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Pathological investigations and molecular characterization of infectious bursal disease virus from poultry farms of western Maharashtra of India

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Abstract
Infectious bursal disease is one of the most important immunosuppressive diseases of birds. Present study was conducted to identify the agent of a suggestive clinical case of Gumboro disease that affected 14-29 day-old broiler chicken flocks in Satara and Pune district of western Maharashtra in the year 2016. The affected birds showed enlarged, hemorrhagic, swollen as well as atrophied bursa of fabricious with cheesy exudate, swollen mottled kidney and muscular hemorrhages. Histopathological evaluation revealed hemorrhages and depletion of lymphoid cells in bursal follicles. The bursal tissue samples were collected for reverse transcriptase-polymerase chain reaction to amplify the VP2 gene having 1.3 kb size, using specific primers. Although, the poultry flocks were vaccinated against IBDv, the occurrence of infectious bursal disease is reported between the age group of 14 to 29 in broiler chicken.

Keywords: Gross, histopathology, Infectious bursal disease, VP2 gene

Introduction
Infectious bursal disease (IBD) is a highly contagious viral infection, affecting the immune system of poultry. The virus usually affects young chickens, and is characterized by the destruction of the lymphoid organs, and in particular the bursa of Fabricius, where B lymphocytes mature and differentiate. Infectious Bursal Disease virus (IBDv) is an important immunosuppressive disease affecting birds. It is an acute and highly contagious illness affecting young chickens from 3 to 6 weeks of age [1, 2]. IBDv is a 60 nm non enveloped virus with icosahedral symmetry, belongs to the Birnaviridae family, and has a genome with two segments of linear double-stranded RNA[3-5]. The Segment A of virus has 3.2 kb and contains two partially overlapping open reading frames (ORFs). The largest ORF encodes for a polyprotein that is proteolytically cleaved to form three polypeptides: VP2 and VP3 are the structural proteins, whereas VP4 is a protease. VP2 contains the major antigenic site, responsible for eliciting neutralizing antibodies. Segment B has 2.8kb and encodes for VP1, an RNA-dependent RNA polymerase. Sequencing of the VP2 hypervariable region, which contains important neutralizing epitopes, has been important to characterize IBDV strains. The virus infects and destroys dividing pre-B lymphocytes in the bursa of Fabricius. However, in spite of intense vaccination schedule followed, the disease caused by vvIBDV strains continues to be a major problem in several parts of the world, causing mortality rate ranging from 3.6% to 50%, or even higher. The vvIBDVs are antigenetically very similar to the classical strains but display a marked increase in virulence, causing high mortality in infected flocks[6]. Current vaccination methods against IBDV are based on individual subcutaneous or intramuscular injection of inactivated or attenuated IBDV into birds. However, other administration routes such as oculonasal, oral or spraying are also common. Vaccination failure was incriminated to be responsible for the emergence of antigenic variation [7]. Presence of vvIBDV in Indian poultry flocks has been recorded as early as 1993 [8]. In India, vvIBDV strains have been implicated in several field outbreaks in the recent past, despite routine vaccination from several part of country [9-11]. Since IBDV continues to evolve through genetic mutation, a continual surveillance for viruses is needed apart from vaccination followed [12]. In-spite of primary vaccination against IBDv in poultry flocks, the mortality of birds was

reported in few broiler poultry farms, wherein booster vaccination was not administered timely leading to occurrence of cases of IBD in such poultry farms. The screened literature for prevalence and occurrence of IBDv in the vaccinated broiler birds from tropical country is scant. The presence investigation reports outbreaks of IBDv in the vaccinated broiler birds from western region of Maharashtra state, India.

Materials and Methods

In the present investigation of 110 birds from 6 different poultry flocks which referred for disease diagnosis through post mortem examination to the Department of Veterinary Pathology, KNPVC, Shirwal were studied. Systemic necropsy examination of dead birds was performed and based on gross lesion(s) in various organs like bursa, liver, kidney, and spleen tissues were recorded and collected in 10% neutral buffered formalin and bursal tissues were also collected in ziplock bag for virus isolation and were preserved at -20̊ C till processing. The formalin fixed tissues were subjected for histological processing using alcohol-xylene protocol in automated tissue processor (*Leica*, Germany) and tissue section were cut on automated microtome (*Medite*, Germany). The tissue sections were stained by routine Hematoxylin and Eosin following standard protocol. The microscopic examination of tissues was undertaken with microphotography on binocular microscope (*Olympus*, Japan) at Central Instrumentation facility of KNP College of Veterinary Science, Shirwal.

The tissue samples of bursa were processed for molecular detection of IBDv using RT-PCR. Reverse transcription-polymerase chain reaction (RT-PCR) was used for detection of viral RNA in homogenates of infected bursa. All the amplicons were confirmed for specific size by using gel electrophoresis[13]. In brief, to detect the fragment of the IBD virus bursal tissue from the suspected birds were used, total RNA was extracted from the bursa of Fabricius using TRIZOL reagent (Thermo Fisher Scientific). Extracted RNA was subjected to c-DNA preparation by Reverse Transcription PCR. The c-DNA and specific primers targeting the conserved region in the IBD virus genome were used for the amplification of viral fragment which yielded about 1.3 kb size amplicons in the 1per cent agarose gel after electrophoresis.

The primer sequence as per reported earlier were used in the present research work. The forward 5'-CCGGAGCTCATGACAAACCTGCAAGATCAAACCC-3'and reverse 5'-GACGGTACCCTACCTTATGGCCCTGATTATGT -3' primer sequence selected from relatively conserved region expected to amplify 1.3 KB were used. About 2.5 µl of cDNA were mixed with 1.0 µl (20 pmol) each of primer forward and reverse. The mixture was heated at 98˚C for 4 minis. The PCR mix containing 2.5 µl of 10X PCR buffer (containing 1.5 mM MgCl2), 0.5µl dNTP's, 0.375 µl Taq DNA polymerase and 17.12 µl of RNAase free water were added. The final reaction volume was 25 µl the tubes were placed in a DNA thermal cycler for 35 cycles of amplification with the following conditions. Denaturation at 95 ˚C for 3 minutes, annealing at 55 ˚C for 1 min and extension time of 5 minutes at end of last cycle a final extension time of 5 minutes at 72 ˚C were given. The amplicon were run on 1% 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 and Discussion

In the present study, 6 poultry flocks of broilers showing mortality with suspected for involvement of IBD were included. The mortality pattern in all the 6 poultry flocks was studied and it was observed that mortality ranged between 1.5 to 6.45% (Table 1). The birds from affected flocks showed clinical sings that included diarrhoea, anorexia, ruffled feather appearance, prostrate and reluctant to move with death subsequently. The IBDv has highest age susceptibility of birds between 3 and 6 weeks of age, when the bursa of Fabricius is at its maximum development. However, a number of other factors like depending the highly virulent strains of IBDv (vvIBDV), the susceptibility age could be broader in the case of vvIBDV strains [14, 15]. Numerous scientific reports are available regarding the clinical signs, pathogenesis, gross and microscopic lesions observed in birds by affected by various strains of IBDv [14-17].

In the present investigation of mortality of broiler birds from 06 different flocks from western Maharashtra, during the systemic postmortem examinations of 110 birds revealed variable pathological lesions in different organs like liver, kidney, spleen, bursa, heart and intestine. However, the bursal lesions were most prominent in the all affected flocks. Birds from three flocks had enlarged (Fig.1), hemorrhagic bursa having blood mixed with cheesy exudate in the lumen (Fig.2). Whereas, few of the birds from different flocks had smaller and atrophic changes of bursa with necrobiotic and hemorrhagic changes.

Along with swollen and hemorrhagic bursa, the other prominent gross lesions from dead birds included swelling of kidneys with degenerative foci of all the lobes with pale discoloration on surface. Characteristic whitish creamy discolorations pattern of renal tissue i.e. mottled appearance (Fig.3) and enlarged ureters due to nephritic changes and accumulation of uric acid was noted in few of the birds. The spleen revealed turgid appearance with degenerative and pale necrotic foci on surface. The liver showed pale and degenerative foci in few of the birds and hepatic lesions were not uniform. These prominent post mortem lesions of bursa and kidney with or without haemorrhages are reported to suspect such cases for IBDv infection in broilers between 2 to 5 weeks of age. [3, 16, 18]. The muscular pathological changes were not uniform in all the flocks studied. Very few of the birds showed presence of pin point hemorrhagic foci on thigh muscles with change in overall texture and colour of muscle fibers.

The detailed histomorphological investigations of affected organs i.e. bursa, kidney, liver, and spleen was carried out for cellular pathological changes. The microscopic pathological alterations in tissue sections of Bursa of Fabricius showed degenerative and necrotic changes of follicles with variable loss of lymphoid tissue (Fig.4). Derangement of bursal follicles with atrophic changes was also noted at several foci. Interstitial edematous lesions with dystrophic and cystic changes of bursal follicles were noted in few tissue sections. Inflammatory mononuclear infiltration was also evident in the interstitial space between the septa of follicles. Congested vasculature and hyperplastic changes of epithelial tissue were also prominent in few bursal tissue. Few sections showed

interstitial hemorrhages in the bursal folds (Fig.5) with presence of eosinophilic edematous exudate. The cellular component of Bursal Follicles was markedly depleted with presence of fragmented cellular debris. The lymphocytolysis was observed in many bursal follicles with pyknotic changes of nucleus. Accumulation of degenerated cellular debris was noted in moderately damaged bursal tissue suggestive of marked destruction of lymphocytes. Apoptotic features with fragmented nucleus were also noted in lymphoid cell population in few bursal follicles. The marked depletion of bursal lymphoid cell is said to occur due to active replication of RNA virus (IBDV) in B-Lymphocytes leading to necrosis and inflammatory changes in bursal follicles [19-25].

Kidney tissues showed marked necrotic and degenerative as well as autolytic features of renal parenchyma. The renal tubules appeared ruptured with swelling of tubular epithelium, necrosis and presence of extensive hemorrhage along with glomerulonephritis (Fig.6). Many of the sections showed interstitial edematous accumulation with focal infiltration of mononuclear cells between the tubules. Mild to moderate tubular degeneration and necrosis indicated by loss of nucleus and soughing of tubular epithelium was noted diffusely in several sections of kidney. Glomerular swelling as well as atrophy was also noted with accumulation of eosinophilic content in glomerular space. The renal parenchyma with collecting tubules towards ureters showed prominent degenerative and dystrophic changes with accumulation of thick cellular debris and clogged urinary content suggestive of uric acid deposits. Many researchers have reported the gross and cellular renal pathological changes in infection with IBDv in birds depicting presence of cellular debris with granular deposits suggestive of accumulation of toxic metabolites in the kidney parenchyma [19-25].

The microscopic examination of liver section showed degenerative changes with cellular swelling and congestion of hepatic parenchyma. Marked degeneration of hepatocytes, karyomegaly, vacuolar changes in cytoplasm and disruption of hepatic cords was noted in few birds. In liver sections from the various flocks showed minimal to mild grade pathological changes, while few sections showed normal hepatic parenchyma. The hepatic parenchyma in few sections showed congested blood vessels with cellular swelling of hepatocytes, focal hemorrhages and degenerative changes (Fig.7) with enlarged nucleus and granular cytoplasm. Vacuolar changes were also noted in many hepatic tissue sections of focal to diffuse nature [19-25]. The hepatic pathology can be induced by the infection due to infectious bursal disease virus (IBDV) as well as mycotoxins [26-28].

These prominent post mortem lesions of bursa and kidney with or without haemorrhages, congested vasculature and hepatomegaly with degenerative changes and congested liver are reported to suspect such cases for IBDv infection in broilers between 2 to 5 weeks of age in the present study of the 6 flocks. The virus replicates primarily in the lymphocytes and macrophages of the gut-associated tissues. Then virus travels to the bursa via the blood stream, where replication occurs. Within 12 hours of post-inoculation, most follicles are positive for virus and by 16 hrs post infection, a second and pronounced viraemia occurs with secondary replication in other organs like liver, spleen, and kidney leading to aggression of disease and death [4].

In the present study 110 bursa of Fabricius from birds showing clinical signs, gross lesions and microscopic lesions of IBDv were used for detection of the viral agent. IBD virus was confirmed in bursal tissues by reverse transcriptase PCR assays with the use of specific primer (Fig.8). The amplicon of 1.3 kb size on 1% agarose gel was reported specific for IBDv as described earlier by scientists. All the bursal tissue homogenate samples showing hemorrhagic lesions were found positive for presence of IBDv on RT-PCR assay.

Table 1: Details of Poultry Flocks examined

Flock Number	Flock Size	Age of birds (days) during study period	Number of birds died (Mortality)	Major gross pathological lesions	% Mortality	Vaccination status	
						IBD	RD
1	500	29 days	12	Swollen Bursa	2.40	+	+
2	480	42 days	20	Swollen Bursa	4.17	+	+
3	250	14 days	5	Swollen and cheesy exudate in Bursa	2.00	+	+
4	600	15 days	7	Hemorrhages and cheesy exudate in Bursa	1.17	+	+
5	400	14 days	6	Swollen and cheesy exudate in Bursa	1.50	+	+
6	930	15 days	60	Hemorrhages and cheesy exudate in Bursa	6.45	+	+

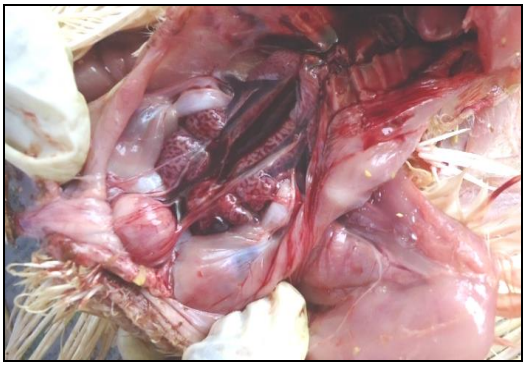

Fig 1: Swollen and hyperemic bursa with mottled look of kidneys with enlargement

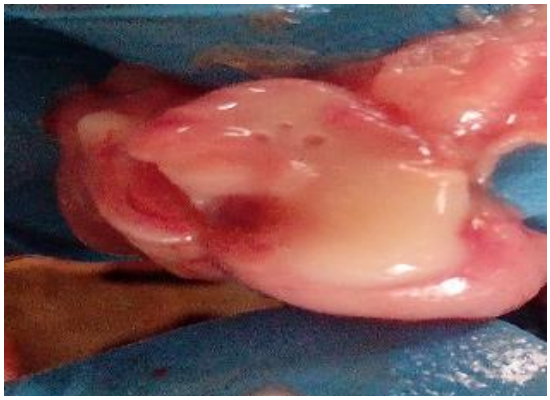

Fig 2: Bursa showing cheesy exudate

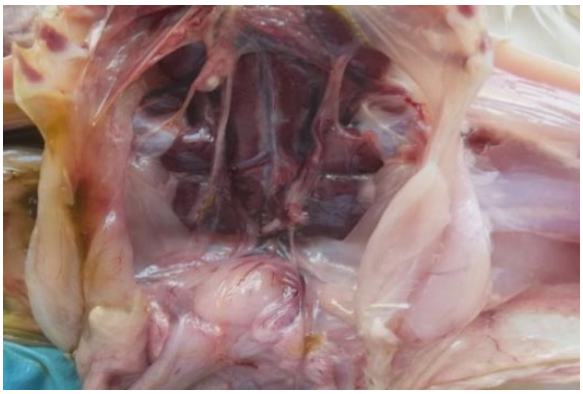

Fig 3: Swollen and hemorrhagic kidneys and enlarged bursa

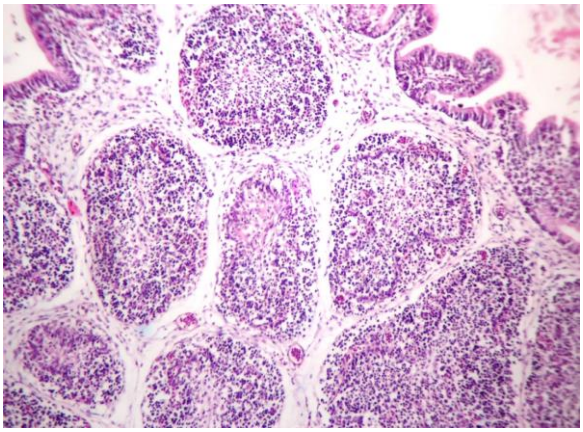

Fig 4: Bursal tissue showing degenerative changes (H&E, x100)

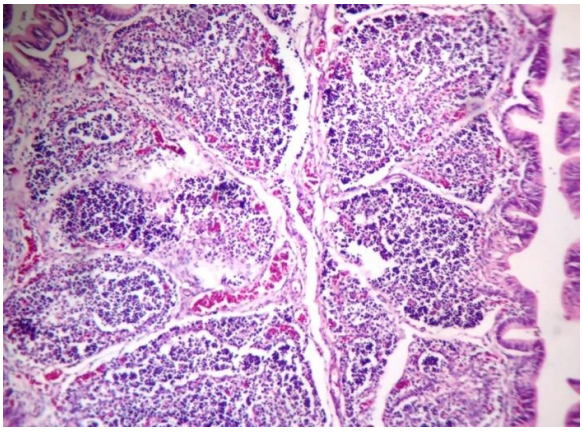

Fig 5: Bursal tissue showing hemorrhagic features and lymphoid depletion (H&E, x100)

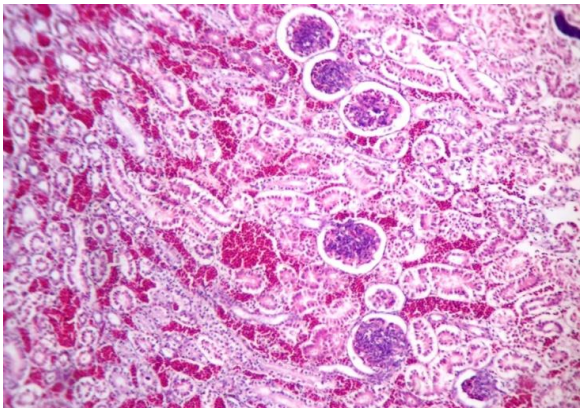

Fig 6: Kidney tissue showing swollen tubules and glomeruli with degenerative and hemorrhagic features (H&E, x100)

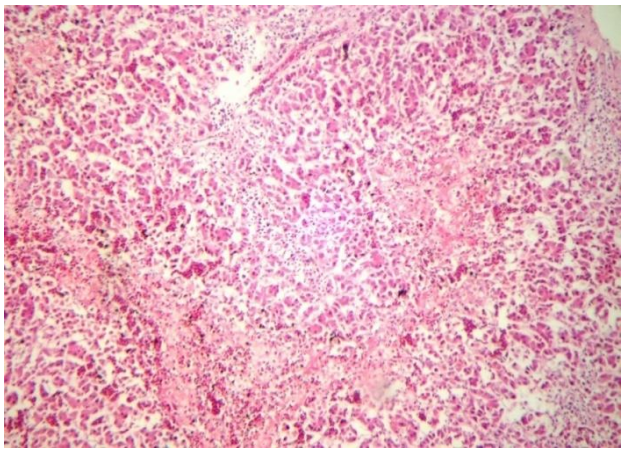

Fig 7: Liver tissue showing degenerative hemorrhagic and necrotic features (H&E, x100)

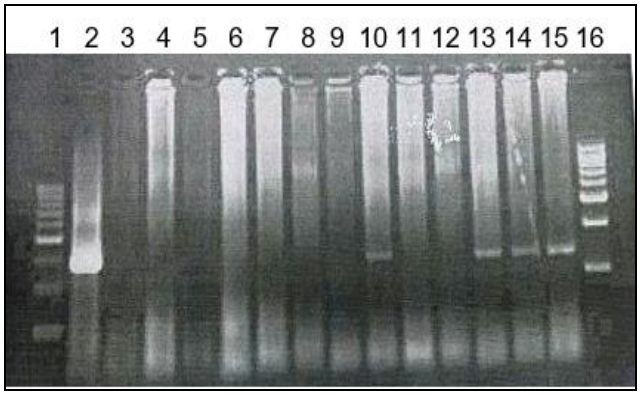

Fig 8: Molecular detection of IBDv depicting 1300bp size amplicons of IBDv. (from Left to right, Lane 1 & 16- DNA ladder Lane 2- Positive control Lane 3- Negative control Lane 10, 13, 14 & 15- Positive field samples for IBDV

Conclusion

The gross and pathological lesions of bursa and kidney with or without haemorrhages, congested vasculature and degenerative changes were reported from various poultry flocks from western Maharashtra. The occurrence of cases for IBDv infection in broilers between age group of 2 to 5 weeks were confirmed by post mortem lesions and molecular characterization of IBD virus using RT-PCR assay in the present study of the 6 flocks.

Our study confirms the involvement of IBDv in the mortality of various farms even after the vaccination against classical IBD. The reason behind such outbreaks recorded could be vaccine strain employed might have not given sufficient protection against existing IBD virus in these farms. Infectious bursal disease virus presents pathological characteristics that are of importance in the diagnosis and control of IBD. The avoidance of booster dose of IBD vaccine is a prominent factor. Infectious bursal disease virus has a high mutation rate and may thus give rise to viruses of modified antigenicity or increased virulence. Although a satisfactory protection could be provided by the induction of high neutralizing antibody titers, interference of parental antibodies in vaccination and avoidance of booster vaccination dose has become the an important obstacle in the establishment of control programmes of IBD specially in broiler chicken.

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