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Original Research

Differentiation of Infectious Bursal Disease Virus (IBDV) Strains in Experimentally Infected Chickens

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Abstract

Infectious bursal disease (IBD) is an acute, highly contagious, and immunosuppressive avian disease caused by IBD virus (IBDV). Differentiation of IBDV strains is crucial for effective vaccination programs and epidemiological investigations. In this study, a combination of clinical, histopathological examinations, immunohistochemistry and indirect immunofluorescence techniques were used to differentiate different IBDV strains. Sixty three weeks old chickens were divided randomly into five equal groups. Chickens of four groups were inoculated orally with different strains of IBDV. Chickens of the fifth group were kept as a control. Three chickens per group were euthanized at 3, 7, 14, 21 days post-inoculation (dpi). Euthanized chickens subjected to autopsy and tissue samples were collected in 10% neutral-buffered formalin for histopathology, immunohistochimestry and immunoflrourescence. The results revealed that clinical examination and histopathology cannot be used alone for differentiation of IBDV strains, while immunohistochemical and immunofluorescence techniques are reliable tools for differentiation of IBDV strains.

Keywords: Infectious bursal disease; Experimental infection; Histopathology, Immunohistochimestry; Immunofluoresence.

Introduction

Infectious bursal disease (IBD) or ‘‘Gumboro disease’’ is a contagious and immunosuppressive disease of young chickens. It was reported for the first time nearly 50 years ago. However, nowadays it is considered to be endemic in most of the poultry-producing countries, and it is one of the major causes for economic losses in the poultry industry (Azad *et al*., 1985; Van den Berg *et al*., 2000). It is caused by infectious bursal disease virus (IBDV), which belongs to the genus Avibirnavirus of the Birnaviridae family and has a non-enveloped and icosahedral capsid. The genome of virus is double-stranded RNA and bi-segmented (Eterradossi and Saif, 2008). There are two serotypes of IBDV (1 and 2) have already been described. Only serotype 1 viruses cause clinical signs and they are classified in increasing order of virulence as mild, intermediate, classical virulent and very virulent strains. Mild and intermediate viruses are used as live virus vaccines (Van den Berg, 2000). On the other hand, serotype 2 viruses may infect chickens and turkeys but they are non-pathogenic for both species (McFerran *et al*., 1980; Jackwood and Saif, 1987; McNulty and Saif, 1988; Muller *et al*., 2003; Sapats *et al*., 2005). IBDV targets the precursors of antibody-producing B lymphocytes in the bursa of Fabricius (BF), leading to lymphoid depletion of B cells and the destruction of BF. Infection by this virus results in a severe immunosuppression in young chickens, inducing an increased susceptibility to other infectious diseases as well as a poor immune response to vaccines (Lukert and Saif, 1997; Van den Berg, 2000).

Vaccination of chickens is the basic strategy to control IBD in poultry farms. The breeding hens are usually hyperimmunised by vaccination in

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order to provide high titers of maternal antibody to offspring. However, variant and vvIBDV strains have been isolated from disease outbreaks, despite the presence of high levels of maternal antibody to classic strains of IBDV (Jackwood and Saif, 1987). Therefore, the use of an appropriate vaccine is critical for effective protection and hence differentiation and identification of IBDV isolates is crucial for the selection of appropriate vaccine. Various laboratory techniques have been used to detect and differentiate IBDV strains/isolates. Differentiation of classic and variant strains has been made by using ELISA and monoclonal antibodies (Snyder *et al*., 1988; Sapats and Ignjatovic, 2000). Differentiation of infectious bursal disease virus (IBDV) strains is crucial for effective vaccination programs and epidemiological investigations (Ghorashi *et al*., 2011).

The aim of the present study was to differentiate of IBDV strains using clinical signs, gross pathology, histopathology, indirect immunofluorescence and immunohistochemistry and to compare among these methods in the differentiation of IBDV.

Materials and methods

Chickens and IBDV strains

Sixty 3 weeks old specific pathogen free (SPF) chickens were obtained from SPF flock were used for this study. Throughout the experimental period, the chickens were kept in cleaned and disinfected isolated rooms under negative pressure and supplied with filtered air. Chickens were housed in stainless steel isolation cabinets and care with provided as required by Institutional Animal Care Committee. Four strains of IBDV; Very virulent IBDV 993 strain (Goryo, 1992) , moderately virulent IBDV J1 strain (Yamaguchi *et al*., 1981), Vaccine strain I: IBDV-BDA vaccine strain (Embrex Inc., Japan) and Vaccine strain II: Nisseiken vaccine strain (Nisseiken Co. Ltd, Japan) was used in the present study.

Experimental design

A total sixty 3 weeks old chickens were divided randomly into five groups, each group is 12 birds and raised separately. Chickens of four groups were inoculated orally with 0.1 ml containing 10^3 embryo infective dose 50 (EID_{50}) of one of the four strains of IBDV used in the study (993, J1, Vaccine strain I, Vaccine strain II). Chickens of the fifth group were inoculated with 0.1 ml phosphate buffer saline and kept as a negative control group. Three chickens per group were euthanized at 3, 7, 14, 21 days post-inoculation (dpi). Euthanized chickens subjected to autopsy and tissue samples of bursa, thymus, spleen, cecal tonsils, duodenum and bone marrow were collected in 10% natural-buffered formalin for histopathology, immunohistochimestry and immunoflrourescence.

Gross pathology

All chickens, which died or euthanized were examined for the presence of gross lesions of IBDV infection. The body weight and weight of bursa of all infected and control chickens were measured. The bursal weight index was calculated by dividing the value of bursal weight (g) by the value of chicken body weight (g).

Histopathology

The bursa, thymus, spleen, cecal tonsils, duodenum and bone marrow tissues of three chickens per group were collected at 3, 7, 14, 21 d.p.i., and then fixed in 10% natural-buffered formalin for 3 days, then were dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in melted paraffin wax. Paraffin sections were cut into 3 µm thick, deparaffinized in xylene, rehydrated by passing through descending grades of ethyl alcohol to distilled water, and stained by hematoxylin and esosin, mounted with neutral balsam. The stained sections were observed under microscope for assessing histopathological changes. The severity of histological lesions was scored as non visible (-), mild (+), moderate (++) or severe (+++).

Immunohistochemistry

Formalin fixed paraffin embedded tissue sections (3 µm thick) of bursa, thymus, spleen, cecal tonsils, duodenum and bone marrow were deparaffinized and hydrated. Tissue sections were covered with 0.1% trypsin Ca (antigen retrieval) for one hour at 37 ºC. The endogenous peroxidase activities were removed with 1% (v/v) hydrogen peroxide (H_2O_2), and the non-specific binding sites were blocked by 10% normal goat serum for 20 minutes

at room temperature. Sections were incubated with polyclonal chicken anti serum to IBD virus (1/10) overnight at 4 ºC in a humidified chamber. After being rinsed three times for 5 min each with TBS, the sections were incubated with peroxidase-labeled rabbit anti-chicken IgG (1/200) for 30 minutes at 37 ºC. After a rinse with TPS, the sections were reacted for 4 minutes with 0.02% (w/v) 3,3-diaminobenzidine (DAB)-4HCL and 0.001% (v/v) H2O2 in 100 ml distilled water, Subsequently, the sections were counterstained with hematoxylin and dehydrated and mounted. The procedure for negative control sections was the same as described above except the primary antibodies were substituted with PBS. The immunohistochemical lesions were scored as none visible (-), mild (+), moderate (++) or severe (+++).

Indirect immunofluorescence

Formalin fixed paraffin embedded tissue sections (3 µm thick) of bursa, thymus, spleen, cecal tonsils, duodenum and bone marrow were deparaffinized and hydrated. Tissue sections were covered with 0.1% trypsin Ca (antigen retrieval) for one hour at 37 ºC. Sections were incubated with polyclonal chicken antiserum to IBD virus (1/10) overnight at 4 ºC in a humidified chamber. After being rinsed three times for 5 min each with TBS, the sections were incubated with Fluorescein isothiocyanate (FITC)-labeled secondary antibody (1/200) for 30 minutes at 37 ºC. After a rinse with TPS, the sections were mounted. The procedure for negative control sections was the same as described above except the primary antibodies were substituted with PBS. The immunofluorescence results were scored as none visible (-), mild (+), moderate (++) or severe (+++).

Results

Clinical signs and mortalities

In the first 24 h after infection, no clinical signs were observed in all chickens; infected with IBDV strains nor control chickens. In chickens infected with 993 strains, clinical signs were first observed at day 2 post inoculation (pi), most of chickens affected showing depression, severe watery diarrhea, ruffled feathers, reluctance to move, anorexia,

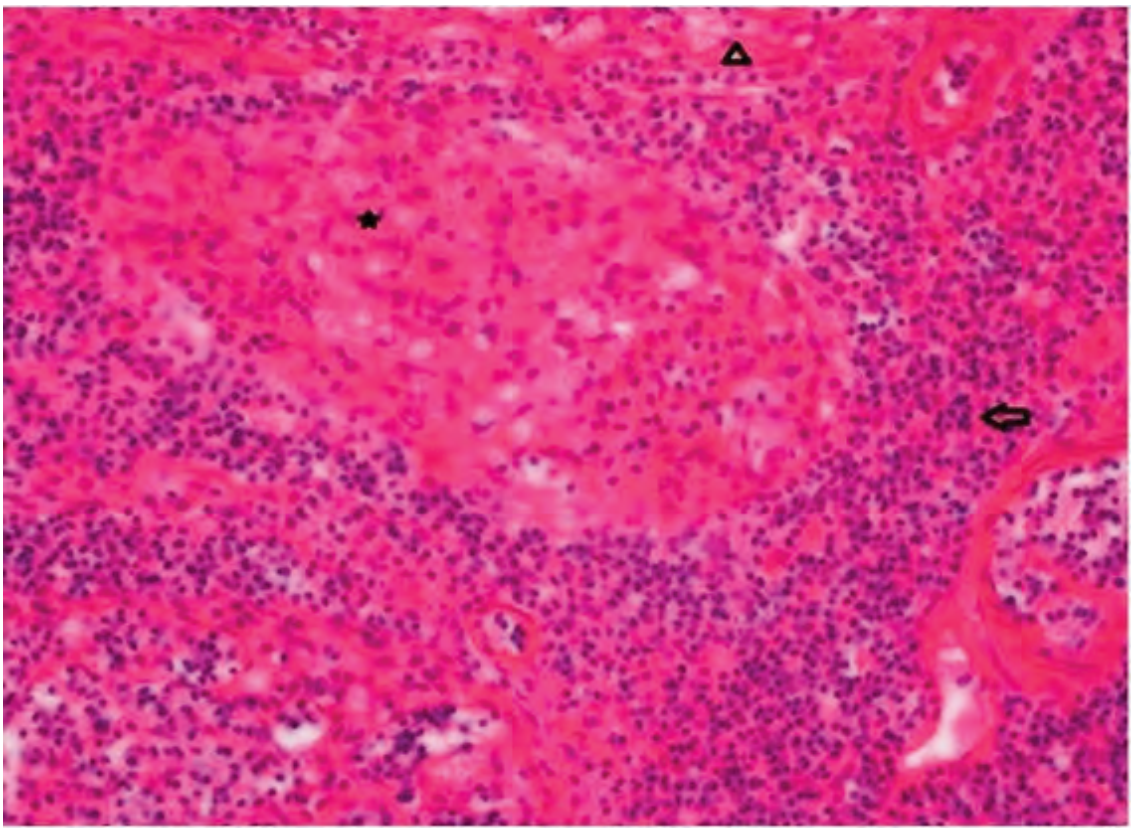

Fig. 1. bursa of chicken 4 days post inoculation with 993 strain, showing severe lymphocytic depletion and necrosis (sticks), monocellular cell infiltration (arrow) and expanding of inter-follicular connective tissue (triangular) (H&E, magnification 40x).

Table 1. Effect of different IBDV strains on SPF chicken body weight and bursa weight

DPI[a]	Strain	Body weight	Bursa weight	BF:BW ratio
	993	97.33	0.5	5.1
	J1	187.33	0.7	3.7
3	Vaccine I	125.66	0.4	3.2
	Vaccine II	144.66	0.4	2.8
	control	99.18	0.2	2.0
	993	110.33	0.26	2.4
	J1	196.33	0.3	1.5
7	Vaccine I	173.33	0.5	2.8
	Vaccine II	169.33	0.3	1.8
	control	181	.03	1.7
	993	191	0.26	1.4
	J1	328	0.3	0.9
14	Vaccine I	304.66	0.6	2.0
	Vaccine II	239.66	0.4	1.7
	control	219.57	0.5	2.3
	993	210.33	0.26	1.2
	J1	391	0.4	1.0
21	Vaccine I	359	0.7	2.0
	Vaccine II	262.33	0.33	1.3
	control	258.33	0.5	1.9

[a] Days post-inoculation, Bf: Bursa of fabrious, BW: body weight. The value presented for the BF:BW ratio were multiplied by 1000

trembling and prostration. Totally 6 chickens out of twelve dead, 3 chickens dead at day 3 pi and other 3 chickens dead at day 4 pi. Chickens recovered gradually from viral infection after 5 day pi. Eventually, clinical signs were hardly seen at 7 days pi. In chickens infected with J1 strain, no mortalities were observed and clinical signs were less severe than those observed in chickens infected with 993 strain. In chickens infected with Vaccine I and Vaccine II strains, no mortality and no clinical signs were observed except slight diarrhea. In chickens of negative control group, no clinical signs and no mortality were observed.

Gross pathology

During the postmortem examination, lesions were observed in chickens of all groups except control group, no gross lesions were observed. Macroscopically, no significant gross lesions were observed except at autopsy in chickens slaughtered at day 3 pi and chickens dead at day 3 and 4 pi.. The basic findings were dehydration of muscles with numerous ecchymotic hemorrhages, enlargement and discoloration of kidneys with ureate in tubules and bursa was severely enlarged and completely hemorrhagic. The bursal weight index of IBDV-infected chickens experienced a gradual decrease from 3 days pi to 21 day pi. The results of body weight, weight of bursa and ratio of bursa to body weight are shown in Table 1.

Histopathology

Results of histopathologic examination expressed as lesion scores in Table 2. No lesions were observed in tissues of control chickens, whereas chickens infected with each of the four strain of IBDV showed lesions. Bursa showed lymphocytic depletion and necrosis, mono-cellular cell infiltra-

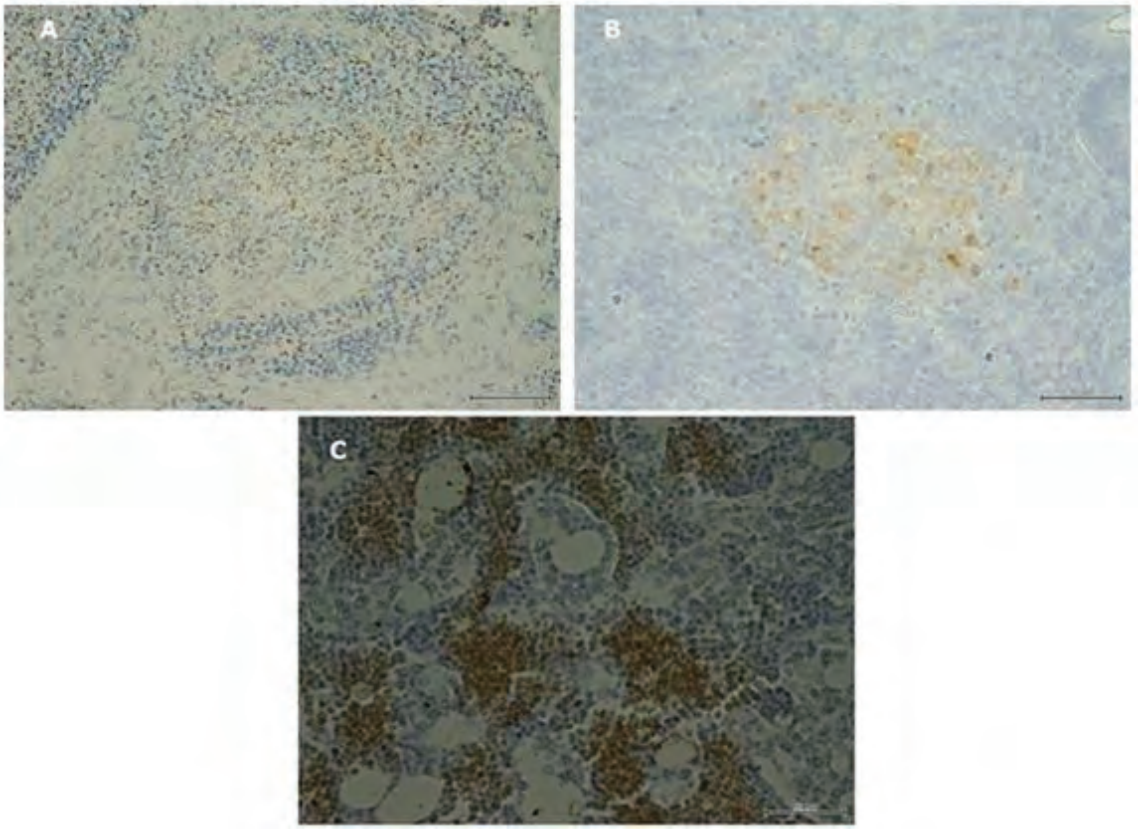

Fig. 2. immunohistochemsitry, A: bursa of chicken 3 days post inoculation with 993 strain (magnification 40x). B: cecal tonsils of chicken 4 days post inoculation with 993 strain (magnification 40x). C: bone marrow of chicken 3 days post inoculation with 993 strain, (magnification 40x), infected cells appear stained with brown color.

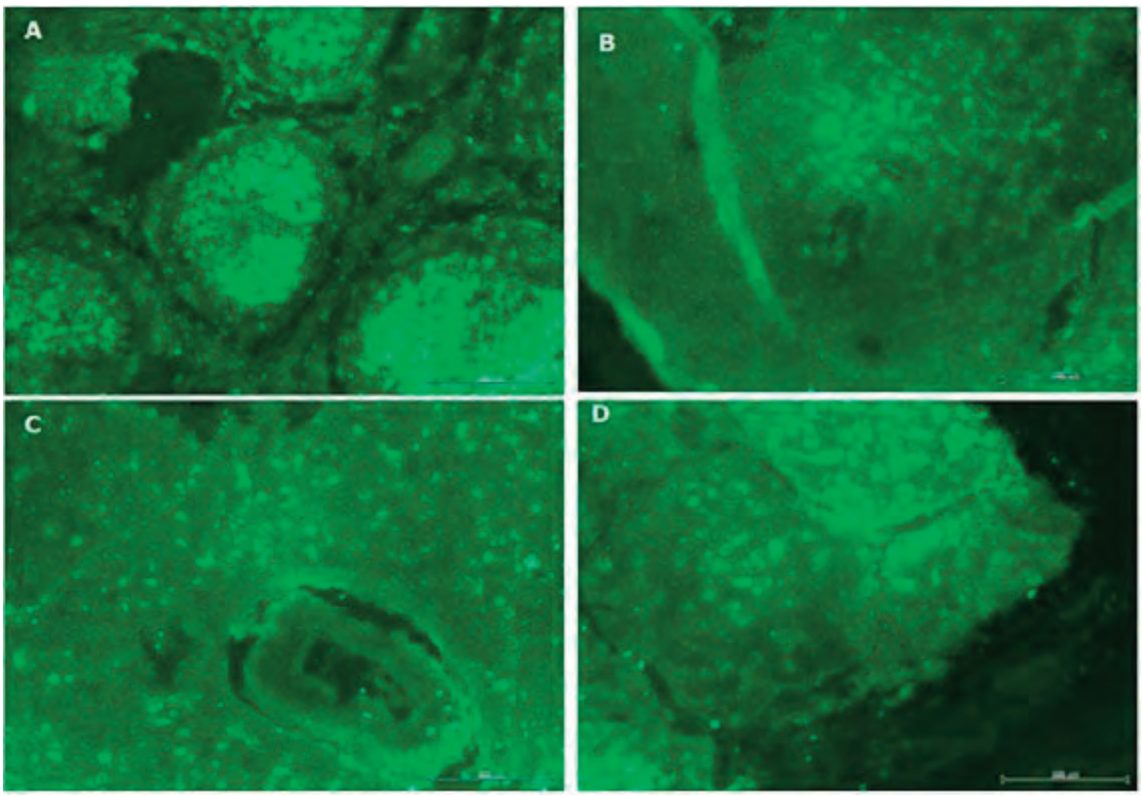

Fig. 3. immunofluorescence, A: bursa of chicken 3 days post inoculation with 993 strain (magnification 20x). B: cecal tonsils of chicken 4 days post inoculation with 993 strain (magnification 20x). C: spleen of chicken 4 days post inoculation with 993 strain (magnification 20x), the viral antigen appears as immunofluoresent green color.

Table 2. Histopathological picture of lymphoid organs of SPF chickens following experimental infection with different strains of IBDV

		Days Post Inoculation[a]																			
		3					7					14					21				
	Organs/Strains	993	J1	BDA	Ni	C	993	J1	BDA	Ni	C	993	J1	BDA	Ni	C	993	J1	BDA	Ni	C
Bursa	Depletion of lymphocytes	+++	++	++	++	-	++	++	+	+	-	+	+	+	+	-	+	+	+	+	-
	Mono-cellular cell infiltration	+++	++	++	+	-	++	+	+	+	-	+	+	+	+	-	+	-	-	-	-
	Expanding of inter-follicular CT	+	++	++	++	-	++	+++	++	++	-	+++	++	++	++	-	++	+++	++	++	-
	Hyperplasia of reticular cells	++	++	++	+	-	++	++	+	+	-	+	+	+	+	-	+	+	+	+	-
	Cystic cavitations	++	+	+	+	-	++	++	+	+	-	++	+	+	+	-	++	+	+	+	-
	Hemorrhage	+++	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spleen	Depletion of lymphocytes	++	++	++	+	-	+++	++	++	++	-	++	+	+	+	-	+	+	+	+	-
	Hyperplasia of reticular cells	+++	++	++	++	-	+++	++	++	++	-	++	++	++	++	-	++	+	+	+	-
Thymus	Depletion of lymphocytes	+++	++	++	+	-	+++	++	+	+	-	++	+	+	+	-	+	+	-	-	-
	Hyperplasia of reticular cells	+++	++	++	++	-	+++	++	++	++	-	++	++	+	+	-	++	+	+	+	-
Cecal tonsils	Depletion of lymphocytes	+++	++	++	+	-	+++	+	+	+	-	++	+	+	+	-	+	+	+	+	-
	Hyperplasia of reticular cells	+++	++	+	+	-	+++	++	+	+	-	++	+	+	+	-	++	+	+	+	-
Duodenum	Depletion of lymphocytes	+++	++	++	+	-	++	+	+	+	-	++	+	+	+	-	++	+	+	+	-
	Hyperplasia of reticular cells	+++	++	++	++	-	+++	++	++	++	-	++	+	+	+	-	++	+	+	+	-
Bone marrow	Hypoplasia or aplasia	+++	+	++	++	-	+++	+	++	++	-	++	+	+	+	-	++	+	+	+	-
	Swelling of cells	++	++	+	+	-	++	++	+	+	-	++	++	+	+	-	++	+	+	+	-
	Hyperplasia of reticular cells	++	++	+	+	-	++	+	+	+	-	++	+	+	+	-	++	+	+	+	-

(-) Non visible, (+) mild, (++)moderate, (+++) severe.
BDA: IBDV- BDA strain (Vaccine I strain), Ni: Nisseiken strain (Vaccine II strain), C: control group,

Table 3. Results of immunohistochemistry of different lymphoid organs of SPF chickens following experimental infection with different IBDV strains.

DPI[a]	strain	Immunohistochemistry					
		Bursa	Spleen	Thymus	Cecal tonsils	Duodenum	Bone marrow
3	993	+++	++	++	++	++	++
	J1	++	++	+	+	+	+
	Vaccine I	+	+	+	+	+	+
	Vaccine II	+	+	+	+	+	+
	control	-	-	-	-	-	-
7	993	++	++	++	++	++	++
	J1	++	+	+	+	+	+
	Vaccine I	+	+	+	+	+	+
	Vaccine II	+	+	-	-	-	+
	control	-	-	-	-	-	-
14	993	-	-	-	-	-	-
	J1	-	-	-	-	-	-
	Vaccine I	-	-	-	-	-	-
	Vaccine II	-	-	-	-	-	-
	control	-	-	-	-	-	-
21	993	-	-	-	-	-	-
	J1	-	-	-	-	-	-
	Vaccine I	-	-	-	-	-	-
	Vaccine II	-	-	-	-	-	-
	control	-	-	-	-	-	-

[a] Days post-inoculation (DPI)

(-) Non visible, (+) mild, (++)moderate, (+++) severe.

tion, cystic cavities in the medulla, hyperplasia of reticular cells in medulla of follicles and expanding of inter-follicular connective tissue (Fig. 1). In some cases bursa show hemorrhage. Spleen showed hyperplasia of epithelial and reticular cells around arteries and lymphoid necrosis specially in peri-arteriolar lymphoid sheath. Thymus, cecal tonsils and duodenum showed lymphoid necrosis and hyperplasia of reticular cells. Bone marrow showed cellular hypoplasia or aplasia, swelling of cells and hyperplasia of reticular and epithelial cells specially around the arteries.

Immunohistochemistry

To determine whether the pathological changes of bursa and other lymphoid organs, including spleen, thymus, cecal tonsils, duodenum and bone marrow in IBDV-infected chickens were related to IBDV proliferation, we detected viral proteins in these organs by immunoperoxidase staining. As a result, abundant viral proteins could be detected in the bursa and other lymphoid tissues at 3 and 7 days p.i of 993 strain, while moderate to few infected cells can be detected at 3 and 7 days pi with J1 and vaccine strains respectively (Table 3). No IBDV antigens were detected in the bursa and other lymphoid organs at 14 and 21 dpi of all strains inoculated in this study. Viral antigens scattered in the cortex and medulla of bursa, as well as in the interfollicular interstitium (Fig. 2A). The positive signals in chicken lymphoid tissue for immunoperoxidaze appear as brown coloration of infected cells (Fig. 2). No IBDV antigens were detected in the bursa and other lymphoid organs from virus-free control chickens.

Immunofluorescence

Immunofluorescence technique was used to detect viral antigen of IBDV in bursa, spleen, thymus, cecal tonsils, duodenum and bone marrow of chick-

Table 4. Detection of viral antigen of IBDV in different organs of SPF chickens following experimental infection with different strains of IBDV using indirect immunofluorescence technique.

DPI[a]	strain	Indirect immunofluorescence					
		Bursa	Spleen	Tthymus	Cecal tonsils	duodenum	Bone marrow
3	993	+++	++	++	++	+	++
	J1	+	+	+	-	-	+
	Vaccine I	++	+	++	+	+	+
	Vaccine II	+	-	-	-	-	+
	control	-	-	-	-	-	-
7	993	+	+	+	+	+	+
	J1	+	+	+	-	-	+
	Vaccine I	+	-	+	-	-	+
	Vaccine II	+	-	-	-	-	+
	control	-	-	-	-	-	-
14	993	-	-	-	-	-	-
	J1	-	-	-	-	-	-
	Vaccine I	-	-	-	-	-	-
	Vaccine II	-	-	-	-	-	-
	control	-	-	-	-	-	-
21	993	-	-	-	-	-	-
	J1	-	-	-	-	-	-
	Vaccine I	-	-	-	-	-	-
	Vaccine II	-	-	-	-	-	-
	control	-	-		-	-	-

[a] Days post-inoculation (DPI)
(-) Non visible, (+) mild, (++) moderate, (+++) severe.

ens infected with 993 (vvIBDV), J1 (moderately virulent IBDV) and vaccine strains of IBDV. Results revealed that IBDV antigen could be detected abundantly, moderately and rarely in bursa and other lymphoid tissues of chickens infected with 993, J1 and vaccine strains of IBDV, respectively at 3 and 7 dpi (Table 4). The positive reaction for immunofluorescense in chicken lymphoid tissue appear as immunofluoresent green color (Fig. 3). Using immunofluorescence, our results indicated that IBDV antigen could not be detected in bursa and other lymphoid organs at 14 and 21 dpi. Bursa and other lymphoid organs of negative control chickens showed negative results for immunofluorescence technique.

Discussion

Detection of infectious bursal disease virus (IBDV) has been extensively studied by many techniques including clinical examination and histopathology, immunohistochemistry and immunofluorescence (Survashe *et al*., 1979; Sharma *et al*., 1989; Tanimura *et al*., 1995; Hassan *et al*., 1996; Dohms *et al*., 1998; Lukert and Saif, 2003; Muller *et al*., 2010). In the present study, these techniques were used not only for detection of IBDV but also to differentiate IBDV strains.

The tissue distribution and severity of lesions of IBDV infection is dependent on the subtype and pathogenicity of the virus (Rosenberger and Cloud, 1986; Tanimura *et al*., 1995). Infection with classic viruses, the bursa increases transiently in size accompanied with inflammation. After the inflammation subsides, rapid bursal atrophy occurs (Sharma *et al*., 1989; Hassan *et al*., 1996). The very virulent infections are characterized by severe clinical signs, high mortality, a sharp death curve followed by rapid recovery (Van den Berg, 2000). The vvIBDV strains cause more severe lesions in the

cecal tonsils, thymus, spleen and bone marrow and a greater decrease in the thymic weight index as compared to the moderately pathogenic strains but bursal lesions are similar (Tanimura *et al*., 1995). In the current study, severe clinical signs with mortality and sever macroscopic lesions were observed in chickens infected with vvIBDV (993 strain), while moderate to mild clinical signs and lesions with no mortalities in other groups infected with other strains (J1, Vaccine I, Vaccine II).

Bursa is the preferred tissue to assess microscopic changes of IBDV infection. There are, however, no marked differences between lesions induced by vvIBDV and other types of IBDV. Identification of the type of IBDV strain involved is not possible based on microscopic examination alone (Tanimura *et al*., 1995, Ignjatovic, 2004). In the present study, vvIBDV (993 strain) showed severe histopathologic lesion scores in all lymphoid organs specially at 3 days pi, while the moderately virulent strain (J1) and vaccine strains showed mild to moderate lesion scores. vvIBDV, unlike other types of IBDV, induce microscopic lesions in tissues such as the thymus, cecal tonsil, spleen and bone marrow. The presence of these lesions has been described in experimental infections only and the lesions have not been used for differential diagnosis (Tanimura *et al*., 1995, Ignjatovic, 2004). Our results indicated that experimental infection with vvIBDV (993 strain) led to severe macroscopic and microscopic lesions not only in bursa, but also in other lymphoid organs including thymus, spleen, cecal tonsils, duodenum and bone marrow.

Immunoperoxidase and immunofluorescent techniques were used to detect and localize infectious bursal disease virus (IBDV) and infectious bronchitis virus (IBV) in fixed, paraffin-embedded chicken tissues (Jonsson and Engstrom, 1986). Detection of IBDV infections, using histopathological methods is not always reliable (Riddle,1987), and immunoperoxidase techniques may be useful for detection and localization of the viral antigen in different tissues and cells comparing to immunofluorescence (Cho *et al*., 1987). Furthermore, immunohistochemical techniques might have advantages over highly sensitive molecular approaches such as RT-PCR and in situ PCR, in studying the early viral pathogenesis, because these techniques can detect minor amounts of virus RNA in any unrelated organs (Siavosh-Haghighi *et al*., 2009). In the present study, immunohischemistry and immunofluorescence techniques were used to detect and differentiate IBDV strains and our results revealed that these two techniques are useful and reliable in differentiation of IBDV strains.

Conclusion

Clinical examination and histopathology can help but not enough alone to differentiate IBDV strains while immunohistochemistry (immunoperixodase) and immunofluorescence techniques can differentiate IBDV strains.

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