svibnja 2023. do studenoga 2024., koja su dovela do uginuća 2946 jediki od ukupno 52 022 oboljele jedinke. Provedena je detaljna obdukcija na 130 ptica, zabilježeni su makroskopski i histopatološki nalazi, a u šest uzoraka hrane fluorimetrijom su određene razine aflatoksina B1. Uzorci koji su, primjenom lančane reakcije polimerazom (PCR), podrvrgnuti amplifikaciji gena za heksone (590 bp) bili su pozitivni na adenovirus peradi (FAdV).Analiza hrane za perad pokazala je razine aflatoksina B1 znatno iznad preporučenih maksimalnih vrijednosti (20 ppb). Akutna smrtnost u jatima brojlera starima između 6 i 37 dana kretala se od 0,44 do 61,9%.Glavni je zahvaćeni organ bila jetra, a zatim bubrezi, slezena i pluća. U svim izbijanjima zaraza zabilježene su patognomonične histopat loške lezije, poput bazofilnih intranuklearnih inkluzijskih tjelešaca u hepatocitima uz prisutnu nekrozu. Ovo istraživanje ističe povezanost imunosupresivnog djelovanja aflatoksina B1 i posljedične IBH infekcije te daje uvid u razvoj odgovarajućih biosigurnosnih strategija i strategija prevencije. Potrebna su daljnja istraživanja radi identifikacije glavnih serotipova FAdV-a i proučavanja razlika u patogenezi IBH-a.

Ključne riječi: hepatitis s inkluzijskim tjelešcima; adenovirus peradi; brojler; molekularna dijagnostika; aflatoksin B1