Pathogenicity Study and Restriction Enzyme Profile of a Recently Isolated Infectious Bursal Disease Virus in Iran

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Summary

The pathogenicity of a recent isolate of Infectious bursal disease virus (IBDV), IR499, isolated from a nonvaccinated flock with 17.5 % mortality rate in susceptible SPF chickens, chickens embryos and broilers was discussed. The molecular characterization of the virus based on the restriction fragment length polymorphism (RFLP) pattern was also investigated. The mortality rates were 85% and 22% in SPF and broiler chickens, respectively. The bursal weight indexes were 4.7 and 1.2 for SPF birds and 1.7 and 0.7 for susceptible broilers four and nine days post inoculation, respectively. The gross lesions were generalized including moderate to sever bursal hemorrhage. Using RT-PCR, a 643bp PCR product was amplified then nested PCR was carried out to amplify a 552bp PCR product. The 552bp product was subjected to *Ssp*I, *Stu*I, *Hha*I and *Sac*I restriction enzymes digestion which was *Ssp*I and *Stu*I double positive and *Hha*I and *Sac*I double negative. The pathogenicity and RFLP pattern finding confirmed that the IR499 virus could be classified as a very virulent IBDV.

***Key words*:** Infectious bursal disease virus, pathogenicity, RT-PCR, RFLP, very virulent

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),

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causes an acute infection of young chickens. The disease is manifested either as immunodeppression or as a clinical illness, depending on the age of the birds (Bayliss *et al* 1990). Of the two IBDV serotypes, only serotype 1 virus is pathogenic for chickens (Lukert & Saif 2003). Based on pathogenic and antigenic studies serotype 1 strains can also be subdivided into four groups classical virulent strains, antigenic variant strains, attenuated strains and very virulent strains (Lim *et al* 1999). Classical virulent strains cause inflammation and sever lymphoid necrosis in bursa of Fabricies (BF) infected chickens resulting in immunodeficiency and moderate mortality (up to 30%) in specific pathogen free (SPF) chickens (Lukert & Saif 2003). Antigenic variant strains are characterized by severe atrophy of the BF without showing inflammation associated with infection by classical strains (Sharma *et al* 1989). Adapting the classical and variant strains to cell cultures or chicken embryonated eggs generates attenuated strains. The attenuated strains cannot cause disease in chickens therefore some of them are being used as live vaccines. Since the late 1980s, outbreaks of newly evolved, very virulent IBD (vvIBD) strain in Europe, Asia, Africa and South America have caused significant economic losses to the poultry industry. Very virulent strains can cause up to 60 to 100% mortality in SPF birds (van den Berg 2000). These cause typical lesions of IBD and are antigenically very similar to the classical strains.

Although, pathogenicity study on SPF chickens is the best criteria for the classification of the IBD viruses (van den Berg 2000) reverstranscriptase polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) analysis for hypervariable region for VP2 gene have been used to diagnose and identify molecular differences in different IBDV pathotypes (Lin *et al* 1993, Ture *et al* 1998, Jackwood & Jackwood 1997, Jackwood & Sommer 1999, Toroghi *et al* 2003).

In Iran the first case of vvIBD was reported in 1996 (Aghakhan *et al* 1996). Unfortunately the early isolate is not available therefore this study was conducted to

characterize a recent vvIBDV isolate *in vivo* (pathogenicity study in SPF and IBDV-antibody free broiler chickens) and *in vitro* (RT-PCR-RFLP analysis).

Materials and Methods

Egg and chicken. SPF embryonated chickens eggs (Cuxhaven, Germany) as the source of the SPF chickens were used. The broiler chickens were progeny of IBDV-antibody-free broiler breeder, aged 3-4 weeks old, which reared for this purpose.

Virus. IR499 isolate after four passages in SPF embryonated eggs via chorioallontoic membrane rout was isolated from a broiler flock in Markazi province with history of no vaccination against IBDV, 17.5% mortality rate and typical lesion of IBD (Bahmaninejad 2004).

Pathogenicity and histopathology studies. The virulence of the IR499 isolate was evaluated by inoculation of the virus in SPF and broiler chickens. Forty chickens of each breed were reared in restricted conditions. Ten birds of each breed served as control group. The rest were inoculated by 10^3EID50/bird via ocular-nasal route. The embryo lesion studied through the four passages in SPF embryonated eggs. Bursal weight indices were calculated 4 and 9 days post inoculation (PI) by the following formula: bursal weight (g)×1000/body weight (g). Bursal, thymus and liver tissues were fixed in 10% buffered formalin, dehydrated in graded ethanol, and embedded in paraffin. The embedded tissues were sectioned and stained with hematoxylin and eosin.

RNA extraction. Total RNA was extracted from IBDV infected bursal tissues by homogenizing approximately 100mg of bursal tissues in 1ml of Tripure (Roche, Germany). After adding 200ml of chloroform, the aqueous phase was separated by centrifugation at 12000*g* for 10min. The supernatant was precipitated by adding 0.5ml of isopropanol at room temperature for 10min. The RNA was pelleted at 12000*g* for 20min, washed with 70% alcohol, dried and dissolved in 10µl of nuclease free water and stored at -20°C until use.

RT-PCR and nested PCR. Segments of IBDV obtained by method of Liu *et al*. (1994). RNA was denatured at 95°C for 5min and chilled on ice. The complementary DNA (cDNA) was synthesized in 20µl reaction mixture containing 6µg RNA, 50ng random primer (hexanucleotide), 4µl of 5×RT-buffer, 0.5µl Rnasin (20U), 1µl of 10mM dNTPs and 1µl MuMLV reveres transcriptase (Roche, Germany). Following the random primers to anneal at 25°C for 10 min, RT was carried out at 37°C for 1h. The RT enzyme was heat inactivated at 95°C for 3min and cDNA was stored at -20°C till further use. The PCR reaction mixture containing 5µl of 10×PCR-buffer (with 15mM $MgCl_2$), 1µl 10mM dNTPs, 15pmol of each forward primer (5′-TCACCGTCCTCAGCTTAC-3′) and reverse primer (5′-TCAGGATTTGGGATCAGC-3′), 0.5µl (2.5U) *Taq* DNA polymerase (Roche, Germany) and 6µl of cDNA. The cDNA was heat denatured in the thermocycler at 95°C for 5min then chilled on ice. The PCR was 94°C (1 min), 52°C (1min) and 72°C (1min) for 35 cycles followed by final extension at 72°C for 5min in the thermocycler (eppendorf, Mastercycler gradient). Nested PCR was carried out to amplify a 552bp PCR product by using the 643bp PCR product and primers (5′-TCAGGATTTGGGATCGC-3′) and (5′-CTCACCCCAGCGACCGATAACGACG-3′) with the cycling profile of previous PCR except annealing temperature was 60°C.

RFLP. RFLP was done by mixing 500ng of 552bp PCR product and 10U of each *Ssp*I, *Stu*I, *Hha*I and *Sac*I restriction enzymes (Roche, Germany). The generated PCR and enzyme digestion products were confirmed by running in 1% agaros gel electrophoresis.

Statistical analysis. The analysis of variance followed by Duncans multiple range test was used to demonstrate the average bursal/body weight index as compared to control groups.

Results

Virulence. Mortality rates were 85% and 22% during 9 days PI among SPF chickens and broilers, respectively The bursal atrophy was observed as early as 4

days PI (Figure 1). The bursal weight indices were 4.7 and 1.2 for SPF birds and 1.7 and 0.7 for ssceptible broilers four and nine days PI. The index was significantly ($P<0.05$) lower than control groups, 6.7 and 5.8 for SPF bird and 2.2 and 2.3 for the other, at that time.

Figure1. *Bursal lesion in a chicken inoculated with IR499 strain (days 3 PI). Sever follicular necrosis and inflamation in the infected bursa were seen*

Gross and histopathological examinations. The course of disease was very short and acute and most of the birds died within 4 days. The first clinical signs in the form of ruffled feather and white or watery diarrhea appeared at 3 days PI. Following depression, anorexia and severe prostration were seen. Mortality was observed as early as 3 days PI. The dead birds showed typical gross lesions at necropsy. Skeletal muscles were darkly discolored with various degree of haemorrhage in the thigh and breast muscles. Proventriculus–gizzard junction in some cases was also affected. The bursa were swollen and surrounded by yellowish, thickened and gelatinous transudate with various degree of hemorrhage in mucosal surface. Some of the bursa were enlarged, turgid with extensive hemorrhagic appearance. Histopathologically, severe lesions were observed specially in the bursa (Figure 2). There was severe edema and follicular lymphocyte necrosis along with various degree of haemorrhage within some follicles. Heavy infiltration of heterophils and macrophages were also observed in the interstitial area. The lymphoid cells in the cortex and medulla were severely reduced with vaculation and

cyst formation which containing pinkish fluids at nine day PI. No lesions were seen in control birds throughout the trial.

Figure2. *Bursal atrophy (day 9 PI). The weight and the size of infected bursa (left) were significantly reduced compared with uninoculate control group (right)*

RT-PCR, nested PCR and RFLP. Specific 643bp and 552bp segments of IBDV were amplified by RT-PCR and nested-PCR, respectively. Digestion of 552bp PCR prouduct with *Hha*I and *Sac*I restriction enzymes revealed that there was no site for these enzymes in VP2 region of IR499 isolate whereas digestion of the PCR product with *Ssp*I and *Stu*I restriction enzymes showed a single site in IR499 isolate for each enzyme (Figure 3).

Figure3. *Agarose gel electerophorosis of the PCR and nested PCR prouducts before and after restriction enzyme digestion. Amplifiction of a 643bp segment identified the IR499 strain as an IBDV, The nested PCR prouduct of 552bp were cleaved by StuI (St) and SspI (Ss) restriction enzymes whereas SacI (Sa) and HhaI (Hh) could not cleaved the 552bp of nested PCR prouduct, M: marker of 100bp*

Discussion

IBD has been a great concern for the poultry industry throughout the world. The re-emergence of the IBDV in the form of antigenic variants and very virulent strains has been the cause of significant losses to poultry industry. The high mutation rate of the RNA polymerase of RNA viruses due to lack of proof reading generates a genetic diversification that could lead to emergence of viruses with new properties, allowing them to persist in immune populations (Lukert & Seif 2003).

Although the molecular methods have provided accurate tools for diagnosis of the pathogen, the most valuable criteria for the classification of IBDV strains as pathotypes should refer to their virulence (mortality or lesions) in 3 to 6 weeks old SPF chicken not to any antigenic specify (van den Berg 2000). The mortality rates of 60% to 100% were reported for various isolates of vvIBDVs (van den Berg 2000, Nunoya *et al* 1992). The extensive distribution, severity of lesions and 85% mortality rates in SPF chickens and 22% in susceptible broilers revealed that the IR499 isolate could belong to vvIBDV. Immunosuppression is known to be more serious consequences since the mortality rate may influence by vaccination or infection. Immunosuppression was more sever in early ages (Faragher *et al* 1974). In our study the immunosuppression effect assessed by monitoring of bursal weight index and histologocal examination of the bursa. The results showed a severe bursal atrophy, which was significant as early as 4 days PI. The histological examination showed that this atrophy occurred due to extensive lymphoid cell necrosis and depletion. The isolate reduced lymphocyte population of BF as a specific source of B-lymphocyte.

Using special primers a 643bp fragment of VP2 was amplified. The nested PCR product was subjected to *Ssp*I, *Stu*I, *Hha*I, and *Sac*I restriction enzymes. *Sac*I and *Hha*I enzymes can cleave PCR products of field classical IBDVs and vaccine strains whereas *Ssp*I and *Stu*I enzymes only cleaved vvIBDV PCR products. Using the enzyme digestion profile vaccine and classical field strains could differentiate from

very virulent strains (Toroghi *et al* 2003). According to RFLP resluts, it seems that IR499 strain completely follows common profile of vvIBDVs, which is *Ssp*I and *Stu*I double positive and *Hha*I and *Sac*I double negative.

The pathogenicity and RFLP studies of IR499 virus showed that this strain belongs to vvIBDVs. Since the first report of occurrence of vvIBDVs in Iran (Aghakhan *et al* 1996) these viruses spread throughout the country, this is not clear why the severity of vvIBDVs decline in comparison to the early occurrences. It may be due to the solid existence of vvIBDVs in the field, which result in early infection of chickens with no clinical signs. On the other hand, the derived maternal antibodies against vaccine strains may reduce the severity of vvIBDVs. Although this is not the first report on occurrence of vvIBDVs in the Iran, the IR499 strain is the only characterized vvIBDV that can be used for antigenicity and immunogenicity studies. Also sequencing of IR499 strain may provide important information about its origin and phylogenic relationship with other vvIBDVs.

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