Bovine Viral Diarrhoea Virus in New Zealand Dairy Cattle: A review of diagnostic methodologies and preliminary data on circulating genotypes

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Abstract

Bovine viral diarrhoea (BVD) is a well-established disease in New Zealand beef and dairy cattle that causes significant economic losses for the industries each year. The pathogenesis of the disease is well understood and there is a wide range of reliable diagnostic tools that have allowed many European countries to successfully eradicate BVD through coordinated national disease control programmes. However, it is difficult to directly apply many of these frameworks in New Zealand due to the unique characteries of the pastoral farming systems that create different logistical and epidemiological challenges. There is a strong need to identify the most cost-effective means of applying existing diagnostic tests to design a feasible national control programme for New Zealand. To support this goal, this thesis has focused on filling two existing knowledge gaps: one around the performance of BVD diagnostics tests in herd and industry level disease control programmes and the other around using molecular diagnostic test methods to characterise the circulating bovine viral diarrhoea virus (BVDV) strains in the cattle populations.

The last formal literature review on BVD diagnostic tests was published more than 15 years ago and there have been significant advances in the availability and performance of the tests since then. In **Chapter 2**, a non-systematic review was conducted on the evolution of BVD diagnostic tests over the past 60 years and how they have been applied to different herd and industry level BVD control programmes. The review includes an in-depth overview of key feature in the pathogenesis of BVD that impact test performance followed by detailed descriptions of the different diagnostic methodologies, their performance and their current applications. The discussion section highlights the remaining limitations and technical gaps in the current BVD diagnostic tests along with suggestions for future research directions.

In particular, molecular epidemiology has been successfully applied in some countries as a part of their BVD control to understand suspected transmission routes but has not yet been applied in New Zealand. As a preliminary investigation, **Chapter 3** was designed to understand current circulating BVDV strains in dairy cattle across New Zealand using a convenience sample virus-positive serum samples that were submitted to commercial diagnostic laboratories during the study time period. Both the 5'UTR and N^{Pro} genes were sequenced from each sample to identify the strain type and phylogenetic analyses were performed to explore the genetic relatedness of each sequence. Although BVDV 1-A was found as the only subtype circulating among dairy cattle, there was a high variation among the sequences within group 1-A. The

phylogenetic analysis showed a fair homogeny of New Zealand dairy isolates with overseas isolates, particularly with BVDV strains previously identified in China. There was also 100% homogeny between the New Zealand dairy isolates and those from cattle that were recently sequenced for another study, which is most likely from the sales of dairy calves into the beef industry for fattening. Several farms were also found to have multiple different phylogenetically distinct BVDV strains, which suggests that they may have been infected from multiple different sources. Molecular sequencing may provide a valuable tool for helping farmers understand the origins of BVDV outbreaks. However, there were some limitations with the sampling and detection methods used. Future research directions were proposed in the discussion including broadening the study into a national survey with adequate representative samples.

Finally, **Chapter 4** provides a brief discussion of how the findings from this work can be used to assist New Zealand in designing a more cost-effective national BVD control programme.

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Abbreviations

BVD Bovine viral diarrhoea
BVDV Bovine viral diarrhoea virus
OIE World Organization for Animal Health
CSFV Classical swine fever virus
BDV Border disease virus
TI Transient infection
PI Persistent infection
MD Mucosal disease
UTR Untranslated region
NAIT National Animal Identification and Tracking system
Ag Antigen
Ab Antibody
ELISA Enzyme linked immunosorbent assay
ORF Open reading frame
VI Virus isolation
PBS Phosphate-buffered saline
DFA Direct fluorescent antibody assay
ACE Antigen capture ELISA
RT-PCR Reverse transcriptase polymerase chain reaction
FAT Fluorescent antibody test
mAb Monoclonal antibodies
VN Virus neutralization
SN Serum neutralization
IPMA Immunoperoxidase monoclonal antibody test
IHC Immunohistochemistry
cDNA Complementary DNA
qRT-PCR Quantitative Reverse Transcriptase Polymerase chain reaction
S/P Serum to sample ratio
BTM Bulk tank milk

1. Introduction

1.1 Overview of bovine viral diarrhoea

Bovine viral diarrhoea (BVD) is a highly contagious disease of cattle that can also affect sheep, goats, deer, camelids, and swine (Abu Elzein & AlKhalyifa, 2012). The disease causes severe economic loses to the dairy and beef industries worldwide and is listed as notifiable by the OIE (World Organization for Animal Health) under cattle diseases and infections (OIE, 2021). Depending on the pathogenic properties of the virus, the virulence of the virus is highly variable with possible outcomes including respiratory, enteric or lethal hemorrhagic conditions accompanied by transient immunosuppression (Baker, 1995). However, the most significant economic impacts occur when pregnant animals get infected and the virus crosses the placenta to attack the fetus. Depending on the time of infection, the outcome could be early embryonic death, abortions, birth defects, stillbirths, or the birth of persistently infected (PI) calves (Volker, Hans, & Ann, 2005). Persistently infected animals are immunotolerant to the virus and have been identified as the main source of transmission because they continuously shed large amounts of virus to the environment throughout their lifespan (Houe, 1999a). Therefore, the fundamental principle of any BVD control or eradication program is to reduce the prevalence of existing PI animals and prevent the creation of new PI animals.

1.2 Genetic diversity of bovine viral diarrhoea virus

Bovine viral diarrhoea virus (BVDV) is a member of family *Flaviviridae*. This family comprises a large group of small (40-50nm) enveloped single–stranded, non-segmented positive sense RNA viruses (Neufeldt, Cortese, Acosta, & Bartenschlager, 2018). The 9-13kb genome of the family *Flaviviridae* contains a single, long ORF flanked by 5'- and 3'-terminal non-coding regions, that forms specific secondary structures required for genome replication and translation (Simmonds, Becher, Bukh, Gould, Meyers, Monath, Muerhoff, Pletnev, Rico-Hesse, Smith, & Stapleton, 2017). These regions encode for approximately 12 structural and non-structural proteins including N^{pro}, C, E^{rns},E1, E2, P7, NS2/3, NS4A, NS4B, and NS5A (Richard E. Booth, Thomas, El-Attar, Gunn, & Brownlie, 2013a).

There are four genera of family *Flaviviridae*: *Flavivirus, Hepacivirus, Pegivirus* and *Pestivirus* and BVDV belongs to genus *Pestivirus*. The genus *pestivirus* comprises many species including viral species such as Bovine Viral Diarrhoea viruses, Border disease virus (BDV), and Classical swine fever virus (CSFV) which are known to be economically more important viruses among the others in the genus. Bovine viral diarrhoea is caused by one on three Pestivirus species: bovine viral diarrhoea virus 1 (BVDV-1/*Pestivirus* A), bovine viral diarrhoea virus 2 (BVDV-2/ *Pestivirus B*) and HoBi-like virus (BVDV-3/*Pestivirus H*/ bovine atypical pestivirus) (Evans et al., 2019).

In cell culture, all pestiviruses show two different biotypes, named cytopathic (cp) and non-cytopathic (non-cp) (Lanyon, Hill, Reichel, & Brownlie, 2014; Tautz, Tews, & Meyers, 2015), where the non-cp strains are commonly found in the field. Non-cytopathic viruses are associated with the majority of BVDV infections (>90%) and cause mild to severe transient infection (TI) as well as persistent infection. Non-cp BVDV is the only biotype that has been observed clinically or experimentally to cause BVDV persistent infection (Sasha R. Lanyon, Fraser I. Hill, et al., 2014; Zhang et al., 2014). CP biotypes cause severe acute and per-acute transient disease as well as mucosal disease (MD) in superinfected PI animals (Khodakaram-Tafti & Farjanikish, 2017; Walz et al., 2010).

Variations among BVDV strains can be evaluated by different methods, including monoclonal antibody reactions, cross-neutralization tests and molecular analysis. The genetic analysis of BVDV has further classified the virus into sub genotypes and identified as BVDV-1 has 21 subtypes (1a-1u), BVDV-2 has three subtypes and BVDV-3 has four subtypes (Yesilbag, Alpay, & Becher, 2017). Partial 5'UTR sequences have been most frequently used for phylogenetic analyses and genotyping of BVDV isolates, followed by N^{pro} and E2 coding sequences. BVDV phylogenies using the 5'UTR alone has shown some limitations for the phylogenetic analyses due to restricted sequence length and lack of diversity (Becher et al., 1997; Xia, Liu, Wahlberg, Baule, & Belák, 2007; Yesilbag et al., 2017). The lack of information does not allow to clearly infer relationships within the major clades, and some branches of the phylogenetic tree. To overcome the above issues, analysis of longer sequences such as complete N^{pro} and E2 coding regions are also recommended along with 5'UTR (Becher et al., 1997; Adam Chernick, Godson, & van der Meer, 2014).

Epidemiological studies have shown that various BVDV subgenotypes predominate in different countries and that has been shown to be invariably dependent on the geographical

location. For example, in Australia, Mexico and the UK, BVDV Type 1C sub-genotype is prevalent (Richard E. Booth et al., 2013a; Gómez-Romero, Basurto-Alcántara, Verdugo-Rodríguez, Bauermann, & Ridpath, 2017; Lanyon & Reichel, 2014; Vilcek, Durkovic, Kolesarova, & Paton, 2005), in Austria, Type 1F, in Germany, Type 1B and Type 1D, in Italy Type 1B and Type 1E, in the USA and Canada, Type 1A and Type 1B, in India Type 1B (Vilcek et al., 2005), and in Switzerland, Type 1H is the most predominant sub-genotype (Stalder et al., 2018).

1.3 Global status of BVDV

BVDV has been detected in 88 countries worldwide (Richter et al., 2019) and it is currently endemic in most cattle producing countries. The direct losses due to BVD has been estimated between NZ$3.45 to NZ$988.04 per animal (Han J-H, 2018; Richter et al., 2017) with the total loss due to the disease predicted to be much higher when considering indirect expenses such as diagnostic testing, vaccination, and the costs of implementing other BVD biosecurity measures. Considering these epidemiological and economical features of BVDV infections, many countries have implemented compulsory or voluntary programs aimed at controlling or eradicating BVD. Several Western European countries including Norway, Sweden, Finland and Denmark have already achieved BVD free status. Recent analysis revealed evidence that the global prevalence of the disease has decreased over time after increasing numbers of countries have successfully implemented control strategies (Scharnböck et al., 2018). However, the high BVD prevalence still remains in countries that have failed to implement any BVD control programmes so far. This includes New Zealand where there have been no coordinated national efforts to eradicate the virus since it was first identified in the 1960s.

1.4 Bovine viral diarrhoea in New Zealand

1.4.1 Prevalence of BVD

The first BVD case in New Zealand was reported in 1960s in a herd of cattle (Salisbury et al., 1961) and additional cases were subsequently found on different farms confirming the active circulation of the virus within the country (Fastier & Hansen, 1966). Since then, the virus has become endemic in both dairy and beef sectors causing more than NZ$150 million each year in direct production losses with an additional NZ$44.5 spent on disease control (C. Heuer,

Healy, & Zerbini, 2007). The latest statistics show more than 65% of dairy herds are conducting annual bulk milk testing through a single diagnostic laboratory (Gates, Han, Evans, Weston, & Heuer, 2019). At present, approximately 45 % dairy herds are classified as having high or very high antibody levels with approximately 5 to 10% of dairy herds believed to be currently actively infected. The prevalence of PCR positive bulk milk samples has dropped from 15% to 5% from 2010 to 2017, which suggests that voluntary control measures implemented by dairy farmers are having a significant impact in reducing disease prevalence in the industry (Gates, Evans, Heuer, Voges, & Weston, 2020).

Data on the epidemiology and economics of BVD in the New Zealand beef cattle industry is much more limited due to the extensive nature of beef production where cattle are often minimally handled and there are many challenges of conducting diagnostic tests (Gates, Evans, Han, Heuer, & Weston, 2020; Han J-H, 2018). However, previous small scale cross-sectional studies have reported around 65% of beef herds had evidence of previous exposure to BVD virus and approximately 50% of herds were actively infected (Cuttance & Cuttance, 2014; C Heuer, Tattersfield, West, & Olson, 2008). However, these statistics were based on limited number of potentially non-representative herds and, to our knowledge, follow-up studies have not been done. Many beef farmers do not perform annual screening tests because of the perceived costs of BVD control as well as the logistical difficulties in yarding extensively grazed beef calves when they are in the appropriate 10–18-month age range to conduct sampling. Previous analysis of National Diagnostic Laboratory Testing Data suggest that fewer than 5 to 10% of beef herds conduct serological screening with limited uptake of individual animal testing in herds with suspected active infections (Gates, Han, et al., 2019). These issues make it difficult to track national trends of disease prevalence in New Zealand beef cattle.

A study conducted in New Zealand cattle in 1998 grouped 20 isolates collected from 1967 to 1997 into the BVDV1 species (Vilček, Björklund, Belák, Horner, & Meers, 1998). Since then, there have only been two studies on the BVDV genotypes present in New Zealand. Both identified the presence of only the BVDV1 genotype, but identified the subtypes 1A and 1C (J. H. Han, Weir, Weston, Heuer, & Gates, 2018). BVDV-2 has not been detected in New Zealand (Ridpath, 2010).

1.4.2 Control strategies

Decades from New Zealand's first case of BVD, the national BVD steering committee was established in 2005 with the purpose of educating veterinarians and farmers about BVD to

improve the voluntary uptake of control measures (Gates, Evans, Weir, Heuer, & Weston, 2019). The committee membership includes veterinary technical experts from academia, clinical practice, and industry who review the science around BVD to make recommendations for controlling the disease in New Zealand and have been driving force behind many BVD research and extension efforts over the past 15 years. One of the main limitations identified by the BVD Steering Committee with controlling BVD in New Zealand was the lack of farmer awareness about the presence of disease in their herds. This BVD awareness has been slowly changing with the introduction of cost effective testing protocols such as bulk tank milk testing in dairy herds introduced in 2010 and the pooled serum antibody test introduced in 2011 which provided an equivalent inexpensive means of screening beef cattle herds for evidence of active viral transmission. With the introduction of the National Animal Identification and Tracing (NAIT) system in New Zealand in 2013, all the tools were now available to support the implementation of a national BVD control programme. Furthermore, there has been evidence that the dairy industry has also made significant voluntary progress in controlling BVD by reducing the prevalence on herds with positive bulk milk PCR tests, which suggests the potential for industry support of a national disease control programme (Gates, Evans, Heuer, et al., 2020). However, there were still uncertainties about which strategy would be the most cost-effective for New Zealand. In July 2017, a three-year BVD research project (www.bvdfree.org.nz) was launched to fill in the remaining knowledge gaps to evaluate the cost-effectiveness of BVD control programmes.

Limitations and Knowledge gaps of BVD control

Although many European countries that have successfully implemented programmes for BVD control, there are several unique features of the New Zealand pastoral farming systems such as its highly seasonal nature and extensive grazing practices resulting in infrequent handling of beef and non-dairy cattle that require consideration when determining which strategies are likely to be the most cost-effective (Han J-H, 2018). An important aspect of this will be developing robust screening programmes at both the herd and national levels based on current knowledge about the performance of BVD diagnostic tests as well as knowledge of the prevalence and distribution of actively circulating strains (J.-H. Han et al., 2018). The last formal literature review on BVD diagnostic tests was published in 2005 (Sandvik, 2005b) and there have since been many advances in the test methodologies that have implications for their use in BVD control programmes. Another critical knowledge gap in New Zealand is around the circulating BVD strains since the last study on BVDV genotypes of BVDV in New Zealand

was published in 1998 (Vilček et al., 1998) and there are likely to have been changes in the molecular epidemiology since that time.

1.5 Summary of objectives

The main aims of the thesis were to (1) provide an overview of diagnostic testing strategies of the world that could serve as a helpful a reference for making decisions about future BVD control programmes in New Zealand and (2) address the current deficits in the understanding of circulating BVDV subtypes in dairy farms across New Zealand as there have been few previous studies in this area.

With those objectives in mind, **Chapter 2** will cover the evolution and application of diagnostic testing strategies for BVDV in cattle as a non-systematic review. Molecular epidemiology has not been applied in national BVD control programmes in New Zealand so far and the main limitation could be recognized as lack of understanding of current circulating BVDV virus strains across the country. To address the knowledge gap, a preliminary phylogenetic analysis has been done across New Zealand dairy animals and has reported in **Chapter 3**. This chapter will cover an overview of circulating BVDV strains in dairy cattle in New Zealand and their relationships with sequences that have been isolated from beef cattle in New Zealand as well as other cattle populations around the world. The general discussion in **Chapter 4** will summarise the overarching findings of the thesis, including the limitations, and suggested directions for further research into BVDV.

2. The evolution and application of diagnostic tests for bovine viral diarrhoea virus in cattle: A non-systematic review

2.1 Abstract

Bovine viral diarrhoea (BVD) is an economically significant disease of cattle with a global distribution. Several European countries have already gained success in eradicating BVD by strategically applying diagnostic tests as well as other disease control measures through coordinated national control programmes. However, it is difficult to apply these frameworks to other countries where differences in the population demographics and regulatory policies create unique logistical challenges. Having up-to-date information on the methodologies and performance of the current diagnostic tests is important for informing decisions about. The last formal review of bovine viral diarrhoea virus (BVDV) diagnostic tests was performed more than 15 years ago and there have been many advances in the field since then. The purpose of this non-systematic review was to highlight evolution and application of diagnostic tests in BVDV in cattle. The first section reviews the epidemiology and pathogenesis of BVD highlighting important features relevant to diagnostic testing. Then chronological evolution of different BVD diagnostic tests starting with virus isolation in the 1940s and working through to the more advanced molecular methods in current use are presented along with an overview of the test methodology, test performance, and practical consideration. The third section then discusses how these tests have been applied in different individual-level, herd-level, and industry-level control programmes. Lastly, the discussion highlights key remaining technical gaps with the current BVD diagnostic tests to provide guidance on directions for future research.

2.2 Introduction

Bovine viral diarrhoea (BVD) is an economically important infectious disease that is widespread in cattle industries throughout the world causing significant impacts on animal health, welfare and production (Chang et al., 2021; Houe, 1999b; Pinior et al., 2017). The economic losses from BVD infections in cattle herds depend on the underlying level of herd

immunity, severity of the clinical signs, duration of infection, and interactions with other pathogens (Santman-Berends, Mars, van Duijn, & van Schaik, 2015). Recent studies have estimated that the production losses associated with BVD could be up to $687.80 US dollars (USD) per animal (Pinior et al., 2017). The disease is primarily spread and maintained in cattle populations by a small number of persistently infected (PI) animals that are created when the fetus gets infected during the mid-stages of pregnancy following an acute infection in the dam (Sasha R. Lanyon, Fraser I. Hill, et al., 2014). Since these PI animals shed large amounts of virus for life, finding and eliminating them from infected herds is key to getting BVD under control. This phenomenon has been highlighted through the successful BVD control programmes in the Scandinavian countries where there has been rigorous detection and elimination of PI animals (Laureyns, Ribbens, & de Kruif, 2010).

One of the key factors behind the success of BVD eradication programmes is the availability of good diagnostic tests that can accurately determine the exposure and infection status of individual animals and herds (Gates, Han, et al., 2019). Like many other infectious diseases, BVD does not have pathognomonic clinical signs and so diagnosis relies heavily on laboratory tests that either look for antibodies (Ab) against bovine viral diarrhoea virus (BVDV) as an indication of previous exposure or for viral genetic material or virus-induced antigens (Ag) as an indication of active infections (Sandvik, 1999). In some national eradication programmes such as the one currently implemented in Scotland, serological screening tests (Ab ELISA) are performed first to identify herds that are likely actively infected with BVDV and should be targeted for further investigation to identify PI animals (Metcalfe, 2019). Other countries such as Germany, Belgium, and Ireland simply test all individual animals after birth rather than using serological screening (Lindberg & Alenius, 1999). Since PI animals can only be created before birth, once an animal tests negative for BVDV, it can be certified as non-PI for life.

The success of any BVD control programme depends on the performance of tests at the both individual animal and herd level, which is in turn affected by choosing the right animals, samples, tests, and frequency of testing as well as making the correct epidemiological inferences about the test results. Although there have been previous review articles about BVDV diagnostic testing strategies (Houe, Lindberg, & Moennig, 2006; Jeremiah T. Saliki & Dubovi, 2004b), the last review was published 15 years ago and there have since been many advances in diagnostic test methodologies as well as better understanding of pathogenesis of BVD. In this review, we first summarise key epidemiological features of BVD relevant to diagnostic testing (Section 1). We then provide an overview of the key milestones in BVDV

diagnostic test development over the past eighty years and how these contributed to our understanding of BVD epidemiology and control (Section 2). We then discuss the performance and application of these tests at the animal, herd, and industry levels to support BVD control (Section 3) Finally, we discuss the remaining technical knowledge gaps that will improve understanding of BVD control (Discussion).

2.3 BVD epidemiology and pathogenesis

BVDV is a member of family *Flaviviridae* and genus *Pestivirus*. The pestiviruses comprise a positive sense, single stranded RNA genome of approximately 12.5 kb in length is flanked at either end by 5' and 3' untranslated regions (5'UTR, 3'UTR) (Meyers & Thiel, 1996; Tautz et al., 2015; Vilček et al., 2001). The 5'UTR contains both highly conserved and variable regions, which has allowed the development of both pan-pestivirus and species-specific molecular diagnostic assays (Ridpath & Bolin, 1998; Toplak, Sandvik, Barlic-Maganja, Grom, & Paton, 2005). The virus contains one long open reading frame (ORF) that is translated into a hypothetical polyprotein of approximately 4000 amino acids which is post-translationally cleaved by viral and cellular proteases to four structural (C, Erns, E1, E2) and eight non-structural proteins (Npro, P7, NS2, NS3, NS4A, NS4B, NS5A, NS4B) (Richard E. Booth, Thomas, El-Attar, Gunn, & Brownlie, 2013b). E2 is a highly-variable, immunologically-dominant glycoprotein in pestiviruses and the main target of neutralizing antibodies (Yesilbag et al., 2017).

Pestiviruses are highly variable both antigenically and genetically, thus each is further classified into distinct species and genera. BVDV-1 is the most widespread species, and 21 genotypes of BVDV-1 (BVDV-1a to BVDV-1u), three genotypes *of* BVDV-2, and four genotypes of HoBi-like pestivirus have been reported (Yesilbag et al., 2017). Depending on the physical presentation in cell cultures, there are two bio types: cytopathic (cp) and non-cytopathic (non-cp) besides cp biotypes induce apoptosis in cultured cells when non-cp biotypes do not cause cell lysis (Gamlen et al., 2010). It should be noted that in the recent updates of International Committee on Virus Taxonomy (ICVT), BVDV-1, BVDV-2 and HoBi-like pestivirus or BVDV-3 were re-named as Pestivirus A, B and H, respectively (Anonymous, 2020). However, to keep the terminology consistent with previous reports, the previous names were retained for use in this review.

The pathogenesis and epidemiology of BVD have several important features that are important for ensuring the tests are applied in a manner that will maximise performance. For susceptible animals, the most frequent route of natural infection is by oronasal uptake of virus from persistently infected (PI) animal. The exposure of naive immunocompetent cattle to non-cp BVDV strains results in transient infections (TI) where the virus is shed in most secretions from day 4 to day 10 post-infection with a measurable antibody response produced within 2 weeks of the initial infection (Sasha R. Lanyon, Fraser I. Hill, et al., 2014; A Meyling, Houe, & Jensen, 1990). The antibodies produced by the recovered animals provide a long term immunity against the particular BVDV strain (Gates, Evans, et al., 2019; Potgieter, 1995).

Transplacental infection is a common event in pregnant cattle exposed to BVDV. During the first 18 days of the pregnancy, the fetus is unattached and no embryonic infection can occur as BVDV does not penetrate the zona pellucida (Moennig & Liess, 1995). After the development of cotyledons (in between 29 to 41 days of post-conception), the virus can be transmitted from the infected dam to the fetus which may result in embryonic infection leading directly to embryonic death (Carlsson, Fredriksson, Alenius, & Kindahl, 1989). Infection of the dam during mid pregnancy between approximately 40 to 120 days of gestation can result in the birth of persistently infected (PI) calves (Brownlie, Hooper, Thompson, & Collins, 1998; Gates, Evans, et al., 2019). During this time, the fetus cannot develop sufficient innate or humoral immune response against the virus and becomes immunotolerant to the infecting BVDV strain (Khodakaram-Tafti & Farjanikish, 2017). After 125 days of the gestation, the fetus is generally considered immunocompetent (Baker, 1995) and fetal infection at this time results either clinically healthy or weak immunocompetent calf with or without congenital defects. In general, PIs shed large quantities of virus in their body secretions throughout their lifespan without developing antibodies while TI animals clear the virus within 2 to 3 weeks of the initial infection and develop antibodies against the infected virus strain that can persist and are detectable for long periods of time (Nettleton & Entrican, 1995).

Maternal antibodies play an important role in protection from BVDV infection in neonatal calves (Goyal & Ridpath, 2005). New-born calves get passive immunity through ingestion and absorption of maternal antibodies from colostrum of the dams that have recovered from natural infection or produced antibodies in response to vaccination. The duration that passively derived antibodies remain in the animal is dependent on the amount of antibodies ingested, intestinal absorption, and the subsequent rate of decay with time (Robert W. Fulton et al., 2004). One study reported that BVDV antibodies decayed to undetectable by 105–230 days of age

(Kendrick & Franti, 1974) while another study indicated that the mean time to for calves to become seronegative was 192 days of age (Robert W. Fulton et al., 2004). In general, the natural immunity from maternal antibodies remains until age of 6 months (Houe, 1994) but can sometimes last for up to 10 months (Gates, Evans, et al., 2019).

Vaccination is another way of giving passive immunity to protect the animals from transient or fetal infection. The overall goal of vaccination is to increase herd immunity so that the incidence of clinical disease and the birth of PI animals are reduced in the herd as a whole. Both modified live and inactivated vaccines are used worldwide and antibodies are detectable 2 to 3 weeks post vaccination and rises until levelling off at 10 to 12 weeks post vaccination (Griebel, 2015; Ridpath, 2013). Estimates of vaccine efficacy in preventing fetal infections varies from 50 to 100% in the literature with an average of approximately 80 to 90% (Goyal & Ridpath, 2005). While some studies suggest that active infection of BVDV by natural infections or vaccinations can prolong persistence of maternally derived antibodies (Robert W. Fulton et al., 2004; Muñoz-Zanzi, Thurmond, Johnson, & Hietala, 2002), others have highlighted that maternally derived immunity may block serum antibody responses against modified live virus vaccines or inactivated vaccines (J. Ellis, West, Cortese, Konoby, & Weigel, 2001; Robert W. Fulton et al., 2004). This is the underlying reason why the vaccine label recommends waiting until calves are over 3 to 4 months of age before giving the vaccine.

Similar to most other infectious diseases, the diagnostic tests for BVD can broadly be divided into two categories: (1) those that determine whether the animal is actively infected (virus-specific antigen, viral RNA or the virus itself) and (2) those that determine if the animal has previously been infected with the virus (virus-specific antibodies) (Jeremiah T. Saliki & Dubovi, 2004a). Table 2.1 shows the animal-level interpretation of positive and negative results for these diagnostic tests. It should be noted that vaccination and maternal antibodies can cause false positive reactions on tests looking for virus-specific antibodies.

Table 2.1 The epidemiological status of animals based on the results from antibody and virus testing regarding BVDV

	Virus positive	**Virus negative**
Antibody positive	• TI animals in the later stages of infection • PI animals with maternal antibodies	• Vaccinated animals • Calves age < 10 months • TI animals after 2weeks of initial infection
Antibody negative	• PI animals • TI animals in the early stages of infection	• Naive animals

2.4 History of BVD diagnostic test development

The diagnostic tests for BVD have evolved significantly over time since the disease was first recognised in the 1940s as highlighted in the timeline for Figure 2.1. In particular, there has been a marked shift from diagnostic techniques that were used mainly to confirm individual clinical cases towards those that allow easier monitoring of herd-level BVD status (Houe et al., 2006). In the remainder of this section, we provide a review of the different methodologies, their performance, and their contributions to advancing BVD control.

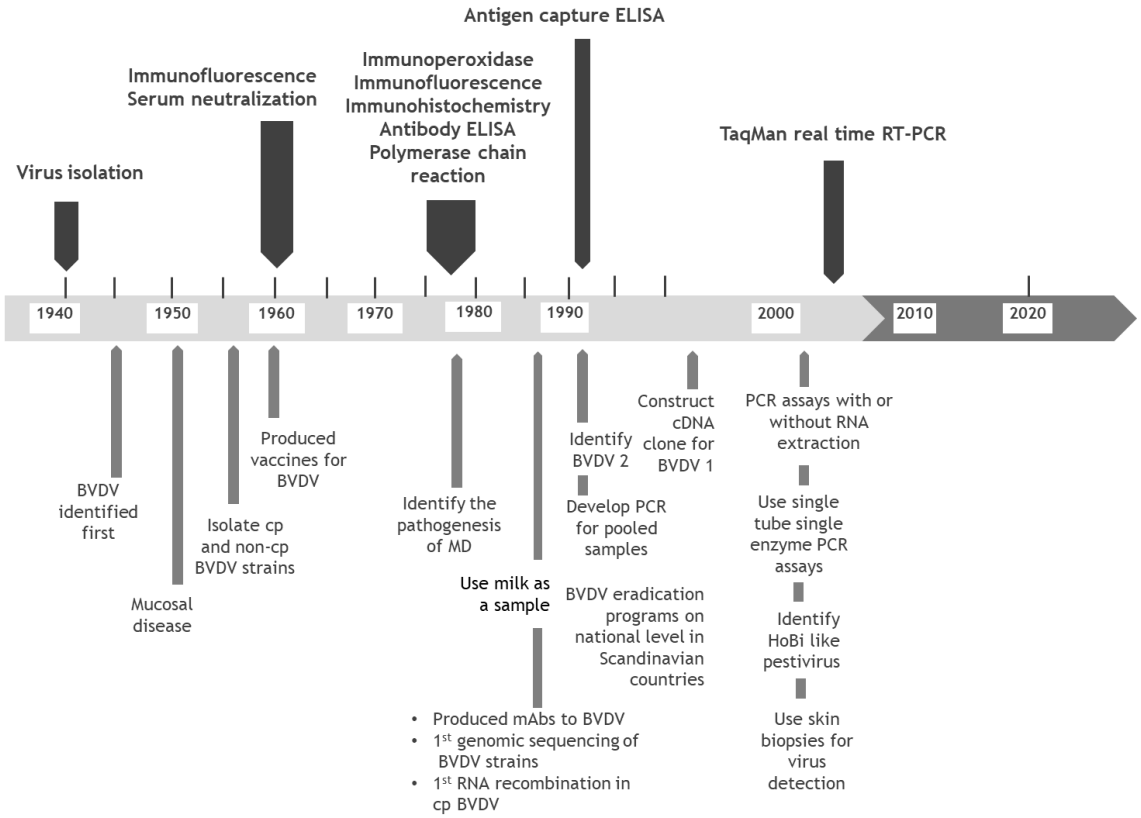

Figure 2.1 Evolution of diagnostic tests and milestones of BVDV

2.4.1 Virus Isolation

The first clinical case of BVD was recognised in 1946 and the virus was subsequently isolated in cell cultures to learn more about its properties. Virus isolation was considered the most reliable virus detection technique at the time and became the gold standard for BVDV diagnostic tests because of the high specificity (Jeremiah T. Saliki & Dubovi, 2004a). A wide range of biological samples can be used to isolate the virus including whole blood, nasal discharge, serum, buffy coat, spleen, lungs, fetus, and semen. The best samples for virus isolation from live animals is from mononuclear cells in the buffy coat obtained from whole blood while the best samples from necropsy are tissues from the lymphoid organs such as spleen, thymus, Payer's patches, and mesenteric lymph nodes (Brock, 1995; Jeremiah T. Saliki & Dubovi, 2004a). The principle of this testing method is to allow virus in the testing sample to grow in a cell culture following isolation and the identification of the virus. Many different cells of bovine origin that support the growth of this virus have been described elsewhere (Sandvik, 1999), but bovine turbinate (BT) cells are widely used for virus isolation as they are

more sensitive to BVDV-induced cytopathic effects that facilitate to differentiate cp from non-cp strains (Goyal & Ridpath, 2005).

In virus isolation, the cell culture plates are inoculated with the sample and are kept in 37^0C for 4 to 5 days for BVDV isolation. After incubation for 4 days, the cells are fixed with 20% acetone in phosphate-buffered saline (PBS). The cytopathic effect can be observed by phase contrast microscope if the sample contains cp biotype (Sandvik, 1999). However, as the majority of BVDV isolates are of the non-cp biotype and cell cultures should be further tested to detect presence of non-cp BVDV strains. This involves fixing and incubating with fluorochrome or enzyme labelled BVDV-specific antibodies to recognize the presence of non-cp BVDV (Brock, 1995). Isolated virus can be confirmed by either direct fluorescent antibody assay (DFA), immunoperoxidase, ACE or RT-PCR.

The most common use of virus isolation is currently as a gold standard to evaluate the sensitivity and specificity of other BVDV diagnostic tests (Belak & Ballagi-Pordany, 1991; S. R. Bolin & Ridpath, 1998; Drew, Yapp, & Paton, 1999; Hamel, Wasylyshen, & Nayar, 1995; R. Renshaw, R. Ray, & E. Dubovi, 2000; Shannon, Richards, Kirkland, & Moyle, 1991). Virus isolation is rarely used in surveillance programmes since the test has several limitations as a diagnostic test. Virus isolation requires specialised cell culturing facilities, is time and labour intensive and careful quality control is required to ensure that the cultured cells and fetal serum used as medium supplement are free of BVDV and its antibodies (Houe et al., 2006). Furthermore, virus isolation is not suitable for detecting young PI animals less than 3 months old because maternal antibodies against BVDV from colostrum can adhere to the virus particles making penetration of cell cultures impossible resulting in false negative test results that directly affects the test sensitivity as well (Dubovi, 2013; Sandvik, 1999; Zimmer, Van Maanen, De Goey, Brinkhof, & Wentink, 2004). However, there are some disagreements regarding considering VI as the gold standard for the virus detection because the test sensitivity is highly depend on the purity of the fetal serum used as a supplement for the cell culture and the sensitivity of a given cell culture system. Fetal calf serum used as a supplement of the cell culture medium should be free from both BVDV and BVDV specific antibodies and further the sensitivity of a given cell culture system will primarily depend on the volume of the inoculum and the period of incubation.

2.4.2 Immunofluorescence

As discussed above, one of the major limitations with viral isolation is VI technique cannot identify non-cp BVDV without a help of another diagnostic test. At the time of BVDV was firstly identified, the confirmatory diagnoses BVDV were originally depended upon transmissions of the disease by calf inoculations to detect and titrate the tissue culture-produced virus or plaque inhibition assays which the cytopathic effect of cp-BVDV was inhibited by non-cp virus as there were no other way of identifying non-cp BVDV (Albert L Fernelius, 1964). Immunofluorescence was firstly applied to healthy PI animals to demonstrate widespread distribution of viral antigen in brain and spinal cord neurons, renal glomeruli, renal tubules, lymph nodes, spleen, small intestine crypts, testicular tubules, and endothelial cells With fluorescence antibody test (FAT), the detection of non-cp BVDV from biological specimens was made much easier (Goyal & Ridpath, 2005).

Immunofluorescence has been used for the direct detection of infectious agents in tissue samples and the test is called direct fluorescent antibody test (DFA). In this procedure, cryostat sections of fresh tissues or smears of buffy coat cells are stained with fluorescein conjugated anti-BVDV antibody and then examined under a fluorescent microscope. The stained cells are examined under a fluorescent microscope to identify positive cells that are characterised by apple green fluorescence (Goyal & Ridpath, 2005). In later adaptations of the methodology, DFA was performed using BVDV specific monoclonal or polyclonal antibodies. However, monoclonal antibodies (mAb) were preferred since that could produce clear FA stain preventing nonspecific background staining. It is important that the mAbs used for the test are broadly reactive against all BVDV strains to prevent false negative results. There are only a few mAbs that have ability to broadly react against all BVDV strains (Jeremiah T. Saliki & Dubovi, 2004a).

The main applications of immunofluorescence techniques since they were first introduced in the 1960s have been use for the confirmation of non-cp BVDV strains from virus isolation and as another gold standard test to evaluate the sensitivity and the specificity of other BVDV diagnostic approaches (Coria, McClurkin, Cutlip, & Ritchie, 1975; A. Fernelius, 1969; Lopez, Osorio, & Donis, 1991; Magar, Minocha, Montpetit, Carman, & Lecomte, 1988; Maisonnave & Rossi, 1982; Roberts, Etchison, & Bond, 1988; Ruckerbauer, Girard, Bannister, & Boulanger, 1971). The test has been optimized and developed to apply for the pooled and individual field samples in late 1970s (Ayanwale, Fahrmann, Johnson, Anderson, & Kaneene,

1979). Initially indirect immunofluorescence has been used for the detection of anti-BVDV antibodies however, ELISA and VN tests appear to have replaced the indirect FAT afterwards considering the cost, time and experienced staff (A. L. Fernelius & Packer, 1969) . The main limitations of DFA are that is that it is agent specific and can only be performed on fresh samples (Dubovi, 2013).

2.4.3 Serum Neutralization

Virus neutralisation (VN) also known as serum neutralisation (SN) is used as the gold standard test for the detection of anti-BVDV antibodies. The first cp-BVDV was isolated in 1960 and that allowed the development of serum neutralization and plaque neutralization assays and this allowed characterization of the antigenic differences of BVDV (Goyal & Ridpath, 2005). The standard test procedure for VN is described extensively elsewhere (Rossi & Kiesel, 1971). The basic steps of VN involve performing two-fold serial dilutions of serum samples that are then incubated with a known amount of the virus followed by the addition of indicator cells. The test is read after 4-5 days of incubation and the highest dilution of the serum that inhibits virally induced cytopathic effects in approximately 50% of inoculated cells is considered to be the antibody titre of the serum (Goyal & Ridpath, 2005). A schematic illustration of the test procedure is shown in figure 2.2. The infectivity for the cp strains is detected by using an inverted light microscope and the infectivity for the non-cp strains is detected by IPMA (R W Fulton et al., 1997).

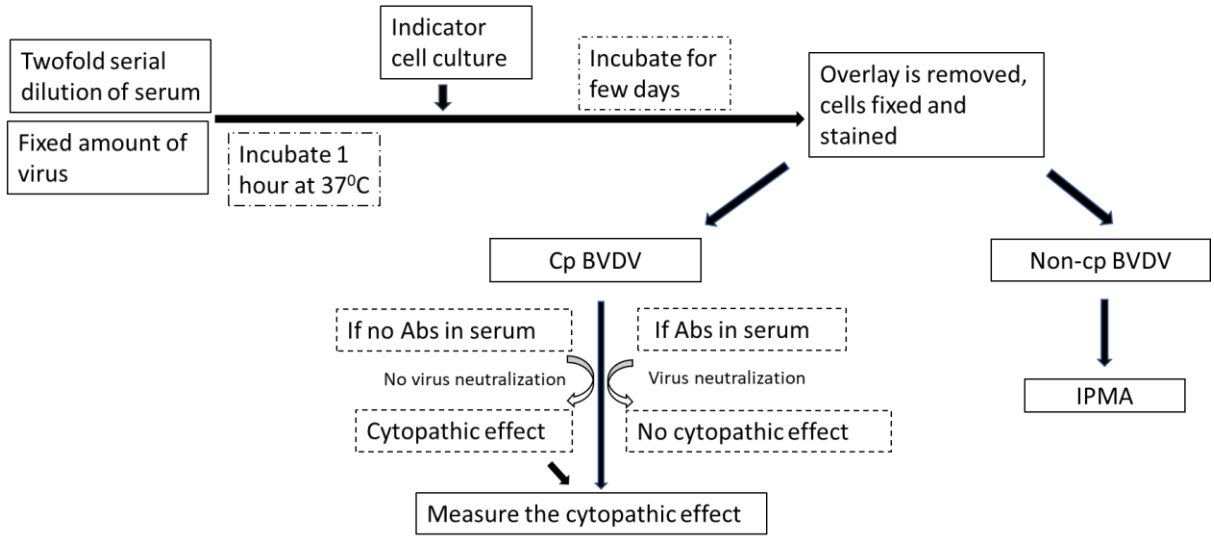

Figure 2.2: Diagrammatic representation of VN testing procedure

VN tests are species independent and cross-neutralization tests can be used to characterize antigenic differences among pestiviruses (Dekker, Wensvoort, & Terpstra, 1995). The causative agent for mucosal disease was first recognized as BVDV in early 1960s using virus neutralizing antibodies (Gillespie, Coggins, Thompson, & Baker, 1961; Thomson & Savan, 1963). The primary use of VN is to determine types of BVDV and to differentiate the antigenic diversity among BVDV sub types (R W Fulton et al., 1997) as it is the only test that can assess antibody status with regard to strain variations of BVDV isolates. A panel of monoclonal antibodies (mAb) were produced for BVDV using structural and non-structural viral genome proteins in early 1990s (S. Edwards, Moennig, & Wensvoort, 1991). Using SN tests and the difference in cross reactivity of mAbs to the different viral proteins, a considerable antigenic diversity among BVDV isolates was demonstrated (Corapi et al., 1990). For example, the cross reactivity of mAbs to the NS3 (a highly conserved non-structural protein of BVDV) was observed with all the pestiviruses but the envelope proteins E2 and E^{rns} appeared to be more specific for the viral species (S Edwards, Sands, & Harkness, 1988)

One of the most common uses for virus neutralization is to determine vaccine efficiency against different circulating BVDV strains. For example, cross-neutralization assays were performed in 1997 to test the cross protection of US vaccines against circulating virus strains in Germany. These studies revealed no significant differences between American and German strains thus indicating that protective vaccines may contain the same strains in these countries (Wolfmeyer et al., 1997). SN tests have also been used to differentiate the antibody titres that were produced by active infection or from vaccination because active BVDV infection by demonstrating a fourfold rise in antibody titres using paired serum samples (Goyal & Ridpath, 2005). The SN tests give endpoint values which may be more biologically relevant than ELISA and the SN test is the only one that can assess antibody status regarding the strain variations that exist among BVDV isolates.

One of the main limitations with VN tests as currently deployed is the significant variation that exists in test results between different diagnostic laboratories, which occurs because laboratories often use locally developed cell systems and different strains of virus without set standards for performing the test. For this reason, it is important that tests conducted on acute and convalescent sera are conducted at the same diagnostic laboratory to ensure that any changes in absolute antibody titres between time points are not simply due to differences in laboratory methodology (Dubovi, 2013). An additional limitation is that VN tests are labour intensive requiring experienced staff and well equipped laboratories to perform the test

(Sandvik, 2005a), which makes it less cost-effective when laboratories are processing only few or sporadic sample submissions (Sandvik, 1999).

2.4.4 Immunoperoxidase

The next advancement in BVDV diagnostic tests came through the introduction of the immunoperoxidase monolayer assay (IPMA) in the 1980s (A. Meyling, 1984) that gave diagnostic laboratories the ability to test many serum samples for BVDV at the same time. The technique was developed for the detection of BVDV in tissues of infected animals. Initially, anti-BVD-virus serum for the IPMA was developed by inoculating laboratory animals. Monoclonal antibodies (mAb) against BVDV were developed in 1985 and thereafter BVDV specific mAbs were used as anti-BVDV-antibodies (Peters, Greiser-Wilke, Moennig, & Liess, 1986; G. H. Smith, Collins, Carman, & Minocha, 1988; Van Zaane, 1984). Monoclonal antibody based IPMA has been compared with polyclonal antibody IPMA and the relative sensitivity and specificity of monoclonal antibody based IPMA have been estimated at close to 100% (Deregt & Prins, 1998). Monoclonal antibodies have been chosen because of their broad reactivity, antigenic avidity to different BVDV proteins and lack of competition for binding sites or binding to unusual BVDV isolates (Deregt & Prins, 1998).

For immunoperoxidase assays, the cell cultures are inoculated and incubated with the BVDV suspected sample and then the anti-BVDV-antibodies or BVDV specific monoclonal antibodies are added for antigen antibody reaction. Then peroxidase -labelled conjugate is used as an immunoperoxidase test to confirm the presence of virus after which the stained cells are examined under light microscope. A developing red colour indicated a positive reaction for infectivity (R W Fulton et al., 1997; Goyal & Ridpath, 2005). Immunoperoxidase tests have some advantages over DFA because Immunoperoxidase can be performed on a large number of samples at once because 96 well assay plates can be used for the test and as the results are read by eye, the assay does not require a fluorescent microscope (Brock, 1995). The immunoperoxidase test is very accurate and reliable for detecting PI animals that are shedding the virus in concentrations that are up to 100 times greater than the viral loads in acutely infected animals. Although this method is not considered to be sensitive enough for diagnosis of acute BVDV infections, this is not a major limitation for control programmes where the focus is on detecting PI animals rather than TI animals (Sandvik, 2005a).

The primary uses of IPMAs have been (1) for determining the virus titre in cell cultures initially, (2) in conjunction with virus isolation (VI) to detect non-cp BVDV (G. H. Smith et

al., 1988), (3) for detection of BVDV contamination in either cell cultures or virus stocks have been previously reported (Castro, Stoffregen, Brigman, & Hillard, 1997), and (4) certification of BVDV free bovine semen for artificial insemination and trading purposes (Afshar, Dulac, Dubuc, & Howard, 1991). The assay has also been used to detect non-cp strains in their study populations (R W Fulton et al., 1997).

2.4.5 Immunohistochemistry

Immunohistochemistry (IHC) is another method used for direct Ag detection and used for formalin fixed tissue samples. The first IHC was developed in the early 1980s using paraffin wax embedded tissue sections biopsied from calves with mucosal disease (Ohmann, 1983). The main advantage of IHC is that this technique can be performed on many different tissue samples collected through necropsy such as thyroid gland, skin, oral mucosa, oesophagus and abomasum samples (Goyal & Ridpath, 2005). The methodology for IHC has been described extensively elsewhere (Wilhelmsen, Bolin, Ridpath, Cheville, & Kluge, 1990). Briefly, the basic steps involve fixing the tissue samples in paraffin wax, mounting a thin slice of paraffin embedded tissue on a slide, and deparaffinizing the sample. Next a specific antibody is applied to the sections to bind to the target epitope prior to chromogenic staining. Peroxidase or alkaline phosphatase staining is done with biotinylated secondary enzyme linked antibody, a substrate is added at the end, and the slide is viewed under the microscope.

In the 1990s, skin biopsies were tested with IHC using panel of mAbs on cryostat sections of skin and this was proven to be a fast and reliable alternative to the conventional virologic methods for the diagnosis of BVDV infection (Thür, Zlinszky, & Ehrensperger, 1996). The ability to perform IHC on skin specimens from live animals resulted in its wider uptake for BVDV diagnosis. Similar to IPMA, however, it has been shown that while IHC staining for BVDV in formalin-fixed, paraffin-embedded skin is an effective method for the diagnosis of PI cattle, it has limited ability to detect transiently infected animals (Njaa, Clark, Janzen, Ellis, & Haines, 2000). IHC has been proposed as an alternative test to the rapid virus isolation method, which utilizes serum as the inoculum for cell cultures in microtiter plates (Brodersen, White, & Smith, 1998). The diagnostic sensitivity and specificity of IHC have been compared with other BVDV diagnostic tests and IHC has shown the best performance with high sensitivity (97%-100%) and specificity (97%-98.8%) (Cornish et al., 2005; J. A. Ellis, Martin, Robert, & Haines, 1995). In particular, one of the main advantages of using IHC on skin samples is that it can be used to screen neonatal calves for persistent BVDV infection since

maternal antibodies do not interfere with the test methodologies (Grooms & Keilen, 2002). However, IHC is a highly labour-intensive process and takes considerable time to get test results, which has limited its current use to support BVD management programmes.

2.4.6 Antibody ELISA

Enzyme link immunosorbent assay (ELISA) was developed in the 1980s as another methodology to identify animals with previous exposure to BVDV which provided some additional advantages over SN for detecting BVDV antibodies particularly with regards to time and cost (Howard, Clarke, & Brownlie, 1985). When the first Ab-ELISAs to be developed were compared with SN tests, there was 97% agreement between the two tests suggesting similar levels of performance (Bock, Burgess, & Douglas, 1986) . Two ELISA techniques were developed in 1987: an indirect ELISA and a competitive ELISA to detect antibodies against BVDV. This marked the first time that monoclonal antibodies were used for an ELISA to detect BVDV and the results showed 100% agreement with SN test results (Juntti, Larsson, & Fossum, 1987). Non-competitive and competitive/blocking ELISAs are currently the two main ELISA techniques that are used to detect Abs against BVDV (Schrijver & Kramps, 1998). The test procedures are described extensively elsewhere (Bhatia, Sood, Mishra, Pattnaik, & Pradhan, 2008; Howard et al., 1985) and a simple illustration of test procedures are shown in figure 2.3. In non-competitive ELISA, specific Abs in the test sample that have been bound to the immobilized Ag are detected by enzyme conjugated IgG-specific Abs. The Ag could be either a whole virus antigen, a non-structural protein, or a peptide. In this test, the amount of Abs bound to antigens is directly proportionate to the enzyme-mediated colour development. In the blocking ELISA, the antibodies present in the sample compete with agent-specific enzyme conjugated antibodies for binding to a specific amount of immobilized antigen. The degree to which the antibodies in the test sample prevent binding of an agent-specific enzyme-conjugated Ab is measured. The amount of Ab in the test sample that is bound to the Ag is inversely proportional to the intensity of the colour development (Schrijver & Kramps, 1998).

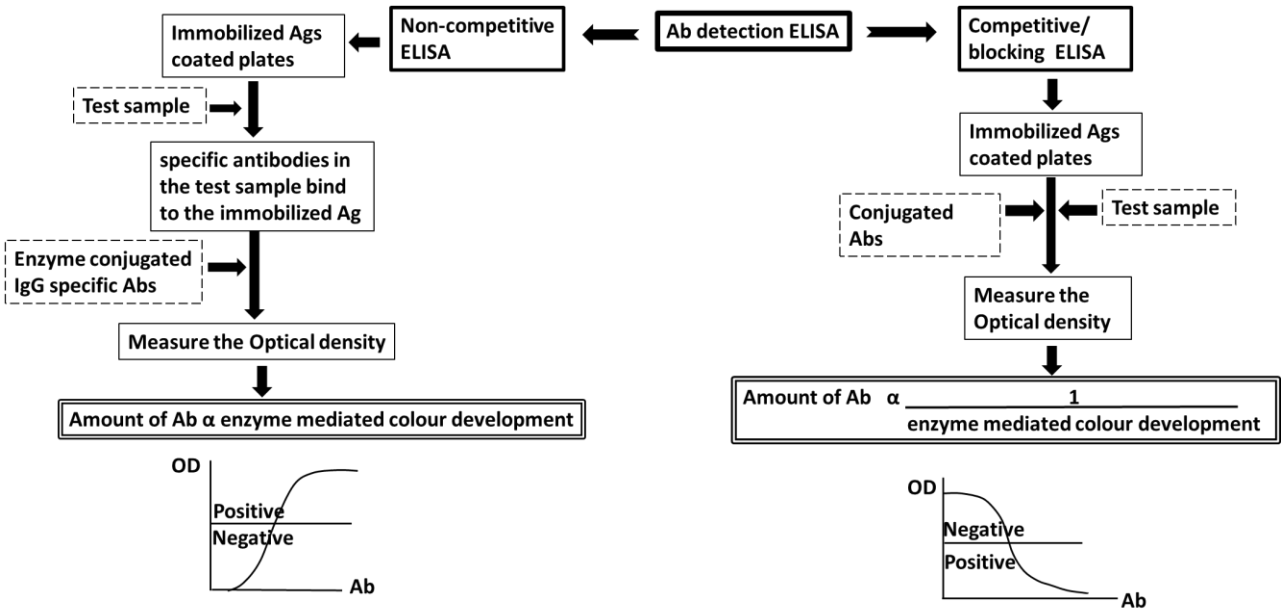

Figure 2.3 Illustration for Ab-ELISA procedure

The Ab-ELISA results can be influenced by the sample type, antigen used for the test, and the conjugated antibodies (Schrijver & Kramps, 1998). The procedure used to prepare the Ag can also affect the specificity and the sensitivity of the test (Goyal & Ridpath, 2005). The most immunogenic proteins of BVDV used for indirect ELISA are E^{rns}, E2, and non-structural protein 3 (NS3) (Collett, 1992). The specificity of serodiagnosis has been enhanced greatly by the use of antigen specific monoclonal antibodies (MoAb) in competitive ELISA systems (S. Bolin, Moennig, Kelso Gourley, & Ridpath, 1988) the best use NS3 mAbs which have shown high sensitivity compared with SN for bovine and ovine serum types (Paton, Ibata, Edwards, & Wensvoort, 1991). The test specificity has been further improved through the use of Ags that are derived from recombinant DNA techniques (G. H. Smith et al., 1988).

Currently, commercially available Ab ELISA kits are widely used to detect BVDV Abs. These test kits reduce inter- and intra-laboratory variations by standardizing the methods and reagents used (Dubovi, 2013). Serum, plasma or milk samples can be used as sample types to detect Abs against BVDV (Beaudeau, Belloc, Seegers, Assié, Sellal, et al., 2001). ELISA tests have more utility due to fast test results, cost effectiveness, and the independence of cell cultures for the test (Sandvik, 1999). However in general Ab-ELISA has been proven to achieve fair sensitivity and the specificity of 99% and 98% in respect to SN tests and highly recommended as a diagnostic test (Sasha R. Lanyon, Fraser I. Hill, et al., 2014).

The main limitation to the Ab-ELISA is that is not possible to distinguish between antibodies produced in response to natural exposure to BVD virus and those acquired from colostrum or

vaccination (Evans et al., 2019). Therefore, it has been recommended that testing for antibodies should not be performed on animals under 10 months of age or on animals that have previously been vaccinated against BVD (Gates, Evans, et al., 2019).

2.4.7 Antigen capture ELISA

After BVDV specific MAbs became available in early 1990s, several antigen capture ELISAs (ACE) were developed for rapid detection of BVDV antigens extracted from different sample types such as blood, serum, milk and tissue samples (Sandvik, 1999). ACE is known as a robust, simple, cost-efficient diagnostic method because it does not require cell culture facilities to perform the test, the test results are minimally affected by prolonged storage of the samples, and the test can generate a positive result even for samples with low concentrations of viral antigens. Although the tests have high analytical sensitivity and specificity (>95%) (Brinkhof, Zimmer, & Westenbrink, 1996; Sasha R. Lanyon, Fraser I. Hill, et al., 2014; Mignon et al., 1992; J. T. Saliki, Fulton, Hull, & Dubovi, 1997; Sandvik & Krogsrud, 1995), the results can be influenced by concentrations of Ag in the sample, the specificity of the Ab reagent, the presence of highly conserved regions in the target Ag (Dubovi, 2013).

The basic principle of ACE uses monoclonal Abs to capture viral Ag followed by detection of antigen-antibody complex with enzyme-conjugated Ab (Ludemann & Katz, 1994). The test procedure is described extensively elsewhere (Sandvik & Krogsrud, 1995). Two BVDV proteins have been identified as potential target antigens, NS3 (p80) and E^{rns} (E0), but NS3 is preferred as it is highly conserved among all BVDV strains and BVDV specific monoclonal antibodies (Mabs) exist that recognize the target antigens (Dubovi, 2013). Antigen capture ELISAs (ACE) have been commercially produced to target NS2-3 protein, a highly immunogenic protein produced in large amounts in PI cattle (Brinkhof et al., 1996).

The rapid diagnostic tests have been introduced by different pharmaceutical companies such as SNAP BVDV Antigen (Ag) Tests that can identify BVDV infected cattle in 20 minutes using ear notch samples. The primary value of these rapid pen-side tests is the ability to manage clinical cases. If the farm has an animal with clinical signs compatible with mucosal disease, it would be useful to rule out BVDV before treating the animal to reduce the risk of spread of the virus. The SNAP tests are also probably useful in situations where animals could be quickly screened to identify PI animals when buying or introducing a previously untested animal to the farm. Moreover, farmers can use this type of on-farm testing which may encourage them to test animals for BVDV and could be used simultaneously with animal identification programs.

This kind of rapid diagnostic test is very convenient for screening new-born calves, replacements heifers, and bulls in the farm without waiting a long time for laboratory test results.

There are several limitations in the use of ACE to detect BVDV infected animals. Although the antigen capture ELISA has previously been considered as the test of choice for eradication programs, occasional false negative test results have been observed with calves that have high levels of colostral antibodies in their blood. In these cases, studies have shown that the maternal antibodies adhere to the surface of the virus which prevents antibodies used in the ACE from binding and leads to false negative results for a small number of likely low-shedding PI animals (Gates, Evans, et al., 2019; Goyal & Ridpath, 2005; Zimmer et al., 2004). It is therefore recommended not to use ACE for testing serum samples in calves less than 3 months of age (Dubovi, 2013). However, skin biopsies or ear notches are still considered acceptable for PI testing since the quantity of maternal antibodies in tissues is much lower than blood and ear notch samples are also convenient to collect at the same time as identification ear tags are placed (Cornish et al., 2005; Kuhne et al., 2005).

2.4.8 Molecular methods

Molecular studies have contributed to understand the epidemiology of BVDV. The basic principle of using molecular methods to diagnose BVD infections is the detection of viral nucleic acid from the animal specimens. Several molecular diagnostic tests have been described for the detection of BVDV infections starting in early 90s with radioactive filter hybridization probes (Sandvik, 1999) and progressing to reverse transcriptase-polymerase chain reactions (RT-PCR) in the early 2000s (Goyal & Ridpath, 2005; Jeremiah T. Saliki & Dubovi, 2004b).

2.4.8.1 Reverse Transcriptase Polymerase chain reaction (RT-PCR)

Detection of virus by RT-PCR has been found to be more rapid and sensitive than virus isolation with the added advantage that the test results are not affected by the presence of Abs in the test sample. RT-PCR has become a frequently used BVDV diagnostic nowadays and can be combined with genetic sequencing to provide a genetic profile or genotype. Genetic typing of pestiviruses has been mostly based on sequence comparisons of the 5’-UTR, Npro and E2 regions (Vilček et al., 2001). It has been concluded that the 5'-UTR is the most conserved region in pestivirus genome (Vilček et al., 1998). The first genomic sequencing of BVDV was done in 1980s and at the same time it was revealed that non-cp and cp strains of BVDV can be

distinguished by genetics (Donis, Corapi, & Dubovi, 1988) and many RT-PCR assays were developed in early 1990s.

RT-PCR can be conducted on almost any sample taken from infected animal including blood, milk, follicular fluid, saliva, and tissue samples (Sasha R. Lanyon, Fraser I. Hill, et al., 2014). The principle of the test involves converting a portion of the viral RNA genome into complementary DNA (cDNA) using target specific DNA oligonucleotides, amplifying the cDNA through cycles of PCR and visualizing the product (amplicon) by gel electrophoresis. Prior to use in the PCR, viral RNA is chemically extracted from cellular material using commercial extraction kits or phenol-chloroform extraction. The RNA is added to a PCR reaction mixture which contains reverse transcriptase enzyme, *Taq* polymerase enzyme, primers specific for the target of interest, and nucleotides. If the target RNA is present, the primers anneal to the RNA strand and then the reverse transcriptase enzyme synthesizes a complementary DNA strand extending from the primer. The *Taq* polymerase enzyme then amplifies the cDNA using multiple cycles of heating and cooling exponentially increasing the number of copies of DNA. The test procedure is described elsewhere (Vilček et al., 2001) and a simple illustration of test procedure of RT-PCR is displayed in figure 2.4. When first developed, a two-step (cDNA + DNA amplification) two-tube RT-PCR assays were used but more recently two-step one-tube RT-PCRs were developed in which cDNA creation and implication occurred in a single tube to minimize sample manipulation and contamination.

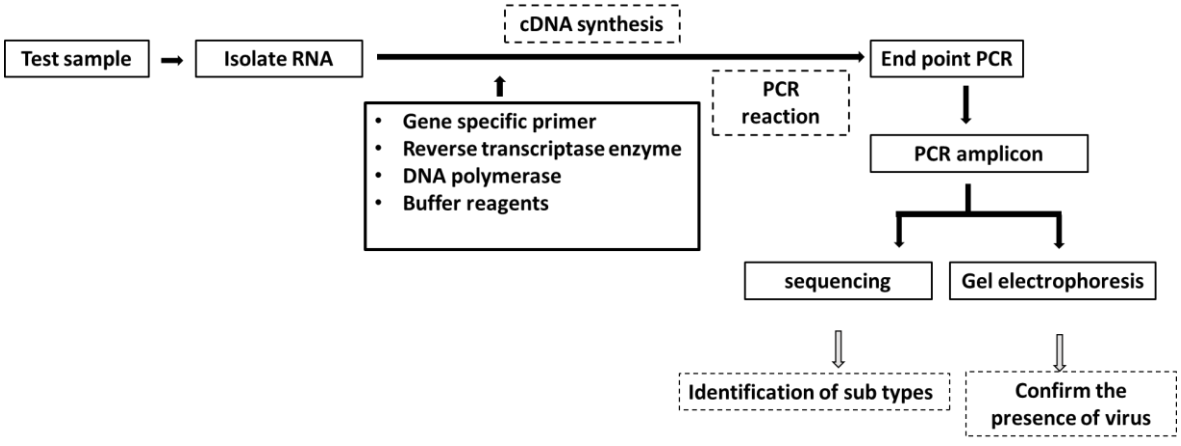

Figure 2.4: Testing procedure for RT-PCR

2.4.8.2 Quantitative Reverse Transcriptase Polymerase chain reaction (qRT-PCR)

The PCR technique was further advanced to develop nested PCR assays with two rounds of PCR to increase analytical sensitivity, and multiplex assays with species-specific primers for genotyping (Goyal & Ridpath, 2005). Quantitative RT-PCR assays determine the actual amount of PCR products present in a given cycle. The probe based qRT-PCR requires a probe labelled with fluorescence that accumulates in each PCR cycle that measures at the end of the PCR (Jia, 2012). More recently, quantitative RT-PCR (qRT-PCR) using TaqMan probes have been developed for rapid detection of BVD virus RNA where sensitivity and specificity have been compared with RT-PCR and have found that TaqMan qRT-PCR was 10-fold more sensitive and 100% specific (Bhudevi & Weinstock, 2001).

Later, qRT-PCR assays were developed to test milk samples of dairy animals with 100% sensitivity compared with VI because PCR test results are not affected by the presence of Abs in milk (Radwan, Brock, Hogan, & Smith, 1995). Since this technique offered high sensitivity, it was suitable to test specimens with potentially low quantities of virus, such as bulk milk, pooled samples of serum or plasma or other biological materials from transiently infected, as well as PI animals (Sandvik, 2005a).

2.4.8.3 Phylogenetic Analysis of BVDV Genomes

The variations of the virus strains within and between farms, viral evolution and transmission patterns have been studied in many countries (Hamers, Lecomte, Kulcsar, Lambot, & Pastoret, 1998). (Vilček et al., 2001). Phylogenetic analyses has provided more detailed information than studies based on reactions with antibodies and has allowed the rapid detection and discrimination of BVDV-1 and BVDV-2 sub genotypes, as well as the identification of novel sub genotypes (Yesilbag et al., 2017).

There are some limitations for molecular diagnostics. For example, a positive RT-PCR does not define the clinical status of an animal as RT-PCR assays detect acutely infected animals, PI animals, and animals vaccinated with modified-live vaccines. Therefore follow-up testing is necessary to define the status of positive animals (Dubovi, 2013). In addition, although PCR can use variety of sample types, the efficiency of the nucleic acid extraction of the specimen depends on the sample type and viral load within the sample (Sasha R. Lanyon, Fraser I. Hill, et al., 2014).

2.5 Application of diagnostic tests to control BVDV at the herd level

The goal of most herd-level BVD control programmes is to eliminate any existing PI animals from the herd and then take measures to prevent the re-introduction of BVDV from outside sources through improved on-farm biosecurity. A BVDV positive herd is defined as a herd that has at least one transiently infected or persistently infected animal present amongst the calves, replacement heifers, mixed-age cows, bulls, foetuses, and/or other fattening cattle on the premises. When trying to eliminate the virus from herd, it is usually necessary to determine the BVD status of all individual cattle through diagnostic testing since there are no pathognomonic clinical signs and PI animals are indistinguishable from their unaffected herd mates. However, testing all individual animals is often prohibitively expensive and logistically challenging to perform especially in large herds and herds that are raising large numbers of stock for fattening. Alternate testing strategies have been proposed that first use serological screening tests to determine whether cattle in the herd or in separately managed groups within the herd have previously been infected with BVDV. If the whole herd test negative for antibodies against BVDV, there are unlikely to be PI animals present and no further action is required. If there is serological evidence of active infections, individual animals in seropositive management groups can then be individually tested to find and eliminate PI animals. In dairy herds, bulk tank milk (BTM) is a convenient sample that allows screening of the herd both for antibodies and for virus to simultaneously determine both the exposure status and infection status of the milking herd, respectively.

In this section, we first review the main serological screening tests used to evaluate herd-level BVD status including (1) individual antibody ELISA (spot test), (2) pooled serum antibody ELISA, and (3) bulk milk antibody ELISA (Houe et al., 2006). We then review the strategies that are used to perform PI hunts in herds with serological evidence of active infections including (1) individual animal testing use ACE or PCR and (2) bulk milk PCR.

2.5.1 Serological screening tests

The majority of herds with PI animals that have been mixing with susceptible cattle for long periods of time are expected to have a large percentage of animals with high antibody levels against BVDV. The seroprevalence of BVDV in affected herds has been estimated from 60% to more than 90% of the cattle population across numerous published studies (Han J-H, 2018; Houe, 1999b; Scharnbock et al., 2018; G. Thobokwe, C. Heuer, & D. P. Hayes, 2004; Voges,

2008). In contrast, only 1-2% of animals in an infected herd may be shedding virus at any given time and it is possible for PI animals to have died or been culled by the time PI hunt is performed (Houe, 1999b; Sasha R. Lanyon, Fraser I. Hill, et al., 2014). Therefore, serological tests are generally more sensitive and cost-effective to use as initial screening tests to determine whether there is evidence of active BVDV transmission within the herd. Previous studies have shown a strong positive correlation between the seroprevalence of BVDV and the likelihood of finding at least one PI animal on subsequent PI hunts (Richard E. Booth & Brownlie, 2016; Lanyon, McCoy, Bergman, & Reichel, 2014; Weir, 2016).

2.5.1.1 Individual Serum Ab-ELISA (Spot Test)

The spot test is an application of individual serum Ab-ELISA that is commonly used to estimate the seroprevalence of BVDV in tested herds (Houe et al., 2006). This screening test involves randomly selecting 5 to 10 animals from a management group and performing individual Ab-ELISA on serum samples from each animal (Houe, 1994; Lindberg & Alenius, 1999). Animals that have only recently been introduced into the herd should be excluded since there may not have been enough time for BVDV to spread and cause an Ab response (Houe et al., 2006). It is also important that the animals are unvaccinated and over 10 months of age to prevent false positive results. Since BVDV antibodies can persist for a long time in recovered animals, the test is most often performed on animals between 10-18 months of age to give a more accurate picture of the current infection status rather than more historical infections. When the tests are being used to make inferences about herd BVD status, it is recommended that the test be performed for each separate management group (defined as groups of cattle that have direct nose-to-nose contact with each other).

A positive result on an individual test indicates that the animal has previously recovered from a BVDV infection and it is not a PI animal (Houe, 1994). Various cut-off points have been suggested to then classify herds as being BVDV positive or negative based on the total number of animals in the sample that returned positive test results. Recent modelling work suggest that the optimal cut-off point appears to be 20% positive animals (Humphry, Reeves, & Gunn, 2018), which is agreement with recommendations published elsewhere (Richard E. Booth & Brownlie, 2016; Houe, 1999b). The herd sensitivity and the specificity for spot testing have been calculated as 93% and 100%, respectively (Richard E. Booth & Brownlie, 2016). However, the results may not be as accurate if the PI animals were only recently introduced into the group or if there are pregnant cattle carrying PI calves (Houe et al., 2006). Although

10 animals are often used as the standard sample size, reducing these numbers does not appear to have a significant effect on the herd-level test performance provided an appropriate cut-point is chosen (Humphry et al., 2018)., For example, a BVD eradication programme in Netherland has performs antibody spot-testing on five animals (Humphry et al., 2018) and it has been suggested that as few as three young stock might need testing especially if the antibody test is done in tandem with antigen testing (Lindberg & Alenius, 1999; Seki, Seimiya, Yaegashi, & Sato, 2006). Testing fewer cattle can make the test more affordable to farmers and potentially increase testing uptake in voluntary programmes.

In addition to predicting the presence or absence of current infection, the spot test can also provide information on the time that has passed since the infection was last present. All animals born after the removal of the last PI animal should theoretically be seronegative by the time they reach 10 months of age (Lindberg & Alenius, 1999). However, false negatives can occur in herds where the PI calves died or were culled before they had the opportunity to infect sufficient numbers of animals in the group to meet the 20% cut-off value. These problems with misclassification can be avoided by repeating the spot test analyses 6 months later (Houe, 1999b). It is recommended that herds with positive spot test results test all new-born animals for BVDV antigen to remove PI animals as soon as possible rather than waiting to see if there are rising titres that may provide stronger evidence of active BVDV transmission occurring in the group (van Duijn, Veldhuis, Mars, de Roo, & Lam, 2019).

One of the significant limitations of spot testing in beef herds is the restriction that animals must be greater than 10 months of age at the time of sampling. By the time the test can be conducted to determine PI animal was born into the calf crop and/or was one of the dams that calving season, it is already too late in the calendar year to implement appropriate vaccination and testing programmes to have prevented more dams from creating PI calves during the breeding season. There are also additional logistical challenges in conducting sampling in beef herds since most calves are weaned at 6 to 8 months of age when they are too young to sample due to potential interference by maternal antibodies, and then minimally handled until 18 to 24 months of age when the results are no longer a good indication of the current BVDV status of the herd. For these reasons, it is often recommended that beef herds with a high risk of BVDV incursions use vaccination and/or calf testing as preventative control measures.

2.5.1.2 Pooled Serum Ab-ELISA

Pooled serum Ab-ELISA is an extension of the spot test where individual samples from 10 to 15 animals are combined into a single pooled sample for testing. The test is commonly used for non-lactating stock such as youngstock, dry dairy stock, and beef cattle (Lanyon, Anderson, & Reichel, 2014; Sasha R. Lanyon, Fraser I. Hill, et al., 2014). This test has the advantage of potentially reducing testing costs by reducing the total number of Ab-ELISAs that must be performed at the diagnostic laboratory. When selecting animals for the test, all the criteria that are followed for individual serum Ab-ELISA (unvaccinated animals greater than 10 months of age) should be followed to get an accurate test result. The total concentration of antibodies is reported as a single S/P (sample to positive) ratio with higher values indicating an increased likelihood of at least one PI animal being present in the group. There is also a strong correlation between the S/P ratio and the seroprevalence of BVDV amongst the individuals contributed to the pool (Evans et al., 2019).

The appropriate sample size for the pooled Ab-ELISA may vary depending on herd size (Sasha R. Lanyon, Malcolm L. Anderson, et al., 2014). Previous studies have shown that pool size had no significant effect on the S/P ratio and pools of any size from 5 to 25 can be tested with the same interpretation (Sasha R. Lanyon, Malcolm L. Anderson, et al., 2014). However, the current recommendation is to collect serum samples from 15 animals (9 minimum) to provide a 95% of chance of finding a seropositive animal among 15 animals. There are three cut-off values used for interpreting the test results If the S/P ratio is < 0.17, animals in the group are unlikely to have been exposed to BVDV and it is very unlikely that there is a PI animal present. If the S/P ratio is between 0.17 and 0.75, animals in the group have been exposed to BVDV, but at lower contact rates. This could be from (1) contact with a PI animal over fence line boundaries, (2) a PI animal that was previously present in the group but subsequently died or was culled, (3) a PI animal that was only recently introduced into the group and has not yet had time to infect many other animals, or (4) a PI animal present in a group that is grazed extensively with low contact rates between animals. If S/P is ≥ 0.75, there is high probability that there is currently or has very recently been at least one PI animal directly mixing with the group. It is recommended that herds with high S/P ratios perform a PI hunt to identify and remove any potential PI animals that may be present.

2.5.1.3 Bulk tank milk Ab-ELISA

Bulk tank milk (BTM) antibody-ELISA is widely used in dairy herds to assess the level of immunity against BVDV in the milking herd (Beaudeau, Belloc, Seegers, Assié, Pourquier, et al., 2001; Beaudeau, Belloc, Seegers, Assié, Sellal, et al., 2001; Eiras, Arnaiz, Sanjuán, Yus, & Diéguez, 2012; C. Heuer et al., 2007; S. R. Lanyon et al., 2014). A BTM sample can be collected for testing at any time when there are lactating cows in the herd. The antibody-ELISA results are expressed as an S/P ratio and various cut-off points are used to make inferences about BVDV seroprevalence in the herd (Beaudeau, Belloc, Seegers, Assié, Pourquier, et al., 2001; Houe et al., 2006; Niskanen, 1993). For example, the cut-off points for S/P ratios in a New Zealand study were reported as S/P ratios <0.25 to be negative, low ($0.25 < S/P \leq 0.5$), moderate ($0.5 < S/P \leq 0.75$), high ($0.75 < S/P \leq 1.0$), and very high ($S/P > 1.0$) (Gates, Evans, Heuer, et al., 2020). In general, dairy herds with bulk milk S/P ratios >0.75 are the considered highly likely to have at least one PI animal amongst the mixed-age cows, replacement heifers, or calves (G. Thobokwe, C. Heuer, & D. Hayes, 2004). .

The main limitation of BTM Ab-ELISA is that a single test result is often difficult to interpret in isolation since it can take several years for antibody levels to drop in herds that have recently cleared BVDV infections depending on the culling and replacement rates in the milking herd (R. E. Booth, Cranwell, & Brownlie, 2013). Furthermore, this test is not particularly sensitive for detecting new BVDV incursions into replacement breeding stock that occur as the result of a small number of susceptible milking cows that were exposed to BVDV during the risk period for generating PI calves (i.e. creating a Trojan dam from transient nose-to-nose contact over fence lines or through contaminated trucks/equipment). In this situation, still there is a potential that a PI calf can be born into the herd, but not enough animals are being infected to trigger a large antibody response in the BTM sample. In addition, the presence of BVD-vaccinated animals contributing to the bulk tank can lead to transient increases in observed antibody levels (Gates, Evans, Heuer, et al., 2020; Gonzalez et al., 2014; Sayers, Sayers, Graham, & Arkins, 2015). Some farmers may be unaware of the vaccination status of animals that were purchased as replacements which could lead to false positive results. Due to these limitations, BTM Ab-ELISA is often used to assess trends in BVDV exposure over time, but it is recommended to be used in conjunction with other tests such as RT-PCR of BTM or annual calf screening to get better information on the current herd BVD status (Evans et al., 2019; Houe et al., 2006; Sasha R. Lanyon, Fraser I. Hill, et al., 2014).

2.5.2 Identify PI animals in positive herds

When a herd-level antibody screening test has indicated that there may be active BVDV transmission within the herd, the next step is to identify and remove any PI animals that may be present since they will continue to serve as an ongoing source of infection to other susceptible animals (Gates, Evans, et al., 2019). These so-called PI hunts typically involve collecting individual blood, tissue, and/or milk samples from all individual cattle on the premises and testing the samples with either ACE or PCR as appropriate for the animals. Although testing all individual cattle is the most reliable means of removing infected animals, it can be prohibitively expensive for many herds and alternative strategies have been employed to help reduce costs.

Firstly, sample collection can easily be timed with routine management events such as disbudding or placing ear tags to make PI hunts logistically easier (Dubovi, 2013). Some farmers will also elect to wait until animals have been removed from the herd through routine culling and selection to reduce the total number of individual animals that must be tested. In herds that conduct genetic parentage testing on calves, the status of the dam can be inferred from the status of the calf. Since PI dams will always produce PI calves, a negative test result for the calf means that the dam was not a PI animal. On the diagnostic laboratory side, RT-PCR can be performed on pooled serum samples from up to 50 animals (R. L. Smith, Sanderson, Walz, & Givens, 2008) and ACE can be performed on pooled saline from ear notch samples with high sensitivity (98%) and the specificity (94%) (Cleveland, Salman, & Van Campen, 2006). If the results from the pool are negative, there is no need to test the individual samples from the pool. It should be noted that ACE is not reliable for use pooled serum samples as the reported sensitivity in pools containing just two serum samples is less than 15% (Cleveland et al., 2006).

In dairy herds, BTM PCR provides an inexpensive means of screening all cattle contributing to the tank for BVDV with a very high diagnostic sensitivity and specificity (Munoz-Zanzi, Johnson, Thurmond, & Hietala, 2000). All animals in the milking herd that were not contributing the bulk milk tank on test date should be individually tested to make sure they are not PI animals (S. R. Lanyon et al., 2014). The maximum theorised herd size in which a single PI cow can be detected has been estimated to be as high as 5000 milking cows (Radwan et al., 1995). There have been published case reports in which detection of PIs in herds has been reported to range from one PI animal in a herd of 132 to two PI cows in a herd of 800 (Sasha

R. Lanyon, Fraser I. Hill, et al., 2014; R. W. Renshaw, R. Ray, & E. J. Dubovi, 2000). If the test result is negative, then no further testing is required. If the test result is positive, some farmers will use a stepwise approach for finding the PI animal where they start by individually testing the bottom 10 to 25% of cows in the herd based on performance and working sequentially upwards in deciles or quartiles until the PI animal is found and the subsequent BTM samples come back negative. For dairy herds that conduct routine herd tests to measure milk yields, milk composition, and somatic cell counts on individual animals, a convenient time to perform bulk milk PCR is on one of the regularly scheduled test dates since this provides an accurate record of all animals contributing to the tank and individual milk samples are available for testing should the BTM results come back positive. It is also important to remember that dairy herds with negative BTM results may still have PI animals amongst replacement heifers and these animals should be tested individually to prevent future incursions into the milking herd.

One of the significant limitations in conducting a PI hunt is that there are currently no diagnostic tests that can accurately determine the BVDV status of foetuses in pregnant cattle. This means (1) that a herd cannot be declared BVD free until those calves have been born and tested for BVDV and (2) that the herd status becomes unknown as soon as there are new pregnant cattle in herd or if animals with an unknown BVD status are introduced into the herd. Another common source of frustration for farmers is when the PI hunt fails to find any PI animals, which is usually the result of the PI animals already having died or been culled by the time testing is performed. However, performing the PI hunt is still advantageous since the animals that test negative can be certified as being non-PI for life.

2.6 Application of diagnostic tests to control BVDV at the industry level

For countries with national BVDV eradication programmes, diagnostic tests have played a central role in facilitating efforts to reduce the prevalence of disease across the cattle industries. In earlier years, diagnostic tests were mainly used to confirm clinical cases of BVDV. However, that approach had no significant impact on lowering national BVDV prevalence since it was not particularly sensitive for detecting infected herds or monitoring herd re-infection. After better characterising both the role of PI animals in BVDV transmission and the economic losses caused by the subsequent BVDV infections, several European countries capitalised on the advances in BVDV diagnostic testing to implement national and regional

control and/or eradication campaigns. The first large-scale BVD eradication programmes were launched in the Scandinavian countries in 1990s with the goal of achieving BVD-free status within 10 years (Hult & Lindberg, 2005; Rikula, Nuotio, Aaltonen, & Ruoho, 2005). Following the success of these programmes, many other European countries have now launched BVDV control programs based on similar targets and using different combinations of control measures. These include performing initial antibody-based screening tests to classify herd status, follow-up tests to identify infected individuals in infected herds, continued monitoring to confirm infection-free status, and vaccination to protect against fetal infections. The general approaches used by different counties are described in the rest of this section.

2.6.1 Approach 1 – Testing all individual cattle for BVD

The most straightforward and efficient method for eliminating PI animals from the population is testing all individual cattle for BVDV. Switzerland performed mass testing of their entire living cattle population for BVD viral antigen within a one-year period (2008-2009) followed by annual calf testing to verify the status of all animals subsequently entering the population. However, this mass-testing approach can be prohibitively expensive and so countries like Ireland, Belgium, and Germany introduced legislation that required farmers to test only new-born calves based on the assumption that older PI animals will eventually leave the population through death or culling. Some countries have further requirements that all cattle moved between locations or sold to a different farm must first be individually tested to prevent BVD spread through animal movements. The approach of calf testing has generally been successful in eliminating PI animals in part because most European counties already have legislation in place that requires new-born calves to have a national identification ear tag placed shortly after birth for traceability purposes. Using tag-and-test devices which collect an individually labelled ear notch sample at the same time as the ear tag is place made it easy to link the BVD test results to the animal's record in national livestock traceability database. In addition, this approach has the advantage that herds can still use vaccination as an adjunctive control measure because the antibodies produced in response to vaccination do not interfere with either PCR or ACE performed on ear notch samples. Germany has implemented systematic vaccination in addition to calf testing as a part of its national BVD control programme to protect pregnant animals against fetal infection. Overall, this general approach of testing individual animals has achieved great success by reducing the PI prevalence from 1.3% to 0.02% in Switzerland 7% to 0.06% in Ireland and 0.48% to 0.01% in Germany (Bachofen et al., 2013; Metcalfe, 2019).

2.6.2 Approach 2 – Using serological screening tests followed by PI hunts

Although testing of all individual cattle against BVDV is the most effective way of identifying and removing PI animals, it can be prohibitively expensive to maintain especially as the prevalence of PI animals in the population declines and most tests would be expected to return negative results. Some countries have therefore employed a two-step approach with initial serological screening to determine whether there is evidence of active BVDV infections followed by PI hunts in herds that are classified as positive based on the antibody levels in tested stock. BTM samples are often used for surveillance in dairy herds while spot tests or pooled serum antibody ELISA remain the tests of choice for beef herds and other dry stock (R. E. Booth et al., 2013). Some countries will still require that all individual cattle moved between locations or sold to other farms are individually tested to prevent BVD spreading through animal movements. The main advantage of this approach to national BVD control is that it can significantly reduce unnecessary testing costs by limiting individual animal testing to herds that are at high risk of having PI animals based on their serological results. The main limitations of this strategy are that vaccination cannot be used as a biosecurity measure because of its potential to interfere with the serological result and that the serological test results may have low sensitivity in herds with recent infections and low specificity in herds with historical infections. Scotland has successfully applied this two-step approach for their BVDV eradication programme and have removed the options of BTM testing in their national program relying instead on serological screening of representative unvaccinated young animals who have been in close contact for at least two months to address these limitations (Metcalfe, 2019).

2.6.3 Approach 3 – hybrid approach of screening + annual calf testing

Some BVDV control programs use a hybrid approach that involves screening of herds to determine the need for a PI hunt plus annual calf testing to catch any potential breakthroughs in herd biosecurity. This approach efficiently controls the two main ways that PI animals can be introduced to the herd: the birth of PI calves from Trojan dams and purchased PI replacement breeding animals. Denmark has applied this combined testing approach for their BVDV eradication program. In early 1994, nearly 39% of dairy herds in Denmark were estimated as persistently infected at which point the hybrid approach was used to accelerate the removal of PIs from the herds. In the Denmark approach, BTM screening and spot testing have been performed with subsequent antigen testing of young stock (Bitsch & Rønsholt, 1995).

2.6.4 Ongoing monitoring

At present, many Western European countries have already either achieved BVD-free status or have regional or national control programmes underway. Once disease prevalence is reduced, the next challenge is identifying the new cases rapidly and cost-effectively to maintain the disease-free status. This is particularly important since unvaccinated BVDV negative herds will lose natural immunity as fewer susceptible animals are getting infected by PI animals, which could lead to more severe consequences if a new BVDV outbreak occurs. Therefore, ongoing monitoring programs are important to identify incursions before they can significantly impact breeding cattle

Different countries have employed different monitoring programs to ensure the BVD free status of their national herds. Countries like Denmark, Sweden and Norway that do not routinely vaccinate animals for BVDV, perform annual serological screening using either bulk milk tests or spot tests as the primary monitoring methods. Serological screening of BTM can be helpful for understanding the temporal trends of BVDV exposure over time but are less widely used due to the previously discussed limitations. Annual calf testing serves as both a monitoring and control tool to catch any potential breakthroughs (Houe et al., 2006; Metcalfe, 2019).

2.6.5 Use of molecular epidemiology in national control programmes

Genetic characterization and phylogenetic analysis contribute to further clarify the relationships between the genetic diversity of the virus and its geographic distribution and also the routes of circulation of the different subtypes in a particular geographical location characterized by a BVDV high genetic variability (Luzzago et al., 2012). Further, it is important to know which subtypes of the virus are circulating and how their prevalence is changing over time to effectively control BVDV by vaccination (Workman et al., 2016). To date at least twenty-five different BVDV-1 and BVDV-2 sub-genotypes have been described (Yesilbag et al., 2017).

Phylogenetic methods have been used by different countries for their national BVDV control campaigns to investigate the source of the infection. Sweden was the first country to apply molecular epidemiology systematically in their BVDV control programme. This approach has sped up the final phase of their BVD-programme to reach complete eradication by facilitating the trace of new infections from previously free herds where the route of transmission was not

clearly understood (Ståhl et al., 2005b). The United Kingdom has linked the molecular epidemiology with cattle movement data for the first time to elucidate potential sources and routes of infection of BVDV at national level (Richard E. Booth et al., 2013b). Similar methods have been used to investigate the molecular epidemiological characteristics of BVDV in Italy (Bazzucchi et al., 2017; Ebranati et al., 2018; Luzzago et al., 2012), South Korea (Choi & Song, 2011), Canada (A. Chernick & van der Meer, 2017), Slovenia (Toplak, Barlic-Maganja, Hostnik, & Grom, 2002), USA (Workman et al., 2016) and China (Wang et al., 2020).

2.7 Discussion

Since the first BVD virus isolation in the 1960s, there have been significant advances in BVDV diagnostic tests that have enabled the disease to be controlled more successfully and cost-effectively at both the herd and national levels. Several techniques for the diagnosis of BVDV infections have been developed recently that have allowed affordable widespread testing of animals for BVDV infection. In particular, the introduction of bulk milk PCR and antibody ELISA has provided a convenient and inexpensive means of screening large dairy herds for evidence of active infection and exposure, while spot tests and pooled serum antibody tests are useful as inexpensive alternatives for screeding non-dairy animals and beef stock. However, it should be noted that although these screening tests are highly sensitive in detecting herds with active infections, there are some issues with specificity in herds where the PI animals have already died or removed at the time of testing could lead false positive results.

There have also been recent improvements with regards of testing individuals including the introduction of tag-and test-devices that enable samples to be collected at the same time as the national animal identification tag is placed, which makes it more convenient to screen the population for PI animals at earlier age and track the status of individual animals at a national level. Further, many commercial diagnostic laboratories now offer BVD testing as an optional add-on to routine herd milk quality testing, which can help dairy herds save on costs of additional sample collection. The introduction of rapid pen-side lateral flow tests have also been useful for diagnosing clinical cases with compatible similar signs of mucosal disease so the farmers can decide whether to treat or remove the animal from the farm based on the test results. Farmers themselves can also use rapid tests for screening new-borns and confirming the BVDV status of purchased animals before bringing them into the herd.

Although national BVDV control programs have made significant progress through the successful application BVDV diagnostic tests, there are remaining limitations with the current BVDV diagnostic test methods to be discussed. Firstly, there are some significant limitations when applying screening tests in cattle populations where vaccination is used as a control measure and also serological tests are unreliable in animals under 10 months of the age. Particularly for seasonal beef herds, by the time the tests can be performed in youngstock, it is generally too late in the calendar year to implement control measures to stop BVDV transmission if the results indicate that a PI animal may have been present. Secondly, there are no BVDV serological tests with true DIVA (differentiating infected from vaccinated animals) test properties, which limits the ability for countries to use vaccination and serological tests together in national control programmes.

Another limitation is that none of the available diagnostic tests can accurately determine the BVDV status of the fetus in pregnant cattle. This means that farmers who purchase pregnant replacement dams risk introducing BVDV to their herds through the birth of PI calves. This is again a greater issue for beef cattle herds where calves remain with their dams until weaning and it can be difficult to individually test and remove PI animals before they have the opportunity to expose pregnant dams during the next mating period. Finally, there are still remaining issues with the gold standard recommendation of waiting three to four weeks to re-test virus positive animals to confirm their PI status. Not only is it expensive to re-test animals, but the suspect animals are also potentially spreading BVDV to other cattle on farm while the confirmation test results are pending. With the current qRT-PCR and antigen ELISA protocols, it is possible to establish cut-off values above which an animal is almost certainly a PI animal and cut-off values below which an animal is almost certainly TI animal. However, there remains a grey zone in the middle where it is impossible to distinguish low shedding PI animals from high-shedding TI animals. In these situations, the options are either to re-test the animal in 3 to 4 weeks or immediately cull with the acknowledgement that some TI animals may be unnecessarily removed.

Advances in molecular sequencing methods have also provided promising tools to improve the efficiency of national BVD eradication programmes by, for example, permitting contact tracing through phylogenetic analysis to identify the likely sources of new BVDV introductions into a herd or monitoring the circulating BVDV strains to ensure that vaccines are targeted to provide adequate protection (Ståhl et al., 2005a). However, few countries have applied molecular epidemiology to their national control efforts with the most likely reason being the

relative expense of molecular methods as well as the need for specialised equipment. However, the costs of sequencing continue to decline and as the technology becomes more available in commercial diagnostic laboratories, there may be more opportunities to apply it widely in national control programmes. There is a need for establishing a BVDV sequence database with clear protocols for the appropriate gene(s) target for sequencing analysis.

Overall, this literature review has highlighted that there is no one-size-fits all approach for applying BVDV diagnostic tests to control programmes at either the herd or national level. However, by integrating knowledge about test performance with an understanding of how the cattle industries work, it is possible to design cost-effective strategies for eliminating the negative impacts of BVD on animal health, welfare, and performance.

3. Preliminary phylogenetic analysis of bovine viral diarrhoea virus (BVDV) subtypes across dairy farms in New Zealand

3.1 Abstract

Bovine viral diarrhoea virus (BVDV) is an important global viral pathogen that causes significant economic losses to the cattle industry. Control of BVDV in New Zealand is reliant on a voluntary test and cull programme. In some countries the application of molecular epidemiology has assisted to trace the routes of transmission of BVDV, yet to date there is very little information regarding the BVDV genotypes circulating in New Zealand. Thus, this study was designed to provide preliminary data on the circulating BVDV subtypes in dairy farms across New Zealand. A convenience sample of BVDV-positive dairy serum samples (n=103) as determined by diagnostic laboratories during routine BVDV screening were used for the study. Of them, 35 samples that were positive for 5'UTR primers and 26 samples that were positive for N^{pro} primers were used for the phylogenetic analysis. The phylogenetic analysis of 5'UTR, and N^{pro} region confirmed all the dairy isolated were belonged to BVDV-1A and showed 95-100% sequence homology with previously identified overseas isolates and 98-100% sequence homology to 1A isolates recently identified in New Zealand beef cattle. Although unique variation among the BVDV isolates from dairy cattle was observed, some isolates did appear to be highly conserved (99-100%) when compared to other isolates from the same farm, among farms in the same region and between regions. Therefore, with further molecular characterization and improved sensitivity of the assays used for phylogenetic analysis it may be possible to use the sequence similarities and variations as a tool in tracing transmission patterns in disease control programs.

3.2 Introduction

Bovine viral diarrhoea (BVD) is an OIE-listed cattle disease that causes significant economic impacts in affected herds and has been detected in more than 88 countries worldwide since 1946 (Pinior, Garcia, Minviel, & Raboisson, 2019; Ridpath, 2010). The virus is a member of the genus *Pestivirus*, which belongs to family *Flaviviridae*. Pestiviruses are highly variable both antigenically and genetically with the genus currently containing many important species

including bovine viral diarrhoea virus 1 (BVDV-1), bovine viral diarrhoea virus 2 (BVDV-2), Border disease virus (BDV), classical swine fever virus (CSFV), and the recently added HoBi-like pestivirus also called BVDV-3 (Anonymous, 2020; Simmonds, Becher, Bukh, Gould, Meyers, Monath, Muerhoff, Pletnev, Rico-Hesse, Smith, Stapleton, et al., 2017; D. B. Smith et al., 2016; Wang et al., 2020). The most prevalent pestivirus species worldwide is BVDV-1 and at least 21 sub-types have been reported at present (Wang et al., 2020; Yesilbag et al., 2017). BVDV-1 has also been detected in diverse domestic and wildlife animal populations including cattle, sheep, goat, pig, deer, buffalo, bison, and alpaca (Evans, Moffat, Hemmatzadeh, & Cockcroft, 2017; D. B. Smith et al., 2016; Yesilbag et al., 2017).

The BVDV genome is a positive single-stranded RNA with the length of approximately 12.3 kb, containing a single open reading frame (ORF) flanked by two untranslated regions at the 5′ and 3′ end (Richard E. Booth et al., 2013b; Wang et al., 2020). The ORF encodes a polyprotein of approximately 4000 amino acids, that can be further cleaved into structural (C, Erns, E1, and E2) and non-structural (Npro, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) polypeptides (Vilček et al., 2001; Wang et al., 2020; Yesilbag et al., 2017). The BVDV-1 5′-UTR is a non-coding region of the genome and is most commonly used for virus genotyping (Wang et al., 2020) because 5' UTR contains highly conserved and variable regions which has allowed the development of both pan-pestivirus and species-specific RT-PCR-based diagnostic assays (Ridpath & Bolin, 1998; Toplak et al., 2002). Further, N^{pro} , NS2/3 and E2 genes are also being used for many assays because of their quality of fine discrimination between closely related viruses (Vilček et al., 2001; Workman et al., 2016).

Understanding the molecular epidemiology of circulating viral strains is important for disease control efforts to describe the genetic diversity of existing viral strains circulating through the population, to track virus evolution over time, and to monitor for the emergence of novel strains (Bazzucchi et al., 2017; Cerutti et al., 2016; A. Chernick & van der Meer, 2017; Ridpath et al., 2011) Previous research has shown this particular importance for BVDV since the subtypes can shift substantially over time (Ridpath et al., 2011). The global studies have shown the distribution of BVDV 1 is greater than BVDV 2. The extensive genetic diversity of BVDV reflected by the number of detected subgenotypes has been described for several European countries, as well as for China and Turkey. High BVDV-1 genetic variation has been found in many European and Asian countries yet less genetic variation of BVDV 1 has found in the Americas, Australia, and Africa (Yesilbag et al., 2017). Analyses over the past two decades

have shown the presence of BVDV-2 in a number of European countries, including Germany, Belgium, France, the United Kingdom, Slovakia, and Austria (Vilcek et al., 2005).

Many countries have used the phylogenetic analysis of virus isolates to investigate the molecular epidemiological characteristics of BVDV (Bazzucchi et al., 2017; A. Chernick & van der Meer, 2017; Choi & Song, 2011; Luzzago et al., 2012; Wang et al., 2020). The Sweden has applied the phylogenetic analysis to trace the unidentified routes of transmission at the latter part of their BVDV eradication program and that has been successful and beneficial in many ways to understand the epidemiological relationship between old/existing and new cases, to trace the chains of infection and to rule out the suspected sources of infections (Ståhl & Alenius, 2012; Ståhl et al., 2005b). A similar approach has been implemented within the Swiss eradication programme too (Stalder et al., 2016). Molecular epidemiology and evolutionary phylodynamics have been used in recent years for BVDV studies since the combined approach of traditional and molecular epidemiology has confirmed links among farms and has provided information useful for the eventual successful control program (Richard E. Booth et al., 2013b; Cerutti et al., 2016). Some studies emphasize the importance of updated knowledge regarding current circulating strains when producing vaccines against BVDV because the vaccines often only cover certain strains and there may be issues with cross-protection if there are other more common strains circulating in the particular geographical region (Evans et al., 2019).

New Zealand is another country that is currently exploring eradication options because it is a prevalent disease with economic impacts for the country. BVDV has become well established and endemic in New Zealand causing the second most impact to the dairy and beef industry (Reichel, Lanyon, & Hill, 2018). The National BVD Streeting committee was established in 2005 with the purpose of improving the knowledge of BVD among farmers and veterinarians (Ellison, 2011). To support this, the BVD Free Project was started on July 2017 and New Zealand is currently evaluating the feasibility of national eradication through the BVD free project.

Several studies have been conducted to identify how the different transmission pathways contribute to the disease spread. Persistently infected animals (PIs) have been identified as the most efficient mode of transmission of the virus under natural circumstances (C. Heuer et al., 2007). In addition to that, the purchase of heifers or cows, mean number and distance of neighbouring farms, sharing a common pasture, or over-the-fence contacts between herds have been shown to significantly increase the risk of BVDV transmission (J.-H. Han et al., 2018;

Obritzhauser, Klemens, & Josef, 2005; Valle, Martin, Tremblay, & Bateman, 1999). These risk factors are highly correlated with the farm management factors and poor farmer compliance with the bio security recommendations (Gates, Woolhouse, Gunn, & Humphry, 2013). The uncertainty of contribution of different transmission pathways to disease spread emphasises the need of finding the most cost-effective way of limiting the transmission of BVDV. Molecular epidemiology is a good approach to address this problem as genetic characterization of isolates and phylogeographical analysis gives a unique opportunity to trace routes of infection (Ebranati et al., 2018). Even though New Zealand has done many research studies to support BVDV control and eradication programs (Gates, Evans, Heuer, et al., 2020; J.-H. Han, Weston, Heuer, & Gates, 2020; C. Heuer et al., 2007)molecular epidemiological approach has not been included. An early study in 1998 identified a BVDV 1a genotype from a New Zealand cow, (Reichel et al., 2018; Vilček et al., 1998), however there is little recent genetic data. Further, recent studies stress the importance of studying the molecular epidemiology due to lack of knowledge of current circulating BVDV strains in New Zealand (Han J-H, 2018). Thus, the aim of this study was to provide preliminary data on the characteristic distribution of currently circulating BVDV in New Zealand dairy cattle.

3.3 Materials and Methods

3.3.1 Sample collection

As part of routine diagnostic screening, serum samples were collected from individual dairy calves by veterinarians and submitted to diagnostic laboratories throughout New Zealand. The blood samples were spun for 10 minutes at 3000 rpm to separate the serum. Serum samples were tested for the presence of BVD viral antigens using either an Idexx BVDV antigen ELISA kits (Berne, Switzerland) or the Applied Biosystems™ VetMAX™ BVDV 4ALL PCR kit from Thermofischer Scientific (Lissieu, France). Those samples that tested positive for BVDV using an antigen ELISA or PCR test were submitted to Massey Veterinary School (Palmerston North) where they were stored at -20 °C while awaiting processing for molecular testing.

3.3.2 Sample processing

3.3.2.1 RNA extraction

Viral RNA was extracted from serum samples by means of Spin protocol of QIAGEN QIAamp Viral RNA Mini Kit according to the manufacturer's instructions (QIAGEN Ltd. Hilden, Germany). The RNA was eluted in 60 µl of buffer and stored at -80 ^{0}C.

3.3.2.2 RT-PCR and DNA sequencing

The 5′-UTR and N-terminal protease (N^{pro}) regions of the RNA genome were amplified and the details of the gene specific oligonucleotide primers used for the RT-PCRs are shown in the Table 3.1. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to amplify the target regions of the BVDV genome by using SuperScript™ III Platinum™ One-Step qRT-PCR Kit (Invitrogen, Waltham, MA, USA).

Table 3.1 Details of oligonucleotide primers used for the RT-PCRs

Gene Target	Primer*	Sequence (5'–3')	Genome position	Size of amplicon
5'UTR	P1-U(forward)	AGAGGCTAGCCATGCCCTTAGT	97–118	299 bp,
	Pest2-L(Reverse)	TCAACTCCATGTGCCATGTAC	395–375	
Npro	B31(Forward)	CCATCTATRCAYACATARATGTGGT	795–771	441 bp,
	B32(Reverse)	TGCTACTAAAAATCTCTGCTGT	355–376	

*The primer sequence, genomic position and predicted amplicon sizes refer to Toplak *et al* (2005). (Toplak et al., 2005)

The 299bp region of 5'UTR was amplified using P1-U and Pest2-L primers (Toplak et al 2005) (Table 1). The reaction mixture for the particular RT-PCR consisted of 25 µl of 2X Reaction buffer, 1 unit of SuperScript™ III Platinum™ *Taq* enzyme mix, 0.2uM of each primer, 19µl of sterile distilled water and 3 µl of up to 1µg eluted RNA to a final volume of 50µl. A BVDV positive confirmed RNA sample was used as the positive control and distilled water was used as a negative control in each PCR run. The PCR conditions were as described by Vilcek et al., (2001) with an initial cDNA synthesis at 42^{0}C for 15 min, a pre-denaturation hold at 99^{0}C for 5 min following 40 cycles of template denaturation at 94^{0}C for 45 s, primer annealing at 60^{0}C for 1 min and extension step at 72^{0}C for 1 min. The final 72^{0}C step was prolonged for 7 min(Vilček et al., 2001).

The 441bp region of N^{pro} of the BVDV genome was amplified using B-31 and B-32 primers (Toplak et al., 2005) (Table 1). The reaction mixture and the PCR conditions were same as described above except the initial two steps of PCR conditions where the cDNA synthesis time changed as 45^0C for 30 min, and then heat inactivation at 94^0C for 5 min (Grom & Barlic-Maganja, 1999; Toplak et al., 2005).

The 5'UTR and N^{pro} amplicons were subjected to 1.5% (w/v) ultra-pure agarose gel (Invitrogen, Waltham, MA, USA) using a100bp molecular marker (Promega, Madison, USA) and visualized which was dyed with RedSafe (iNtRON Biotechnology, Kirkland, WA, USA) and inspected under a UV light, using a transilluminator, for bands that matched the expected sizes of the genes of interest. Positive amplicons were purified using PureLink PCR purification kit (Invitrogen, Waltham, MA, USA) and sent for Sanger Sequencing using the 3130xl Genetic Analyser (Applied Biosystems Inc, California, USA) to confirm their genomic sequences. The electropherograms were viewed in Geneious R9 (Biomatters, Auckland, NZ) (Kearse et al., 2012) and checked for overlapping nucleotide peaks. Successful sequence results were submitted to the NCBI Blast and compared to published sequences available from GenBank.

3.3.3 Phylogenetic analysis

The sequences from our survey and reference sequences from the GenBank database including representatives of the Types 1A-U and Type 2 BVDV genotypes were trimmed to the same length (227 bases for UTR and 337 bases for N^{PRO}) using Geneious^{TM} (Biomatters, Auckland, New Zealand) and aligned using Claustral W (Higgins, Thompson, & Gibson, 1994; Thompson, Higgins, & Gibson, 1994). A phylogenetic tree was generated in MrBayes version 3.1 using Bayesian phylogenetics. A general time-reversible model including invariable sites (GTR+I) was used. The Bayesian phylogeny was obtained using one cold and three hot Monte Carlo Markov chains, which were sampled every 1000 generations over 1,500,000 generations; 1500 trees were generated. Of these trees, 25% were discarded as burn-in material. The remaining 1125 trees were used to construct a majority consensus tree. Bootstrap percentages were added to the tree at the appropriate nodes.

The sequence divergence between and within the different lineages was calculated using a Jukes-Cantor model of substitution implemented in the program PAUP* 4.0 Beta version 10 (Swofford, 2002).

3.4 Results

3.4.1 Sample collection

Serum samples were received from both North Island and South Island. A total of 174 serum samples were received from 11 regions including Auckland (n = 12 from 5 farms), Otago (n= 22 from 8 farms), Manawatu (n= 37 from 16 farms), Gisborne (n=5 from 3 farms), Waikato (n= 37 from 20 farms), Bay of Plenty (n= 24 from 10 farms), Northland (n= 7 from 3 farms), Canterbury(n = 6 from 2 farms), Hawke's Bay (n = 4 from 4 farms) , Southland (n=7 from 3 farms) and Wellington (n =13 from one farm). However, 42 of these samples were excluded because they were from beef cattle (n=7), unknown region (n=30) or samples that were broken or damaged in transport (n=5).

3.4.2 RT-PCR analysis

In general, only one to two samples from each farm were sent for genotyping and repeat samples were stored for later testing except for one occasion where seven samples were taken from a same farm to determine on-farm transmission. In total, 103 dairy serum samples were used for RNA extraction and subsequent RT-PCR for the detection of complimentary DNA of BVDV. Out of 103 RNA extractions, 48 samples were recognised by the P1-U/Pest2-L primers and amplified the 5'UTR products of size 299bp (Table1). Thirty-five samples that had strong bands of suitable intensity in gel electrophoresis sent for the sequencing. Of the 35 samples that demonstrated a strong positive band for the 5'UTR, 26 samples also amplified with the B32/B31 Npro primers generating a PCR product of the expected size of 441 bp.

3.4.3 Phylogenetic analysis of UTR sequences

The geographic distribution of the samples that were successfully sequenced and used for the phylogenetic analysis are displayed in Figure 3.1. Of 35 samples that were sent for Sanger sequencing, only 31 samples were successful and were used for the phylogenetic analysis and representing 22 different farms in 8 regions.

Blast analysis of the 5'UTR region revealed that the New Zealand dairy isolates were BVDV Type 1A and shared some sequence homology with previously identified BVDV Type 1A isolates from around the world, including China (range 96.9%-100%, GenBank MN417860), South Korea (range 96.4% - 100%, GenBank MK509773) and Ireland (98.2%- 99.6%,

GenBank MW114245). Moreover, the 5'UTR region of the dairy isolates showed similarity to previously identified BVDV isolates from New Zealand beef cattle, such as isolates #445 and #603 (98.2- 100% GenBank MN961225 and MN961226). Importantly, dairy isolates # 370, #381, #911 and #960 showed 99.1% sequence homology with previously reported isolates from Korea (GenBank MK509773), China (GenBank MH490942), New Zealand pestivirus (AF026785) and New Zealand beef (GenBank MN961227) suggesting these isolates many be closely related to each other. Similarly, dairy isolates #7-30, #6-87 and #1-27 all showed 98.2% sequence homology with a Korean (GenBank MK509773) and a Chinese isolate (GenBank MH490942) but showed lower sequence homology (96.8%) with the New Zealand beef # 603 (MN961226) suggesting that there may be a second group of similar isolates among diary sequences. Further, dairy isolates #25-196, # 4-320 were 99.11% similar to a third Chinese isolate (GenBank MK170071), suggesting a third group.

After BLAST analysis, the 5'UTR sequences were used for further phylogenetic analysis from which a phylogenetic tree was generate using BVDV Types 1A and 1C reference sequence (Figure 3.2). Analysis confirmed that all the New Zealand dairy sequences grouped with BVDV subtype 1A reference sequences. The sequence homology of 5'UTR phylogenetic analysis is displayed in Table 3.2. Overall, the dairy isolates in group 1A showed 92.4%-100% homogeny when all the sequences where compared with each other. Within this group, there appeared to be at least five distinct clusters, named from 1A-1 to 1A-5 (Figure 3.2). Isolates within group 1A-1 included two samples from the same Manawatu farm which shared 99.6% sequence homology. Isolates within group 1A-2 included two samples from two different farms located in Waikato and Manawatu with 96.9% homogeny. Isolates within A1-3 showed 100% sequence homogeny with two samples from same farm and one sample from a different farm, all within the Auckland region. Isolates within A1-4 showed 98.3%-99.1% sequence homogeny with three different samples from three different locations in South Island. Finally, all the samples from A1-5 came from a same farm located in Otago that had 99.1% -100% sequence homogeny.

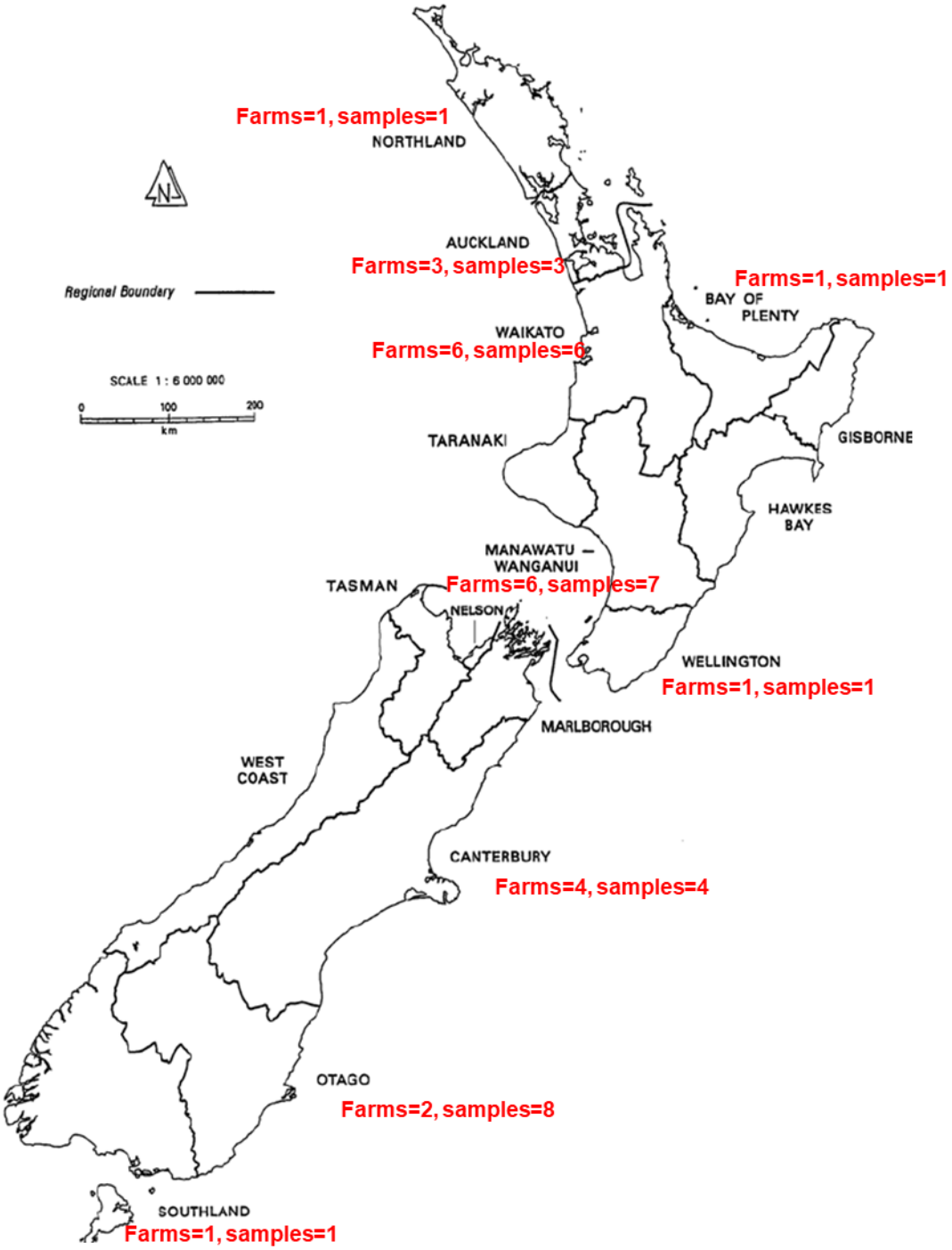

Figure 3.1 Geographical distribution of 5'UTR and N^{Pro} isolates of BVDV in New Zealand analysed in the study

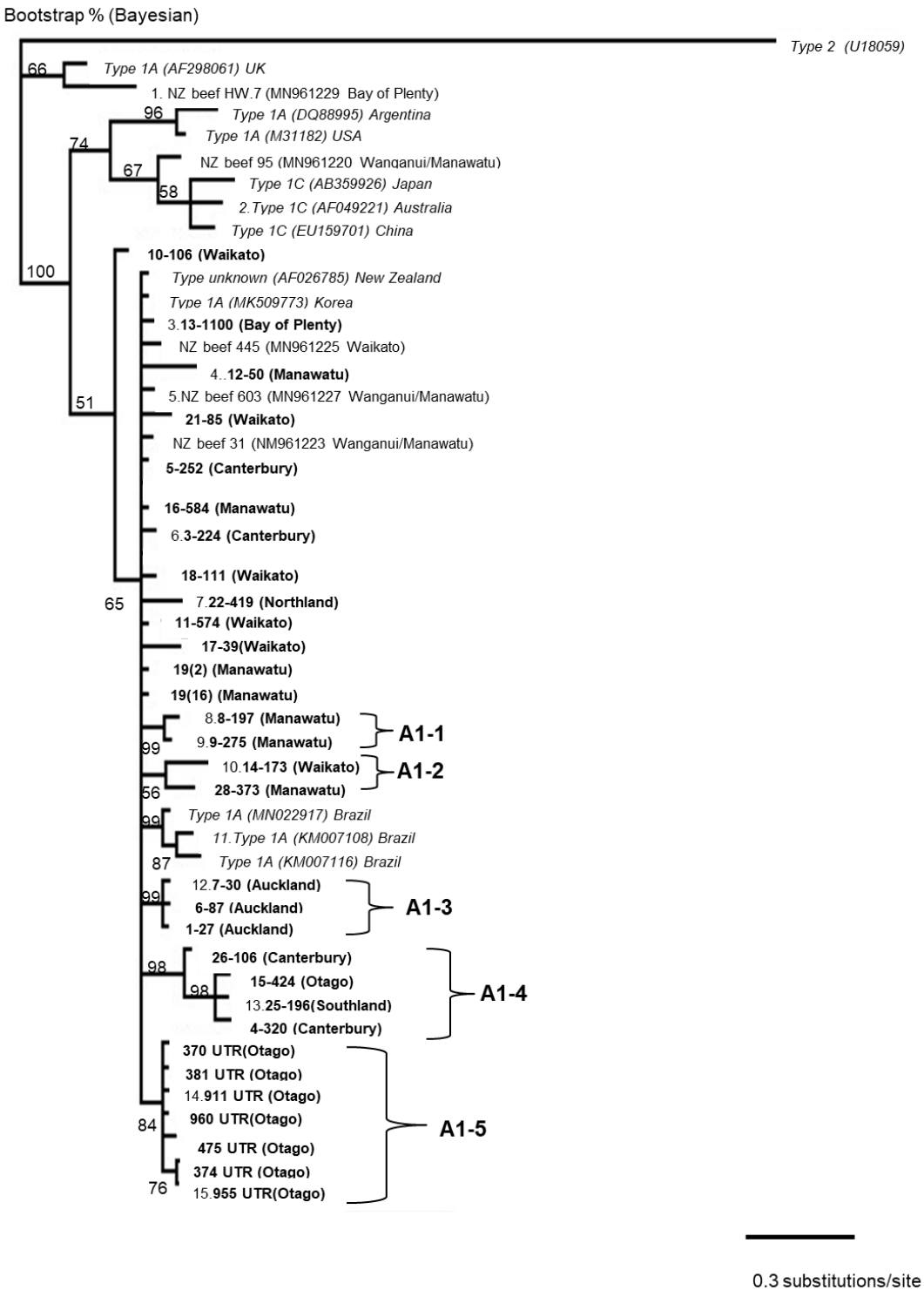

Figure 3.2 Phylogenetic analysis and comparison 5'-UTR sequences

*Twenty-one representative BVDV isolates from New Zealand dairy cattle (bold) and previously published BVDV sequences representing type1A-U present in the GenBank. Names of the lineages (when available) and GenBank accession numbers of the sequences are given after the virus type grouping and location isolated is identified in within brackets. The branch lengths are drawn proportionally to the amount of changes (scale bar is shown). The clusters of New Zealand dairy sequences are named A1-1 to A1-5.

Table 3.2 The sequence homology (in percentage) between 5 5' UTR gene representative isolates and known BVDV types

VDV type	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1.*NZ beef HW.7*	-														
2. *Type 1C Australia*	91.1	-													
3. **1100 (Bay of Plenty**)	92.4	94.2	-												
4. **50 (Manawatu)**	90.3	92.0	96.5	-											
5. *NZ beef 603*	98.0	93.7	99.2	97.3	-										
6. **224 (Canterbury)**	91.5	93.7	99.1	96.4	99.1	-									
7. **419 (Northland)**	91.5	93.2	96.9	94.7	97.3	96.9	-								
8. **197 (Manawatu)**	90.2	93.6	97.8	96.0	97.8	97.8	95.6	-							
9. **275 (Manawatu)**	90.7	94.1	98.3	96.5	98.3	98.3	96.0	95.6	-						
10. **173 (Waikato)**	89.0	91.4	96.4	95.6	96.4	96.4	94.2	96.4	96.9	-					
11. *Type A Brazil*	92.4	93.4	97.3	95.5	97.3	97.3	95.5	95.9	96.7	94.6	-				
12. **30 (Auckland)**	90.7	93.2	98.2	96.9	98.2	98.3	96.0	97.8	98.2	96.4	96.8	-			
13. **196 (Southland)**	91.2	92.8	96.0	92.4	95.1	95.1	93.8	93.8	94.2	93.4	95.5	94.2	-		
14. **911 (Otago)**	91.5	93.2	98.2	95.5	98.2	98.3	96.9	96.9	97.3	95.6	97.7	97.3	96.0	-	
15. **955 (Otago)**	91.1	92.7	97.8	95.1	97.8	97.8	96.4	96.4	96.9	96.0	97.2	96.9	96.4	99.6	-

[a]Numbers correspond to the numbers of BVDV Types in Figure 2 in which the phylogenetics of the BVDV isolates anf the GenBank assession numbers are given. Sequence homogeny was calculated with the use of a Jakes-Cantor model of submission. The new isolates are given in bold.

3.4.4 Phylogenetic analysis of N^{pro} sequences

To confirm the groupings found with the 5'UTR sequences, partial Npro gene sequences were analysed. Twenty-six samples showed the presence of an amplicon at the expected size (441 bp). Upon sequencing, only nine samples had positive sequence (Table 3.4).

Blast analysis revealed that these nine New Zealand dairy isolates were similar to previously identified BVDV isolates from China (96.1 %- 98.5% GenBank MH490942 and MH417939), and South Korea (96.1% - 97.9%, GenBank JQ418633 and MK509773) as well as previously identified isolates from New Zealand beef cattle, such as isolates #CH4 and #603 (96.1- 97.5%, GenBank MN954522 and MN954521) and an isolate from New Zealand goat (96.4%, GenBank U80900). Moreover, dairy isolates # 275 and #197 showed 98.5%-98.6% sequence homology with previously reported isolates from China (GenBank MN417939.1) and 98.6%-98.8% homology with New Zealand beef cattle #603 (GenBank MN954521)suggesting that there may be a sub group of similar isolates among diary sequences.

In order to perform further phylogenetic analysis, four of the nine were removed as the resulting sequence was too short at approximately 200 base pairs. The five remaining sequences represented five farms located in four regions (Canterbury, Wellington, Auckland and Manawatu). Phylogenetic analysis revealed that these five isolates clustered only with BVDV 1A reference sequences (Figure 3.3), consistent with the 5'-UTR phylogenetic analysis and similar to previously identified BVDV type 1A isolates from a New Zealand goat isolate (range 94.3-96.3%, GenBank U80900), and New Zealand beef subtype 1-A # 354 (92.0%-93.4%, GenBank MN 954523). Further, when the five sequences are compared to each other, they displayed a fairly high level of nucleotide homology ranging from 96.9 – 99.7% with the samples # 9-275 and # 10-197 from a same farm located in Manawatu showing 99.7% of sequence homology (Table 3.3).

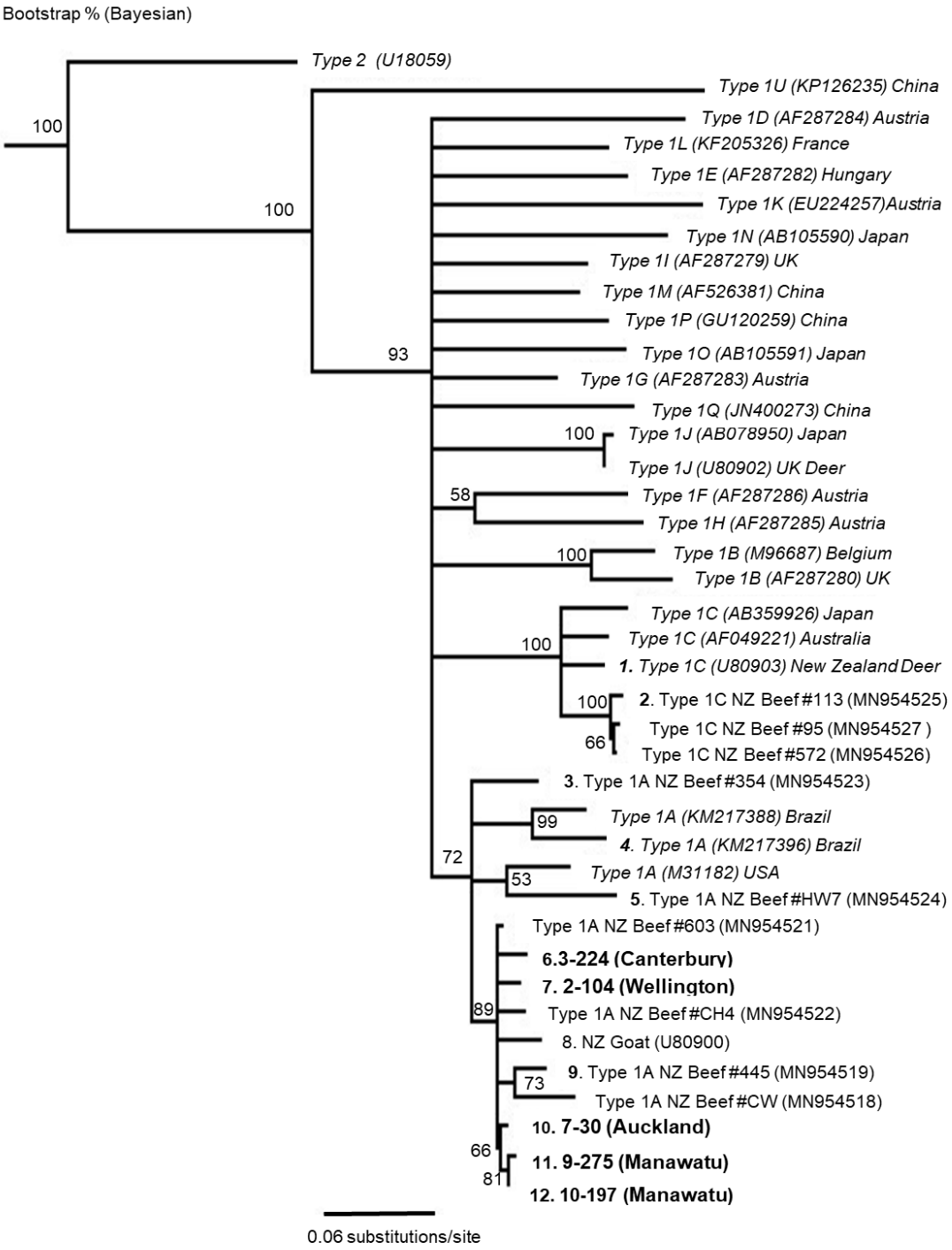

Figure 3.3 Phylogenetic analysis and comparison N^{Pro} sequences

*five representative BVDV isolates from New Zealand dairy cattle (bold) and previously published BVDV sequences representing type1A-U present in the GenBank. Names of the lineages (when available) and GenBank accession numbers of the sequences are given after the virus type grouping and location isolated is identified in within brackets. The branch lengths are drawn proportionally to the amount of changes (scale bar is shown).

Table 3.3 The sequence homology (in percentage) between 12 N^{Pro} gene representative isolates and known BVDV types

BVDV type	1	2	3	4	5	6	7	8	9	10	11	12
1. *Type1CNew Zealand Deer*	-											
2. Type 1C NZ Beef 113	93.4	-										
3. Type 1A NZ Beef 354	82.2	80.8	-									
4. *Type 1A Brazil*	78.7	77.4	89.5	-								
5. Type 1A NZ Beef HW7	81.2	80.5	89.2	86.1	-							
6. **3-224 (Canterbury)**	84.3	82.6	92.3	87.5	86.4	-						
7. **2-104 (Wellington**	86.1	84.3	93	89.2	88.2	96.9	-					
8. NZ Goat (U80900)	84.5	82.8	92.2	87.6	86.6.2	94.9	95.3	-				
9. Type 1A NZ Beef 445	83.6	81.9	92	87.1	87.1	95.1	95.5	94.3	-			
10. **7-30 (Auckland)**	85	83.3	93.4	88.9	87.8	97.2	97.6	96.3	97.2	-		
11. **9-275 (Manawatu)**	85.4	82.9	93.4	89.2	87.5	96.9	97.2	96	96.9	99	-	
12. **10-197 (Manawatu)**	85.7	83.3	93.7	89.5	87.8	97.2	97.6	96.3	97.2	99.3	99.7	-

[a]Numbers correspond to the numbers of BVDV Types in Figure 3 in which the phylogenetics of the BVDV isolates anf the GenBank assession numbers are given. Sequence homogeny was calculated with the use of a Jakes-Cantor model of submission. The new isolates are given in bold.

Table 3.4 Summary of 5'UTR and N^{PRO} isolates of BVDV from different locations in New Zealand analysed in the study

Region	Farm #	Isolate	Screening assay PCR	Grouping in 5`UTR	Grouping in Npro	GenBank accession number 5`UTR	GenBank accession number Npro
Otago	Farm 1	15-424	PCR	1A	-	Pending	
	Farm 2	370	PCR	1A	?	Pending	
	Farm 2	374	PCR	1A	+	Pending	
	Farm 2	381	PCR	1A	+	Pending	
	Farm 2	911	PCR	1A	+	Pending	
	Farm 2	955	PCR	1A	-	Pending	
	Farm 2	960	PCR	1A	-	Pending	
	Farm 2	475	PCR	1A	-	Pending	
	Farm 3	Black	PCR	+	-		
	Farm 4	443	PCR	+	?		
Southland	Farm 5	25-196	PCR	1A	?	Pending	
	Farm 5	205	PCR	+	-		
Canterbury	Farm 6	3-224	PCR	1A	1A	Pending	Pending
	Farm 7	4-320	PCR	1A	-	Pending	
	Farm 8	5-252	PCR	1A	-	Pending	
	Farm 9	130	PCR	+	-		
	Farm 10	26-106	PCR	1A	+	Pending	
Wellington	Farm 10	104	PCR	?	1A		Pending
Manawatu	Farm 11	16-584	PCR	1A	1A	Pending	
	Farm 12	12-50	PCR	1A	?	Pending	
	Farm 13	1	PCR	+	-		
	Farm 14	A15864	PCR	?	?		
	Farm 15	8-197	PCR	1A	1A	Pending	Pending
	Farm 15	9-275	PCR	1A	1A	Pending	Pending
	Farm 16	19(2)	PCR	1A	-	Pending	
	Farm 16	19(16)	PCR	1A	-	Pending	
	Farm 17	28-373	PCR	1A	-	Pending	

Waikato	Farm 18	189	PCR	+	-		
	Farm 19	11-574	PCR	1A	?	Pending	
	Farm 20	14-173	PCR	1A	?	Pending	
	Farm 21	18-111	PCR	1A	?	Pending	
	Farm 22	445	PCR	+	-		
	Farm 23	21-85	PCR	1A	+	Pending	
	Farm 24	739	PCR	+	-		
	Farm 25	17-39	PCR	1A	?	Pending	
	Farm 26	10-106	PCR	1A	?	Pending	
Auckland	Farm 27	1-27	PCR	1A	?	Pending	
	Farm 27	86	PCR	+	-		
	Farm 28	7-30	PCR	1A	1A	Pending	Pending
	Farm 28	6-87	PCR	1A	1A	Pending	
	Farm 29	29	PCR	+	-		
	Farm 29	7	PCR	+	-		
Bay of Plenty	Farm 30	17-50	PCR	+	-		
	Farm 31	13-1100	PCR	1A	-	Pending	
	Farm 32	84	PCR	+	-		
Northland	Farm 33	22-419	PCR	1A	-	Pending	
	Farm 34	80	PCR	+	-		
	Farm 35	18-127	PCR	+	-		

"+" indicates a positive PCR amplification but sample was not sequenced to determine BVD type

"?" indicates a positive PCR amplification but sequencing results was too poor quality to determine BVD type

"-" indicates unsuccessful PCR amplifications

3.4.5 Sensitivity of PCR vs ELISA/qPCR diagnostic lab

Out of 103 RNA extractions, only 48 (47%) samples resulted in a positive 5'UTR products of size 299bp after RT-PCR amplification. In order to identify the reason for the poor amplification rate of <50%, five samples that were negative for our RT-PCR sent to IDEXX laboratories (NZ) ULC, Palmerston North for confirmation of their positive BVDV status using the antigen ELISA. Results confirmed that the five samples were considered to be highly positive for BVDV, suggesting an issue with sensitivity and/or specificity resulting in poor test agreement between the diagnostic assays (qPCR and ELISA) and the RT-PCR assay and the primers used in this study.

3.5 Discussion

The preliminary results of this study revealed that only the BVDV subtype 1A was present in dairy cattle across New Zealand. These findings are constant with the 1A genotype identified in a study by Vilcek *et al* 1998 (Vilček et al., 1998) and a more recent study in beef cattle (Lal, 2019). To date, only BVDV 1-A and 1-C have been identified circulating in New Zealand cattle (Lal, 2019; Packianathan et al., 2017; Vilček et al., 2001) . Given the presence of BVDV 1-A throughout the country, it would appear that it could be considered an endemic genotype. This is in contrast to BVDV 1C which appears to be restricted to the Waikato region (Lal 2019). In this study, phylogenetic analyses of both the 5'UTR and N^{Pro} were examined. Even though all the dairy isolates studied belonged to the BVDV 1-A subtype a high degree of variation among the sequences within group 1-A were observed. However, whilst unique variation among isolates was observed, some isolates did appear to be highly conserved when compared to other isolates from the same farm (5'UTR Group 1A-1), among farms in the same region (5'UTR Group 1A-3 and -5) and between regions (5'UTR Group 1A-2 and -4). Therefore, it may be possible to use the sequence similarities and variations as a tool in tracing transmission patterns in disease control programs. For example, the genetic similarities of isolates from the same farm and different farms suggests on farm transmission and in between farm transmission. The direction of transmission using animal movement is important to identify and eliminate the source of the infection. Further, transmission data could be used to recommend biosecurity measures for individual farms that will help them target the specific pathways of re-introduction of BVD virus to the farm. Another use of having a good

understanding about the circulating BVDV strains on farms is that it may be possible to evaluate vaccine efficiencies as a control strategy.

In addition, some dairy BVDV isolates showed 100% sequence homology with New Zealand beef BVDV isolates which may be evidence there is virus transmission among beef and dairy cattle. Even though the direction of the transmission cannot be confirmed without historical animal movement data, the transmission could be suspected as from dairy industry into the beef industry since many surplus dairy calves in New Zealand are sold to the beef industry for fattening (Gates, 2021). About 20% of BVDV isolates in the world are recognized as BVDV-1A (Yesilbag et al., 2017). Therefore, it is not surprising that many of our dairy sequences showed genetic similarities with sequences that were originated from Korea, Brazil, UK, USA. In particular, some of the New Zealand dairy BVDV sequences showed high sequence similarity up to 100% with isolates of China. China remains one of the biggest markets of importing live cattle/ breeding stock from New Zealand the genetic similarities could be a result a of animal trading of breeding stock mixed with Trojan Dams (MPI, 2021).

The main limitations of this study were limitations in the samplings and detection methods. Considering the experimental expenses, serum samples were not collected specifically for this study therefore the timing and the number of samples for the study was dependent with a third party, in this case, diagnostic laboratories undertaking routine BVDV testing. The study was designed as a one-year project and the samples were received during COVID-19 outbreak period. This resulted in the sample size not being as large as expected and not representative of all the regions. An additional limitation was the analytical sensitivity and specificity of the RT-PCR used to amplify the viral isolate RNA for sequencing purposes. Since the performance of the RT-PCR has not been evaluated, it was difficult to predict the exact reason why almost 50% of the serum samples which were recorded as BVDV antigen positive by the commercial diagnostic laboratories, did not amplify in the RT-PCR used for sequencing.. The literature shows evidence that the two main types of test which are used by the New Zealand diagnostic laboratories, qPCR using TaqMan hydrolysis and IDEXX antigen ELISA tests, have 100 % sensitivity and specificity (Bhudevi & Weinstock, 2001; Dubovi, 2013; Hill, Reichel, McCoy, & Tisdall, 2007; MacPherson, 2019). Therefore, a possible reason for our negative test results could be low sensitivity of the primers and/or reaction conditions used for the study. It is also possible that the serum samples were not appropriately stored by the diagnostic laboratories whilst awaiting shipment to Massey University. Ideally, serum should be stored at -70^{0}C to

prevent damage to temperature sensitive RNA. Extended periods at 4^0C or frequent freeze-thaw cycles could affect the viability of RNA for later analysis.

Despite these limitations, this study remains the first to provide preliminary data on the BVDV genotypes circulating among New Zealand dairy cattle. It is hoped that the results of this study could be used to inform the use of molecular epidemiology with current BVD control strategies to develop system to eradicate the BVDV from New Zealand.

4. General Discussion

4.1 Introduction

This research was done with the purposes of (1) providing an overview of BVD diagnostic testing strategies to serve as a reference for making decisions about future BVD control programmes in New Zealand and (2) addressing the knowledge gaps of understanding the current circulating BVDV strains in New Zealand.

In **Chapter 2**, the non-systematic review broadly discussed the chronological evolution of BVD diagnostic tests over the past 60 years and how they have been applied to assist with herd level and industry level BVDV control strategies around the world. While advances in screening tests such as BTM testing and pooled serum antibody ELISA testing have had a significant impact on improving the cost-effectiveness of control programmes, there is clearly not a one-size-fits-all approach for their application. There are unique challenges in New Zealand due to the different demographic structure of pastoral farming systems including the highly seasonal nature of reproduction and the widespread use of extensive grazing practices. For beef herds, the young stock screening tests have more limited value because by the time the tests can be performed on animals over 10 months of age, it is generally too late in the calendar year to implement control measures to stop BVDV transmission if the results indicate that a PI animal may have been present. To overcome this problem, routine vaccination of replacement heifers and cows is highly recommended (J.-H. Han et al., 2020). In contrast, the dairy sector has shown progress with the widespread uptake of voluntary annual BTM screening tests (Gates, Evans, Heuer, et al., 2020), but without accurate information on the vaccination programmes used by each herd it can be difficult to interpret trends in the BTM antibody ELISA results. Another challenge in New Zealand compared with European countries is that cattle are not required to be ear tagged until they reach 6 months of age so although there are good diagnostic tests to screen calves for PI animals almost immediately after birth, there has not been significant uptake of these tests. Since dairy calves are handled more intensively after birth, there may be opportunities to push for increased calf testing to catch breakthroughs in biosecurity instead of needing to rely on vaccination to prevent the birth of PI animals. For both herd types, testing all the purchased breeding cattle for BVD and double fencing to prevent nose-to-nose contact with cattle from other herds over shared fence

lines remain important elements of BVD control to block the major routes of BVD transmission (J.-H. Han et al., 2018). The application of molecular epidemiology to assist with contact tracing may further aid in national BVD control in New Zealand and should be more thoroughly evaluated as part of future national disease control programmes if the cattle industries decide to progress towards a compulsory control programme.

In **Chapter 3**, a preliminary phylogenetic analysis was undertaken to determine the circulating BVDV strains among dairy cattle in New Zealand. In this study we used both UTR and N^{Pro} phylogenetic analyses to understand sequence patterns. The study showed that only the BVDV-1A genotype was circulating among the sampled dairy cattle. Since the study samples were non-representative of all the regions, it was difficult to conclude that BVDV1-A was the only circulating subtype among dairy cattle in New Zealand. Previous phylogenetic analyses have shown BVDV-1A among cattle in general and BVDV 1-C among beef cattle in one region of New Zealand (Lal, 2019; Vilček et al., 1998). These subtypes are the second and third most frequently-reported genotypes in the world (Yesilbag et al., 2017) and are highly associated with subtypes found in neighbouring countries (Robesova, Kovarcik, & Vilcek, 2009; Yesilbag et al., 2017). For example, the phylogenetic analysis showed a fair homogeny of New Zealand dairy isolates with previously isolated overseas isolates that were originated from Korea, Japan, UK, USA and Brazil, amongst all the BVDV strains of our study have shown more than 95% of homogeny (100% homogeny with some sequences) with previously identified China BVDV isolates. A recent study shows that BVDV 1-A and 1-C are the most frequent subtypes in China (Chang et al., 2021). China remains one of the biggest markets of importing live cattle breeding stock from New Zealand (MPI, 2021) from which unidentified Trojan dams could be a possible mode of transmission. Therefore, it will be very important to include a reliable screening approach for live imported cattle is New Zealand is to develop and maintain a BVDV-free status. It is also possible that New Zealand may have very few subtypes due to the isolated geographical location of the country and the existing strict rules of importation of live production animals to the country.

In this study, BVDV-1A was the subtype found among dairy cattle, but there was high sequence variation among the sequences within group 1-A. In the 5'UTR analysis, some isolates from the same farm appeared to be highly conserved and some had 100% sequence homology with isolates from different farms both within and outside of their region of origin. This finding suggests that there may have been a single introduction of BVD virus at one location which subsequently spread to other animals on both the same and other farms. However, this was not the case for all isolates where other farms had evidence of multiple strains which suggests that

BVD may have been introduced from multiple different sources. The genetic similarities of isolates from the same farm and different farms suggest that disease transmission takes place both on farm and between farms. These sequence similarities and variations can be used as a tool to trace transmission routes. Further molecular tracing is useful in BVD control programmes for disease free herds to infer whether new outbreaks are from exposure to positive animals within the herd or from new introductions from outside sources, which may help herds establish better biosecurity recommendations to prevent re-introduction.

In 5'UTR analysis, in particular, it was interesting to note that some dairy Isolates showed 100% homogeny with previously identified New Zealand beef cattle isolates. Although the transmission directions cannot be confirmed without having historical animal movement data, it is more likely that the strains are spreading from the dairy industry into the beef industry since many surplus dairy calves in New Zealand are sold to the beef industry for fattening (Gates, 2021) . There may be some transmission in the reverse direction since beef bulls are often used in dairy breeding programmes as clean-up bulls for animals that failed to conceive to artificial insemination (Gates, 2021).

4.2 Limitations

The main limitations of this study were limitations in the samplings and detection methods. As there were limitations in receiving adequate number of samples to represent the entire country, the findings of this study could not be used to reflect the national status of existing circulating BVDV strains in New Zealand dairy cattle. Therefore, to expand this study into a national level survey, the sample number should be increased so that it represents all regions of the country. Overseas, national phylogenetic surveys have been undertaken in England and China using approximately 100 confirmed virus positive samples from all the regions of the country in a particular time frame (Richard E. Booth et al., 2013b; Chang et al., 2021). However, this approach is more challenging in New Zealand due to the lack of required BVDV testing and restricted to only those farms already aware of potential BVDV issues and thus a biased sample. Ideally, to identify on-farm and inter-farms transmission in New Zealand dairy herds, an average of five BVDV positive animals from each farm as suggested (Gates, 2021). Another limitation of this study was the lack of clinical histories of the sampled animals with only the region where the samples were collected recorded. If the phylogenetic analysis is used as a tool

to trace the routes of the disease transmission, demographic and animal movement data should be included.

There were also considerable limitations of the RT-PCR used for this study. The performance of the of the primers, reagents, analytical sensitivity and specificity of the test have not tested in advance were a major limitation of the testing methods. Since we have not evaluated the performance of the test, it was difficult to confirm the exact reason why 50% of the serum samples which were recorded as BVDV antigen positive by the commercial diagnostic laboratories, did not amplify in the RT-PCR used for sequencing. However, one possible reason could be low sensitivity of the primers. There are several different primers identified in the literature for the amplification of the Npro and 5'UTR BVDV regions. Future studies should examine these other primer sets in the reaction mix used in this study to determine if primers are the reason for the poor sensitivity. Another reason could be the denaturation of the samples if that were not appropriately stored by the diagnostic laboratories whilst awaiting shipment to Massey University. It will be important for future work ensure that sample storage and management are optimal and consistent in order to limit potential storage related sample degradation.

4.3 Future directions

In order to make better inferences of the circulating BVDV strains across the country, this research should be extended into a national phylogenetic survey that includes both dairy and beef samples with adequate number that represents all the regions of the country. A national gene bank should be established and updated to understand the strain variation patterns, new infections and virus evolutions that would be helpful to develop vaccines and to determine the vaccine efficiencies in disease control programs. Standard procedures and protocols for virus sequencing should be introduced so that it could minimize the inconsistencies of virus isolates and will be helpful to use as references in future genetic studies. As BVDV has also been shown to infect a range of other ruminant species including sheep and deer, genetic typing could be applied in future studies to understand the transmission patterns among cattle and other ruminants in New Zealand.

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6. Appendices

Appendix 1: Purification of viral RNA (spin protocol)

The protocol is for purification of viral RNA from 140 µl serum. Larger volumes up to 560 µl can be processed by increasing the initial volumes proportionally and loading the QIAamp Mini spin column multiple times, as described below in the protocol.

1. 650 µl of prepared Buffer AVL containing carrier RNA was pipetted into a 1.5 ml microcentrifuge tube.
2. 140 µl of plasma was added to the Buffer AVL-carrier RNA into a microcentrifuge tube and was mixed for 15 seconds by pulse-vortexing.
3. The mixture was incubated for 10 minutes and briefly centrifuged the tube to remove drops from the inside of the lid.
4. 560 µl of ethanol (96-100%) was added to the sample and mixed by pulse-vortexing for 15 seconds.
5. 630 µl of the lysate was added into the QIAamp Mini column without touching the Mini column membrane with the pipette tip and was centrifuged at 6000 x g for 1 minute and placed the QIAamp mini column into a clean 2 ml collection tube after discarding the tube containing filtrate.
6. The remaining solution of step 4 was added to the mini column and centrifuged for 6000 x g for a one minute and the filtrate was removed, and the mini column was placed into a 2 ml collection tube.
7. 500 µl of Buffer AW 1was added to the Mini column and centrifuged at 6000 x g for one minute and the filtrate was discarded, and the mini column was placed into a 2 ml collection tube provided.
8. 500 µl of Buffer AW 2 was added to the Mini column and centrifuged for 20000 x g for three minutes and the Mini column was placed into a new 1.5 ml centrifuge tube (not provided) after discarding the filtrate.
9. 60 µl of Buffer AVE was added to the Mini column placed in 1.5 ml centrifuge tube and incubated in room temperature for one minute.

10. The tube was centrifuged at 6000 x g for one minute and the Mini column was discarded and the filtrate (RNA) was stored in -810C until use for the PCR.

Appendix 2: RT-PCR for BVD-5'UTR using primers P1/PEST

Nucleic acid extraction	Kit
RNA	Qiagen QIAamp Viral RNA extraction kit.

Primers	Name	Sequence (5'-3')	Size	Target
Forward	PI-U	5'-AGAGGCTAGCCATGCCCTTAGT-3'	300 bp	5'UTR
Reverse	PEST	5'-TCAACTCCATGTGCCATGTAC-3'		

PCR kit: Invitrogen Superscript III RT/Platinum Taq kit

Reagent mix	RT-PCR Round Volume (50µL)
Sterile distilled water	19.0
2 X reaction buffer	25
10 µM P1 Primer	0.2 uµ
10 µM PEST Primer	0.2 uµ
RT/Taq enzyme	0.2 uµ
RNA	1 µg

PCR Controls	Description
Positive	BVD viral isolates in -80^{0}C freezer
Negative	Nuclease free water

PCR Program for 5'UTR

Cycling parameters: RT-PCR	Temp (ºC)	Time	No. cycles
Hold	42	15 min	1
Hold	99	5 min	1
Denature	94	45 sec	40
Anneal	60	1 min	

Extension	72	1 min	
Hold	72 4	7 min ∞	1

Electrophoresis	**Description**	**Size of amplicons(bp)**
Agarose gel	1.5%	300 bp
MW marker	100 bp	

References: Grom, J. and Barlic-Maganja D. (1999) Bovine viral diarrhoea (BVD) infections-control and eradication programme in herds in Slovenia. Veterinary Microbiology, 64:259-264.

Appendix 3: RT-PCR for BVD-N^{Pro} using primers B31/B32

Nucleic acid extraction	Kit
RNA	Qiagen QIAamp Viral RNA extraction kit.

Primers	Name	Sequence (5'-3')	Size	Target
Forward	PI-U	5'- CCATCTATRCAYACATARATGTGGT-3'	441 bp	N^{Pro}
Reverse	PEST	5'- TGCTACTAAAAATCTCTGCTGT -3'		

PCR kit: Invitrogen Superscript III RT/Platinum Taq kit

Reagent mix	RT-PCR Round Volume (50µL)
Sterile distilled water	19.0
2 X reaction buffer	25
10 µM P1 Primer	0.2 uµ
10 µM PEST Primer	0.2 uµ
RT/Taq enzyme	0.2 uµ
RNA	1 µg

PCR Controls	Description
Positive	BVD viral isolates in -80^0C freezer
Negative	Nuclease free water

PCR Program for 5'UTR

Cycling parameters: RT-PCR	Temp (ºC)	Time	No. cycles
Hold	45	30 min	1
Hold	94	5 min	1
Denature	94	45 sec	40
Anneal	60	1 min	

Extension	72	1 min	
Hold	4	∞	

Electrophoresis	**Description**	**Size of amplicons(bp)**
Agarose gel	1.5%	441 bp
MW marker	100 bp	

References: Grom, J. and Barlic-Maganja D. (1999) Bovine viral diarrhoea (BVD) infections-control and eradication programme in herds in Slovenia. Veterinary Microbiology, 64:259-264.

Appendix 4: Gel electrophoresis procedure

1. 1.5 % ultra-pure agarose gel (Invitrogen, Waltham, MA, USA)was used for the gel preparation. 0.6 g of Agarose was used for 40 ml of gel preparation.
2. 10 x TBE buffer was used to make agarose gel (0.6 g Agarose with 40 ml of x TBE)
3. The mixer was put in a microwave to dissolve properly and placed the mixture in a water bath to make it slightly cold.
4. 0. 2 µl of RedSafe (iNtRON Biotechnology, Kirkland, WA, USA) was added as the staining solution.
5. The gel combs were placed in the gel plate and the solution was poured into the plates and left for about one hour to set the gel properly.
6. 1 x TBE was poured into the electrophoresis tank and the gel was placed in the tank after removing the comb.
7. The gel loadings were prepared and loaded to the gel as below.
    a. Ladder was prepared (3 µl of loading dye + 5 µl of distilled water + 5 µl of molecular marker) and 10 µl of the mix was added to the first well.
    b. Samples were prepared (3 µl of loading dye + 10 µl of sample) and 10 µl of the mixer was added to the wells one by one and the last two wells were loaded with negative control and positive control respectively.
8. The gel was run in 100 V for an hour and the gel was inspected under a UV light, using a transilluminator for bands that matched the expected sizes of the genes of interest.
9. Positive amplicons were purified using PureLink^{R} PCR purification kit.

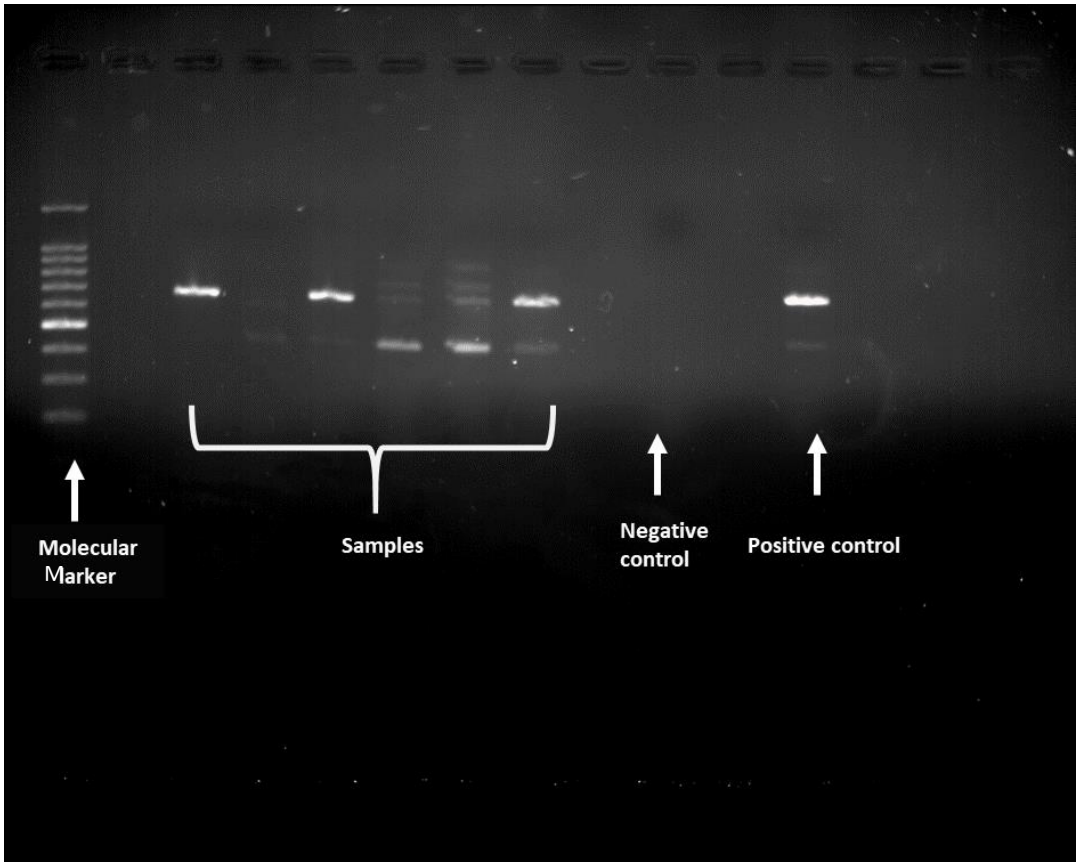

Figure: An image of gel electrophoresis

Appendix 5: Protocol for purification of PCR products

PureLink^{R} PCR Purification kit was used for cleaning PCR products

1. 200 ml of binding buffer was added to B2 bottle (supplied with the kit), of it 160 µl of B2 was added to 40 µl of PCR products.
2. All the mix was put into the PureLink^{R} Spin column in the collecting tube and centrifuged at 10,000 x g for one minute and the flow-through was discarded.
3. The column was re-inserted into the collection tube and 650 µl of wash buffer (W1) was added and centrifuged for 10000 x g for one minute and
4. The flow-through was discarded and the column was placed in the same centrifuge tube and centrifuged at 20000 x g for three minutes.
5. The column was placed into a 1.7 µl elution tube that comes with the kit and 50 µl of elution buffer was added to the centre of the column.
6. The elution tube was incubated in room temperature for one minute and was centrifuged at 20000 x g for two minutes.
7. The sip column was removed and the purified PCR products in the elution tube was stored at -20^{0}C until send for sequencing.