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Detection of infectious bursal disease virus in tissue samples by immunoperoxidase staining technique

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Abstract
Chicken is the only host that manifests clinical signs and its pathogenicity is correlated with the distribution of antigens in organs. IBDV caused lymphoid depletion which can be detected by Immunohistochemistry. In this study, Immunohistochemistry was used to detect the viral antigen in frozen sections. A hyper-immune serum produced in rabbits through injection of IBD vaccine and used as a primary antibody in present study. Positive IBDV antigen staining of lymphoid cell population in bursa of Fabricius, Spleen Caecal tonsils, Thymus and Muscles was observed by using indirect Immunohistochemtry. The total number of sample in this study was 183 and out of which 165 showed positive characteristic golden brownish colour.

Keywords: immunohistochemistry, IBD, hyper-immune serum and IBD vaccine

Introduction
Infectious bursal disease is an acute, highly contagious and immunosuppressive avian lymphotropic viral disease of poultry primarily affecting the chicks of 3-6 weeks of age [1, 2]. However it may affect day old chick, where it produced subclinical infection resulting immunosuppression [3]. The disease is caused by IBD virus belongs to genus *Avibirnavirus* under family *Birnaviridae* [4] and characterized by vent picking, whitish or watery diarrhoea with soiled vent feathers, anorexia, depression, ruffled feathers, trembling, severe prostration, and finally death. Mortality commences on the third day of infection, reaches a peak by day four thereafter mortality drops rapidly, and the surviving chickens recover after five to seven days [5-7].
Macroscopically, bursa of Fabricius is the target organ of the disease where the organ becomes swollen, oedematous with yellowish gelatinous exudates on the surface in the initial stage. Gradually the swollen bursas are hemorrhagic with a black berry appearance [6, 7]. There are haemorrhages in the extra bursal lymphoid organs like spleen, thymus and caecal tonsils. The breast and thigh muscle also become hemorrhagic [6, 7].
Microscopically, there are intra and inter follicular haemorrhage, oedema, depletion of lymphocytes and heterophilic infiltration in follicles of bursa of Fabricius, spleen, thymus and caecal tonsils. Complete depletion of the lymphoid cells formed cystic cavity. Infiltration of fibrous connective tissue in the inter-follicular and intra-follicular areas [8, 6, 9].
Routine diagnosis of IBD has been conducted by using several techniques using specific hyper-immune serum. It is also important for studying the distribution of antigen tropism. Therefore, present study was aimed to understand the distribution of IBDV antigen in lymphoid and non lymphoid organs.

Materials and Methods
Field samples
One hundred and eighty three (183) numbers of samples were collected from 29 different outbreaks of different farms from in and around Guwahati, Kamrup during this study.

Raising of Hyper immune serum against IBD in Rabbit
A total of five adult dewormed New Zealand white rabbits were used. Each rabbit was injected with 0.5 ml IBDV vaccine (Georgia strain) intramuscularly in multiple sites. After the priming

immunization, two further booster immunizations were administered at 2-weeks interval by the same route of injection. The rabbits were bled 14 days after the last immunization and the blood samples were collected for sera preparation. The serum was separated and stored at -20 °C.

Tissue section preparation

In the present study, the IBD suspected tissue samples were from bursa of Fabricius, spleen, thymus, caecal tonsils and muscles collected during the post-mortem examination and preserved in -20°C to demonstrate the viral antigen. Briefly, the tissue samples were cut in to a small piece of approximately 1 X 1 X 0.5cm and were mounted with a cryoembedding compound on a cryostat mounting block. The piece of tissue was freezed onto the cryostat chuck. Sections of maximum 5µm thickness were cut and mounted onto micro slides, which have been previously cleaned with alcohol. Several slides with sections from the same tissues were prepared. The mounted sections were dried at room temperature for 20 minutes and fixed in acetone (analytic grade) at -20 ºC for 10 minutes.

Staining procedure of Indirect IPT

Frozen tissue sections are stained for IBD viral antigen detection following the method of [10]. The steps are as follows.

i. The fixed tissue section was treated with 0.5% hydrogen peroxide for 10 minutes in methanol to block the endogenous peroxidase.
ii. Sections were washed twice with PBS-T for 5 minutes each and were treated with 0.4% triton X-100 (in PBS) for 15 minutes.
iii. Then the tissue section was treated with 2% BSA for 30 minutes in PBS-T as buffer for blocking of non-specific sites.
iv. Thereafter, add 50µl IBD hyper immune serum diluted @ 1:100 in 1% BSA to the sections. The sections were then incubated for 1 hour at 37 ºC in water bath.
v. After that, the sections were washed twice with PBS-T for 5 minutes each.
vi. Then, add 50µl rabbit anti chicken horse radish peroxidase (HRPO) conjugate to all the sections and incubated at 37ºC for 1 hour in water bath.
vii. Washing the tissue section with PBS-T 3 times.
viii. The sections were treated with 50µl of Chromogen substrate solution (DAB) and stained for 15-30 minutes at room temperature.
ix. After discarding the substrate the sections were washed with 1/3 PBS: H2O and observed under the microscope.

Results and Discussion

In the present study, IBD viral antigen was demonstrated by indirect Immunoperoxidase staining technique in the samples of bursa of Fabricius, Spleen and Thymus collected during post mortem examination. A total of one hundred and eighty three (183) numbers of samples from different organs were used for the study. Out of which, 165 no's showed presence of viral antigen in the affected tissues samples.

Viral antigens were identified inside the cells of the rarefied areas of bursal follicles, inner lining of the cystic cavities in affected follicles (Fig. 1, 2) as well as in the fibrotic follicle of the affected bursal sections (Fig. 2). The positive staining in the cytoplasm of infected lymphocyte and macrophages in the lymphoid follicles was characterized by golden brownish, fine or coarse granules (Fig. 3). The positive staining indicating the presence of bound and stained IBDV antigen (Fig.4). Present finding coinciding with the findings recorded by several workers [11-17]. Positive staining in the lymphocytes and macrophages indicate the primary target cells of infection of the virus in these cells [18, 12, 14]

Variations in the intensity of staining were observed in different bursal smears. Variation in the intensity of staining depends on the amount of antigen localized in that particular sample [12, 13, 15]. The antigen-positive cells were found to be more frequent in the medullary region then the cortex of the bursal follicle. Present findings coincide with the findings of other previous workers [12, 14].

In addition, antigens positivity were also recorded in inter follicular connective tissue as well as in the bursal epithelium (Fig. 3 & 4). Present findings are in agreement with the findings of other earlier workers [13, 14, 16, 8].

Development of colour at the site of antigen antibody reaction is due to the oxidation of horse redish peroxidase by Chromogen (Diamino benzidine) in presence of hydrogen peroxide (https://www.bosterbio.com/protocol-and-troubleshooting/immunohistochemistryihcprinciple# reagents). Similar description was also made by [19]. Blocking of endogenous peroxidase activity by 0.5per cent (%) hydrogen peroxide in methanol reduced the background colouration. BSA in diluting solution blocks the non reactive site and triton X100 helps in the disruption of cell for virus interaction with enzyme labelled antibody. Quenching the endogenous peroxidase activity by with methanol and hydrogen peroxide suggested by [20] appeared to be effective for better retention of virus antigenicity in the tissue. No background staining was evident in this study which may be due to the localization of the IBD virus in the lymphocytes. Similar observation was also recorded by [19].

In the present study, short fixation time was given because, very short fixation time prevent the reduction of viral antigenicity of the cell. Similar observation was also described by [21].

The immunoperoxidase stained particles, which represent areas of viral replication, can be co-related with the microscopic lesions of bursa of Fabricius, suggesting that the IBDVs were the cause for bringing such changes. More severe the lesions, stronger was the stain intensity and greater the number of positively stained infected cells were noticed in the section of that particular sample. These observations were in agreement with the findings recorded by [22] using monoclonal antibody in the formalin fixed paraffin embedded tissue samples.

IBD virus antigens have also been observed in lymphoid cells of spleen and cortical lymphocytes of thymus, caecal tonsils and muscle. Similar observation was also recorded by [16, 14, 15, 23].

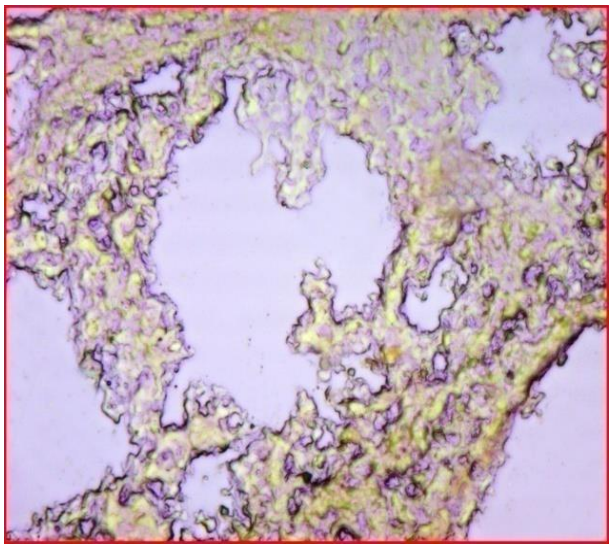

Fig 1: Photomicrograph of a section of bursa of Fabricius showing IBDV antigen positive in inner lining epithelium of cystic cavity of follicle, (Indirect IPT X100).

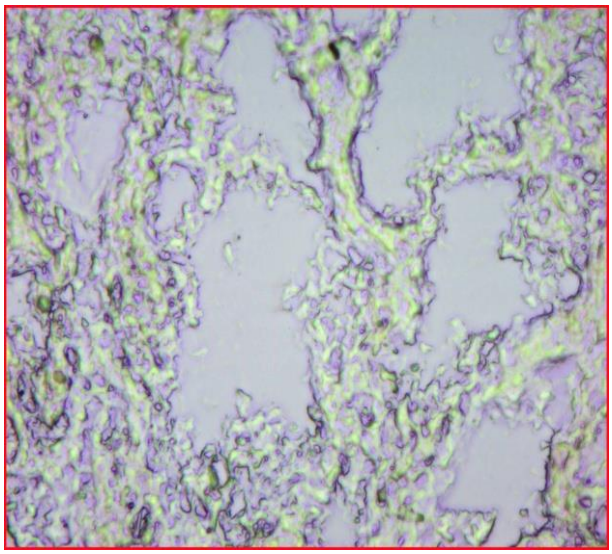

Fig 2: Photomicrograph of a section of bursa of Fabricius showing IBDV antigen positive in inner lining epithelium of rarefied follicle, X400.

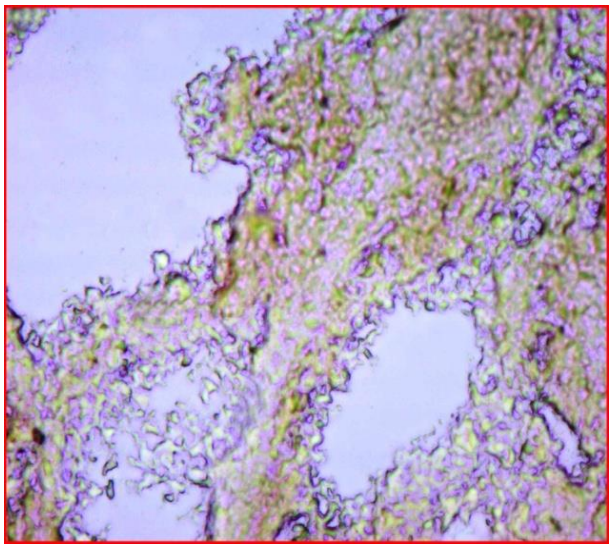

Fig 3: Photomicrograph of a section of bursa of Fabricius showing inter-follicular connective tissue as well as the bursal epithelium showing the presence of IBDV antigen (Indirect IPT X100).

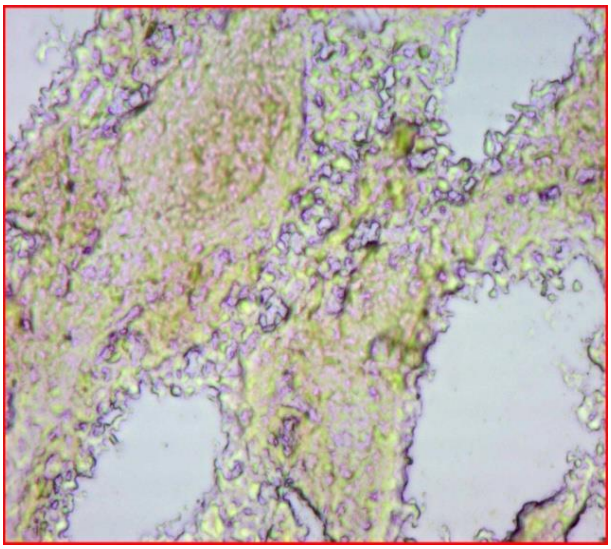

Fig 4: Photomicrograph of a section of bursa of Fabricius showing intra-follicular connective tissue as well as the bursal epithelium showing the presence of IBDV antigen (indirect IPT X100).

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