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RESEARCH ARTICLE

Hepato-nephropathy associated with aflatoxicosis complicated with inclusion body hepatitis in Broilers

H.U. Chandan, V.S. Dhaygude*, C.S. Mote, U.M. Tumlam[1], Surabhi Bhandekar, Prajakta Dethe and G.S. Sonawane[2]

Department of Veterinary Pathology, KNP College of Veterinary Science, Shirwal, Distt. Satara-412 801, MAFSU, Nagpur, [1]Department of Veterinary Microbiology, [2]Instructional Livestock Farm Complex

Address for Correspondence

V.S. Dhaygude, Professor, Department of Veterinary Pathology, KNP College of Veterinary Science, Shirwal, Distt. Satara-412 801, MAFSU, Nagpur, India; E-mail: vithaldhaygude@mafsu.ac.in

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ABSTRACT

Poultry continues to be one of the fastest growing segments of the agricultural sector in India today. However, this sector faces greater degree of economic losses due to mycotoxicosis. The present study investigated the aflatoxicosis complicated with inclusion body hepatitis. A total of 64 birds from 10 different broiler farms were presented for necropsy. The carcasses revealed large areas of cooked or parboiled appearance in the pectoral muscles. The liver showed diffuse swelling with multifocal, small to large, pale whitish necrotic stripes/patches. In many birds, liver appeared yellowish and fragile and in few cases, focal hemorrhages were also observed. Kidneys were diffusely swollen, necrotic and mottled. The cardiac muscles showed multifocal to diffuse whitish areas. Hydropericardium was recorded in carcasses from 2 flocks. The spleen was diffusely swollen, pale and focally congested. The histopathological examination of liver revealed moderate to severe multifocal round vacuoles in cytoplasm, multifocal areas of necrosis leading disruption of hepatic cord arrangement, individualization of hepatocytes and multifocal areas of infiltration predominantly by PMNs with a few MNCs. In 2 out of 10 flocks, the liver also revealed typical intranuclear inclusion bodies in addition to above changes. Kidneys showed mild to moderate degenerative and necrotic changes with tubular swelling, thickening of glomerular basement membranes and increased number of mesangial cells. The cardiac muscles exhibited interstitial infiltration. Spleen showed multifocal areas of lymphoid depletion and associated reticulo endothelial cell hyperplasia. Bursa of fabricius showed moderate to severe lymphoid depletion. DNA was extracted from liver and spleen samples and the conventional PCR targeting hexon gene specific for FAdV yielded expected product of 897 bp in 3 flocks. Toxicological analysis of feed samples confirmed the presence of toxic levels of aflatoxin (AFB1-181, 196 and 241 ppb, AFB2-15, 15 and 20 ppb) from 3 different farms including those 2 farms showing intranuclear inclusions in hepatocytes and PCR positivity for FAdV. The gross and histopathological lesions, toxicological analysis and PCR confirmed the occurrence of aflatoxicosis and IBH together.

Keywords: Aflatoxin, fowl adenovirus, hepato-nephropathology

INTRODUCTION

Poultry farming plays a vital role in the global food industry, with broilers forming a significant portion of meat production due to their rapid growth rate and feed efficiency. However, one of the persistent challenges in broiler production is the contamination of feed with mycotoxins, particularly aflatoxins. Aflatoxins are important mycotoxin which are hepatotoxic, mutagenic and carcinogenic compounds produced mainly by *Aspergillus* spp., which are more prevalent in tropical regions. Among the aflatoxins, aflatoxin B1 is considered as a most potent hepatotoxic agent and induces high morbidity, mortality and production losses. Extended and improper storage of chicken feed leads to increased aflatoxin production. The clinical symptoms of aflatoxicosis include fatigue, loss of appetite, reduced growth rate, microbial stress, economic losses and toxicity[1]. In field conditions, most of the time, high levels of mycotoxins in feed result in immunosuppression that exposes birds to infectious diseases. Fowl adenoviruses are common pathogens secondary to mycotoxicosis. IBH is the most common concomitant disease along with aflatoxin B1 when present in feed at higher than permissible levels, *i.e.* 20 ppb[2].

Fowl adenovirus (FAdV) infection in broilers is a disease that leads to significant economic losses for poultry farmers due to increased mortality and decreased productivity from poor chicken performance. FAdVs cause various diseases, including inclusion body hepatitis (IBH), hydropericardium syndrome (HPS), respiratory infections, gizzard erosions, arthritis and pancreatitis[3]. In addition to its direct impact, the virus acts as an immunosuppressive agent, this can result in secondary complications. Although most FAdVs are considered non-pathogenic due to their widespread presence

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in poultry flocks, certain Group I FAdVs, such as serotypes 4 and 8, are pathogenic and linked to IBH in chickens. While it was previously thought that immunesuppression caused by pre-infection or concurrent infection with Infectious Bursal Disease Virus (IBDV) or Chicken Infectious Anemia virus (CIAV) contributed to IBH, recent studies suggest that these infections or other immunosuppressive factors may not be necessary for the onset of IBH caused by FAdV[4]. This indicates that FAdV is emerging as a primary pathogen capable of causing significant diseases, with or without predisposing factors.

The concurrent impact of aflatoxicosis and IBH in broilers has a synergistic and devastating effect on health and productivity. Field data from India underscore that FAdV particularly serotypes 11 and 8b is rapidly becoming a primary threat to flock health, independent of secondary immunosuppressive triggers. However, this investigation reports concurrent occurrence of inclusion body hepatitis and aflatoxicosis in commercial broiler chickens.

MATERIALS AND METHODS

The current study examined aflatoxicosis complicated by inclusion body hepatitis (IBH) in spontaneous field cases. A total of 64 birds from 10 different broiler farms were brought to the Department of Veterinary Pathology, KNPCVS, Shirwal, for necropsy during September and October, 2024.

Necropsy

Necropsy examinations were performed on the deceased birds and the gross findings were documented. Representative tissue samples were collected and preserved in 10% neutral buffered formalin for histopathological analysis and some stored at -80^{O}C for molecular studies. Feed samples were collected for toxicological analysis.

Histopathology

The tissue samples were fixed in 10% neutral buffered formalin and processed using the routine paraffin-embedding technique. In brief, 4 µm thick sections were deparaffinized and stained using the Hematoxylin and Eosin staining method for detailed microscopic examination.

DNA extraction & polymerase chain reaction (PCR)

The tissue samples stored at -80^{O}C were processed for DNA extraction using the DNeasy Blood and Tissue Kit, according to the manufacturer's instructions. PCR was carried out using primers targeting the hexon gene of FAdV Group I (Forward primer: 5'-CAARTTCAGRCAGACGGT-3'; Reverse primer: 5'-TAGTGATGMCGSGACATCAT-3'). The PCR reaction mixture of 25 µl consisted of 3.0 µL DNA (5 ng/µL), 12.5 µL DreamTaq PCR Master Mix (Thermo Scientific, USA), 1.0 µL forward primer (10 pmol/µL), 1.0 µL reverse primer (10 pmol/µL) and 7.5 µL nuclease free water. The amplification conditions included an initial denaturation at 95^{O}C for 15 minutes, followed by 35 cycles of 94^{O}C for 45 seconds, 57^{O}C for 45 seconds, 72^{O}C for 45 seconds and a final extension at 72^{O}C for 10 minutes.

The PCR products were then analyzed using electrophoresis on a 1.5% agarose gel (in 0.5 Tris-Borate EDTA buffer), stained with ethidium bromide (0.5 µg/mL). After the dye had migrated sufficiently, the gel was visualized under ultraviolet light (302 nm) and documented using Alpha Imager software. The relative size of the amplified product was determined by comparing it with a standard DNA molecular weight marker run along side the PCR products (Thermo Scientific, USA).

Toxicological analysis

Feed samples were collected from different flocks. Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2, Ochratoxin, T-2 toxin, Citrinin, Zearalenone were estimated in the feed samples by the method described by Romer (1975)[5]. The toxins were extracted with acetone, treated with cupric carbonate and ferric gel to eliminate fluorescent materials other than aflatoxin, then washed with acid and alkali and extracted with chloroform, dried, rediluted with chloroform and spotted in an activated thin layer chromatography (TLC) plate with standards and ascertained the concentration by visual comparison method in a UV viewing cabinet[6].

RESULTS

Gross and Histopathological findings

A detailed necropsies showed large areas of cooked or partially cooked appearance in pectoral muscles (Fig. 1). The liver exhibited diffuse swelling with multifocal, small to large, pale, whitish necrotic streaks or patches on the surface. In several birds, the liver appeared yellowish (fatty) and fragile with focal hemorrhages (Fig. 2). The kidneys were consistently swollen, necrotic and mottled (Fig. 3). The cardiac muscles showed areas of whitish discoloration both multifocally and diffusely. Hydropericardium was recorded in carcasses of three flocks. The spleens were enlarged, pale and occasionally congested in focal areas (Fig. 4).

The histopathological examination of the liver revealed moderate to severe multifocal round vacuoles (clear cell areas) in the cytoplasm (Fig. 5), along with multifocal necrotic areas causing disruption of the hepatic cord arrangement, individualization of hepatocytes and multifocal infiltration primarily by polymorphonuclear cells (PMNs). Focal congestion and mild periarteriolar infiltration of MNCs were also observed (Fig. 5). In two out of the ten flocks, the liver showed typical intranuclear

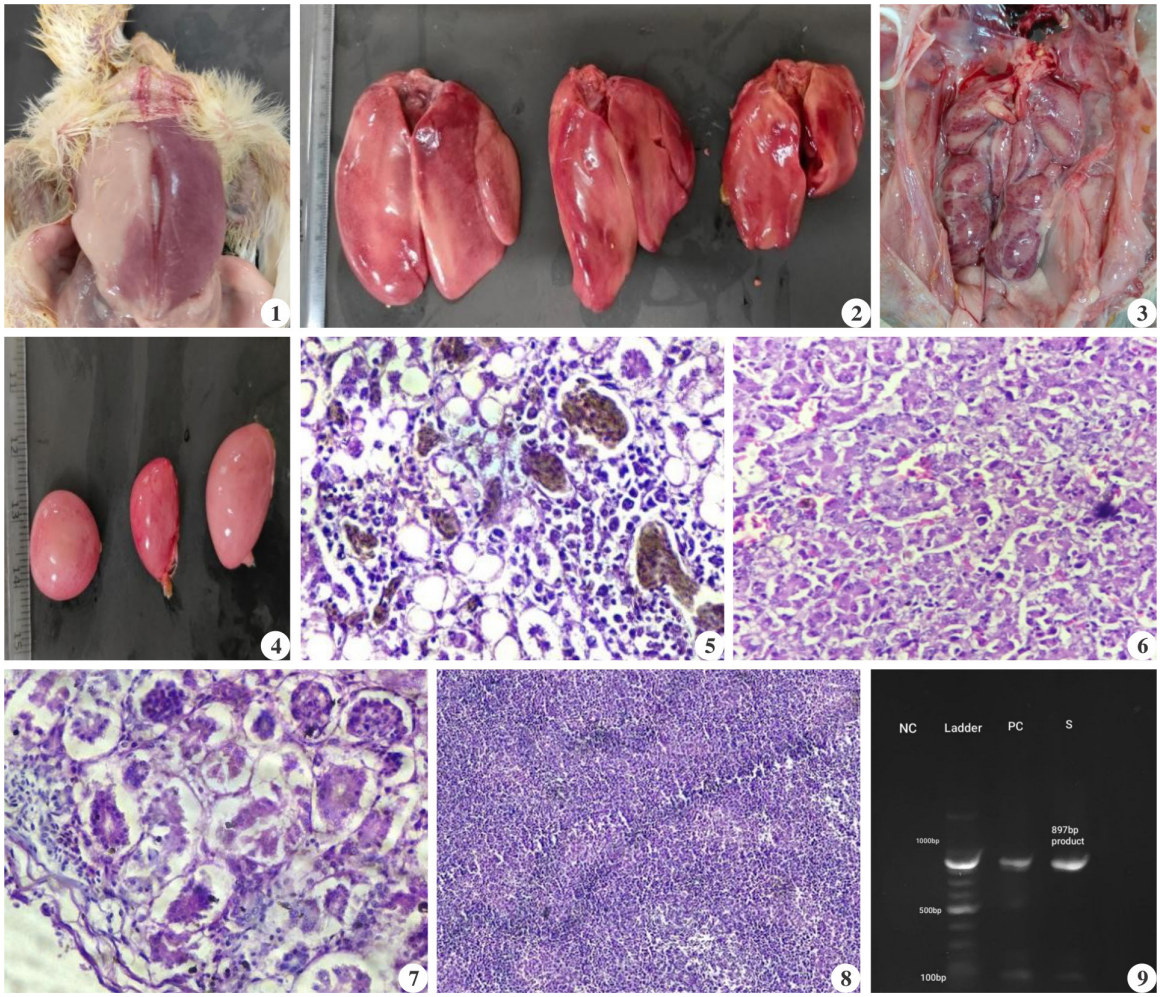

Fig. 1. Cooked or parboiled large area in pectoral muscles; **Fig. 2.** The liver showed diffuse swelling, multifocal small to large pale whitish necrotic stripes/patches; **Fig. 3.** Kidneys were diffusely swollen, congested, necrotic and mottled; **Fig. 4.** Spleen were diffusely swollen, pale and focally congested; **Fig. 5.** Liver: Moderate to severe multifocal round vacuoles (fatty change) in cytoplasm & focal congested areas (H&E x400); **Fig. 6.** Liver: Degenerative & necrobiotic changes in hepatocytes & basophilic intranuclear inclusion bodies (H&E x400). **Fig. 7.** Kidney: Tubular degeneration, necrosis and infiltration (H&E x400); **Fig. 8.** Spleen: Multifocal areas of lymphoid depletion and reticulo endothelial cell hyperplasia (H&E x100); **Fig. 9.** Agarose gel electrophoresis showing PCR amplification of "hexon" gene (897 bp) (1000 bp ladder, NC: Negative control, PC: Positive control, S: Sample).

Flock no.	Aflatoxin B1	Aflatoxin B2	Aflatoxin G1	Aflatoxin G2	Ochratoxin	T-2 toxin	Citrinin	Zearalenone
1	181ppb	15ppb	ND	ND	ND	ND	ND	ND
2	196ppb	15ppb	ND	ND	ND	ND	ND	ND
3	241ppb	20ppb	ND	ND	ND	ND	ND	ND

ND: Not Detected

inclusion bodies in addition to the other changes (Fig. 6). The kidneys exhibited mild to moderate degenerative and necrotic changes, including tubular swelling and increased number of mesangial cells (Fig. 7). Cardiac muscles showed interstitial infiltration. The spleen displayed multifocal lymphoid depletion along with reticulo endothelial cell hyperplasia (Fig. 8).

Genome detection studies

DNA was extracted from liver and spleen samples and conventional PCR targeting the hexon gene specific to FAdV produced the expected 897 bp product in three flocks (Fig. 9).

Toxicological analysis of feed samples

Toxicological analysis confirmed the presence of toxic levels of aflatoxin, with AFB1 levels of 181, 196 and 241 ppb and AFB2 levels of 15, 15 and 20 ppb, in feed samples from three different farms which were also found positive for IBH histopathologically and confirmed by PCR. The

other toxins like Aflatoxin G1, Aflatoxin G2, Ochratoxin, T-2 toxin, Citrinin, Zearalenone were not detected in any of the tested flocks.

DISCUSSION

The simultaneous detection of high aflatoxin levels and histopathological lesions typical of IBH (e.g., intranuclear inclusion bodies) along with PCR confirmation suggests a synergistic role of aflatoxins and FAdV in exacerbating hepatic damage and immunosuppression. The observation of cooked or partially cooked pectoral muscles is unusual in aflatoxicosis + IBH, since this appearance is classically associated with heat stroke or deep pectoral myopathy due to ischemic necrosis. In this case, the presence of aflatoxin induced hepatic dysfunction and IBH associated circulatory disturbance may have predisposed to severe ischemic myopathy, producing the "cooked" appearance. Reduced detoxification capacity of the liver could exacerbate muscle hypoxia, leading to protein denaturation in muscles. The hepatic failure and anemia in IBH could impair oxygen delivery, contributing to muscular ischemia and the cooked like gross change. Macroscopic and microscopic examination of the liver showed hallmark changes of both aflatoxicosis (fatty change, fragility, hemorrhages) and IBH (intranuclear inclusion bodies, necrosis, PMN infiltration). This dual pathology confirms co-morbidity and emphasizes the need for differential diagnosis. Aflatoxin B1 levels in all three affected flocks (181-241 ppb) exceeded acceptable limits, indicating feed contamination and an important predisposing factor. The absence of other mycotoxins supports the conclusion that AFB1 was the primary toxin involved. Aflatoxins are known to suppress the immune system, likely facilitating FAdV replication and worsening the clinical severity of IBH. This highlights the importance of toxin control in preventing viral opportunism. Lesions in kidneys, cardiac muscles and spleen, along with hydropericardium and cooked muscle appearance were pointing to systemic toxicity[7]. These findings align with both aflatoxin poisoning and systemic IBH, making diagnosis more complex. PCR amplification of the hexon gene results revealed the presence of a DNA band measuring 897 base pairs (bp) and same findings were correlated with the previous findings[8] confirmed the presence of FAdV in affected tissues, validating the IBH diagnosis and its co-occurrence with aflatoxicosis. Aflatoxins cause oxidative stress, impair detoxification and filtration processes and suppress immunity, while FAdV-induced IBH exacerbates hepatic injury through viral replication and inclusion body formation. The combined effect leads to marked histopathological changes, immunosuppression and increased mortality, highlighting the need for early diagnosis, mycotoxin control and FAdV monitoring in broiler production. The presence of aflatoxins in feed not only impacts poultry health but also poses a potential public health risk *via* the food chain. Economic losses due to mortality and reduced productivity are significant, particularly in commercial poultry operations. Regular testing of feed for aflatoxins and implementation of mycotoxin binders or detoxifiers is essential in disease prevention, especially in areas with known FAdV prevalence. A comprehensive approach including biosecurity, virological monitoring, feed hygiene and farmer education is required to prevent recurrence of such compounded outbreaks.

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