Genetic characterization of bovine viral diarrhoea (BVD) viruses: confirmation of the presence of BVD genotype 2 in Africa

HG Ularamu [1,2], KP Sibeko [1], AB Bosman [1], EH Venter [1], M van Vuuren [1]

1.Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa

2.Virology Research Department, National Veterinary Research Institute P.M.B 01, Vom, Plateau State Nigeria

E-mail: ularamuhussaini@yahoo.co.uk +234 80 3700 5070)

Abstract

Bovine viral diarrhoea virus (BVDV) has emerged as one of the economically important pathogens in cattle populations with a worldwide distribution causing a complex of disease syndromes. Two genotypes BVDV 1 and 2 exist and are discriminated on the basis of the sequence of the 5′ non-coding region (5′ NCR) using real-time PCR. The use of the real-time PCR is more sensitive, specific, and less time consuming than a conventional PCR, and has reduced the risk of cross-contamination of samples. Limited information exists on BVDV genetic subtypes in South Africa. The aim of this study was to determine the genotypes of BVDV currently circulating in South African feedlots. A total of 279 specimens (219 tissue samples, 59 trans-tracheal aspirates and 1 blood sample) were collected from dead and living cattle with lesions or clinical signs compatible with BVDV infection. Pooled homogenates from the same animals were prepared and total RNA extracted. A screening test was performed on the pooled samples and positive pools were investigated individually. The Cador BVDV Type 1/2 RT-PCR Kit (Qiagen, Hilden, Germany) was used for the real-time PCR assay on a LightCycler® V2.0 real-time PCR machine (Roche Diagnostics, Mannheim, Germany). The results were read at 530 and 640 nm for BVDV 1 and 2 respectively. Bovine viral diarrhoea virus was detected in a total of 103 samples that included 91 tissue samples, 1 blood sample and 11 trans-tracheal aspirates. Eighty five (82.5%) of the strains were genotype 1 and 18 (17.5%) were genotype 2. Comparing the sequencing data, genotype 1 and 2 from the field strains did not cluster with vaccine strains currently used in feedlots in South Africa. The present study revealed the presence of BVDV genotype 2 in cattle in South Africa based on the high sequence similarity between genotype 2 field strains and strain 890 from North America. The presence of genotype 2 viruses that phylogenetically belong to different clusters and coexist in feedlots is consistent with the possibility of multiple virus introductions. These results represent the first documented evidence for the presence of BVDV genotype 2 in African cattle.

Key words: Bovine viral diarrhoea virus; Genotypes; Cador BVDV Type 1, Cador BVDV Type 2; Real-time polymerase chain reaction; genetic characterization; 5′ non-coding region

1. Introduction

Bovine viral diarrhoea virus (BVDV) is a member of the pestivirus genus that has been shown to consist of two different genotypes known as BVDV 1 and BVDV 2 [22, 26]. One of the significant biological differences between these two genotypes is the increased virulence observed among some BVDV2 isolates [28]. Initial reports of BVDV described a clinically severe disease that was rarely fatal, highly contagious and characterized by fever, diarrhoea, mucosal lesions and leukopenia [21]. Subsequent to these initial reports, descriptions that followed pointed to acute uncomplicated BVDV as a mild or sub-clinical disease of short duration with negligible mortality in all ages of cattle [1-2, 9, 10]. During the past two decades clinically severe disease outbreaks associated with acute BVDV infection have been reported in the US, Britain, Canada and Germany [11,12,18,24].

The genome of pestiviruses consists of a single-stranded, positive-sense RNA molecule, with a length of 12.3 Kb, comprising one large open reading frame (ORF) which encodes about 4 000 amino acids [3]. The BVDV open reading frame, which starts with the Npro viral autoprotease, is flanked at the 5′ and 3′ termini by untranslated regions (5′-NCR, 3′-NCR) [20]. The 5′ non-coding region (5′NCR) of the genome is considered to be highly conserved among pestiviruses, allowing the selection of specific primers that amplify all known pestiviruses. Therefore, the 5′NCR has been the target region when studying differences between and within pestiviruses [8, 13, 14, 23, 25]. Reverse transcriptase polymerase chain reaction (RT-PCR) has been used by various researchers for the detection of pestiviruses using oligonucleotide primers based on the 5′NCR of the viral genome [16-17]. Most of these primers recognize pestivirus isolates but failed to differentiate BVDV from other pestiviruses [25, 31].

Since the early 1970s the presence of BVDV has been known in southern Africa through serological surveys but only a few documented reports confirming its presence by means of virus isolation and correlation with clinical disease are available [30]. The first isolates that were obtained in the early 1970s, were antigenically similar to the prototype Oregon (C24V), and the NADL strains, and were isolated from diseased cattle with enteric or respiratory signs [30]. Although several studies have been done in Europe and the USA on different aspects of BVDV, limited documented information is available in southern Africa

on the nature of the virus, prevalence of different strains (C24V and NADL) and the economic importance of the bovine viral diarrhoea/mucosal disease complex [15]. BVDV genotype 1 strains NADL and C24V were isolated during the 1990s were subsequently characterized genetically [3, 15].

The presence of BVDV genotype 2 had been suspected by veterinarians in South Africa based on clinical signs compatible with the described haemorrhagic syndrome [22, 27]. However, studies by Baule et al., [3] could not confirm the presence of genotype 2 in South Africa. Since 1997, no further effort has been made to characterize strains in South Africa. Such information can assist in the diagnosis, epidemiology and control of the BVDV-associated disease and may assist *inter alia* feedlot veterinarians doing risk assessments for vaccination programmes. The aims of this research project were therefore to investigate the genetic heterogeneity of BVDV in South Africa and to molecularly characterize BVDV isolates obtained from feedlots in South Africa using a real-time RT-PCR assay. Part of the genome of the virus was also sequenced and phylogenetically grouped by comparison with existing sequencing data available on GenBank.

2. Materials and Methods

2.1 Specimen collection.

A total of 276 specimens were collected from dead and living cattle from different feedlots in South Africa during 2009 and submitted by private practitioners and feedlot consultants. Tissues [spleen (SP) and tracheal lymph nodes (LN)] from dead animals (n=216), trans-tracheal aspirates (TTAs) (n=59) from living cattle, and one blood sample submitted as a routine diagnostic sample were collected.

Samples collected from necropsy (n = 197) were obtained from Karan Beef feedlot close to Heidelberg in Gauteng Province. Karan Beef feedlot is the largest single-owner feedlot in the world (www.karanbeef.com) and buys and processes 1200 animals per day throughout the year. Karan Beef buys cattle from all areas of South Africa as well as Namibia and transports them by road to the feedlot near

Heidelberg. Necropsy samples were also obtained from a feedlot in KwaZulu-Natal (n = 19) that buys cattle almost exclusively from that region.

Necropsy samples were taken from animals that showed significant respiratory pathology that included chronic lesions or in some cases acute lesions due to either an overwhelming acute pneumonia or rarely from animals that presented with a shock lung appearance. In addition, some samples were submitted from cattle that displayed "unusual" pathology e.g. atypical tick-borne disease or suspected mycotoxicoses. One hundred and seven of the Karan Beef samples were also examined histologically (data not shown but on record at the feedlot).

Variables such as treatment history, origin, purchase history, entrance weight or days on feed were not considered when selecting animals for sampling purposes. Only the gross pathology as observed during necropsies was considered. Most animals had a history of chronic, advanced pneumonia and had been in the feedlot for longer than 30 days when they died or were sacrificed. A summary of the clinical signs of the animals, the geographical origin, number and type of specimens collected are given in Table i. Specimens classified under "others" included animals that died acutely without manifestation of obvious clinical signs.

The sixty specimens collected from living cattle were limited to sick animals. The parameters for selection to undergo trans-tracheal aspirates included: first time pull for treatment, fever >40 ºC, nasal discharge, fast and/or shallow breathing, hanging ears, dragging of feet when walking. These were all animals that had been present in the feedlot for at least 2 weeks when pulled the first time for diagnosis and treatment. Samples were collected at 15 different feedlots situated in the Gauteng, North West, Limpopo, Free State and Northern Cape provinces.

2.2 Reverse transcriptase real-time PCR

2.2.1 Processing of specimens

The tissue specimens (spleen & lymph nodes) and transtracheal aspirates were stored at -70 °C before processing. A 20% suspension was prepared from 1 g of spleen and lymph node from one animal, homogenized in 4 ml of phosphate buffered saline with calcium (Ca^{++}) and magnesium (Mg^{++}) (PBS plus) pH 7.2, using a sterile pestle and mortar. The suspension was centrifuged at 1500 x g for 10 minutes and the supernatant decanted into 2 ml micro-centrifuge tubes for storage at -70 °C until used.

In order to reduce the number of samples to be tested individually, aliquots from 10 homogenates were pooled for screening. A 25 µl aliquot of each homogenate was withdrawn from each tube using a pipette with sterile disposable tips to avoid cross-contamination and nucleic acid extraction was performed on the pooled samples. All samples from positive pools were subsequently tested individually.

2.2.2 Viral RNA extraction

Extraction of RNA was performed on pooled samples using the RNeasy® Mini Kit (Qiagen®) according to the manufacturer's instructions. Briefly, 350 µl of Buffer RLT was added to 200 µl tissue homogenate and centrifuged at 11,200 X g for 3 minutes in an Eppendorf centrifuge (Centrifuge 5417 R, Eppendorf AG. 22331 Hamburg, Germany). The supernatant was transferred into a new 2ml micro-centrifuge tube. A volume of 350 µl 70% ethanol was added to 350 µl supernatant (lysate) and mixed by pipetting up and down after which 700 µl of the mixture was transferred into an RNeasy® spin column placed in a 2 ml collection tube. The mixture was centrifuged at 8000 x g for 15 seconds using a refrigerated Eppendorf centrifuge (Eppendorf 5417R), followed by three washing steps. Firstly 700 µl buffer RW1 was added and centrifuged for 15 seconds at 8000 x g. Secondly 500 µl of buffer RPE was added and centrifuged at 8000 x g for 15 seconds and finally another 500 µl of RPE buffer was added and centrifuged at 8000 x g for 2 minutes. A volume of 40 µl of RNase-free water was finally used to elute the RNA. The RNA was quantified in a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) at 260 nm. The RNA purity was confirmed as a 260/280 nm ratio above 1.8 and the integrity was verified by agarose gel electrophoresis. The extracted RNA was stored at -70 °C until used.

2.2.3 Characterization of BVDV Type1/2 by reverse transcriptase real-time PCR (rtRT-PCR)

The Qiagen Cador BVDV Type 1/2 rtRT-PCR Kit (QIAGEN GmbH, QIAGEN Strasse 1, D-40724 Hilden) was used for the differentiation of BVDV genotypes 1 & 2 and border disease virus (BDV). The test was standardized in the laboratory on a LightCycler® V2.0 (Roche Diagnostics, Mannheim, Germany) machine, with minor variations from the published Applied Biosystems ABI-PRISM 7900HT Real-Time PCR System recommended by the kit. The Qiagen Cador BVDV Type1/2 rtRT-PCR master mix contains reagents and enzymes for the reverse transcription and specific amplification of the highly conserved 5′NCR region of the BVDV and BDV genomes. The amplicons are detected by measuring the FAM™ (BVDV genotype 1), and JOE (BVDV genotype 2) signals in channels 530 nm and 640 nm of the Lightcycler® Real-Time PCR system, respectively. The field samples were analyzed against the standardized positive (genotypes 1 and 2) and negative controls from the Qiagen Cador BVDV Type 1/2 rtRT-PCR kit. Genotype 1 and 2 controls could both be detected at 530 nm wavelength but not distinguished. Genotype 2 was exclusively detected by the JOE hydrolysis probe in the 640 nm channel.

The rtRT-PCR was initially performed in a final volume of 50 µl containing 38 µl of BVDV master mix (kit), 2 µl BVDV Mg-sol (commercial name from kit) and 10 µl of extracted RNA. It was then optimized for the 25 µl reaction using 19 µl of BVDV master mix, 1 µl of BVDV Mg-sol and 5 µl of the template. The following reaction steps were used: the RNA was transcribed to cDNA at 50 °C for 30 minutes followed by a denaturation cycle at 95 °C for 10 minutes. The amplification programme included 45 cycles of three steps each comprising of denaturation at 95 °C for 30 seconds, primer annealing at 60 °C for 1 minute and product extension at 72 °C for 10 seconds where after the results were recorded.

2.2.4 Sequencing

To confirm the genotypes of the different BVDV isolates, strains were randomly selected and sequenced. A cDNA synthesis from extracted RNA was first performed using the GeneAmp® Gold RNA PCR reagent kit (Applied Biosystems) as specified by the manufacturer. An oligo $d(T)_{16}$ primer was used for cDNA synthesis in a reaction including 1 µl of the extracted RNA in 0,2 ml thin walled tubes in the GeneAmp PCR system 9700HT (Applied Biosystems).

PCR primers Forward F2 [5' CTC GAG ATG CCA TGT GGA C 3'] and Reverse PESTR [5' CTC CAT GTG CCA TGT ACA GCA 3'] (Letellier et al., 2003) were used for sequencing of an approximate 245 bp product. The PCRs were performed in 0,2 ml thin-walled tubes in the GeneAmp PCR system 9700HT (Applied Biosystems). The following reaction mixture was used: 0.2 µM oligonucleotide primer PESTR, 0.2 µM oligonucleotide primer F2, 2.5 µl cDNA, 12.5 µl High Fidelity PCR master mix (Roche Diagnostics, Germany) in a total volume of 25 µl. The PCR was performed for 40 cycles (one cycle = 30 seconds at 94 °C, 1 minute at 55 °C, 1 minute at 72 °C). The reaction had an initial denaturation step at 94 °C for 10 minutes and a final elongation step at 72 °C for 7 minutes. To remove from the amplicon all primers, nucleotides, enzymes, salts, and other impurities that may interfere with the sequencing reaction, the 245 bp amplicons were purified using the QIAgen PCR purification kit (QIAgen) and the concentration of the purified products were determined by spectrophotometry using a NanoDrop® ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA).

Sequencing reactions were performed by Inqaba Biotechnology (Pretoria, South Africa) using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and the samples were subjected to automated sequencing using an ABI Prism model 3130 sequencer (ABI Advanced Biotechnological institute, Perkin-Elmer Corporation, Foster City, USA). The nucleotide sequence comparisons and phylogenetic analysis were done using the Staden software and analyzed data was subjected to BLAST analysis using the NCBI website www.ncbi.nlm.nih.gov .

2.2.5 Phylogenetic analysis

Nucleotide sequences used for phylogenetic analysis were aligned using Clustal X [29]. The reliability of the phylogenetic tree obtained from the 5′NCR region was evaluated by running 1000 replicas in the bootstrap test and a consensus tree was plotted, using classical swine fever virus strain HLJ (08) as an out-group. The nucleotide sequences derived from 245 bp of the 5′NCR gene region were obtained for 20 isolates, but only 12 viruses showed good sequences from cattle that originated from different feedlots across the region were used in the phylogenetic analysis. The isolates were randomly selected from the total of 103 BVDV positive samples to be representative of feedlots in the different regions. Sequences were aligned and compared to the corresponding region of sequences of pestiviruses of bovine, porcine and ovine origin and vaccine strains as published by other authors and listed in GenBank. These include *inter alia* BVDV genotype 1, NADL and Osloss as reference strains from GenBank and the following vaccine strains: Pfizer vaccine strain reference number 53637UTR, Oregon C24V, Singer and BVDV genotype 2 strain 296nc.

3. Results

3.1 detection and characterization of BVDV Types 1 & 2 by RT real-time PCR

The presence of BVDV in tissues of feedlot cattle was demonstrated by rtRT-PCR using a commercial Qiagen Cador BVDV Type1/2 Kit. A total of 276 RNA samples were tested and results are summarized in Table ii. Ninety one of 216 (42%) lymph node/spleen samples, 11 of 59 (19%) trans-tracheal aspirates and one blood sample were positive by nucleic acid detection using real-time PCR.

Positive samples were obtained from 63 of 103 (61.2%) animals that showed respiratory signs prior to death or sampling. Acute deaths occurred in 23 of 103 (23.3%) of animals, animals with enteric signs totaled 12 of 103 (11.7%) and those with a shock lung appearance 5 of 103 (4.8%). The disease conditions associated with cattle from which viral nucleic acid was detected is presented in Table iii.

The geographical origin of the cattle sampled and the genotypes of BVD virus obtained during this study are presented in Table iv.

The amplification curves for both genotype 1 and 2 were observed at 530 nm when using the FAM hydrolysis probe were (Figure 1). However, when JOE hydrolysis probe was used the result observed at 640 nm could distinguish between genotype 1 and 2 (Result not shown).

3.2 5'NCR sequence analysis

The nucleotide sequences derived from part of the 245 bp 5'NCR of the 12 selected BVDV strains detected in this study was compared to sequencing data obtained from GenBank using GAP 4 of the Staden Package.

A phylogenetic tree was generated from BVDV genotype 1 isolates (FS160, FS50, FS138, DGV2, NC3, FS38 and KZN608) by comparative alignment of sequences of the 245 bp of the 5′NCR of the BVDV genome, using the Clustal X package [29]. The numbers on each branch represent the number of times the group or subgroup was picked in 1000 re-runs in the bootstrap analysis (Figure 2). In figure 2 the phylogenetic analysis of these field strains are compared to reference strains of pestiviruses (Osloss accession number AJ558196; Singer accession number L32875B; NADL accession number AF3039181) and to sequences from previously published work on BVDV isolates from South Africa [3]. The branch distances were calculated using the neighbor-joining method. The virus types identified were phylogenetically discriminated into two distinct groups, namely Groups A and B, within BVDV genotype 1 group. Group A isolates (FS160; FS50; FS138; DGV2; NC3) clustered with BVDV strain Osloss (accession number AJ558196) and BVDV isolate 23-15 (accession number AF298059) classified as subgroup Ib [3], whereas, group B viruses (KZN608; FS38) clustered with strain S-ALT7/K (accession number U97470), Singer (accession number L35852) and NADL (accession number AF039181) under subgroup Ia [3].

Results of the phylogenetic analysis of BVDV genotype 2 isolates (FS161; FS164; FS170; FS175; FS208) compared to reference strains and vaccine strains obtained from Genbank are shown in figure 3. The phylogenetic tree was generated based on a comparison of a 245 bp section of the 5′NCR of the BVDV

gene. The distances were also calculated using the neighbor-joining method. The field isolates analyzed were phylogenetically discriminated and clustered with reference strain 890 accession number L32886 and a pestivirus type 2 strain (accession number AF039180) [7] separately from the vaccine strains. The field strains did not cluster with vaccine sequences (Figure 3 indicated in green, the Pfizer vaccine strain reference number 53637UTR) obtained from GenBank.

4. **Discussion**

The presence or absence of genotype 2 BVD viruses in cattle in South Africa have been the subject of discussion since the 1990s when the first genotype 2 viruses were described in North America. Limited studies of the genetic heterogeneity of local BVDV strains have so far only revealed the presence of genotype 1 viruses. Interest in this topic also came from private practitioners who were provided with marketing material recommending vaccination with vaccines containing both genotypes following the marketing authorization of the first attenuated BVDV 2 vaccine in South Africa (Titanium® 5, Virbac RSA (Pty) Ltd) in 2004.

Several real-time RT-PCR assays have been used to detect and genotype BVDV viruses. The Cador BVDV real-time PCR kit from Qiagen (Hilden, Germany) was developed for detection and genotyping of bovine pestiviruses [19]. The kit uses primers directed at the 5′NCR of the genome. This region has been shown to be highly conserved among the four pestivirus species [20] and the 5′NCR is suitable for common primer design. It also contains less homologous regions suitable for design of species-specific probes [4].

The detection of both BVDV genotype 1 and 2 can also be accomplished using the Cador kit with modifications. Separate probes must be used to distinguish between the two genotypes. Detection of BVDV genotype 1 was accomplished using the FAM labelled hydrolysis probe and similarly BVDV genotype 2 detection was accomplished with the use of the JOE labelled hydrolysis probe.

Bovine viral diarrhoea viruses were first identified within the pestiviruses based on sequence comparison of the 5′NCR [22, 26]. Later, hybridization and more extensive sequence analysis revealed that the 5′NCR was highly conserved in BVDV genomes. This led to the suggestion that the 5′NCR region might not serve

as a good target sequence for phylogenetic studies because of its highly conserved nature [5]. However, numerous investigators demonstrated that the 5′ NCR region provides a useful tool in genotyping BVDV isolates [6, 32, 22, 26]. The hypervariable region 2 of the 5′ NCR was also used for phylogenetic analysis in this study. Nucleotide sequences of the 5′ NCR of 12 viruses obtained from cattle that originated from different feedlots in the country were used together with sequences obtained from GenBank.

Among the 103 BVD viruses detected in this study, 85 were classified as genotype 1 and 18 as genotype 2. The 18 genotype 2 viruses that were obtained were detected in cattle originating from different regions within the country. However, the majority of the samples (n=197) were obtained from cattle during their feeding period at Karan Beef feedlot in Gauteng Province. It is reasonable to assume that most of the animals became infected either during transport over long distances, or during mixing with cattle from various regions after arrival. No association could therefore be inferred in terms of the geographic origin of the cattle and the genotypes of the virus. Detection of genotype 2 BVDV during this study represents the first documented evidence of its presence in cattle on the African continent.

The origin of BVDV 2 in South African cattle is not clear. The most realistic explanation would be that it might have been due to introduction of infected animals from other countries where outbreaks have been recorded. It is also possible that the sample sizes used in previous studies by Baule et al., [3] and Van Vuuren, [30] were too small to detect genotype 2 BVDV. A concern that requires explanation is the question whether genotype 2 viruses detected in South Africa may have been of vaccine origin when cattle were vaccinated during processing shortly after arrival at the feedlot. Two commercial vaccines that contain attenuated type 2 viruses are registered in South Africa for use in cattle, namely Titanium (Virbac) and Bovishield (Pfizer). These two products are widely used in the cattle industries in South Africa. Sequencing revealed that the Virbac vaccine strain 296 and Bovishield (Pfizer) are not closely related to the five genotype 2 viruses sequenced in this study (Figure 2). Lack of evidence for the type 2 viruses to be of vaccine origin was further supported by the fact that TTAs were collected from live cattle that had been in the feedlot for at least 2 weeks before diagnosis and treatment.

Similarly, samples collected from dead cattle all had a history of chronic, advanced pneumonia, or had been in the feedlot for longer than 30 days when they died or were sacrificed. More importantly, the BVDV genotype 2-containing Pfizer vaccine was not used at Karan Beef feedlot (where all the BVDV genotype 2

viruses were detected) prior to the beginning of 2010. Before 2010, the older Pfizer Bovishield 4 vaccine that does not contain genotype 2 was used. It is therefore reasonable to assume that the genotype 2 virus detected in necropsy samples were not of vaccine origin.

Bovine viral diarrhoea virus infection in cattle has no pathognomonic clinical signs. From the results obtained involvement of the respiratory system, animals that died acutely and gastro-intestinal system involvement predominated. A previous study completed in South Africa reported that BVDV was mostly identified in outbreaks of respiratory disease, usually in association with other pathogens [15].

In conclusion, the present study revealed the presence of BVDV genotype 2 in cattle in South Africa. Based on the high sequence similarity supported at a confidence level of 81.5% by the bootstrap analysis between the genotype 2 field strains with strain 890 of North American origin (fig 3), it may reflect the introduction of the virus in the country from animals imported from the northern hemisphere. The presence of genotype 2 viruses that phylogenetically belong to different clusters and coexist in feedlots is consistent with the possibility of multiple virus introductions. Movement of cattle and/or the use of contaminated equipment and infected products such as needles, gloves and semen could be responsible for more than one introduction of the virus [3].

There is continuing interest in control strategies for BVDV infection in cattle. The rapid and reliable diagnosis of both persistently and acutely infected cattle is imperative. The use of real-time RT-PCR methods to establish the presence or absence of BVDV RNA in cattle is contributing meaningfully to diagnostic screening and control strategies [33].

Acknowledgements

Funding for the research was provided by Pfizer® South Africa, Vision pharmaceuticals, South Africa, Design Biologix, South Africa..

References

1. Baker JC (1990) Clinical aspects of bovine virus diarrhoea virus infection Rev. Sci. Tech., 9 (1) 25-41.
2. Baker JC (1995) The clinical manifestation of Bovine Viral Diarrhoea Infection, Vet. Clin. of Nrth Ame: Food Ani. Prac., 11:425-445.
3. Baule C, Van Vuuren M, Lowings JP, Belák S(1997) Genetic heterogeneity of bovine viral diarrhea viruses isolated in Southern African. Vir. Res., 52: 205–220.
4. Baxi M, Mcrae D, Baxi S, Greiser-Wilke I, Vilcek S, Amoako K, Deregt D, (2006) A one-step multiplex real-time RT-PCR for detection and typing of bovine viral diarrhoea viruses. Vet. Microbiol., 116: 37-44.
5. Becher P, Orlich M, Shannon AD, Horner G, Konig M, Thiel HJ(1997) Phylogenetic analysis of pestiviruses from domestic and wild ruminants. J. of Gen. Virol., 78: 1357-1366.
6. Beer M, Wolf G, Kaaden OR (2002) Phylogenetic analysis of the 5′ -untranslated region of German BVDV type II isolates. J. of Vet. Med. B. Infect. Dis. Vet. Pub. Hlth., 49: 43-47.
7. Bolin, Ridpath (1992) Difference in virulence between two noncytopathic bovine viral diarrhoea viruses in calves. Ame. J. of Vet. Res. Vol.53(11).
8. Boye M, Kamstrup S, Dalsgaard K (1991) Specific sequence amplification of bovine virus diarrhea virus (BVDV) and hog cholera virus and sequencing of BVDV nucleic acid. Vet. Microbiol., 29: 1-3.
9. Brock K V (1995) Diagnosis of bovine viral diarrhoea virus infection. Vet. Clin. of Nrth. Ame. food Anim. pract: 11(3) 549-561.
10. Brownlie J (1990) The pathogenesis of bovine virus diarrhoea virus infection. Rev. Sci. Tech., 9 (1) 43-59.
11. Carman S, Van Druemel T, Ridpath J, Hazlett M, Alves D, Dubovi E, Tremblay R, Bolin S, Godkin A, Anderson N (1998) Severe acute bovine viral diarrhoea in Ontario, 1993-1995. J. of Vet. Diag. Invest., 10(1) 27-35.

12. David, GP, Crawshaw TR, Gunning RF, Hibberd RC, Lloyd GM, Marsh PR (1994) Severe disease in adult diary cattle in three UK diary herds associated with BVD virus infection Vet. Rec., 134(18)468-472.
13. De Moerlooze L, Lecomte C, Brown-Shimmer S, Schmetz D, Guiot C, Vandenbergh D, Allaer D, Rossius M, Chappuis G, Dina D, Renard A, Martial JA (1993) Nucleotide sequence of the bovine viral diarrhea virus Osloss strain: comparison with related viruses and identification of specific DNA probes in 5′ untranslated region. J. of Gen. Virol., 74: 1433-1438.
14. Hofmann MA, Brechtbühl K, Stäuber N (1994) Rapid characterization of new pestivirus strains by direct sequencing of PCR- amplified cDNA from the 5′ noncoding region. Arch. of Virol., 139: 217-229.
15. Kabongo PN (2001) The isolation and characterization of bovine viral diarrhoea viruses from cattle in South Africa. Dissertation University of Pretoria, Pretoria.
16. Letellier P, Kerkhofs P, Wellemans G, Vanopdenbosch E(1999) Detection and genotyping of bovine diarrhoea virus by reverse transcription polymerase chain amplification of the 5′ untranslated region. Vet. Microbiol., 64: 155-167.
17. Letellier C, Kerkhofs P, (2003) Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhoea virus. J. of Virol. Methods., 114: 21-27.
18. Liebler E, Johannsen U, Pohlenz J (1995) The haemorrhagic form of acute bovine virus diarrhoea. Literature Review and Case Report. Tierarzti prac. 23 (1) 18-25.
19. Liu L, Xia H, BelÅk S, Baule C (2008) A TaqMan real-time RT-PCR assay for selective detection of atypical bovine pestiviruses in clinical samples and biological products. J. of Virol. Methods., 154: 82-85.
20. Meyers G, Thiel HJ (1996) Molecular characterization of pestiviruses. Advan. Virus Res., 47: 53-118.
21. Olafson P, Maccallum AD, Fox FH (1946) An apparently new transmissible disease of cattle. *Cornell Veterinarian* 36: 2205-213.

22. Pellerin C, Van Den Hurk J, Lecomte J, Tussen P (1994) Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virol., 203: 260–268.

23. Qi F, Gustad T, Lewis TL, Berry ES (1993) The nucleotide sequence of 5′-untranslated region of bovine viral diarrhea virus: its use as a probe in rapid detection of bovine viral diarrhea viruses and border disease viruses. Mol. Cell Prob. 7: 349-356.

24. Rebhun WC, French TW, Perdrizet JA, Dubovi EJ, Dill SG, Karcher L.F (1989) Thrombocytopenia associated with acute bovine virus diarrhoea infection in cattle. J. Vet. Intern. Med., 3 (1) 42-46.

25. Ridpath JF, Bolin SR, Katz J (1993) Comparison of nucleic acid hybridization and nucleic acid amplification using conserved sequences from the 5′ noncoding region for detection of bovine viral diarrhea virus. J. Clin. Microbiol., 31: 986±989.

26. Ridpath J F, Bolin SR, Dubovi E.J (1994) Segregation of bovine viral diarrhoea virus into genotypes. *Virol.,* 205: 66- 74.

27. Ridpath JF (1996) Sequence diversity and genotyping. In symposium on bovine viral diarrhoea virus. A 50 year review. College of Veterinary Medicine Cornell University: 39.

28. Stroffregen B, Bolin SR, Ridpath JF, Pohlenz J (2000) Morphologic lesions in type 2 BVDV infections experimentally induced by strain BVDV2- 1373 recovered from a ®eld case Vet. Microbiol., 77: 157-162.

29. Thomson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nuc. Acid Res., 25: 4876-4882.

30. Van Vuuren M (2006) A review of bovine viral diarrhea virus infection in livestock in Southern Africa. Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 2005 1, (9): http://www.cababstractsplus.org/cabreviews Online ISSN 1749-8848

31. Vilcek S, Herring AJ, Herring JA, Nettleton PF, Lowings JP, Paton DJ (1994) Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Arch. of Virol., 136: 309-323.

32. Vilcek S, Paton DJ, Durkovic B, Strojny L, Ibata G, Moussa A(2001) Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch. of Virol., 146: 99-115.
33. Young NJ, Thomas CJ, Collins ME, Brownlie J (2006) Real-time RT-PCR detection of bovine viral diarrhoea virus in whole blood using an external RNA reference. J. of Virol. Methods., 138: 218-222.

Figure Legends

Figure1: Amplification curves showing BVDV genotype 1 and 2 positive controls, several BVDV genotype 1 isolates (NC3, LD2, MV1 and MU2) and a negative control viewed at 530 nm with the use of the FAM hydrolysis probe.

Figure 2: Phylogenetic analysis of seven field strains of BVDV genotype 1 in relation to published South African (S-ALT7/K, S-ALT2/K, S-ALT1/K) and some other sequences of pestiviruses obtained from Genbank.

Figure 3: Phylogenetic tree showing the positioning of 5 field strains of BVDV genotype 2 (FS161, FS175, FS208, FS170, and FS164 in red) obtained during this study in relation to Pfizer vaccine strain reference number 53637UTR (in green).

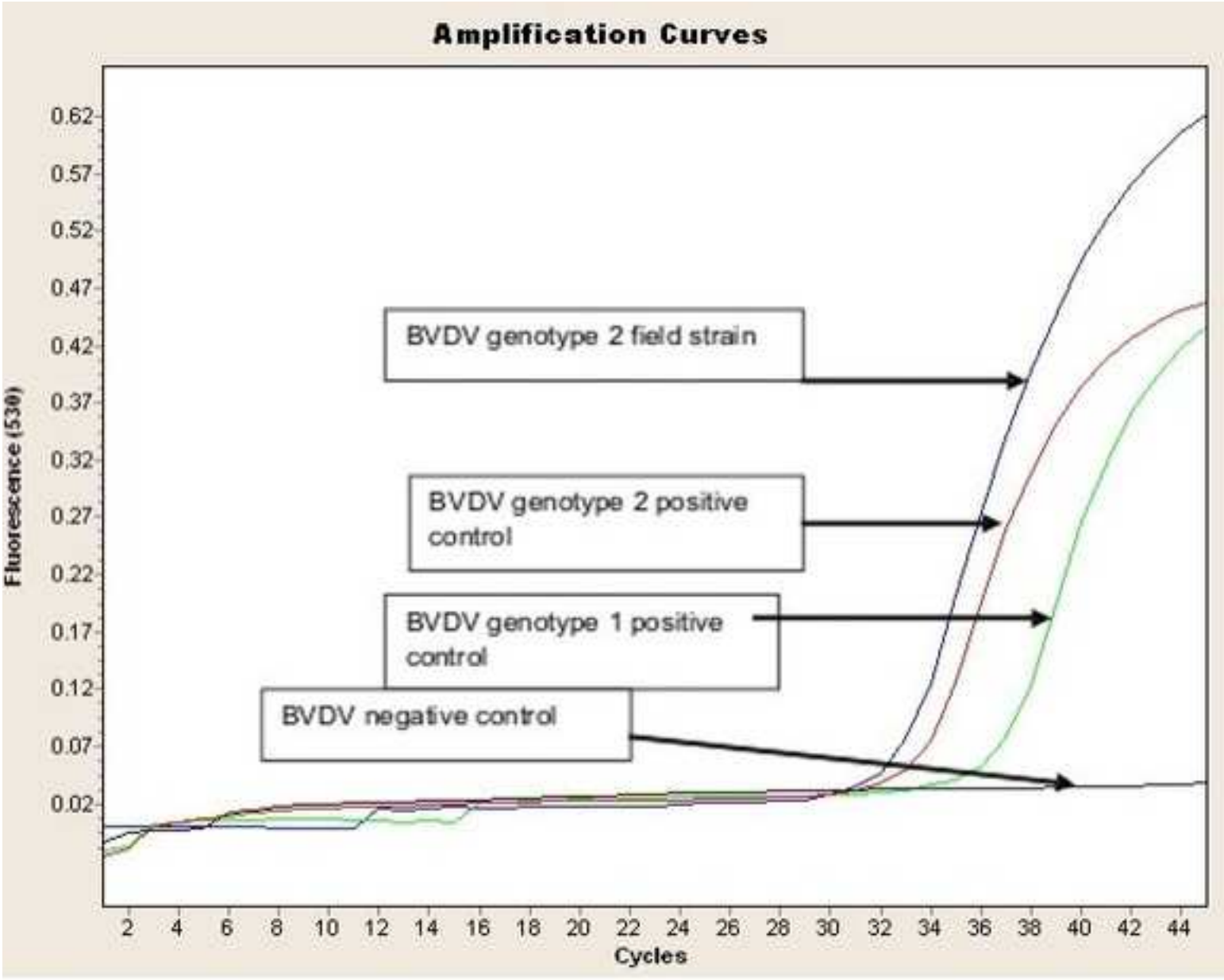

Figure 2a: **Amplification curve of a known BVDV genotype 2 strain (FS 208); a BVDV genotype 2 positive control; a BVDV genotype 1 positive control; and a BVDV negative control viewed at 530 nm using the FAM hydrolysis probe.**

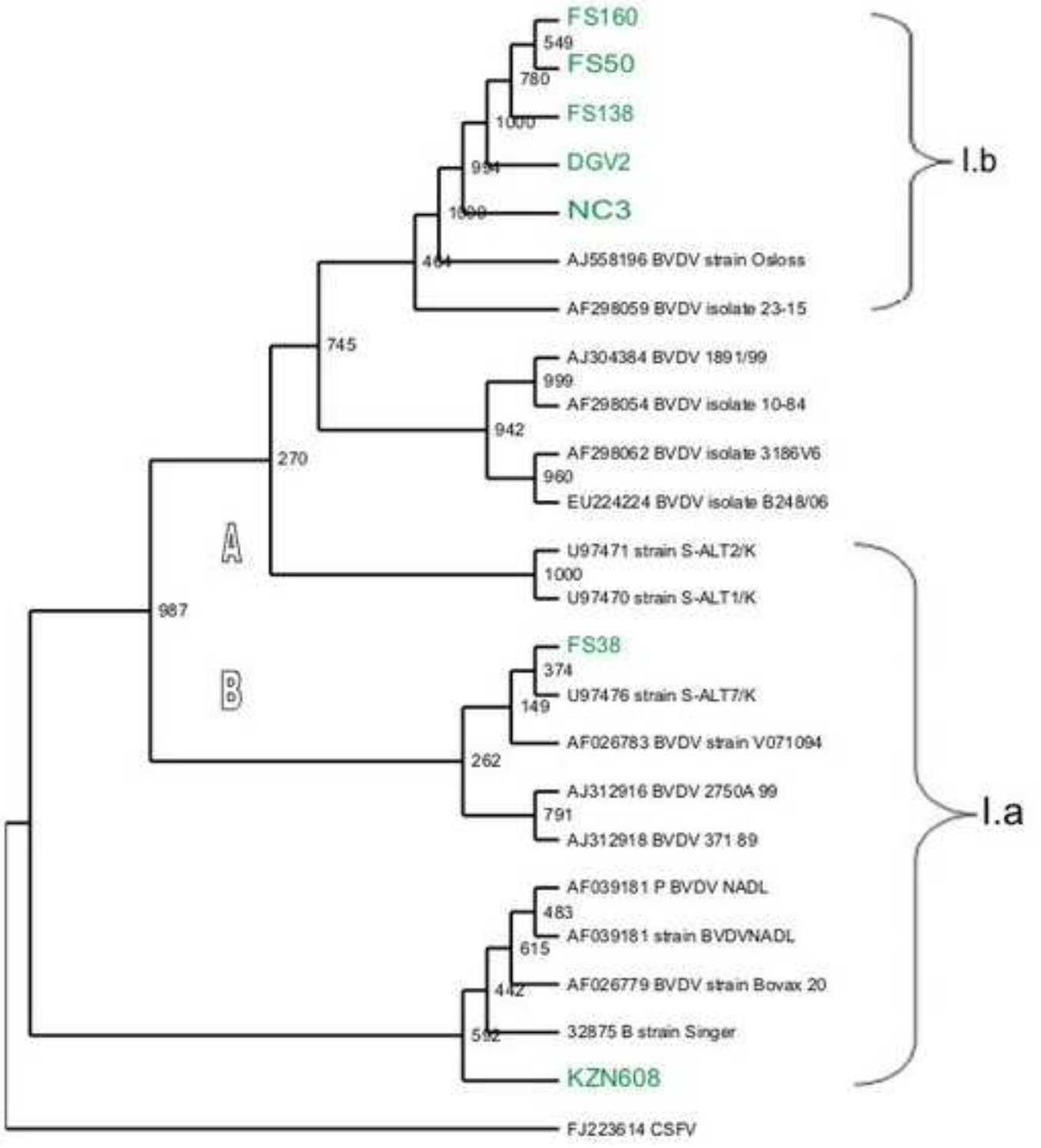

Figure 3: ***Phylogenetic analysis of*** **seven field strains of BVDV genotype 1 in relation to published South African (S-ALT7/K, S-ALT2/K, S-ALT1/K) and some other sequences of pestiviruses obtained from Genbank.**

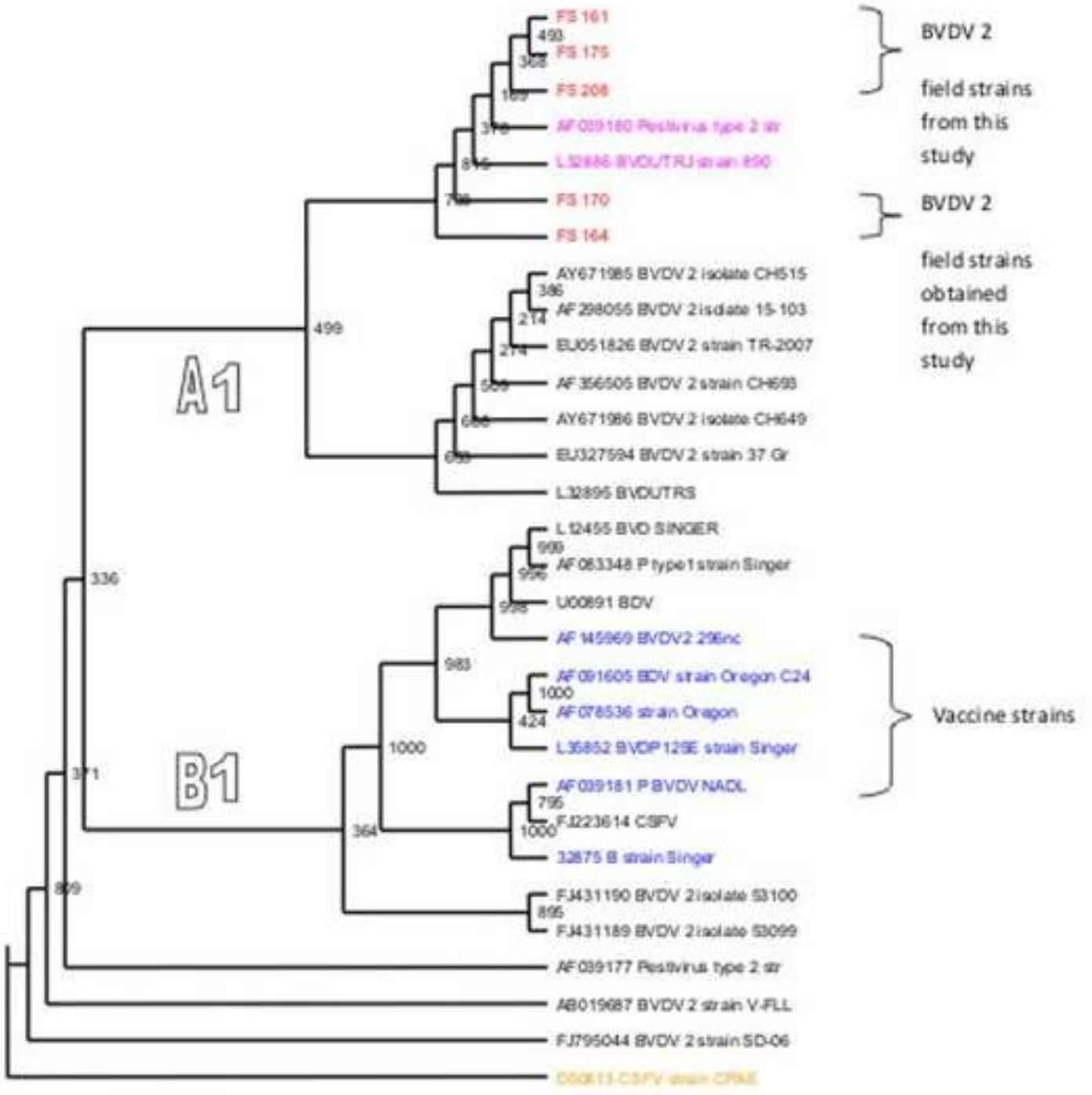

Figure 4: ***Phylogenetic tree showing the positioning of 5 field strains of*** **BVDV genotype 2 (FS161, FS175, FS208, FS170, and FS164 in red) obtained during this study in relation to published sequences of pestiviruses and**

Table i: **Main history/clinical signs and province of origin of the cattle from which specimens were collected**

History/clinical signs	KZN	NW	FS	EC	WC	LIM	NC	GA	Total
Respiratory signs	4	7	15	11	1	5	10	56	109
Digestive system signs (diarrhea)	1	2	2	4	0	1	1	0	11
Others (Acute death)	21	1	2	0	0	0	0	127	151
Shock lung appearance	0	1	1	1	0	0	1	1	5
Total	26	11	20	16	1	6	12	188	276

Abbreviations represent provinces within South Africa. KZN: KwaZulu-Natal; NW: North West; FS: Free State; EC: Eastern Cape; WC: Western Cape; LIM: Limpopo; NC: Northern Cape; GA: Gauteng.

Table ii: Number of samples analyzed and test results

Specimen	No. tested	No. positive genotype 1	No. positive genotype 2	Negative
Lymph node/Spleen	216	73	18	125
Trans-tracheal aspirates	59	11	0	48
Whole blood	1	1	0	0
Total	276	85	18	173

Table iii: Number of BVDV positive animals detected in relation to the predominant clinical signs

History/Clinical Signs	Number BVDV positive
Respiratory	63 (61.2%)
Enteric	12 (11.7%)
Shock lung	5 (4.8%)
Others (Acute death)	23 (22.3%)
TOTAL	103 (100%)

Table iv**:** Distribution of BVDV genotypes in relation to the Province of origin of the cattle from which the sample were collected

Location of origin	Genotype 1	Genotype 2	Total number of isolates
Kwazulu-Natal	15	1	16
Limpopo	12	1	13
Northern Cape	9	3	12
Eastern Cape	9	9	18
Western Cape	1	0	1
North West	9	2	11
Free State	18	2	20
Gauteng	12	0	12