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BIOTYPIC CHARACTERISATION OF BOVINE VIRAL DIARRHOEA VIRUS (BVDV) USING REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) IN CLINICAL SAMPLES

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In this study, bovine viral diarrhoea virus (BVDV) was detected and biotypically characterised in clinical samples using reverse transcriptase-polymerase chain reaction (RT-PCR). The RT-PCR technique produced two different amplicons (402 and approx. 680 bp in size) in case of the presence of both biotypes (cp and ncp) in the sample. The mixture of the biotypes as detected by RT-PCR was verified by the immunoplaque assay (IPA). Purification of biotypes was carried out by native plaque isolation and subsequent RT-PCR revealed single products (402 or approx. 680 bp in size) in each clone. The results showed that RT-PCR can be used for accurate molecular differentiation between the BVDV biotypes.

Key words: BVDV, RT-PCR, biotype characterisation

Bovine viral diarrhoea virus (BVDV) is a member of the *Pestivirus* genus, *Flaviviridae* family and closely related to other members of the genus, namely border disease virus (BDV) and hog cholera virus (HCV) (Franchi et al., 1991).

The genome of these viruses consists of a positive single-stranded RNA, which is 12.5 kb in size (Meyers et al., 1989*b*). Two biotypes of BVDV, cytopathic (cp) and non-cytopathic (ncp), have been distinguished according to their effects on cultured cells. It is well known that interactions between the two biotypes play a key role in the pathogenesis of mucosal disease (MD) (Brownlie et al., 1984; Baker, 1987). A major difference between the biotypes on the molecular level is the synthesis of NS2-3 protein in cells infected with the ncp biotype, which protein is antigenically related to the NS3 protein synthesised in cells infected with the cp biotype (Donis and Dubovi, 1987).

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In nature predominantly the non-cytopathic biotype is present and it has been shown that cp strains are generated by recombination between the genome of ncp BVDV and viral or cellular polynucleotides within persistently infected animals (Meyers and Thiel, 1996). The most common type of insertions has been detected in two reference BVDV strains. The first type of insertion was identified as ubiquitin-coding sequence within the genome of strain Osloss (Meyers et al., 1989*a*) while a second type (cINS) was found in strain NADL (Meyers et al., 1991). Native and immunoplaque tests have formerly been reported as conventional techniques for the biotypic differentiation of BVD viruses. Previously, the RT-PCR protocols, which were primed from flanking sides of the possible cellular or viral insertions in NS2-3 coding regions, have been described by numerous research groups and these attempts provided the understanding of the differentiation mechanisms between the biotypes (Meyers et al., 1991; Greiser-Wilke et al., 1993).

The aim of this study was to determine the suitability of single-step RT-PCR for biotypic differentiation of BVDV in clinical specimens as an alternative and accurate technique in comparison to the combination of cultural isolation and immunoplaque assay.

Materials and methods

Cell culture and viruses

Madin Darby Bovine Kidney (MDBK) cells, which had been proved to be free of intrinsic BVDV by previous tests, were used in the study. The cells were maintained in Dulbecco's Minimum Essential Medium (DMEM) enriched with 5% BVDV-free fetal calf serum (FCS), 50 IU/ml penicillin and 50 mg/ml streptomycin.

Viruses used in the study were reisolated from the departmental collection of clinical samples which had been diagnosed during previous studies. For this purpose, a total of 8 buffy coat samples obtained from persistently infected animals in a previous study and one faeces and one buffy coat sample from a mucosal disease case (Yeşilbag and Burgu, 2003) were selected from the collection. In addition, NADL and a ncp isolate from Germany (0712/Hannover) were also included, serving as cp and ncp controls.

Isolation and identification of BVDV isolates

The faecal sample was prepared for virus isolation using routine technique and together with buffy coat samples they were inoculated onto MDBK cells grown in roller tubes. Following adsorption of inoculation materials for 1 h, the cells were incubated in CO_2 atmosphere for 5 days and were evaluated for the presence of CPE indicative of pestiviruses by daily microscopic examination.

The viruses harvested by one cycle of freezing and thawing of culture tubes were then subjected to immunoperoxidase (IPX) assay in order to identify the viruses as described elsewhere (Ozkul et al., 2001).

Biotyping of BVDV isolates, immunoplaque assay and cloning of the biotypes

A combination of immunoplaque and native plaque assay was performed as described by Liess et al. (1993) for the characterisation and subsequent cloning of the biotypes.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The test was carried out as described by Greiser-Wilke et al. (1993). RNA extraction was performed as described elsewhere (Chomczynski and Sacchi, 1987) from either the clinical samples or the isolated viruses before (when both biotypes are in mixture) and after biotypic cloning (when the biotypes were separated). Complementary DNA to genomic RNAs was synthesised using Moloney Murine Leukaemia Virus (MMLV) RT in presence of random hexamer primers, as described by Ozkul et al. (2001). The primer set used in the PCR was:

NS2-3-F → 5'–GCA GAT TTT GAA GAA AGA CAC TA- 3' (Position 4937-4960)
NS2-3-R → 5'–TTG GTG TGT GTA AGC CCA- 3' (Position 5339-5321).

The primers locate within NS2-3 coding genomic region and amplify a product around 402 bp in size in ncp biotypes and a product bigger than 650 bp in size in cp biotypes (Fig. 1).

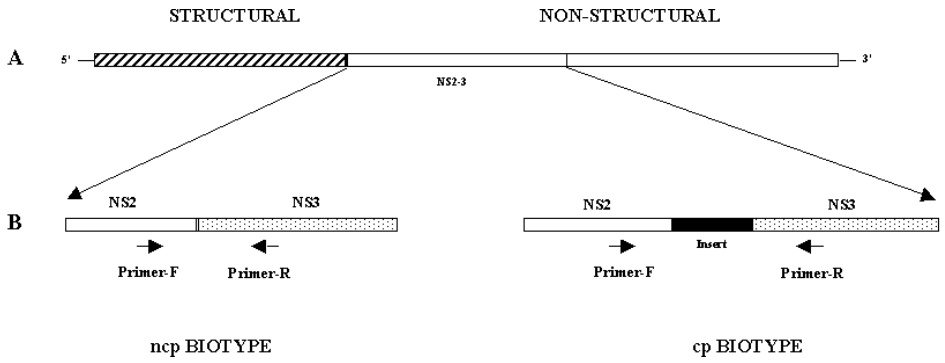

Fig. 1. Schematic presentation of the positions of the primers used. A) Single-stranded positive polarity RNA genome of a pestivirus. Shaded area indicates genes in which structural proteins are encoded, empty box indicates non-structural protein coding regions. B) Genetic structure of NS2-3 region in ncp and cp biotypes of pestiviruses. Empty box indicates NS2 region, dotted box indicates NS3 region and black box shows cellular insert in case of cp biotype. Arrows indicate positions of the forward and reverse primers used

Results

Virus isolation and IPX

Daily observation of the MDBK cell cultures revealed pestivirus-specific CPE on the second day of incubation and progressed quickly in buffy coat and faecal samples obtained from MD. There was no CPE in cultures infected with 8 buffy coat samples obtained from persistently infected animals. Cultures were frozen and thawed in order to harvest the viruses on the fourth day and were stored at –80 °C in small volumes until further use. The viruses were identified as pestivirus by detecting brown-reddish cytoplasmic staining with IPX technique.

Immunoplaque assay and cloning of the biotypes

Immunoplaque assay performed after the first passage revealed the presence of both biotypes (cp+ncp) in the isolated viruses. Both biotypes were purified by immunoplaque and native plaque combination repeated three times. The purity of the cloned biotypes was proved by repeated analysis using immunoplaque assay and RT-PCR separately.

RT-PCR

Two different sizes of DNA products (approx. 402 and 680 bp) were detected at the end of the RT-PCR performed with specimens from MD indicating the possible presence of a mixture of both biotypes (Fig. 2). DNA products of the same size were also determined by RT-PCR performed after the first passage of the specimens on MDBK cells. The RT-PCR revealed a product of about 680 bp in size with the purified cp clone and with NADL, whereas all remaining buffy coat samples and the ncp control (0712/Hannover isolate) produced an amplicon of about 402 bp in size.

Discussion

Biotypic identification of ruminant pestivirus infections is of critical importance for control of the infection by eliminating the persistently infected animals. Although many techniques including molecular diagnostic assays have been reported so far, cell culture based systems have obvious superiority in the practice with regard to the biotypic differentiation of pestivirus isolates. The primary aim of this study was to investigate the possibility of using NS2-3 targeted RT-PCR for immediate differentiation of BVDV biotypes directly in clinical samples in comparison to virus isolation and subsequent immunoplaque assay.

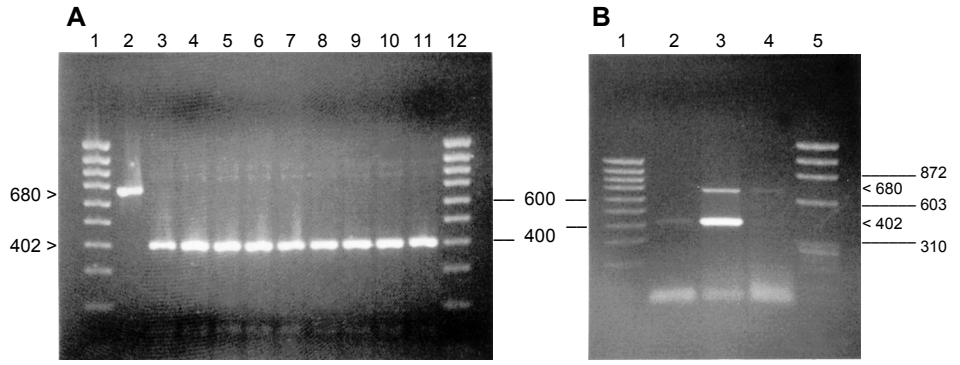

Fig. 2. Agarose gel appearance of RT-PCR results of the pestivirus isolates before and after biotypic cloning in cultured MDBK cells. Panel A: blood samples from persistently infected animals – Lanes 1 and 12: 100 bp DNA ladder (MBI, Fermentas, Lithuania); Lane 2: NADL; Lanes 3–10: buffy coat samples from PI animals; Lane 11: ncp isolates 0712/Hannover. Panel B: samples from mucosal disease – Lane 1: DNA ladder 100 bp (MBI, Fermentas, Lithuania); Lane 2: ncp clone (402 bp); Lane 3: mixed biotypes as detected in clinical samples before cloning (ncp, cp; 402, 680 bp, respectively); Lane 4: cp clone (680 bp); Lane 5, PhiX 174 DNA/HaeIII (MBI, Fermentas, Lithuania). Arrowheads indicate DNA products, in size of 402 and 680 bp, yielded from mixed and cloned pestiviruses

Major biochemical alterations between BVDV biotypes are primarily seen in the nonstructural p125 coding region (NS2-3). The main difference between the two biotypes is the synthesis of nonstructural p80 (NS3) protein antigenically related to p125 in cells infected by cp BVD viruses (Donis and Dubovi, 1987). It has been proposed that the generation of cp biotypes is dependent on recombination between the viral genome and cellular sequences (Meyers and Thiel, 1996) or most commonly insertions of cellular ubiquitin sequences into the NS2-3 genomic region (Meyers et al., 1989*a*; Meyers et al., 1991).

In this study, RT-PCR revealed successful biotypic differentiation in clinical samples (i.e. buffy coats and a faecal sample) based on the molecular approach of the occurrence of cp BVD viruses. The results obtained from RT-PCR were confirmed by the combination of virus isolation and immunoplaque assay. RT-PCR produced two amplicons of different size (about 400 and 680 bp in size) when the procedure started from the clinical sample. This result indicated the presence of both biotypes in the faecal sample of the affected animal. The detected viruses were then propagated in MDBK cells and cloned on the basis of their cytopathogenicity. The RT-PCR attempt performed at the end of biotypic cloning revealed a single band for each virus (i.e. 402 bp for the ncp biotype and approximately 680 bp for the cp biotype). This result indicated that RT-PCR products from NS2-3 region cp genomes have nucleotide excess of about 300 bp in size, which might be caused by the insertion of extra genetic material or by gene duplication. This observation was in agreement with those from previous

reports (Meyers et al., 1989*a*; Meyers et al., 1991; Greiser-Wilke et al., 1993; Vilcek et al., 2000) on the NS2-3 region. However, sequence analysis of the NS2-3 region was not performed in this research since detection of the origin of cp biotypes has only epidemiological but no diagnostic importance.

Recently, Vilcek et al. (2000) investigated additional sequences in the NS2-3 region of 32 biologically defined (in cell culture) but not cloned cp BVDV isolates. Amplification of sequences with RT-PCR and subsequent amino acid sequence analysis revealed that a 402 bp DNA product was detected in all isolates except three of the isolates and the Indiana strain, and 13 of the isolates produced a DNA fragment between 600 and 850 bp in size. The results of nucleotide sequence analysis revealed that all insertions were of cINS type and were located in a very similar position to that found in NADL genome. The results of the first part of the research (Vilcek et al., 2000) are found to be similar to those observed in our study. Detection by RT-PCR of a 402 bp DNA fragment from non-cloned cp virus indicated the mixture of the two biotypes and, in combination with the clinical details, led us to the indirect diagnosis of MD.

The use of quick and reliable diagnostic techniques offers great advantages in the differentiation of critical animal diseases such as rinderpest (RP) and MD. The RT-PCR used in this research seems to be adaptable for diagnosis of pestivirus-related clinical manifestations such as MD in addition to single-step biotype characterisation in clinical and/or postmortem samples.

In conclusion, RT-PCR was successfully used for the differentiation of cp and ncp biotypes in the same samples and provided valuable data in the diagnosis of an MD case on the basis of the molecular evaluation of results. The procedure was found to be time-saving, accurate and less expensive in comparison to cell culture based conventional methods (virus isolation and immunoplaque assay). However, nucleotide analysis of the NS2-3 junction in BVDV isolates still needs to be performed in order to characterise external sequences and subsequently to explain the natural source of within-herd cp biotypes.

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