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Differentiation of infectious bursal disease virus strains at a genomic level

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ABSTRACT

Reverse transcription-polymerase chain reaction (RT-PCR) followed by restriction fragment-length polymorphism (RFLP) analysis was tested in order to determine its accuracy and sensitivity for differentiation of infectious bursal disease virus (IBDV) strains. Two commercial vaccine strains and two Croatian field isolates were analysed. RT-PCR assay was used for amplification of a variable part of VP2 gene (422 bp) encoding for major neutralizing epitopes. Amplified VP2 fragments were further characterized by RFLP analysis using the SacI and TaqI restriction endonucleases. Both field isolates showed RFLP pattern similar to highly virulent European and Asian strains. Both vaccine strains had RFLP profiles different from that of the field isolates. Vaccinal strain 2512 had RFLP pattern similar to mild vaccinal strain pbg-98, and strain VMG-91 was similar to other classical vaccinal strains of intermediate pathogenicity.

Key words: infectious bursal disease, RT-PCR, RFLP, vaccinal strains, field isolates

Introduction

Infectious bursal, or Gumboro, disease is an acute, highly infectious viral disease of immature chickens. The etiological agent, infectious bursal

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disease virus (IBDV), has a tropism to actively dividing B-lymphocytes (INOUE et al., 1994; TANIMURA et al., 1995), primarily in the bursa of Fabricius, but also in other organs of the immune system. This results in marked immunosuppression (SAIF, 1991; SHARMA et al., 1994) making the birds prone to various other diseases such as Newcastle and Marek's diseases, infectious bronchitis, fowl cholera, coccidiosis, colibacillosis. Increased susceptibility to respiratory viruses and immunosuppression lead to depression in egg production, hatchability and improper egg quality.

IBDV belongs to the *Avibirnavirus* genus of the *Birnaviridae* family (MURPHY et al., 1999). Strains are divided into serotypes 1 and 2 (McFERRAN et al., 1980). Type 1 comprises six subtypes with a broad range of strains from apathogenic to very virulent for chickens (JACKWOOD and SAIF, 1987) and includes both standard and variant strains (ROSENBERGER and CLOUD, 1986). Seroype 2 strains are non-pathogenic (CUMMINGS et al., 1986; ISMAIL et al., 1988).

Non-enveloped IBDV encodes in its bi-segmented double-stranded RNA (dsRNA) genome four structural virion proteins, VP1, VP2, VP3, and VP4, as well as a non-structural protein, VP5. Polyprotein VP2-VP4-VP3 and VP5 are encoded by a large A segment of viral genome. Polyprotein is autocatalytically processed rendering three proteins: structural proteins VP2 and VP3 and viral protease VP4 (HUDSON et al., 1986). Non-structural VP5 polypeptide plays an important role in the release of the virus (LOMBARDO et al., 2000), but it is not responsible for the difference in pathogenicity among serotypes 1 and 2 (SCHRODER et al., 2001). VP1, RNA dependent RNA polymerase (RdRp), is encoded by a small genomic segment B (MORGAN et al., 1988).

The region responsible for the different phenotypes was located in VP2. It has been identified as the major host-protective antigen of IBDV containing conformationally dependent protective epitopes. Most of them are located in a small variable region between amino acids 206 and 350 (YAMAGUCHI et al., 1996; VAKHARIA et al., 1994).

The syndrome of IBD emerged at the end of the 1950s. By the mid-1970s IBD was present in most countries with established poultry industries. The prevalence of clinical IBD was reduced following the

introduction of live vaccines from 1966 onwards. Inactivated emulsified vaccines were introduced at the end of the 1970's. Despite vaccination, an increase in the severity of clinical IBD was diagnosed in the early 1980s in the U.S.A. New isolates, causing severe disease and high mortality even in older birds, occurred without any major alteration in the antigenic structure of viral proteins (CHETTLE et al., 1989). Despite the fact that variants are results of minor changes in some epitopes located on VP2 protein of standard "classical" IBDV (BROWN et al., 1994; SNYDER et al., 1988; WHETZEL and JACKWOOD, 1995), a standard vaccinal strain of type 1 induces only partial protection against variant strains. In the period between 1987 and 1993 a very virulent (vv) IBDV strain of type 1 attained heavy losses in broilers, commercial egg pullets and replacement breeders in Europe, and subsequently in Africa and Asia (VAN DEN BERG and MEULEMANS, 1991; LASHER and SHANE, 1994; NUNOYA et al., 1992). The emergence of vvIBDV was diagnosed in Croatia in 1995 (SAVIĆ et al., 1997).

Classical detection of IBD viruses, like that of virus isolation on chick embryos and histopathological examination, is a time-consuming and costly procedure. The application of serological, and especially widely used molecular methods (JACKWOOD and JACKWOOD, 1997; JACKWOOD and NIELSEN, 1997; JACKWOOD and SOMMER, 1997; JACKWOOD et al., 2001; LIU et al., 1994), enables a more exact differentiation of IBDV strains. Molecular-based techniques may cover all aspects of IBD diagnosis: detection of virus, characterization - including inference of virulence - and information on the origin and spread of virus. Rapid identification of IBDV strains is a prerequisite for the effective control of this economically important infection of commercial poultry. This presents an opportunity for the application of appropriate immunoprophylactic measurements and development of vaccines protecting efficiently against field isolates.

The possibility of differentiation of Croatian field isolates from vaccinal IBDV strains at a genomic level, by the reverse transcription (RT)/ polymerase chain reaction (PCR)/restriction fragment-length polymorphism (RFLP) technique, is reported in this paper. The assay was carried out with a 422 base pair (bp) fragment of the variable region of VP2 gene using the restriction enzymes SacI and TaqI and primers designed to amplify the selected VP2 segment. According to the literature nucleotide

sequences data for various IBDV strains, the restriction enzymes were selected in order to enable the differentiation of vaccinal from field IBDV strains. The suitability of the RT-PCR/RLFP technique for molecular identification of IBDV strains was preliminarily tested using two field and two vaccinal strains. The results gained so far of analyses of vaccinal strains of known molecular composition should confirm the accuracy of the applied technique.

Materials and methods

Viruses. Mild Winterfield 2512 and intermediate VMG 91 vaccinal IBDV strain are components of the live, attenuated, commercially-available vaccine GUMBOKAL® SPF (Veterina, Zagreb, Croatia), and GUMBOKAL® IM SPF (Veterina), respectively. Both strains were replicated in embryonated specific-pathogen-free (SPF) hen eggs. The field strains were isolated from the bursa of Fabricius of broilers exhibiting most of the signs and symptoms characteristic of IBD. The first field isolate IBDV/96/01 was obtained from broilers that had died at 30 days of age (Koka, Varaždin, Croatia). The other, IBDV/97/02, was obtained from broilers that had died at 33 days of age (PZ Bistra, Bistra, Croatia).

Reverse transcription. Total RNA from infected bursal tissues and from lyophilized vaccine samples was extracted by using Trizol reagent (Life Technologies Inc, U.S.A.) according to the manufacturer's protocol. RNA samples were dissolved in nuclease-free water. The RNA was denaturised at 95 °C for 5 minutes and then submitted to the reverse transcription procedure. A 20 µl reaction mixture containing 1 µg of heat-denatured total RNA, 2.5 µM of random hexamer primers, 0.5 mM of each dNTPs, 5.0 mM $MgCl_2$, 1 U/µl of an RNase inhibitor and 2.5 U/µl of Moloney murine leukemia virus reverse transcriptase (Perkin Elmer Cetus, Forster City, U.S.A.), was incubated at 42 °C for 45 minutes. Reverse transcriptase was then inactivated by heating at 95 °C. The cDNA obtained by the RT reaction was diluted to a volume of 100 µl.

Polymerase chain reaction. Amplification was carried out with 10 µl of a sample of heat-denatured cDNA in 50 µl of a reaction mixture containing 0.2 mM of each primer, 0.2 mM of each dNTPs, 1.5 mM of

$MgCl_2$ and 0.5 U/µl Taq polymerase (Perkin Elmer Cetus, Forster City, U.S.A.) The primers were designated to amplify a 422 bp fragment of the VP2 gene from bp 733 to bp 1155 (BAYLISS et al., 1990). The incubation temperatures and durations of each cycle of the PCR were 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 45 sec, with a final extension at 72 °C for 7 mins. Products obtained by PCR amplification were analysed by electrophoresis on 2% agarose gel. In order to determine molecular masses of DNA segments, DNA marker XIII (Roche Diagnostic, Mannheim, Germany) ranging from 50 to 750 bp was used.

Restriction analysis. 10 µl of unpurified RT-PCR products were incubated overnight with 0.2 U/µl of the restriction enzyme SacI at 37 °C, and with TaqI at 65 °C (Roche Diagnostic, Mannheim, Germany). Products obtained by restriction enzyme digestion were separated electrophoretically on both 3% agarose and 8% polyacrylamide gel, followed by ethidium bromide and silver staining, respectively.

Results

The main genetic differences among IBDV strains are located in a variable region of the gene-encoding VP2 protein. In order to differentiate

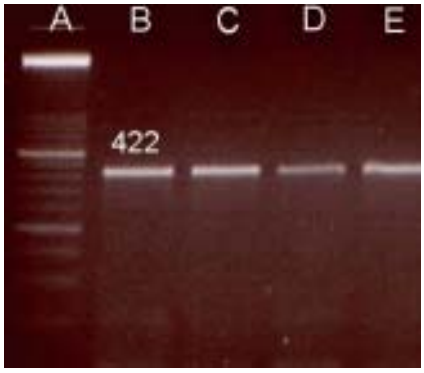

Fig. 1. Agarose gel electrophoresis of unpurified RT-PCR products obtained by treatment of RNAs of four IBDV strains. Lane A: DNA molecular-mass marker. Lane B: field isolate IBDV/96/01. Lane C: field isolate IBDV/97/02. Lane D: intermediate vaccinal VMG-91 strain. Lane E: mild vaccinal Winterfield 2512 strain.

IBD viral strains, oligonucleotide primers complementary to DNA, segments of VP2 gene ranging from 733 to 1155 nucleotides (422 bp) were evaluated. Reverse transcription yielded a cDNA which could be amplified by PCR. Agarose gel electrophoresis confirmed that PCR-amplified fragments of DNAs of two vaccinal and two field strains prepared by us were 422 bp segments (Fig. 1).

RT-PCR products were further submitted to RLFP analysis. For this purpose the RT-PCR products were digested with restriction enzymes SacI and TaqI. Two electrophoretic bands of 137 and 285 bp, obtained after SacI digestion of DNA segments of vaccinal strains, revealed the presence of a single restriction site (Fig. 2). One 422 pb RFLP band, obtained after SacI digestion of DNA segment from field isolates, indicated the absence of the restriction site (Fig. 2).

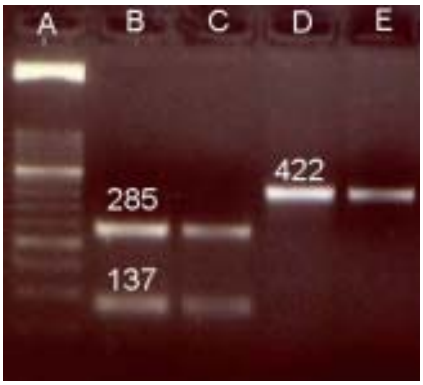

Fig. 2. Agarose gel electrophoresis of 422 bp DNA segments of IBDV digested by SacI restriction enzyme. Lane A: DNA molecular-mass marker. Lane B: mild vaccinal Winterfield 2512 strain. Lane C: intermediate vaccinal VMG-91 strain. Lane D: field isolate IBDV/96/01. Lane E: field isolate IBDV/97/02.

Two electrophoretic bands were obtained after digestion of DNA segments of field isolates and vaccinal 2512 strain with TaqI. Agarose gel electrophoretic bands of 101 and 321 bp were obtained after TaqI digestion

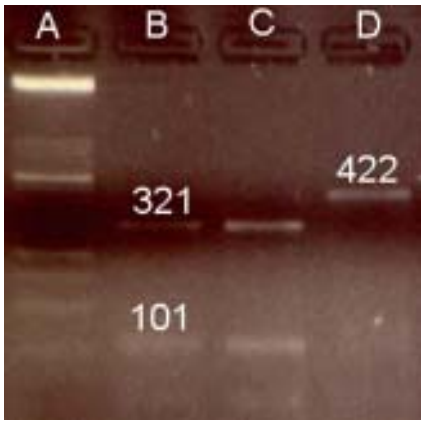

Fig. 3. Agarose gel electrophoresis of 422 bp DNA segments of IBDV digested by TaqI restriction enzyme. Lane A: DNA molecular-mass marker. Lane B: field isolate IBDV/96/01. Lane C: field isolate IBDV/97/02. Lane D: intermediate vaccinal VMG-91 strain.

Fig. 4. Polyacrylamide gel electrophoresis of a 422 bp DNA segment of the mild vaccinal Winterfield 2512 strain digested by TaqI restriction enzyme (lane B). Lane A represents DNA molecular-mass marker.

of DNA segments of field strains (Fig. 3). Polyacrylamide gel electrophoresis showed that RFLP bands of 144 and 278 bp were obtained as products of TaqI digestion of DNA segment isolated from vaccinal 2512 strain (Fig. 4). These results revealed that a single TaqI restriction site is present at a different position on 422 bp DNA segments of field isolates and vaccinal 2512 strain (Figs. 3 and 4). One agarose gel band of 422 bp, indicating restriction site absence, was obtained after TaqI digestion of DNA segment of vaccinal VMG 91 strain (Fig. 3).

Discussion

The possibility of differentiation of IBDV strains by RT-PCR/RFLP analysis of the hypervariable region of VP2 gene was tested using two vaccinal and two field strains. A 422 bp nucleotide sequence (733 – 1155 nucleotides) from genome coding for the variable region of VP2 (Fig. 1)

was amplified by RT-PCR and submitted to RFLP analysis using restriction endonucleases TaqI and SacI.

The presence of a SacI restriction site in both vaccinal strains, and no SacI restriction sites in field isolates, were found (Fig. 2). This result agreed with literature-cited data that SacI restriction site is present in the majority of classic vaccinal IBDV strains (JACKWOOD and NIELSEN, 1997; SELLERS et al., 1999). The recognition sequence for SacI is located on codons 245 and 246 of IBDV VP2 gene (BAYLISS et al., 1990). The absence of SacI restriction site between codons 245 and 246 in several vvIBDV strains was attributed to the point mutation in the third base of the codon 245 (GAG, glutamic acid › GAA, glutamic acid). The fact that this mutation does not cause the amino acid change presentss advances to molecular-based analyses over serological tests for differentiation of IBDV strains. The loss of the SacI restriction site offers a rapid method for the RFLP analysis, proving that RT-PCR/RFLP is a simple and sensitive method for detection of genetic variations among isolates that are closely related serologically, which could not be differentiated using current serologic methods.

Two segments were obtained after TaqI digestion of 422 bp RT-PCR products of both field strains (Fig. 3). TaqI digestion of a RT-PCR product of vaccinal 2512 strain also yielded two fragments, but different from that obtained after digestion of the same cDNA segments of field isolates (Fig. 4). Fragments of 101 and 321 bp, obtained by cleavage of RT-PCR products of field isolates (Fig. 3), indicated that the TaqI restriction site was located at the third base of codon 234 (ATC, isoleucin). This restriction site was found in vvIBDV strains distributed worldwide, such as European UK661 strain (BROWN et al., 1994), Japanese OKYM (YAMAGUCHI et al., 1997), Chinese HK46 (CAO et al., 1998) and Indian MH1/97 (KATARIA et al., 1999) strains.

Fragments of 144 and 278 bp (Fig. 4) obtained after TaqI digestion of RT-PCR product of vaccinal 2512 strain indicated that the TaqI restriction site was located on codon 249 (CGA, arginine). Arginine at position 249 in mild pbg-98 vaccinal strain (BAYLISS et al., 1990), as well as in a strain used for preparation of Gumbovax vaccine (IKUTA et al., 2001), was referred. Using monoclonal antibodies, VAKHARIA et al. (1994) proved that arginine

is present at position 249 in pbg-98 strain. TaqI restriction site was not found in 422 bp fragment of cDNA of intermediate VMG-91 strain (Fig. 3), indicating the absence of arginine in position 249. Analysis of intermediate vaccinal D78 strain with monoclonal antibodies showed that arginine in position 249 is replaced by glutamine (CAA) (VAKHARIA et al., 1994). The presence of glutamine at position 249 in intermediate as well as vvIBDV strains is also indicated by numerous nucleotide sequence data (BAYLISS et al., 1990; ETERRADOSSI et al., 1997; IKUTA et al., 2001; KATARIA et al., 1999). Consequently, the absence of arginine in position 249 in VMG-91 strain suggested that it belongs to intermediate rather than to mild vaccinal strains. This result confirmed once again that RFLP analysis using restriction endonuclease TaqI is a powerful diagnostic tool in differentiation IBDV strains, in that it enables the detection of the point mutation in the second base of codon 249 influencing the pathogenicity of IBDV strains.

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SAŽETAK

Procijenjena je točnost i osjetljivost metode temeljene na razlici dužina restrikcijskih odsječaka DNA (RFLP, *restriction fragment lenght polymorphism*) za razlikovanje sojeva virusa zarazne bolesti burze (VZBB). Lančanom reakcijom polimerazom (PCR, *polymerase chain reaction*) uz prethodnu reverznu transkripciju (RT, *reverse transcription*) umnožen je dio promjenljivog dijela VP2 gena koji kodira glavne virusne neutralizacijske epitope. Nastali produkti veličine 422 parova baza (bp, *base pair*) pocijepani su restrikcijskim endonukleazama SacI i TaqI. Restrikcijski profili nastali enzimskim cijepanjem uspoređeni su s podatcima navedenim u banci gena. Metoda je testirana na dva komercijalna cjepiva proizvedena u Hrvatskoj (Gumbokal® SPF, soj 2512 i Gumbokal® IM SPF, soj VMG 91) te dva hrvatska terenska izolata VZBB (IBDV/96/01 i IBDV/97/02). Oba su terenska izolata pokazala RFLP profil jednak vrlo virulentnim europskim i azijskim sojevima VZBB. Oba cijepna soja imala su različit RFLP profil od terenskih izolata. Cijepni soj 2512 ima RFLP profil jednak blagim cijepnim sojem pbg-98, a VMG 91 je sličan poznatim intermedijarnim sojevima VZBB.

Ključne riječi: zarazna bolest burze, RT-PCR, RFLP, cijepni sojevi, terenski izolati