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Clinico-Pathological and Serological Characterization of Two Indian Field Isolates of Infectious Bursal Disease Virus

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Abstract: The clinicopathological and serological characterization of two field isolates of infectious bursal disease virus (IBDV) recovered from vaccinated flocks that had been vaccinated against infectious bursal disease in Hoshiarpur (Punjab) and Hisar (Haryana) has been undertaken. Broiler chicks (26 days old) were inoculated with 10^5 p.f.u of Hoshiarpur isolate (group I) and 10^4 p.f.u. of Hisar isolate (group II) by oral plus intraocular routes. Although both field viruses produced lesions characteristic of IBDV, some differences in the nature and extent of damage were observed. Gross changes included: haemorrhages in the skeletal muscles, atrophy of bursa, haemorrhages in the intestine. The major histopathological lesions observed were: depletion of lymphocytes in both cortical and medullary regions of bursa, depletion of lymphocytes and reticular hyperplasia in spleen, coagulative necrosis in kidney, depletion of plasma cells in Harderians gland, and haemorrhagic foci in thymus. Both the isolates induced high levels of neutralizing antibodies in the sera of IBDV inoculated birds at 21 d.p.i. ($\log_{10}$ 4.10 and $\log_{10}$ 4.25 in group I and II respectively).

Key words: Infectious bursal disease virus, neutralizing antibodies

Introduction

Infectious bursal disease (IBD) is an acute, contagious disease caused by infectious bursal disease virus (IBDV), an *avibirnavirus*. Young chicks in the age group of 3-6 weeks are more susceptible although the virus can infect older birds. The immature B cells in the bursa of Fabricius are the main targets of the virus resulting in immunosupression and vaccination failure against other important pathogens such as Newcastle disease virus, chicken anemia virus, Marek's disease virus, coccidia etc. Several vaccines are now available to control IBD but outbreaks have been reported in vaccinated flocks. This may be attributed to: different pathotypes (Sharma *et al.*, 1989; Alamsyah *et al.*, 1993; Rosales *et al.*, 1989) and antigenic variants (Wood *et al.*, 1984; Snyder *et al.*, 1988; van der Marel *et al.*, 1990). The present study aims at characterization of two field isolates recovered from flocks that had been vaccinated from different geographical locations in northern India based on pathological changes induced by them and the neutralization antibody responses generated in experimentally infected broiler chicks.

Materials and Methods

Chicks: One week old chicks of Comb breed from unvaccinated parent flock were procured from Poultry farm of CCS Haryana Agricultural University, Hisar and reared in cages. Feed and water were supplied to them *ad lib* till 26 days of age.

Virus isolates: The Hoshiarpur isolate was generously provided by Prof. M.S. Oberoi (Department of Veterinary Microbiology, Punjab Agricultural University, Ludhiana). The virus was revived in CEF cell culture. The Hisar isolate designated as Hisar 97'IBDV was recovered in CEF cell culture by us in our laboratory and was serially passaged three times. The viruses were stored at -20°C until their use.

Experimental design: The chicks were divided into three groups which were housed in separate rooms on the day of inoculation and afterwards. Random serum samples from birds before inoculation were obtained and examined for the presence of IBD-specific antibodies. In addition, two birds were sacrificed from the control group on day 0 in order to look for the presence of IBD-specific gross lesions. Groups I and II comprised of 21 chicks each which were inoculated with $1x10^5$ p.f.u/bird of Hoshiarpur isolate and $1x10^4$ p.f.u/bird of Hisar'97 isolates of IBDV. The route of inoculation was oral plus ocular. Group III comprised of 16 uninoculated control birds. Sampling was done on day 0 and subsequently on 1, 3, 5, 7, 10, 15 and 21 d.p.i. Birds were weighed on the day of sampling and blood was drawn directly from heart. Three birds from each group and two from the control group were sacrificed by cervical dislocation on the specified days. Carcass was observed for gross lesions. Tissue specimens (bursa, spleen, kidney, liver, thymus and Harderian gland) were collected and fixed in 10% neutred buffered formalin for histopathological examination.

Histopathology: Formalin-fixed, paraffin-embedded tissue sections from different organs were stained with

Table 1: Gross lesions following IBDV infection with field isolates

Days post infection		1	2	3	5	7	10	15	16
Hemorrhage in skeletal muscles	GI	+	+	+	+	+	+	-	-
	GII	+	+	+	+	+	+	+	+
Bursal changes	GI	+	-	-	-	-	-	-	-
	GII	-	-	-	-	-	-	-	-
Enlargement	GI	-	+	+	+	+	+	+	+
	GII	+	+	+	+	+	+	+	+
Atrophy	GI	-	-	-	-	-	-	-	-
	GII	+	+	+	+	-	-	-	-
Gelatinous film on serosa	GI	+	+	-	-	-	-	-	-
	GII	+	+	+	+	+	+	-	-
Creamy exudate in lumen	GI	-	-	-	-	-	-	-	-
	GII	-	-	-	-	-	-	-	-
Hemorrhhage	GI	-	+	+	+	+	+	+	-
	GII	+	+	+	+	+	+	+	-
Enlargement of spleen	GI	-	-	-	-	-	-	-	-
	GII	-	-	-	-	-	-	-	-
Presence of urates/Enlarge ment of kidney	GI	+	+	+	+	+	+	+	-
	GII	+	+	+	+	+	+	+	+
Hemorrh ages in intestine	GI	+	+	+	+	+	+	+	-
	GII	+	+	+	+	+	+	+	+

GI denotes Group I chickens inoculated with Hoshiarpur isolate of IBDV. GII denotes Group II chickens inoculated with IBDV- Hisar '97. + and – indicate presence or absence of lesions.

haematoxylin and eosin by the method of Luna (1968).

Serum neutralization test: For detecting neutralization antibody levels, the serum sample were analyzed by Constant virus and varying serum technique after heat-inactivating them at 56ºC for 30min. Serial two fold dilution of each sample were made in tissue culture medium, M-199 containing 20 mM of HEPES buffer. In a 96 well tissue culture plate, a volume of 50µl of each dilution was added to the wells in triplicate followed by addition of 100 $TCID_{50}$ of the virus in 50 µl culture medium. The serum virus mixture was incubated for one h at 37°C followed by addition of 100µl of cell suspension (CEF in a concentration of 5 x 10^5 cells). The plates were then incubated in a humidified environment for 96 h at 37°C and observed daily for CPE. After 96 h, the plates were stained with 0.5% crystal violet for 10 min., washed, air-dried and examined for CPE. The SN_{50} titer was determined as per method of Reed and Muench (1938).

Results

Clinical signs: The infected birds in both the groups were dull but signs of depression were not apparent. Most of the birds had elevated body temperatures till 5 d.p.i. as compared to the control birds. Greenish watery diarrhoea was observed during acute phase in most cases. Increased water and decreased feed intakes were observed in the infected groups. The uninoculated control birds were apparently healthy without clinical signs of IBD.

Gross pathology: The details of gross pathological lesions in various organs are presented in Table 1. The lesions included: haemorrhages in the thigh muscles regularly till 10 d.p.i. and 21 d.p.i. in groups I and II, respectively. The haemorrhages observed at 1 d.p.i. in both the groups are presented in Fig. I a,b. The bursa was slightly enlarged only at day 1 in Group I. Progressive bursal atrophy was observed during the course of infection in both the groups. Additionally, group II birds exhibited the presence of yellowish gelatinous fluid on serosal surface till 7 d.p.i. (Fig. 2). Bursa occasionally contained a creamy exudate (Table 1).
Splenomegaly was observed in birds of group I from 3 to 15 d.p.i. and in group II, regularly till 15 d.p.i. (Table 1). Haemorrhages in the small intestine were observed in both the groups (Fig. 3) and not in the control group throughout the period of observation.

Histopathology: The following histopathological lesions were observed in sections obtained from virus inoculated birds and were not demonstrable in sections from control birds.

Bursa of fabricius: Gradual depletion of lymphocytes was observed in both cortical and medullary regions of bursa. Inflammatory reaction was characterized by the presence of heterophils at 1 d.p.i. and reticulo-endothelial cells at 3 d.p.i. in the interfollicular space. The medulla of bursa exhibited vacuolation and formation of cysts (Fig. 4a). The normal architecture of

Table 2: SN_{50} titres (log_{10}) on different days post inoculation of IBDV (Means ±SD)

Days post inoculation	Group I	Group II
5	1.72±0.02	1.90±0.07
7	2.48±0.24	2.25±0.07
10	3.38±0.90	3.55±0.03
15	3.37±0.14	3.85±0.01
21	4.10±0.07	4.25±0.06

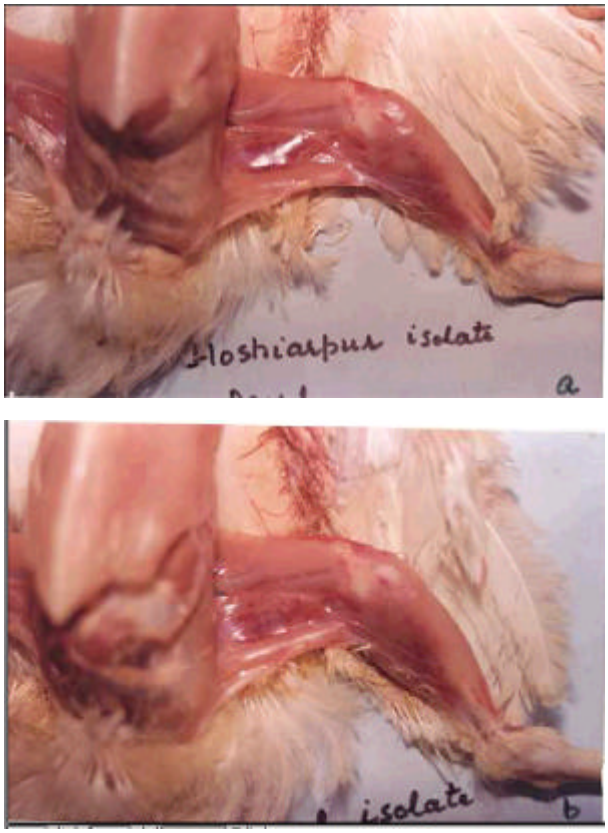

Fig. 1: Haemorrhages in thigh muscles at 1 d.p.i with (a) Hoshiarpur isolate and (b) Hisar isolate

bursa from control bird is shown in Fig. 4b for comparison.

Spleen: Presence of heterophils in the white pulp of spleen was observed at 3 d.p.i. and 1 d.p.i., respectively in groups I and II. Appreciable depletion of lymphocytes was observed from 3 d.p.i. onwards in both the groups. Reticular cell hyperplasia was observed by 5 and 3 d.p.i. in groups I and II, respectively. The architecture of spleen was normal by 21d.p.i.

Liver: Necrotic foci marked by the infiltration of heterophils were observed on 7 d.p.i. in group I and 5 to 10 d.p.i. in group II in the liver parenchyma.

Kidney: Coagulative necrosis was observed in kidney by

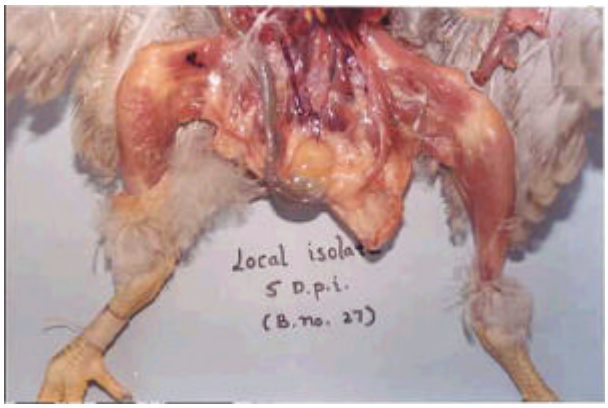

Fig. 2: Yellowish gelatinous fluid on serosal surface of bursa at 5 d.p.i with Hisar isolate

Fig. 3: Diffused haemorrhages on the mucosal surface of small intestine of bird inoculated with Hoshiarpur isolate at 5 d.p.i

3 d.p.i. onwards till 10 d.p.i. in both the groups. Focal areas of lymphocytic infiltration were also observed in both the groups.

Harderian gland: Mild depletion of plasma cells was observed in this gland at 5 to 15 d.p.i. (Fig. 5a presents changes at 10 d.p.i. in group I birds and Fig. 5b

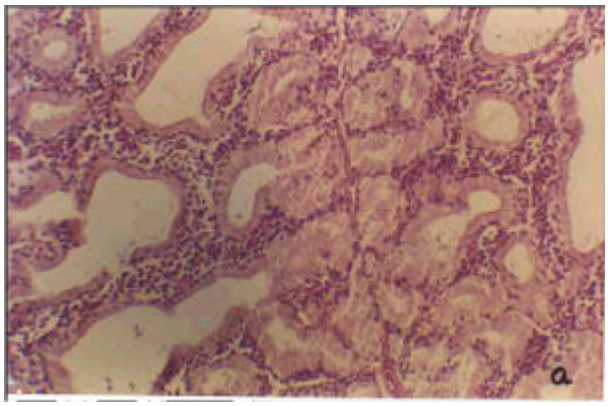

Fig. 4a: Vacuolation and presence of acidophilic mass in bursa in birds inoculated with Hisar isolate at 10 d.p.i (33X)

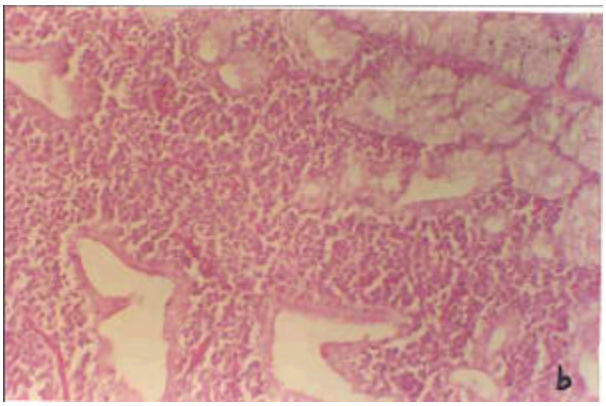

Fig. 4 b: Bursal section of control bird (33X)

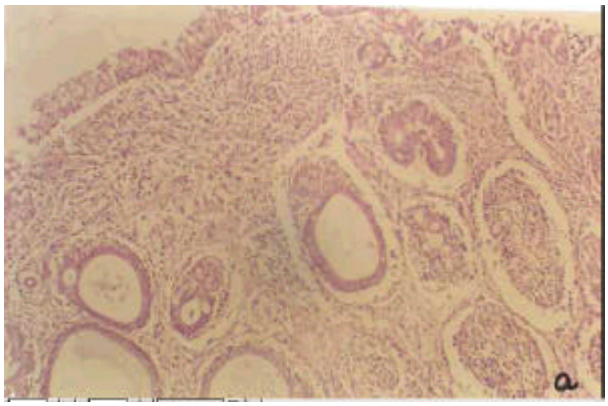

Fig. 5 a: Depletion of lymphocytes in follicles of Harderian gland in bird inoculated with Hoshiarpur isolate at 10 d.p.i (66X)

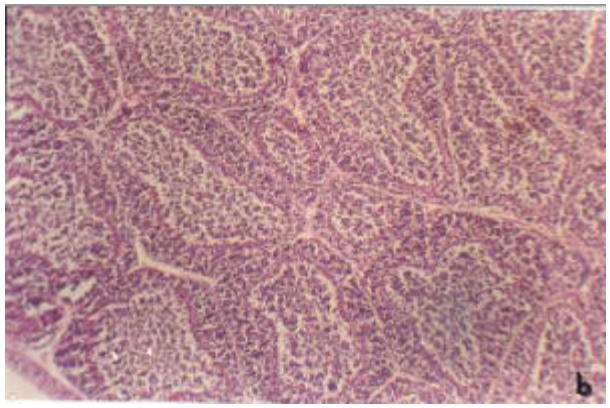

Fig. 5 b: Section of Harderian gland of a control bird at 10 d.p.i (66X)

represents gland from control bird). The sections obtained on other days of sampling could not be examined.

Thymus: Haemorrhagic foci and infiltration of heterophils and macrophages were seen in the cortex of thymus at 10 d.p.i.

Serum neutralization test: The trends in the SN titers are presented in Table 2. Mean SN titers (log_{10}) of 1.72 and 1.90 in groups I and II, respectively were observed on 5 d.p.i. which peaked to maximum values of log_{10} 4.10 and log_{10} 4.25, respectively on 21 d.p.i.

Discussion

The field isolates used in the present study were confirmed as IBDV isolates by precipitation reaction in the AGPT and neutralization with hyperimmune serum against Georgia strain of IBDV. Besides, the convalescent serum from the birds inoculated with field strains cross-neutralized Georgia virus. The isolates were obtained from field outbreaks which were reported in the poultry flocks that had been vaccinated with Georgia virus, a strain of intermediate pathogenicity. Such failures can be attributed to a number of factors - (i) the field strains could be antigenically different from the vaccine strains, (ii) the field strains could be more virulent as compared to the Georgia strain, (iii) inadequate protection afforded by the vaccine strain. The studies on the pathogenesis of isolates have been undertaken in order to characterize them primarily on the basis of pathological lesions produced in the chicks and the immune responses induced by the isolates to begin with. In CEF cell culture, the Hoshiarpur isolate produced milder and slower cytopathogenicity as compared to that produced by the IBDV Hisar-'97 isolate. The broiler chicks used for the experimental infection were obtained from an organized farm where the flocks had no previous history of IBD and the practice of vaccinating the parent flock with IBDV was not followed. The unvaccinated chicks obtained from the farm were reared at the premises of the institute till 26 days prior to inoculating them with the field isolates. The IBDV-free

status of the chicks was further ensured by absence of IBDV-specific antibodies in their sera by SNT and AGPT prior to inoculation. All the three groups, i.e. both the inoculated and uninoculated control groups were housed separately wide apart so as to avoid infection of the control group and cross infections between the two groups.

Following inoculation, the birds in both the virus inoculated groups were dull but not depressed. Other clinical signs included: elevated body temperatures, greenish watery diarrhoea during the acute phase, increased water and decreased feed intakes as compared to control birds. With the specified dose of each field virus which was determined arbitrarily in a pilot experiment so as to study the sequential changes, mortality was not observed in either of the two groups. The typical clinical signs referred to in the text books in context with the field outbreaks are in fact not exactly reproducible under experimental conditions (Cho and Edgar, 1972). This is not the only case with IBDV infection but other viruses too such as equid herpesvirus-1 (Burrows and Goodridge, 1979). One probable reason to account for this could be the flock size and intensive rearing conditions would promote heavy environmental load of the virus. Although the main route of entry is oral cavity, the poultry dust containing IBDV can infect through routes such as ocular, respiratory and cloaca. The heavy environmental load might explain increased mortality in birds reared under intensive conditions. Although *In vitro* attenuation of the virus during its adaptation in CEF has been considered a factor for low mortality without affecting the immunogenicity of the virus (Yamaguchi *et al*., 1996), this does not apply to the present study where the isolation and subsequent revival and passages were made in CEF cell culture.

The typical gross lesions, the hemorrhages in thigh muscles and occasionally in breast muscles observed in both the infected groups lasted longer in group II till 21 d.p.i. as compared to 10 d.p.i in group I. A progressive regression first and subsequently repopulation of the bursa is evident in our study.

The presence of yellowish gelatinous fluid on the serosal surface of bursa between 1 to 7 d.p.i. regularly has been a peculiar feature of the IBDV Hisar-97 isolate infection. A straw colored viscous transudate surrounding the organ was also observed by Ley *et al.* (1979). We did not observe petechial hemorrhages and necrotic foci on the mucosal surface of bursa with both the field isolates. However, we did observe such changes in the bursa from which IBDV-Hisar'97 was isolated and bursae from Georgia strain inoculated birds in a pilot study. Splenomegaly has been observed till 15 d.p.i. in both the groups. This observation is consistent with that of others (Morales and Boclair, 1993; Helmboldt and Garner, 1964).

One of the peculiar gross pathological changes we observed was; the presence of diffuse hemorrhage on the mucosal surface in the proximal part of small intestine in both the groups throughout the course of experiment. This is probably the first such report although, nonspecific enteritis has been observed by Winterfield (1963). The belief that such hemorrhage is a sequel to IBDV infection is based on that observation that such a change was not observed in uninoculated control birds throughout the experiment. Whether the hemorrhage resulted due to flair up of enteric coccidial organisms due to immunosuppressive effect or a direct consequence of IBDV infection requires further investigations.

Vervelde and Davison (1997) reported SN titres between 4500 to 6500 in 21 days old chicks. High SN titres in the serum of infected birds in both the groups in our experiment may be imputed to higher immunogenicity of the field virus.

Although the aspects covered in the present study regarding characterization of the field viruses are preliminary, bursal indices, pathogenicity to chick embryos and CEF cell culture have been described (Gupta, 1999), further studies for their molecular characterization viz nucleotide sequencing and reactivity with monoclinal antibodies need to be undertaken in order to determine relatedness between them *vis a vis* vaccine strain (Georgia strain of IBDV). These studies would reflect whether the field viruses originated from the vaccine strain or emerged due to selection pressure.

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