THE ISOLATION AND CHARACTERIZATION OF BOVINE VIRAL DIARRHOEA VIRUSES FROM CATTLE IN SOUTH AFRICA

by

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DEDICATION

Happy is the man who gains understanding

Proverbs 3:13

Dedicated to my children

Axel and Benissa

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DECLARATION

With the exception of the gene sequence analyses, this dissertation is the candidate's own original work. It has not been previously submitted and is not currently being submitted in candidature for any other degree.

Candidate_____

P. N. Kabongo

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P N KABONGO

SUPERVISOR: PROFESSOR M VAN VUUREN

ABSTRACT

A limited number of scientific publications dealing with aspects of BVDV infection have emanated from southern Africa. This study describes the isolation of BVD viruses, gene sequence analysis of the 5' non-translated region (5' NTR) of the genome, the generation of phylogenetic data of local strains and the recording of clinical signs associated with each isolate.

Specimens (n=352) collected during 1998-1999, from live and dead animals from different farming systems, were obtained from private practitioners, feedlot consultants and abattoirs throughout the country. Specimens from buffaloes (*Syncerus caffer*) in the Kruger National Park were included as specimens from dead animals. Three cell lines and 200 tubes of pooled foetal bovine sera were also processed. Standard cell culture techniques to isolate virus were followed. Techniques designed to detect BVDV antigen or nucleic acid such as antigen capture enzyme-linked immunosorbent assay and polymerase chain reaction, were used on blood, organs and cell lines. The indirect fluorescent antibody test was used for antibody detection.

Twenty-five isolates from cattle were confirmed as BVDV with PCR and after analysis of the 5'NTR, the most conserved part of the genome, a phylogenetic tree was constructed. All strains were noncytopathic and were identified as BVDV I, either BVDV Ia (NADL-like) or BVDV Ic or BVDV I* subgroups. BVDV was not detected in 37 lymph nodes obtained from 37 buffaloes in the Kruger National Park.

Of the clinical signs in cattle from which virus was isolated, pyrexia and respiratory distress was the most frequent (46,7%), followed by pyrexia and diarrhoea (20%), respiratory disease without pyrexia (20%) and diarrhoea without pyrexia (13,3%). Abortion, congenital malformations, haemorrhagic syndrome and poor growth were also included as criteria for selection of animals for specimen collection.

ISOLASIE EN KARAKTERISERING VAN BEES VIRUS DIARREE VIRUSSE (BVDV) VAN BEESTE IN SUID AFRIKA

P N KABONGO

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OPSOMMING

'n Beperkte aantal wetenskaplike publikasies ten opsigte van BVDV infeksies in suidelike Afrika het tot dusver die lig gesien. Hierdie studie beskryf die isolasie van BVD-virusse, analise van die basis volgorde van die 5' nie-getransleerde area (5' NTA) van die genoom, die inwin van filogenetiese gegewens van plaaslike stamme en die kliniese tekens wat met elke stam verband hou.

Gedurende 1998-1999 is 352 monsters van lewende en dooie diere uit verskillende boerderystelsels van privaat praktisyns, voerkraalkonsultante en abattoirs deur die hele land verkry. Monsters afkormstig van buffels (*Syncerus caffer*) in die Kruger Nasionale wildtuin is ingesluit onder monsters afkomstig van dooie diere. Drie selkulture en 200 buise gepoelde fetale bees serum is ook ondersoek. Die virus is volgens standaard weefselkultuurtegnieke geïsoleer. Bloed, orgaan-en selkulture is onderwerp aan tegnieke soos ELISA en polimerase ketting reaksie (PKR) om die antigeen of nukleinsuur van BVDV op te spoor. Teenliggame is met die fluoresserende teenliggaamtoets uitgeken.

Deur die PKR-tegniek is 25 isolate van beeste as BVDV uitgeken. 'n Filogenetiese boom is opgestel na analise van die 5' NTA, 'n goed bewaarde gedeelte van die genoom. Al die stamme was nie-sitopaties vir selle en is uitgeken as BVDV tipe I, hetsy die subgroep BVDV Ia (NADL-agtig), BVDV Ic of BVDV I*. Limfknope van 37 buffels uit die Krugerwildtuin was negatief vir BVDV.

Van die kliniese tekens wat waargeneem was in beeste waaruit viruse geïsoleer is, was koors en asemhalingsiekte die mees algemeenste, nl. 46,7%, gevolg deur koors en diarree (20%), asemhalingsiekte sonder koors (20%) en diarree sonder koors (13,3%). Aborsie, kongenitale abnormaliteite, bloedings en swak groei is ook ingesluit as maatstawe vir die seleksie van diere vir monsterversameling.

CHAPTER 1. GENERAL INTRODUCTION

Bovine viral diarrhoea (BVD) was first described by Olafson and co-workers in New York State (Olafson *et al.* 1946) as an acute, highly contagious disease characterized by fever, diarrhoea and leukopaenia. They gave it the name of "virus diarrhoea" (VD) because of the most prominent clinical sign, diarrhoea. Later in 1946, Childs reported on the occurrence of a similar disease of cattle in Saskatchewan, Canada. A few years later, a disease was described in various states of the USA that affected the mucous membranes of the alimentary and respiratory tracts of cattle and was called "mucosal disease" (MD) (Ramsey & Chivers 1953). It appeared to have some similarities to virus diarrhoea, although it was less contagious and of a lower morbidity but it had a higher mortality rate. Mucosal disease is another clinical manifestation of bovine viral diarrhoea virus (BVDV) infection. It occurs specifically in immunotolerant carrier animals that are persistently infected (PI) with noncytopathic (ncp) BVDV and subsequently become superinfected with a cytopathic (cp) BVDV strain (Brownlie *et al.* 1984a,b; Bolin 1995a). A serological comparison between strains causing BVD and MD revealed that these two viruses are closely related. Today, both diseases are regarded as clinical manifestations caused by the same virus.

Most BVDV infections are inapparent, though acute and chronic forms of the disease exist. Following the initial description of the disease, BVDV infections have been found to be associated with abortion and neonatal death. The virus was isolated from aborted foetuses (Gillespie *et al.* 1967). Experimental infections in pregnant cattle proved that the virus could act as a teratogenic agent. Congenitally infected calves do not always succumb shortly after birth, but may

survive for years in a clinically healthy state, while remaining PI (Bezek & Mechor 1992).

Before BVD was recognized, hog cholera (HC), also referred to as classical swine fever (CSF), was reported in the midwest of the USA as a cause of devastating epidemics among pig populations (Benner 1928). The discovery of a close antigenic relationship between HC and BVD viruses (Darbyshire 1960; Mengeling *et al.* 1963) led to the classification of both viruses in the genus *Pestivirus* of the family *Togaviridae* (Horzinek 1973).

Hughes *et al.* 1959 was the third group of researchers that described a disease caused by another *Pestivirus* namely border disease (BD) of sheep, also referred to as hairy shaker disease. The infectious nature and the close relation between border disease virus (BDV) and BVDV were discovered later by several groups (Acland *et al.* 1972; Gardiner & Barlow 1972). Bovine viral diarrhoea virus, HCV and BDV were only classified as members of the *Flaviviridae* family in the last decade (Wengler *et al*. 1995).

Bovine viral diarrhoea virus is the prototype of the *Pestivirus* genus. Together with border disease virus (BDV) and hog cholera virus (HCV) they are antigenically and structurally closely related, but are not strictly species-specific. HCV and BVDV cross-react in immunodiffusion (Darbyshire 1960), immunofluorescence (Mengeling *et al.* 1963) and neutralization assays (Corthier *et al.* 1974). Pigs exposed to certain strains of HCV or BVDV can develop neutralizing antibody to both HCV and BVDV (Carbrey *et al.* 1976). Immunization of pigs with BVDV can

confer some protection against challenge with virulent HCV. BDV immunized pigs also appear to be cross-protected against HCV infection (Laude & Gelfi 1979).

Sheep naturally or experimentally infected with BDV produce neutralizing antibody to all 3 pestiviruses (Acland *et al.* 1972) and BDV antigen can be demonstrated in cell culture or tissue sections by immunofluorescence with a BVDV or HCV antiserum (Harkness *et al.* 1977; Terpstra 1978). Sheep inoculated with a virus isolated from a lamb showing signs of BD produced higher neutralizing antibody titres to BVDV than to BDV (Barlow *et al.* 1979). In addition, BVDV and BDV may cross-infect the ovine and bovine species and induce comparable congenital defects (Terlecki *et al.* 1980).

Virus isolation with confirmation by immunofluorescence has been the usual method used to detect BVDV in specimens from clinical cases, in contaminated cell cultures and in biological products (Mengeling *et al*. 1963; Meyling 1984; Laamanen *et al.* 1997). Virus may be isolated from several organs and its isolation is a sensitive method for confirming infection. The *in vitro* cultural characteristics of BVDV are relevant to an understanding of the epidemiology and control of BVD and MD (Bezek & Mechor 1992). Two biotypes are found; cp BVD viruses rapidly induce cytoplasmic vacuolation in susceptible cell monolayers and produce an 80 000 Daltons polypeptide whereas ncp BVD viruses do not induce cytopathic effects in susceptible cell monolayers and do not produce the 80 000 Daltons polypeptide and must be detected by indirect means (Dubovi 1992). Viruses isolated from newborn calves that have survived foetal infections and from

asymptomatic PI cattle are always ncp, while those from the tissues of cattle suffering from MD represent both cp and ncp types (Bolin 1995a).

Serological subgroups of BVDV are not currently recognized, but several reports document significant genomic and antigenic heterogeneity among BVDV. The antigenic diversity of BVDV has been observed (Howard *et al*. 1987; Dubovi 1992; Flores *et al.* 2000). Howard *et al.* (1987) suggested that there is an antigenic spectrum within a single related group for BVDV rather than distinct serotypes whereas Dubovi (1992), stipulated that experimental data do not support unique serotypes for BVDV. This variation may permit foetal infections even in animals assumed to be well vaccinated (Dubovi 1992). Practical consequences of BVDV antigenic diversity may have implications for diagnostic test methodologies and for control by vaccination; monoclonal antibodies have demonstrated a high degree of diversity within the pestivirus population (Corapi *et al*. 1990a).

The use of nucleic acid probes as diagnostic reagents has been compromised by the nucleic acid sequence variation found in the BVDV isolates tested (Dubovi 1992). In cattle, the emergence of new variants (Pellerin *et al.* 1994; Ridpath *et al.* 1995; Ridpath *et al.* 2000) which are poorly neutralized by antisera to standard reference strains of BVDV, raises the possibility that existing vaccines based on single virus strains may not stimulate protective immunity. It could be postulated that immune pressure from the widespread use of such vaccines might have favoured the emergence of new viruses (Edwards & Paton 1995).

Bovine viral diarrhoea virus has been grouped into two genotypes by comparison of genomic sequences from the 5' non-translated region (NTR) of the genome which is the most conserved part of the genome when compared to the 3' NTR (Pellerin *et al.* 1994; Paton *et al.* 1995b; Vilcek *et al.* 1999). More than 800 ruminant pestiviruses from the USA and Canada and 28 porcine pestiviruses from North America, South America, Europe and Asia were compared (Ridpath 1996). All the viruses isolated from pigs were similar to HCV reference strain Alfort (HCV genotype) and isolates from small ruminants were similar to the reference strain BD-31 (BDV genotype). The majority of viruses from cattle were similar to the BVDV reference strain NADL (BVDV 1 genotype) and others, including a few from small ruminants, were similar to virus 890 (BVDV II genotype) (Pellerin *et al.* 1994; Ridpath *et al.* 1996).

Viruses from the BVDV I and BVDV II genotypes have been associated with reproductive failure, persistent infections, respiratory disease and enteric disease in both small ruminants and cattle. Outbreaks of mucosal disease have been associated with both viruses BVDV I and BVDV II genotypes. In other words, both genotypes have cp and ncp biotypes. Outbreaks of haemorrhagic syndrome in cattle have been associated with BVDV genotype II (Pellerin *et al*. 1994; Ridpath *et al.* 1996; Ellis *et al*. 1998). The emergence of the BVDV genotype II viruses in North America where BVDV genotype I vaccination has been widely practised, raised the question whether the current generation of vaccines would cross-protect against both types (Pellerin *et al.* 1994).

Bovine viral diarrhoea virus is one of the most economically important viral pathogens of cattle causing a wide range of clinical syndromes in Great Britain, USA, Sweden, Denmark, Canada, Poland, Australia and Belgium (Duffel *et al.* 1986; Baker 1987; Alves *et al.* 1996; Kita 1996; Kirkland 1996; Saliki 1996). Losses are mostly due to reproductive failure as infection during pregnancy can result in embryonic resorption, abortion, stillborn calves, teratogenesis or birth of PI animals (Badman *et al.* 1981; Baker 1987; Anderson *et al.* 1990).

The presence of BVDV in South Africa has been known since the early 1970s (Thomson & Blackburn 1972; Theodoridis & Boshoff 1974) and was found in association with diarrhoea, MD, abortion, teratogenic defects, stillbirth and respiratory disease. During the last decade, several 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. Several serological surveys have indicated that infection with BVDV is widespread in cattle, sheep, goats and wild ruminants (Thomson 1971; Theodoridis *et al.* 1973; Barnard 1977; Depner *et al.* 1991; Giangaspero *et al.* 1991; Van Vuuren 1991; Baule & Banze 1994; Muvavarirwa *et al.* 1995; Ferreira *et al.* 2000). During a study conducted in Namibia (Depner *et al.* 1991), the prevalence of antibodies to pestiviruses was found to be 58% in cattle sera, 14% in sheep, 4,6% in goats and more than 40% in game that included giraffe (*Giraffa camelopardalis*), eland (*Taurotragus oryx*) and kudu (*Tragelaphus strepsiceros*).

Although in Europe and USA several studies have been conducted on different aspects of the BVDV, in southern Africa, little is known about the nature of the virus, prevalence of its different strains and the economic significance of the BVD/MD complex. Some veterinary practitioners have suspected the presence of BVDV genotype II based on clinical signs compatible with the described haemorrhagic syndrome (Pellerin *et al.* 1994; Ridpath *et al.* 1996). However, in a previous study, Baule *et al.* (1997) could not confirm the presence of genotype II. Considering the implications of the genomic diversity in the diagnosis, epidemiology and control of BVDV infections it is deemed important to characterize the BVD viruses occurring in a region.

Knowledge of the virus in terms of genetic and antigenic composition is therefore important to provide information for the production or importation of efficient vaccines for use in conjunction with other biosecurity measures.

The aims of this research project were:

i) To isolate BVD viruses to enlarge the pool of local strains for characterization

ii) To identify the genotypes of BVDV that circulate in South Africa and obtain phylogenetic data of local strains for comparison with imported vaccine strains

iii) To record the clinical signs associated with each isolate in order to establish the relationship between the isolates and clinical disease under South African conditions

CHAPTER 2. LITERATURE REVIEW

2.1 Classification and characterization of BVD viruses

BVDV is presently classified in the family *Flaviviridae*. Genera of the family include *Pestivirus* and *Flavivirus* (Wengler *et al.* 1995).

Pestiviruses are among the smallest enveloped animal RNA viruses and possess a nucleocapsid of non-helical, probably icosahedral symmetry (Horzinek 1991). They consist of a single-stranded, positive-sense, RNA genome, approximately 12.5 Kb long, comprising one large open reading frame (ORF) that encodes about 4000 amino acids (Collett 1992). This open reading frame is preceded by a short untranslated region, the 5' non-translated region (5' NTR) of the genome which is considered to be highly conserved among pestiviruses, allowing the selection of specific primers that amplify all known pestiviruses. The 5' non-translated region has been the target region for studying differences between and within pestivirus species (Boye *et al.* 1991; De Moerlooze *et al.* 1993; Ridpath *et al.* 1993; Hofmann *et al.* 1994).

Investigators have shown that the 5'NTR of pestiviruses is composed of highly conserved regions intercalated by three variable regions, termed I, II and III (Deng & Brock 1993). These regions are located in positions corresponding to nucleotides 1-73 (I), 209-223 (II) and 284-323 (III) in the genome of the National Animal Diseases Laboratory (NADL) reference strain. Nucleotide substitutions accounting for differences between strains are located within these variable regions, and are largely of the covariant type to preserve RNA secondary structure (Deng & Brock 1993).

The BVDV particle is spherical with a diameter of 40-60 nm and consists of an icosahedral nucleocapsid surrounded by an outer envelope containing 10-12 nm ring-like subunits on their surface (Moennig 1990).

Bovine viral diarrhoea virus can be classifed into two genotypes, BVDV I and BVDV II based on comparison of the 5'NTR of the viral genome. The 5' non-translated region of several bovine viral diarrhoea viruses isolated from a severe outbreak was amplified by polymerase chain reaction and sequenced (Harpin *et al*. 1995). Sequences of the BVDV type II isolates revealed the loss of an internal PstI restriction site, which is present in all known BVDV type I 5' NTR sequences (Harpin *et al.* 1995). BVDV type I shows great intragenotypic variability (Kwang *et al.* 1991; Lewis *et al*. 1991) and can be divided into, BVDV Ia that comprises isolates from North America and BVDV Ib that includes isolates from Europe (Vilcek *et al.* 1999). Another genotype distinguishable from Ia, Ib and II is Ic. An isolate from a human buffy coat sample from a Belgian patient and three other bovine field isolates of BVDV originating from Germany were found to belong to BVDV Ic (Baule *et al*. 1997; Giangaspero *et al.* 1997).

Genotype I (BVDV I) is represented by the reference strains NADL and Osloss and involves the majority of BVDV strains. Genotype II (BVDV II) is represented by strain 890 as reference strain and comprises mainly isolates associated with haemorrhagic syndrome of cattle, a form of acute BVDV infection described in North America (Pellerin *et al.* 1994; Ridpath *et al.* 1996). BVDV II also comprises isolates of ovine origin (Becher *et al.* 1995; Vilcek *et al.* 1997).

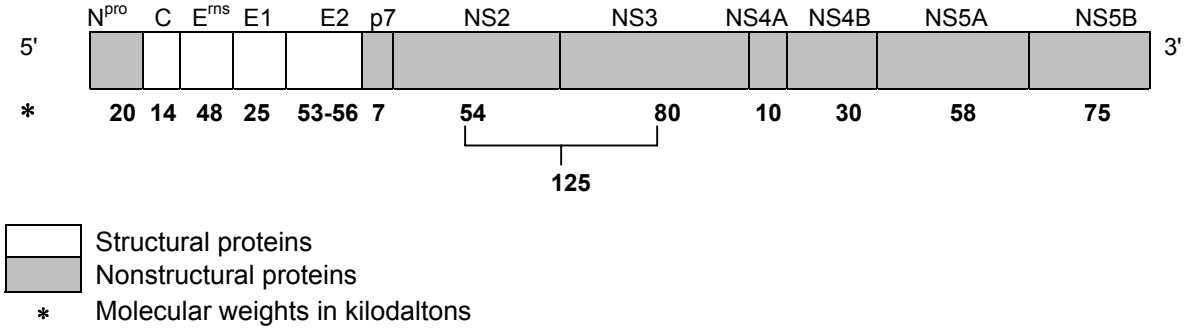
On the basis of the natural transmission of pestiviruses between various host animal species, the genus *Pestivirus* was believed to contain three species of viruses that include BVDV, BDV and CSFV. This nomenclature based on host of origin may no longer be appropriate; the proposed classification which is still under discussion, divides the genus into four genotypes: genotype I would include the present BVDV I strains, mainly of cattle origin; genotype 2 would include isolates of HCV; genotype 3 would include sheep and pig isolates with characteristics of "true BDV" that share less than 71% nucleotide similarity with other pestiviruses. This suggests that true BDV is distinct from BVDV, CSFV as well as other ovine and bovine pestiviruses currently referred to as BVDV type II. The latter represents genotype 4 in the proposed classification (Sullivan *et al.* 1997; Vilcek *et al.* 1997). The division is based on a panel of monoclonal antibodies supported by genetic sequencing and also virus neutralization with polyclonal sera (Becher *et al.* 1995; Paton *et al*. 1995c; Vilcek *et al.* 1997).

The occurrence of heterogeneous strains among BVDV I has been revealed by nucleotide sequencing and nucleic acid hybridization (Kwang *et al.* 1991; Lewis *et al.* 1991) supporting evidence previously shown by polyclonal and monoclonal antibody analysis (Howard *et al.* 1987; Bolin *et al.* 1988; Corapi *et al.* 1990a). The practical significance of the heterogeneity among BVDV strains is still under consideration. It may have implications in the design of broad reactive diagnostic assays based on serological and molecular methods (Kwang *et al.* 1991; Lewis *et al.* 1991) as well as in the development of vaccines conferring protection against a wide range of strains (Bolin *et al.* 1991a). Knowledge of the type of strain occuring

in a region and the epidemiology of the virus can help to establish effective strategies to control BVDV infections.

Pestivirus proteins are designated either by molecular mass (e.g.: gp53-57; p80) that often varies from one laboratory to another one, or by letter and number abbreviations (C, E1, E2) (Collett 1996, Fig.1). Proteins of BVDV coded for in the open reading frame (ORF) are composed of structural proteins and nonstructural proteins. Except for the first protein encoded by the BVDV genome, the structural proteins are encoded in the first third of the genome (5' end) whereas the nonstructural proteins are encoded in the last two thirds of the genome (3' end). The exact function of these proteins is still hypothetical. There are four structural proteins present in virions that include P14/C which is the capsid (C) protein and three envelope (E) glycoproteins (gp48/E^{rns}, gp25/E1 and gp53/E2) (Fig.1). Most strains have been shown to contain the conserved non-structural protein NS3 and a lack of the more strain-specific E2 surface glycoprotein (Canal *et al.* 1998).

Fig.1: Graphical representation of BVDV genes and the proteins encoded by the gene sequences



P14/C is well conserved among different pestiviruses. It is found in the cytoplasm of infected cells. It is not known whether it migrates to other compartments. The function of the protein is to package the genomic RNA and to provide necessary interactions for formation of the enveloped virion. Sera from convalescent cattle do not contain antibodies to p14/C (Donis 1995).

Experimental data and computer analyses indicated that the envelope gp48/E^{rns} (=envelope soluble Rnase) does not possess a transmembrane domain, and the nature of its virus association remains unclear (Silva-Krott *et al.* 1994). It appears to be secreted to the extracellular space by exocytosis and much of E^{rns} is found free in the culture medium of virus-infected cells (Donis 1995). This glycoprotein induces considerable levels of antibodies in infected cattle but these antibodies have limited virus-neutralizing activity. Gp25/E1 is a transmembrane glycoprotein, that contains two hydrophobic domains that serve to anchor the protein in the membrane and initiate translocation of the adjacent polypeptide gp53/E2. Gp25/E1 is found in virions covalently linked to gp53/E2 and forms a heterodimer with E2 whereas E2 forms a homodimer. Convalescent cattle serum does not contain significant levels of antibody to gp25/E1. Gp53/E2 is the major viral glycoprotein and principal target of neutralizing antibodies (Donis & Dubovi 1987; Bolin *et al.* 1991).

The remainder of the BVDV encoded proteins is non-structural in nature and includes p125/NS2-NS3, p10/NS4A, p32/NS4B, p58/NS5A and p75/NS5B. The identity of p32/NS4B is still speculative. P125 is cleaved into P54 and P80 when cytopathic strains replicate (Collett *et al.* 1988). P54/NS2 is exclusively found in

some but not all cytopathic isolates of BVDV. The first translation product protein of the ORF (open reading frame), P20/N^{pro} (amino terminal proteins) is a proteinase responsible for its autocatalytic cleavage from the nascent polyprotein.

P7 is often found fused with E2 but not found in virions and may play a role in virus assembly maturation. The role of non-structural protein P54/NS2, proteolytic product of P125/NS2-NS3, is unclear; this protein possesses a zinc finger motif suggestive of some involvement in nucleic acid binding or RNA recognition. This fact, coupled with the observation that the genetic rearrangements found in cytopathic viruses occurs in this region of the ORF, suggests a recombinagenic role for NS2. This part of P125/NS2-NS3 is poorly immunogenic and does not induce humoral antibodies in infected cattle (Bolin & Ridpath 1989; Donis 1995).

P80/NS3 is a multifunctional protein, exhibiting three enzymatic activities: a serine proteinase activity responsible for viral polyprotein processing at all sites downstream of its position within the ORF, an ATPase activity and RNA helicase activity. The latter two activities are presumed to be important in RNA replication by the virus (Donis 1995).

While both biotypes, cp and ncp express the non-structural protein NS2-NS3, cytopathogenicity of BVDV strains is correlated strictly with the appearance of the non-structural protein p80/NS3 that represents the marker protein of cp BVD viruses. The production of NS3 is usually caused by cytopathic specific genome alterations that were found to be due to RNA recombination. Molecular analyses of the cp BVDV strain Oregon revealed that it does not possess such genome

alterations but it is able to generate NS3 via processing of NS2-NS3 (Kummerer *et al.* 1998). Expression p80/NS3 in BVDV infected cells correlates with induction of cytopathic changes in cells (Donis & Dubovi 1987; Pocock *et al.* 1987). These changes could result from a direct interaction of p80/NS3 with cellular macromolecules, with perturbation of cell physiology. Alternatively, p80/NS3 may enhance BVDV replication.

P80/NS3 is the most conserved protein in the genus *Pestivirus*. This polypeptide is very stable in infected cells and highly immunogenic (Donis & Dubovi 1987; Bolin & Ridpath 1989). Infected cattle including cattle vaccinated with modified live BVDV vaccine develop a strong humoral antibody response to p125/NS2-NS3. Cattle vaccinated with killed vaccine develop negligible humoral antibody to this nonstructural polypeptide. Antibodies to BVDV p125/NS2, 3 cross-react with hog cholera p125/NS2-NS3 and vice versa. A similar situation of immune cross-reactivity occurs in border disease.

P10/NS4A is a cofactor to the serine proteinase activity of NS3. The functions of p32/NS4B and p58/NS5A are unknown but p32/NS4 B is postulated to serve as a membrane anchor for the virus replication complex whereas p58/NS5A (hydrophilic protein) is likely to involve RNA replication. Cattle infected with BVDV fail to produce humoral antibodies to p32/NS4B. The p75/NS5B protein is the viral RNA directed RNA polymerase. No serum antibodies to p75/NS5B are found in cattle recovered from BVDV infection (Donis 1995).

Replication occurs in association with membranes. There is no subgenomic mRNA in infected cells. The genomic RNA is believed to be translated into a polyprotein that is rapidly processed co-translationally and post-translationally. Differences exist in polyprotein processing by ncp and cp biotypes of BVDV. Both cellular and virus encoded proteinases are probably involved in polyprotein processing. Candidate virus proteins possessing proteolytic activity for cytopathic BVDV are p20 and p80.

Pestiviruses and in particular BVDV are unique viruses. The novel N^{pro} and E^{rns} proteins, the cytopathic and noncytopathic biotypes of BVDV, the propensity for genetic recombination and the factors involved in persistent infection, mucosal disease and acute disease all contribute to the uniqueness of BVDV.

2.2 Epidemiology

2.2.1 Geographical distribution

Bovine viral diarrhoea virus was first recognized in the USA in 1946 in association with an epizootic of acute and rarely fatal disease characterized by diarrhoea and erosive lesions of the digestive tract. The virus has a worldwide distribution. Surveys in many parts of the world have shown that the prevalence of BVDV serum neutralizing antibodies is often more than 60% (Taylor *et al.* 1977; Giangaspero *et al.* 1991; Moerman *et al.* 1993; Harkness & Van der Lugt 1994).

2.2.2 Sources of infection

Sources of the BVDV infection are generally the same for different countries but may vary in importance from one area to another depending on farm structure and

management systems. Animals that are infected post-natally often develop acute BVDV infections that, in the majority of cases, are inapparent and transient. It has been shown that transmission of virus in these cases is relatively inefficient (Meyling *et al.* 1990). This could be explained by the small amounts of virus shed in excretions and secretions, together with the limited stability of the virus in the environment. The persistent infection is a generalized infection and the virus is present in many tissues, especially in epithelial and endothelial cells and in cells of the mononuclear phagocytic system. The virus is shed continually and may be isolated from virtually any secretion or excretion including nasal discharge, saliva, semen, urine, tears and milk (Radostits & Littlejohns 1988). Persistently infected animals have a life-long viraemia, and are efficient transmitters of the virus.

Serum neutralizing antibodies to BVDV have been found in goats, sheep, pigs, humans, equids and wild animals (Stöber *et al*. 1984; Depner *et al*. 1991; Giangaspero *et al.* 1993). Experimental infection of pregnant goats with BVD virus has resulted in a higher rate of embryonic deaths, abortion and stillbirth than in pregnant ewes (French *et al.* 1974). Goats also appear to be susceptible to HCV; intravenous inoculation of pregnant goats with virulent HCV resulted in the infection of all offspring. The goats gave birth to normal and stillborn kids, and also produced mummified and oedematous kids. In pigs, *in utero* infection with BVDV has been reported (Terpstra & Wensvoort 1997). Specific antibodies to BVD virus can be found in humans in many countries (Giangaspero *et al.* 1988) and data on emerging infections in humans has been recorded (Giangaspero *et al.* 1997). There is data that indicate that the majority of ungulate species are infected by BVD or BVDV-like viruses (Nettleton *et al.* 1980; Anon. 1981).

2.2.3 Transmission

The predominant means of transmission from one herd to another is the introduction into a herd of a PI animal. Transmission may be direct through inhalation or ingestion of infected saliva, oculonasal discharge, urine and faeces. Respiratory infection is probably the most important mode of direct transmission (Potgieter *et al.* 1984a,b; Kelling *et al.* 1995). Many field cases have been traced to the movement of animals through areas where cattle are congregated such as public stockyards and sale barns, or where infected cattle, especially PI animals that continually shed large quantities of BVDV in all body fluids, are introduced into susceptible herds (Barber *et al.* 1985; Ames & Baker 1990).

Although direct contact is the primary method of spread, BVDV can spread through indirect contact. Veterinarians and stockmen have spread the disease from farm to farm through contaminated footwear, clothing or veterinary instruments. BVDV can be transmitted on rectal sleeves when the same sleeve is used to examine susceptible cattle immediately after a PI animal was examined (Lang-Ree *et al.* 1994; Roeder 1994). Transmission occurs even if no blood is seen on the sleeve. Fomites such as nose tongs that carry body fluids can also transmit the virus between cattle. Water troughs, feed bunks, feed buckets and nipple feeders recently used by infected animals may serve to transmit the virus.

Bovine viral diarrhoea virus can be experimentally transmitted to susceptible animals by drenching them with faecal material, by intravenous, intramuscular, subcutaneous or intranasal inoculation with blood collected from animals during the viraemic stage or with a splenic emulsion from an animal destroyed during the febrile stage. Olafson *et al. (*1946) demonstrated that 5 ml of blood injected

subcutaneously was regularly infective. The potential for transmission via hypodermic needles if they are not sterilized before and after use among animals was described (Roeder & Harkness 1986). High doses of virus and multiple routes of inoculation promoted the development of clinical and haematological changes typical for BVDV infection (Polak & Zimudzinski 2000).

Transmission also may occur through semen, uterine secretions, amniotic fluid or placenta containing virus (Meyling *et al.* 1987; Revell *et al.* 1988; Bolin 1995a). The virus can pass transplacentally from the dam to the foetus. Embryos transferred from cattle persistently infected with ncp BVDV are a potential vehicle for the transmission of the virus.

On the basis of *in vitro* and *in vivo* experiments, it is generally accepted that if the zona pellucida is intact and the embryos are washed adequately, the risk of virus transmission is non-existent (Singh *et al.* 1982). It is also important that foetal calf serum free from BVDV in washing fluids is used (Singh *et al.* 1982). The threat of transmission of BVDV by embryo transfer is minimal if the proportion of oocysts that are infected with ncp BVDV are non-viable and if non-infected embryos are externally disinfected as recommended by the treatment protocol. Since transmission is possible for *in vitro* fertilization one should only use animals certified BVDV free (Brownlie *et al.* 1997).

High levels of BVDV have been observed in the entire genital tract of PI bulls including their testes (Revell *et al*. 1988; Voges *et al.* 1998). Semen from immunocompetent bulls undergoing acute BVDV infection may be transiently

infected for at least two weeks (Revell *et al.* 1988). Acutely infected animals are inefficient transmitters of the virus due to the brief and limited viral excretion (Kirkland *et al*. 1997).

It is generally accepted that a PI animal is a potent source of virus transmission (Voges *et al.* 1998) therefore, bulls entering artificial insemination facilities are routinely screened for persistent infection with BVDV in Canada, the United States, Europe and also South Africa.

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 nonbiting flies for 96 hours after they feed on PI cattle. Nonbiting flies such as face flies can harbor the virus but it is not clear if they can transmit it (Gunn 1993).

2.2.4 Host susceptibility

Although BVDV most commonly infects cattle, the virus can also be found in pigs, sheep, goats and wild ruminants for which it is also pathogenic (Stöber 1984; Depner *et al.* 1991; Giangaspero *et al.* 1991). The virus is also shed by these species and interspecies transmission is easily achieved experimentally. *Pestivirus* isolates from sheep can infect cattle and those isolates from cattle can infect sheep. Experimental infection of pregnant ewes with BVD virus and of pregnant cattle with BD virus has produced clinical and pathological signs that were similar to those in the species from which the viruses were isolated (Richards

et al. 1971). Serological surveys have shown that the ewes had naturally occuring antibodies against BVDV (St George 1971).

Experimentally, there have been attempts to adapt pestiviruses to infect a variety of other hosts. Only rabbits appear to support virus replication (Fernelius *et al.* 1969). Reports of antibodies to pestiviruses in human sera (Giangaspero *et al.* 1988; Wilks *et al.* 1989), of pestivirus antigens in human stools (Yolken *et al.* 1989) and human leukocyte (Giangaspero *et al.* 1997) require further investigations. There are indications of a possible impact of infection in humans such as microencephalic infants and gastroenteritis associated with respiratory illness in children (Yolken *et al*. 1989; Giangaspero *et al.* 1997).

Among free-living ruminants, pestiviruses have been recovered from roe deer (*Capreolus capreolus*) (Baradel *et al.* 1988), fallow deer (*Dama dama*) (Frölich 1995), dromedary camel (*Camelus dromedarius*) (Hegazy *et al.* 1996); African buffalo (*Syncerus caffer*) (Hamblin & Hedger 1979); giraffe (*Giraffa camelopardalis*) (Soine *et al.* 1992) and wildebeest (*Connochaetes spp*.) (Doyle & Heuschele 1983) but in some cases, the contribution of the virus to the cause of death was uncertain. Serological surveys have shown that many species of free-living ruminants in North America, Europe and Africa have varying prevalence rates of antibodies to pestiviruses. Hamblin & Hedger (1979) cited by Nettleton (1990) detected antibody to BVDV in sera from ruminants and other species in nine African countries. Seroprevalence rates of between 30 and 87% were detected in African buffalo, kudu (*Tragelaphus strepsiceros*), eland (*Taurotragus oryx*), waterbuck (*Kobus ellipsiprymnus*) defassa waterbuck (*Kobus defassa*),

reedbuck (*Redunca arundinum*), sable antelope (*Hippotragus niger*) and oryx (*Oryx gazella*).

2.3 Pathogenesis

The wide variety of clinical signs observed during bovine viral diarrhoea virus infections demonstrates the complexity of the pathogenesis. Bovine viral diarrhoea virus infection in cattle ranges from transient acute infections that may be inapparent or mild to mucosal disease that is inevitably fatal (Brownlie 1984a,b). The immunosuppressive effect of acute BVDV infections can enhance the clinical disease caused by other pathogens. Chronic mucosal disease is defined as progressive wasting and usually diarrhoeic condition; it is suggested that this may develop following superinfection of persistently viraemic cattle with a heterologous cytopathic biotype (Bolin 1995a)

During acute infections, it is likely that the initial infection takes place in the oronasal mucosa and that the virus spreads from this site systemically. Field strains capable of rapid growth in the oronasal mucosa have been involved in the acute disease (Liebler *et al.* 1997; Bruschke *et al.* 1998). These viruses may account for the limited oculonasal discharge and shallow ulcerations seen in some of the acute infections (Baker 1987). Systemic spread of infection may occur as free virus in serum or as virus associated with the cells in the blood; the lymphocytes and monocytes are generally regarded as being particularly sensitive to BVDV infections (Bruschke *et al.* 1998). After infection, BVDV antigen can be recovered from nearly all tissues and different biotypes can also be isolated from different sites whether it is mucosal disease or an acute infection or persistent

infection. In a case of mucosal disease, Spagnuolo *et al.* (1997a,b) observed that large amounts of BVDV antigen were detected mainly in tissue samples from lymphoid and gastrointestinal tissues, with only small amounts in the respiratory tract. Abundant ncp BVDV antigen was detected in the pituitary gland and in pancreatic Langerhans islets, whereas cytopathic BVDV was isolated mainly from mesenteric lymph nodes and Peyer' s patches.

Immunosuppression of cattle infected with BVDV either postnatally or *in utero* is recognized (Reggiardo & Kaeberle 1981; Roth *et al.* 1986). A further complication of acute infections occurs when there is invasion of BVDV along with another pathogen. The basis of mixed infections is the immunosuppression consequent on the transient leukopenia and on a neutrophil dysfunction following acute infection (Brownlie 1990; Potgieter 1995). Lymphocytes and macrophages constitute important target cells for the replication of BVDV. Lymphoid destruction results in varying degrees of lymphocyte depletion in infected calves even in those that remain clinically normal (Reggiardo & Kaeberle 1981; Bolin *et al.* 1985a). A neutropenia in infected animals is often present (Roth & Kaeberle 1983). The depletion of these immunocompetent cells in cattle results in reduced resistance to pathogens. The clinical consequence of BVDV induced immunosuppression depends on several factors such as managemental or environmental stress and intercurrent infections.

Intercurrent BVDV infection appears to enhance the virulence of other pathogens or change the character of the disease caused by such pathogens in cattle (Reggiardo & Kaeberle 1981; Potgieter 1995; Penny *et al.* 1996). Interference

with the clearance of endogenous bacteria from the blood and increased shedding of BHV-1 in calves after challenge while suffering from experimentally induced BVD are indications of depression of host defences. Under intensive or semi-intensive management systems, there is a significant association between BVDV infection and acute respiratory disease (Ames & Baker 1990). In many BVDV infected calves, apparent opportunistic infections with *Mannheimia haemolytica* during the period of immunosuppression that follows BVDV infection have resulted in lung disease of increased severity (Reggiardo & Kaeberle 1981; Potgieter *et al*. 1984b). Simultaneous infections with BVDV and bovine herpesvirus type 1 (BHV-1) have resulted in severe clinical disease in which respiratory, ocular and alimentary tract lesions developed (Grieg *et al.* 1981). Other infectious diseases that may be exacerbated by BVDV infection include actinomycosis, papular stomatitis, enteritis caused by *Salmonella*, *E coli*, acute helminthiasis, metritis and mastitis (Bohac & Yates 1980; Penny *et al.* 1996).

In mucosal disease, the immune status of PI and seronegative animals must be considered. A proportion of PI animals may have a permanently-impaired immune response (Roth *et al.* 1986). This immunological dysfunction could be the inability to secrete immunoglobulin which explains the impaired cellular immunity in chronically diseased PI, seronegative calves (Johnson & Muscoplat 1973) or a result of the effect of the virus on the *in utero* development of the thymus (Duffel & Harkness 1985). Unlike primary postnatal infections of BVDV, which is associated with decreased lymphocyte numbers, immunosuppression in PI seronegative cattle is not characterized by a reduction in the absolute numbers of lymphocytes.

It is now well established that BVDV has the ability to cross the placenta and infect the foetus as well as to cross the blood-brain barrier (Woodard 1994; Bolin 1995a). The most important determinant of the outcome of infection of pregnant animals with BVDV is the age of the foetus when infection occurs, but other parameters such as host genotype, immune status and the biological characteristics of the virus strain may also influence it. The ability of bovine foetuses to mount immune responses to BVDV develops after 90 days of gestation and nearly all foetuses are capable of anti-BVD virus antibody production at 180 days of gestation (Casaro *et al.* 1971; Bielefeldt Ohmann *et al.* 1982; Bolin 1995a). Persistently infected calves could be produced when seronegative heifers or cows are exposed to BVD virus at ± 40 to 120 days of gestation (Liess 1984; Brownlie *et al.* 1989; Woodard 1994). Persistently infected animals are incapable of mounting a protective immune response to the ncp BVDV and its deficiency is specific but its immune system may be capable of recognizing and responding to antigens induced by heterologous virus (Bolin 1995a).

There has been no evidence that infection *in utero* with cytopathic virus could result in a persistent viraemia or immunotolerance. It was suggested that cells able to support a persistent viraemia with cytopathic virus might not be developed in the young foetus (Brownlie *et al.* 1989). Calves born persistently infected may die prematurely from secondary infections or environmental stress (Ross *et al.* 1986; Woodard *et al.* 1994). They constitute important sources of BVDV in the environment and they are at high risk of developing mucosal disease (acute or chronic) later in life (Bolin 1995a; Fritzemeier *et al.* 1997). Less than 10% of PI

calves may grow up to produce more infected calves and serve as constant sources of infection for other cattle (Ross *et al.* 1986; Woodard *et al.* 1994).

Mucosal disease because of its notable features has attracted much interest in terms of its pathogenesis by several research workers (Brownlie *et al.* 1984a; Bolin *et al*. 1985b; Bolin 1995a; Fritzemeier *et al.* 1997; Tautz *et al.* 1998). Cattle persistently infected with non-cytopathic BVDV developed MD after superinfection with homologous cytopathic BVDV (Brownlie *et al*. 1984a; Bolin 1995a). It became apparent that superinfection with cytopathic BVDV did not always induce MD. When for example persistently infected animals were vaccinated with cytopathic BVDV, they did not develop MD but produced viral neutralizing antibody and remained healthy (Bolin 1995a). In nature, antigenically closely related ncp BVDV and cp BVDV have been isolated from cattle with fatal MD and are termed homologous pairs. Heterologous viral pairs have also been isolated from some cases of MD (chronic cases). Non-cytopathic and cytopathic BVDV isolated from an outbreak of postvaccinal MD were analyzed and it was found that the cytopathic vaccine virus was different genetically and antigenically from the resident noncytopathic BVDV (Bolin 1995a).

The genomes of several 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 onset MD are the consequence of different pathogenic mechanisms (Fritzemeier *et al.* 1997). Experimental MD was induced by superinfection of calves persistently viraemic with a ncp BVDV using an

antigenically similar cp BVDV. From 3 cases, one animal developed clinical signs 2 weeks after superinfection (early onset MD) while the onset of the disease in the other 2 cases occurred with a delay of months (late onset MD). Isolated viruses were characterized using a panel of monoclonal antibodies against the E2 glycoproteins and RT-PCR was also used to amplify specific insertions and duplications in the NS2-3 genomic region. The results showed that the cp BVDV isolated during the early onset of the disease was identical to the one used for superinfection. In contrast, the cp BVDV from the late onset MD was the result of genetic recombination between the persistent ncp virus and the superinfecting cp BVDV (Fritzemeier *et al.* 1997).

The presence of antigenically closely related cp BVDV in PI animals does not necessarily lead to the development of MD. Apart from the antigenic relatedness between the persisting ncp BVDV and cp BVDV other factors, for instance the number of circulating lymphocytes might determine whether or not PI animals might develop MD (Bruschke *et al*. 1998). Before superinfection of PI animals with the antigenically closely related cp BVDV, lymphocyte subsets were determined by flow cytometric analysis and the percentage of gammadelta subsets were much higher in the PI animals that did not develop MD than in non-viraemic control animals and in the PI animals that died of MD (Bruschke et al. 1998).

2.4 Clinical signs and pathology

Numerous clinical forms of BVDV infection occur in cattle, affecting either a single or, more commonly, multiple organ systems (Fulton *et al.* 2000). Immunity can be

transiently impaired due to primary post-natal infections and may allow secondary infections with opportunistic microorganisms to take place, often in the respiratory tract (Potgieter 1995). Clinical manifestations of BVDV infection in cattle have been grouped into acute bovine virus diarrhoea infection, congenital infection, chronic infection and mucosal disease. Acute forms of the disease may include subclinical infections, bovine viral diarrhoea, transplacental infections and immunosuppression.

Subclinical infection is the most common form; it has been estimated that 70 to 90 % of BVDV infections in susceptible immunocompetent cattle are subclinical (Ames & Baker 1990) or mild cases also called “sleepers” in which clinical signs may not be detected.

Bovine viral diarrhoea represents an acute infection in seronegative, immunocompetent cattle ranging in age from six months to two years (Brownlie 1984a,b). The incubation period is 5 to 7 days and is followed by transient fever and leukopaenia (Duffel & harkness 1985). Viraemia occurs 4 to 7 days (Brownlie *et al.* 1987) after infection and in some cases may persist up to 15 days (Duffel & Harkness 1985). A rapid respiratory rate may develop secondarily to pyrexia, which may be incorrectly diagnosed as pneumonia (Perdrizet *et al.* 1987). Animals if pregnant may abort within ten days to three months; others would be partly off feed, have a slight elevation in temperature, a mild drop in milk production and pass soft faeces for a day or two (Perdrizet *et al.* 1987). In severe cases, affected animals run a high temperature, scour profusely, show depression, complete anorexia, ptyalism and develop ulcers in the mouth, nose and on the

muzzle. Animals may become dehydrated with sunken eyes. Milk secretion and rumination may be suspended (Perdrizet *et al.* 1987). Weak animals may be recumbent. Mortality varies from 4 to 8%. In case of abortion after acute infection, death could occur due to septic metritis (Kendrick 1971; Whitmore *et al.* 1981).

Persistently infected calves may have a death rate of 50% in the first year of life (Duffel & Harkness 1985) and may be smaller at birth, have a slower rate of growth and may die or be culled from the herd for being a "poor doer". Persistently infected animals are predisposed to infection by other microorganisms that manifest most often as enteritis and pneumonia (Barber *et al.* 1985). Immunosuppression has been reported in PI calves (Roth & Kaberle 1983; Potgieter 1988) which may account for the increased susceptibility to diseases such as enteritis and pneumonia. Subclinical disease in the form of glomerulonephritis and encephalitis has been described in PI, but otherwise normal appearing cattle (Cutlip *et al.* 1980).

Mucosal disease is a sporadic form of BVDV infection in cattle generally between the age of six months and two years. The disease is characterized by severe clinical signs, low morbidity and high case fatality. It includes acute and chronic forms. In the acute MD less than 5% of the herd is affected but case fatality rate approaches 100%. Occasionally epizootics may involve up to 25% of the animals in a herd. It is characterized by pyrexia of 40.5 ºC to 41 ºC, depression, weakness and anorexia. Heart and respiratory rates are elevated. Emaciation and dehydration with acidosis develop as the disease progresses. Milk production decreases in lactating cows (Bolin *et al.* 1985b). Necrosis of mucous membranes

of the lips, cheeks, tongue, pharynx, larynx and oesophagus and thrombocytopaenia resulting in haemorrhages in various tissues have been described (Olafson *et al.* 1946; Bolin *et al.* 1985b; Rebhun *et al.* 1989; Corapi *et al.* 1990b; Flores *et al*. 2000). Ptyalism often accompanies the oral lesions, which are generally found in 75 to 80% of the cases. Mucopurulent nasal discharge is often observed with lacrimation and corneal oedema. Other lesions that include interdigital dermatitis, conjunctivitis, arthritis, laminitis and coronitis were observed (Flores *et al.* 2000). Profuse watery diarrhoea generally develops two to three days after the onset of clinical signs, but in peracute cases death may occur prior to the onset of diarrhoea. Faeces are foul smelling and may contain variable amounts of fresh or clotted blood. Fibrinous intestinal casts may be passed. Straining at defecation is often observed. Ruminations are decreased and mild to moderate bloat may develop. Erosions throughout the alimentary tract, catarrhal enteritis and congestion of the large intestine mucosa in a striping pattern is often seen (Brownlie *et al.* 1984a,b).

A small proportion of cattle that develop mucosal disease do not die acutely, and become chronically affected (McClurkin *et al.* 1985). These cases have been referred to as chronic mucosal disease. Clinical signs include inappetence, weight loss, progressive emaciation and an overall unthrifty appearance. Diarrhoea may be continual or intermittent. Chronic bloat may be observed. Nasal discharge is a frequent finding. Areas of alopecia and hyperkeratinisation may develop, usually on the neck. Chronic erosives lesions in the mouth and the skin are common findings and failure of skin lesions to heal is an important feature in chronic cases

of MD. Affected cattle may survive up to 18 months and die from severe debilitation (Brownlie 1984a,b; McClurkin *et al*. 1985).

After an *in utero* infection with BVDV, the clinical picture covers a broad spectrum of illness ranging from an apparently normal newborn to individuals with extensive lesions and malformations. The affected newborn may die or recover from the disease but neonatal mortality is relatively high (Whitmore *et al.* 1981). Low birth weight is a fairly constant feature and it is frequently associated with a failure to thrive postnatally (Whitmore *et al.* 1981; Bolin 1990). A variety of clinico-pathological signs have been recorded including embryo loss, abortion, foetal mummification, stillbirth, intrauterine growth retardation, selective organ stunting, congenital malformations and clinically inapparent PI animals (Casaro *et al.* 1971; Whitmore *et al.* 1981; Anderson *et al.* 1990; Bolin 1990). Congenital malformations that have been reported after *in utero* infections with BVD virus include cerebellar degeneration or hypoplasia, microencephaly, internal hydrocephalus, optic neuritis, chorioretinopathy, cataracts, skeletal defects, skin abnormalities and spinal dysmyelination (Scott *et al.* 1973; Terlecki *et al.* 1980; Whitmore *et al.* 1981; Roeder & Drew 1984; Ross *et al.* 1986; Bolin 1990; Woodard *et al.* 1994; Spagnuolo *et al.* 1997a,b). The course of the disease depends on the virus biotype and genotype involved and the susceptibility of the host.

2.5 Diagnosis

Clinical signs are not reliable indicators of BVDV infections. It has been observed that BVDV has the capability of changing its clinical manifestations and often

serves as a challenge to the clinician (Brown *et al.* 1973; Brownlie *et al.* 1984a,b; Potgieter *et al.* 1984b; Perdrizet *et al.* 1987). Diagnosis of BVDV was first 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). The first parameter that led to a suspicion of a viral origin for the disease was a severe leukopaenia.

Early in 1957, Lee & Gillespie described the replication of a BVDV in cell culture without a 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

Various cell cultures have been used for the propagation of BVD virus strains. Cytopathic BVD viruses were first isolated and propagated in bovine foetal kidney (BFK) cell cultures (Gillespie *et al.* 1960) and these are still the most extensively used to date. Cells such as bovine foetal lung cells, testis cells, thymus cells, turbinate cells and tracheal cells are also used (Lee & Gillespie 1957; Goldsmit 1975; Meyling 1984; Deregt & Prins 1998).

Although natural hosts of pestiviruses include only cloven-hooved ungulates, it has been demonstrated that pestiviruses can adapt *in vitro* to grow in mammalian cells of heterologous species including canine, feline and primate cells (Bolin *et al.* 1994). Monkey cells produced variable results (Wellemans & Van Opdenbosch 1987; Bolin *et al.* 1994). 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). Extreme care must be taken to ensure that both cells and serum used in cell cultures are screened for freedom from pestivirus to avoid secondary contamination of cells of both human and animal origin. Serum should also be free of antibody to pestivirus.

In the live animal, blood is the most useful specimen for BVDV isolation. Virus may be found free in serum, or released by grinding up blood clots in the laboratory (Kirkland & MacKintosh 1992) but the highest sensitivity can be obtained by co-culture of leukocytes in the buffy coat of blood collected in heparin-containing tubes. BVDV is very stable in the serum; it may survive at room temperature for at least 7 days (Saliki *et al.* 1997).

Virus isolation may detect transient viraemia in immunocompetent cattle that must be differentiated from persistent infections through consecutive testing. A PI animal will remain virus-positive and generally seronegative, whereas acutely infected cattle will have seroconverted and be virus-negative at the time of the second sampling.

Where the virus is isolated from tissues at necropsy, the result must be related to the pathology to determine whether acute BVD or MD has occurred. Persistently infected cattle, including those suffering from MD, have continuously high titres of virus in the blood and other tissues (Duffel & Harkness, 1985). In the case of PI calves under about 5 months of age, maternal antibody in blood or serum may mask the seronegative status encountered in most carrier animals and may

interfere with virus isolation; they should be re-examined later in life. This problem can be overcome by collecting eye and nasal swabs for virus isolation (Kirkland & MacKintosh 1992).

Persistently infected bulls shed virus continuously in semen (Revell *et al.* 1988). Virus isolation may also be carried out on semen if blood is not available and this would also reveal any transient virus contamination due to acute BVD infection of the bull. From dead animals, the virus may be found in many organs, particularly the lymphoid tissues, lung and kidney (Kirkland & MacKintosh 1992).

Cytopathic strains of BVDV cause characteristic *in vitro* cell changes such as cytoplasmic vacuolations that are evident in inoculated cell cultures within 24-48 hours. Most commonly, BVDV isolates obtained from field cases are noncytopathic in cell culture (Brock 1995). Isolation of ncp BVDV generally requires 3-5 days of culture. Identifying viral antigen in positive cell cultures requires recognition of the isolated virus by immunofluorescence or immunoenzyme techniques (Edwards 1990; Bolin *et al.* 1991b; Brock 1995).

2.5.2 Antigen detection

2.5.2.1 Immunofluorescence staining

Immunofluorescence staining is a specific and rapid means of identifying an unknown virus isolate (Vickers & Minocha 1990). The fluorescent antibody technique (FAT), which has been applied in the diagnosis of a large number of viral agents, has also been used for the staining of BVDV infected cells.

It was first used by Mengeling *et al.* (1963) who demonstrated the antigenic relationship between hog cholera and BVD viruses using immunofluorescence. Later, others used the technique for demonstration of ncp and cp strains of BVDV in tissue culture (Fernelius 1969; Baker 1987; Dubovi 1990). Immunofluorescence staining of susceptible cell cultures inoculated with suspected material is the best way of detecting viruses that are not cytopathic. Either the direct or the indirect method can be used to stain the infected cell monolayer (Vickers & Minocha 1990).

Direct fluorescent antibody testing of the buffy coat has proved reliable in detecting viraemia in animals experimentally infected with cp BVDV. It has also been used in tissue specimens; the most widely applied being immunofluorescence on acetone-fixed sections or smears of nasal epithelial cells (Silim & Elazhary 1983; Roeder & Drew 1984). The technique is rapid and can be done in laboratories lacking cell culture facilities. Positive staining was also found in sections of thyroid and salivary glands, spleen, lymph nodes and around the sites of lesions in the intestine (Duffel & Harkness 1985).

2.5.2.2 Immunoperoxidase staining

Immunoperoxidase staining for detection of BVDV in leukocyte smears or cell cultures, in frozen tissues sections, skin biopsies have been described (Meyling 1984; Njaa *et al.* 2000). Bovine viral diarrhoea virus antigen has been demonstrated in conventional formalin-fixed, paraffin-embedded sections by a combination of protease XIV digestion of the sections and biotin-streptavidin amplification of the labelling signal (Allan *et al.* 1989).

Castro *et al.* (1997) described a rapid labelled streptavidin-biotin immmunoperoxidase staining procedure for the detection of ncp BVDV antigen in cell cultures using another approach of fixation of cells in suspension with subsequent adherence to slides before staining. This gentle method preserves cellular morphology for the screening of contaminants in cell culture by immunoperoxidase staining. The technique offers the advantage that stained wet mounts can be viewed immediately by light microscopy with subsequent preservation into permanent mounts.

A recent method used for the detection of BVDV in persistently infected animals is the immunohistochemistry of sectioned paraffin-embedded skin biopsies, mounted on poly-L-lysine-coated slides and stained for BVDV using monoclonal antibody 15 C5 and diaminobenzidine as chromogen. This method was found an easy, accurate, less expensive antemortem diagnostic test for the detection of PI animals when compared to virus isolation. Further study is still needed to determine the reliability of this test for differentiating between cattle that are persistently infected and those with severe, acute BVDV infection (Dubois *et al.* 2000; Njaa *et al.* 2000).

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 was performed using

a biotinylated polyclonal porcine antiserum to BVDV followed by avidin-FITC-conjugate. This method requires sophisticated equipment and qualified personnel.

2.5.2.4 Enzyme linked immunosorbent assay

With the availability of BVDV-specific monoclonal antibodies several different antigen capture enzyme-linked immunosorbent assays (ELISA) have been developed (Fenton *et al.* 1991; Brinkhof *et al.* 1996; Graham *et al.* 1998) and have now become commercially available. A monoclonal antibody capture ELISA to detect BVDV antigen in white blood cells of BVDV-infected animals was developed, as was an antigen capture ELISA using a polyclonal goat anti-BVDV serum to trap BVDV antigen and a combination of three different monoclonal antibodies for the detection of the trapped antigen from white blood cells, blood clots and tissue samples.

None of the antigen capture methods equaled the sensitivity of virus isolation (Brinkhof *et al.* 1996). Antigen capture ELISA (Shannon *et al.* 1991) has been valuable for the examination of blood samples and tissues especially when the tissues are unsuitable for virus isolation. This type of test can be rapidly performed, is suitable for automation and does not require expensive investments in laboratory facilities. For these reasons, ELISA is of great benefit in diagnosing BVDV carriers during eradication campaigns.

Until recently, the tests were designed to detect pestivirus-specific antigens in leukocyte preparations from heparinised blood samples, whole blood clots and tissue. A BVDV antigen test kit for detecting the virus in serum samples from PI animals recently became available (Syracuse Bioanalytical, Inc). Both the antigen

capture ELISA and the antibody ELISA are the tests of choice in eradication programmes for BVDV when many animals have to be tested (Fenton *et al.* 1991; Shannon *et al.* 1991).

2.5.3 Antibody detection

Serum neutralizing antibody was shown to be a good indication of protection. An active immunity produces a long-term immunity lasting for years. Complement fixing antibodies appear before neutralizing antibodies and reach their peak level at 15 weeks after infection. Many serological surveys were conducted in USA, Europe, Africa and other parts of the world with the availability of a serum neutralization test (Gutenkust & Malmquist 1963; Fernelius *et al.* 1971; Hafez & Frey 1973; Harkness *et al.* 1978).

Antibody detection may also be used for herd screening as part of a general health monitoring programme and specifically to identify seronegative cattle, which can be subjected to further testing if they are persistently infected. Not all persistent carriers are seronegative. It is known that small numbers of PI cattle have BVD virus specific antibody in their bloodstream (Duffel & Harkness 1985).

Agar gel immunodiffusion (AGID), virus neutralization (VN) and antibody ELISA (Kirkland & Mackintoch 1992) are serological tests commonly used for testing the presence of *Pestivirus* antibody. The choice of one or more of these tests depends to a large extent on the purpose for testing.

2.5.3. 1 Agar gel immunodiffusion test

Compared with neutralization tests or enzyme immunoassays, the immunodiffusion in gel technique has a low sensitivity and, because it does not yield a quantitative result, interpretation of tests for seroconversion on paired sera is more difficult. It is cheap, easy to perform and, provided antigen can be obtained, offers a useful screening technique for laboratories lacking advanced technical and virological facilities. The antibodies detected are directed mainly against a soluble, non-structural antigen (Gutekunst & Malmquist 1963) and correlate better with neutralization test results than with complement fixing (CF) antibodies (Harkness *et al.* 1978).

2.5.3. 2 Virus neutralization test

Virus neutralization is the most common serological method used for determining levels of BVDV antibody (Brock 1995). Serum VN tests commonly require 3-5 days to perform. Recovered cattle show a strong virus neutralizing antibody response in their sera, and this is the accepted reference test for antibodies to BVDV (Edwards 1990). There is no international reference standard for BVDV and the result of VN tests may vary widely depending on the strain of virus used, the cell type and the test conditions. It is very important that the cell culture, whether primary, secondary or a cell line, is screened for the absence of contaminating BVDV (Hassan & Scott 1986); and that any serum supplement used in the cell culture medium is of a quality suitable for the growth of BVDV.

The important principle is to establish a satisfactory combination of cell type and virus strain to give a clear and consistent distinction between wells where the virus

grows, and those where it is neutralized. Variation in VN results between different batches of cell cultures is a problem (Fernelius *et al.* 1971) that can be reduced by the use of uniform cell passage levels from frozen stocks. For this reason, acute and convalescent sera being examined for seroconversion should always be tested together on the same day. The most widely used virus strains are Oregon C24V and NADL (Brownlie *et al.* 1989).

2.5.3.3 Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA) is now established as a sensitive, rapid, reliable and economical test for BVD virus serology. The results correlate well with those of the VN test (Chu *et al.* 1985). It is assumed that ELISA is less affected than VN by the strain of virus used in the test (Radostits & Littlejohns 1988). A positive correlation has been demonstrated between VN and ELISA prepared with purified BVDV test antigen-coated plates (Howard *et al.* 1985).

The competitive ELISA that was developed to detect antibody against the p80 protein and the VN test detected high antibody titres to BVDV three years after BVDV infection (Justewicz *et al.* 1987). BVDV ELISA has been associated with high levels of background reading. To decrease background levels, several different methods of antigen preparation have been used (Bolin *et al.* 1988). Both indirect and blocking types of tests can be used (Juntti *et al.* 1987; Katz *et al.* 1987). The indirect ELISA has also been used to evaluate levels of antibodies to BVDV in individual and bulk milk samples (Juntti *et al.* 1987). A correlation was found between the level of BVDV antibodies in bulk tank milk samples and the

presence of PI animals. This type of screening can be applied only to nonvaccinated herds.

2.5.3.4 Indirect immunofluorescence

The IFT has been used for detecting BVDV antibody in foetal fluids (Lucas *et al.* 1986) that can give irregular results with CF and VN tests. The indirect immunofluorescence test (IFT) provides a simple, rapid and highly sensitive test for the detection of BVDV antibodies and is particularly useful for laboratories that have the proper equipment (McCullough *et al.* 1987). It is also useful for laboratories doing tests on small numbers of sera. Fixed capture antigen slides can be stored at -70ºC and used when required. Like AGID and ELISA, the IFT detects both group and type-specific antibodies reducing the problem of strain selection. Infected cells cultures are fixed to a multi-well glass slide with acetone, then reacted with a dilution of test serum.

2.5.3. 5 Peroxidase-linked assay

Although originally devised for screening pig sera for antibodies to hog cholera and BVD viruses (Holm 1981), PLA can also be used for serum from cattle. Peroxidase-linked assay (PLA) is similar in concept to immunofluorescent; 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 Nucleic acid detection methods

2.5.4.1 Nucleic acid hybridization probes and polymerase chain reaction (PCR)

These methods involve the direct detection of nucleic acids of the viral genomic RNA. The advantage over virus isolation is the lack of potential interference with neutralizing antibody and the sensitivity and specificity. They also detect genomic RNA of infectious as well as defective or inactivated virus particles. The sensitivity is equal if not greater than virus isolation (Belák *et al.* 1991).

Hybridization probes for the detection of BVDV were developed with the availability of BVDV cDNA clones and nucleic acid sequence information. This method confirmed the genetic diversity of BVDV isolates as determined by monoclonal antibody analysis (Bolin *et al.* 1988; Xue *et al.* 1990). By comparative sequence analysis of various BVDV sequences, the regions of the genome, such as the nonstructural p80/125 protein region and the 5' non-translated region (5' NTR), were identified as highly conserved (Kwang *et al.* 1991; Brock *et al.* 1993).

Viral nucleic acids can be isolated from infected cells as well from the virion itself. One of the main problems with the isolation of viruses in some specimens is the low concentration of infective virus or the presence of specific antibody (Alansari *et al.* 1993). This problem can be overcome by using a single PCR to amplify virus sequences from infected cell cultures and a nested double PCR to detect low concentrations of the virus. The use of a double PCR amplification strategy using two sets of degenerate nested primers from the 3' region of the genome that enhance the sensitivity of detection of BVDV in serum has been described (Alansari *et al.* 1993).

A PCR bulk milk test for the detection of BVDV RNA as a screening test to identify herds infected with BVDV has been developed (Brock *et al*. 1992). PCR amplification relies on the ability of specific DNA oligonucleotides to bind to complementary target sequences with a high level of specificity. The ability to detect BVDV RNA by reverse transcription PCR (RT-PCR) has been reported by many laboratories (Belák *et al.* 1991; Hooft van Iddekinge *et al.* 1992).

Polymerase chain reaction can detect the genomic sequences regardless of the virus infectivity and especially if the specimens had been frequently frozen and thawed. The presence of masking antibodies in pooled commercial serum does not affect PCR detection of viral sequences, as it does with virus isolation methods (Alansari *et al.* 1993, Ellis *et al.* 1995; Laamanen 1997). After acute infection, BVDV is generally detected from 3-10 days post infection by virus isolation with PCR amplification, but BVDV can be detected up to 12-14 days post infection during the initial development of BVDV-specific antibody. This method is fast, reliable, sensitive and less vulnerable to strain variability. Factors such as cost, technical expertise, equipment and automation and RNA extraction methods remain considerations to be taken into account when comparing this test with the standard methods of virus isolation and serology.

This technique, although suited to leukocytes or serum samples, can become cumbersome due to the required RNA purification steps if applied to solid, often autolytic tissues, e.g cases of abortion.

Compared with viral isolation, PCR is much faster, especially if the biotype in hand is non-cytopathic as it takes longer to propagate and it is the prevalent form in nature. PCR can tolerate strain variation when using primers designed from a conserved genomic region such as the 3' end.

2.5.5 Electron microscopy

Bovine viral diarrhoea virus has been tentatively identified by electron microscopy in purified virus preparations, in infected cells cultures and in tissues and cells from infected animals (Bielefeldt Ohman 1990). Factors such as the lack of shut-down of cellular protein synthesis in infected cells, a density of the viral particle similar to that of the host cell, growth to comparatively low titres in cell culture, virion fragility in purification procedures and lability of antigenic sites have hindered the use of EM on negatively stained preparations of purified or partially purified virus (Magar & Lecomte 1987; Bielefeldt Ohmann 1990).

Thin sectioning has been more helpful. Mature virus particles are easily detected in circulating mononuclear leukocytes and in the intestinal crypt epithelial cells. These studies have revealed a spherical membrane-bound structure with a diameter of 40-60 nm. The membrane is smooth, bilaminar and surrounds a dense or semi-dense core of 20-25 nm. The core particle may be isometric or hexagonal (Bielefeldt Ohmann 1990). EM is not used as a routine test for the presence of BVDV.

2.6 Control

In many research publications and review articles, the importance of BVD transmission by PI cattle has been emphasized (Baker *et al.* 1954; Bezek *et al.* 1992; Brock *et al.* 1998). In addition, much has been learnt about the specificity of the immune response as it relates to biotypes of BVD virus in PI and immunocompetent cattle (Donis & Dubovi 1987; Meyling *et al.* 1987; Bolin 1995b). The specificity of the immune response may influence prevention and control programmes, including the type of vaccine used or the diagnostic tests used to identify PI cattle (Houe *et al.* 1995).

Management practices that rely on natural exposure to provide immunity should be considered ineffective despite the high prevalence of seropositive cattle. Up to 30% of immunocompetent cattle may be seronegative in the USA and, as a result are at risk of infection that could subsequently result in transplacental spread of the virus to foetuses if they are pregnant (Ames & Baker 1990; Bolin *et al.* 1995b). A similar situation is more than likely present in southern Africa.

Considering the epidemiology of BVDV infection, the introduction of BVD virus in a herd can take place by means of movement of other ruminants such as sheep and goats, or introduction of new cattle. Wild ruminants, such as deer and antelope may also serve as reservoirs for BVD virus (Frölich 1995).

The use of contaminated modified live BVDV vaccines may introduce the virus to a farm (Ames & Baker 1990; Bezek & Mechor 1992). The potential for contamination with ncp BVDV exists with any procedure that uses foetal calf

serum (e.g. manufacture of vaccines, embryo transfer). Strict biosecurity systems with isolation and testing of all cattle entering the farm are necessary to ensure no virus enters the farm (Kelling *et al.* 2000). In reality, this is generally impractical for most dairy and cow/calf operations. A reasonable compromise would be to limit movement of cattle onto the farm and to avoid relocating pregnant animals (Rauff *et al.* 1996). If pregnant cattle are purchased then the offspring should be tested before introduction to the herd (Kelling *et al*. 2000).

Replacement animals (including breeding bulls) should be purchased from herds for which accurate records are kept about the disease history and vaccination programme (Ames & Baker 1990). Semen used for artificial insemination should be obtained from bulls that have tested negative for BVDV infection (Meyling 1988).

Exposure of cattle to small ruminants and wild ruminants should be limited by means of separate housing for sheep and goats and well-maintained fences to decrease contact with deer or antelope (Ames & Baker 1990).

The need for a vaccine against BVD was raised soon after the description of the disease by Olafson *et al.* (1946). The early vaccines contained ncp BVDV that was attenuated by serial passage in rabbits (Baker *et al.* 1954). These vaccines were considered protective, but they were not commercialized. Vaccines for BVDV were not produced commercially until cytopathic BVDV was isolated (Gillespie *et al.* 1960). The goal of any BVD virus vaccination programme is to

induce immunity that will limit viral replication following infection and thus prevent the subsequent effects of viral infection (Houe *et al.* 1995).

Practitioners have concerns about the commercial BVD virus vaccines that are available (Ames & Baker 1990; Anon. 1993; Anon. 1994; Brownlie *et al.* 1995; Dean & Leyh 1999; Ridpath *et al.* 2000). Both modified live virus (MLV) and inactivated virus vaccines for BVDV type I and II are available in several countries. Both biotypes are found in vaccines; frequent cp strains include NADL, Singer and Oregon and ncp such as New York. There are combination vaccines, containing BVDV and other viral agents such as bovine herpes 1 virus, parainfluenza 3 virus and bovine respiratory syncytial virus or bacterial agents such as *Haemophilus*, *Leptospira* and *Mannheimia*. Monovalent vaccines containing only BVDV are also available (Ames & Baker 1990).

Commercially available live and killed BVD vaccines in South Africa are derived from genotype I and generally contain strains NADL and Oregon. They contain either both biotypes, or cytopathic only. They are available either in combination with viral agents such as bovine herpes virus 1, parainfluenza 3 virus and bovine respiratory syncytial virus or with *Mannheimia haemolytica.* Vaccines that include genotype II are not yet in use in South Africa.

Modified live virus BVD vaccines stimulate a rapid immune response after vaccination and antibody is detected in serum that neutralizes an antigenically diverse array of BVDV (Bolin & Ridpath 1989; Dean & Leyh 1999). The duration of antibody in serum after vaccination with MLV products is not known but it is

likely to be similar to that elicited by field virus during a natural infection. In most cattle naturally infected, antibody is detected at high concentrations for more than 1 year and may persist for several years (Coria & McClurkin 1978). Immunization of calves with a MLV vaccine is not inhibited by colostral antibody at viral neutralizing up to 1:32 (Menanteau-Horta *et al.* 1985).

Immunization should be successful in most calves that are 4 to 6 months of age. There are several disadvantages associated with MLV BVD vaccine that include failure of immunization due to improper storage and handling of the vaccine, postvaccinal MD (Bittle & House 1973; Ames & Baker 1990; Anon. 1993; Bolin 1995a), immunosuppression (Roth & Kaeberle 1983) and foetal infection resulting in abortion and congenital anomalies (Liess *et al.* 1984; Trautwein *et al.* 1986).

Although many PI cattle survive vaccination with cytopathic modified live vaccine without adverse effects, some PI cattle may develop mucosal disease when injected with MLV containing a cytopathic biotype antigenically similar to the non cytopathic biotype that induced the persistent infection (Bolin 1995a). Immunosuppression and genetic recombination are other potential problems associated with MLV vaccines. Vaccine strains of BVDV apparently retain some immunosuppressive properties that are manifested briefly after vaccination (Roth & Kaeberle 1983). This immunosuppression might enhance the pathogenicity of other infectious agents, resulting in post vaccinal disease. MLV vaccines can undergo genetic recombination with the nucleic acid of other viruses or of the vaccinate. The RNA of BVDV has been shown to recombine with RNA of both cellular and viral origin (Meyers *et al.* 1992; Qi *et al.* 1992). A recombination event

between the RNA of a vaccine virus and RNA from the vaccinate or from another virus can create a virus capable of causing disease. Because BVDV causes foetal death and congenital defects, MLV vaccines are not recommended for use in pregnant cattle (Cortese 1994; Bolin 1995b).

Killed BVDV vaccines are commercially available and they lack several of the disadvantages associated with MLV vaccines. Reversion to virulence or genetic recombination with another virus is not likely to happen. The methods used to inactivate the vaccine virus are likely to inactivate any other adventitious agents as well, which reduces the potential for postvaccinal disease arising from a contaminated vaccine. Because inactivated virus cannot infect the foetus, it is safe to administer to pregnant cows. It will not result in vaccine breaks, and does not cause immunosuppression or alter the numbers of circulating lymphocytes within immune cell populations (Ellis *et al.* 1988; Larsson & Fossum 1992).

Disadvantages associated with most inactivated vaccines for BVD include cost of the vaccine and the fact that a single initial dose of inactivated vaccine is inadequate to stimulate a protective immunity even if vaccination is boosted annually. Adequate vaccination appears to protect a cow from severe disease and death, but it may not always protect her foetus. Killed-vaccines require a two-dose priming vaccination series, followed by annual revaccination.

Adverse reactions may occur and include localized inflammatory reaction at the site of injection and anaphylaxis. Also, a transient decrease in milk production may occur in dairy cattle shortly after vaccination. Colostrum-derived antibody in

calves may also interfere with the immunity induced by inactivated vaccines. To overcome this situation, such vaccines are administered from 6 months of age.

Another concern is that the immune response generated by the vaccine virus whether modified or killed may not include the antigenic spectrum of all field strains. Vaccination is more effective if vaccine strains are antigenically closely related with the strain present on the farm (Ames & Baker 1990). There are still concerns whether the current BVDV genotype I derived vaccines do cross-protect against BVDV genotype II. Current vaccines, when administered properly, appear to protect against different strains of BVDV (Cortese 1994; Dean & Leyh 1999). Ridpath *et al* (2000) reported that BVDV infection continues to have a significant impact upon US cattle despite the availability of more than 140 federally licensed vaccines.

CHAPTER 3. DETECTION OF BOVINE VIRAL DIARRHOEA VIRUS IN SPECIMENS FROM CATTLE IN SOUTH AFRICA AND CORRELATION OF ISOLATES WITH CLINICAL DISEASE

3.1 Introduction

Detection of BVDV in cattle was undertaken to broaden information on BVDV biotypes and genotypes circulating in South Africa and their correlation with clinical disease. Few studies have been conducted in this field in southern Africa (Theodoridis *et al.* 1973; Theodoridis & Boshoff 1974; Van Vuuren 1991; Baule *et al.* 1997). The gold standard for the diagnosis of virus infections remains virus isolation in cell cultures (Lee *et al.* 1957; Gillespie *et al.* 1963; Fernelius *et al.* 1969; Horner *et al.* 1995).

Virus isolation in the live animal is best achieved during the viraemic stage as early as 3 days after infection to 8 or 10 days after infection. In some animals the virus may be fleetingly present in the blood for only 2-3 days during the course of BVDV infection. The presence of BVDV in infected animals can be demonstrated by inoculation of susceptible cells with serum, buffy coat cells or tissues homogenates and subsequent detection of BVDV antigens in infected cells by immunofluorescence or immunoperoxidase staining (Meyling 1984; Dubovi 1990). Whole blood is the specimen of choice for BVDV isolation to identify acutely infected animals. A viraemia would also be detectable in the serum of PI animals (Brock 1995).

Tissue culture procedures are labour intensive and time consuming and require technical expertise and the use of appropriate culture systems. Non-cytopathic

biotypes of BVDV may require 2 or 3 passages in cell culture before detectable amounts of viral antigen are produced. In spite of these constraints, virus isolation in tissue culture with confirmation by immunofluorescence has been a sensitive method used to detect BVDV in specimens from clinical cases and in contaminated cells cultures or biological products (Dubovi 1990; Bolin *et al.* 1991b; Horner *et al.* 1995). Other sensitive and quick methods for the detection of BVDV include antigen capture ELISA and nucleic acid detection by means of PCR (Brinkhof & Westenbrink 1996; Laamanen *et al.* 1997).

Singly or in combination, the two biotypes of BVDV induce clinical disease of the lymphoid, enteric, respiratory and reproductive systems (Corapi *et al.* 1990b; David *et al.* 1993; Baule & Banze 1994; Fulton *et al.* 2000). Infections with either biotype of virus may cause a variety of clinical disease syndromes in cattle. Fulton *et al.* (2000) have compared biotypes and genotypes of BVDV based on clinical signs and necropsy lesions and found that more ncp biotypes and type 1 genotypes were reported from cattle with respiratory disease at clinical presentation. Cattle with lesions of fibrinous pneumonia at necropsy had more type 1 than type 2 genotypes while cattle with necropsy lesions of enteritis/colitis and systemic lesions had more cp strains than ncp strains. In South Africa, no studies correlating isolates with clinical manifestations of BVDV infection have been done.

A limited number of studies have been conducted in African buffaloes (*S. caffer*) and in water buffaloes (Bubalus bubalis) and were based on serological surveys (Thomson & Blackburn 1972; Hafez & Frey 1973; Amber *et al.* 1988; Zaghawa

1998). The presence of the infection in water buffaloes (*Bubalus bubalis*) was confirmed in Egypt (Hafez & Frey 1973; Amber *et al.* 1988) and in India (Sudharshana *et al.* 1999) by the presence of neutralizing antibodies, but in Zimbabwe, Thomson & Blackburn (1972) obtained negative results in African buffaloes. In South Africa, there is no report available on BVDV infection in African buffaloes.

3.2 Materials and methods

3.2.1 Specimen collection

Specimens were obtained from private practitioners and feedlot consultants who make use of the laboratory services of the Department of Veterinary Tropical Diseases and the Veterinary Academic Hospital, Faculty of Veterinary Science, University of Pretoria and were received between January 1998 and October 1999. Mesenteric lymph nodes collected from 37 African buffaloes culled in the Kruger National Park were transported to the Section for Exotic Diseases, Onderstepoort Veterinary Institute where they were stored frozen prior to processing.

Specimens obtained from living cattle were limited to sick animals. The following clinical criteria were used to define "sick animals": Neurological defects, pyrexia, mild to severe diarrhoea, especially in calves, haemorrhagic syndrome, infertility, abortion, erosions on the feet, nasal discharge and oral ulcers.

The specimens from live animals included blood in heparin-containing tubes for virus isolation and for antigen capture ELISA tests.

Necropsy specimens were collected from cattle that died in feedlots and in commercial beef and dairy herds and from foetuses and included spleen, lymph nodes and lung. All specimens were chilled during transport to the laboratory.

Five hundred clinical specimens from 368 living and dead animals were received for virus isolation. Specimens (n=27) from 16 animals were discarded due to either haemolysis, advanced autolysis, broken tubes or bacterial proliferation. Only one specimen per animal was processed in those cases where more than one specimen was received. The remaining 352 specimens from 352 animals were subjected to virus isolation procedures in tissue culture. Of the latter, 154 specimens were also tested by means of an antigen capture ELISA (IDEXX). From 66 dead animals, 66 specimens representing 10 spleens, 9 lungs and 47 lymph nodes were also processed for virus isolation. The origin of the specimens, number of animals and the predominant clinical signs in the cattle from which the specimens were collected as well as other specimens from non-clinical cases are listed in table 1. Specimens classified under "others", included antibody negative sera, specimens from the culled African buffaloes, cell lines and all other cases that did not fall under the selected headings.

Table 1: Main clinical signs, origin and number of selected specimens subjected to virus isolation

History	Provinces								
	NW	KZN	F	EC	WC	G	N	MPG	Total
Reproductive disease	0	0	1	2	5	3	0	2	13
Pyrexia	0	5	1	6	0	1	0	0	13
Gastro-enteric disease	11	2	7	2	1	15	1	0	39
Respiratory disease	6	8	25	53	5	7	1	0	105
Haemorrhagic syndrome	0	1	2	0	1	0	0	0	4
Poor growth	0	0	1	0	0	0	0	0	1
Others	45	0	38	21	10	11	37	18	180
Total	62	16	75	84	22	37	39	20	355

NW: NorthWest; KZN: KwaZulu-Natal; F: Free State; EC: Eastern Cape; WC: Western Cape; G: Gauteng; MPG: Mpumalanga; N: Northern province

3.2.2 Processing of specimens for virus isolation

3.2.2.1 Blood

Blood in heparin-containing tubes was centrifuged at 1500 g for 10 minutes, and the buffy coat that contained the white blood cell fraction was collected and stored in 2 ml Nunc freezing tubes at - 70 ºC.

Blood without anticoagulant was centrifuged at 1000 g for 10 minutes and the sera collected and stored in 2 ml volumes in Nunc tubes at –20 ºC until used within two weeks. The temperature for long storage was –70 ºC.

3.2.2.2 Necropsy tissues from cattle and buffaloes

If not processed immediately, tissues were stored at –70 ºC. Tissues specimens collected during necropsies were processed as follow: 2 g of pooled organs were ground with sterile sand in a mortar and resuspended in 10 ml phosphate buffered saline with calcium (Ca^{++}) and magnesium (Mg^{++}) (PBS plus). The suspension was centrifuged at 1500 g for 10 minutes and the supernatant passed through a 0,22 µm filter. The filtrate was then poured into 2 ml Nunc tubes and stored at –20 ºC until inoculation into cell cultures within two weeks.

3.2.3 Reference viruses and cell cultures

Both cytopathic and noncytopathic BVDV strains were used as controls in cell culture work. The cp reference strain C24V (Oregon) was obtained from the National Veterinary Services Laboratories, Ames, Iowa, USA. The ncp strain was a local strain (ALT3) obtained from Allerton Laboratories, Pietermaritzburg, South Africa. The passage number of C24V was unknown when received. The ALT3

strain was obtained at the 8th passage. After receipt, it was passaged more than 12 times in Madin Darby bovine kidney (MDBK) cells, stored in 2 ml Nunc tubes and kept at –70 ºC as stock virus.

Primary and secondary cells from calf foetal kidney and MDBK line cells were used. The primary and secondary cells were used between passages 2 and 10. For the preparation of calf foetal kidney cell cultures, the kidneys were collected aseptically from foetuses obtained from a local abattoir. The serosal surface of each intact uterus containing a foetus was disinfected with alcohol, opened with a sterile blade and the foetus removed and placed in a clean plastic basin. The skin of the foetus was similarly disinfected before opening its thoracic and abdominal cavities with sterile scissors and forceps. Kidneys were collected and immediately immersed in a glass container containing Ca^{++} and Mg^{++} free phosphate buffered saline (PBS minus) and an antibiotic (1ml gentamycin per 1000 ml of PBS minus). The kidneys were then taken to the laboratory and processed under a sterile work hood for the preparation of primary cell cultures. They were cut finely with scissors in sterile petri dishes and rinsed in PBS minus containing gentamycin.

The tissues were transferred to a glass container with PBS and washed with the help of a magnetic stirrer for 30 minutes. The buffer was discarded and replaced with 0,25 % trypsin solution and rinsed to get rid of any remaining buffer solution. Fresh trypsin solution was added and stirred for 30 minutes. Cells from the first trypsinisation were seeded into tissue culture flasks as were those of the second trypsinisation. For fragments of tissues not well digested, additional trypsin

solution was added and the container placed in a refrigerator overnight for harvesting the next morning.

Once the cell monolayer had formed, the cells were trypsinised, centrifuged and resuspended in a freezing medium consisting of 50 % minimum essential medium, 40 % foetal calf serum (FCS) and 10 % sterile glycerol. Cells from a 75 cm^2 tissue culture flask were resuspended in 3 ml freezing medium and stored in two 1,5-ml cryotubes in liquid nitrogen. Freezing of cells was done at a uniform controlled rate of 1 ºC per minute.

Before use, cells were rapidly thawed for 2 to 3 minutes in a water bath, and were then grown as monolayers in 75 cm^2 flasks in modified Eagle's medium with gentamycin (1ml per 1000 ml of MEM) supplemented with 5 % FCS (Highveld Biological Products). The maintenance medium for inoculated cell cultures contained a concentration of 2 % FCS. The serum had been filtered and gamma irradiated at 28-30 KGY under conditions that preserve its biological integrity. Before use, it was inactivated at 56 ºC for 30 minutes. Horse serum at 5% was also used to rule out any BVDV contamination either in cells or in each batch of foetal calf serum used.

3.2.4 Detection of bovine viral diarrhoea virus in cell cultures by immunofluorescence

3.2.4.1 Inoculation of cell cultures

Prior to inoculation, flasks of MDBK cells or secondary calf foetal kidney (CFK) cells containing MEM supplemented either with FCS or horse serum were tested by FAT to rule out BVDV contamination that could have occurred through contaminated cells or foetal calf serum. Each specimen was then inoculated into a 25 cm^2 plastic tissue culture flask containing a subconfluent monolayer of MDBK cells or secondary CFK cells. The volume of the inoculum was one-tenth (1ml) of the volume of the maintenance medium. Flasks were then placed in a 37ºC incubator. Uninoculated flasks used, as negative controls were included in each run. The cells were checked daily for CPE (cytoplasmic vacuolation and detachment of cells). Blind passages were done between 4-8 days.

Before each blind passage, 25 cm^2 flasks containing cells inoculated 4-5 days previously with suspected material from clinical specimens were frozen and thawed and the contents used as a new inoculum on a fresh cell monolayer. Before preparing monolayers in chamber slides, cells were counted using a microscope counting chamber (Hemocytometer). The number of cells aimed for was 4 x 10^5 per ml. An 8-well chambered glass slide system (Lab-Tek, Nalge Nunc International) was used to assay samples. Each chamber was seeded with 20 µl MDBK cells in 400 µl of Eagle's minimum essential medium and 5 % heated FCS per well and then inoculated with 10 µl of inoculum 24 hours later. A known BVD virus was added to 1 well of the 8-chambered slide to act as a positive control. Chambered slides were then incubated for 24 hours at 37ºC in a

humidified atmosphere with 5 % carbon dioxide in air before immunofluorescence staining with conjugated antibodies.

3.2.4.2 Direct immmunofluorescent antibody test

The presence of ncp BVDV in cell cultures was detected either by direct or indirect immunofluorescent staining in chamber slides. For the direct method, slides containing cell monolayers were fixed in a solution containing 75 parts acetone and 25 parts methanol at room temperature for 10 minutes. They were washed for 5 minutes in PBS and 5 minutes in distilled water with the help of a magnetic stirrer and dried. Slides were stained for BVDV antigen using 20µl of fluorescein-labelled specific antibody and placed in the incubator at 37 ºC for 35 minutes. Slides were washed as previously described in PBS and distilled water, dried and examined by fluorescence microscopy.

The BVDV conjugate was produced from antibodies obtained from pigs that were immunized with 3 type 1 and 3 type 2 bovine strains of BVDV. It was obtained from National Veterinary Services Laboratory, Ames, Iowa, USA. It was tested for efficacy and titrated to determine the optimal working dilution using cells infected with the cytopathic strain C24V. The working dilution was not tested with other strains. It was used at a working dilution of 1:30 to 1:40. The mounting fluid was used at pH 7,4 and was composed of:

NaHCO3	0,715 grams
Na2CO3	0,16 grams
Distilled water	10ml

Add glycerol to make 100ml, pH: 7,4

The direct fluorescent antibody test was also used on buffy coat smears. Buffy coat smears were made on glass slides on which they were left to dry before being examined under a light microscope for areas of white cell accumulation that were encircled with a diamond pencil. Smears were fixed in a solution containing 75 parts acetone and 25 parts of methanol at room temperature for 10 minutes. The rest of the procedure was followed as mentioned above.

3.2.4.3 Indirect immunofluorescent antibody test

Chamber slides containing cell monolayers were fixed in cold acetone at -20 ºC for 10 minutes. They were washed twice for 5 minutes in PBS and then in distilled water and dried. Twenty microlitres of a known anti-BVDV serum diluted 1:10 in PBS was added to each well and the slide incubated at 37 ºC for 35 minutes. The slides were again washed twice for 5 minutes in PBS and then in distilled water and dried. Finally, each well was covered with 20µl rabbit polyclonal anti-bovine immunoglobulin G conjugated to fluorescein isothiocyanate diluted with 0,05 % Evans blue stain to a working dilution of 1:40. The antibovine conjugate used was obtained from SA Scientific Products. It was tested for efficacy and a working dilution determined using cells infected with cytopathic strain C24V. The slides were incubated at 37 ºC for 35 minutes and finally, they were washed twice for 5 minutes in PBS and then in distilled water and dried. The slides were examined by fluorescence microscopy.

3.2.5 Detection of bovine viral diarrhoea virus in specimens using antigen capture ELISA

3.2.5.1 Bovine viral diarrhoea virus p80 antigen test kit (HerdChek™: IDEXX)

The IDEXX commercial kit was designed to detect a non-structural BVDV protein NS3 with a molecular mass of 80 000 Daltons (p80) in peripheral blood leukocytes, blood clots, whole heparinized blood, tissue samples, nasal swabs and cell cultures.

Whole heparinized blood and cell cultures were tested during this project and were processed according to the manufacturer's guidelines. The assay is a sandwich ELISA that utilizes microtitre strips coated with polyclonal antiserum that binds pestivirus antigens. A monoclonal antibody cocktail forms the top of the sandwich. Unbound material is removed by washing. A horseradish-peroxidase conjugate and a substrate reactive with the enzyme detect the bound specimen-monoclonal sandwich.

The absorbance of the samples and control samples were measured at 450 nm on a microplate reader using air as blank. Before the results could be interpreted, the mean absorbance value (OD) of the duplicate of the positive control (OD pos) and of the negative control (OD neg) was calculated and the absorbance value of all samples were corrected using the formula: Corrected absorbance value = sample absorbance value (OD test) minus the negative control absorbance value (OD neg).

The following criteria defined the validity of the assay: The positive control should have a mean absorbance value more than 0,800 and the negative control should have a mean absorbance value less than 0,200. The validity of the test was confirmed since the mean absorbance value of the duplicate positive control was 2,1415 which was more than 0,800 and the mean absorbance value of the duplicate control negative was 0,115 which was less than 0,200. A positive result was indicated by colour development. The optical density was measured using a microplate at a single wavelength either at 450 nm, or on a dual wavelength of 450 nm and 620 nm.

3.3 Results

3.3.1 Detection of bovine viral diarrhoea virus in cell cultures

Results of attempted BVDV isolation from specimens from calves and adult cattle with clinical signs suggestive of BVDV infection and from the African buffaloes are summarized in table 2. Four sera out of 132; 3 white cell specimens out of 154; 3 spleen specimens out of 10; 2 lung specimens out of 9; and 3 lymph node specimens out of 47 (including 10 lymph nodes from cattle and 37 from the buffaloes) were positive on virus isolation in cell culture (n=15).

Table 2: BVD viruses detected by means of virus isolation in cell cultures

	Serum	Whole blood	Spleen	Lung	Lymph nodes	Total
Total number of specimens tested	132	154	10	9	47	352
Number positive	4	3	3	2	3	15
Number doubtful	2	3	2	1	0	8
Number negative	126	148	5	6	44	329

3.3.2 Detection of bovine viral diarrhoea virus using antigen capture ELISA (IDEXX)

Six out of 157 specimens that included whole blood from 3 clinical cases and 3 cell lines were positive with corrected absorbance values that ranged from 0,801 to 1,525 (Table 3). They were also positive on virus isolation. Pyrexia, diarrhoea, respiratory tract infection, abortion, congenital malformations/reproductive failure, haemorrhagic syndrome and poor growth were the criteria of selection for the collection of specimens. The number of isolations associated with the particular disease condition is presented in table 4. Respiratory distress with pyrexia were the most frequent clinical signs (46,7 %), followed by cattle with diarrhoea and pyrexia (20%) and respiratory disease without pyrexia (20 %) and diarrhoea without pyrexia (13,3%).

Table 3: Positive values of samples tested by means of Ag capture ELISA (IDEXX)

Samples	OD test values	OD corrected
Blood heparin	0,992	0,817
Blood Heparin	1,640	1,525
Blood Heparin	0,964	0,849
Cell culture	1,280	1,165
Cell culture	0,998	0,883
Cell culture	1,001	0,886

Table 4: Frequency of clinical syndromes associated with BVDV isolates during 1998-1999 (n=15)

Disease conditions	Number of positive specimens	% of positive specimens
Respiratory plus pyrexia	7	46,7
Respiratory tract infection	3	20
Diarrhoea plus pyrexia	3	20
Diarrhoea	2	13,3

Table 5 represents the age distribution of cattle infected with BVDV and from which 15 isolates were obtained. The age predilection was based on the farming system. Most isolates were from cattle in feedlots accounting for the mean age of 7 months at arrival at the feedlot and 12 months at departure. The predominant age group of cattle infected with BVD virus and expressing clinical signs during this project was less than 12 months.

Table 5: Age distribution of BVDV-positive cattle

Age	Number of animals	% of animals
7 months to 1 year	10	66,7
Above one year	5	33,3

3.4 Discussion

For BVDV isolation, a variety of specimens may be used including blood, urine, nasal mucous, vaginal mucous, uterine fluids and internal organs. Faeces have been found to be an undesirable specimen (Dubovi 1990) because of risk of bacterial contamination of cell cultures.

In this study, private practitioners and feedlot consultants submitted bovine clinical samples (sera, blood in heparin-containing tubes and specimens of spleen, lymph nodes and lung) as requested. In live animals, blood samples in heparin were the cleanest and easiest to handle when sent on ice, and buffy coat cells were one of the preferred specimen for virus isolation since BVDV has an affinity for lymphoid tissue and thus for peripheral blood leukocytes. Sera have been shown to be an excellent diagnostic sample in persistently infected animals. PI animals usually have large amounts of BVDV, e.g. 10^3-10^5 CCID50/ml (Bolin et al. 1991b). Furthermore, the BVDV is very stable in serum, surviving at room temperature for at least 7 days (Saliki et al. 1997).

Although, in some cases more than one sample was submitted, only one sample per animal was processed due to budget limitations.

For practical purposes, samples were stored at –20 ºC for short storage not more than two weeks and at –70 ºC for long storage. For best long term survival, storage at –70 ºC has been proven to be the best while at –20 ºC, a drop in virus titre may occur after a while (C. Baule, Veterinary Faculty, Swedish University, 2001, personal communication).

The cell cultures used, namely MDBK and CFK, were also routinely monitored for BVDV to rule out any adventitious contamination that could have happened in the laboratory during cultivation of the cells. It has been reported that the infectivity titres of BVDV were considerably higher in medium supplemented with horse serum than with ox or two different batches of commercial foetal calf serum (Hyera *et al.* 1987).

During this study, foetal calf serum was used, as it was readily available. The foetal calf serum used was obtained from a commercial supplier, Highveld Biological Products. The serum had been filtered and gamma irradiated at 28-30 KGY under conditions that preserve its biological integrity. All batches obtained from the distributor were tested and certified BVDV free after gamma irradiation. Nevertheless, during this study, serum bottles once opened were routinely tested for BVDV.

Buffy coats were subjected to both antigen capture ELISA and virus isolation (n=103). Whole blood specimens (n=51) were tested only by means of an antigen capture ELISA. ELISAs have been used for rapid detection of both BVDV antibodies and antigens in blood, but should be backed up by other methods such

as virus isolation in cell cultures or amplification of viral nucleic acid. Others were subjected only to virus isolation (n=198). Twenty-seven specimens were judged unfit for virus isolation due to either complete haemolysis, advanced decomposition (organs not on ice), broken tubes or bacterial proliferation. Only one specimen per animal was processed, the rest was stored at –70 ºC.

Staining of infected MDBK and CFK cells with FITC-conjugated antibodies resulted in specific fluorescence in the cytoplasm of infected cells. Antigen was diffusely distributed throughout the cytoplasm of most infected cells. Initially, the DFA and the IFA tests were not equally effective in detecting BVDV. Nonspecific staining and difficulties in distinguishing clearly between infected and non-infected cells complicated the DFA test.

In this study, all the viruses isolated were noncytopathic. This is the most common biotype found in nature. Most BVDV isolated during other studies were also noncytopathic biotypes (Baule *et al.* 1997; Carman *et al.* 1998; El-kholy *et al.* 1998; Fulton *et al.* 2000).

An opportunity to obtain specimens for virus isolation arose during investigations into tuberculosis in African buffaloes and other species in the Kruger National Park (Keet *et al.* 1996). A randomized epidemiological survey was carried out during September and October 1998 to assess the prevalence and distribution of infection in the buffalo population. Six-hundred-and-eighteen African buffaloes were culled during this survey. Collection sites were spread throughout the park (B. Penzhorn, Veterinary Faculty, University of Pretoria, 1999, personal

communication). BVDV has been diagnosed in water buffaloes elsewhere (Hafez & Frey 1973; Amber *et al.* 1988; Sudharshana *et al.* 1999). No significance could be attributed to the absence of BVDV in the 37 lymph nodes obtained from 37 African buffaloes other than that the number of specimens was small in comparison to the number of buffaloes (30 000) in the Kruger National Park.

There are no pathognomonic clinical signs of infection with BVDV in cattle (Baker 1987; Carman *et al.* 1998). In the USA (Saliki 1996) various cases of BVDV infection were classified into syndromes based on the predominant clinical manifestation: Reproductive disease comprising abortion, repeat breeding, stillbirth, weak calves; acute/peracute BVD in animals of all ages often resulting in death; classical BVD represented by gastroenteritic disease, pyrexia and respiratory disease; haemorrhagic syndrome comprising bloody secretions and petechial haemorrhages; MD characterized by gastroenteritis and digital erosions and ulcers; respiratory disease that included pyrexia, bronchopneumonia and weakness. Results compiled from different reports showed that the association with enteric and respiratory disease predominated (Evermann *et al.* 1993; Fulton *et al.* 2000). Bovine viral diarrhoea virus has been identified in outbreaks of respiratory disease, usually in association with other pathogens (Potgieter 1977; Yates 1982; Howard *et al.* 1987; Van Vuuren 1991).

Bovine viral diarrhoea should be considered as a differential diagnosis in outbreaks of diarrhoea, especially in yearlings or adults, reduced milk yield and of general reproductive problems (infertility, embryonic loss, abortion) especially where there is evidence of congenital abnormalities in calves. During this study,

BVD virus was not isolated from 2 calves with congenital abnormalities, 13 cases of haemorrhagic syndrome, 42 cases of abortion and 5 animals with poor growth. Based on the main clinical signs recorded from case histories, the diagnosis of BVD on clinical grounds is not possible.

BVDV has the capacity of continually changing its clinical manifestations and often serves as a challenge to the most astute clinician. Diagnostic investigations therefore rely on laboratory detection of the virus, or of virus-induced antigens or antibodies in submitted samples. The haemorrhagic form is more common in the northern hemisphere (Pellerin *et al.* 1994; Flores *et al.* 2000; Ridpath *et al.* 2000) and has not yet been recorded in Africa. From the few haemorrhagic cases from which specimens were submitted during this study, some were suspected to be mucosal disease and others were suspected to be caused by BVDV type II. Neither BVDV type II nor the virus pair, cp BVDV and ncp BVDV were isolated. It was concluded that no case of mucosal disease was encountered during this study. From cattle with clinical signs such as abortion, poor growth and pyrexia no BVDV was isolated. Among the 15 isolates, 10 were from feedlots, 4 from commercial beef cattle and 1 was from a dairy herd. The most frequent sign in feedlot cattle was pyrexia with respiratory distress (46,7%), followed by diarrhoea and pyrexia (20%), respiratory tract infection without pyrexia (20%) and diarrhoea without pyrexia (13,3%). It appeared from the duration of clinical signs provided by owners or veterinarians that most of the cases were acute infections.

CHAPTER 4. MOLECULAR ANALYSIS OF BVDV ISOLATES FROM SOUTH AFRICA

4.1 Introduction

The genome of BVDV is a single-stranded, positive-sense, non-polyadenilated RNA of approximately 12.5 Kb in length. It has two non-translated regions at the 5' end (5'NTR) and at the 3' end (3' NTR) of the genome. Translation occurs in a cap-independent manner from a single large open reading frame (ORF) that encodes a polyprotein of about 4000 amino acids. The polyprotein is co- and post-translationally processed by viral and host cell proteases into mature structural and non-structural proteins. The BVDV genome encodes 4 structural proteins: capsid protein (C), envelope protein with intrinsic RNase activity (E^{rns}), transmembrane glycoprotein (E1) and envelope glycoprotein (E2). At least 7 non-structural proteins (Npro, p7, NS2-NS3, NS4A, NS4B, NS5A and NS5B) are found (Collett *et al.* 1996; Thiel *et al.* 1996).

The 5' NTR of the genome is highly conserved among pestiviruses and for this reason it has been used as a target for molecular detection of pestiviruses and for genetic discrimination among and within pestivirus genotypes (Pellerin *et al.* 1994; Paton *et al.* 1995a; Lowings *et al.* 1996; Baule *et al.* 1997; Becher *et al.* 1997; Vilcek *et al.* 1997; 1999).

Two genotypes of BVDV have been discriminated on the basis of the 5'NTR analysis, namely BVDV type I, which is subdivided into subgroup Ia and represented by the reference strain NADL, and subgroup Ib, represented by the reference strain Osloss. BVDV type II has strain 890 as reference and comprises especially isolates associated with a new form of acute infection in cattle, the

haemorrhagic syndrome, originally described in North America (Pellerin *et al.* 1994). BVDV type II has also been shown to include ovine isolates (Paton *et al.* 1995b; Ridpath *et al.* 1995; Vilcek *et al.* 1997) and recently, to be present outside North America (Canal *et al.* 1998; Nagai *et al.* 1998, Dean & Leyh 1999; Flores *et al.* 2000).

The natural transmission of pestiviruses between host species has prompted a new classification of the members of the *Pestivirus* genus. The suggested classification takes into account the antigenic and genomic relationship rather than the species of origin (Paton *et al.* 1995c; Becher *et al.* 1997; Sullivan *et al.* 1997; Vilcek *et al.* 1997). Accordingly, the *Pestivirus* genus is divided into 4 genotypes: genotype 1 (*Pestivirus* type 1) that includes the present BVDV type I strains; genotype 2 (*Pestivirus* type 2) represents isolates of HCV; genotype 3 (*Pestivirus* type 3) includes sheep and pig isolates defined as "true BD" viruses, and genotype 4 (*Pestivirus* 4) includes isolates of cattle and sheep currently defined as BVDV type II.

The presence of BVDV in South Africa has been confirmed by several serological surveys (Theodoridis *et al.* 1973; Theodoridis & Boshoff 1974; Ferreira *et al.* 2000). However, little is known about their biological properties. Only one study in southern Africa by Baule *et al.* (1997) has described the nature of some isolates and found that they were all genotype I. The aim of this section of the project was to broaden knowledge on the genetic characteristics of local isolates and the findings from the previous study by Baule *et al.* (1997).

4.2 Materials and methods

4.2.1 Specimens

Specimens (n=33) consisting of 18 viruses obtained by means of virus isolation (Chapter 3), 7 FCS specimens that tested positive on ELISA (p.80), 8 specimens that yielded doubtful results with the FA (p.64) and tissue specimens taken from the original submissions consisting of tissue filtrates and serum were submitted to PCR. The latter also included specimens that tested negative for BVDV with FA or antigen ELISA. Some specimens were tested in duplicate, which accounts for the total number of 117. Twenty two of the 25 positive specimens mentioned above, were sent in duplicate and consisted of the frozen original submission and cell cultures. The remaining 3 specimens were submitted as single specimens only.

The seven FCS specimens mentioned above, were part of a set of 156 pooled serum specimens tested by means of an antigen ELISA test different from the one described on p.64 namely the USDA-licenced Syracuse Bioanalytical test. These specimens were obtained from Highveld Biological Products, a commercial supplier of FCS. The serum was collected at different abattoirs that slaughter dairy and beef cattle. The supplier was not able to keep records for identification of cows or the farm of origin of the cows. Sera were generally obtained from foetuses with a body length of 55 to 60 cm and approximately 180 to 210 days of gestation. Whole blood was collected from each foetus and allowed to clot. Serum from each foetus was randomly poured into a 2-litre bottle that could be filled with sera from four big foetuses or up to 10 small foetuses (E. Bey, Highveld Biological (Pty) Ltd, Kelvin, 1999, personal communication). Ten ml aliquots of the pooled sera were then provided in 200 tubes of 10 ml each and kept frozen. The

specimens were provided before sterilization by irradiation. This collection of sera therefore represented a minimum of 800 foetuses.

4.2.2 Indirect fluorescent antibody test

The 200 hundred tubes of pooled sera were tested for the presence of BVDV antibody by means of the IFA test. Each tube represented at least 4 foetuses. The indirect fluorescent antibody test for antibody detection was performed on multiwell slides. Slides prepared in the Department of Veterinary Tropical Diseases containing fixed cell monolayers infected with cytopathic strain C24V Oregon were used. Ten microlitres of serum to be tested was added to each well and the slide incubated at 37°C for 35 minutes. Slides were washed twice for 5 minutes in phosphate buffered saline (PBS) and then in distilled water and dried. Each well was covered with 10 µl rabbit antibovine immunoglobulin G conjugated to fluorescein isothiocyanate (SA Scientific Products) diluted with 0,05 % Evans blue stain to a working dilution of 1:40. Finally, slides were washed twice for 5 minutes in PBS and then in distilled water and dried. The slides were examined by fluorescence microscopy.

4.2.3 Antigen capture ELISA

All antibody negative sera were tested for BVDV antigens. The test used was a USDA-licenced BVD antigen capture ELISA test kit (Syracuse Bioanalytical, Inc.) but only 156 pooled serum specimens representing at least 4 foetuses per specimen could be tested with available reagents. In contrast to the IDEXX test described in Chapter 3, the Syracuse test can detect BVDV antigens in serum. It employs a BVDV antigen-specific monoclonal antibody as the capture antibody,

goat polyclonal anti-BVDV antibody as the detector, and a horseradish peroxidase anti-goat conjugate. Before the results could be interpreted, the average optical density (OD) value for each control and sample assayed was calculated. The OD value obtained for the negative control (background) was subtracted from each of the other OD values to obtain "blank-corrected" OD values for the positive control and samples. Results were then normalized by calculating the ratio of each "blank-corrected OD to the "blank-corrected" OD obtained with the positive control.

To determine the status of the animal, the "normalized" OD results were compared with the manufacturer's guidelines. For a run to be acceptable, the raw OD values (unblanked) obtained for the kit controls should fall within the following ranges: The positive control should have a raw OD value more than 0,8 and the negative control should have a raw OD value less than 0,5. The optical density was measured at 450 nm against air and negative control absorbancy values on a microplate reader. Positive tubes were kept frozen for virus isolation.

4.2.4 Virus isolation with immunofluorescent staining

Tubes of pooled sera that tested positive with the antigen capture ELISA were subjected to virus isolation in tissue culture. Sera from frozen tubes were thawed and centrifuged at 1000 x g for 10 minutes, passaged through a 0.22 µm filter and 1 ml of each specimen was inoculated into a 25 cm^2 flask containing MDBK cells and 10 ml of Eagle's minimum essential medium (MEM). Cells were passaged after 4-8 days into chamber slides, and the indirect fluorescent antibody test for antigen detection was then performed on the chamber slides as described in Chapter 3.

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

Total RNA was extracted from supernatants of infected cells, tissue homogenates, whole blood and serum specimens, using TRIzol (Gibco, Life Technologies), according to the manufacturer's instructions. cDNA was synthesized by random priming with pdN6 (Amersham-Pharmacia, Uppsala, Sweden) using Moloney murine leukaemia virus reverse transcriptase (M-MLV RT) (Gibco, Life Technologies), as follows: 5 µl of total RNA were mixed with 0.02 U of pdN6 and 3 µl of ddH_2O and denatured at 65 ºC for 10 minutes, then quickly chilled on ice. A reaction mix containing 4 µl of 5 x 1st strand buffer, 2 µl of 0.1 M DTT, 0.5 µl of each dNTP (10 mM each), 24 U of RNAse inhibitor (RNA guard, Amersham-Pharmacia) and 200 U of M-MLV RT was added. Synthesis was carried out at 37 ºC for 90 minutes, followed by the inactivation of the enzyme at 95 ºC for 5 minutes.

A PCR was used to amplify specific sequences from the 5' NTR of the genome. The oligonucleotide primers were obtained from the National Veterinary Institute, Uppsala, Sweden and used as follows: forward, 5'-AGCCATGCCCTTAGTAGGACT-3', reverse, 5'-ACTCCATGTGCCATGTACA-3' (corresponding to positions 105-125 and 399-378, respectively, in the sequence of BVDV strain NADL). Amplification was carried out in a total volume of 50 µl containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1 µg/µl of BSA, 0.2 mM of each deoxynucleotide, 15 pmol of each primer, 2.5 mM $MgCl_2$, 2.5% Formamide, 1 U of Taq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CA, USA), and 5 µl of cDNA. The reaction mixes were overlaid with 2 drops of mineral oil. PCR cycles were as follow: 5 cycles of denaturation at 94 ºC for 45 seconds, annealing at 55 ºC for 45

seconds and extension at 72 ºC for 1 minute, followed by 30 cycles of denaturation at 94 º C for 45 seconds, annealing at 50 ºC for 45 seconds and extension at 72 ºC for 1 minute. A final extension step at 72 ºC for 7 minutes was included. Precautions to avoid contamination as described by Belák & Ballagi-Pordany (1993) were followed throughout the RT-PCR. The PCR products were visualized by ethidium bromide staining, after electrophoresis on 2 % agarose gels.

4.2.6 Sequencing and sequence analysis

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

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

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

4.3 Results

The results of tests for the detection of BVDV antigens and BVDV antibodies from pooled foetal calf sera are summarized in table 6.

Table 6: Results of three different assays for BVDV antigens and BVDV antibodies in pooled calf serum

Foetal calf serum	Indirect fluorescent antibody test	Antigen capture ELISA	Virus isolation
Number tested	200	156	7
Positive	6	7	0
Negative	194	149	7

4.3.1 Indirect fluorescent antibody test

Six of 200 pooled samples of bovine FCS (3 %) were positive for antibodies to BVDV.

4.3.2 Antigen capture ELISA

Seven of 156 pooled samples of bovine FCS (4.4 %) were positive for BVDV antigens. The normalized OD values obtained from 3 out of 7 samples had inconclusive values that ranged from 0, 31-0,36 (Table 7).

Table 7: Normalized OD values in "Gray Zone"

Samples	OD values	Normalized values
Tube 32	0, 529	0, 32
Tube 46	0, 522	0, 316
Tube 81	0, 595	0, 36

The remaining 4 specimens were positive with normalized values that ranged from 0,42-1,22 (Table 8).

Table 8: Normalized values obtained from 4 positive tubes of pooled calf sera

Samples	OD Values	Normalized values
Tube 7	1, 141	0, 69
Tube 8	1, 605	0, 97
Tube 77	2, 019	1, 22
Tube 200	0, 695	0, 42

The 3 specimens that yielded inconclusive results were retested according to the test protocol and the new normalized values ranged from 0,42-0,48 (Table 9).

Table 9: Normalized OD values after re-assay of samples from table 7

Samples	OD values	Normalized values
Tube 32	0, 695	0, 42
Tube 46	0, 678	0, 41
Tube 81	0, 794	0, 48

All samples that yielded normalized OD values greater than 0,39 were then considered positive according to the parameters of the test. These 7 positive samples were inoculated in cell cultures. The normalized OD values for the remaining samples (n=149) ranged from 0,02-0,1 and were all below 0,20 which according to the cut-off value for this particular test were negative. The six tubes that were positive for antibodies on IFA test were negative on antigen ELISA (Table 10).

Table 10: Negative normalized OD values of 6 tubes that were negative on IFA test for antibody

Samples	OD values	Normalized values
Tube 1	0, 033	0, 02
Tube 3	0, 033	0, 02
Tube 4	0, 115	0, 07
Tube 9	0, 148	0, 09
Tube 20	0, 165	0, 10
Tube 35	0, 182	0, 11

4.3.3 Virus isolation with immunofluorescent staining

The 7 pooled FCS specimens that were positive with the antigen ELISA were negative for virus isolation in tissue culture after the first passage. Further passages were not attempted.

4.3.4 PCR

Forty-seven (including 22 isolates submitted in duplicate and 3 as single specimens) out of 117 specimens tested positive for BVDV (Table 11).

Eight other specimens that included 2 sera, 3 buffy coats, 2 spleens and 1 lung gave inconclusive readings with the FA test in cell cultures. Two were confirmed negative and 6 yielded a weak band with PCR. All of the strains were identified as BVDV I, either BVDV Ia (NADL-like) or BVDV Ib (Osloss-like) subgroups or BVDV I*.

Table 11: Results of the PCR assay for BVDV on selected specimens

Specimens	PCR	
	No. tested	No. Positive
Serum	54	11
Spleen	10	3
Buffy coat	32	3
Lung	8	3
Lymph nodes	10	2
Cell lines	3	3
Total	117	47

The 25 isolates obtained after virus isolation (n=18) and antigen ELISA (n=7) were confirmed as BVDV with PCR (Table 12).

Table 12: Comparison of the Ag capture ELISA test, virus isolation and PCR for the detection of BVDV

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

NT: Not tested

*: Three buffy coat that tested positive on both tests

Table 13 shows isolates, that were confirmed by PCR and the predominant clinical signs associated with them. Seven isolates obtained from 156 pooled serum specimens and 3 cells lines of unknown history were included under the heading "others", since no clinical syndrome could be ascribed to them.

Table 13: Predominant clinical syndrome associated with BVDV isolates and their origin

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

ncp: noncytopathogenic

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

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

The phylogenetic assignment of these isolates, compared to reference strains of pestiviruses and to sequences from a previous investigation with BVDV isolates from southern Africa is shown in Fig.2. The phylogenetic tree was generated based on a comparison of 245 nucleotide long sequences in the 5' NCR. The distances were calculated using the neighbor-joining method. The BVDV isolates listed in table 8 were determined to be BVDV type I. The 25 isolates analyzed were phylogenetically discriminated as follows: 2 (ST22F/99, ST21G/99) segregated clearly as subgroup Ia; none was found under subgroup Ib; 3 (ST25G/99, ST23F/99, ST24G/99G) were included in a cluster preliminarily termed Ic (Baule *et al.* 1997), whilst the remaining isolates formed a separate cluster named I*.

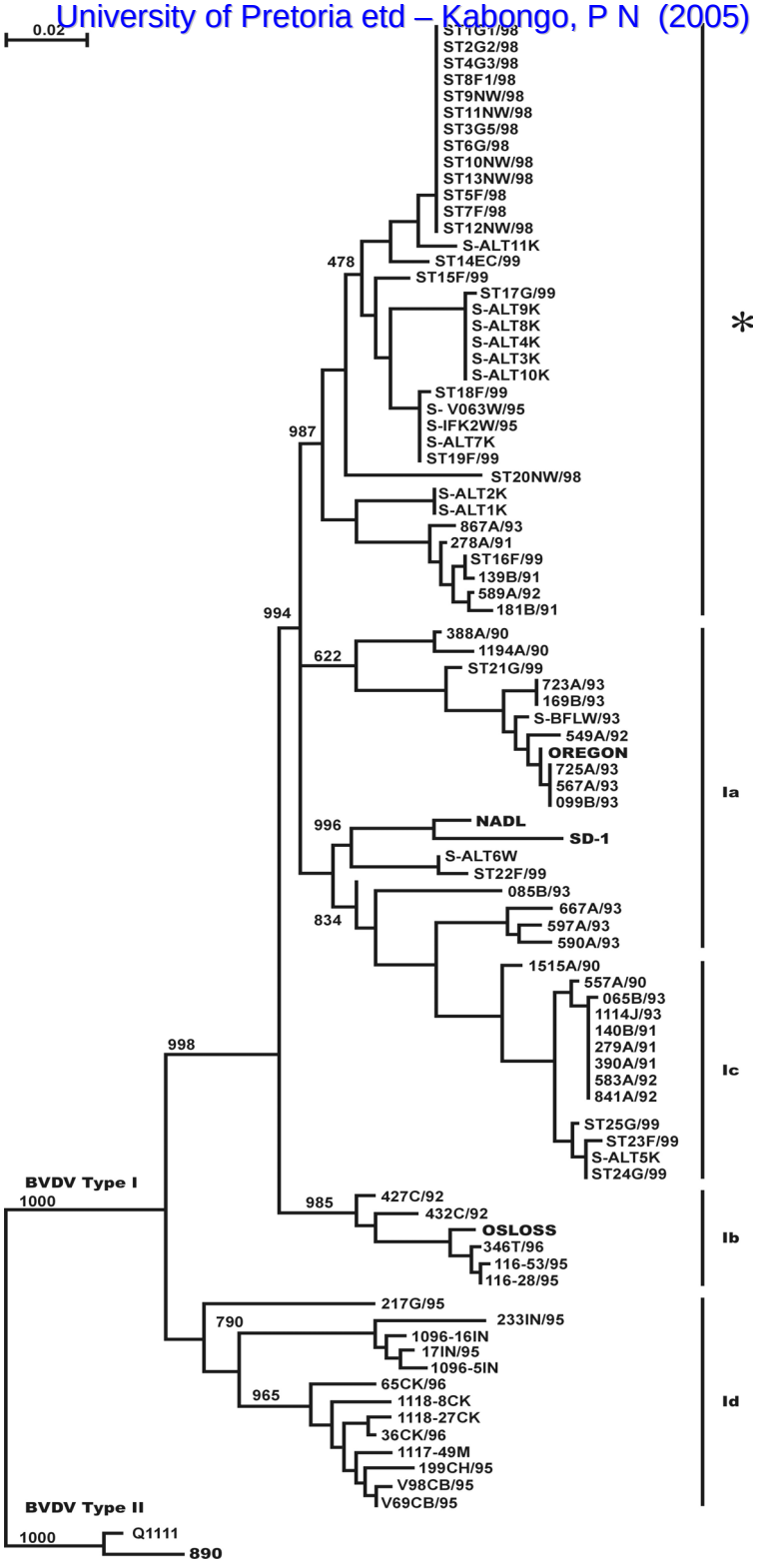


Fig.2: Phylogenetic analysis of BVDV isolates and sequences from Genbank

Reference strains of BVDV type I (NADL, OSLOSS), BVDV type II (890), BDV, HCV and isolates from a previous investigation on BVDV are included. The phylogeny is based on analysis of 5'NTR of the genome. The isolates (see table 13) cluster into subgroup Ia, subgroup Ic and subgroup I*

4.4 Discussion

It can be reliably estimated that each container with pooled sera represented at least 4 foetuses. Therefore, 149 pooled sera that were negative for BVDV antigens represented at least 596 foetuses. Since, it was not possible to assess the number of foetuses that could have been infected in a tube of pooled sera, no particular conclusion can be made about the possible number of virus positive foetuses.

Several reports have described contamination of veterinary biological products with BVDV (Wensvoort *et al.* 1988). These findings indicate that large pools of serum obtained from many foetuses are not likely to be free of BVDV and /or antibodies to BVDV. The presence of BVDV means that these foetuses were infected in utero with the potential to maintain the virus in the bovine population after birth (Scott *et al.* 1973). Since this virus has the ability to cross the placenta, it is to be expected that a small proportion of foetal sera will contain it (Kniazzeff *et al.* 1967) and these findings re-emphasize the dangers inherent in the use of untreated foetal calf serum for tissue culture production. In addition to infecting several foetal cell types, BVDV is found free in FCS (Kniazeff *et al.* 1975; Rossi *et al.* 1980; Bolin *et al.* 1991b; Bolin & Ridpath 1998) creating a potential problem for diagnostic laboratories because FCS is frequently used to supplement media used for cell culture, and a variety of cell cultures from several species are susceptible to infection with BVDV (Nuttall *et al*. 1977; Wellemans & Van Opdenbosch 1987). Sera from infected animals, if used for cell cultures, will be of particular significance for the production of viral vaccines. Vaccination with such vaccines

may give rise to abortions since transplacental infection by BVDV can cause death and malformations of the foetus (Bolin 1990; Cortese 1994).

Certain tests employing cell cultures in veterinary diagnostic laboratories are dependent on the use of cell cultures free of contamination with ncpBVDV. Such tests include virus and chlamydia isolations, serum neutralization assays and the microplate virus isolation technique for detection of BVDV in PI. Contamination of viral stocks also has important implications for experimental virology. In a trial designed to assess the clinical responses of calves to infection by respiratory syncytial (RS) virus, the inoculum containing this virus grown in CFK cells was found to contain BVDV (Stott *et al.* 1987). If BVDV had not been detected, the clinical reactions of inoculated animals would have been attributed solely to RS virus. Regular screening of bovine cell cultures is essential if viral stocks are to be kept free from BVDV.

Artificial breeding procedures, either through the movement and aggregation of animals or more directly, through the use of infective materials such as semen or foetal calf serum, have great potential to facilitate the spread of infection and disease.

Foetal calf serum may also contain antibodies that can interfere with the titre of BVDV propagated for use in diagnostic tests. Also, antibodies against BVDV cross-react with other pestiviruses (Darbyshire 1960; Harkness *et al.* 1977) namely BDV and HCV and may interfere with diagnosis of the diseases caused by these viruses. Neither BVDV antigens nor antibodies were detected in the

commercial FCS obtained from Highveld Biological Products that was routinely used as a supplement in cell culture media during this study.

Foetal calf sera that were positive for BVDV with the antigen capture ELISA, were negative after passage in tissue culture. It has been shown that virus isolation protocols using cell cultures cocultivated with washed leukocytes (Frey *et al.* 1991) or inoculated with blood clots (Barber *et al.* 1985) are more sensitive than those in which cell cultures are inoculated with serum. An incidental observation suggesting a negative correlation between virus titre in serum and level of antigen in leukocytes (Gottschalk *et al.* 1992) supports possible occurrence of antigen-positive and virus isolation negative samples. Since the presence of antibody was not detected serologically, the most reasonable explanation for the negative virus isolation results could have been the loss of infectivity of the virus.

There was no relationship between the geographic origin, the nature of the clinical signs and the typing of the BVDV isolates. Animals from the 4 provinces: Northwest (NW); Free State (F); G (Gauteng); Eastern Cape (EC) were infected with the same strain. This can possibly be explained by the movement of animals, the absence of closed herds and perhaps vaccination. Throughout South Africa, there is a diversity of farming systems from extensive to intensive, including closed herds such as those where artificial insemination is used. Isolates were obtained from samples collected in feedlots, dairy herds and commercial beef farms in all provinces, indicating the ubiquitousness of BVDV in South Africa.

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

Six clinical samples from which virus had not been isolated; showed a weak band with RT-PCR although the weak band was situated at the correct molecular weight position. The weak band was detected in the second round of amplification in nested PCR, since the DNA from the first cycle (product used for sequencing) was not enough to use in direct sequencing without further enrichment by cloning or by cultivation of the original material. These six specimens were not molecularly analyzed and were not included in the phylogenetic tree. The weak bands were most likely detected as a result of the more sensitive nested PCR. It has been shown in a previous study (Baule *et al.* 1997) that analysis of a short fragment in the 5' NTR (such as the 119 bp product from a nested PCR) may exclude sequence information important for strain distinction within genotypes.

PCR was in agreement with virus isolation in all the negative cases except in 7 out of 156 pooled sera that were negative for virus isolation after one passage but

tested positive on antigen ELISA. This confirms the need for more than one passage before virus becomes detectable with the FA test.

All the BVDV strains obtained during this study were BVDV I (BVDV Ia (NADL-like), BVDV Ic subgroups or BVDV I*). No association could be demonstrated between the geographic origin of the isolates and branch discrimination. The 3 groupings formed by the South African isolates (subgroup Ia, cluster Ic and cluster provisionally called*) included BVD viruses from different regions: F, G, NW and EC. It is worth noting the similarity with isolates of the BVDV cluster preliminarily termed Ic in a previous investigation (Baule *et al.* 1997) and which did not segregate with either the Ia or the Ib subgroups. The presence of isolates of this cluster in South Africa may reflect a local genetic subgroup that is spreading in the region since genotype I shows an intragenotyping diversity. This might have occurred by means of cattle movements or the use of biologicals such as cell culture-derived vaccines.

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

A clustering of isolates with regards to farms of origin was not observed with the isolates investigated, as reported by others (Paton *et al.* 1995a; Vilcek *et al.* 1999). Differences in farming practices, i.e., extensive farming versus intensive farming may contribute to this difference in virus ecology. Closed herds and restricted contact among cattle may be a determinant to establish BVDV in a herd-specific manner. Most herds from which the samples originated were managed extensively.

CHAPTER 5. GENERAL DISCUSSION

Pestiviruses are causative agents of important diseases in cattle (BVD/MD), pigs (hog cholera) and sheep (border disease) worldwide. They are antigenically related to each other and infection of pigs and sheep with cattle isolates occurs. Therefore, there is a need for more information on the epidemiology of these viruses.

Bovine viral diarrhoea virus is considered in some countries as the single most important virus infection of cattle directly causing or significantly contributing to a wide range of clinical syndromes (Childs 1946; Corapi *et al.* 1990b; Bolin & Ridpath 1992; Evermann *et al.* 1993). BVD is an economically important disease. The most important cause of BVDV-associated economic losses appears to be reproductive disorders (Brownlie *et al.* 1989; Woodard *et al*. 1994; Saliki 1996). There are several infectious diseases of cattle of which the clinical signs are compatible with BVDV infections: diseases with oral lesions and diarrhoea that include rinderpest and bovine malignant catarrhal fever; diseases with oral lesions but without diarrhoea that include foot-and-mouth disease, vesicular stomatitis, bluetongue, bovine papular stomatitis, necrotic stomatitis; and diseases with diarrhoea but without oral lesions that include salmonellosis, winter dysentery, paratuberculosis, copper deficiency, or molybdenum excess, intestinal helminthiasis, coccidiosis and arsenic poisoning (Baker 1987)

Reproductive disorders, such as unexplained abortions, infertility, stillbirth and teratogenesis, may also indicate the presence of BVDV in a herd. Therefore, the

diagnosis of BVD on the basis of clinical signs alone is not possible and requires laboratory support.

In Europe and USA several studies have been conducted on different aspects of the virus, but some aspects still remain elusive: functions of BVDV proteins are still hypothetical; the function of NS4B/P38 in viral replication remains to be identified (Van Olphen & Donis 1997). Further investigation at the molecular level is still needed; the impact of pestivirus infection on the health of wild populations needs further investigation and the nature of the immunotolerance to BVDV remains unproven. During the mutational event, it is still not clear how the virus kills the cell. The precision of the tolerance to "homologous" virus appears sufficiently restricted to allow the immune recognition and clearance of heterologous virus or viral variants.

In South Africa, although the presence of BVDV was recognized by serological means during the early 1970s (Thomson & Blackburn 1972; Theodoridis & Boshoff 1974) no viruses were isolated and registered in the genetic bank. It was during the last decade that several strains were isolated at Allerton Laboratories in Pietermaritzburg, Onderstepoort Veterinary Institute and Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. Some veterinary practitioners have suspected the presence of BVDV genotype II based on clinical signs in cattle compatible with the haemorrhagic syndrome described in North America. However, in a previous study, Baule *et al.* (1997) could not confirm the presence of genotype II.

Considering the implications of the genomic diversity in the diagnosis, epidemiology and control of BVDV infections, it is deemed important to characterize the BVDV viruses occurring in a region. Knowledge of the virus in terms of genetic and antigenic composition is therefore important to provide information for the production or importation of efficient vaccines for use in conjunction with other biosecurity measures. With the resources available to us during the study period, the aims of this research were to isolate BVD viruses in order to enlarge the pool of local strains for characterization, to identify the genotypes of BVDV that circulate in South Africa, to obtain phylogenetic data of local strains for comparison with imported vaccine strains, and to record the clinical signs associated with each isolate in order to establish the relation between isolates and clinical disease under South African conditions.

Two biotypes of BVDV are found: Cytopathic BVD viruses that induce cytoplasmic vacuolation in susceptible cell monolayers, and noncytopathic viruses. The ncp biotype grows slowly in cell culture if low titre of the virus is present; it may require several passages before sufficient viral antigen is expressed to allow detection with the fluorescent antibody test (Smith *et al.* 1988; Roberts *et al.* 1991). During this study, some specimens only tested positive after 2 to 3 passages. The virus can be inactivated by serum neutralizing antibodies or by physical means, such as heat during transport, and therefore, it may lose its infectivity. This may have happened with some of the specimens that formed part of this investigation where the cold chain was not maintained. This may also explain weak bands found in the second round of amplification with the nested PCR.

Isolation of BVDV can be complicated by contamination of cell cultures with ncp BVD virus (Hassan & Scott 1986; Smith *et al.* 1988; Dubovi 1990). The source of the contaminant BVDV is often foetal calf sera used to supplement cell culture media. Cell cultures used for BVDV isolation were tested frequently to rule out any adventitious noncytopathic BVDV contamination. Viral antigen or nucleic acid were detected using antigen capture ELISA or PCR tests in cases where virus isolation in tissue culture was unsuccessful. This may be related to either a loss of infectivity of the virus or presence of toxic elements for the cells. The PCR detects both viable and non-infectious or incomplete viral particles since it amplifies specific nucleotide sequences that are generally more stable in a clinical specimen (Alansari *et al.* 1993; Schmitt *et al.* 1994; EL-Kholy 1998). Antigen capture ELISA for BVDV allows more rapid confirmation of a suspected BVDV infection avoiding the usual delay in isolating ncp BVDV. Serological subgroups of BVDV are not currently recognized, but several reports document significant genomic and antigenic heterogeneity among BVDV (Howard *et al.* 1987; Dubovi 1992; Flores *et al.* 2000).

Veterinary diagnostic laboratories should avoid non-irradiated FCS in diagnostic procedures for pestiviral infections. Secondly, there is a significant risk that adventitious BVDV from FCS may lead to contamination of vaccines. There are reports (Dubovi 1990; Bolin *et al.* 1991b) of vaccines that have been contaminated with BVDV. Results obtained during this study from different batches of pooled foetal calf serum collected at different abattoirs in the country and prior to irradiation, indicate widespread infection with BVDV in South Africa and the need to irradiate all raw serum used for commercial purposes. To prevent

contamination of cell cultures, gamma irradiated foetal calf serum free from BVDV was used during this project.

Foetal calf serum may contain antibodies against BVDV (Virakul *et al.* 1985; Bolin *et al.* 1991b). Since, there is no procedure to get rid of the presence of antibodies, serum-free media or alternative supplements such as horse serum, should be considered for cell cultures used for BVDV isolation. Horse serum has also been found to increase the detection of BVDV in specimens (Hyera *et al*. 1987). Foetal calf sera used during this project was tested for antibodies to BVDV and were found negative on all occasions.

There are no pathognomonic clinical signs of infection with BVDV in cattle. Multitude clinical manifestations that included abortion, acute/peracute disease, classical BVD, diarrhoea, haemorrhage, MD and pneumonia were reported in south central USA (Saliki 1996).

Base sequences of amplified fragments of the viral genome can provide detailed information on the degree of amino acid homology between different strains (Boye *et al.* 1991; Brock *et al.* 1995). Such information could be relevant in epidemiological studies and approaches to vaccination (Hooft van Iddekinge *et al.* 1992; Brock 1995). In this project, PCR confirmed BVDV in all cases where the presence of virus was detected by either virus isolation or antigen capture ELISA, or both. It detected virus in 7 cases where the virus was not isolated in tissue culture. In this study, 25 isolates obtained were analyzed on the basis of nucleotide sequencing of the 5' non-coding region (5'NTR) of the BVDV genome.

The 245 base nucleotide sequences derived from the 5'NTR were aligned and compared to the corresponding positions from published sequences of BVDV type I and type II strains of pestiviruses of ovine and porcine origin. The phylogenetic tree generated from the comparison, showed that the southern African isolates belong to BVDV I genotype and showed similarity to BVDV reference strain NADL. They were placed in the subgroup Ia and subgroup Ic of BVDV genotype I. Other isolates formed a cluster that was provisionally called * (Fig 2).

Strains of BVDV fall within two subgroups, BVDV type I and BVDV type II. BVDV type I strains can be divided into two antigenic subgroups on the basis of reactivity to a panel of monoclonal antibodies (subgroups I a and I b). This subdivision is also supported by genetic analysis, mainly of the 5'NCR and the E2 coding region. Genetic analysis of several strains have revealed the existence of a number of strains not fitting genetically within the Ia and Ib subgroups (eg Ic subgroup). Further subgroups have not been serologically and genetically characterized to clarify whether they also represent new antigenic types. It also applies to the 20 isolates from this study that belong to a new cluster * and might need further investigation. This also confirms the intragenotypic variability that exists within BVDV type I.

Phylogenetic analysis and differences in virulence suggest that BVDV type II are heterogeneous; some isolates from Brazil were genotypically different from the BVDV type 2 isolates identified in North America and Europe (Flores *et al.* 2000; Ridpath *et al.* 2000). The antigenic segregation seen between type I and type II in cross-neutralization tests using polyclonal antibodies illustrates the need for further

studies to determine the significance of the antigenic variations as they pertain to the clinical expressions of BVDV infection.

The spectrum of genetic and antigenic variation is bound to have implications in diagnosis and also in the need for designing protective vaccines, taking the different types into account. The predominant pestiviruses found in particular hosts are not the same in all countries. Most are usually maintained in nature in one particular host species, from which they may occasionally be transmitted into a heterologous host. The HCV type has only been isolated from pigs, but the other two main types have been obtained from at least two hosts. BVD viruses are mainly found in cattle but also occur in sheep in some countries and are occasionally isolated from pigs. The BDV types are mainly ovine isolates but have been recovered from pigs. Where HCV has been eradicated, other pestiviruses may actually be more common in pigs. Cattle in most countries appear to be mainly infected with BVDV type I but in North America, BVDV type II has been increasingly isolated. Sheep appear to be readily infected by pestiviruses from cattle, including BVDV type I and type II. In some countries both these viruses are present, whereas in others only BVDV type I has been detected.

The antigenic variation among pestiviruses needs more investigation to understand how much a natural infection with one type of pestivirus is likely to protect against reinfection with a second variant. Diagnostic tests should ideally be able to detect a wide range of strains. For genetic detection, this is achieved by choosing highly conserved regions of the genome for the selection of diagnostic primers and probes. For serological tests, using viruses of subgroups Ia and Ib

and some type II strains as capture antigens should be representative enough. But, it has also been recorded that serological tests using only one virus may result in a significant number of false negative results (Flores *et al.* 2000). Raising new monoclonal antibodies against some of the viruses would be one way to determine whether they represent one or several new groups.

In South Africa where closed herds are uncommon, vaccination against BVDV infection should form an integral part of a biosecurity programme to control BVDV, but deciding which type of vaccine to use can be confusing to users. The main objective of a vaccination programme would be to protect against as many field strains as possible and to protect the foetus from transplacental infection. Both modified live and inactivated vaccines are available. Inactivated vaccines are safe and can be used in pregnant animals. However, they take longer to stimulate an antibody response and depend on a high antigenic content, multiple vaccinations and the use of effective adjuvants to enhance the immune response. The same level of safety cannot be reported for modified live BVD vaccines as breaks in immunity, immunosuppression, MD and abortions have been reported in the past in Europe and the USA. Modified live vaccines however, provide better immunogenicity at the expense of safety.

The emergence of BVDV genotype II in North America where BVDV genotype I derived vaccines were widely used raised questions about the cross protection between type I and type II. A similar situation may arise in South Africa as a result of intercontinental movement of animals and of biological products. Vaccines should ideally be produced using local virus strains. Vaccines imported into South

Africa contain strains such as NADL and Singer. Since the isolates from this study are closely related to the strains Oregon and NADL, it can be concluded with some reservation, that the current generation of imported BVDV vaccines are still appropriate for use in South Africa in spite of the antigenic variability among BVDV isolates.

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Annexe 1. Detailed summary of the origin and nature of specimens tested and the results obtained.

Date, origin, number of specimens, particulars of the animals, main history, tests and results						
Date	Sender/Area	Specimens	Animal/age	History	Tests	Results
15/01/98	Clocolan (F)	*BH (n=1) *S (n=1)	F, 24 mo (n=1)	Pyrexia, 40ºC, soft diarrhoea	DFA ELISA	Neg
19/03/98	Vryheid (KZ)	*S (n=4)	F, 24 mo (n=4)	Pyrexia, 39, 8ºC	DFA PCR	Doubtful (n=1); Neg (n=3)
7/04/98	Ventersdorp (NW)	*S (n=1)	F, 18 mo (n=1)	Cough	DFA PCR	Neg
20/04/98	Brandfort (F)	*BH (n=1) S (n=1)	M, 6 mo (n=1)	Poor growth	DFA ELISA	Neg
22/04/98	Benoni (G)	*Lu,Sp,Ln (n=3)	F, 7 mo (n=1)	Pyrexia 40ºC, diarrhoea, anorexia and death	IFA DFA PCR	Pos (n=1) ST1G1/98
24/04/98	Medunsa (G)	*S (n=1)	M, 6mo (n=1)	Nervous signs Incoordination	DFA PCR	Doubtful Neg
23/04/98	Vryburg (NW)	*BH (n=44)	F (n=40), M (n=4), 24 mo (n=44)	Herds screened by antibody ELISA	ELISA	Neg
21/05/98	Warmbad (NW)	*S (n=2)	F, 24 mo (n=2)	Nasal discharge + cough	IFA PCR	Neg
21/05/98	Onderstepoort (G)	BEDTA (n=2) BH (n=2) *S (=2)	F, 24 mo (n=2)	Screening test: Ab ELISA	ELISA IFA DFA	Neg
26/05/98	Potchefstroom (NW)	B H (n=11) *S (n=11)	F, 24 mo (n=11)	Pyrexia 41ºC and diarrhoea	IFA PCR	Neg
26/5/98	Benoni (G)	*Lu (n=4)	F, 18 mo (n=4)	Pneumonia	IFA	Neg
5/6/98	Secunda (MPG)	*BH (n=18)	F, 24 mo (n=18)	Herds seroneg by Ab ELISA	IFA (n=11) ELISA (n=7)	Neg Neg
26/6/98	Vryburg (NW)	*BH (n=2)	F, M, 18 mo (n=2)	Pyrexia 41ºC and 39, 7ºC respiratory distress	IFA ELISA PCR	Neg

26/6/98	Clocolan (F)	Lu, *Sp (n=2)	M, 1 week (n=1)	Malformed (Microphtalmia)	IFA ELISA	Neg Neg
26/6/98	Onderstepoort (G)	*BH (n=1)	M, 9 mo (n=1)	Fever: 40,3 ºC, nasal discharge + cough	IFA ELISA PCR	Neg
26/6/98	Stellenbosch (WC)	*BH (n=4) S (n=4)	F, 30-36 mo (n=4)	Abortion	IFA ELISA	Neg
26/6/98	Bethal (MPG)	*BH (n=2)	F, 36 mo (n=2)	Abortion	IFA ELISA	Neg
26/6/98	Benoni (G)	*BH (n=11)	F, 24 mo (n=11)	Fever 39, 9 ºC and diarrhoea (n= 9); diarrhoea with soft faeces(n=2) (n=11)	IFA PCR	Pos (n=3) ST2G2/98, ST4G3/98 And ST3G5/98
26/6/98	Stellenbosch (WC)	Lu, K, Ln,*Sp (n=4)	F, 24 mo (n=4)	Pneumonia	IFA ELISA	Neg
7/7/98	Medunsa (G)	*BH, S BEDTA (n=3)	M, few weeks (n=1)	Nervous signs (Incoordination)	IFA ELISA	Neg
4/8/98	Marburg (KZN)	*BH, S (n=2)	F, 26 mo (n=1)	Erosive stomatitis and haemorragic diarrhoea	IFA ELISA	Neg
5/8/98	Bronkhorstspruit (G)	*Lu (n=1)	F, 18mo (n=1)	Fever 41ºC, dyspnoea and death; pneumonia	IFA PCR	Pos (n=1) ST21G/98
7/8/98	Brits (NW)	*Cells		Check up	IFA ELISA PCR	Pos (n=1) ST20NW/98
12/8/98	Bethlehem (F)	BH (n=1) *S (n=1)	M, 16 mo (n=1)	Fever, cough	IFA	Neg
13/8/98	Ga-Rankuwa (G)	*Sp (n=1)	M, 7 mo (n=1)	Fever 41ºC, dyspnoea and sudden death	IFA ELISA PCR	ST6G/98
24/8/98	Highveld biological products (F+EC)	*S (n=200 tubes of pooled sera)	Foetuses	Each tube contains sera from at least 5 foetuses. Sera were collected at different abattoirs for a	IFA (Ag) (Ab) ELISA (Syrac	Neg.Ag Pos Ab (n=6) ELISA and PCR: Pos ST14EC/99,

				survey purpose on BVDV antigens and antibodies	use bioanal ytical) PCR	ST15F//99, ST16F/99, ST18F/99, ST19F/99, ST22F/99 and ST23F/99
2/9/98	Knysna (WC)	*S (n=1)	M, 24 mo (n=1)	Pyrexia, cough and nasal discharge	IFA ELISA	Neg
4/9/98	Clocolan (F)	*S (n=2)	F,M, 12 mo (n=2)	Slight fever 39, 4° C and initial respiratory signs	IFA PCR	Pos. (n=1) ST7F/98
9/9/98	Stutterheim (EC)	*BH (n=4); S (n=4)	F, 24 mo (n=4)	Neg on Ab ELISA	IFA ELISA	Neg
10/9/98	Bethlehem (F)	*S (n=9)	F (7) M (2), 8 mo (n=9)	Fever 41ºC, nasal discharge and dyspnoea	IFA PCR	Pos (n=1) ST8F1/98
15/09/98	Swartruggens (NW)	Lu, Sp, *Ln (n=3)	M, 12 mo (n=1)	Pneumonia	IFA PCR	Pos (n=1) ST11NW/98
18/9/98	Ventersdorp (NW)	S (n=2) *BH (n=2)	M , F 12 mo (n=2)	Pyrexia 41ºC reluctant to eat , lacrymation and nasal discharge	IFA ELISA PCR	Neg
21/9/98	Parys (F)	BEDTA (n=3) BH (n=3) *S (n=3)	F (n=3), 12 mo	Fever 40ºC, cough and respiratory distress	IFA PCR	Pos (n=1) ST5F/98
22/9/98	Potchefstroom (NW)	* Sp, K (n=2)	M, 7 mo (n=1)	Pyrexia 42ºC, dyspnoea and acute death	IFA PCR	ST9NW/98
23/9/98	Warmbad (N)	Sp, Lu, *Ln (n=3)	M (n=1), 24 mo	Erosive stomatitis , petechial haemorrhages and ulcers in the digestive tract	IFA	Neg
8/10/98	Onderstepoort (G)	*Lu (n=1)	Foetus (n=1)	Abortion	IFA PCR	Doubtful Weak band
9/10/98	Qweenstown (EC)	K, *Sp, Lu (n=3)	Calf (n=1)	Calf weak and died	IFA	Neg

		*BH (=3)	Heifers (n=3), 18 mo	Heifers for screen purpose	IFA ELISA	Neg Neg
19/10/98	Vryburg (NW)	Lu, *Ln, Sp (n=3)	F (n=1), 9 mo	Pneumonia	IFA PCR	Pos (n=1) ST12NW/98
20/10	Stutterheim (EC)	*S (n=9-2): 2 broken tubes	F, 24 mo(n=7)	Antibody ELISA neg	IFA	Neg (n=7) NT (n=2)
28/10/98	Ventersdorp (NW)	*Ln, L (n=2)	F, 24 mo (n=1)	Pneumonia	IFA PCR	Pos (n=1) ST13NW/98
30/10/98	Molteno (EC)	BH (n=1) *S (n=1)	M, 30 mo (n=1)	Respiratory distress	IFA	Neg
3/11/98	Dordrecht (EC)	BH (n=1)	F, 24 mo (n=1)	Totally haemolysed, not processed	NT	-
3/11/98	Queenstown (EC)	BH (n=1) S (n=1)	F, 30 (n=1)	Advanced autolysis, not processed.	NT	-
5/11/98	Rustenburg (NW)	*S (n=2) BH (n=2)	F, 12 mo (n=2)	Pyrexia 40ºC and cough	IFA PCR	Pos (n=1) ST10NW/98
12/1/99	(G)	*Cell's suspension		Check up	IFA ELISA PCR	Pos (n=1) ST24G/99
29/1/99	Dundee (KZN)	S (n=2) BH (n=2)	F, 24 mo (n=2)	Advanced autolysis, not processed	IFA	Neg
30/1/99	Molteno (EC)	BH totally haemolysed (n=1)	F, 28 mo (n=1)	Advanced autolysis, not processed	NT	-
5/2/99	Dundee (KZN)	*S, Ln	F, 8 mo (n=1)	Acute diarrhoea and death	IFA	Neg
9/2/99	Dundee (KZN)	BH (n=1) *S (n=1)	M, 26 mo (n=1)	Chronic soft diarrhoea	IFA	Neg
9/2/99	Dundee (KZN)	*BH (n=5)	F (n=3) M (n=2) (n= 5), 9 mo	Pyrexia 40ºC, cough, nasal discharge	IFA	Neg
26/2/99	Cascades (WC)	*Sp, Ln	F, 12 mo (n=1)	Diarrhoea followed by death	IFA PCR	Doubtful Weak band
15/3/99	Bisho	*S (n=3)	F, 30 mo	Fever 39, 9 ºC	IFA	Neg

	(EC)	BH (n=3)	(n=3)		PCR	
17/3/99	Alberton (G)	*Sp, Ln (n=2)	F, 8 mo (1)	Diarrhoea and death	IFA PCR	Pos (n=1) ST17G/99
26/3/99	Dordrecht (EC)	*BH (n=3)	F, 24 mo (n=3)	Fever, 40, 2ºC	IFA PCR	Doubtful Weak band
26/3/99	Dundee (KZN)	*S (n=1) BH (n=1)	M, 24 mo (n=1)	Acute fever, 41ºC	IFA	Neg
29/3/99	Onderstepoort (G)	Lu (n=2), *Sp (n=2), Acqueous humour (n=1)	M, few days (n=2)	Microphtalmia	IFA PCR	Doubtful Weak band
30/04/99	Grahamstown (EC)	*Ln, Lu (n=2)	M, 12 mo (n=1)	Pneumonia	IFA	Neg
30/4/99	Dordrecht (EC)	BH (Tube broken during centrifugation) (n=1)	M, 12 mo (n=1)	Not processed	NT	-
16/5/99	Winburg (F)	*Ln	F, 7 mo (n=1)	Death after severe melena and anaemia	IFA	Neg
22/5/99	Middelburg (EC)	*B H (n=6)	F, 18 mo (n=8)	Cough (n=6)	IFA ELISA PCR (n=1)	Neg
		*Lu (n=2)		Pneumonia (n=2)		
26/5/99	Queenstown (EC)	Ln, Lu, Sp, K, L (n=5)	M, 7 mo (n=1)	Advanced autolysis and no proper submission form, not processed	NT	-
1/6/99	Heidelberg (WC)	*BH (n=10)	F, 24 mo (n=10)	Screening test	IFA ELISA	Neg
3/6/99	Brandfort (F)	Lu, L, *Sp (n=3)	M, 24 mo (n=1)	Snotty nose, cough and lacrymation	IFA PCR	Doubtful Weak band
4/6/99	Molteno (EC)	*BH (n=2)	M, 30 mo (n=2)	For screening purpose	IFA ELISA	Neg
17/6/99	Burgersdorp (EC)	*S (n=4)	F, 24 mo (n=4)	Cough and nasal discharge	IFA	Neg
20/7/99	Onderstepoort	*BH (n=1)	F, 6 mo	Pyrexia 40 ºC and	IFA	Neg

	(G)		(n=1)	anorexia	ELISA	
20/7/99	Dundee (KZN)	Sp,Lu,K (n=3)	F, 7 mo (n=1)	Advanced autolysis, no proper submission form, processed: Bacterial proliferation in TC	NT	-
21/7/99	(G)	Cell culture suspension (n=1)		Check up	IFA ELISA PCR	Pos (n=1) ST25G/99
21/7/99	Pietermaritzburg (KZN)	*Ln, Lu (n=2)	M, 18 mo (n=1)	Pyrexia 40ºC, nasal discharge	IFA PCR	Neg
27/7/99	Middelburg (EC)	BH (n=1) *S (n=1)	M, 24 mo (n=1)	Cough	IFA	Neg
10/08/99	Winburg (F)	*BH (n=1)	M, 24 mo (n=1)	Respiratory distress	IFA ELISA	Neg
16/8/99	Bothaville (F)	BH (n=6) *S (n=6)	M, 12 mo (n=6)	Fever 40 ºC Nasal discharge, lacrymation	IFA	Neg
18/8/99	Parys (F)	BEDTA (n=20) *S (n=20)	F,18 mo; M, 24 mo M (2) (n=20)	Herds screened by antibody ELISA	IFA	Neg
18/8/99	Qweenstown (EC)	Br (n=1) *Sp (n=1)	F, 12 mo (n=1)	Nervous signs, diarrhoea, emaciation and death	IFA PCR	Doubtful Weak band
20/8/99	Qweenstown (EC)	Sp (n=1); *Ln (n=1); BH+*Ln (n=1)	F, 12 mo (n=3)	Found dead (n=2) Anorexia and died (n=1)	IFA	Neg
24/8/99	Magaliesburg (G)	*BH (n=3)	F, 7 mo (n=3)	Animals with diarrhoea	IFA ELISA	Neg
27/8/99	Steynsburg (EC)	*BH (n=22)	F(n=20), M (n=2), 24 mo (n=22)	Fever 39, 9ºC- 40, 2ºC	IFA ELISA	Neg
27/8/99	Aberdeen (EC)	*BH (n=1)	M, 24 mo (n=1)	Fever 41ºC	IFA ELISA	Neg
30/8/99	Parys (F)	*S (n=18)	F, 18 mo (n=18)	Neg antibody ELISA	IFA	Neg
30/8/99	Parys	*S (n=8)	F, 24 mo	Diarrhoea	IFA	Neg

	(F)		(n=8)			
2/9/99	Queenstown (EC)	*S (n=11)	F, 30 mo (n=11)	Screening test	IFA	Neg
10/9/99	Onderstepoort (G)	*BH (n=1)	F, 30 mo (n=1)	Abortion	IFA	Neg
16/9/99	Qweenstown (EC)	Pl, Ln, Lu, Sp (n=4)	F, 30 mo; + M calf, 7 mo (n=1)	Abortion, advanced autolysis, no complete history; processed: Bacterial proliferation in TC	NT	-
23/9/99	Queenstown (EC)	*S (n=1)	F, 24 mo (n=1)	Herd with history of abortion	IFA	Neg
8/10/99	Queenstown (EC)	*BH (n=7-4)	F, 24 mo (n=3)	(n=4) not processed. Animals with fever 40, 5ºC (n=3)	NT IFA ELISA	- Neg
26/10/99	Queenstown (EC)	BH (n=9) *S (n=9)	F, 30 mo (n=9)	No history	IFA	Neg
27/10/99	Dundee (KZN)	Br, Sp, *Ln (n=3)	M, 8mo (n=1)	Pneumonia and septicaemia (general congestion)	IFA	Neg
29/10/99	Onderstepoort (G)	S (n=3), BH (n=3), *Ln (n=3)	M, 7 days (n=3)	Weak born calves and died few days later	IFA	Neg
27/11/99	Louis Trichardt (N)	*BH (n=1)	M, 24 mo (n=1)	Respiratory	IFA	Neg

Ab: Antibody; Ag: Antigen; BH: Blood in heparinized tubes; BEDTA: Blood in EDTA tubes; Br: Brain; EC: Eastern Cape; F: Female; F: Free State; G: Gauteng; Lu: Lung; Ln: Lymph nodes; S: Serum; Sp: Spleen; TC: Tissue culture; M: Male; Mo: Month; MPG: Mpumalanga; N: Northern; NW: North West; Neg: Negative; NT: Not tested; Pl: Placenta; Pos: Positive; K: Kidney; KZN: Kwazulu Natal; WC: Western Cape and *: Single selected specimen which was processed.