



Molecular analysis of bovine viral diarrhoea virus isolates from South Africa

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ABSTRACT

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The presence of bovine viral diarrhoea virus in South Africa has been confirmed by several serological surveys. However, little is known about its biological properties. Twenty five isolates obtained by isolation in tissue culture and detected by means of the antigen capture ELISA from clinically sick cattle and from foetal calf serum in South Africa were characterized on the basis of analysis of the 5' non-translated (NTR) region of the genome. A reverse-transcription polymerase chain reaction (RT-PCR) was used to amplify specific sequences from the 5'NTR of the genome. The oligonucleotide primers corresponding to positions 105–125 and 399–378, respectively, in the sequence of BVDV strain NADL were used to generate the PCR products. Both strands were sequenced directly with these primers and fluorescence-labelled dideoxynucleotides in an automated nucleic acid sequencer. Reference strains of pestiviruses [(BVDV type I, BVDV type II, border disease virus (BDV) and hog cholera virus (HCV)] and isolates from a previous investigation on BVDV in southern Africa were included for comparative purposes.

All the BVDV strains obtained during this study belong to subgroups of BVDV genotype I. No association could be demonstrated between the geographic origin of the isolates. A number of isolates formed another branch separate from the existing branches Ia, Ib and Ic. These findings suggest that extensive genetic diversity can be found within BVDV type I isolates from southern Africa. Isolates that group with the classical BVDV type I strains, particularly of American origin, coexist with variants that appear to represent a local genetic pool and or variants evolving from the classical strains.

Keywords: BVDV 5'NTR, phylogeny, reverse-transcription polymerase chain reaction, sequencing, South Africa

INTRODUCTION

Bovine viral diarrhoea virus (BVDV) is a pathogen of cattle with a worldwide distribution. It causes a

variety of prenatal and postnatal clinical syndromes. Together with the viruses of border disease (BD) and hog cholera (HC) it forms the genus *Pestivirus* within the family *Flaviviridae* (Horzinek 1991; Collett 1992).

The genome of BVDV is a single-stranded, positive-sense, non-polyadenilated RNA of approximately 12.5 Kb in length. It has two non-coding regions at the 5' end (5'NTR) and at the 3' end (3'NTR) of the genome. Translation occurs in a cap-independent manner from a single large open reading frame (ORF) that encodes a polyprotein of about 4 000 amino acids. The polyprotein is co- and post-translationally processed by viral and host cell proteases

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into mature structural and non-structural proteins. The BVDV genome encodes four structural proteins: capsid protein (C), envelope protein with intrinsic RNase activity (E^{ms}), transmembrane glycoprotein (E1) and envelope glycoprotein (E2). At least seven non-structural proteins (N pro, p7, NS2-NS3, NS4 A, NS4 B, NS5 A and NS5 B) are found (Collett 1996; Thiel, Plagemann & Moennig 1996). The 5'NTR of the genome is highly conserved among pestiviruses and for this reason it has been used as a target for molecular detection of pestiviruses and for genetic discrimination among and within pestivirus genotypes (Pellerin, Vandenhurk, Lecomte & Tijssen 1994; Lowings, Iyata, Needham & Paton 1996; Baule, Van Vuuren, Lowings & Belák 1997; Becher, Orlich, Shannon, Horner, König & Thiel 1997; Vilcek, Alenius, Paton, Mittelholzer & Belák 1999).

Two genotypes of BVDV have been described on the basis of the 5'NTR analysis, namely BVDV type I, which is subdivided into subgroup Ia and represented by the reference strain NADL, and subgroup Ib, represented by the reference strain Osloss. BVDV type II has strain 890 as reference and comprises especially isolates associated with a new form of acute infection in cattle, the haemorrhagic syndrome, originally described in North America (Pellerin *et al.* 1994). BVDV type II has also been shown to include ovine isolates (Vilcek, Nettleton, Paton & Belák 1997; Ridpath, Neil, Frey, Landgraft & Thiel 2000); and, recently, to be present outside North America (Canal, Strasser, Hertig, Masuda & Peterhans 1998; Dean & Leyh 1999; Flores, Weiblen, Gil, Tobias, Lima, Garcez & Botton 2000).

The natural transmission of pestiviruses which are not highly host specific between hostspecies has prompted a new classification of the members of the *Pestivirus* genus. The suggested classification takes into account the antigenic and genomic relationship rather than the species of origin (Becher *et al.* 1997; Sullivan, Chang & Akkina 1997; Vilcek *et al.* 1997). Accordingly, the genus *Pestivirus* is divided into four genotypes: genotype 1 (*Pestivirus* type 1) that includes the present BVDV type I strains; genotype 2 (*Pestivirus* type 2) represents isolates of HCV; genotype 3 (*Pestivirus* type 3) includes sheep and pig isolates defined as "true BD" viruses and genotype 4 (*Pestivirus* type 4) includes isolates of cattle and sheep currently defined as BVDV type II.

The presence of BVDV in southern Africa has been confirmed by several serological surveys (Theodoridis, Boshoff & Botha 1973; Theodoridis & Boshoff 1974; Barnard 1977; Depner, Hubschle & Liess 1991; Giangaspero, Alders, Baer, Blondel & Mor-

gan 1991; Van Vuuren 1991; Baule & Banze 1994; Muvavarirwa, Mudenge, Moyo & Javangwe 1995; Ferreira, Lourens & Van Vuuren 2000). However, little is known about its biological properties.

The aim of this paper was to expand knowledge on the genetic characteristics of local isolates and those from the previous study by Baule *et al.* (1997).

MATERIALS AND METHODS

Specimens

Specimens of blood, organs and lymphoid tissues from sick and dead cattle were obtained from feedlots, commercial beef farms and dairy farms or were submitted by private practitioners and feedlot consultants. Other specimens comprised cell lines ($n = 3$) submitted for testing for the presence of adventitious viruses, and pooled serum obtained from fetuses ($n = 7$) at an abattoir. Some specimens were tested in duplicate, which accounts for the total number of 117 (Table 1).

Tissue filtrates of the original specimens or sera were inoculated either on Madin Darby Bovine Kidney (MDBK) line cells or primary and secondary cells calf foetal kidney cells (CFK). The viruses that were isolated, were identified by means of specific fluorescein-conjugated antisera and antigen capture ELISA tests.

RT-PCR of the 5'NTR of the BVDV genome

Total RNA was extracted from supernatants of infected cells, tissue homogenates and serum specimens, using TRIzol (Gibco, Life Technologies), according to the manufacturer's instructions. cDNA was synthesized by random priming with pdN6 (Amersham-Pharmacia, Uppsala, Sweden) using Moloney murine leukaemia virus reverse transcriptase (M-MLV RT) (Gibco, Life Technologies), as follows: 5 $\mu\ell$ of total RNA were mixed with 0.02 U of pdN6 and 3 $\mu\ell$ of ddH₂O and denatured at 65°C for 10 min, then quickly chilled on ice. A reaction mix containing 4 $\mu\ell$ of 5x 1st strand buffer, 2 $\mu\ell$ of 0.1 M DTT, 0.5 $\mu\ell$ of each dNTP (10 mM each), 24 U of RNase inhibitor (RNA guard, Amersham-Pharmacia) and 200 U of M-MLV RT was added. Synthesis was carried out at 37°C for 90 min, followed by the inactivation of the enzyme at 95°C for 5 min.

A polymerase chain reaction (PCR) was used to amplify specific sequences from the 5'NTR of the genome. The oligonucleotide primers used were as

follows (corresponding to positions 105–125 and 399–378, respectively, in the sequence of BVDV strain NADL):

Forward – 5'-AGCCATGCCCTTAGTAGGACT-3'

Reverse – 5'-ACTCCATGTGCCATGTACA-3'

Amplification was carried out in a total volume of 50 μl containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1 $\mu\text{g}/\mu\text{l}$ of BSA, 0.2 mM of each deoxynucleotide, 15 pmol of each primer, 2.5 mM MgCl_2 , 2.5% Formamide, 1 U of Taq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CA, USA), and 5 μl of cDNA. The reaction mixes were overlaid with two drops of mineral oil. PCR cycles were as follows: 5 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s and extension at 72 °C for 1 min. A final extension step at 72 °C for 7 min was included. Precautions to avoid contamination as described by Belák & Ballagi-Pordány (1993) were followed throughout the RT-PCR. The PCR products were visualized by ethidium bromide staining, after electrophoresis on 2% agarose gels.

Sequencing and sequence analysis

The amplicons were purified using the QIAquick DNA purification kit (Qiagen), according to the manufacturer's instructions and spectrophotometrically quantified. Both strands were sequenced directly with the same primers used to generate the PCR products and fluorescence-labelled dideoxynucleotides in an automated nucleic acid sequencer (ABI PRISM 377). The primers were selected based on alignments of sequences of various pestiviruses (BVDV type I, CSFV and BDV). Highly conserved parts of the 5'NTR were used for the selection of primers. These primers have also been evaluated for the amplification of BVDV type II as well and were therefore suitable for the detection of all known pestiviruses.

Nucleotide sequence editing, analysis and alignments were done using multiple programmes from the DNASTAR package (DNASTAR Inc., Madison, WI.). The phylogenetic analysis presented was completed following alignment of nucleotide sequences using the Megalign. Reference strains of pestiviruses, NADL-BVDV type I, subgroup Ia (American type), Osloss-BVDV type I, subgroup Ib (European type), 890-BVDV type II, BDV and HCV and isolates Ic from a previous investigation on BVDV in southern Africa (Baule *et al.* 1997) were included for comparative purposes. The criteria for assign-

ment of genotype were based on sequence similarity as shown in the phylogenetic tree. Strains branching with or similarly to NADL are considered subgroup Ia, with Osloss subgroup Ib and so forth. The EMBL/Genbank/DDBJ for the nucleotide sequences corresponding accession numbers are: AF041040, M31182, M96751, M96687, L32885, L32888 and sequences selected from U97409-U97481. The phylogenetic tree was edited with the Deneba Canvas (5.0) graphic programme.

RESULTS

A total of 117 specimens were subjected to molecular characterization of which 25 were confirmed positive with PCR (Table 1). Eighteen isolates obtained by isolation in tissue culture and seven isolates detected in foetal calf sera by means of the antigen capture ELISA were confirmed as BVDV with PCR. Eight other specimens that included two sera, three buffy coats, two spleens and one lung gave inconclusive readings with the FA test in cell cultures. Two were confirmed negative and six yielded a weak band with PCR. They were not molecularly analyzed and were not included in the phylogenetic tree (Fig. 1).

All of the strains were identified as BVDV I, either subgroups BVDV Ia (NADL-like) or BVDV Ib (Osloss-like) or BVDV I*.

Table 2 shows isolates that were confirmed by PCR and the predominant clinical signs associated with them. Seven isolates obtained from 156 pooled serum specimens and three cell lines of unknown history were included under the heading "others", since no clinical syndrome could be ascribed to them.

The phylogenetic assignment of these isolates, compared to reference strains of pestiviruses and to sequences from a previous investigation with BVDV isolates from southern Africa is shown in Fig. 1. The phylogenetic tree was generated based on a comparison of 245 nucleotide long sequences in the 5'NTR. The distances were calculated using the neighbor-joining method. The BVDV isolates listed in Fig. 1 were determined to be BVDV type I. The 25 isolates analyzed were phylogenetically discriminated as follows: two (ST22F/99, ST2G/99), segregated clearly as subgroup Ia; none was found under subgroup Ib; three (ST25G/99, ST23F/99, ST24G/99) were included in a cluster provisionally termed Ic (Baule *et al.* 1997), whilst the remaining isolates formed a separate cluster named I*.

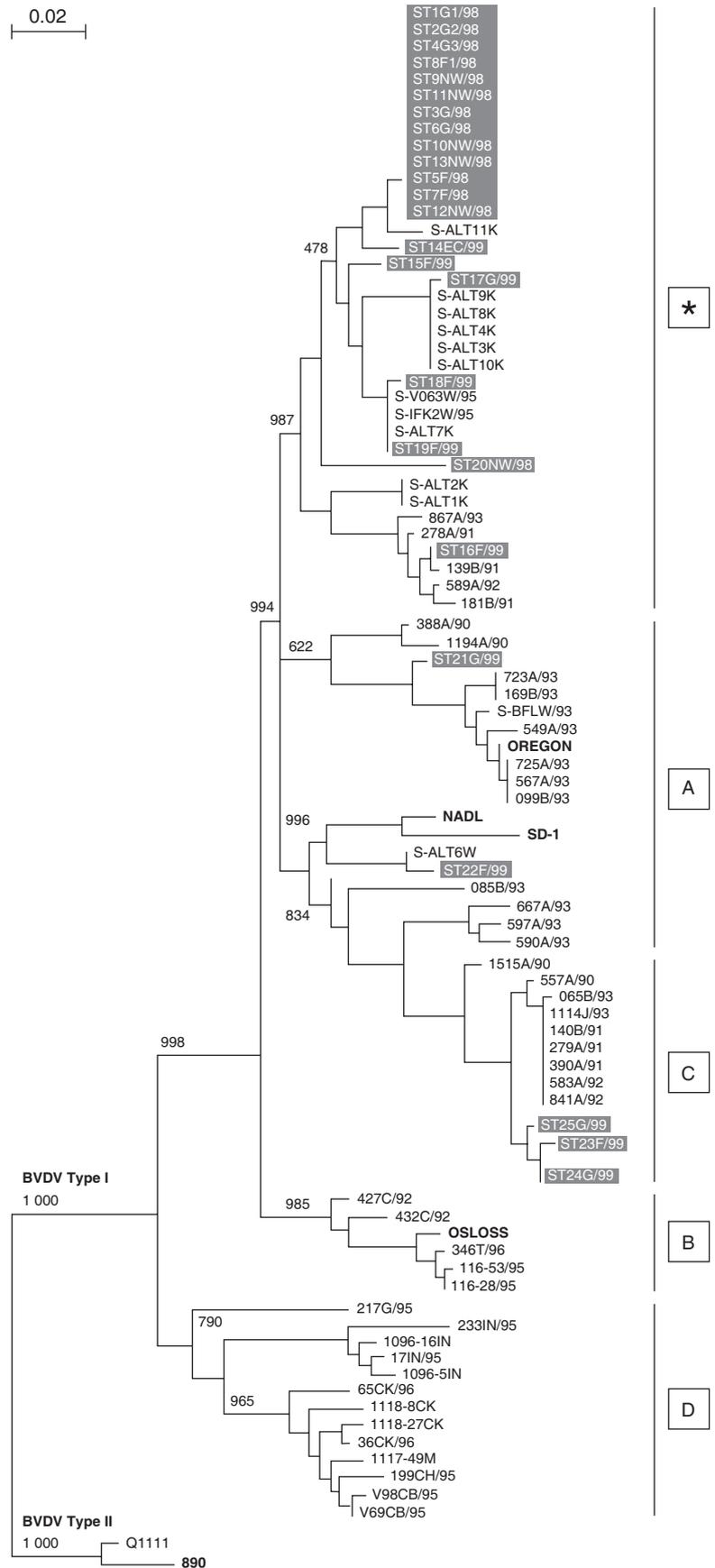


FIG. 1 Phylogenetic analysis of BVDV isolates and sequences from Genbank Reference strains of BVDV type I (NADL, OSLOSS), BVDV type II (890), BDV, HCV and isolates from a previous investigation on BVDV are included. Isolates obtained during the present investigation are indicated in bold. The phylogeny is based on analysis of 5'NTR of the genome. The isolates (see Table 2) cluster into subgroup Ia, subgroup Ic and subgroup I*

TABLE 1 BVDV isolates obtained by virus isolation, ELISA and confirmed with PCR

Specimens	Number	ELISA positive	Virus isolation positive	PCR positive
Serum	54	7	4	11
Whole blood	32	3	3	3
Spleen	10	NT	3	3
Lung	8	NT	2	2
Lymph nodes	10	NT	3	3
Cell lines	3	NT	3	3
Total	117	7	18	25

NT: Not tested

* Three buffy coats that tested positive on both tests

TABLE 2 Predominant clinical syndrome associated with BVDV isolates and their origin

Respiratory +Pyrexia (n = 7)	Respiratory (n = 3)	Diarrhoea +Pyrexia (n = 3)	Diarrhoea (n = 2)	Others (n = 10)
ncpST5F/98 ncpST6G/98 ncpST7F/98 ncpST8F/98 ncpST21G/98 ncpST9NW/98 ncpST10NW/98	ncpST11NW/98 ncpST12NW/98 ncpST13NW/98	ncpST1G1/98 ncpST2G2/98 ncpST4G/98	ncpST3G/98 ncpST17G/99	ncpST14EC/99 ncpST15F/99 ncpST16F/99 ncpST18F/99 ncpST19F/99 ncpST20NW/98 ncpST22F/99 ncpST23F/99 ncpST24G/99 ncpST25G/99

ncp: noncytopathogenic

* This classification is based on the predominant clinical signs observed by the clinician in naturally infected animals. The enteric syndrome manifests as acute or chronic diarrhoea; and the respiratory syndrome as nasal discharge, respiratory distress, sneezing and coughing, while "others" include those of unknown history and those in which no clinical syndrome ascribed to the case

** Identification of isolates: ncp: noncytopathic biotype, followed by S for South Africa, T for tropical diseases, the isolate ID and the area where it came from, the number after province of origin, where applicable, represents number of samples from the same sender in order of submission, which is followed by year of isolation. The letters that represent the province of origin: NW: North-West; F: Free State; EC: Eastern Cape; G: Gauteng

DISCUSSION

There was no relationship between the geographic origin, the nature of the clinical signs and the typing of the BVDV isolates. Animals from the North-West (NW); Free State (F); G (Gauteng) and Eastern Cape (EC) Provinces were infected with the same strain. This may inter alia be the result of the free movement of animals, the absence of closed herds or vaccination. Throughout South Africa, there is a diversity of farming systems from extensive to intensive, including closed herds where artificial insemination (AI) is used. Isolates were obtained from samples collected in feedlots, dairy herds and commercial beef farms in all provinces, indicating the ubiquitousness of BVDV in South Africa.

The reverse-transcription PCR based on the 5'NTR of the virus genome and further sequencing enabled differentiation of BVDV genotypes and subgroups; this is of epidemiological importance and might be of value in control programmes. It has been reported that direct detection of the virus in serum or homogenized tissue specimens clinical samples by RT-PCR is often unsuccessful (El-Kholy, Bolin, Ridpath, Arab, Abou-Zeid, Hamman & Platt 1998). This might be due to either the presence of certain elements in the clinical specimens that are inhibitory to reverse transcriptase or taq polymerase enzymes or to masking of the target template by proteins coagulated during extraction of nucleic acids in the clinical specimens.

Six clinical samples from which virus had not been isolated showed a weak band with RT-PCR although it was situated at the correct molecular weight position. These six specimens were not molecularly analyzed nor were they included in the phylogenetic tree. The results obtained with PCR were in agreement with those obtained by virus isolation in all the negative cases except in seven out of 156 pooled sera that were negative for virus isolation after one passage but tested positive on antigen capture ELISA. This confirms the need for more than one passage before virus becomes detectable with the FA test.

All the BVDV strains obtained during this study were ncp BVDV I (BVDV Ia (NADL-like), BVDV Ic subgroups or BVDV I* although Theodoris isolated cp BVDV in 1974. No association could be demonstrated between the geographic origin of the isolates and branch discrimination. The three groupings formed by the South African isolates (subgroup Ia, cluster Ic and cluster provisionally called I*) included BVD viruses from different regions: F, G, NW and EC. It is worth noting their similarity to isolates of the BVDV cluster provisionally termed Ic in a previous investigation (Baule *et al.* 1997) which did not segregate with either the Ia or the Ib subgroups. The presence of isolates of this cluster in South Africa may reflect a local genetic subgroup that is spreading in the region since genotype I shows an intragenotyping diversity. This might have occurred because of cattle movement or the use of biological such as cell culture-derived vaccines. No type BVDV type II were found, however the vaccine appears to be protective against both types I and II.

A number of isolates I* ($n = 20$) formed another branch separate from Ia, Ib or Ic. This branch was, however; distinct from the one defining a cluster preliminary termed Id by Baule *et al.* (1997) and was found to comprise isolates particularly distinct from the Ia and Ib subgroups. These findings suggest that an extensive genetic diversity can be found within BVDV type I isolate from southern Africa. Isolates that group with the classical BVDV type I strains, particularly of American origin, coexist with variants that appear to represent a local genetic pool and/ or variants evolving from the classical strains.

A clustering of isolates with regards to farms of origin was not observed with the isolates investigated, as has been reported by others (Paton 1995; Vilcek *et al.* 1999). Differences in farming practices, i.e. extensive farming versus intensive farming may con-

tribute to this difference in virus ecology. Closed herds and restricted contact among cattle may be a determinant factor to establish BVDV in a herd-specific manner. Most herds from which the samples originated were managed extensively.

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