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Genetic characterization of bovine viral diarrhoea viruses isolated from cattle in South Africa

BY

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Submitted in partial fulfillment of the requirements for the degree of

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DEDICATION

To the praise of the glory of His grace, by which he made us accepted in the Beloved.

Ephesians 1:6

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DECLARATION

I declare that this dissertation hereby submitted to the University of Pretoria for the degree of Magister Scientiae (Veterinary Science) has not been previously submitted by me for the degree at this or any other University, that it is my own work in design and in execution, and that all material contained therein has been duly acknowledged.

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LIST OF ABBREVIATIONS

AGID	Agar gel immunodiffusion
BD	Border disease
BVDV	Bovine viral diarrhoea virus
CCV	Canine coronavirus
CDNA	Complementary deoxyribonucleic acid
CF	Compliment fixation
Cp	Cytopathic
CSFV	Classical swine fever virus
EP2	Envelope protein 2
HC	Hog cholera
IETS	International embryo transfer society
IPX	Immunoperoxidase
MD	Mucosal disease
MLV	Modified live vaccine
NADL	National animal diseases laboratory
NCR	Non-coding region
Ncp	Non-cytopathic
NS	Non-structural protein
OD	Optical density
ORF	Open reading frame
OVI	Onderstepoort Veterinary Institute
OVAH	Onderstepoort Veterinary Academic Hospital
PCR	Polymerase chain reaction
PI	Persistently infected
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RTrt-PCR	Real-time reverse transcriptase polymerase chain reaction
SNT	Serum-virus neutralization test
VI	Virus isolation

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ABSTRACT

Bovine viral diarrhoea virus (BVDV) has emerged as one of the economically important pathogens in cattle populations with a worldwide distribution and causing a complex of disease syndromes. It is a single-stranded RNA virus of the genus *Pestivirus* in the family *Flaviviridae*. Two genotypes (1 and 2) of BVDV exist and can be distinguished on the basis of the 5′ non-coding region (5′ NCR) of the genome using real-time PCR. This technique is more sensitive, specific, less time consuming and has reduced risks of cross contamination of samples compared to a conventional PCR. 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 one blood sample) were collected from dead and living cattle. Pooled homogenates from the same animals were prepared and total RNA was extracted from 200 µl of the homogenates using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. 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. The PCR was performed on a Lightcycler® V2 (Roche Diagnostics, Mannheim, Germany) real-time PCR machine and the amplified products were detected via fluorescent dyes. 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, one blood sample and 11 trans-tracheal aspirates. Eighty five of the strains were genotype 1 strains and 18 were genotype 2. These results represent the first documented evidence for the presence of BVDV genotype 2 in South African cattle.

CHAPTER 1 : INTRODUCTION

Bovine viral diarrhoea virus (BVDV) has emerged recently as one of the pestivirus species that belong to two different genotypes known as BVDV1 and BVDV2 (Pellerin *et al.*, 1994; Ridpath *et al.,* 1994). One of the significant differences biologically between these two genotypes is the increased virulence observed among some BVDV2 isolates (Stroffregen, *et al.,* 2000). Initial reports of bovine viral diarrhoea virus described a clinically severe disease that was rarely fatal, highly contagious and characterized by fever, diarrhoea, mucosal lesions and leukopenia (Olafson *et al.*, 1946). Subsequent to these initial reports, veterinary references and reviews frequently described acute uncomplicated BVDV as a mild or sub-clinical disease of short duration with negligible mortality in all ages of cattle (Baker, 1990, 1995; Brock, 1995; Brownlie, 1990). Pathology studies of BVDV infection predominately focused on the establishment of persistent infections and the development of mucosal disease (MD). There is little information in the literature regarding the pathology associated with acute BVDV infections and that which is available is based primarily on research with BVDV1 isolates (Stroffregen, *et al.,* 2000). Recently, clinically severe disease outbreaks associated with acute, BVDV infection have been reported in the US, Britain, Canada and Germany (Carman *et al.,* 1998; David *et al.,* 1994; Liebler *et al.,* 1995; Rebhun *et al.,* 1989).

In North America these outbreaks of clinically severe disease were associated with infection with noncytopathic BVDV2 (Carman *et al.,* 1998; Pellerin *et al.*, 1994; Ridpath *et al.,* 1994). Infection with these highly virulent BVDV2 was associated with prolonged fever, leukopenia, severe thrombocytopenia and diarrhoea (Bolin and Ridpath, 1992; Corapi *et al.*, 1989). In 1993, widespread BVDV2 outbreaks resulted in unprecedented losses in dairy, beef and veal herds in Ontario, Canada (Carman *et al.,* 1998). Fever, pneumonia, diarrhoea and sudden death occurred in all age groups of cattle. Abortions were frequently observed in pregnant animals. In all during that period, 150 diary, 600 beef and 100 veal herds were affected with losses estimated at $40,000 ± $100,000 per herd (Stroffregen, *et al.,* 2000). This economically important pathogen of cattle can be characterized based on cell culture analysis, into cytopathic (cp) and non-cytopathic

(ncp) biotypes (Pellerin *et al*., 1994; Ridpath & Bolin, 1995). This is an important characteristic of the virus. It refers to the capacity of strains to produce cellular damage *in vitro* and not the behaviour of the virus in an animal (Ferreira *et al.,* 2000), and plays a pivotal role in the unique pathogenesis of mucosal disease.

In cattle, naturally acquired or experimentally induced primary infection with noncytopathic or cytopathic biotypes of BVDV are transient and usually result in clinically inapparent or mild disease (Bolin and Ridpath, 1992). Noncytopathic BVDV is isolated from cattle with naturally acquired infections more frequently than cytopathic BVDV, and is considered the predominant viral biotype (Bolin and Ridpath, 1992).

Cattle persistently infected (PI) with BVDV are the major virus reservoirs for the spread of the virus. Cattle become persistently infected when the virus infects the foetus transplacentally between approximately 40 & 120 days of gestation with non-cytopathic BVDV (Shimazaki *et al*., 1998). During this period, the foetal immune system is still developing and circulating BVDV is perceived as self-antigen, preventing the immune response from eliminating the virus. Persistently infected (PI) animals born alive are constantly producing and shedding large quantities of virus into the environment through nasal and oral secretions, urine and faeces. Congenitally infected calves do not always succumb shortly after birth, but may survive for years in a clinically healthy state, notwithstanding persistent infection (Bezek & Mechor, 1992).

Hog cholera (HC) or classical swine fever (CSF) virus was first reported in the nineteen thirties in the Mid-west of the USA (Moennig, 1990). The close antigenic relationship discovered between HC and BVD viruses by Darbyshire (1960) was the ultimate justification to group both viruses together using the term “pestiviruses” (Horzinek, 1973). Later the genus *Pestivirus* was officially recognized by the International Committee for the Taxonomy of Viruses.

The third pestivirus-mediated disease termed “border disease” (BD) of sheep was described by Hughes *et al*. (1959). Bovine viral diarrhoea virus, HCV and BDV were only classified as members of the family *Flaviviridae* during the early 1990s (Wengler *et al.,* 1995).

Bovine viral diarrhoea virus belongs to the genus *Pestivirus* that include border disease virus (BDV) of sheep and classical swine fever (CSFV). The virus classification was based on the host species that was infected (Letellier *et al.,* 1999). However, pestiviruses are able to cross the species barrier. Bovine viral diarrhoea virus can cross-infect cattle, sheep, goats, pigs and wild ruminants (Carlsson, 1991; Paton *et al*., 1992). Border disease virus is an ovine pathogen that occasionally infects pigs (Roehe *et al*., 1992; Edwards *et al*., 1995; Vilcek & Belåk, 1996). Differentiation between CSFV and other pestiviruses can be accomplished by the use of CSFV-specific monoclonal antibodies (Mabs) but the search for ruminant pestivirus-specific Mabs has failed due to the great antigenic diversity (Edwards *et al.,* 1991).

Virus isolation (VI) procedures for BVDV have been established using different cell culture formats, periods of incubation and serial passage of the inoculum. Since most field isolates of BVDV in samples from both healthy and sick animals are noncytopathic, the inoculated cells are routinely fixed after three to five days and examined for the presence of pestivirus antigens either by immunofluorescence or immunoperoxidase (IPX) staining (Sandvik, 2005). Although the microplate virus isolation assay (IPX-test) is generally regarded as the reference test for detection of BVDV, the sensitivity can be increased significantly by scaling up the volume of the sample under investigation, and by serial passaging before immuno-detection (Sandvik, 2005). For routine detection of PI animals, the standard IPX assay has proven to be useful for detection of BVDV in the sera of PI animals, in which the virus may be present in concentrations two to three logs higher than in acutely infected animals. Cytopathic and non-cytopathic BVDV biotypes are a characteristic of BVDV that describes how the virus interacts with cells in an artificial cell culture system. The cytopathic BVDV causes characteristic visible cell damage and death. Non-cytopathic BVDV does not cause visible cell damage and death.

Different serological assays have been used for BVDV antibody detection such as serum-virus neutralization (SN), agar gel immunodiffusion (AGID), complement fixation (CF), indirect immunofluorescence (IF), western blotting (WB) and various enzyme linked immunosorbent assays (ELISAs) (Sandvik, 1999; Saliki & Dubovi, 2004). With few exceptions, only two of these are currently in routine use in diagnostic laboratories - the serum-virus neutralization test and ELISA. The SNT is a biological *in vitro* system which quantifies the inhibitory effect of specific antibodies on virus replication in cell

cultures. The antibodies detected by the VNT are predominantly against the envelope protein E2, which may result in different titres depending on which virus strain is used in the assay (Sandvik, 2005). Two principally different ELISA formats are commonly in use; indirect (activity amplification) and blocking (competitive or activity modulation) assays (Tijssen, 1985; Schrijver & Kramps, 1998). In the indirect format, viral antigen is immobilized on the solid phase, to which specific antibodies and subsequently detecting enzyme-conjugated antiglobulins bind. A positive reaction is recognized by colour development in the substrate solution, which is read optically and reported as optical density (OD) values (Sandvik, 2005). In the blocking ELISAs, the presence of specific antibodies prevents the trapping of test antigen between a layer of immobilized captured antibodies and a reporting layer of enzyme-labeled antibodies, which also are virus specific (Sandvik, 2005). Thus, a positive sample causes a reduction of the OD, which is expressed as percentage inhibition, relative to the OD of a negative reference serum (Sandvik, 2005). Many kits of both versions are available commercially.

Reverse transcriptase polymerase chain reaction (RT-PCR) has been shown to be a rapid and sensitive technique for detection of viral nucleic acid and it has been used by various researchers for the detection of pestiviruses using oligonucleotide primers based on conserved regions of the viral genome (Letellier *et al.*, 1999). Most of the polymerase chain reaction (PCR) primers that were selected in the 5' non-coding region (5′NCR) recognized the greatest number of pestivirus isolates but failed to differentiate BVDV from other pestiviruses (Ridpath *et al.*, 1993; Vilcek *et al.*, 1994).

Based on the sequence comparison of 5′NCR, the BVDV isolates were subdivided in two genotypes. Sequence homology within each group was very high (>93%) while homology between group I and II dropped close to 74% (Pellerin *et al.,* 1994; Ridpath *et al.*, 1994). Over 800 ruminant pestiviruses from USA and Canada, and 28 porcine pestiviruses from North America, South America, Europe and Asia were compared (Ridpath *et al.,* 1996). From this analysis the viruses isolated from pigs were similar to the HCV reference strain Alfort (HCV genotype) and isolates from small ruminants were similar to the reference strain BD-31 (BDV-genotype). Several isolates from cattle were similar to the BVDV reference strain NADL (BVDV 1 genotype) and others, including a few from small ruminants were similar to strain 890 (BVDV 2 genotype) (Pellerin *et al.*, 1994; Ridpath *et al.*, 1996).

Bovine viral diarrhoea virus infection was classified into syndromes based on the predominant clinical manifestation: reproductive disease comprising abortion, repeat breeding, stillbirth, birth of weak calves; acute/peracute BVD in animals of all ages often resulting in death; classical BVD manifested by gastroenteritic disease, pyrexia and respiratory disease; haemorrhagic syndrome with bloody secretions and petechial haemorrhages; MD characterized by gastroenteritis, digital erosions and ulcers; and respiratory disease including pyrexia, bronchopneumonia and weakness (Van Vuuren, 2006).

The occurrence of genotype 2 in North America where genotype 1 vaccination has been widely practiced raised the question whether the current generation of vaccines would cross-protect against both types (Pellerin *et al.,* 1994). However, several studies have been conducted to assess the ability of inactivated and modified-live vaccines (MLV) to protect foetuses against either natural or experimental exposure to BVDV (Ficken *et al*., 2006). Results of further studies have indicated that monovalent BVDV vaccines containing only a type 1 BVD strain are capable of conferring a good level of cross-protection against clinical disease caused by type 2 BVDV (Ficken *et al*., 2006). More recently, modified- live bivalent BVDV vaccines containing types 1 and 2 BVDV strains have been associated with a higher degree of foetal protection against type 2 BVDV than that achieved by use of the aforementioned monovalent type 1 BVDV vaccine. However, the results of bivalent BVDV vaccine administration were not compared with the effects of the same vaccine containing only the type 1 BVDV strain (Ficken *et al*., 2006). The impact of BVDV on bovine health has gradually become more apparent, and control programmes aiming at eradicating BVDV have consequently attracted increasing interest, compared to the BVDV management by vaccination (Brock, 2003).

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 through virus isolation and correlation with clinical disease are available (Van Vuuren, 2006). The first isolates that were obtained in the early 1970s, were antigenically similar to the prototype Oregon (C24V) strain, and the NADL strain, and were isolated from diseased cattle with enteric or respiratory signs (Van Vuuren, 2006). Isolates of different strains have been isolated at Allerton Laboratories in Pietermaritzburg, the Onderstepoort Veterinary Institute (OVI) and virology laboratory of the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Science, University of

Pretoria during the last decade (Kabongo, 2001). Serological surveys conducted in South Africa during the mid-1990s indicated a seroprevalence in both dairy and beef cattle of 66.02, 62.49 and 60.31% for the years 1994, 1995 and 1996 respectively, with the seroprevalence in individual herds varying from 26-81% (Gerdes, 1997). In Zimbabwe, a similar survey in the mid-1990s that included sera from cattle from several provinces yielded a seroprevalence of 79.2% (Muvavarirwa *et al*., 1995).

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 its different strains and the economic importance of the BVD/MD complex (Kabongo, 2001). The presence of BVDV genotype 2 has been suspected by some veterinarians in South Africa based on clinical signs compatible with the described haemorrhagic syndrome (Pellerin *et al.,* 1994; Ridpath *et al.,* 1996). However, early studies by Baule *et al.* (1997) could not confirm the presence of genotype 2. Considering the implications of the genomic diversity in the diagnosis, epidemiology and control of BVDV infections it is important to characterize the BVD viruses occurring in the region (Baule *et al.,* 1997). Since this statement was made in 1997, no effort has been made to characterize the strains of BVDV strains in South Africa. Updated information may assist *inter alia* feedlot veterinarians doing risk assessments for vaccination programmes, and contribute to a better understanding by production animal veterinarians of the epidemiological profiles of the type of strains present and the infections they cause.

The aims of this research project were:

- To investigate the genetic heterogeneity of BVDV in southern Africa.
- Molecular characterization of BVDV isolates obtained from feedlots in southern Africa using a real-time reverse-transcriptase PCR.
- To sequence part of the genome of the virus and to phylogenetically group the viruses by comparison with existing sequencing data available on GenBank.
- To provide information for effective strategies for the control of BVDV infection in southern Africa.

CHAPTER 2 : LITERATURE REVIEW

2.1 Virus characteristics

Bovine viral diarrhoea virus is a member of the genus *Pestivirus* in the family *Flaviviridae* and is closely related to classical swine fever and ovine border disease viruses (Donis, 1995). Two genetically distinct genotypes of BVDV exist, namely types 1 and 2, with further subdivisions discernable by genetic analysis (Vilcek *et al.,* 2001). The genome of pestiviruses consists of a single-stranded, positive-sense RNA molecule, with a typical length of 12.3 kbp, comprising one large open reading frame (ORF) which encodes about 4000 amino acids (Baule *et al.,* 1997). The polyprotein is subsequently cleaved into the structural and non-structural proteins by viral and cellular proteases. 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) (Meyers & Thiel, 1996). 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. It has, therefore, been the target region when studying differences between and within pestivirus species (Boye *et al.,* 1991; De Moerlooze *et al.,* 1993; Qi *et al.,* 1993; Ridpath *et al.,* 1993; Hofmann *et al.,* 1994). The 5′NCR of pestiviruses is composed of highly conserved regions intercalated by three variable regions, termed 1, 2 and 3 (Deng & Brock, 1993). These regions are located in positions corresponding to nucleotides 1-73 (1), 209-223 (2) and 284-323 (3) in the genome of the National Animal Diseases Laboratory (NADL) reference strain (Baule *et al.,* 1997). The nucleotide substitution resulting in differences between strains are located within these variable regions, and are largely of the covariant type to preserve RNA secondary structure (Deng & Brock, 1993). Pestiviruses are among the smallest enveloped animal RNA viruses, about 40 nm in diameter and possess an icosahedral nucleocapsid (Moennig, 1990).

Bovine viral diarrhoea virus genotypes can be differentiated from each other, and from other pestiviruses, by the use of monoclonal antibodies (MAbs) directed against the envelope proteins E2 and E^{RNS} or by genetic analysis (Pellerin *et al.,* 1994; Ridpath *et al.,* 1994). Classification of BVDV isolates on the basis of nucleotide sequences of the viral genome became a standard procedure, especially following the identification of BVDV genotype 2 in North America in the early 1990s (Van Vuuren, 2006). Bovine viral diarrhoea virus isolates were segregated into two genotypes based on phylogenetic analysis of the nucleotide sequences (245nt) of the 5′-non-translated region (5′-NCR) of the viral genome (Van Vuuren, 2006). Genetic analysis of BVDV in southern Africa, by means of analysis of the 5′-NCR, revealed that all strains tested so far belong to the genotype 1 subgroup of BVDV (Van Vuuren, 2006). The southern African isolates were originally described as belonging to four groups (1a-1d) (Baule *et al.,* 1997), but a subsequent study has shown that BVDV type 1 isolates have evolved from a common ancestor in at least 11 genetic directions, and that a selected group of southern African strains could be divided between six of the 11 groups (Van Vuuren, 2006). These findings suggest that extensive genetic diversity can be found within BVDV type 1 isolates from southern Africa.

Genotype 1 (BVDV 1) is represented by the reference strains NADL and Osloss and involves the majority of BVDV strains. The 890 strain represents BVDV genotype 2 and the isolates are mainly associated with a haemorrhagic syndrome in cattle, a form of peracute infection described in North America (Baule *et al.,* 1997). A similar outbreak occurred in Canada in early 1993, when Ontario reported multiple herds with peracute disease and high death loss in young and adult cattle (Anonymus, 1994). Acute and peracute non-mucosal clinical manifestations appeared to be associated with a BVD virus that had major genomic differences from the virus that causes classic BVD (“JAVMA” 1994). Researchers at the National Animal Disease Center of the USDA Agricultural Research Service have tentatively labeled the one genomic form classic BVD type 1, and the other type 2 (Anonymus 1994).

The genus *Pestivirus* was believed to have three species on the basis of the natural transmission between various animal hosts that include BVDV, BDV and CSFV. The classification based on host origin may no longer be appropriate; the current classification which is still under discussion, divides the genus into four genotypes: genotype 1 includes the BVDV 1 strains, mainly of cattle origin; genotype 2 includes

isolates of HCV; genotype 3 includes sheep and pig isolates with characteristics of "true BDV" that share 71% nucleotide similarity with other pestiviruses (Kabongo, 2001). Border disease virus is different from BVDV, CSFV as well as other ovine and bovine pestiviruses currently referred to as BVDV type 2. The latter represents genotype 4 in the proposed classification (Sullivan *et al.,* 1997; Vilcek *et al.,* 1997). The classification was based on a panel of monoclonal antibodies supported by genetic sequencing and also SN tests with polyclonal sera (Becher *et al.,* 1995; Paton *et al*., 1995c; Vilcek *et al.,* 1997).

The nucleotide sequencing and nucleic acid hybridization has revealed the occurrence of heterogeneous strains among BVDV 1 (Kwang *et al.,* 1991; Lewis *et al.,* 1991; Ridpath & Bolin, 1991a, b). The practical significance of the heterogeneity among BVDV strains is still under assessment. However, it is considered to have implications for the design of broad reactive diagnostic assays based on serological and molecular methods (Kwang *et al.,* 1991; Lewis *et al.,* 1991; Ward & Misra, 1991) as well as the development of vaccines conferring protection against a wide range of strains (Bolin *et al.,* 1991; Ridpath *et al.,* 1994). The development of an effective strategy for the control of BVDV infections will rely on the knowledge of the type of strains present and the epidemiological profiles of the infections they cause.

The pestiviral ORF is translated into one polyprotein, which is processed co- and post-translationally in infected cells into the viral structural proteins C, E^{RNS}, E1 and E2 and the non-structural proteins N^{pro}, p7, NS2/3, (NS2, NS3), NS4A, NS4B, NS5A and NS5B (Kadir *et al.,* 2008). Differences exist in polyprotein processing among ncp and cp biotypes of BVDV. Both cellular- and virus-encoded proteinases are probably involved in protein processing. Candidate virus proteins possessing proteolytic activity for cytopathic BVDV are P^{20} and P^{80} (Kabongo, 2005). Cytopathic (cp) pestiviruses produce a lot of NS3, whereas non-cytopathic (ncp) pestiviruses only produce a small amount.

Both the cp and ncp-BVDV can cross the placenta and infect calves *in utero*. However, ncp BVDV can lead to immunotolerance if it crosses the placenta between 40 and 120 days of gestation which is not the case with cp strains. Depending on the stage of gestation the foetus may be reabsorbed, aborted or continue to develop (Mahony *et al*., 2005). If the development of the PI foetus continues the calf may be born with no

apparent clinical signs. The calf, however, will be persistently infected (PI) with BVDV and continue to shed the virus to susceptible animals (Roeder & Harkness, 1986). The PI calf may continue to develop to maturity but at some stage the ncp-BVDV may spontaneously mutate to the cp biotype, resulting in the onset of fatal mucosal disease (Mahony *et al*., 2005). It has now been established that mucosal disease occurs only when cattle that are born immunotolerant to, and persistently infected with a noncytopathic BVDV become superinfected with a cytopathic BVDV (Baker, 1995).

The envelope protein E2 is the main immunogen of pestiviruses. After pestivirus infection antibodies against E^{RNS}, E2 and NS3 are produced, but only E2 specific antibodies have neutralizing properties. For the development of a cellular immunity NS3 and NS4A are important (Sandvik, 2005).

2.2 Epidemiology

2.2.1 Distribution

Bovine viral diarrhoea virus is distributed worldwide and the causative agent of pre- and post-natal infections accounting for a variety of economically important syndromes (Letellier & Kerkhofs, 2003). Bovine viral diarrhoea virus was first recognized in the USA in 1946 and was associated with an epizootic of a fatal disease characterized by diarrhoea and erosions in the digestive tract. The presence of BVDV in southern African was also known since the early 1970s through serological surveys with only a few documented reports confirming its presence through virus isolation and correlation with clinical disease (Thomson & Blackburn 1972; Theodoridis *et al.,* 1973; Theodoridis & Boshoff, 1974).

2.2.2 Sources of infection

Bovine viral diarrhoea virus infections in different countries are generally the same but the significance may vary from one area to another depending on the farm structure and management systems. Infection with BVDV can result in a wide spectrum of clinical diseases ranging from subclinical infections to a highly fatal form known as mucosal disease (MD) (Baker, 1995). The clinical response to infection depends on multiple interactive factors. Host factors that influence the clinical outcome of BVDV infection include whether the host is immunocompetent or immunotolerant to BVDV, pregnancy

status, gestational age of the foetus, immune status (passively derived or actively derived from previous infection or vaccination), and concurrent level of environmental stress (Baker, 1995). In pregnant, susceptible animals pestiviruses are readily transmitted to the foetus. Depending on the age of the foetus and its immunological competence, the infection may lead to foetal death, malformations, persistent viraemia throughout gestation and postnatal life, or at least frequently in older ruminant foetuses, to a specific immune response with elimination of the virus. In all three host species which are cattle, sheep and pigs, persistently infected animals continuously shedding virus have a major impact on the epidemiology of the disease (Moennig, 1990).

2.2.3 Transmission

Pestiviruses are able to cross species barriers with relative ease. The most frequent means of transmission from one herd to another is the introduction of persistently infected animals into the herd. Infection may be direct through inhalation or ingestion of infected saliva, oculonasal discharges, urine and faeces. Respiratory infection is probably the most important route of direct transmission (Kabongo, 2001). Exposure of susceptible animals in places where cattle are congregated such as public stockyards and sale barns, especially PI animals that will shed large quantities of BVDV in secretions, will enable the transmission of the disease.

Apart from direct contact as a method of spread, BVDV can also be spread through indirect contact. Veterinarians and stockmen have been incriminated in the spread of the disease from farm to farm through contaminated fomites such as clothing or veterinary equipment. It can also be transmitted on gloves when the same glove is used to examine susceptible cattle immediately after a PI animal was examined (Lang-Ree *et al.,* 1994; Roeder, 1994). Experimentally, BVDV can be transmitted to susceptible animals by drenching them with infected faecal material or by inoculation with blood collected from animals during the viraemic stage or with a splenic emulsion from an animal destroyed during the febrile stage (Kabongo, 2001). Olafson *et al.* (1946) demonstrated that 5 ml of blood injected subcutaneously was regularly infective. Spread by means of the use of non-sterilized hypodermic needles among animals was described (Roeder & Harkness, 1986).

In pregnant, susceptible animals infection with pestiviruses is readily transmitted to the fetus. Infected semen, uterine secretions, amniotic fluid or placenta may cause transmission (Kabongo, 2001). Transmission of BVDV as embryo-associated viruses during embryo transfer has always been a concern. Although a body of published research work suggests that the International Embryo Transfer Society's (IETS) standard washing procedure is sufficient to remove BVDV from a zona pellucida-intact bovine embryo (Gregg *et al.,* 2009), other studies have demonstrated that a proportion of *in vivo* and *in vitro*-derived zona pellucida-intact embryos exposed to certain high affinity isolates of BVDV can retain infectious virus even after the embryos have been thoroughly washed (Gregg *et al.,* 2009). Since transmission is possible with *in vitro* fertilization one should only use animals certified BVDV free (Brownlie *et al.,* 1997).

Acutely infected animals are relatively inefficient transmitters of the virus due to the brief and limited viral excretion (Kirkland *et al.,* 1997). Insects may transmit BVDV under experimental conditions (Gunn, 1993). Biting flies can transmit BVDV to cattle and sheep. The virus can survive within biting and non-biting flies for 96 hours after they fed on PI cattle (Kabongo, 2001). Non-biting flies such as face flies can harbour the virus but it is not clear if they can transmit it (Gunn, 1993).

2.2.4 Host susceptibility

Natural pestivirus infections and disease do not only occur in cattle, but also in sheep, pigs, goats and a wide range of captive and free-living wild ruminants (Van Vuuren, 2006). The isolation and identification of BVDV or related pestiviruses in southern Africa from species other than cattle have not been documented until recently. An outbreak of border disease in 2001 in a flock of sheep in South Africa was confirmed by means of histopathology and virus isolation and identification (Van Vuuren, 2006). In addition, classical swine fever re-emerged in South Africa in 2005 following the feeding of contaminated swill off-loaded from a ship from South-east Asia.

Confirmation of the presence and effects of BVDV infections were also confirmed in other southern African countries (Muvavarirwa *et al.,* 1995; Baule & Banze 1994; Depner *et al.,* 1991). In addition, the role of wild animals in the epidemiology of BVDV in the region was examined. Antibodies to BVDV were found in the sera of wild animal species from southern Africa countries (Hamblin & Hedger 1979). Pestiviruses are

however, able to cross the species barrier. Bovine viral diarrhoea virus can cross-infect cattle, sheep, goats and pigs (Carlsson, 1991; Paton *et al.,* 1992). Border disease virus is an ovine pathogen that occasionally infects pigs (Letellier *et al.,* 1999).

Serological surveys have shown that many species of free-living ruminants in North America, Europe and Africa have a varying prevalence rate of pestivirus-reactive antibodies. Pestiviruses have been detected in free-living ruminants such as roe deer (*Capreolus capreolus*) (Baradel *et al.,* 1988), fallow deer (*Dama dama*) (Frölich, 1995), dromedary camel (*Camelus dromedaries*) (Hegazy *et al.,* 1996); African buffalo (*Syncerus caffer*) (Hamblin & Hedger 1979); giraffe (*Giraffa camelopardalis*) (Soine *et al.,* 1992) and wildebeest (*Connochaetes* spp.) (Doyle & Heüschele, 1983). However, the contribution of the virus to morbidity and mortality was uncertain in many cases.

2.3 Pathogenesis

An important aspect of the biology and pathogenesis of bovine viral diarrhoea virus (BVDV) is persistent infection that occurs following *in utero* infection of the bovine foetus (Brock *et al*., 1997). The development of persistently infected animals contributes significantly to the high prevalence of BVDV infections (Brock *et al*., 1997). Because persistently infected animals are a continuous source of virus, the identification and removal of persistently infected animals is an essential component of current prevention and control measures (Brock *et al*., 1997). The complex pathogenesis of pestivirus infections is manifested by a wide variety of clinical signs observed during BVDV infection, including digestive disorders, reproductive disorders and respiratory tract infections. Clinically BVD is a very diverse condition ranging from asymptomatic or mild and transient signs of upper respiratory tract infection to severe acute disease with signs associated with the digestive, haematopoietic, reproductive or respiratory organ systems, often exacerbated by super-infection with other pathogens (Sandvik, 2005).

Fatal mucosal disease (MD), one of the consequences of BVDV infection of cattle could not previously be reproduced experimentally. Later it was accomplished provided that cows infected during the first four months of gestation with ncp BVDV gave birth to healthy, persistently viraemic calves (Orban *et al.,* 1983; Leiss *et al.,* 1984; McClurkin *et al.,* 1984). Superinfection later in life with a homologous cp strain of BVDV will result

in the severe disease (Brownlie *et al.,* 1984a; Bolin, 1995). It has also been suggested that MD may be a consequence of the *in vivo* mutation of the ncp strain to a cp strain (Brownlie *et al.,* 1986; Howard *et al.,* 1987), which would partially explain the erratic and sporadic occurrence of MD in a cattle population. The erratic nature of MD is likely more the result of the fact that cp viruses are rare in nature and for an animal to become infected with homologous ncp and cp strains is therefore equally rare.

2.3.1 Acute BVDV infections

Most infections of animals with BVDV go unnoticed and are confirmed only on serological evidence. Viraemia occurs four to seven days (Brownlie *et al*., 1986) after infection and in some may persist up to 15 days (Duffel & Harkness, 1985) but is likely less in most cases. There is a specific antibody response which develops slowly, starting at about the second week and reaching a maximum around 10-12 weeks. Once immune, the animal appears to have life-long resistance to further disease caused by BVDV but may show evidence of subsequent exposures by seroconversions (Brownlie *et al.,* 1986). Systemic spread of infection may occur as free virus in serum or as virus associated with the cells in the blood; the monocytes and lymphocytes are generally regarded as being particularly permissive to BVDV infections (Bruschke *et al.,* 1998).

2.3.2 Persistent Infection

Cattle persistently infected (PI) with BVDV are the major virus reservoir for the spread of the virus. Cattle become persistently infected when exposed as a foetus between the first and fourth month of gestation with non-cytopathic BVDV (Shimazaki *et al*., 1998). During this period, the foetal immune system is still developing and circulating BVDV is distinguished as self-antigen, preventing the immune response from eliminating the virus. Persistently infected animals that survive are constantly producing and shedding virus into the environment through nasal and oral secretions, urine and feaces.

2.3.3 Mucosal disease

The experimental reproduction of fatal mucosal disease supports the hypothesis that both non-cytopathic and cytopathic BVDV are required for the pathogenesis of this disease (Brownlie *et al.,* 1984). The proposed mechanism for pathogenesis is that initial infection of the pregnant cow occurs with a non-cytopathic form of BVDV before 120 days of gestation and induces immunotolerance to the virus; this permits the virus to persist in the calf after it is born. Animals that survive this period can later (usually six to 24 months) be superinfected with a cytopathic strain of the same virus (Brownlie *et al.,* 1986). This virus grows unhindered by any immunity as a result of the tolerance and the animal invariably dies demonstrating diarrhoea and severe damage to the lining (the mucosa) of the gut.

Several genomes of BVD viruses isolated from animals with MD were sequenced. A linkage between RNA recombination, generation of NS3 and the onset of fatal MD was demonstrated (Tautz *et al.,* 1998). It has also been observed that early and late onsets of MD are the consequence of different pathogenic mechanisms (Fritzemeier *et al.,* 1997).

2.4 Clinical signs and economic effects with special reference to southern Africa

Bovine viral diarrhoea virus in cattle induces no pathognomonic clinical signs during infections. Various cases of BVDV infection were classified into syndromes based on the predominant clinical manifestation in the USA (Saliki, 1996). These important syndromes are reproductive disease comprising abortion, repeat breeding, stillbirth, birth of weak calves; acute/peracute BVD in animals of all ages often resulting in death; classical BVD manifested by gastroenteritic disease, pyrexia and respiratory disease; haemorrhagic syndrome with bloody secretions and petechial haemorrhages; mucosal disease characterized by gastroenteritis, digital erosions and ulcers; and respiratory disease including pyrexia, bronchopneumonia and weakness (Van Vuuren, 2006). Previous reports have shown that the association with enteric and respiratory disease pre-dominates.

The range and severity of clinical signs documented in cattle in southern Africa are similar to those described in other parts of the world, and emphasize the varied clinical manifestations that can be associated with BVD/MD. Enteric signs vary from mild diarrhoea to acute haemorrhagic diarrhoea, dehydration and death (Van Vuuren, 2006). Other clinical signs described include pyrexia, nasal discharge, decrease in milk production, anaemia, depression, reduced appetite, salivation (sometime blood-stained), varying degrees of erosion and ulceration of the buccal mucosa and tongue, varying degrees of bloat, and emaciation (Ferreira *et al.,* 2000; Kabongo & van Vuuren, 2004). In terms of the effects of BVDV infection on reproduction, a significant number of cows in some infected dairy herds that were confirmed by rectal palpation to be in the first trimester of pregnancy, subsequently returned to oestrus. Repeat breeders were evident in several herds with confirmed BVDV infections (Van Vuuren, 2006). Congenital malformation and abortion have not been associated with BVDV in the few studies that focused on the clinical effects of BVDV infections, but specimens from abnormal or aborted calves or foetuses are in general, seldom submitted to diagnostic laboratories in southern Africa. Respiratory signs followed by diarrhoea were the most frequent signs following investigations by Kabongo & Van Vuuren (2004) in southern Africa cattle.

Necropsies done on animals subsequently confirmed as having died from MD yielded lesions typical of the disease. These included *inter alia*, erosions and shallow ulcerations on the buccal mucosa, external nares and abomasum, congestion and petechial haemorrhages in the abomasum, and evidence of catarrhal enteritis with enlargement of mesenteric lymph nodes and Peyer's patches (Ferreira *et al.,* 2000).

2.5 Diagnosis

Diagnosis of BVDV infections based on clinical signs is not reliable because of its capability to manifest a variety of clinical signs and often serves as a challenge to the clinician (Kabongo, 2001). Bovine viral diarrhoea virus diagnosis in the USA was initially based on clinical signs and necropsy findings but was confused with winter dysentery and foot-and-mouth disease when severe mucosal lesions were present (Olafson *et al.,* 1946).

Early in 1957, Lee & Gillespie described the replication of a BVDV in cell culture without cytopathic effects and its response in cattle, and later Gillespie (1960) described the cytopathogenicity. Serum neutralization tests were also described during the same period.

2.5.1 Virus isolation

Bovine viral diarrhoea virus strains have been propagated in various cell cultures. Bovine foetal kidney (BFK) cell cultures were first used for isolation and propagation of cytopathic BVD viruses (Gillespie *et al.,* 1960) and to date are still extensively used. Other cells used are bovine foetal lung cells, testis cells, thymus cells, turbinate cells and tracheal cells (Kabongo, 2001).

Although the natural hosts of pestiviruses include only cloven-hooved ungulates, it has been established that pestiviruses can adapt *in vitro* in mammalian cells of heterologous species including canine, feline and primate cells (Bolin *et al.,* 1994). Monkey cells produced variable results (Kabongo, 2001). Human cells may essentially be susceptible to BVDV infection because serum antibodies reactive to pestiviruses have been detected in humans whom had no contact with potentially infected animals (Giangaspero *et al.,* 1993).

Blood is an important specimen for BVDV isolation in live animals. In serum samples virus may be found free or they may be released during grinding of blood clots from clotted blood in the laboratory (Kirkland & Mackintosh, 1992), but highest sensitivity can be obtained by co-culture of cell cultures and leukocytes in the buffy coat of blood collected from sick animals in heparin-containing tubes. Bovine viral diarrhoea virus is very stable in serum, surviving at room temperature for at least seven days (Bolin, *et al*., 1991).

2.5.2 Antigen detection

2.5.2.1 Immunofluorescence staining

Immunofluorescence staining is a rapid and specific technique used for identifying unknown virus isolates (Vickers & Minocha, 1990). The use of fluorescence antibody techniques has been applied in the diagnosis of a large number of viral agents, including staining of BVDV infected cells.

Mengeling *et al*. (1963) demonstrated the antigenic relationship between hog cholera and BVD viruses using the immunofluorescence technique. However, the use of other techniques for demonstration of ncp and cp strains of BVDV in tissue culture was established (Fernelius, 1969; Baker, 1987; Dubovi, 1990). An effective way of detecting susceptible cell cultures inoculated with suspected materials that are cytopathic is through immunofluorescence staining. Infected monolayer cells can be stained either by use of the direct or the indirect method (Vickers & Minocha, 1990).

In animals infected experimentally with cp BVDV, the use of direct fluorescent antibody testing of the buffy coat has proved reliable in detecting viraemia. It has also been used in tissue specimens and immunofluorescence on acetone-fixed sections or smears of nasal epithelial cells being the most widely applied (Kabongo, 2001). The techniques are rapid and can be applied in laboratories lacking cell culture facilities. Positive staining was also found in sections of thyroid and salivary glands, lymph nodes and around the sites of lesions in the intestine (Duffel & Harkness, 1985).

2.5.2.2 Immunohistochemical staining

The use of immunohistochemical staining for detection of BVDV in leukocyte smears or cell cultures, in frozen tissues sections, and skin biopsies have been described (Meyling, 1984; Njaa *et al.,* 2000). The technique offers the advantage that stained wet mounts can be viewed immediately by light microscopy with subsequent preservation into permanent mounts.

Skin biopsies can be obtained from live animals with relative ease, especially young calves. In PI animals, BVDV antigens can readily be detected in tissue sections by immunohistochemical staining (Thur *et al*., 1996; Brodersen, 2004). Although more labour intensive, this method can be applied for detection of viral antigen in young PI calves which otherwise could have tested negatively by virus isolation or antigen ELISA due to inhibition by maternal antibodies (Zimmer *et al*., 2004).

2.5.2.3 Flow cytometry

Flow cytometry can be used for the direct identification of BVDV antigen in clinical samples as an alternative to virus isolation (Ellis *et al.,* 1988; Qvist *et al.*, 1990). The sensitivity of flow cytometry analysis of lysates of whole blood samples was found to be equivalent to virus isolation (Qvist *et al.,* 1991). It is performed by using a biotinylated polyclonal porcine antiserum to BVDV followed by avidin-FITC-conjugate (Allan *et al.*, 1987). This method requires sophisticated equipment and qualified personnel.

2.5.3 Serology

Among different serological assays that have been used for BVD over the years are the serum neutralization test (SNT), agar gel immunodiffusion, western blotting, complement fixation test, indirect immunofluorescence and various enzyme linked immunosorbent assays (ELISAs) (Sandvik, 1999; Saliki & Dubovi, 2004). However, only the SNT, I FA and ELISAs are used routinely.

2.5.3.1 Serum-virus neutralization test

The SNT is an *in vitro* system, which quantifies the inhibitory effect of specific antibodies on virus replication in cell cultures. Thus, its use is limited to virological laboratories, which need to control potential problems with pestivirus contamination of the cell cultures and/or inhibitory specific antibodies in the media (Sandvik, 2005). The antibodies detected by the SNT are predominantly against the envelope protein E2 (Sandvik, 2005). It is fairly labour intensive, requires experienced staff and well equipped laboratories, and will typically take 5-6 days to perform. It is therefore mostly used as a reference test for back-up and calibration purposes (Sandvik, 2005).

2.5.3.2 Antibody ELISA

For testing of large numbers of samples, the ELISA has many advantages over the SNT. They are independent of cell cultures and challenge viruses, give test results within a few hours, are relatively inexpensive both to establish and run, and are suitable for automation. Two different ELISA formats are commonly in use; indirect (activity amplification) and blocking (competitive or activity modulation) assay as described by Sandvik (2005).

In the indirect format, viral antigen is immobilized on the solid phase, onto which specific antibodies and subsequent detecting enzyme-conjugated antiglobulins bind. A positive reaction is recognized by colour development in the substrate solution, which is read optically and reported as optical density (OD) values (Sandvik, 2005).

In blocking ELISAs, the presence of specific antibodies prevents the trapping of test antigen between a layer of immobilized capture antibodies and a reporting layer of enzyme-labeled antibodies, which also are virus specific. Thus, a positive sample causes a reduction of the optic density (OD), which is expressed as a percent inhibition, relative to the OD of a negative serum.

2.5.3.3 Agar gel immunodiffusion

The agar gel immunodiffusion test has low sensitivity compared to the SNT and ELISA. It is a qualitative test and interpretation of tests results for seroconversion on paired sera is more difficult. It is a good screening test, easy to perform and can be carried out even in laboratories lacking advanced technical and virological facilities. The antibodies detected are directed mainly against a soluble, non-structural (NS) antigen (Gutekunst & Malmquist, 1963) and correlates better with the SNT than with complement fixing (CF) antibodies (Harkness *et al.,* 1978).

2.5.3.4 Peroxidase-linked assay

Although originally devised for screening pig sera for antibodies to hog cholera viruses (Holm, 1981), PLA can also be used for serum from cattle. Peroxidase-linked assay (PLA) is similar in concept to immunofluorescence; subsequent addition of insoluble chromagen produces a visible colour reaction. This can be read by light microscopy and enables the test to be performed in microtitre plates.

2.5.4 Molecular characterization

Reliable assays are needed to detect all pestiviruses, such as BVDV-1, BVDV-2, BDV, and atypical bovine pestiviruses described recently (Liu *et al*., 2008). Several real-time reverse transcriptase polymerase chain reaction assays have been developed for rapid and sensitive detection of this virus (Letellier & Kerkhorfs, 2003; Young *et al.,* 2006; Baxi *et al*., 2006).

Several methods have been applied for the detection of pestivirus RNA, but only the reverse transcription-polymerase chain reaction (RT-PCR) seems to have been adapted for diagnostic purposes. Detection of RNA by RT-PCR includes four different steps; extraction of RNA, reverse transcription to cDNA, primer-directed amplification and lastly detection of amplified products. In the first protocols, these steps were carried out separately, which is time-consuming and increase the risk of cross-contamination of samples. More recent TaqMan RT-PCRs combine the last three steps in a single tube, and by eliminating the need for gel electrophoresis the risk for carry-over contamination with previously amplified DNA and false positives is greatly reduced (McGoldrick *et al.,* 1999). The use of separate sets of primers and probes allows for discrimination between BVDV genotypes 1 and 2 in the same assay (Letellier & Kerkhofs, 2003).

2.5.4.1 Reverse transcriptase polymerase chain reaction

Genetic typing of pestiviruses is mostly based on the genetic diversity of the 5′-NCR, N^{pro} and E2 genomic regions. The 5′-NCR provides meaningful phylogenetic inferences (Vilcék *et al.,* 2001) as this region has the highest degree of sequence conservation and is efficiently amplified by RT-PCR. It is also the most frequently analyzed portion of the genome. Total RNA is extracted from samples using extraction kits based on the manufacturer's instructions and RT-PCR is carried out to amplify the extracted RNA. Amplification is performed for a total of 35 cycles to achieve denaturation, annealing and extension Yamamoto *et al.* (2008). After amplification, the PCR products will be electrophoresed on 1-2% agarose gels in tris-borate-EDTA buffer.

2.5.4.2 Real-time transcriptase polymerase chain reaction

Real-time PCR is now being used to identify, classify and quantify many viral pathogens as it is a highly sensitive and rapid method for detecting viral nucleic acid sequences in clinical specimens. Furthermore, real-time PCR is a quantitative technique and as such may be used to assess viral RNA levels (Young *et al.,* 2006). Real-time PCR methods for genotyping BVDV have been described previously (Bhudevi & Weinstock, 2001, 2003; Letellier & Kerkhofs, 2003).

Using real-time PCR to quantify the viral RNA levels in BVDV-infected animals presents particular technical and biological challenges. Collection and processing of samples from different animals over a number of different days inevitably results in some degree of sample to sample variation in the technical detection of the target nucleic acids (Young *et al.,* 2006). This may arise from differences in efficiency of RNA extraction, reverse transcription or PCR reactions which is made particularly significant by the sensitivity of this technique. These technical variations are often "controlled" for by normalization of the signal to an internal standard, typically a "housekeeping" gene, facilitating the comparison of different data sets (Young *et al.,* 2006). This presents two further problems: Firstly, this is a common practice that such housekeeping genes are rarely truly static and may alter under experimental conditions (Thellin *et al.,* 1999; Whelan *et al.,* 2003). To take account of this, the best approach would be to recommend assessing the validity of a number of different housekeeping gene targets and comparing them under the test conditions used. This laborious task is the only way to objectively identify more stable mRNA sets. However, this process is not relevant to the BVDV situation during infection as, secondly, during acute infection with BVDV animals experience a measurable leukopaenia and the number of cells per volume of blood changes over time (Young *et al.,* 2006). Traditional methods of virus isolation quantify the amount of virus recovered from a fixed volume of blood irrespective of the cell numbers in this sample, which during acute BVDV infection will fluctuate (Polak and Zmudzinski, 2000). By standardizing the viral RNA measured by real-time PCR to a housekeeping gene set, the quantification is essentially done on a per cell basis rather than a per volume basis rendering the comparison of samples from leukopaenic calves more difficult and precluding a simple comparison with more traditional virological methods (Young *et al.,* 2006).

To overcome these difficulties a novel two-step real-time RT-PCR method using an external RNA reference standard was developed, which has been evaluated on samples from experimentally infected cattle. Each sample was spiked with a known amount of a second RNA virus, namely canine coronavirus (CCV), prior to RNA extraction (Young *et al.,* 2006). While the external standard is not an exact mimic of the true target it is subject to the same experimental procedures and provides an external standard for correction of differences in the efficiency of RNA extraction or RT-PCR. Furthermore, to facilitate ease of sample handling the method was based on quantification of BVDV RNA per volume of frozen whole blood (Young *et al.,* 2006).

The method is fast, reliable, sensitive and specific, but is very expensive, requires technical expertise, equipment and automation and RNA extraction methods should be considered when comparing this test with the standard methods of virus isolation and serology.

2.6 Control of BVDV

Advances in the understanding of the epidemiology, pathogenesis and immunity of BVD have enabled development of rational strategies for controlling disease syndromes caused by the virus. In South Africa there is a growing consensus that the identification of PI calves, in combination with a proper vaccination programme, is essential for the successful control of BVDV (Van Vuuren, 2006).

Earlier approaches made use of the fact that PI calves are in most cases seronegative as a result of immune-tolerance. In herds where the majority of cattle were found to be seropositive for BVDV with a small number of seronegative animals, the latter were culled on the premise that they were most likely PI (Van Vuuren, 2006). The subjectivity of this approach was realized and efforts to screen all cattle for the virus including all calves born in the herd for nine months after initiating the programme are now encouraged (Ferreira *et al.,* 2000). Where virus isolation was not practicable for individual animals on the scale necessary to identify carriers directly, detection and elimination of persistently viraemic animals can be accomplished by serological testing of the whole herd. If there are few negatives, their blood is subjected to virus isolation procedures or antigen detection techniques (Van Vuuren, 2006). One difficulty in vaccinated herds is that viraemic animals will seroconvert with neutralizing antibodies when the vaccine virus is antigenically different from the virus responsible for the natural infection (Van Vuuren, 2006).

It is advisable that animals introduced into a herd should be tested prior to introduction. If an animal is to be introduced into a non-vaccinated herd, it is recommended that the animal should be quarantined during the testing of two consecutive samples taken approximately 30 days apart. Calves should be tested immediately after birth before they have consumed colostrums which might contain specific antibodies that might interfere with recovery of the virus and before they have made contact with susceptible animals (Potgieter, 2004).

There are both live (usually in combination with other viruses) and inactivated BVDV (in combination with other viruses, or BVDV alone) vaccines available in South Africa. Although the majority are imported from Europe and USA, and contain well-known type 1 BVDV strains, a multivalent vaccine that includes the BVDV valency became available in South Africa during the late 1980s. In the early 1990s, cow/calf producers were encouraged to ensure that all young stock destined for feedlots be vaccinated with the modified-live combination vaccines (MLV) at least 14 days before transport. This pre-conditioning approach has become established during recent years, and currently many producers receive better prices as a result of their reputations as producers of stock that perform better in feedlots (Van Vuuren, 2006).

Ideally, veterinarians are encouraged through continuing professional development and local veterinary publications to approach the control of BVDV holistically by means of biosecurity measures that involve enhancement of immunity, prevention of exposure of cattle at risk to BVDV infection, and elimination of PI carries from the herd. These should be accomplished by vaccination, prevention of exposure by means of quarantine, adequate fencing, utilization of sentinel animals, diligent testing, and cleaning and disinfection of fomites such as vehicles and equipment (Van Vuuren, 2006).

CHAPTER 3 :
MATERIALS AND METHODS

3.1 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.

Necropsy samples (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 1 200 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 that are typically associated with BVD 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. These animals all 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 1. 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, Northwest, Limpopo, Free State and Northern Cape provinces.

Table 1 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 (diarrhoea)	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.

3.2 Reverse transcriptase real-time PCR

3.2.1 Processing of specimens

The tissue specimens (spleen & lymph nodes) and transtracheal aspirates were stored at -70 ℃ 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 (PBS) 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 ℃ 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.

3.2.2 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 three minutes in an Eppendorf centrifuge (Centrifuge 5417 R, Eppendorf AG. 22331 Hamburg, Germany). The supernatant was transferred into a new 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 two 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 ℃ until used.

3.2.3 Cador BVDV Type 1/2 RTrt-PCR Kit

The Qiagen Cador BVDV Type 1/2 RTrt-PCR Kit (QIAGEN GmbH, QIAGEN Strasse 1, D-40724 Hilden) is a ready-to-use system for the differentiation of BVDV genotypes 1 & 2 and border disease virus (BDV). The test was standardized in the laboratory of the DVTD with the aid of a Lightcycler® V2.0 (Roche Diagnostics, Mannheim, Germany) machine with small 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 signal in the FAM™ (BVDV genotype 1), and JOE (BVDV genotype 2) channels representing 530 nm and 640 nm respectively in the Lightcycler® Real-Time PCR System. The field samples were analyzed against the standardized positive and negative controls of the 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 at 640 nm which was the criteria used to differentiate between genotype 1 and 2.

3.2.4 Real-time PCR

The reverse transcriptase real-time PCR (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 (template). It was then optimized to 25 µl final volume per reaction using 19 µl of BVDV master mix, 1 µl of BVDV Mg-sol and 5 µl of the template. The Qiagen Cador BVDV Type 1/2 RTrt-PCR Kit (described in 3.2.3) includes negative and positive controls for BDV, BVDV genotype 1 and genotype 2. The following reaction steps were used: the RNA was transcribed to cDNA at 50 ℃ for 30 minutes; followed by a denaturation period at 95 ℃ for 10 minutes. The amplification programme included 45 cycles of three steps each, comprising of denaturation at 95 ℃ for 30 seconds, primer annealing at 60 ℃ for one minute and product extension at 72 ℃ for 10 seconds where after the results were recorded.

3.2.5 Sequencing

To confirm the genotypes of the different BVDV isolates, selected samples were 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 $d(T)_{16}$ was used as primer for cDNA synthesis, which was performed using 1 µl of the extracted RNA in 0.2 ml thin walled tubes in the GeneAmp PCR system 9700HT (Applied Biosystems).

Nucleotide sequences (5'-3') of the PCR primers used for sequencing in this study are as follows:

Forward F2: 5' CTC GAG ATG CCA TGT GGA C 3'

Reverse PESTR: 5' CTC CAT GTG CCA TGT ACA GCA 3' (Letellier *et al*., 2003.)

The PCR primers F2 and PESTR were used to amplify an approximate 245 bp product, which was then subjected to direct sequencing using the same primers (Letellier *et al*., 2003). 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 ℃, one minute at 55 ℃, one minute at 72 ℃). The reaction had an initial denaturation step at 94 ℃ for 10 minutes and a final elongation step at 72 ℃ for seven minutes. To remove all primers, nucleotides, enzymes, salts, and other impurities from the amplicon 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 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.

3.2.6 Phylogenetic analysis

Nucleotide sequences used for phylogenetic analysis were aligned using Clustal X (Thompson *et al.*, 1997). 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 viruses but only 12 were used in the phylogenetic analysis. The viruses were 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 vaccine strains Pfizer 53637, Oregon C24V, Singer and BVDV genotype 2 strain 296nc (Meyers *et al.*, 1989).

Table 2 A list of samples used for sequencing/phylogenetics and origin of the cattle from which the samples were collected

Sample ID No.	Type of specimen	Origin	RTrt-PCR result
FS38	LN/SP	FS	Genotype 1
FS50	LN/SP	NC	Genotype 1
FS138	LN/SP	NW	Genotype 1
FS160	LN/SP	GA	Genotype 1
NC3	TTA	KZN	Genotype 1
DGV2	TTA	KZN	Genotype 1
KZN608	LN/SP	KZN	Genotype 1
FS161	LN/SP	EC	Genotype 2
FS164	LN/SP	EC	Genotype 2
FS170	LN/SP	EC	Genotype 2
FS175	LN/SP	NC	Genotype 2
FS208	LN/SP	EC	Genotype 2

CHAPTER 4 : RESULTS

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 as summarized in Table 3. Ninety one of 216 lymph node/spleen samples, 11 of 59 trans-tracheal aspirates and one blood sample were positive by nucleic acid detection using real-time PCR.

Table 3 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**

Positive samples were obtained from 61.2% animals (n=63) that showed respiratory signs prior to death or sampling. Acute deaths accounted for 22.3% (n=23), animals with enteric signs 11.7% (n=12) and those with a shock lung appearance 4.8% (n=5).

The disease conditions associated with cattle from which nucleic acid was detected is presented in Table 4. Respiratory signs were the most frequent clinical signs (63/103), followed by cattle that died acutely (23/103).

Table 4 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%)**

The geographical origin of the cattle sampled and the genotypes of BVD virus obtained during this study are indicated in Table 5. The number of positive samples from cattle originating from Kwazulu Natal Province totaled 16 of which only one was genotype 2. Cattle from the Free State Province yielded 18 genotype 1 and two genotype 2 viruses, while nine genotype 1 and nine genotype 2 viruses were detected in cattle from the Eastern Cape. Others included are twelve genotype 1 and one genotype 2 viruses from cattle from Limpopo Province, nine genotype 1 and three genotype 2 viruses from cattle from the Northern Cape. Only one genotype 1 virus from an animal from the Western Cape, nine genotype 1 and two genotype 2 viruses from animals hailing from the Northwest Province and 12 genotype 1 viruses from cattle in Gauteng Province were detected.

Table 5 Distribution of BVDV genotypes in relation to the province of origin of the cattle from which the samples 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
TOTAL	**85**	**18**	**103**

The amplification curves of the RTrt-PCR used in this study are illustrated in Figures 1, 2 and 3. Figure 1 shows the amplification curve viewed at a wavelength of 530 nm using the FAM hydrolysis probe and both genotype 1 and 2 are observed at this wavelength as indicated. Figure 2 also shows genotype 1 and 2 controls with the genotype 2 field strain FS 208 at the same 530 nm wavelength, but when the same field strain is viewed at 640 nm (Figure 3) using the JOE hydrolysis probe it clearly distinguishes it as genotype 2. Figure 2 and 3 represent the same amplification curve viewed at different wavelengths.

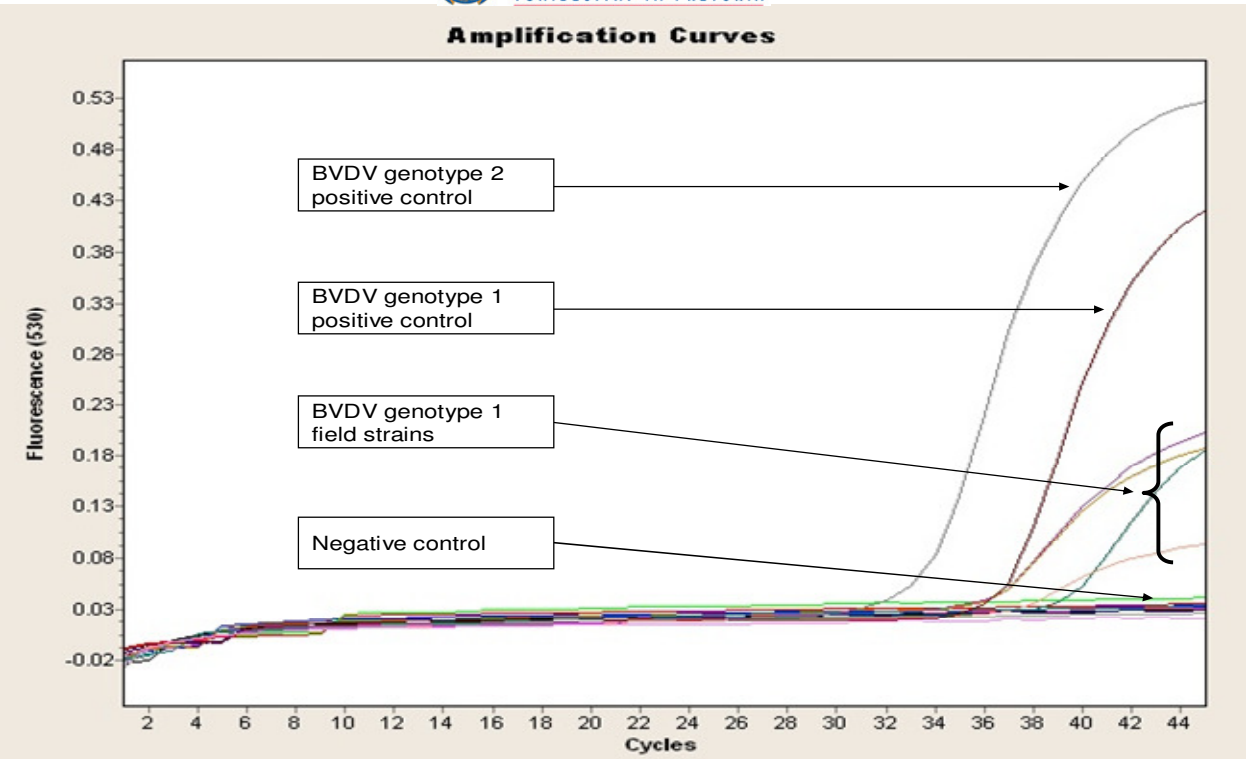

Figure 1 Amplification curves showing BVDV genotype 1 and 2 positive controls, several BVDV genotype 1 isolates (NC3, LD2, MV1 and MU2) and a negative control viewed at 530 nm with the use of the FAM hydrolysis probe.

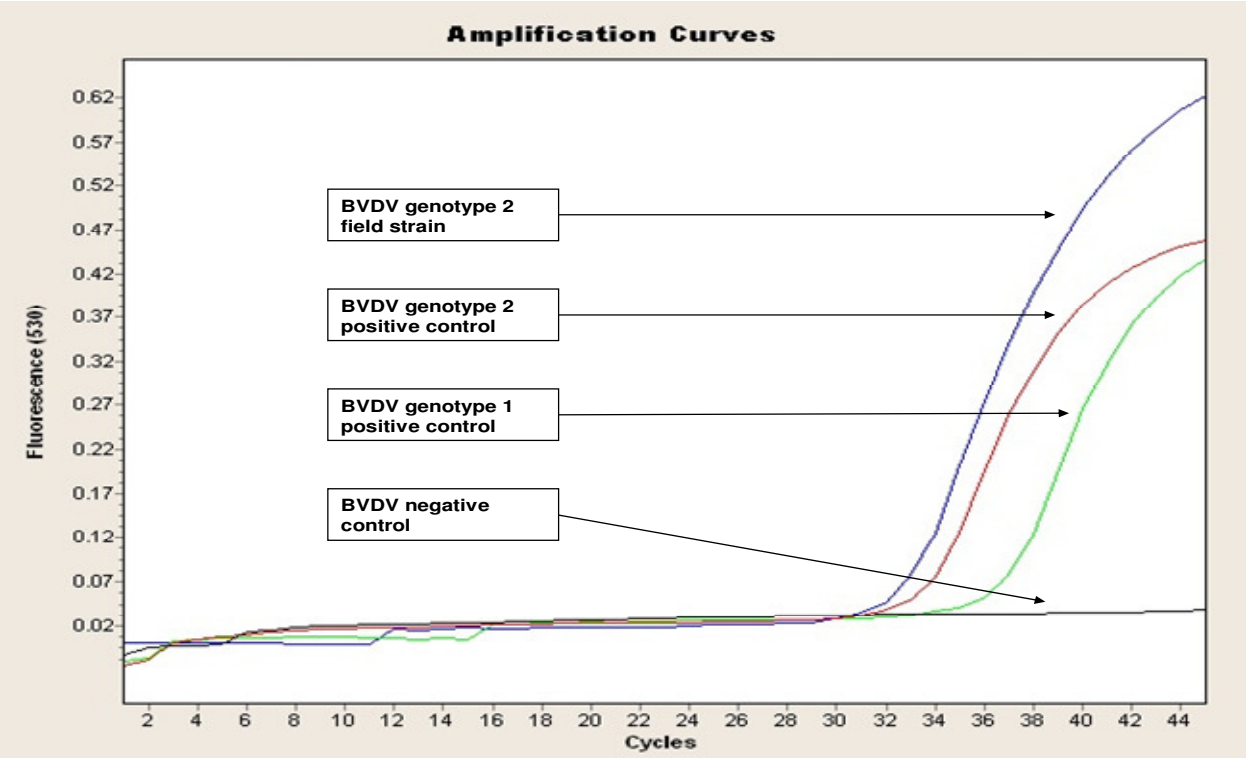

Figure 2 Amplification curve of a known BVDV genotype 2 strain (FS 208); a BVDV genotype 2 positive control; a BVDV genotype 1 positive control; and a BVDV negative control viewed at 530 nm using the FAM hydrolysis probe.

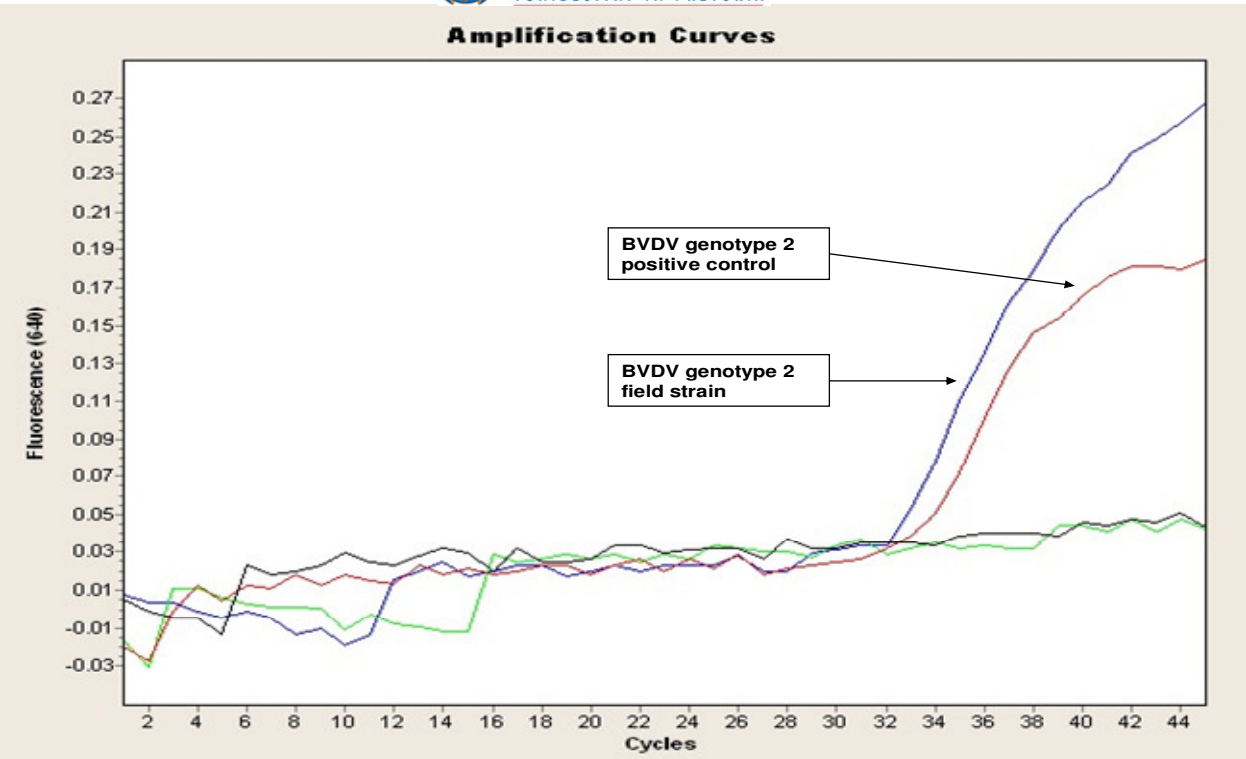

Figure 3 A BVDV genotype 2 positive control and a BVDV genotype 2 field strain (FS 208) viewed at 640 nm using the JOE hydrolysis probe.

The nucleotide sequences derived from 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 positive isolates (FS160, FS50, FS138, DGV2, NC3, FS38 and KZN608) by comparative alignment of sequences from part of the 245 bp of the 5′NCR of the BVDV genome, using programmes of the Clustal X package (Thompson *et al*., 1997). The numbers on each branch represent the number of times the group or subgroup was picked in 1000 re-runs in the bootstrap analysis. In Figure 4 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. The distances were calculated using the neighbor-joining method. The viruses analyzed were phylogenetically discriminated into two distinct groups, namely Groups A and B, within the BVDV genotype 1. Group A isolates (FS160; FS50; FS138; DGV2; NC3) clustered

with BVDV strain Osloss and BVDV isolate 23-15 accession number AF298059 classified as subgroup 1b (Baule *et al*., 1997), whereas, group B viruses (KZN608; FS38) clustered with strain S-ALT7/K accession number U97470, Singer and NADL under subgroup 1a (Baule *et al*., 1997).

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 Figures 5 and 6. 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 as group A clustering with reference strain 890 accession number L32886 and a pestivirus type 2 strain accession number AF039180 (Bolin and Ridpath, 1992) separately from the vaccine strain in group B. The field strains did not cluster with vaccine sequences obtained from GenBank as indicated in blue in Figure 5 and green in Figure 6 showing the Pfizer vaccine strain reference number 53637. All Genbank sequences used in the trees are summarized in Annexure 2.

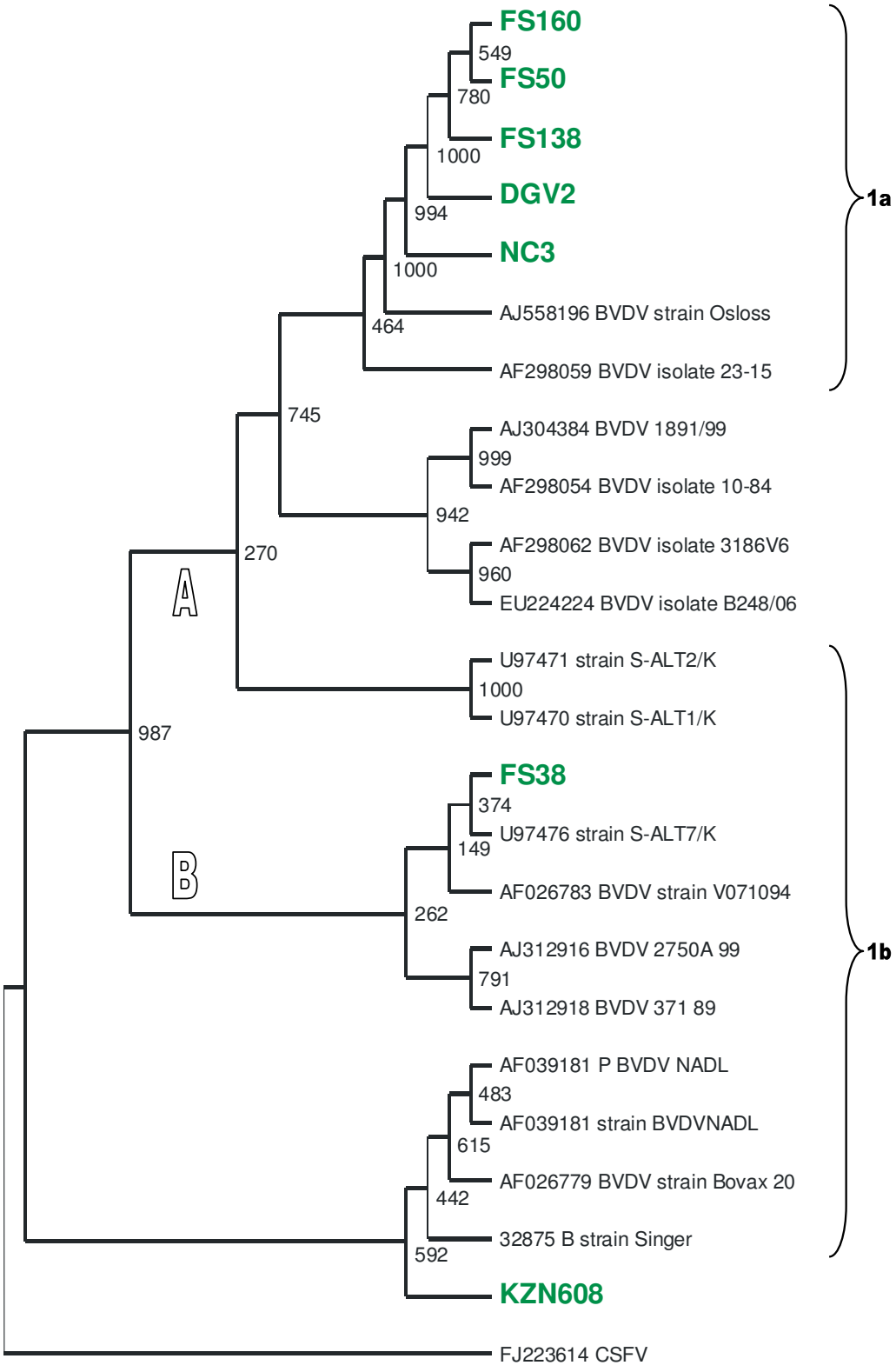

Figure 4 Phylogenetic analysis of seven field strains of BVDV genotype 1 in relation to published South African (S-ALT7/K, S-ALT2/K, S-ALT1/K) and some other sequences of pestiviruses obtained from Genbank.

Figure 5 Phylogenetic tree showing the positioning of five 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 vaccine sequences (in blue). The classical swine fever virus (D50813 CSFV strain CPAE) in yellow was used as an out-group.

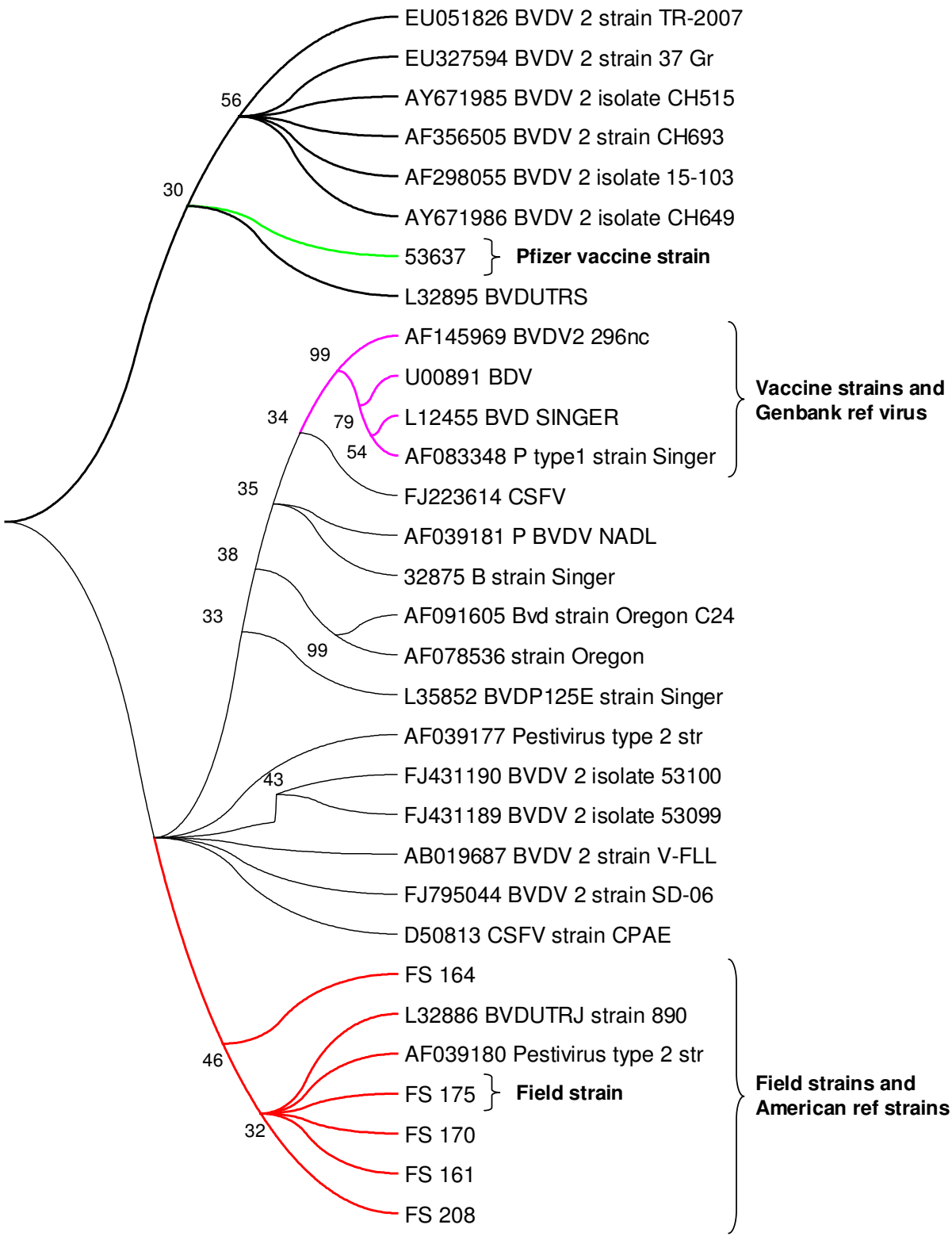

Figure 6 Phylogenetic tree showing the positioning of five local field strains of BVDV genotype 2 (FS161, FS175, FS208, FS170, and FS164 in red) obtained during this study in relation to the Pfizer vaccine strain reference number 53637 (in green).

The frequency distribution of cattle that were necropsied and tested positive for BVDV virus were compared with the time spent on feed in the feedlot and is indicated in Figure 6. Most mortalities occurred during the first five to six weeks on feed.

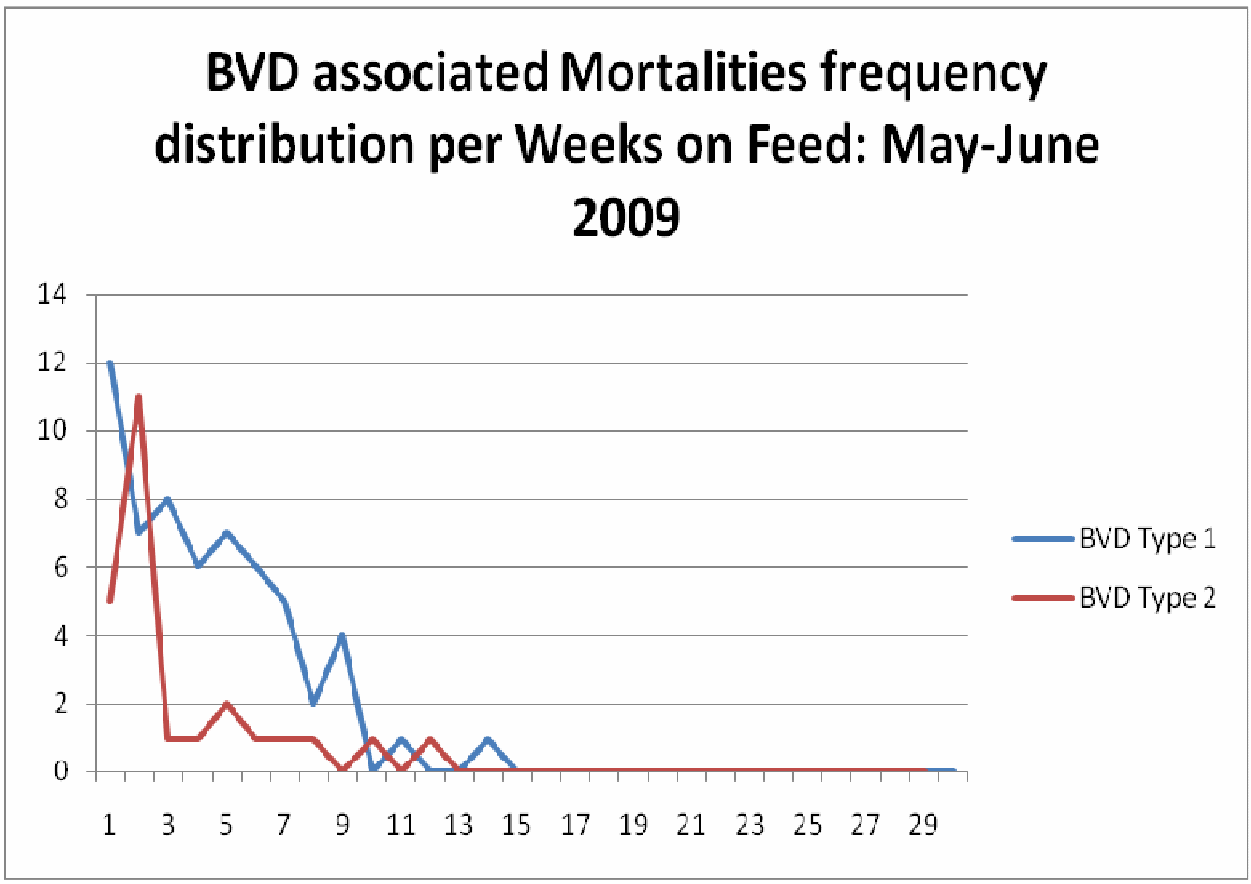

Figure 7 Karan Beef samples: Frequency distribution of BVD rtPCR-positive cases per week on feed.

CHAPTER 5 : DISCUSSION

Bovine viral diarrhea virus is an important pathogen of cattle that causes major economic losses in the cattle industry. It causes acute infections with various clinical outcomes and transplacental infections of foetuses. A significant characteristic of this pestivirus is the ability to cause persistent infection (PI) in calves. Persistently infected cattle are the major virus reservoirs for the spread of BVDV. The PI animals that survive are continuously shedding virus into the environment through oral and nasal discharges, urine and faeces. Effective control of BVDV requires the identification and removal of PI calves in combination with a vaccination programme (Letellier and Kerkhofs, 2003).

The presence or absence of genotype 2 BVD viruses in cattle in southern 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 type 2 vaccine in South Africa (Titanium® 5, Virbac RSA (Pty) Ltd) in 2004.

During this research project 276 samples were collected from various feedlots distributed all over South Africa. BVD viruses detected were genotyped using a commercial real-time rtPCR assay. The Cador BVDV RTrt PCR kit is rapid, highly sensitive, and specific and therefore offers a useful approach for BVDV detection and genotyping. For the extraction of BVD viral RNA, a variety of tissue specimens were processed, including blood, trans-tracheal aspirates, and internal organs such as spleen and lymph nodes.

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 read in the 530 nm channel and similarly BVDV genotype 2 detection was accomplished with the use of the JOE labelled hydrolysis probe, read in the 640 nm channel.

Several real-time RT-PCR assays have been developed for rapid and sensitive detection of BVDV (Letellier and Kerkhofs, 2003; Young *et al*., 2006; Baxi *et al*., 2006). The Cador BVDV real-time PCR kit from Qiagen (Hilden, Germany) was developed for detection and genotyping of bovine pestiviruses (Liu *et al*., 2008). 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 (Meyers and Thiel, 1996) and the 5′NCR is suitable for common primer design. It also contains less homologous regions suitable for design of species-specific probes (Baxi *et al*., 2006).

The real-time PCR assay has several advantages over conventional PCR. Real-time reactions use fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR (Letellier and Kerkhofs, 2003). The technique is less time consuming and decreases the contamination risk of post PCR product analysis on gels. The TaqMan chemistry was used in this study for the amplification of the PCR product that was detected during each cycle by the release of a fluorescent reporter dye from a hybridization probe (Letellier and Kerkhofs, 2003; Bhudevi and Weinstock, 2001). The technique has been reported as a rapid detection and genotyping technique for BVDV which makes it well suited for the removal of PI animals in herds for the control of BVDV infection (Letellier and Kerkhofs, 2003).

Bovine viral diarrhoea viruses were first identified within the pestiviruses based on comparison of the 5′NCR (Pellerin *et al*., 1994; Ridpath *et al*., 1994). Later, hybridization and more extensive sequence analysis revealed that the 5′ NCR was highly conserved in BVDV genomes. This led some to suggest that the 5′ NCR region might not serve as a good target sequence for phylogenetic studies because of its highly conserved nature (Becher *et al*., 1997). However, numerous investigators demonstrated that the 5′ NCR region provides a useful tool in genotyping BVDV isolates (Beer *et al*., 2002; Vilcek *et al*., 2001; Pellerin *et al*., 1994; Ridpath *et al*., 1994). 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 as listed in Annexure 2.

Phylogenetic trees were generated from comparative alignment of sequences from part (245 bp) of the 5'NCR of the BVDV genome, using multiple programmes of the Clustal X package. The numbers on each branch represent the number of times the group or subgroup was picked in 1000 re-runs in the bootstrap analysis. The genotype 1 field strains (FS 160, FS 50, FS 138, DGV 2, NC 3, KZN 608) and selected sequences obtained from GenBank and shown in Figure 4 were arbitrarily divided into two groups, Group A and B within the BVDV 1 genotype (pestivirus type 1) showing a distinction between the two groups which was supported at a confidence level of 98.7% by the bootstrap analysis. Genotype 1 viruses are further divided in phylogenetic subtypes 1a and 1b which was designated according to the expanded proposed nomenclature for BVDV 1 as described by Pellerin *et al*. (1994), using reference strains such NADL, Osloss, SD-1 Singer and Oregon C24V. Five of the strains found in this study (FS 160, FS 50, FS 138, DGV 2, NC 3) grouped with the Osloss strain, which placed them in subgroup 1b of BVDV as defined by Pellerin *et al*. (1994). In Group B, field strain KZN 608 (cluster 1a) grouped with NADL the American reference strain and Singer from Argentina. Also, field strain FS 38 (cluster 1a) grouped with the South African strain S-ALT7/K from Kwazulu Natal (Figure 4).

Viruses of genotype 2 were also divided in two groups, namely Group A and B. The field viruses (FS 161, FS 164, FS 170, FS 175, FS 208) in Group A in Figure 5 displayed a bootstrap value of 37.8% with the American BVD UTRJ strain 890, indicating that the sequence homology is high. Strain 890 is a reference strain and the virus is associated with a haemorrhagic syndrome of cattle, a form of BVDV infection first described in North America (Ridpath *et al*., 1994; Pellerin *et al*., 1994). Four of the genotype 2 strains that clustered with strain 890 are from cattle that originated from the Eastern Cape Province (FS161, FS164, FS170 and FS208) and one (FS175) from Northern Cape Province. None of the BVD strains identified as BVDV-2 were associated with severe haemorrhagic diarrhoea in this study. None of the vaccine strains (BVDV-2 296nc; Oregon C24; strain Oregon; P125E strain Singer; B strain

Singer and Pfizer strain 53637) obtained from GenBank grouped with the BVDV type 2 strains (FS161; FS164; FS170; FS175; FS208) detected in this study.

Among the 103 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 on 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 in South Africa.

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 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. 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) vaccine strain are not closely related to the five type 2 viruses sequenced. The type 2 viruses obtained in this study are therefore not originally vaccines strains as none of the vaccine sequences clustered with the five type 2 sequenced viruses. However, as indicated in Chapter 3 under Material and Methods, TTAs were collected from live cattle that had been present in the feedlot for at least two weeks when pulled the first time for 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 genotype 2-containing Pfizer vaccine was not used at Karan Beef feedlot (where all the type 2 viruses were detected) prior to the beginning of

2010. Before that time, 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. BVD-positive cattle in this study did not show the trends reported in North American feedlot literature where most BVD positive cases died later in the feeding period than what was observed here (Figure 7). However, causal conclusions cannot be drawn from this data. A previous study completed in South Africa reported that BVDV was mostly identified in outbreaks of respiratory disease, usually in association with other pathogens (Kabongo & Van Vuuren, 2004).

In conclusion, the present study revealed the presence of BVDV genotype 2 in cattle in South Africa. Based on the high sequence similarity between the genotype 2 field strains with strain 890 of North American origin, 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 (Baule *et al.,* 1997).

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. Molecular diagnostic methods are established tools for the detection of numerous viral pathogens. 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.

REFERENCES

ACHENBACH, J.E., TOPLIFF, C.L., VASSILEV, V.B., DONIS, R.O., ESKRIDGE, K.M & KELLING, C.L. (2004). Detection and quantitation of bovine respiratory syncytial virus using real-time quantitative RT-PCR assays. *Journal of Virological Methods*, 121:1-6

ALLAN, G.M., MCNULTY, M.S., BRYSON, D. & MACKIE, P.M. (1989). Demonstration of bovine virus antigen in formalin-fixed, paraffin-embedded tissue using a streptavidin/biotin technique. *Research in Veterinary Science*, 46:416-418.

ANONYMUS. (1994). "Peracute bovine viral diarrhoea reportedly spreading". *Journal of the American Veterinary Medical Association,* 205(3):391-392.

BAKER, J.C. (1987). Bovine viral diarrhoea virus: A review. *Journal of the American Veterinary Medical Association*, 190:1449-1458.

BAKER, J.C. (1990). Clinical aspects of bovine virus diarrhoea virus infection. *Rev. Sci. Tech,* 9(1):25-41.

BAKER, J.C. (1995). The clinical manifestation of Bovine Viral Diarrhoea Infection. *Veterinary Clinics of North America: Food Animal Practice,* 11:425-445.

BARADEL, J.M., BARRAT, J., BLANCOU, J., BOUTIN, J.M., CHASTEL, C., DANNACHER, G., DELORME, D., GERARD, Y., GOURREAU, J.M., KIHM, U., LARENAUDIE, B., LE GOFF, C., PASTORET, P.P., PERREAU, P., SCHWERS, A., THIRY, E., TRAP, D., UILENBERG, G. & VANNIER, P. (1988). Results of a serological survey of wild mammals in France. *Revue Scientifique et Technique de l' Office International des Epizooties,* 7:861-883.

BAULE, C. & BANZE, J. (1994). Bovine virus diarrhoea virus infections in calves from selected farms in Mozambique. *Bulletin of Animal Health and Production in Africa,* 42:280-286.

BAULE, C., VAN VUUREN, M., LOWINGS, J.P. & BELÅK, S. (1997). Genetic heterogeneity of bovine viral diarrhea viruses isolated in Southern African. *Virus Research,* 52:205-220.

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. *Veterinary Microbiology,* 116:37-44.

BECHER, P., KÖNIG, M., PATON, D.J. & THIEL, H.J. (1995). Further characterization of border disease virus isolates: evidence for the presence of more than three species within the genus pestivirus. *Virology*, 209:200-206.

BECHER, P., ORLICH, M., SHANNON, A.D., HORNER, G., KONIG, M. & THIEL, H.J. (1997). Phylogenetic analysis of pestiviruses from domestic and wild ruminants. *Journal of General Virology,* 78:1357-1366.

BEER, M., WOLF, G. & KAADEN, O.R. (2002). Phylogenetic analysis of the 5′ -untranslated region of German BVDV type II isolates. *Journal of Veterinary Medical B. Infectious Diseases Veterinary Public Health,* 49:43-47.

BEZEK, D.M. & MECHOR, G.D. (1992). Identification and eradication of bovine viral diarrhoea virus in a persistently infected dairy herd. *Journal of the American Veterinary Medical Association*, 201:580-586.

BHUDEVI, B. & WEINSTOCK, D. (2001). Fluorogenic RT-PCR assay (TaqMan) for detection and classification of bovine viral diarrhoea virus. *Veterinary Microbiology*, 83:1-10.

BHUDEVI, B. & WEINSTOCK, D. (2003). Detection of bovine viral diarrhoea virus in formalin fixed paraffin embedded tissue sections by real time RT-PCR (TaqMan). *Journal of Virological Methods*, 109:25-30.

BOLIN & RIDPATH. (1992). Difference in virulence between two noncytopathic bovine viral diarrhoea viruses in calves. *American Journal of Veterinary Research* Vol. 53(11).

BOLIN, S.R. (1995). The pathogenesis of mucosal disease. *Veterinary Clinics of North America: Food Animal practice,* 11:489-497.

BOLIN, S.R., MATHEWS, P.J. & RIDPATH, J.F. (1991). Methods for detection and frequency of contamination of foetal calf serum with bovine viral diarrhoea virus and antibodies against bovine viral diarrhoea virus. *Journal of Veterinary Diagnostic Investigation,* 3:199-203.

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. *Veterinary Microbiology*, 29:1-3.

BROCK, K.V. (1995). Diagnosis of bovine viral diarrhoea virus infection. *Veterinary Clinics of North America: Food Animal Practice,* 11(3):549-561.

BROCK, K.V., GROOMS, D.L., RIDPATH, J. & BOLIN, S.R. (1998). Changes in levels of viremia in cattle persistently infected with bovine viral diarrhoea virus. *Journal of Veterinary Diagnostic Investigation,* 10:22-26.

BROCK, K.V. (2003). The persistence of bovine viral diarrhoea virus. *Biologicals*, 31:133-135.

BRODERSEN, B.W. (2004). Immunohistochemistry used as a screening method for persistent bovine viral diarrhoea virus infection. *Veterinary Clinics North America: Food Animal Practice,* 20:85-93.

BROWNLIE, J., CLARKE, M.C. & HOWARD, C.J. (1984). Experimental production of fatal mucosal disease in cattle. *Veterinary Record*, 114:535-536.

BROWNLIE, J., CLARKE M.C. & HOWARD, C.J. (1986). Clinical and experimental mucosal disease - defining a hypothesis for pathogenesis. *Procedures of an EEC conference on pestivirus infections of ruminants*, Brussels, September 1985.

BROWNLIE, J. (1990). The pathogenesis of bovine virus diarrhoea virus infection. *Rev. Sci. Tech.,* 9(1):43-59.

BROWNLIE, J., BOOTH, P.J., STEVENS, D.A. & COLLINS, M.E. (1997). Expression of non-cytopathic bovine viral diarrhoea virus (BVDV) in oocysts and follicles of persistently infected cattle. *Veterinary Record*, 141:335-337.

BRUSCHKE, C.J.M., HAGHPARAST, A., HOEK. A., RUTTEN, V.P.M.G., WENTINK, G.H., VAN RIJN, P.A. & VAN OIRSCHOT, J.T. (1998). The immune response of cattle, persistently infected with non-cytopathic BVDV, after superinfection with antigenitically semi-homologous cytopathic BVDV. *Veterinary Immunology and Immunopathology*, 62:37-50.

CALLAHAN, J.D., BROWN, F., OSORIO, F.A., SUR, J.H., KRAMER, E., LONG, G.W., LUBROTH, J., ELLIS, S.J., SHOULARS, K.S., GAFFNEY, K.L., ROCK, D.L. & NELSON, W.M. (2002). Use of a portable real-time reverse transcriptase polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *Journal of American Veterinary Medical Association*, 220:1636-1642.

CARLSSON, U. (1991). Border disease in sheep caused by transmission of virus from cattle persistently infected with BVDV. *Veterinary Record,* 128:145-147.

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. *Journal of Veterinary Diagnostic Investigation*, 10(1):27-35.

CORAPI, W.V., FRENCH, T.W. & DUBOVI, E.J. (1989). Severe thrombocytopenia in young calves experimentally infected with noncytopathic bovine viral diarrhoea virus. *Journal of Virology,* 63(9):3934-3943.

COUVREUR, B., LETELLIER, C., COLLARD, A., QUENON, P., DEHAN, P., HAMERS, C., PASTORET, P.P. & KERKHOFS, P. (2002). Genotyping, genetic and antigenic variability in bovine viral diarrhoea field isolates. *Virus Research*, 85:17-28.

DARBYSHIRE, J.H. (1960). A serological relationship between swine fever and mucosal disease of cattle. *Veterinary Record*, 72:331.

DAVID, G.P., CRAWSHAW, T.R., GUNNING, R.F., HIBBERD, R.C., LLOYD, G.M. & MARSH, P.R. (1994). Severe disease in adult diary cattle in three UK diary herds associated with BVD virus infection. *Veterinary Record,* 134(18):468-472.

DEPNER, K., HUBSCHLE, O.J.B. & LIESS, B. (1991). Prevalence of ruminant pestivirus infections in Namibia. *Onderstepoort Journal of Veterinary Research,* 58:107-109.

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, J.A. (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. *Journal of General Virology,* 74:1433-1438.

DENG, R. & BROCK, K.V. (1993). 5′ and 3′ untranslated regions of pestivirus genome: primary and secondary structure analyses. *Nucleic Acid Research*, 21:1949-1957.

DONIS, R.O. (1995). Molecular biology of bovine viral diarrhoea virus and its interactions with the host. *Veterinary Clinics of North America Food Animal Practice,* 11:393-423.

DOYLE, L.G. & HEUSCHELE, W.P. (1983). Bovine viral diarrhea virus infection in captive exotic ruminants. *Journal of the American Veterinary Medical Association,* 183:1257-1259.

DUBOVI, E.J. (1990). The diagnosis of bovine viral diarrhoea infections: A laboratory view. *Veterinary Medicine,* 85:1133-1139.

DUFFELL, S.J. & HARKNESS, J.W. (1985). Bovine virus diarrhoea-mucosal disease infection in cattle. *Veterinary Record*, 117:240-245.

EDWARDS, S., MOENNIG, V. & WENSVOORT, G. (1991). The development of an international reference panel of monoclonal antibodies for the differentiation of hog cholera virus from other pestiviruses. *Veterinary Microbiology*, 29:101-108.

EDWARDS, S., ROEHE, P.M. & IBATA, G. (1995). Comparative studies of border disease and closely related virus infections in experimental pigs and sheep. *British Veterinary Journal*, 151:181-187.

ELLIS, J.A., DAVIS, W.C., BELDEN, E.L. & PRATT, D.L. (1988). Flow cytofluorimetric analysis of lymphocyte subset alterations in cattle infected with bovine viral diarrhoea virus. *Veterinary Pathology*, 25:231-236.

FERNELIUS, A.L., LAMBERT, G. & HENNESS, G.J. (1969). Bovine viral diarrhoea virus. Host cell interactions: Adaptation and growth of virus in the cell lines. *American Journal of Veterinary Research*, 1561-1572.

FERREIRA, G.M., LOURENS, D.C. & VAN VUUREN, M. (2000). The prevalence of bovine viral diarrhoea antibodies in selected South African dairy herds and control of the disease. *Journal of the South African Veterinary Association*, 71:10-13.

FICKEN, M.D., ELLSWORTH, M.A., TUCKER, C.M. & CORTESE, V.S. (2006). Effects of modified-live bovine viral diarrhoea virus vaccines containing either type 1 or type 1 and 2 BVDV on heifers and their offspring after challenge with noncytopathic type 2 BVDV during gestation. *Journal American Veterinary Medical Association,* 228(10):1559-1564.

FRITZEMEIER, J., HAAS, L., LIEBLER, E., MOENNIG, V. & GREISER-WILKE, I. (1997). The development of early vs late onset mucosal disease is a consequence of two different pathogenic mechanisms. *Archives of Virology*, 142:1335-1350.

FRÖLICH, K. (1995). Bovine virus diarrhoea and mucosal disease in free-ranging and captive deer (Cervidae) in Germany. *Journal of Wildlife Diseases,* 31:247-250.

GERDES, T. (1997). Bovine viral diarrhoea virus. Proceedings of a workshop on BVDV. *Pfizer Animal Health, Bakubung, South Africa.*

GIANGASPERO, M., VACIRCA, G., BUTTNER, M., WOLF, G., VANOPDENBOSCH, E. & MUYDERMANS, G. (1993). Serological and antigenic findings indicating pestivirus infection in man. *Archives of Virology,* 7:53-62.

GILLESPIE, J.H., BAKER, J.A. & MCENTEE, K. (1960). A cytopathogenic strain of bovine virus diarrhoea virus. *Cornell Veterinarian,* 50:73-79.

GREGG, K., CHEN, S.H., GUERRA, T., SADEGHIEH, S., XIANG, T., MEREDITH, J. & POLEJAEVA, I. (2009). A sensitive and efficient detection method for bovine viral diarrhoea virus (BVDV) in single pre-implantation bovine embryos. *Theriogenology,* 71:966-974.

GUNN, H.M. (1993). Role of fomites and flies in the transmission of bovine viral diarrhoea virus. *Veterinary Record,* 132:584-585.

GUTEKUNST, D.E. & MALMQUIST, W.A. (1963). Separation of a soluble antigen and infectious particles of bovine viral diarrhoea viruses and their relationship to hog cholera. *Canadian Journal of Comparative Medicine and Veterinary Science*, 27:121-123.

HAMBLIN, C. & HEDGER, R.S. (1979). The prevalence of antibodies to bovine viral diarrhoea/mucosal disease virus in African wildlife. *Comparative Immunology Microbiology and Infectious Diseases*, 2:295-303.

HARKNESS, J.W., SANDS, J.J. & RICHARDS, M.S. (1978). Serological studies of mucosal disease virus in England and Wales. *Research in Veterinary Science*, 24:98-103.

HEGAZY, A.A., FAHMY, L.S., SABER, M.S., ABOELLAIL, T.A., YOUSIF, A.A. & CHASE, C.C.L. (1996). Bovine virus diarrhoea virus infection causes reproductive failure and neonatal deaths in dromedary camels. In: *Symposium: bovine viral diarrhoea virus*. A 50 year review. College of Veterinary Medicine, Cornell University, pp. 205.

HOFMANN, M.A., BRECHTBÜHL, K. & STÄUBER, N. (1994). Rapid characterization of new pestivirus strains by direct sequencing of PCR- amplified cDNA from the 5′ noncoding region. *Archives of Virology,* 139:217-229.

HOFFMANN, B., BEER, M., SCHELP, C., SCHIRRMEIER, H. & DEPNER, K. (2005). Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. *Journal of Virological Methods,* 130:36-44.

HOLM-JENSEN, M. (1981). Detection of antibodies against hog cholera virus and bovine viral diarrhoea virus in porcine serum. *Acta Veterinaria Scandinavica*, 22:85-98.

HORZINEK, M.C. (1973). Comparative aspects of Togaviruses. *Journal of General Virology*, 20:87-103.

HOWARD, C.J., BROWNLIE, J. & CLARKE, M.C. (1987). Comparison by the neutralization assay of pairs of non-cytopathic strains of bovine virus diarrhoea virus isolated from cases of mucosal disease. *Veterinary Microbiology*, 13:361-369.

HUGHES, L.E., KERSHAW, G.P. & SHAW, I.G. (1959). Border disease: An undescribed disease of sheep. *Veterinary Record*, 71:313-317.

KABONGO, N. & VAN VUUREN, M. (2004). Detection of bovine viral diarrhea virus in specimens from cattle in South Africa and possible association with disease. *Journal of the South African Veterinary Association,* 75(2):90-93.

KABONGO, P.N. (2001). The isolation and characterization of bovine viral diarrhoea viruses from cattle in South Africa. MSc. Thesis. Department of Veterinary Tropical Diseases Faculty of Veterinary Science, University of Pretoria, Pretoria.

KADIR, Y., CHRISTINE, F., BARBARA, B., ZEKI, Y., FERAY, A., AYKUT, O., IBRAHIM, B., SIBILINA, C.R., HEINZ-JÜRGEN, T. & MATTHIAS, K. (2008). Genetic heterogeneity of bovine viral diarrhoea virus (BVDV) isolates from Turkey: Identification of a new subgroup in BVDV-1. *Veterinary Microbiology*, 130:258-267.

KWANG, J., LITTLEDIKE, E.T., BOLIN, S. & COLLETT, M.S. (1991). Efficiency of various cloned DNA probes for detection of bovine viral diarrhoea viruses. *Veterinary Microbiology*, 28:279-288.

KIRKLAND, P.D. & MACKINTOSH, S.G. (1992). Bovine pestivirus infections, virology and serology. *Australian Standard Diagnostic Techniques for Animal Diseases,* 3-16.

KIRKLAND, P.D., MCGOWAN, M.R., MACKINTOSH, S.G. & MOYLE, A. (1997). Insemination of cattle with semen from a bull transiently infected with a pestivirus. *Veterinary Record*, 140:124-127.

LANG-REE, J.R., VANT, T., KOMMISRUD, E. & LOKEN, T. (1994). Transmission of bovine viral diarrhoea virus by rectal examination. *Veterinary Record,* 135:412-413.

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. *Veterinary Microbiology*, 64:155-167.

LETELLIER, C. & KERKHOFS, P. (2003). Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhoea virus. *Journal of Virological Methods,* 114:21-27.

LEWIS, T.L., RIDPATH, J.F., BOLIN, S.R. & BERRY, E.S. (1991). Detection of BVD viruses using synthetic oligonucleotides. *Archives of Virology*, 117:269-278.

LIESS, B., ORBAN, S., FREY, H.R., TRAUTWEIN, G., WIEFEL, W. & BLINDOW, H. (1984). Studies on transplacental transmissibility of bovine virus diarrhoea (BVD) vaccine virus in cattle. *Zentralblatt für Veterinärmedizin B*, 31:669-681.

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.

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. *Journal of Virological Methods*, 154:82-85.

MAHONY, T.J., MCCARTHY, F.M., GRAVEL J.L., CORNEY, B., YOUNG, P.L. & VILCEK S. (2005). Genetic analysis of bovine viral diarrhoea viruses from Australia. *Veterinary Microbiology,* 106:1-6.

MCCLURKIN, A.W., LITTLEDIKE, E.T., CUTLIP, R.C., FRANK, G.H., CORIA, M.F. & BOLIN, S.R. (1984). Production of cattle immunotolerant to bovine viral diarrhoea virus. *Canadian Journal of Comparative Medicine,* 48:156-161.

MCGOLDRICK, A., BENSAUDE, E., IBATA, G., SHARP, G. & PATON, D.J. (1999). Closed one - tube reverse transcription nested polymerase chain reaction for the detection of pestiviral RNA with fluorescent probes. *Journal of Virological Methods,* 79:85-95.

MENGELING, W.L., GUTEKUNST, D.E., FERNELIUS, A.L. & PIRTLE, E.C. (1963). Demonstration of an antigenic relationship between hog cholera and bovine viral diarrhoea viruses by immunofluorescence. *Canadian Journal of Comparative Veterinary Science,* 162-164.

MEYERS, G. & THIEL, H.J. (1996). Molecular characterization of pestiviruses. *Advances in Virus Research*, 47:53-118.

MEYERS, G., RÜMENAPF, T. & THIEL, H.J. (1989). Molecular cloning and nucleotide sequence of the genome of hog cholera virus. *Virology*, 171:555-567.

MEYLING, A. (1984). Detection of BVD virus in viraemic cattle by an indirect immunoperoxidase technique. In: McNulty, M.S., MacFerran, J.B. & Martinus Nijhoff (Eds). Recent Advances in Virus Diagnosis, Boston, MA, pp 37-46.

MOENNIG, V. (1990). Pestiviruses: a review. *Veterinary Microbiology*, 23:35-54.

MUVAVARIRWA, P., MUDENGE, D., MOYO, D. & JAVANGWE, S. (1995). Detection of bovine viral diarrhoea virus antibodies in cattle with enzyme-linked immunosorbent assay. *Onderstepoort Journal of Veterinary Research,* 62:241-244.

NJAA, B.L., CLARK, E.G., JANZEN, E., ELLIS, J.A. & HAINES, D.M. (2000). Diagnosis of persistent bovine viral diarrhoea virus infection by immunohistochemical staining of formalin-fixed skin biopsy specimens. *Journal of Veterinary Diagnostic Investigation*, 12:393-399.

OLAFSON, P., MACCALLUM, A.D. & FOX, F.H. (1946). An apparently new transmissible disease of cattle. *Cornell Veterinarian,* 36:2205-213.

ORBAN, S., LEISS, B., HAFEZ, S.M., FREY, H.R., BLINDOW, H. & SASSE-PATZER, B. (1983). Studies on transplacental transmissibility of a bovine virus diarrhoea (BVD) vaccine virus. I. Inoculation of pregnant cows 15-90 days before parturition (190^{th}-265^{th} day of gestation). *Zentralblatt für Veterinärmedizin, Reihe B*, 30:619-634.

PATON, D.J., SIMPSON, V. & DONE, S.H. (1992). Infection of pigs and cattle with bovine viral diarrhoea virus on a farm in England. *Veterinary Record,* 131:185-188.

PATON, D.J., SANDS, J.J., LOWINGS, J.P., SMITH, J.E., IBATA, G. & EDWARDS, S. (1995). A proposed division of the pestivirus genus using monoclonal antibodies supported by cross-neutralization assays and genetic sequencing. *Veterinary Research*, 26:92-109.

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. *Virology,* 203:260-268.

POLAK, M.P. & ZMUDZINSKI, J.F. (2000). Experimental inoculation of calves with laboratory strains of bovine viral diarrhoea virus. *Comparative Immunology Microbiology and Infectious Diseases*, 23:141-151.

POTGIETER, L.N.D. (2004). Bovine viral diarrhoea and mucosal disease. In: Coetzer, J.A.W., Tustin, R.C., editors. Infectious Diseases of livestock. Oxford University Press, Cape Town, South Africa, p. 946-969.

QI, F., GUSTAD, T., LEWIS, T.L. & BERRY, E.S. (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. *Molecular Cell Probes,* 7:349-356.

QVIST, P., HOUE, H. & AASTED, B. (1991). Comparison of flow cytometry and virus isolation in cell culture for identification of cattle persistently infected with bovine viral diarrhoea virus. *Journal of Clinical Microbiology*, 29:660-661.

QVIST, P., AASTED, B. & BLOCH, B. (1990). Flow cytometric detection of bovine viral diarrhoea virus in peripheral blood leukocytes of persistently infected cattle. *Canadian Journal of Veterinary Research,* 54:469-472.

RAMSY, F.K. & CHIVERS W.H. (1953). Mucosal disease of cattle. *North American Veterinarian*, 34:629-633.

REBHUN, W.C., FRENCH, T.W., PERDRIZET, J.A., DUBOVI, E.J., DILL, S.G. & KARCHER, L.F. (1998). Thrombocytopenia associated with acute bovine virus diarrhoea infection in cattle. *Journal of Veterinary Internal Medicine,* 3(1):42-46.

REID, S.M., FERRIS, N.P., HUTCHINGS, G.H., KING, D.P. & ALEXANDERSEN, S. (2004). Evaluation of real-time reverse transcription polymerase chain reaction assays for the detection of swine vesicular disease virus. *Journal of Virological Methods,* 116:169-176.

REID, S.M., FERRIS, N.P., HUTCHINGS, G.H., ZHANG, Z., BELSHAM, G.J. & ALEXANDERSEN, S. (2002). Detection of all serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. *Journal of Virological Methods*, 105:67-80.

RIDPATH, J.F. & BOLIN, S.R. (1991). Antigenic and genomic comparison between non-cytopathic and cytopathic bovine viral diarrhoea viruses isolated from cattle that had spontaneous mucosal disease. *Journal of General Virology*, 72:725-729.

RIDPATH, J.F. & BOLIN, S.R. (1991). Hybridization analysis of genomic variability among isolates of bovine viral diarrhoea virus using cDNA probes. *Molecular Cell Probes,* 5:291-298.

RIDPATH, J.F., BOLIN, S.R. & 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. *Journal of Clinical Microbiology,* 31:986-989.

RIDPATH, J.F., BOLIN, S.R. & DUBOVI, E.J. (1994). Segregation of bovine viral diarrhoea virus into genotypes. *Virology,* 205:66-74.

RIDPATH, J.F. & BOLIN, S.R. (1995). The genomic sequence of a virulent bovine viral diarrhoea virus (BVDV) from the type 2 genotype: Detection of a large genomic insertion in a non-cytopathic BVDV. *Virology*, 212:39-46.

RIDPATH, J.F. (1996). Sequence diversity and genotyping. In: *Symposium on bovine viral diarrhoea virus*. A 50 year review. College of Veterinary Medicine Cornell University, 39.

RISATTI, G.R., CALLAHAN, J.D., NELSON, W.M. & BORCA, M.V. (2003). Rapid detection of classical swine fever virus by a portable real-time reverse transcriptase PCR assay. *Journal of Clinical Microbiology*, 41:500-505.

ROEDER, P.L. & HARKNESS, J.W. (1986). Bovine viral diarrhoea virus infection: Prospects for control. *Veterinary Record,* 118:143-147.

ROEDER, P.L. (1994). Transmission of BVD virus by rectal examination. *Veterinary Record*, 135:606.

ROEHE, P.M., WOODWARD, M.J. & EDWARDS, S. (1992). Characterization of p^{20} sequences from a border disease-like pestivirus isolated from pigs. *Veterinary Microbiology*, 33:231-238.

SALIKI, J.T. (1996). Bovine viral diarrhoea: Clinical picture in South Central United States. In: *Symposium on bovine viral diarrhoea virus*. A 50 year review. College of Veterinary Medicine, Cornell University, 159.

SALIKI, J.T. & DUBOVI, E.J. (2004). Laboratory diagnosis of bovine viral diarrhoea virus infections. *Veterinary Clinics of North America: Food Animal Practice*, 20:69-83.

SANDVIK, T. (1999). Laboratory diagnostic investigations for bovine viral diarrhoea virus in cattle. *Veterinary Microbiology,* 64:123-134.

SANDVIK, T. (2005). Selection and use of laboratory diagnostic assays in BVD control programmes. *Preventive Veterinary Medicine*, 72:3-16.

SCHRIJVER, R.S. & KRAMPS, J.A. (1998). Critical factors affecting diagnostic reliability of enzyme-linked immunosorbent assay formats. *Revue Scientifique et Technique*, 17:550-561.

SHIMAZAKI, T., SEKIGUCHI, H., NAKAMURA, S., TAGUCHI, K., INOUE, Y. & SATOH, M. (1998). Segregation of bovine viral diarrhoea viruses isolated in Japan into genotypes. *Journal of Veterinary Medical Science*, 60:579-583.

SOINE, C., UATANAUA, G. & DEPNER, K.R. (1992). Prevalence of antibodies to bovine viral diarrhoea virus in Namibian wildlife. *Tropical Animal Health and Production,* 24:125-126.

STROFFREGEN, B., BOLIN, S.R., RIDPATH, J.F., POHLENZ, J. (2000). Morphologic lesions in type 2 BVDV infections experimentally induced by strain BVDV2-1373 recovered from a field case. *Veterinary Microbiology*, 77:157-162.

SULLIVAN, D.G., CHANG, G.J. & AKKINA, R.K. (1997). Genetic characterization of ruminant pestiviruses: sequence analysis of viral genotypes isolated from sheep. *Virus Research*, 47:19-29.

TAUTZ, N., MEYERS, G. & THIEL, H.J. (1998). Pathogenesis of mucosal disease, a deadly disease of cattle caused by a pestivirus. *Clinical and Diagnostic Virology*, 10:121-127.

THELLIN, O., ZORZI, W., LAKAYE, B., DE BORMAN, B., COUMANS, B., HENNEN, G., GRISAR, T., IGOUT, A. & HEINEN, E. (1999). Housekeeping genes internal standards: use and limits. *Journal of Biotechnology*, 75:291-295.

THEODORIDIS, A., BOSHOFF, SET. & BOTHA M.J. (1973). Mucosal disease in Southern Africa. *Journal of the South African Veterinary Association,* 44:61-63.

THEODORIDIS, A. & BOSHOFF, SET. (1974). Isolation of cytopathogenic strains of bovine viral diarrhea virus in Southern Africa. *Journal of the South African Veterinary Association,* 445:203-205.

THOMSON, G.R. & BLACKBURN, N.K. (1972). Bovine virus diarrhea mucosal disease in Rhodesian cattle. *Zimbabwean Veterinary Journal,* 3:15-19.

THOMSON, J.D., GIBSON, T.J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D.G. (1997). The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Research,* 25:4876-4882.

THUR, B., ZLINSZKY, K. & EHRENSPERGER, F. (1996). Immunohistochemical detection of bovine viral diarrhoea virus in skin biopsies: a reliable and fast diagnostic tool. *Zentralblatt für Veterinärmedizin, Reihe B*, 43:163-166.

TIJSSEN, P. (1985). Laboratory Techniques in Biochemistry and Molecular Biology: Practice and Theory of Enzyme Immunoassay, Elsevier, Amsterdam. ISBN 0-444-80633-4.

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.

VICKERS, M.L. & MINOCHA, H.C. (1990). Diagnosis of bovine viral diarrhoea virus infection using monoclonal antibodies. *Journal of Veterinary Diagnostic Investigation*, 2:300-302.

VILCEK, S., HERRING, A.J., HERRING, J.A., NETTLETON, P.F., LOWINGS, J.P. & PATON, D.J. (1994). Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. *Archives of Virology*, 136:309-323.

VILCEK, S. & BELÅK, S. (1996). Genetic identification of pestivirus strain Frijters as a border disease virus from pigs. *Journal of Virological Methods,* 60:103-108.

VILCEK, S., NETTLETON, P.F., PATON, D.J. & BELÅK, S. (1997). Molecular characterization of ovine pestiviruses. *Journal of General Virology*, 78:725-735.

VILCEK, S., PATON, D.J., LOWINGS, P., BJÖRKLUND, H., NETTLETON, P. & BELÅK, S. (1999). Genetic analysis of pestiviruses at the 3-prime end of the genome. *Virus Genes* 18:107-114.

VILCEK, S., PATON, D.J., DURKOVIC, B., STROJNY, L., IBATA, G. & MOUSSA, A. (2001). Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. *Archives of Virology*, 146:99-115.

WARD, P. & MISRA, V. (1991). Detection of bovine viral diarrhoea virus, using degenerate oligonucleotide primers and the polymerase chain reaction. *American Journal of Veterinary Research*, 52:1231-1236.

WENGLER, G., BRADLEY, D.W., COLLET, M.S., HEINZ, F.X., SCHLESINGER, R.W. & STRAUSS, J.H. (1995). Family *flaviviridae*. In: Murphy, F.A., Fauquet, C.M., Bishop, C.M., *et al*. (Eds.), Viral Taxonomy. Springer, New York, pp. 27-45.

WHELAN, J.A., RUSSELL, N.B. & WHELAN, M.A. (2003). A method for the absolute quantification of cDNA using real time PCR. *Journal of Immunological Methods,* 278:261-269.

YAMAMOTO, T., KOZASA, T., AOKI, H., SEKIGUCHI, H., MORINO, S. & NAKAMURA, S. (2008). Genomic analyses of bovine viral diarrhoea viruses isolated from cattle imported into Japan between 1991 and 2005. *Veterinary Microbiology*, 127:386-391.

YOUNG, N.J., THOMAS, C.J., COLLINS, M.E. & BROWNLIE, J. (2006). Real-time RT-PCR detection of bovine viral diarrhoea virus in whole blood using an external RNA reference. *Journal of Virological Methods*, 138:218-222.

ZIMMER, G.M., VAN MAANEN, C., DE GOEY, I., BRINKHOF, J. & WENTINK, G.H. (2004). The effect of maternal antibodies on the detection of bovine virus diarrhoea virus in peripheral blood samples. *Veterinary Microbiology*, 100:145-149.

Annexure 1 :
Results

Summary of the origin of the cattle, test used and the result of specimens tested

		ID. No.	Specimens	Test	Result	Province of origin of animal
1	8/7/2009	KZN606	LN/SP	RT-rtPCR	Neg	KZN
2	8/7/2009	KZN607	LN/SP	RT- rt PCR	Neg	KZN
3	8/7/2009	KZN608	LN/SP	RT- rt PCR	Pos.genotype 1	KZN
4	8/7/2009	KZN609	LN/SP	RT- rt PCR	Neg	KZN
5	8/7/2009	KZN610	LN/SP	RT- rt PCR	Neg	KZN
6	8/7/2009	KZN611	LN/SP	RT- rt PCR	Neg	KZN
7	8/7/2009	KZN613	LN/SP	RT- rt PCR	Neg	KZN
8	8/7/2009	KZN615	LN/SP	RT- rt PCR	Neg	KZN
9	8/7/2009	KZN616	LN/SP	RT- rt PCR	Neg	KZN
10	8/7/2009	KZN617	LN/SP	RT- rt PCR	Neg	KZN
11	8/7/2009	KZN618	LN/SP	RT- rt PCR	Neg	KZN
12	8/7/2009	KZN619	LN/SP	RT- rt PCR	Neg	KZN
13	8/7/2009	KZN620	LN/SP	RT- rt PCR	Neg	KZN
14	8/7/2009	KZN621	LN/SP	RT- rt PCR	Neg	KZN
15	8/7/2009	KZN622	LN/SP	RT- rt PCR	Neg	KZN
16	8/7/2009	KZN623	LN/SP	RT- rt PCR	Neg	KZN
17	8/7/2009	KZN624	LN/SP	RT- rt PCR	Neg	KZN
18	8/7/2009	KZN625	LN/SP	RT- rt PCR	Neg	KZN
19	8/7/2009	KZN627	LN/SP	RT- rt PCR	Pos. genotype 1	KZN
20	3/8/2009	KA1	TTA	RT- rt PCR	Neg	
21	3/8/2009	KA2	TTA	RT- rt PCR	Neg	
22	3/8/2009	KA3	TTA	RT- rt PCR	Neg	
23	3/8/2009	KA4	TTA	RT- rt PCR	Neg	
24	3/8/2009	KA5	TTA	RT- rt PCR	Neg	
25	3/8/2009	KA6	TTA	RT- rt PCR	Neg	
26	3/8/2009	KA7	TTA	RT- rt PCR	Neg	
27	3/8/2009	KA8	TTA	RT- rt PCR	Neg	
28	3/8/2009	KA9	TTA	RT- rt PCR	Neg	
29	3/8/2009	KA10	TTA	RT- rt PCR	Neg	
30	3/8/2009	TH1	TTA	RT- rt PCR	Neg	
31	3/8/2009	TH2	TTA	RT- rt PCR	Neg	
32	3/8/2009	TH3	TTA	RT- rt PCR	Neg	

33	3/8/2009	TH4	TTA	RT- rt PCR	Neg	
34	3/8/2009	TH5	TTA	RT- rt PCR	Neg	
35	3/8/2009	BH1	TTA	RT- rt PCR	Neg	
36	3/8/2009	BH2	TTA	RT- rt PCR	Neg	
37	3/8/2009	BH3	TTA	RT- rt PCR	Neg	
38	3/8/2009	BH4	TTA	RT- rt PCR	Neg	
39	3/8/2009	BH5	TTA	RT- rt PCR	Neg	
40	3/8/2009	SIS1	TTA	RT- rt PCR	Neg	
41	3/8/2009	SIS2	TTA	RT- rt PCR	Neg	
42	3/8/2009	SIS3	TTA	RT- rt PCR	Neg	
43	3/8/2009	SIS4	TTA	RT- rt PCR	Neg	
44	3/8/2009	SIS5	TTA	RT- rt PCR	Neg	
45	3/8/2009	NC1	TTA	RT- rt PCR	Neg	
46	3/8/2009	NC2	TTA	RT- rt PCR	Neg	
47	3/8/2009	NC3	TTA	RT- rt PCR	Pos. genotype 1	NW
48	3/8/2009	NC4	TTA	RT- rt PCR	Neg	
49	3/8/2009	EAC1	TTA	RT- rt PCR	Neg	
50	3/8/2009	EAC2	TTA	RT- rt PCR	Neg	
51	3/8/2009	EAC3	TTA	RT- rt PCR	Neg	
52	3/8/2009	EAC4	TTA	RT- rt PCR	Neg	
53	3/8/2009	NN1	TTA	RT- rt PCR	Neg	
54	3/8/2009	NN2	TTA	RT- rt PCR	Neg	
55	3/8/2009	NN3	TTA	RT- rt PCR	Neg	
56	3/8/2009	NN4	TTA	RT- rt PCR	Neg	
57	3/8/2009	NN5	TTA	RT- rt PCR	Neg	
58	3/8/2009	LD1	TTA	RT- rt PCR	Pos. genotype1	Limpopo
59	3/8/2009	LD2	TTA	RT- rt PCR	Pos. genotype1	Limpopo
60	3/8/2009	LD3	TTA	RT- rt PCR	Pos. genotype1	Limpopo
61	3/8/2009	MV1	TTA	RT- rt PCR	Pos. genotype1	Limpopo
62	3/8/2009	MV2	TTA	RT- rt PCR	Pos. genotype1	Limpopo
63	3/8/2009	MV3	TTA	RT- rt PCR	Pos. genotype1	Limpopo
64	3/8/2009	MV4	TTA	RT- rt PCR	Pos. genotype1	Limpopo
65	3/8/2009	MU1	TTA	RT- rt PCR	Pos. genotype1	NW
66	3/8/2009	MU2	TTA	RT- rt PCR	Pos. genotype1	NW
67	3/8/2009	WB1	TTA	RT- rt PCR	Neg	
68	3/8/2009	WB2	TTA	RT- rt PCR	Neg	
69	3/8/2009	TAB1	TTA	RT- rt PCR	Neg	
70	3/8/2009	TAB2	TTA	RT- rt PCR	Neg	
71	3/8/2009	TAB3	TTA	RT- rt PCR	Neg	
72	3/8/2009	KG1	TTA	RT- rt PCR	Neg	
73	3/8/2009	KG2	TTA	RT- rt PCR	Neg	

74	3/8/2009	KG3	TTA	RT- rt PCR	Neg	
75	3/8/2009	KG4	TTA	RT- rt PCR	Neg	
76	3/8/2009	DGV1	TTA	RT- rt PCR	Neg	
77	3/8/2009	DGV2	TTA	RT- rt PCR	Pos. genotype1	NC
78	15/09/2009	OVAH	BL	RT- rt PCR	Pos. genotype1	GA
79	3/8/2009	DGV3	TTA	RT- rt PCR	Neg	
80	9/1/2009	614020	LN/SP	RT- rt PCR	Pos. genotype2	KZN
81	15/4/2009	612182	LN/SP	RT- rt PCR	Neg	
82	7/5/2009	301610	LN/SP	RT- rt PCR	Neg	
83	7/5/2009	503914	LN/SP	RT- rt PCR	Neg	
84	7/5/2009	826950	LN/SP	RT- rt PCR	Neg	
85	7/5/2009	532226	LN/SP	RT- rt PCR	Neg	
86	7/5/2009	115004	LN/SP	RT- rt PCR	Neg	
87	11/5/2009	606275	LN/SP	RT- rt PCR	Neg	
88	13/5/2009	101528	LN/SP	RT- rt PCR	Neg	
89	14/5/2009	pre-pro	LN/SP	RT- rt PCR	Neg	
90	15/5/2009	612067	LN/SP	RT- rt PCR	Pos. genotype1	LIM
91	15/5/2009	620985	LN/SP	RT- rt PCR	Neg	
92	16/5/2009	615934	LN/SP	RT- rt PCR	Pos. genotype1	NC
93	16/5/2009	606275	LN/SP	RT- rt PCR	Neg	
94	16/5/2009	419595	LN/SP	RT- rt PCR	Neg	
95	17/5/2009	417405	LN/SP	RT- rt PCR	Pos. genotype1	GA
96	17/5/2009	615069	LN/SP	RT- rt PCR	Neg	
97	17/5/2009	611766	LN/SP	RT- rt PCR	Pos. genotype1	NW
98	17/5/2009	620219	LN/SP	RT- rt PCR	Pos. genotype1	FS
99	19/5/2009	624696	LN/SP	RT- rt PCR	Pos. genotype1	EC
100	20/5/2009	529214	LN/SP	RT- rt PCR	Neg	
101	20/5/2009	503545	LN/SP	RT- rt PCR	Neg	
102	20/5/2009	204061	LN/SP	RT- rt PCR	Neg	
103	20/5/2009	618605	LN/SP	RT- rt PCR	Pos. genotype1	NW
104	20/5/2009	625079	LN/SP	RT- rt PCR	Pos. genotype1	WC
105	20/5/2009	613964	LN/SP	RT- rt PCR	Neg	
106	21/5/2009	616317	LN/SP	RT- rt PCR	Neg	
107	21/5/2009	608919	LN/SP	RT- rt PCR	Neg	
108	21/5/2009	305458	LN/SP	RT- rt PCR	Neg	
109	22/5/2009	916129	LN/SP	RT- rt PCR	Neg	
110	23/5/2009	610588	LN/SP	RT- rt PCR	Neg	
111	23/5/2009	522141	LN/SP	RT- rt PCR	Neg	
112	25/5/2009	614118	LN/SP	RT- rt PCR	Pos. genotype1	FS
113	25/5/2009	625062	LN/SP	RT- rt PCR	Neg	
114	26/5/2009	208460	LN/SP	RT- rt PCR	Pos genotype1	GA

115	26/5/2009	627214	LN/SP	RT- rt PCR	Pos. genotype1	NW
116	27/5/2009	609346	LN/SP	RT- rt PCR	Neg	
117	27/5/2009	610609	LN/SP	RT- rt PCR	Neg	
118	28/5/2009	419609	LN/SP	RT- rt PCR	Neg	
119	28/5/2009	526473	LN/SP	RT- rt PCR	Neg	
120	28/5/2009	509168	LN/SP	RT- rt PCR	Neg	
121	28/5/2009	409068	LN/SP	RT- rt PCR	Neg	
122	29/5/2009	308638	LN/SP	RT- rt PCR	Neg	
123	29/5/2009	325987	LN/SP	RT- rt PCR	Neg	
124	29/5/2009	106204	LN/SP	RT- rt PCR	Neg	
125	29/5/2009	521176	LN/SP	RT- rt PCR	Neg	
126	29/5/2009	522734	LN/SP	RT- rt PCR	Neg	
127	29/5/2009	501426	LN/SP	RT- rt PCR	Neg	
128	30/5/2009	701200	LN/SP	RT- rt PCR	Pos. genotype 1	NW
129	30/5/2009	625895	LN/SP	RT- rt PCR	Neg	
130	30/5/2009	610597	LN/SP	RT- rt PCR	Pos. genotype1	FS
131	30/5/2009	620867	LN/SP	RT- rt PCR	Pos. genotype1	FS
132	31/5/2009	611084	LN/SP	RT- rt PCR	Neg	
133	6/6/2009	610609	LN/SP	RT- rt PCR	Pos. genotype1	FS
134	6/6/2009	613374	LN/SP	RT- rt PCR	Neg	
135	6/6/2009	609450	LN/SP	RT- rt PCR	Pos. genotype1	FS
136	6/6/2009	517289	LN/SP	RT- rt PCR	Neg	
137	6/6/2009	614793	LN/SP	RT- rt PCR	Pos. genotype1	NC
138	6/6/2009	609835	LN/SP	RT- rt PCR	Pos. genotype1	FS
139	6/6/2009	620181	LN/SP	RT- rt PCR	Neg	
140	10/6/2009	609429	LN/SP	RT- rt PCR	Pos. genotype1	FS
141	10/6/2009	702878	LN/SP	RT- rt PCR	Pos. genotype1	NC
142	10/6/2009	627449	LN/SP	RT- rt PCR	Pos. genotype1	FS
143	10/6/2009	427748	LN/SP	RT- rt PCR	Pos. genotype1	GA
144	10/6/2009	427749	LN/SP	RT- rt PCR	Pos. genotype1	GA
145	10/6/2009	616803	LN/SP	RT- rt PCR	Pos. genotype1	FS
146	10/6/2009	617367	LN/SP	RT- rt PCR	Pos. genotype1	KZN
147	10/6/2009	526641	LN/SP	RT- rt PCR	Pos. genotype1	GA
148	10/6/2009	621089	LN/SP	RT- rt PCR	Neg	
149	10/6/2009	609587	LN/SP	RT- rt PCR	Neg	
150	11/6/2009	626494	LN/SP	RT- rt PCR	Neg	
151	11/6/2009	612144	LN/SP	RT- rt PCR	Pos. genotype1	FS
152	11/6/2009	NO no.	LN/SP	RT- rt PCR	Pos. genotype1	GA
153	11/6/2009	NO no.	LN/SP	RT- rt PCR	Neg	
154	11/6/2009	218934	LN/SP	RT- rt PCR	Neg	
155	11/6/2009	XTIAN	LN/SP	RT- rt PCR	Neg	

156	12/6/2009	533746	LN/SP	RT- rt PCR	Neg	
157	12/6/2009	710710	LN/SP	RT- rt PCR	Pos. genotype1	NW
158	12/6/2009	623830	LN/SP	RT- rt PCR	Neg	
159	12/6/2009	415207	LN/SP	RT- rt PCR	Neg	
160	12/6/2009	626501	LN/SP	RT- rt PCR	Neg	
161	13/6/2009	704803	LN/SP	RT- rt PCR	Pos. genotype1	NC
162	13/6/2009	423009	LN/SP	RT- rt PCR	Pos. genotype1	GA
163	13/6/2009	617187	LN/SP	RT- rt PCR	Pos. genotype1	NC
164	13/6/2009	101528	LN/SP	RT- rt PCR	Pos. genotype1	GA
165	13/6/2009	127002	LN/SP	RT- rt PCR	Pos. genotype1	GA
166	14/6/2009	623848	LN/SP	RT- rt PCR	Neg	
167	14/6/2009	623598	LN/SP	RT- rt PCR	Neg	
168	14/6/2009	616703	LN/SP	RT- rt PCR	Neg	
169	14/6/2009	628960	LN/SP	RT- rt PCR	Neg	
170	15/6/2009	624745	LN/SP	RT- rt PCR	Pos. genotype1	EC
171	15/6/2009	623335	LN/SP	RT- rt PCR	Neg	
172	15/6/2009	614952	LN/SP	RT- rt PCR	Neg	
173	16/6/2009	608587	LN/SP	RT- rt PCR	Neg	
174	17/6/2009	614022	LN/SP	RT- rt PCR	Neg	
175	17/6/2009	718018	LN/SP	RT- rt PCR	Pos. genotype1	GA
176	18/6/2009	608559	LN/SP	RT- rt PCR	Neg	
177	18/6/2009	609107	LN/SP	RT- rt PCR	Neg	
178	18/6/2009	629695	LN/SP	RT- rt PCR	Neg	
179	18/6/2009	629575	LN/SP	RT- rt PCR	Pos. genotype1	NC
180	18/6/2009	521109	LN/SP	RT- rt PCR	Pos. genotype1	GA
181	18/6/2009	710704	LN/SP	RT- rt PCR	Neg	
182	18/6/2009	529089	LN/SP	RT- rt PCR	Neg	
183	18/6/2009	619526	LN/SP	RT- rt PCR	Neg	
184	19/6/2009	818298	LN/SP	RT- rt PCR	Neg	
185	19/6/2009	621703	LN/SP	RT- rt PCR	Neg	
186	19/6/2009	615080	LN/SP	RT- rt PCR	Neg	
187	20/6/2009	617299	LN/SP	RT- rt PCR	Pos. genotype1	FS
188	20/6/2009	704823	LN/SP	RT- rt PCR	Pos. genotype1	KZN
189	21/6/2009	705368	LN/SP	RT- rt PCR	Neg	
190	21/6/2009	620609	LN/SP	RT- rt PCR	Neg	
191	21/6/2009	624589	LN/SP	RT- rt PCR	Pos. genotype1	EC
192	21/6/2009	612745	LN/SP	RT- rt PCR	Neg	
193	21/6/2009	623246	LN/SP	RT- rt PCR	Neg	
194	21/6/2009	624747	LN/SP	RT- rt PCR	Neg	
195	21/6/2009	703032	LN/SP	RT- rt PCR	Pos. genotype1	NW
196	21/6/2009	610197	LN/SP	RT- rt PCR	Neg	

197	22/6/2009	620486	LN/SP	RT- rt PCR	Neg	
198	22/6/2009	610572	LN/SP	RT- rt PCR	Pos. genotype1	FS
199	22/6/2009	612417	LN/SP	RT- rt PCR	Neg	
200	22/6/2009	608632	LN/SP	RT- rt PCR	Neg	
201	22/6/2009	711046	LN/SP	RT- rt PCR	Neg	
202	24/6/2009	625331	LN/SP	RT- rt PCR	Neg	
203	24/6/2009	7069..	LN/SP	RT- rt PCR	Neg	
204	24/6/2009	610283	LN/SP	RT- rt PCR	Neg	
205	25/6/2009	616165	LN/SP	RT- rt PCR	Neg	
206	27/6/2009	621662	LN/SP	RT- rt PCR	Neg	
207	28/6/2009	614022	LN/SP	RT- rt PCR	Neg	
208	28/6/2009	626501	LN/SP	RT- rt PCR	Pos. genotype1	FS
209	28/6/2009	610312	LN/SP	RT- rt PCR	Neg	
210	29/6/2009	427830	LN/SP	RT- rt PCR	Neg	
211	29/6/2009	710455	LN/SP	RT- rt PCR	Neg	
212	29/6/2009	573172	LN/SP	RT- rt PCR	Pos. genotype1	GA
213	29/6/2009	622067	LN/SP	RT- rt PCR	Pos. genotype1	NC
214	29/6/2009	513172	LN/SP	RT- rt PCR	Neg	
215	30/6/2009	710162	LN/SP	RT- rt PCR	Neg	
216	30/6/2009	616151	LN/SP	RT- rt PCR	Pos. genotype1	KZN
217	30/6/2009	622030	LN/SP	RT- rt PCR	Neg	
218	1/7/2009	710179	LN/SP	RT- rt PCR	Pos. genotype2	NC
219	2/7.2009	615960	LN/SP	RT- rt PCR	Pos. genotype1	KZN
220	3/7/2009	625803	LN/SP	RT- rt PCR	Pos. genotype1	EC
221	4/7/2009	709724	LN/SP	RT- rt PCR	Pos. genotype1	GA
222	5/7/2009	614829	LN/SP	RT- rt PCR	Pos. genotype2	NC
223	6/7/2009	624846	LN/SP	RT- rt PCR	Pos. genotype1	EC
224	6/7/2009	613187	LN/SP	RT- rt PCR	Pos. genotype1	NC
225	6/7/2009	620548	LN/SP	RT- rt PCR	Pos. genotype1	LIM
226	6/7/2009	612007	LN/SP	RT- rt PCR	Pos. genotype1	LIM
227	7/7/2009	609853	LN/SP	RT- rt PCR	Pos. genotype2	FS
228	8/7/2009	627675	LN/SP	RT- rt PCR	Pos. genotype1	NW
229	8/7/2009	702277	LN/SP	RT- rt PCR	Pos. genotype2	EC
230	9/7/2009	624377	LN/SP	RT- rt PCR	Pos. genotype2	NC
231	10/7/2009	630059	LN/SP	RT- rt PCR	Pos. genotype2	EC
232	11/7/2009	706604	LN/SP	RT- rt PCR	Pos. genotype2	EC
233	11/7/2009	711079	LN/SP	RT- rt PCR	Pos. genotype2	EC
234	20/7/2009	711024	LN/SP	RT- rt PCR	Pos. genotype2	EC
235	21/7/2009	704423	LN/SP	RT- rt PCR	Pos. genotype1	NW
236	22/7/2009	703962	LN/SP	RT- rt PCR	Pos. genotype1	EC
237	23/7/2009	704074	LN/SP	RT- rt PCR	Pos. genotype1	EC

238	23/7/2009	710426	LN/SP	RT- rt PCR	Pos. genotype1	NC
239	24/7/2009	909524	LN/SP	RT- rt PCR	Pos. genotype1	GA
240	25/7/2009	623164	LN/SP	RT- rt PCR	Pos. genotype2	LIM
241	26/7/2009	628341	LN/SP	RT- rt PCR	Pos. genotype1	EC
242	31/7/2009	611190	LN/SP	RT- rt PCR	Pos. genotype1	LIM
243	1/8/2009	608914	LN/SP	RT- rt PCR	Pos. genotype1	FS
244	7/8/2009	608308	LN/SP	RT- rt PCR	Pos. genotype1	NW
245	24/8/2009	612219	LN/SP	RT- rt PCR	Pos. genotype1	FS
246	25/8/2009	622543	LN/SP	RT- rt PCR	Pos. genotype2	FS
247	27/8/2009	625638	LN/SP	RT- rt PCR	Pos. genotype2	EC
248	30/8/2009	710553	LN/SP	RT- rt PCR	Pos.genotype2	NW
249	4/9/2009	702542	LN/SP	RT- rt PCR	Pos. genotype2	EC
250	5/9/2009	706978	LN/SP	RT- rt PCR	Pos. genotype2	NW
251	7/9/2009	621420	LN/SP	RT- rt PCR	Pos. genotype2	EC
252	8/9/2009	627842	LN/SP	RT- rt PCR	Pos. genotype2	EC
253	30/9/2009	627864	LN/SP	RT- rt PCR	Pos. genotype1	EC
254	NO date	528260	LN/SP	RT- rt PCR	Neg	
255	NO date	Zastron	LN/SP	RT- rt PCR	Neg	
256	NO date	708198	LN/SP	RT- rt PCR	Pos. genotype1	LIM
257	NO date	GEEN	LN/SP	RT- rt PCR	Neg	
258	NO date	609832	LN/SP	RT- rt PCR	Pos. genotype1	FS
259	NO date	430080	LN/SP	RT- rt PCR	Neg	
260	NO date	600122	LN/SP	RT- rt PCR	Neg	
261	NO date	130205-1	LN/SP	RT- rt PCR	Pos. genotype1	GA
262	NO date	422601	LN/SP	RT- rt PCR	Neg	
263	NO date	503363	LN/SP	RT- rt PCR	Neg	
264	NO date	533455	LN/SP	RT- rt PCR	Neg	
265	NO date	505476	LN/SP	RT- rt PCR	Neg	
266	NO date	533587	LN/SP	RT- rt PCR	Neg	
267	NO date	130205-2	LN/SP	RT- rt PCR	Neg	
268	NO date	107504	LN/SP	RT- rt PCR	Neg	
269	NO date	528260	LN/SP	RT- rt PCR	Neg	
270	NO date	525149	LN/SP	RT- rt PCR	Neg	
271	NO date	603302	LN/SP	RT- rt PCR	Neg	
272	NO date	106755	LN/SP	RT- rt PCR	Neg	
273	NO date	614543	LN/SP	RT- rt PCR	Pos. genotype1	FS
274	NO date	500460	LN/SP	RT- rt PCR	Neg	
275	NO date	302697	LN/SP	RT- rt PCR	Pos. genotype1	GA
276	14/09/2009	NAM	LN/SP	RT- rt PCR	Neg	

Annexure 2 :
Genbank Sequences

List of Genbank sequences, virus name, accession number and Country/region of origin

Virus Name	Accession Number	Country/region	Reference
BVDV 2 strain 890	L32886	Iowa, USA	Pellerin *et al.*, 1994
BVDV 2-296nc	AF145969	Ames Iowa USA	Ridpath and Waltz, 1999
BVDV-strain Singer	L35852	Canada	Pellerin *et al.*, 1995
CSFV	AF039180	USA	Topliff and Kelling, 1998
Oregon C24V	AF091605	England	McGoldrick *et al.*, 1998
BVDV 1 strain Oregon	AF078536	Canada	Gilbert *et al.*, 1999
S-ALT1/K	U97470	Kwazulu-Natal	Baule *et al.*, 1997
S-ALT2/K	U97471	Kwazulu-Natal	Baule *et al.*, 1997
S-ALT7/K	U97476	Kwazulu-Natal	Baule *et al.*, 1997
Strain Osloss	AJ558196	Western India	Swamy *et al.*, 2003
BVDV 1	AF298059	Slovakia	Vilcek *et al.*, 2001
BVDV 1	AJ304384	Germany	Tajima *et al.*, 2001
BVDV 1	AF298054	Slovakia	Vilcek *et al.*, 2001
BVDV 1	AF298062	Slovakia	Vilcek *et al.*, 2001
BVDV 1	EU224224	Western Austria	Hornberg *et al.*, 2009
BVDV strain V071094	Af026783	New Zealand	Vilcek *et al.*, 1997
BVDV 2750A99	AJ312916	Northern Ireland	Graham *et al.*, 2001
BVDV 2	AF298055	Slovakia	Vilcek *et al.*, 2001
BVDV 2	EU051826	Turkey	Oguzoglu and Muz, 2007
BVDV 2 CH649	AF356505	Chile	Pizarro *et al.*, 2006
BVDV 2 CH649	AY671986	Chile	Pizarro *et al.*, 2006
BVDV 2 strain 37Gr	EU327594	Austria	Vilcek *et al.*, 2003
BVDV 1 strain waters	L32895	USA	Pellerin *et al.*, 1994

BVD Singer	L12455	Canada	Yu *et al.,* 1994
BVD Singer-AE2	AF083348	Canada	Deregt *et al.*, 1998
BDV	U00891	Germany	Becher *et al.*, 1994
BVDV 1	FJ223614	Ukraine	Gerilovych, 2008
BVDV 1 strain Singer	L32875	Canada	Pellerin *et al.*, 1994
BVDV 2	FJ431189	USA	Kim *et al.*, 2008
BVDV 2	FJ431190	USA	Kim *et al.*, 2008
CSFV	AF039177	USA	Topliff and Kelling, 1998
BVDV 2 strain V-FLL	AB019687	Japan	Sakoda *et al.*, 1999
BVDV 2 SD-06	FJ95044	China	Zhu *et al.*, 2009
CSFV strain EBTr(CCL44)	D50813	Japan	Harasawa and Mizusawa, 1995
BVDV NADL	AF039181	USA	Topliff and Kelling, 1998