

An experimental study on early pathogenesis of a very virulent isolate of infectious bursal disease virus, employing immunohistochemistry

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Summary

In this study, immunohistochemistry was used to clarify the early stages of viral kinetics and cyclic course of IBDV, IR499, which has been described earlier as a very virulent strain (vvIBDV). Fifteen, 4-week-old SPF chickens were inoculated with 10^3 EID₅₀ of vvIBDV, IR499, via oculo/nasal route. Five birds served as controls, and inoculated with phosphate buffered saline (PBS). The birds were then bled, and tissue samples from bursa of Fabricius, cecal tonsils, liver, spleen, thymus and thigh muscle were harvested at 3, 6, 12, 24 and 48 h post-inoculation (p.i.). Typical positive signals were first observed as early as 3 h p.i. in lymphoid cells of cecal tonsils (the organ of primary affinity) and Kupfer cells of liver. Viral antigens in bursa were first found at 6 h p.i. which represents the occurrence of primary viraemia. After secondary viraemia, the virus appeared in spleen and thymus at 12 h p.i. These findings at early stages of viral infection, represented IBDV, IR499, as a very virulent strain with a rapid and generalized course, at *in vivo* level.

Key words: Infectious bursal disease, Very virulent infectious bursal disease virus, Pathogenesis, Immunohistochemistry

Introduction

Infectious bursal disease virus (IBDV), a member of the genus *Avibirnavirus* in the family *Birnaviridae* (Dobos *et al.*, 1979; Muller *et al.*, 1979; Kibenge *et al.*, 1988), causes an acute contagious infection in young chickens and has been of great concern for the poultry industry for a long time. The disease is manifested either as immunodepression or as a clinical illness, depending on the age of the bird (Bayliss *et al.*, 1990). Of the two IBDV serotypes, only serotype I is pathogenic for chickens (Lukert and Saif, 2003). Until 1987, the strains of the virus were of low virulence, causing less than 2% mortality, and efficiently controlled by vaccination. In 1986, vaccination failure was described in different parts of the world due to the reemergence of the very virulent strains.

Very virulent infectious bursal disease

virus (vvIBDV) was first described in Europe at the end of the 1980s (Van den Berg, 2000).

The acute forms of the disease were then described in Japan in the early 1990s, (Nunoya *et al.*, 1992) and rapidly spread all over Asia and other major parts of the world.

The sudden reemergence of very virulent IBD strains created the need for a better characterization of the circulating local and native isolates. IBDV genome possesses a high mutation rate which might lead to viruses with new properties such as antigenic variations or increased virulence. This situation markedly accentuated the necessity for characterization of newly local isolates of the virus and application of more efficient tools for constant field monitoring, which is helpful to put the disease under control. Studies on the pathogenesis and pathogenicity of the virus is of great importance for classification and

characterization of an IBDV isolate (Van den Berg, 2000). Pathogenesis studies are also important to evaluate different propagation systems for strains that can be used for vaccine preparation (Hassan and Saif, 1996; Abdel-Alim and Saif, 2002).

The first case of very virulent IBDV in Iran was reported in 1996 (Aghakhan *et al.*, 1996). Unfortunately, the early isolate is not available, but many new strains were isolated and characterized later.

This experimental study was conducted on a recent local isolate of a very virulent IBDV (IR499) (which was previously characterized by Shoushtary *et al.* 2004), to investigate the early pathogenesis and cyclic process of the virus in a 48 h course. Immunoperoxidase technique helps to clarify the sequence of appearance and chronological distribution of IBD viral antigen in different organs under *in vivo* systems.

Materials and Methods

Virus

Cloned IBDV, IR499 (GeneBank accession number: EU091536), was isolated and described as a very virulent local and native strain of Iran by Shoushtary *et al.* (2004).

The inoculum of the virus had a titre of 10^3 mean egg infective dose (EID₅₀) per 100 µl phosphate buffered saline (PBS).

Experiment design

Fifteen, 4-week-old SPF white leghorn chickens (Lohmman) were housed in an isolated chamber which had not been exposed to common poultry pathogens and were inoculated with an inoculum of vvIBDV, IR499 (a titre of 10^3 EID₅₀ per 100 µl), via oculo/nasal route. Five birds which served as controls, were also inoculated with the same amount of PBS and were kept separately. The birds were euthanized and bled, and tissue samples from bursa of Fabricius, cecal tonsils, liver, spleen, thymus and thigh muscle were harvested at 3, 6, 12, 24 and 48 h post-inoculation. Tissue samples were fixed in 10% neutral buffered formalin for a maximum of 2 days, to prevent conformational changes in viral antigens.

All birds of both treatment and control

groups were tested serologically for other common poultry pathogens such as New-Castle disease virus, infectious bronchitis virus and influenza virus, using ELISA.

Immunohistochemistry

Infectious bursal disease viral antigen was demonstrated by indirect immunoperoxidase system, using a Histomark Orange KPL (Gaithersburg MD USA) kit.

The sections were deparafinized and hydrated. To quench endogenous peroxidase activity, hydrated samples were incubated for 5 min in 3% H₂O₂ in methanol (blocking solution). The slides were treated with primary antibody: anti IBDV (SPAFAS, G0118), at 1/100 dilution for an overnight, then the sections were treated with 0.3% trypsin for 20 min and 5% bovine serum albumin for 30 min and incubated with peroxidase conjugated rabbit anti-chicken (Sigma MO. USA) as the secondary antibody at a dilution of 1/2000 for 30 min. Optimal primary and secondary antibodies were determined prior to the experiments.

Antigen was visualized with 3,3'-diaminobenzidine (DAB) solution and counterstained with haematoxylin. All incubations were carried out at room temperature and the sections were thoroughly washed between incubations.

To assess the specificity of immunoperoxidase staining, two negative controls were included for each experiment. In one negative control, the primary antibody was substituted with antibody diluent and in the other, tissue sections were devoid of any IBDV antigens.

Results

Bound and stained IBDV antigen was observed as brownish, fine or coarse granules in the cytoplasm of infected cells, while the nuclei stained bluish.

Positive reactions (stained cells) were absent in tissue sections of control birds and also in tissue sections stained as negative controls.

The results are summarized in Table 1.

All 3 birds in treatment groups, at each sampling time showed completely similar pathologic and immunohistochemical

distribution results.

At 3 h p.i., tissue sections of cecal tonsils and liver of the treatment group, showed slight positive reactions especially in lymphoid cells of diffused sub-epithelial lymphatic tissue Fig. 1 and Kupfer cells, respectively. At this time, sections of bursa, spleen, thymus and thigh muscle were all negative and the birds appeared quite normal at necropsy.

At 6 h p.i., lymphoid cells and macrophages in the cortices of a few lymphoid follicles of the bursa showed IBD virus antigen and also the signals were observed in the same cells of cecal tonsils and liver. Other tissue samples of the treatment group, did not show any positive reactions. There were no signs of infection at necropsy.

At 12 h p.i., tissue samples of cecal tonsils, bursa of Fabricius, spleen and thymus showed typical positive signals in lymphoid cells and macrophages. Positive signals were observed in less than 50% of follicles of the bursa, both in cortex and medulla. Clinical signs and necropsy findings were still absent.

At 24 h p.i., viral antigens observed in lymphoid cells and macrophages of cecal tonsils, bursae (about 50% of the follicles), thymus, and spleen. At this time, the population of macrophages and reticular epithelial cells in the follicles of the bursae was considerable, due to the increasing lymphoid cells depletion. In addition, the appearance of reactive lymphoid nodules and germinal center formation adjacent to a central artery, in the spleen, were first observed at 24 h p.i. At this time, petechial haemorrhages and hyperaemia in cecal tonsils and thigh muscle were observed at necropsy, besides clinical symptoms such as slight depression. The bursae were apparently normal in gross examinations.

At 48 h p.i., strong positive signals due to the presence of viral antigen were observed in more than 80% of follicles of the bursa (Figs. 2 and 3). At this time, bursal follicles showed extensive lymphocyte depletion, intrafollicular cyst formation, interfollicular edema and replacement of lymphoid cells by macrophages and reticular epithelial cells. Positive signals were also observed in lymphoid cells and macrophages of cecal tonsils (Fig. 4), spleen (Fig. 5) and thymus, which were unevenly distributed throughout the sections. Considering immunoperoxidase staining intensity and histopathological lesions of cecal tonsils, spleen and thymus, these organs showed milder and more delayed reactions to the virus, comparing to the bursae at the same time. In addition, haemorrhages in cecal tonsils, hyperaemia of the thymus, bursal edema and hyperaemia, enlarged and hyperaemic kidneys and hyperaemia of the round-edged livers were observed in gross examination.

At 48 h p.i., clinical signs consisting of depression, ruffled feathers and watery diarrhoea were more prominent.

Liver sections did not demonstrate any IBD virus antigen after 6 h p.i., and the results for the thigh muscle sections were negative in all sampling time intervals.

The viral antigen distribution within the cells of different tissues did not manifest any specific arrangement, and in most cases, viral antigens dispersed as fine to coarse granules within the infected or degenerated cells including lymphoid cells, macrophages and reticular epithelial cells.

Discussion

In the present study, the initiation of the early stages of vvIBDV, IR499, infection and its propagation course, along with

Table 1: IBDV, IR499, viral antigen appearance in tissue sections at different time intervals

Time*	Tissue					
	Bursae	Cecal tonsils	Liver	Spleen	Thymus	Thigh muscle
3	-	+	+	-	-	-
6	+	+	+	-	-	-
12	+	+	-	+	+	-
24	+	+	-	+	+	-
48	+	+	-	+	+	-

*Hours post-inoculation

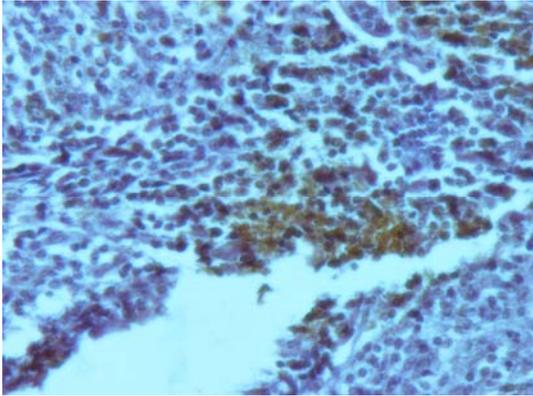


Fig. 1: Cecal tonsils harvested 3 h p.i.; typical positive signals aggregate locally within lymphoid cells in diffused subepithelial lymphatic tissue ($\times 400$)

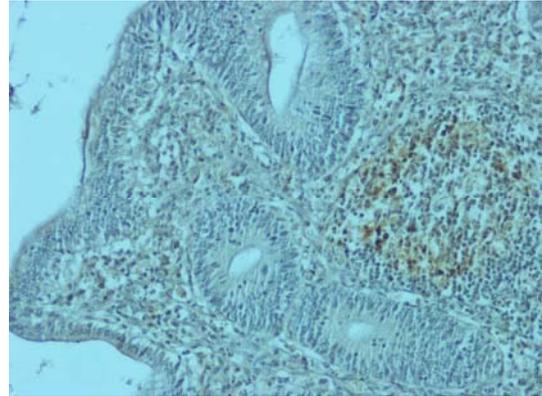


Fig. 4: Cecal tonsils at 48 h p.i.; more intense specific signals comparing to earlier viral antigen detections in the cortex of a lymphoid follicle ($\times 200$)

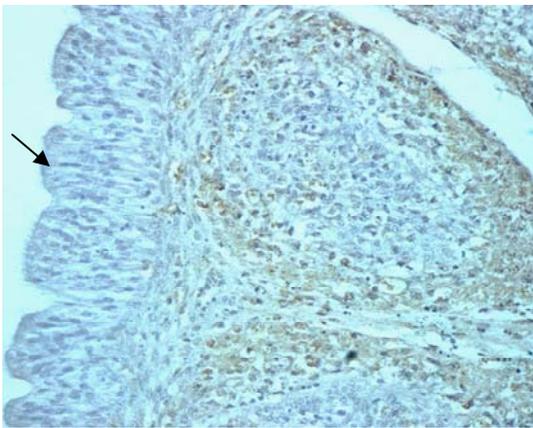


Fig. 2: Follicles of the bursa, harvested at 48 h p.i.; pseudo stratified ciliated columnar epithelium (arrow). Brownish viral antigens present in the cortices of two adjacent follicles ($\times 400$)

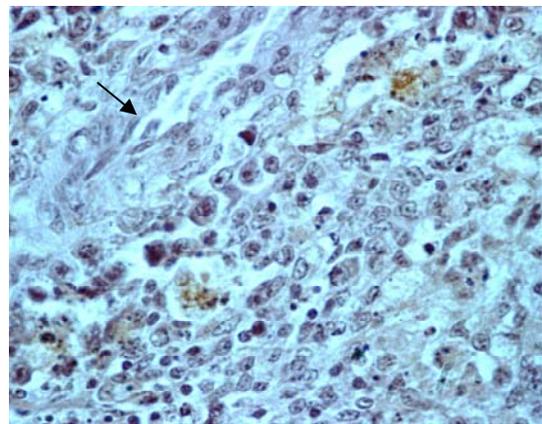


Fig. 5: Spleen at 48 h p.i.; viral antigens within degenerated lymphoid cells in a lymphoid follicle formed around a central artery (arrow; $\times 400$)

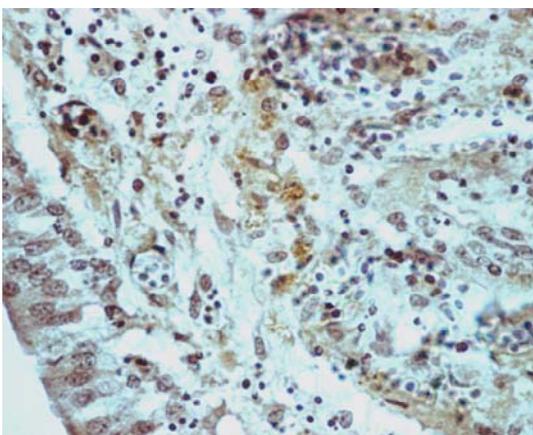


Fig. 3: Bursa of Fabricius at 48 h p.i.; antigen positive cells within the cortex of a lymphoid follicle and edematous sub-epithelial area ($\times 1000$)

specific histopathological lesions were demonstrated using immunoperoxidase method.

This study also showed that vvIBDV, IR499, has a rapid and generalized cyclic course and multiplication at each time interval was more rapid than that of milder strains; the incubation period which is usually considered as about 4 days for classical and very virulent strains (Van den Berg, 2000), took less than 48 h p.i. for IBDV, IR499.

Although the pathogenesis of infectious bursal disease virus has been extensively reviewed (Muller *et al.*, 1979; Van den Berg, 2000; Zhang *et al.*, 2002), little is known about early pathogenesis and viral kinetics of very virulent strains.

Typical positive signals were first

observed at 3 h p.i. in cecal tonsils and to a lesser extent in liver, however, in similar studies by Muller *et al.* (1979) and Tanimura *et al.* (1995), IBD virus antigen was not detected as early as 3 h p.i. This early appearance of the viral antigen could be an indication of primary replication of the virus in lymphoid cells of cecal tonsils, which seems to be the organ of primary affinity of the virus. IBDV subsequently reaches the liver through portal circulation (Muller *et al.*, 1979)

Detection of IBDV infections, using histopathological methods is not always reliable, (Riddle, 1987), and immunoperoxidas 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.

Becht (1980) suggested that the development of clinical signs during IBDV infection depend on the rate and amount of virus replication within differentiating B-lymphocytes in the bursa. Also it was shown that the pathogenicity of virulent field strains of IBDV, might be associated with viral antigen distribution in non-bursal lymphoid organs such as spleen and thymus (Tanimura *et al.*, 1995), in other words, IBD virus strains differ in tissue tropism which had been proved to be dependent on virulence. In this study, severe depletion of lymphoid cells was observed not only in the bursa of Fabricius, but also in the non-bursal lymphoid tissues such as cecal tonsils and spleen.

It has been shown that using various immunostaining methods, a higher frequency of antigen-positive cells could be demonstrated in birds with vvIBDV compared with other milder strains, in the spleen (Tanimura *et al.*, 1995) and thymus (Nunoya *et al.*, 1992). Furthermore, rapid depletion of bursal lymphocytes was reported to occur mainly during the first 24 h after IBDV infection (Jackwood *et al.*,

1992; Ojeda *et al.*, 1997). Some investigators have suggested that the sequence of the pathogenesis and viral multiplication is more pronounced in very virulent strains comparing to the milder strains (Jackwood *et al.*, 1992; Nunoya *et al.*, 1992; Cruz-Coy *et al.*, 1993; Tanimura *et al.*, 1995; Ojeda *et al.*, 1997; Van den Berg, 2000).

For vvIBDV, IR499, the lymphoid cells depletion in bursal and non-bursal lymphoid organs such as cecal tonsils, spleen and thymus, along with the cyclic course of viral antigen appearance, were more rapid and severe in the present study comparing to the results of the previous studies on milder strains (Muller *et al.*, 1979). Furthermore, in this study the immunoperoxidase stained particles, which represent areas of viral replication, were associated with microscopic lesions; the more severe the lesions were the stronger was the intensity of immunoperoxidase stain.

Our results also showed that after viral inoculation through oculo/nasal route, vvIBDV, IR499, reached the bursa of Fabricius 6 h p.i., where mass replication occurred probably via the blood circulation (transient primary viraemia). Muller *et al.* (1979), using immunofluorescent test for a classical virulent IBDV strain, found that the first viral antigen appearance in the bursa (about 10 h p.i.), might be due to primary viraemia after propagation of the virus in cecal tonsils.

According to our study, the immunoperoxidase signals first observed in spleen and thymus at 12 h p.i., which corresponds well with the occurrence of the secondary viraemia. Muller *et al.* (1979), found that by 16 h p.i., a second and pronounced viraemia occurs with secondary replication of a classical virulent strain of IBDV in non-bursal lymphoid organs such as spleen and thymus. It should be noted that although the oculo/nasal route was used in this study, virus was detected first in cecal tonsils as early as 3 h p.i., and primary and secondary viraemia occurred at 6 and 12 h p.i., respectively. This shows that the virus cyclic course and kinetics did not differ with the oral route, which was used by other investigators (Muller *et al.*, 1979).

Appearance of viral antigen in bursa

accompanied by an infiltration of T cells, while IgM⁺ cells undergo a precipitous decrease and the immunoglobulin level remains the same (Kibenge *et al.*, 1999; Muller *et al.*, 2003; Rautenschlein *et al.*, 2007). This is supported by the fact that thymus undergoes the process of lymphoid depletion in a more delayed manner, comparing to the bursa. In our study, the bursal lymphoid cell depletion, initiated as early as 12 h p.i. whereas for thymus, slight lymphoid cell depletion started at about 24 h p.i. The considerable delay between viral antigen detection in thymus and initiation of the histopathologic lesions in this organ, corresponds well with the fact that thymus could be involved in defense mechanisms against the disease, particularly in the early stages of infection.

From 12 h p.i. the cells containing viral antigen, were more prominent and frequent in other lymphatic tissues such as spleen and thymus. Strong positive signals were found in almost all tissues harvested at 48 h p.i. (except for liver and thigh muscle), which were distributed diffusely, but the intensity of the positive signals in thymus, spleen and cecal tonsil were much lower than those of the bursae.

Gradually onwards, macrophages and reticular epithelial cells containing viral antigen form the dominant cell population in the lymphoid tissues including bursa, spleen and thymus to replace the depleted lymphoid cells. As in previous studies, our results showed specific positive signals in reticular epithelial cells (Nunoya *et al.*, 1992; Tanimura *et al.*, 1995).

Other studies using *in situ* RT-PCR demonstrated viral antigens in thigh muscle, this shows that the disease could be spread by chicken meat, so that it has important consequences for the control and distribution of the disease (Zhang *et al.*, 2002). Based on our results, no positive signals were observed using immunoperoxidase in thigh muscle. The contradiction explained by the fact that pathologic lesions observed in thigh muscle infected by IBDV, is due to immune complex formation (Ley *et al.*, 1983). It should be noted that such involved viral antigens can not react with specific primary antibodies in immunostaining, since the antigenic epitopes were already masked.

According to the findings of this study, IBDV, IR499, characterized as *in vivo* level as a typical and very virulent isolate with a rapid and pronounced generalized course.

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