DEVELOPMENT OF PROBES FOR DIFFERENTIATION OF INFECTIOUS BURSAL DISEASE VIRUS STRAINS OF VARIOUS VIRULENCE BY DOT-BLOT HYBRIDIZATION

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Summary. – Two different radio-labeled nucleic acid probes, prepared from reverse transcription–polymerase chain reaction (RT-PCR) amplified variable region of VP2 and VP1 gene sequences of a highly virulent infectious bursal disease virus (IBDV), were tested for their ability to detect field isolates of IBDV directly in clinical bursal tissue specimens and vaccine strains of IBDV in tissue cultures. The VP2 gene probe was able to detect both field isolates and vaccine strains of IBDV under high as well as low stringency while the VP1 gene probe could differentiate under high stringency field isolates from vaccine strains, hybridizing only with RNA of field isolates. The sensitivity of both the probes was found to be 4 ng of purified viral RNA.

Key words: infectious bursal disease virus; detection; dot-blot hybridization; field strains; virulence; vaccine strains

Introduction

IBD is a highly contagious, immuno-suppressive viral disease of young chickens. The major problems in affected flocks are due to depletion of B lymphocytes in bursa of Fabricius leading to increased susceptibility to concurrent bacterial and viral diseases as well as poor immune response to vaccines. Emergence of antigenic variant strains in USA and highly virulent pathotypes in Europe, Asia and Africa in recent past resulted in heavy economic losses to poultry industry (Jackwood and Saif, 1987; Chettle *et al.*, 1989; Cao *et al.*, 1998; Eterradossi *et al.*, 1999). IBD virus (IBDV), the causative agent of the disease, is classified into the *Avibirnavirus* genus, the *Birnaviridae* family (Murphy *et al.*, 1995). Out of two serotypes of the virus reported, only serotype 1 produces clinical disease in chicken. The virus has a double stranded, bi-segmented RNA genome. The larger A segment encodes the polyprotein VP2-VP4-VP3, which is auto-cleaved into major structural proteins VP2, VP3 and VP4, a viral protease. Another protein of unknown function, VP5 is encoded by an overlapping ORF at the 5′-end of A segment. The smaller B segment encodes VP1 protein, a viral RNA polymerase (Kibenge *et al.*, 1990; Mundt *et al.*, 1995).

IBDV strains of intermediate virulence are being used as vaccines to protect birds against highly virulent viruses. These vaccines produce mild lesions in bursa and are partially immuno-suppressive. Therefore, it is very important to differentiate them from highly virulent IBDV isolates. For the diagnosis of IBD, a highly sensitive technique of nucleic acid hybridization has been employed by many workers using both radio- and non-radio-labeled probes to detect virus directly in clinical specimens (Davis and Boyle, 1990; Jackwood, 1990; Jackwood *et al.*, 1992; Hathcock and Giambrone, 1992). Using nucleic acid probes prepared from conserved and variable regions of VP2 gene, both serotypes of IBDV have been differentiated (Kibenge, 1992).

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Abbreviations: CEF = chicken embryo fibroblast; IBD = infectious bursal disease; IBDV = IBD virus; MMLV = Moloney murine leukemia virus; nt = nucleotide; RT-PCR = reverse transcription–polymerase chain reaction

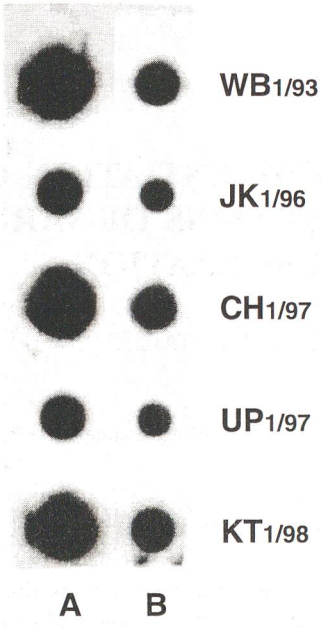

Fig. 1
Detection of highly virulent IBDV in clinical specimens using the VP1 gene probe
Low (A) and high (B) stringency conditions.

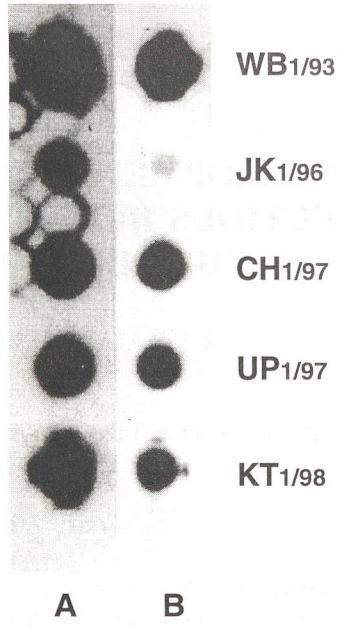

Fig. 2
Detection of highly virulent IBDV in clinical specimens using the VP2 gene probe
Low (A) and high (B) stringency conditions.

However, there are no reports on differentiation of vaccine strains from highly virulent field isolates of serotype 1 viruses by nucleic acid hybridization. Here, we report on differentiation of highly virulent field isolates from vaccine strains and detection of IBDV directly in clinical specimens using ^{32}P-labeled probes, prepared from VP1 and VP2 gene sequences.

Materials and Methods

Viruses and cell culture. Five clinical specimens (WB1/93, JK1/96, CH1/97, UP1/97 & KT1/98) obtained as bursal tissues infected with highly virulent IBDV from different parts of India, three cell culture-adapted vaccine strains (Georgia, Im+ and Lukert) and an Indian classical strain (Poona) were tested. The cell culture-adapted viruses were propagated in primary chicken embryo fibroblasts (CEFs) grown in the medium 199 supplemented with 10% of fetal calf serum.

Extraction of RNA from purified virus was performed according to Kataria *et al.* (1999). Briefly, after clarification at 3,000 x g for 20 mins, the virus in an infected cell culture supernatant was pelleted at 100,000 x g for 2 hrs and purified by a discontinuous 30–60% sucrose gradient centrifugation at the same speed for 3 hrs. RNA from purified virus was extracted with 1% SDS and 250 µg/ml proteinase K. After phenol-chloroform extraction viral RNA was purified by differential precipitation using 2 mol/l and 4 mol/l lithium chloride according to Diaz-Ruiz and Kaper (1978). The purified viral RNA was used in testing the sensitivity of nucleic acid probes.

Extraction of total RNA from bursal tissue was performed according to Lin *et al.* (1993). Approximately 100 mg of a chloroform-extracted bursal tissue homogenate was treated with 0.5% SDS and 1 mg/ml proteinase K at 37°C for 2 hrs, ethanol-precipitated, dissolved in nuclease-free water and stored at -20° C.

Extraction of total RNA from CEFs infected with vaccine strains was performed by the Trizol reagent (Life Technologies Inc., USA) following the manufacturer's protocol.

Probes for dot-blot hybridization were prepared by labeling RT-PCR-amplified VP1 and VP2 gene sequences of the field isolate WB1/93. Two sets of primers were used. Primers for the VP1 gene were 5′-ACCCTTGTGCTAGACCAGTG-3′ (forward, nt 1518–1537) and 5′-GAACCCCTTTGCCTCCAG-3′ (reverse, nt 1997–1980), while primers for the VP2 gene were 5′-CGCTATAGGGCTTGACCCAAAAA-3′ (forward, nt 651–673) and 5′-CTCACCCCAGCGACCGTAACGACG-3′ (reverse, nt 1202–1179). The RT-PCR for the VP2 gene was carried out as described by Kataria *et al.* (1998). For RT, approximately 4 µg of total RNA was heat-denatured in the presence of 100 ng of random hexamer primers and reverse transcribed at 37°C for 1 hr using Moloney murine leukemia virus (MMLV)-reverse transcriptase in a 20 µl reaction mixture. For PCR, 5 µl of cDNA was amplified using 10 pmoles of each primer, 200 µmol/l dNTPs, 2.5 U of Taq DNA polymerase in a 50 µl reaction mixture in 35 cycles of 94°C, 60°C and 72°C each for 1 min for the VP2 gene primers. For the VP1 gene primers, the annealing temperature was 52°C, keeping other conditions identical. The PCR products generated from VP1

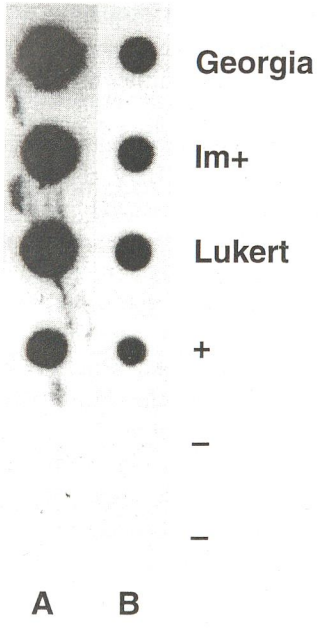

Fig. 3
Dot-blot hybridization of IBDV vaccine strains using the VP2 gene probe
Low (A) and high (B) stringency conditions. Positive (+) and negative (–) controls.

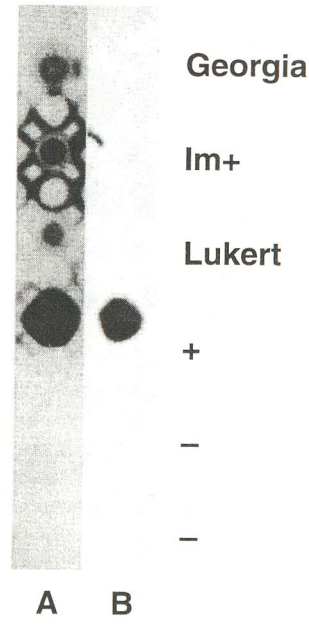

Fig. 4
Dot-blot hybridization of IBDV vaccine strains using the VP1 gene probe
Low (A) and high (B) stringency conditions. Positive (+) and negative (–) controls.

and VP2 genes were labeled by [α^{32}P] dCTP, using the random priming method described by Sambrook *et al.* (1989). The labeled products were purified using Sephadex G25 columns.

Dot-blot hybridization. The protocols described by Jackwood *et al.* (1990) and Kibenge (1992) were used with some modifications. Total RNA extracted from 100 mg of IBDV-infected bursal tissue or a 25 cm^2 flask was denatured with equal volume of denaturing buffer (2 parts of formaldehyde plus 3 parts of the 20 x SSPE buffer) at 68°C for 15 mins. After adding an equal volume of formamide, the sample was further heat-denatured at 100°C for 3 mins. The same quantity of denatured RNA was dot-blotted on three different pieces of nylon membrane (0.2 µm, Nytran, Schleicher and Schuell) using the 96 well Manifold I microsample filtration device (Schleicher and Schuell). To test sensitivity of the probes, the RNA extracted from purified virus was diluted serially 5-fold (500 ng to 0.8 ng) and blotted onto nylon membrane. Approximately 10 ng of homologous PCR product (positive control) and RNA from uninfected bursa and CEFs (negative controls) was also blotted. After cross-linking the nucleic acid by exposure to ultraviolet light, the blots were pre-hybridized in a pre-hybridization buffer containing 50% formamide at 42°C for 4 hrs in a hybridization oven (HB-1, Techne, U.K.). The heat-denatured ^{32}P-labeled, VP1 and VP2 gene probes were allowed to hybridize separately in the pre-hybridization buffer at 42°C for 16 hrs. After hybridization, the blots were subjected to low stringency washings (1 x SSPE and 0.1% SDS for 30 mins followed by 0.1 x SSPE and 0.1% SDS for 2x 30 mins each at 25°C) and autoradiographed. The same blots were further subjected to high stringency washings (0.1 x SSPE and 0.1% SDS for 2x 30 mins each at an increased temperature of 68°C) and exposed to X-ray film.

Results and Discussion

In this study, the VP1 and VP2 gene probes were prepared by ^{32}P-dCTP labeling of RT-PCR products generated from the field isolate WB1/93. Both the probes detected under low as well as high stringency, RNA of all the five IBDV field isolates tested (Figs. 1 and 2). The VP2 gene probe was able detect RNA of all the vaccine strains both under low and high stringency (Fig. 3). However, the VP1 gene probe hybridized with RNA of all the vaccine strains only under low stringency, while there were no signals under high stringency. Thus the VP1 gene probe could differentiate the field isolates from the vaccine strains (Fig. 4). Both the probes produced strong signals with homologous PCR products but no signals with RNA of uninfected CEFs or bursal tissues (Figs. 3 and 4). The sensitivity of both the probes was tested on purified IBDV RNA extracted from the classical IBDV strain under high stringency conditions; it was 4 ng of RNA (Fig. 5).

Nucleic acid probes prepared from cDNA or RT-PCR products, generated from both genome segments of IBDV, have been used widely to detect virus directly in clinical specimens (Davis and Boyle, 1990; Jackwood *et al.*, 1992;

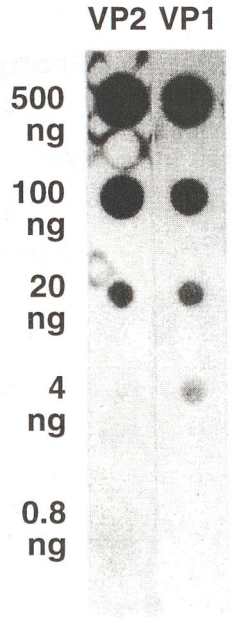

Fig. 5
Sensitivity of the VP2 and VP1 gene probes for purified IBDV RNA under high stringency

Hathcock and Giambrone 1992). In this study, two probes were prepared from a field IBDV isolate to test their ability to detect only field isolates. The probe prepared from the VP1 gene sequence was found to differentiate field isolates from vaccine strains. Both the VP1 and VP2 gene probes were able to detect IBDV in all the five clinical bursal tissue specimens tested, giving strongest signals with homologous RNA (WB1/93) under low as well as high stringency. The VP2 gene probe could also detect all the three vaccine strains both under high and low stringency. However, the VP1 gene probe hybridized with vaccine strains of IBDV only under low stringency; no signals were observed under high stringency conditions. For the vaccine strains, even under low stringency conditions, the signals with the VP1 gene probe were weaker than those with the VP2 gene probe, indicating specificity of the VP1 gene probe for virulent field isolates. A sequence variation with in the VP1 gene of vaccine strains and field isolates of IBDV has been reported by Brown and Skinner (1996). This was also evident as the RT-PCR-amplified VP1 gene sequence (used as probe in this study) had different restriction profile for vaccine strains and field isolates indicating sequence variation in this region (unpublished data). This sequence variation could be responsible for the specificity of the VP1 gene probe for either vaccine strains or field isolates. In this study, vaccine strains and field isolates of serotype 1 viruses could not be differentiated using the probe prepared from the variable region of VP2 gene. Using a probe prepared from the same variable region of VP2 gene, Kibenge (1992) could not differentiate even IBDV serotype 1 from serotype 2 viruses.

In this study the probes could not be tested for their ability to differentiate the two IBDV serotypes, as there is no authentic report on the presence of serotype 2 IBD viruses in India. Earlier, Henderson and Jackwood (1990) and Jackwood (1990), using probes prepared from both genome segments of classical serotype 1 STC strain have not been able to differentiate IBDV serotypes 1 and 2 under low stringency. On the other hand, Kibenge (1992), using a VP2 gene probe prepared from the conserved C-terminal region of OH strain of IBDV serotype 2, was able to differentiate the IBDV serotypes under high stringency. In the present study, a VP1 gene probe differentiating attenuated vaccine strains and highly virulent field isolates of IBDV is being reported for the first time.

The sensitivity of the ^{32}P-labeled VP1 and VP2 gene probes used in this study was 4 ng under high stringency, while Jackwood *et al.* (1989) and Lee (1992) reported a sensitivity of their nucleic acid probes up to 0.1 ng and 0.04 ng, respectively, but under low stringency. The probes used in our study were not tested under low stringency, which might further increase their sensitivity. The low sensitivity observed in our experiments could be due to the fact that the probes prepared from the genome of a highly virulent field isolate were tested on purified viral RNA of IBDV classical strain under high stringency, as it was easier to purify viral RNA from a cell culture-adapted virus than from field isolates which are difficult to adapt to cell culture (Lim *et al.*, 1999). The reason for the high level of background signals with the VP2 gene probe in the sensitivity experiment is not known. Probes prepared from two different genomic regions of a field isolate might detect viral RNA of all the field isolates, even under high stringency, indicating their similarity at genetic level. The variation in signals with different field isolates could be due to different concentrations of virus present in clinical specimens, as total RNA isolated from the same amount of bursal tissue (100 mg) was blotted onto membranes. Strong signals with the homologous WB1/93 PCR product and no signals with the negative controls (uninfected bursal tissue or CEF RNA) indicate specificity of the probes.

Summing up, the VP1 gene probe can be used under high stringency to differentiate virulent field isolates from attenuated vaccine strains of IBDV, while the VP2 gene probe can help in diagnosis of IBD in general.

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References

Brown MD, Skinner MA (1996): Coding sequences of both genome segments of European very virulent infectious bursal disease virus. *Virus Res.* **40**, 1–15.

Cao YC, Yeung WS, Law M, Bi YZ, Leung FC, Lim BL (1998): Molecular characterization of seven Chinese isolates of infectious bursal disease virus: Classical very virulent and variant strains. *Avian Dis.* **42**, 340–351.

Chettle N, Stuart JC, Wyeth PJ (1989): Outbreak of virulent infectious bursal disease in East Anglia. *Vet. Rec.* **125**, 271–272.

Davis VS, Boyle JA (1990): Random cDNA probes to infectious bursal disease virus. *Avian Dis.* **34**, 329–335.

Diaz-Ruiz JR, Kaper JM (1978): Isolation of viral double stranded RNAs using LiCl fractionation procedure. *Prep. Biochem.* **8**, 1–17.

Etteradossi N, Arnauld C, Tekaia F, Toquin D, Lecoq H Le, Rivallan G, Guittet M, Domenech J, van den Berg TP, Skinner MA (1999): Antigenic and genetic relationships between European very virulent infectious bursal disease viruses and an early West African isolate. *Avian Pathol.* **28**, 36–46.

Hathcock TL, Giambrone JJ (1992): Tissue-print hybridization using a non-radioactive probe for the detection of infectious bursal disease virus. *Avian Dis.* **36**, 202–205.

Henderson KS, Jackwood DJ (1990): Comparison of the dot-blot hybridization assay with antigen detection assays for the diagnosis of infectious bursal disease virus infections. *Avian Dis.* **34**, 744–748.

Jackwood DJ (1990): Development and characterization of nucleic acid probes to infectious bursal disease viruses. *Vet. Microbiol.* **24**, 253–260.

Jackwood DJ, Kibenge FSB, Mercado CC (1989): Detection of infectious bursal disease viruses by using cloned cDNA probes. *J. Clin. Microbiol.* **27**, 2437–2443.

Jackwood DJ, Kibenge FSB, Mercado CC (1990): The use of biotin-labelled cDNA probes for the detection of infectious bursal disease viruses. *Avian Dis.* **34**, 129–136.

Jackwood DH, Saif YM (1987): Antigenic diversity of infectious bursal disease viruses. *Avian Dis.* **31**, 766–770.

Jackwood DJ, Swayne DE, Fisk RJ (1992): Detection of infectious bursal disease viruses using *in situ* hybridization and non-radioactive probes. *Avian Dis.* **36**, 154–157.

Kataria RS, Tiwari AK, Bandyopadhyay SK, Kataria JM, Butchaiah G (1998): Detection of infectious bursal disease virus of poultry in clinical samples by RT-PCR. *Biochem. Mol. Biol. Int.* **45**, 315–322.

Kataria RS, Tiwari AK, Butchaiah G, Kataria JM (1999): Differentiation of infectious bursal disease virus strains by RT PCR-restriction enzyme digestion. *Acta Virol.* **43**, 245–249.

Kibenge FSB (1992): Differential detection of infectious bursal disease virus serotype using cDNA probes to VP2 coding region. *Am. J. Vet. Res.* **53**, 1337–1342.

Kibenge FSB, Jackwood DJ, Mercado CC (1990): Nucleotide sequence analysis of genome segment A of infectious bursal disease virus. *J. Gen. Virol.* **71**, 569–570.

Lee LH (1992): Characterization of non-radioactive hybridization probes for detecting infectious bursal disease virus. *J. Virol. Methods* **38**, 81–92.

Lim B-L, Cao Y, Yu T, Mo C-W (1999): Adaptation of very virulent infectious bursal disease viruses to chicken embryo fibroblast by site directed mutagenesis of residues 279 and 284 of viral coat protein VP2. *J. Virol.* **73**, 2854–2862.

Lin Z, Kato A, Otaki Y, Nakamura T, Saamaz E, Ueda S (1993): Sequence comparisons of a highly virulent infectious bursal disease virus prevalent in Japan. *Avian Dis.* **37**, 315–323.

Mundt E, Beyer J, Muller H (1995): Identification of a novel viral protein in infectious bursal disease virus infected cells. *J. Gen. Virol.* **76**, 437–443.

Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD (1995): Virus taxonomy Classification and Nomenclature of Viruses. Sixth Report of the International Committee on Taxonomy of Viruses. *Arch. Virol.* (suppl.) **10**, 240–244.

Sambrook J, Fritsch EF, Maniatis T (1989): *Molecular Cloning: A Laboratory Manual.* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor.