1-1-2012

Diagnosis and Characterization of Bovine Viral Diarrhea Virus

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DIAGNOSIS AND CHARACTERIZATION OF BOVINE VIRAL DIARRHEA VIRUS

By

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A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctorate of Philosophy
in Veterinary Medical Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

May 2012

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Title of Study: DIAGNOSIS AND CHARACTERIZATION OF BOVINE VIRAL DIARRHEA VIRUS

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Bovine viral diarrhea virus (BVDV) is an important viral pathogen affecting all ages of cattle, resulting in significant economic losses worldwide. BVDV infection is associated with a diverse array of symptoms including gastrointestinal disorder, respiratory distress, fetal malformation, stillbirth, abortions, and mucosal disease (MD). Transplacental infections of fetuses between 42 and 125 days of gestation can result in immune-tolerance and the surviving fetuses become persistently infected (PI). PI animals are major reservoir of BVDV and it becomes problematic to control the disease. The objectives of this dissertation were to: 1) develop a cost-effective testing scheme to detect BVDV PI animals from exposed herds, 2) characterize two virulent BVDV-2 Mississippi isolates associated with severe hemorrhagic diseases, and 3) perform phylogenetic analysis based on sequences of 5'UTR, E2, and NS5B regions. First, we developed a BVDV testing scheme by combining pooled real-time RT-PCR with antigen capture enzyme-linked immunosorbent assay (ACE) to screen cattle herds. From positive pools individual positives were identified using ACE. Data from a three year period indicated that 92.94% PI animals were infected with BVDV-1, 3.53% with BVDV-2, and 3.53% with both BVDV-1 and BVDV-2. Analysis of the 5'UTR of 22 isolates revealed

the predominance of BVDV-1b followed by BVDV-2a. Second, two virulent BVDV isolates, M10-3432 and M10-5347, were successfully recovered from an adult beef breeding cow and feedlot calf respectively. When compared to the reference strain BVDV-2 125c, five and three unique amino acids in E2 regions were different from M10-5347 and M10-3432 respectively. Phylogenetic analysis of E2 region grouped both Mississippi isolates in BVDV-2a, a subtype containing high virulent strains. M10-3432 was clustered with high virulent strain 890 while M10-5347 was clustered with high virulent strain CD87. Third, we compared the phylogenetic analyses of BVDV based on the sequences of 5'UTR, E2, and NS5B at either nucleotides or amino acids level. Although slight differences were observed, the virulent BVDV isolates were consistently classified into BVDV-2a cluster regardless of region of sequences used. Furthermore, phylogenetic tree constructed using combined two or more regions had higher posterior probability and bootstrap value than phylogenetic trees constructed using a single region

DEDICATION

I would like to dedicate this dissertation to my parents, Shengyu Yan and Yingying Wang.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to the people who spend time and effort in supporting as well as critiquing this dissertation. First I would like to thank my major professor Dr. Chinling Wang and co-major professor Dr. Lanny Pace to make it possible to finish this dissertation on time. Especially I would like to thank my committee member Dr. Mike Zhang, my former major professor for the past few years, who guided my research work in the Mississippi Veterinary Research and Diagnostic Laboratory. Second, I would also like to express my gratitude to all my committee members: Dr. Alejandro Banda, Dr. Henry Wan, and Dr. Andy Perkins for their suggestions and help for my dissertation. Also I would like to give special thanks Dr. Shuping Zhang who gave me a lot of suggestions and helped my research and paper writing. Finally, I would like to thank pathologists Dr. Brittany Baughman and Dr. Floyd D. Wilson who supplied the pathology and necropsy data and interpreted immunohistochemistry slides for me. At last, I would like to thank the staff at the Mississippi Veterinary Research Diagnostic and Laboratory – Anthony Liu, Tina Hay, Candy Zhang, and Gabriel Senties-Ramirez, Jay Kay Thornton, Latoya Sly, Corey Williams, Abdul Tariq Muhammad, Calvin Vaughn, and all other CVM students and staff who helped me in preparation of my dissertation.

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Yan L, Zhang S, Pace L, Wilson F, Wan H, Zhang MZ. 2011. A combination scheme of real time RT-PCR and antigen captured ELISA screens the BVDV infected animals. J Vet Diagn Invest 23(1): 16-25

Zhang M, Yan L, Zhu G, Holifield M, Todd D, Zhang S. Mar 2012. Isolation and characterization of Streptococcus troglodytidis sp. nov., from a foot abscess of a chimpanzee (Pan troglodytes). Int J Syst Evol Microbiol.

Aldwairi TA, Yan L, Weerasinghe KS, Perkins AD. Mar 2012. Comparison of miRNA target prediction algorithm using computational methods. 4th International Conference on Bioinformatics and Computational Biology (BICoB). Las Vegas, Nevada

Li S, Zhang MZ, Yan L, Lillehoj H, Pace LW, Zhang S. 2011. Induction of CXC chemokine messenger-RNA expression in chicken oviduct epithelial cells by *Salmonella enterica* serovar Enteritidis via the type three secretion system-1. Avain Dis 53(3): 396-404

Baughman B, Zhang S, Jin L, Pace LW, Cooley J, Yan L, Zhang M. 2011. Laboratory diagnosis of deerpox virus infection in a white-tailed deer fawn. In press in J Vet Diagn Invest 23(5): 965-970

ABBREVIATIONS

Akaike information criterion: AIC

Antigen capture enzyme linked immunoabsorbent assay: ACE

Blue tongue virus: BTV

Border disease virus: BDV

Bovine viral diarrhea virus: BVDV

Classical swine fever virus: CSFV

Cytopathic: CP

Infectious Bovine rhinotracheitis virus: IBR

Immunohistochemistry: IHC

Indirect fluorescent antibody: IFA

Maximum parsimony: MP

Maximum likelihood: ML

Mucosal disease: MD

Neighboring Joining: NJ

Non cytopathic: NCP

Persistently infected: PI

Real-time reverse transcription polymerase chain reaction: real-time RT-PCR

Serum neutralization: SN

Virus isolation: VI

CHAPTER I

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a major viral pathogen of cattle and results in significant economic losses worldwide by infecting cattle and contaminating biological products. *Classical swine fever virus* (CSFV), *Border disease virus* (BDV), BVDV, and tentative pestivirus giraffe-1 virus are members of genus *Pestivirus* of the family *Flavivirdae*. BVDV is classified into two biotypes cytopathic (CP) and noncytopathic (NCP) based on the presence or absence of visible cytopathic effect (CPE) in infected cell cultures. BVDV is also divided into two genotypes - BVDV-1 and BVDV-2 - based on genetic variation.

BVDV infection is associated with a diverse array of symptoms including gastrointestinal disorder, respiratory distress, fetal malformation, stillbirth, abortion, and mucosal disease (MD). BVDV can infect all ages of cattle including fetuses. Trans-placental infections of the fetuses between 42 and 125 days of gestation can result in immune-tolerance and the surviving fetuses become persistently infected (PI). PI animals are associated with higher incidence of respiratory diseases and associated risk for treatment, and PI animals shed a large amount of virus from their body secretions and excretions as the reservoir of BVDV (McClurkin et al. 1984; Baker 1995; Loneragan et al. 2005b). Therefore, identification and subsequent removal of PI animal(s) from exposed herds plays an important role in BVDV prevention and eradication.

Clinical manifestations caused by BVDV vary from clinically unapparent to clinically severe depending on the virulence of the BVDV-2 strains. Most of virulent BVDV strains belong to BVDV-2 genotype, whereas the avirulent BVDV-2 isolates occurred more frequently than did the virulent BVDV-2. However, little is known about the virulence of BVDV-1 strains. Genetic mutations in envelop proteins may lead to antigenic variations as well as the virulence of BVDV, which complicate the BVDV diagnosis and prevention. In this study, we isolated two virulent BVDV-2 isolates from Mississippi herds, which were not detected in fresh tissues using direct fluorescent antibody. There is a need for evaluating the effectiveness of the commonly used detection methods for BVDV.

Phylogenetic analysis of BVDV has been widely used for revealing the evolutionary history of new isolates and classifying them on the basis of the molecular sequencing data. The highly virulent strains of BVDV associated with hemorrhagic syndrome discovered in North American in recent years are genotyped as BVDV-2 using phylogenetic analysis of 5'UTR region. Currently many new computational methods are available to be used in phylogenetic analysis of BVDV.

The main objectives of this study were:

1) To develop a BVDV testing scheme by using a combination of real-time RT-PCR and antigen capture enzyme-linked immunosorbent assay (ACE) for the detection of persistently infected animals

2) To characterize two virulent BVDV isolates which were not detectable using direct fluorescent antibody test from tissue samples of animals with severe clinical disease and fatal outcome

3) To perform phylogenetic analysis of BVDV based on sequences of 5'UTR, E2, and NS5B regions

CHAPTER II

REVIEW OF LITERATURE

Bovine viral diarrhea virus (BVDV) is a major viral pathogen affecting cattle and resulting in significant economic worldwide losses. In the 1940s, BVDV was described as "X disease" in western Canada and as "Bovine viral diarrhea" (BVD) in Cornell University. Symptoms included severe diarrhea, depression, anorexia, ulceration of oral mucosa, a high rate of morbidity, and a relatively low rate of mortality (Olafson et al. 1946). BVDV was first classified into the *Togaviridae* family in the 1970s (Fenner and Maurin 1976). Together with classical swine fever virus (CSFV), border disease virus (BDV), and tentative pestivirus giraffe-1 virus, BVDV is classified into the genus *Pestivirus* of the *Flaviviridae* family in the VIIIth report of the International Committee on the Taxonomy of Viruses (Thiel et al. 2005).

2.1 Physical properties and taxonomy

Pestivirus virions are composed of a lipid bilayer envelop with embedded transmembrane glycoproteins that surrounded a protein-RNA nucleocapsid with a diameter of 40-to-60 nm and an inner core with an approximate density of 30-nm electrons (Zuckerman 1981). Pestiviruses can survive over a relatively broad pH range, but they can be inactivated by heat, organic solvents, and detergents (Liess 1981; Rümenapf et al. 1991). BVDV and CSFV were able to be purified at buoyant sucrose of 1.134 g/ml and sediment at 100 Svedberg units (Laude 1987).

Based on the presence or absence of visible cytopathic effect (CPE) in infected cell cultures, BVDV is distinguished into two biotypes: cytopathic strain (CP) and noncytopathic strain (NCP) (Lee and Gillespie 1957). Both CP and NCP BVDV express the nonstructural proteins (NS) 2-3, but the generation of NS3 strictly correlates with the CP BVDV (Meyers and Thiel 1996; Kümmerer et al. 2000). BVDV is also classified into two genotypes - BVDV-1 and BVDV-2 - based on the genetic variation (Ridpath et al. 1994b). BVDV-1 was isolated more frequently from cattle with respiratory symptoms than BVDV-2 (Fulton et al. 2000). In early studies, BVDV-1 was typed into two groups - BVDV-1a and BVDV-1b - in the USA and Canada (Pellerin et al. 1994; Ridpath and Bolin 1998). With the increase of new BVDV-1 isolates, BVDV-1 was classified into at least 11 genetic groups around the world based on the phylogenetic analysis of 5'-untranslated region (5'UTR) and N-terminal protease fragment (N^{pro}) (Vilcek et al. 2001). BVDV-1b can be clustered further into 1b1 and 1b2 subtypes based on the variation of E2. Strains CP7 and NY1 were clustered into 1b2, whereas Osloss strain was clustered into 1b1. The predominant BVDV-1b strain is 1b2 in the United States, and there has been relatively small change of 1b2 during a 50-year period (Tajima and Dubovi 2005). Based on the genetic variation of 5'UTR and NS2/3, BVDV-2 was typed into two groups 2a and 2b (Flores et al. 2002). However, symptoms vary from clinically unapparent to clinically severe depending on the virulence of the BVDV-2 strains. The hemorrhagic syndrome of BVDV infections was associated with BVDV-2; however, the CPE of BVDV does not correlate with virulence. In nature, the avirulent BVDV-2 isolates occurred more frequently than did the virulent BVDV-2 (Ridpath et al. 2000). New BVDV-2 isolates were identified from South America, and these new isolates were typed into 2b, 2c, and 2d. Limited sequence information exists for 2c and 2d (Giangaspero et al.

2008). BVDV-1a, BVDV-1b, and BVDV-2a are major genotypes isolated in North America (Ridpath et al. 2000; Ridpath 2010).

2.2 Molecular characteristics

The BVDV genome is single-stranded and positive-sense RNA with ~12.5 kb, possesses a single open reading frame (ORF), and encodes about 4000 amino acids. The BVDV long ORF was flanked by 5'UTR (372-385 nucleotides in length) and 3'UTR (229-273 nucleotides in length) (Collett et al. 1988b; Lindenbach et al. 2007) . The BVDV genome lacks a cap structure at 5' terminus and terminates with a short poly (C) instead of a poly (A) at 3' terminus (Brock et al. 1992). Among coding regions, the first third of the ORF encodes an autoprotease N^{pro} and four structural proteins C, E^{rns}, E1, E2, while the 3' end of the RNA genome encodes the other presumably nonstructural proteins: p7, NS2-3, (NS2), (NS3), NS4A, NS4B, NS5A, and NS5B (Meyers and Thiel 1996; Lindenbach et al. 2007) (Figure 2.1).

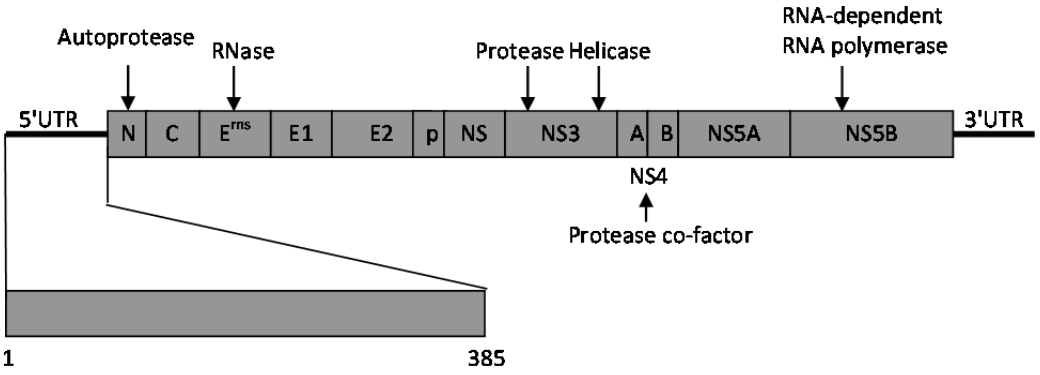

Figure 2.1 BVDV genome structure

Pestivirus-unique nonstructural protein N^{pro} refers to an N-terminal autoprotease that is released from the polyprotein precursor, and pestivirus-unique envelope glycoprotein E^{rns} refers to the RNase activity (Wiskerchen et al. 1991; Ridpath and Bolin

1995). The activity of host signal peptidases gives rise to C, E^{rns}, and E2, which are destined to form the capsid and the envelope of the virion (Lindenbach et al. 2007). Both E1 (gp33) and E2 (gp55) are integral membrane proteins, which contain two-to-three and four-to-six N-linked glycosylation sites, respectively (Weiland et al. 1990). The glycoproteins E^{rns}, E1, E2 are composed of the envelope of BVDV, and the E^{rns} and E2 form a disulphide-linked homodimer. E1 is associated with E2 by forming a heterodimer, and E1 is assumed to be a membrane anchor of E2. All glycoproteins - E^{rns}, E1, and E2 - are associated with virus attachment and/or cell entry (Weiland et al. 1990; Rümenapf et al. 1993b). Both CP and NCP BVDV express NS2-3, but the generation of NS3 strictly correlates with the CP phenotype. The study of the genetic characterization of NCP-CP pairs indicates that most CP BVDV strains are generated from NCP pestivirus by RNA recombination (Meyers et al. 1996; Pellerin et al. 1995). RNA recombination includes host cell derived insertions, sometimes combined with large duplications or deletions of viral sequences (Meyers et al. 1996). NS3 possesses multiple enzymatic activities - serine protease, NTPase, and helicase activity - and NS5B represents the RNA-dependent RNA polymerase (RdRp) (Pankraz et al. 2005). Two hydrophobic proteins, NS4A and NS4B, are similar in size and composition. NS4A possesses serine protease cofactor activity (Xu et al. 1997), and NS4B associates with the rearranged host membranes and Golgi apparatus (Weiskircher et al. 2009). A large and hydrophilic phosphor-protein, NS5A, possesses the zinc-binding properties required for RNA replication (Tellinghuisen et al. 2006). Replication of the virus genome occurs in the cytoplasm of the infected cell along with the translation process. The virus-encoded RdRp of NS5B is necessary for the replication of the RNA genome, and RdRp is a key enzyme among members of the virus family *Flaviviridae* (Steffens et al. 1999).

2.3 Replication, transcription, assembly, and release of virus particles

BVDV infection of the host cell involves multiple steps, including the attachment of virions, interaction with specific receptors, internalization, and membrane fusion (Hulst and Moormann 1997; Liang et al. 2003; Lecot et al. 2005). BVDV replication takes place in the cytoplasm; the viral RNA functions as a messenger RNA in the cytoplasm of the host cell and directs the synthesis of an unstable polyprotein which is co- and post-translational processed into at least 11 mature structural and nonstructural viral proteins (Gray and Nettleton 1987; Collett et al. 1988a; Rümenapf et al. 1993a; Rümenapf et al. 1993b). BVDV enters the host cell by clathrin-dependent receptor-mediated endocytosis and decapsidation of the nucleocapsids; the viral genome serves as a template for the translation of viral proteins (Poole et al. 1995; Grummer et al. 2004). No cap structure exists among pestiviruses, which rely on internal ribosomal entry sites (IRES) of 5'UTR to mediate the initiation of translation. The 5'UTR contains essential structural or functional components that may extend into nucleotide sequences within the ORF, and three domains and a pseudoknot structure are required for IRES activity (Gong et al. 1996; Lindenbach et al. 2007). During the translation of Pestivirus, nonstructural proteins assemble into a functional replicate complex to catalyze transcription of positive-sense RNA into complementary strand negative-sense RNA, which provides a template for the replicate complex to synthesize nascent positive-sense RNA using a semi-conservative asymmetric replication model (Warrilow et al. 2000). The model includes three virus-specific RNAs: a double-stranded replicative form (RF), a partially single-stranded and partially double-stranded replicative intermediate form (RI), and a single-stranded viral RNA. The process of BVDV replication begins with a positive strand replicate complex comprised of viral and cellular components formed at the 3’

terminus of the genome (Hietala and Crossley 2005). Through semi-conservative asymmetric replication, BVDV replication can be detected in cell culture models within four-six hours of infection by the finding of negative-strand RNA and nascent positive-strand RNA. The peak of BVDV titer is detected at 12-24 hours post-infection (Gong et al. 1996).

Little information is available on the assembly and release of virions from infected cells. Electron microscopic examination shows that BVDV virions appear to mature in intracellular vesicles and are released through exocytosis (Gray and Nettleton 1987; Hietala and Crossley 2005; Lindenbach et al. 2007).

2.4 Pathogenesis of BVDV infections

BVDV has two biotypes, CP and NCP, and two genotypes, BVDV-1 and BVDV-2. A study screening cattle with respiratory disease indicates that NCP BVDV was isolated more frequently than was CP BVDV, and BVDV-1 genotypes were isolated more frequently than BVDV-2 genotypes in cattle with respiratory disease, based on necropsy samples of calves with fibrinous pneumonia (Fulton et al. 2000).

BVDV infection is associated with a diverse array of manifestations, including gastrointestinal disorders, respiratory distress, fetal malformations, stillbirth, abortions, persistent infections (PI), and mucosal disease (MD) (Baker 1995). BVDV can cross the placenta and infect fetuses of all ages, and the result of infection varies depending on the stage of gestation in which infection occurs. Trans-placental infections of a fetus between 42 and 125 days of gestation can result in abortion, stillbirth, weak calf, and immune-tolerance. The calf's immature immune system lacks the ability to recognize the persisting NCP virus as foreign, and the animals are considered immune tolerant. If a

fetus survives after infection, they will develop as animals persistently infected by BVDV and will not produce neutralizing antibodies (McClurkin et al. 1984). Fetal exposure to BVDV before 42 days of gestation may result in embryonic death, and cattle infected with BVDV during this period will have lower fertility than non-infected cattle (Muñoz-Zanzi et al. 2004). Conversely, exposure after day 125 of gestation may result in a fetus that is able to mount an immune response to a BVDV infection because the immune system develops during the later stages of gestation (McClurkin et al. 1984).

Persistently infected animals, reservoirs of BVDV, continually shed large amounts of virus throughout their lives via body secretions and excretions (McClurkin et al. 1984; Baker 1995; Loneragan et al. 2005). Although CP BVDV is able to cross the placental, PI animals were caused with the infection of NCP BVDV strain. Most BVDV isolates are NCP but yield CP variants after repeated passage in cell culture, but NCP BVDV appeared to cause abortion more readily than did the CP BVDV (McClurkin et al. 1984; Brownlie et al. 1989).

Acute infection of BVDV ranges from subclinical to highly fatal depending on the viral strain, host immune status, host reproductive status, and the presence of other pathogens. In most cases, NCP viruses cause the acute BVDV infections (McClurkin et al. 1984; Houe 1999). Virus isolation is generally possible between three and ten days post-infection. Most clinical results are mild following the acute infection, which includes diarrhea (most of time present following acute infection) and leucopenia.

In the late-1980s, a severe hemorrhagic syndrome in adult cattle and veal calves was reported in several countries, and this severe hemorrhagic syndrome was associated with NCP BVDV without an accompanying CP strain. This BVDV hemorrhagic syndrome was characterized by fever and marked thrombocytopenia resulting in bloody

diarrhea, epistaxis, petechial and ecchymotic hemorrhages on mucosal surfaces, and bleeding from infection sites (Corapi et al. 1989).

Mucosal disease is the most severe disease associated with BVDV infection and is usually fatal; it often occurs between the ages of six months and two years (Baker 1995). Persistent infected animals are immunotolerant and recognize BVDV as self-antigens. When NCP PI animals were superinfected with CP BVDV that matches the antigen pattern of the NCP, the PI animals will develop mucosal disease. Therefore, both CP and NCP biotypes of BVDV can be isolated from mucosal disease (McClurkin et al. 1984).

2.5 Virulence

The most virulent BVDV strains were characterized as the BVDV-2 genotype, and limited information is available regarding the virulence of BVDV-1 strains (Bolin and Ridpath 1992; Corapi et al. 1989; Pellerin et al. 1994; Ridpath et al. 2007). BVDV-2 was isolated from bovine with hemorrhagic syndrome characterized by thrombocytopenia and high mortality (Rebhun et al. 1989; Bolin and Ridpath 1992; Pellerin et al. 1994) and bovine with mild clinical forms (Topliff and Kelling 1998). Research indicated that the hemorrhagic syndrome caused by BVDV-2 is noncytopathic; therefore, the virulence of BVDV is not associated with cytopathic effect *in vivo* (Ridpath et al. 2000). Highly virulent BVDV infections associated with fever, pneumonia, diarrhea, abortion, death, and hemorrhages on mucosal surfaces and various internal organs have been reported among all ages of cattle in North America (Corapi et al. 1989; Alves et al. 1996). Compared to the highly virulent infection, the low virulence isolates caused mild respiratory tract diseases without diarrhea (Odeon et al. 1999; Kelling et al. 2002). Calves infected with highly virulent BVDV-2 isolates (23025 or 17583) develop more noticeable

lesions and more severe signs of clinical disease than do calves inoculated with low virulence BVDV-2 such as 713, 5521, or 17011 isolates (Kelling et al. 2002). BVDV infection forms were recognized as mucosal disease, chronic BVD (a form of MD), mild-acute BVD, and severe BVD. BVDV-2 high virulence isolates were isolated from severe acute BVDV infections in North America in the early 1990s. BVDV-2 strains 1373 (NCP) and p24515 were involved in the Ontario epidemic, and strain IAF_103 was involved in the Quebec epidemic. BVDV-2 NY93 was isolated from the severe acute BVDV outbreak of a dairy herd in New York in 1993 (Corapi et al. 1989; Pellerin et al. 1994; Ridpath et al. 1994a). BVDV-2 CD87 was isolated from a severe acute BVD outbreak in New York, and its infection was associated with high fever, bloody diarrhea, hemorrhages, and prolonged bleeding from venepuncture sites (different from severe acute BVD, called hemorrhagic syndrome) (Corapi et al. 1989). BVDV-2 strain 890 (NCP) was also isolated from a yearling heifer in Canada that died with extensive internal hemorrhages (Bolin and Ridpath 1992). BVDV-2 strain JZ05_1 was a low virulence and cytopathic strain isolated in China (Li et al. 2010a), and limited information is available regarding the virulence of BVDV-1 strains

The translation efficiency of high virulence isolates is greater than low virulence, resulting in variation in the regulation of transcription and translation between high virulence and low virulence isolates. Primary and secondary structure differences may influence the translation efficiencies (Topliff et al. 2005). Unlike DNA motifs, RNA motifs are more conserved in structures than they are in sequences. The conserved region of pestiviruses 5'UTR contains the IRES (Goens 2002), which is involved in the cap-independent translation initiation of viral RNA (Rijinbrand et al. 1997). The 3'UTR also harbors primary and secondary RNA structures, which probably function *in cis* to direct

minus-strand initiation (Yu et al. 2000; Isken et al. 2004; Pankraz et al. 2005). The genome structure and mechanism of viral translation initiation in pestivirus shows strong similarity with *Picornaviridae* (Poole et al. 1995). Research of the 5'non coding region of poliovirus, a member of *Picornaviridae*, indicates that point mutation within the one stem loop of an RNA structure is sufficient to attenuate the neurovirulence of poliovirus (Skinner et al. 1989; Stewart and Semler 1998). Secondary structure analysis of the 5'UTR of BVDV divides the region into four domains: I-IV (NADL as the reference). Domain I spans from one to 70 (Ia and Ib), domain II spans 77-140, domain III spans 153-323, and domain IV spans from 370-385 (Brown et al. 1992). The proper folding of the Ia structure is important to the initiation of efficient translation. However, Ia motif is not critical for RNA replication. The stem of this structure is constituted mainly of conserved nucleotides of pestivirus, but the loop region is predominantly composed of variable residues (Yu et al. 2000).

High virulence BVDV-2 was reported and characterized as NCP based on its activity in cultured epithelial cells (Bolin and Ridpath 1992). Research indicates that the 5'UTR of BVDV relates to the virulence among BVDV-2 genotype; two nucleotide positions, 219 and 278, were identified in the internal ribosomal entry site that distinguishes the high virulence from the low virulence genotype 2 isolates. The low virulence strains have a cytosine (C) and a uracil (U), respectively. However, the high virulence strains have a U and a C, respectively. The substituted bases are virulence markers used to differentiate BVDV-2 isolates (Topliff and Kelling 1998).

The activity of host signal peptidases gives rise to C, E^{rns}, and E2, which are destined to form the capsid and the envelope of the virion (Lindenbach et al. 2007). The glycoproteins E^{rns}, E1, and E2 are composed of the envelope of BVDV, and the E^{rns} and

E2 form a disulphide-linked homodimer. E1 is assumed to be a membrane anchor for E2, all glycoproteins E^{rns}, E1, and E2 are associated with virus attachment and/or cell entry (Weiland et al. 1990; Rümenapf et al. 1993a). E2 is composed of approximately 370 amino acids, which are the immunodominant protein (Weiland et al. 1990; Hulst and Moormann 1997). E1 is associated with E2 by forming a heterodimer, which depends on the charged residues of transmembrane domains and is essential for virus entry (Ronecker et al. 2008). CSFV and BVDV share a common E2 receptor or co-receptor for binding and entry of these pestiviruses because recombinant CSFV E2 can bind to cells and block infection of CSFV and BVDV (Hulst and Moormann 1997). Research of the CSFV indicates that the structural protein E2 is the virulence factor of CSFV, which was proven by constructing the infectious clone without E2 to generate an attenuated strain (Risatti et al. 2005). Both the carboxyl terminal region of E2 and the N-linked glycosylation of E2 influence the virulence of CSFV Brescia strain in swine (Risatti et al. 2007). The most variable protein of pestiviruses is immunodominant protein E2, which is the location of the majority of neutralization epitopes and PCR, tested to differentiate pestivirus species. This highly variable region of E2 is suitable for identifying and classifying pestiviruses (Meyers and Thiel 1996; Mingala et al. 2009).

2.6 Diagnostic Methods

BVDV is a major viral pathogen that affects cattle. It causes significant economic losses worldwide by infecting cattle and contaminating biological products. BVDV viral antigens were found in epithelial cells at the site of entry, endothelial cells, lymphoreticular cells, and macrophages. BVDV can be detected from most tissues, including peripheral blood mononuclear cells (PBMC), the gastrointestinal tract, and

neurons (Lindenbach et al. 2007). BVDV can be detected from tissue, serum, semen, and somatic cells of milk (Afshar et al. 1991; Schmitt et al. 1994; Radwan et al. 1995; Renshaw et al. 2000; Beaudeau et al. 2001; Driskell and Ridpath 2006).

As instances of BVDV infection and subsequent economic losses increase, detection of the pathogen becomes essential to prevent and eradicate BVDV infections. Different diagnostic methods have been developed based on antibodies, antigens, and nucleic acid at both the herd and individual level. BVDV has been detected through spot hybridization with probes prepared from cloned cDNA sequences and RNA hybridization (Potgieter and Brock 1989; Roberts et al. 1991). BVDV has also been detected using enzyme-linked immunosorbent assay (ELISA), virus isolation (VI), immunohistochemistry (IHC), serum neutralization (SN), and direct and indirect fluorescent assay (FA) (Afshar et al. 1991; Cornish et al. 2005; Roberts et al. 1988; Schmitt et al. 1994; Saliki et al. 1997; Weiland et al. 1990). PCR amplification was developed to detect BVDV from bulk milk samples; and single-tube single-enzyme RT-PCR, TaqMan PCR, and real time RT-PCR were used to identify BVDV from tissue, serum, semen, and milk (Weinstock et al. 2001; Mahlum et al. 2002; Bhudevi and Weinstock 2003; Baxi et al. 2006; Daliri et al. 2007).

Among the variety of diagnostic methods, however, VI is still perceived as the gold standard, but it is laborious, time-consuming, and unsuitable for whole-herd testing (Saliki et al. 1997; Renshaw et al. 2000). VI detection relies on the existence of viable virus in tissues. Autolysis, especially for aborted fetuses or stillborn calves, reduces the recovery of live viruses, which can lead to false negative results (Ellis et al. 1995; Ridpath et al. 2009). PCR detection is 14.6 times more sensitive than virus isolation in

detecting BVDV RNA in the purified milk somatic cells of PI animals (Radwan et al. 1995).

A variety of ELISA diagnostic tests have been developed and widely used to detect BVDV from serum, or the presence of antigen from ear notch tissue, milk, etc. (Cornish et al. 2005). ELISA is suitable for screening samples in large volumes due to its time savings, but the cost for testing individual animals is relatively high (Schmitt et al. 1994; Driskell and Ridpath 2006). Although VI and ELISA seem sufficient to identify PI among older animals, the colostral antibodies may interfere with both methods for detection of BVDV in young calves, especially for serum testing. PCR is a nucleic acid based assay, which should not be subjected to interfere from colostral antibodies, and may be a better method for screening young PI calves. Colostral antibodies in PI animals decline rapidly, and they will be absent by the time a calf reaches two months of age. Available VI negative and PCR-positive calves are retested for BVDV at or after this age. Generally, the recommendation stands that calves younger than three months tested by VI should be retested again at three months (Brinkhof et al. 1996; Brock et al. 1998). Real-time RT-PCR has been widely accepted to identify, type, and detect BVDV from pooled samples due to its specificity, high sensitivity, and rapid turnaround time (Letellier and Kerkhofs 2003; Yan et al. 2011).

IHC detection is limited to tissue samples, which must be fixed using formalin and embedded using paraffin. Therefore, IHC detection normally takes three to four days; such length of time is inappropriate for urgent cases. ACE is performed on fresh skin (ear notch) specimens, and IHC is performed on skin specimens fixed in neutral-buffered 10% formalin, but real-time RT-PCR and VI can be performed on both tissue and serum samples.

Rapid tests, such as ACE, permit identification and segregation of PI cattle pending results of further tests, which reduces the animal's contact with the rest of the feedlot population. Cattle that are vaccinated with BVDV-1a and 2a may not induce adequate protective immunity against BVDV. Therefore, control of BVDV necessitates an enhancement of immunity against BVDV through vaccination and biosecurity by identification and removal of PI cattle. This process will maintain the BVDV-negative status among the animal population. A rapid test for detection of PI cattle may be beneficial so that PI cattle can be removed as quickly as possible from the feedlot, minimizing continuous exposure to BVDV. Compared with the cost of VI and real-time RT-PCR on individual samples among a population, IHC and ACE are cost friendly. However, ACE can be accomplished in less than five hours of laboratory time, rather than three to five days for IHC. This quick turnaround time allows animals to be removed sooner and lessens the potential threat of acute infection. Moreover, ACE provides totally objective results, whereas IHC is subjective and has the potential for misinterpretation from inexperienced pathologists.

2.7 Phylogenetic analysis

Phylogenetic analysis of pestivirus has been widely used to genotype the new isolates and to study their evolutionary history. Multiple ways have been used to infer the phylogeny of a species, they all fall into two fundamental approaches to construct the phylogenetic tree by either single genetic element or concatenating multiple genetic sequences (de Queiroz et al. 1995; Gadagkar et al. 2005; Huelsenbeck et al. 1996; Nei et al. 2001; Yang 1996). Different genomic regions of pestivirus have been used to type new isolates and 5'UTR is the most commonly used (Pellerin et al. 1994; Baule et al.

1997; Wolfmeyer et al. 1997; Ridpath et al. 2000; Vilcek et al. 2003); N^{pro} (Vilcek et al. 2001), E2 (Mingala et al. 2009; Oguzoglu et al. 2010), NS3 (Xia et al. 2007), and NS5B are also used to phylogenetic analysis (Nagai et al. 2004). 5'UTR harbors internal ribosomal entry site, which is highly conserved among all members within the genus *pestivirus*, thus being used extensively for the characterization of BVDV genotypes (Pestova et al. 1998). Immunodominant protein E2 is the location of the majority of neutralization epitopes, and therefore E2 can as the target for serological tests to differentiate pestivirus species. BVDV can be classified into two genotypes BVDV-1 and BVDV-2 based on the genetic variations of 5'UTR (Ridpath et al. 1994). BVDV-1 can be further divided into at least 11 subtypes (1a to 1l) based on the phylogenetic analysis of 5'UTR and N^{pro} (Jackova et al. 2008; Vilcek et al. 2001) while BVDV-2 can be divided further into four subtypes BVDV-2a, BVDV-2b, BVDV-2c, and BVDV-2d (Giangaspero et al. 2008). BVDV-1a, 1b and 2a are the major subtypes in North America (Ridpath 2010).

Secondary structure prediction and phylogenetic analysis are the most common methods for the classification of the pestivirus isolates. Sequence alignment is the basis of the phylogenetic analysis, and sequence alignment is also important for the prediction of consensus structure from multiple sequences. Sequence alignment indicates the differences between two (pair-wise alignment) or more (multiple-alignment) sequences by searching individual characters or patterns of the sequences. Multiple sequence alignment (MSA) has been used to identify the conserved sequence region or to establish the evolutionary history by aligning a set of three or more sequences (Wang and Jiang 1994). Progressive alignment and iterative alignment are commonly used in MSA.

Different programs were developed to perform MSA, such as ClustalW, T-coffee, Muscle (Notredame et al. 2000; Edgar 2004; Larkin et al. 2007), and others.

The evolutionary history of genes and species can be revealed by using the phylogenetic tree, which is also used in viral classification. Phylogenetic trees include rooted trees and unrooted trees, and rooted trees can tell the common ancestor. Phylogenetic trees can be constructed using four methods: distance, maximum parsimony, maximum likelihood, and Bayesian. The distance approach is based on the genetic distance of sequences, but this method is unable to use local high-variation regions effectively (Mount 2004). Both neighbor-joining (NJ) and un-weighted pair group method with arithmetic mean (UPGMA) use distances between sequences to construct the phylogenetic tree (Saitou and Nei 1987; Murtaugh 1992). Nonetheless, the constant rate of evolution (molecular clock hypothesis) assumed in UPGMA is not appropriate for inferring relationships. UPGMA is no longer commonly used to establish phylogenetic trees.

The phylogenetic tree produced from maximum parsimony is the tree with the least evolutionary change or the least position changes between sequences. Maximum parsimony is a very simple and popular method appropriate for use with highly similar sequences and small group sequences. But maximum parsimony cannot guarantee the statistical consistency necessary to produce the high probability phylogenetic tree (Felsenstein 1978).

The phylogenetic tree with the highest probability or likelihood can be found using maximum likelihood. Maximum likelihood has lower variance than other methods because it evaluates different tree topologies and uses all sequence information fully. Maximum likelihood is also robust to many violations of the assumption in the

evolutionary model, and it outperforms all other methods, such as parsimony or distance methods. However, maximum likelihood consumes much time and memory, and the result is dependent on the model of evolution used (Aldrich 1997; Cam 1990). Moreover, the phylogenetic trees constructed using distance methods, maximum parsimony, and maximum likelihood was supported using bootstrap analysis (Felsenstein 1978).

Bayesian inference is another phylogeny approach and can be performed using the MrBayes. The reliability of Bayesian inference was estimated using posterior probabilities, which was approximated using a simulation technique: Markov chain Monte Carlo (Ronquist and Huelsenbeck 2003; Mar et al. 2005). Bayesian allows implementation of complex parameter-rich evolution models whereas maximum likelihood does not.

Phylogenetic analysis can be performed on the single gene or concatenated on multiple genes. Phylogeny inference from a set of species is known as the species tree, and the phylogeny inference from gene(s) is called the gene tree. Phylogenetic trees can be established from concatenating multiple genes into a super-gene alignment, which is then analyzed to generate the species tree. Phylogenies are also inferred separately from each gene, and consensus of these gene phylogenies is used to represent the species tree.

2.8 Secondary structure prediction

2.8.1 RNA secondary structure prediction

RNA secondary structure analysis is an important approach in efficient structure prediction, and it will supply the important guides for biological investigations. For example, mRNA secondary structure prediction is able to identify in vivo binding sites of RNA-binding proteins (Li et al. 2010b). The 5 UTR of pestivirus possesses the IRES,

which is conserved among all members of pestiviruses and involved in the translation initiation of the viral genome. BVDV 5 UTR harbors extensive RNA structure motifs, which are related with cap-independent translation initiation and replication of viral RNA. RNA secondary structure is composed of duplexes, stems, hairpins, internal loops, bulge loops, and junctions. The secondary structure fold of RNA is based on base pairing using hydrogen bonds between complementary RNA nucleotides: G:C, A:T, and G:U.

With the development of computational methods, secondary structure prediction falls into two general categories: one uses multiple sequence alignments to predict consensus structure and the other predicts the structure of a single sequence. For multiple sequences, the comparative sequence analysis was used to predict the consensus structures of a group of sequences by base-pairing canonical pairs common to multiple sequences. This method is efficient when multiple homologous sequences are available (Pace et al. 1999). For single sequences, dynamic programming algorithms are used to predict the secondary structure to minimize free energy. The most popular algorithm is the minimum free energy (MFE) method, which has been used in the Mfold and RNAfold to predict the secondary structure of a single sequence (Zuker and Stiegler 1981; Hofacker et al. 1994). The accuracy of secondary structure predictions from a single sequence is limited using free energy minimization because of the incomplete free energy values listed in nearest neighbor models, the failure of conformity with the thermodynamic minimum due to unknown RNA folds, and multiple biologically active conformations of some RNA sequences (Zuker 2003). However, identification of the conserved structure using phylogenetic information may be helpful to remedy this defect.

RNA motifs are more conserved in structures than in sequences. Therefore, the consensus structure of a set of homologous or functionally related RNA sequences may

represent the RNA motifs, which may provide a deeper insight of regulator activities. Currently, three approaches can be used to predict consensus secondary structures: approach A, a consensus secondary structure was inferred from the evolutionary and energetic information of sequence alignment; approach B, simultaneously align and infer a consensus structure using "Sankoff algorithm"; and approach C, align the secondary structures to infer the consensus structure. Among these three approaches, approach A is the most successful approach, but it is limited by the accuracy of the alignment. Approach B is time-consuming and requires extreme memory, and approach C is limited to the existence of a reliable structure (Gardner and Giegerich 2004). Generally, the secondary structure may contain pseudoknots, which are ignored by most tools due to the complexity and scarcity of these motifs. Therefore, some programs as described below were designed to predict the secondary structure with pseudoknots.

Web based server programs which can predict consensus RNA secondary structures from an RNA alignment such as loops, stems, and pseudoknots. Web based servers are able to predict consensus structures:

Pfold. Pfold integrates both the energy-based and evolution-based approaches to predict the folding of multiple aligned RNA sequences. The former depends on the physical properties of single sequences, while the latter uses evolutionary information in the form of compensatory base pair substitutions. Pfold was able to fold an alignment with fewer than 40 sequences, 500 nucleotides in length (Seemann et al. 2008).

Probabilistic evolutionary and thermodynamic (PETfold). PETfold was developed by combining evolutionary and thermodynamic information in one probabilistic model to predict consensus RNA secondary structure from an RNA alignment. PETfold is better than Pfold and RNAalifold because of its wide range of parameter settings. The default

parameters of PETfold is thought to be a reasonable approximation for the RNA family alignments with different levels of diversity (Seemann et al. 2011).

RNAalifold. RNAalifold predicts consensus structure with a pseudoknot from aligned RNA sequences by calculating their minimum free energy (MFE), partition function (pf), and base pairing probability matrix. Clustal format alignment will be acceptable in the RNAalifold, which is a part of Vienna package. The original RNAalifold combines thermodynamic energy minimization with a simple scoring model to assess evolutionary conservation. Gaps within unpaired regions are simply ignored because the alignment positions appear as indices and loop sizes. RNAalifold performance has now been improved to treat gaps more accurately and evaluate covariation of a sequence using an elaborate model. The improved performance does not dramatically change implementation and has negligible extra computational cost (Bernhart et al. 2008).

Comparing RNA secondary structure is a key in bioinformatics when analyzing the functional similarities of different RNA secondary structures. Dot bracket notation is the dominant RNA secondary structure format. Dot represents unpaired bases and brackets represent base pairs in RNA stems, both dot and bracket notations have become an unofficial standard.

2.8.2 Protein secondary structure prediction

Regulatory functions related to RNA performance were formerly assigned only to proteins. These functions are also associated with evolutionary conserved motifs that contain specific sequence and structure properties (Huelsenbeck et al. 1996).

The primary sequence of protein is a polypeptide chain, which is composed of amino acids using peptide bonds. The spatial arrangement of these amino acids will form the secondary structure of a protein. Protein secondary structures are composed of alpha helices, beta strands, beta sheets, loops, and coils. Secondary structure prediction of a protein is helpful for tertiary structure prediction.

Structural protein E2 of BVDV is the main target of the BVDV neutralizing antibody, and E2 is related to BVDV virulence (Collett et al. 1988a; Driskell and Ridpath 2006; Risatti et al. 2007). Understanding the secondary structure of E2 may be helpful in identifying the relatedness of E2 and BVDV virulence. X-ray crystallography and NMR are two major experimental methods for determining protein structures. However, both methods are time consuming and incapable of satisfying the needs of increased protein sequences. Therefore, efficient prediction programs were developed to predict the secondary structure of proteins.

Protein secondary structure prediction approaches were developed based on simple stereochemical or statistics principles in the 1970s (Lim 1974; Chou and Fasman 1974; Garnier et al. 1978). With the development of secondary structure prediction approaches, pattern matching, homology or nearest neighbor, neural network, evolutionary methods, and combined approaches have been used in protein secondary structure prediction (Taylor 1988; Rost 1996; Levin 1997; Jones 1999). Protein secondary structure prediction programs have been extended to predict structures from a single sequence to a sequence alignment.

Chou-Fasman. As an empirical approach, Chou-Fasman was developed using statistical principles and was developed originally in the 1970s. This method predicts protein secondary structures by analyzing relative frequencies of amino acid in alpha

helices, beta sheets, and turns based on known structures. The application of Chou-Fasman has been limited because of low accuracy (55%), lacking account of long-range information, and assumption of simple additive probability (Chou and Fasman 1974).

Web based server programs which can predict protein secondary structures such as α-helix, β-sheet and coils. Below are web server approaches with accuracy of more than 70% in predicting protein secondary structure.

PredictProtein. PredictProtein is a widely used automatic web server for protein secondary structure prediction. PredictProtein was developed based on neural network with accuracy of more than 72% (Rost 1996; Rost et al. 2004).

JPred. JPred is a web server for predicting consensus protein secondary structures based on evolutionary information. This approach simplifies the algorithms and identifies the conservation patterns that are associated with structure and function. Consensus structure predictions from JPred improve the prediction accuracy (Q3), which indicates the total accuracy for all alpha helix, beta strand, and coil. JPred is a well-defined secondary structure prediction program for single sequences and multiple sequences with accuracy of up to 73–75% (Cuff et al. 1998).

SAM–T02. SAM-T02 is a Hidden Markov model (HMM)-based protein secondary structure prediction program with accuracy of up to 75%. However, SAM–T02 is only able to process one sequence at a time and the amino acids must all be in upper case (Karplus et al. 2003).

PSIPRED. The PSIPRED server incorporates three methods – PSIPRED, GenTHEADER, and MEMSAT2 – to predict the protein secondary structure from amino acid sequences. PSIPRED is a highly accurate approach that incorporates two feed-forward neural networks. The performance of these programs was evaluated using

stringent cross-validation procedure. PSIPRED is a highly accurate approach with an average Q3 score of 76.5% (McGuffin et al. 2000).

2.9 Control, prevention, and vaccination

BVDV can be transmitted through horizontal and genetic vertical transmission. Horizontal transmission occurs by direct contact with a BVDV-carrier, infected secretion, saliva, feces, urine, semen, et al., and vertical transmission is transmitted from the BVDV-carrier to her fetus (Meyling et al. 1990). BVDV infects all ages of cattle, from fetuses to adult cattle. PI animals are more efficient transmitters than are transient infected animals because of their long-lasting virus shedding (Larson 2005). Therefore, the major strategy behind preventing the spread of BVDV is to identify and remove PI animals. More than 90% of PI cattle were produced due to the BVDV acute infection of susceptible pregnant female between 45 and 120 days of gestation, and less than 10% of PI cattle were produced by PI animals that were pregnant (Thomas 2009). To control and prevent the spread of BVDV, common control programs focus on identification and removal of PI cattle, biosecurity measures to prevent the import of BVDV, isolation of new herd additions and avoiding contact with infected feed and instruments, and vaccination of new calves and heifers/cows to prevent infection of fetuses and calves (Fulton 2005).

Vaccination is important to prevent the occurrence of PI calves. The effective vaccine must take into account the diversity of both the antigenic and genetic makeup. The developed BVDV vaccines include the killed or modified live vaccine (MLV) to BVDV-1a, BVDV-1b, and BVDV-2a (Cortese et al. 1998; Fulton et al. 1995; Fulton et al. 2000; Fulton et al. 2002). Over 180 vaccines are based on either BVDV alone or

BVDV in combination with bovine herpesvirus-1 and parainfluenza virus type 3. Bovine respiratory syncytial virus was developed and licensed by the USDA, and these vaccines have been listed in the *Compendium of Veterinary Products* since 2006. Most of the vaccines were developed to protect the infection of BVDV-1a from the 1960s to the 1990s. However, the BVDV-1 vaccine is not able to efficiently protect from BVDV-2 infection, and BVDV-1b is the predominant subtype in the United States (Fulton et al. 2000; Fulton et al. 2002; Yan et al. 2011). Moreover, limited BVDV-1b, BVDV-2a commercial vaccines are available. Due to the increase of the new subtypes, a broad-spectrum vaccine is needed to efficiently protect animals from BVDV's multiple subtypes. Some companies have attempted to develop either an MLV or a killed BVDV vaccine with both BVDV-1 and BVDV-2 immunogens.

The BVDV MLV vaccine is similar to other MLV vaccines which require smaller amounts of virus than killed vaccines, and require only one dose for immunization. However, the handling of MLV requires more rigid precautions than killed vaccines because it is susceptible to inactivation by chemicals and/or exposure to higher temperatures. Although the killed vaccines are safer than the MLV, the efficiency of killed vaccine is lower than MLV (Fulton 2005). Viruses may be transmitted to the fetus if the pregnant cow is vaccinated using MLV, therefore, the development of an efficient and safe MLV is essential for the future.

2.10 References

1. Afshar A, Dulac GC, Dubuc C, Howard TH. 1991. Comparative evaluation of the fluorescent antibody test and microtiter immunoperoxidase assay for detection of bovine viral diarrhea virus from bull semen. Can J Vet Res 55(1):91-93.

2. Aldrich J. 1997. RA Fisher and the making of maximum likelihood 1912-1922. Statistical Science:162-176.

3. Alves D, Tremblay R, Godkin A, Anderson N, Carman S, McEwen B, Hazlett M. 1996. Update on bovine virus diarrhea in Ontario. Can Vet J 37(3):177.

4. Baker JC. 1995. The clinical manifestations of bovine viral diarrhea infection. Vet Clin North Am Food Anim Pract 11(3):425-445.

5. Baule C, Van Vuuren M, Lowings JP, Belak S. 1997. Genetic heterogeneity of bovine viral diarrhoea viruses isolated in Southern Africa1. Virus research 52(2):205-220.

6. Baxi M, McRae D, Baxi S, Greiser-Wilke I, Vilcek S, Amoako K, Deregt D. 2006. A one-step multiplex real-time RT-PCR for detection and typing of bovine viral diarrhea viruses. Vet Microbiol 116(1-3):37-44.

7. Bernhart S, Hofacker I, Will S, Gruber A, Stadler P. 2008. RNAalifold: improved consensus structure prediction for RNA alignments. BMC bioinformatics 9(1):474.

8. Bhudevi B, Weinstock D. 2003. Detection of bovine viral diarrhea virus in formalin fixed paraffin embedded tissue sections by real time RT-PCR (Taqman). J Virol Methods 109(1):25-30.

9. Bolin SR, Ridpath JF. 1992. Differences in virulence between two noncytopathic bovine viral diarrhea viruses in calves. Am J Vet Res53(11):2157-2163.

10. Brinkhof J, Zimmer G, Westenbrink F. 1996. Comparative study on four enzyme-linked immunosorbent assays and a cocultivation assay for the detection of antigens associated with the bovine viral diarrhoea virus in persistently infected cattle. Vet Microbiol 50(1-2):1-6.

11. Brock KV, Deng R, Riblet SM. 1992. Nucleotide sequencing of 5'and 3'termini of bovine viral diarrhea virus by RNA ligation and PCR. J Virol Methods 38(1):39-46.

12. Brock KV, Grooms DL, Ridpath J, Bolin SR. 1998. Changes in levels of viremia in cattle persistently infected with bovine viral diarrhea virus. J Vet Diagn Invest 10(1):22-26.

13. Brown EA, Zhang H, Ping LH, Lemon SM. 1992. Secondary structure of the 5 nontranslated regions of hepatitis C virus and pestivirus genomic RNAs. Nucleic Acids Res 20(19):5041-5045.

14. Brownlie J, Clarke MC, Howard CJ. 1989. Experimental infection of cattle in early pregnancy with a cytopathic strain of bovine virus diarrhoea virus. Res Vet Sci 46(3):307-311.

15. Cam LL. 1990. Maximum likelihood: an introduction. Int Stat Rev 58(2):153-171.

16. Chou PY, Fasman GD. 1974. Prediction of protein conformation. Biochemistry 13(2):222-245.

17. Collett MS, Larson R, Belzer SK, Retzel E. 1988a. Proteins encoded by bovine viral diarrhea virus: the genomic organization of a pestivirus. Virology 165(1):200-208.

18. Collett MS, Larson R, Gold C, Strick D, Anderson DK, Purchio AF. 1988b. Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhea virus. Virology 165(1):191-199.

19. Corapi WV, French TW, Dubovi EJ. 1989. Severe thrombocytopenia in young calves experimentally infected with noncytopathic bovine viral diarrhea virus. J Virol 63(9):3934-3943.

20. Cornish TE, van Olphen AL, Cavender JL, Edwards JM, Jaeger PT, Vieyra LL, Woodard LF, Miller DR, O'Toole D. 2005. Comparison of ear notch immunohistochemistry, ear notch antigen-capture ELISA, and buffy coat virus isolation for detection of calves persistently infected with bovine viral diarrhea virus. J Vet Diagn Invest 17(2):110-117.

21. Cortese VS, Grooms DL, Ellis J, Bolin SR, Ridpath JF, Brock KV. 1998. Protection of pregnant cattle and their fetuses against infection with bovine viral diarrhea virus type 1 by use of a modified-live virus vaccine. Am J Vet Res59(11):1409-1413.

22. Cuff JA, Clamp ME, Siddiqui AS, Finlay M, Barton GJ. 1998. JPred: a consensus secondary structure prediction server. Bioinformatics 14(10):892-893.

23. Daliri M, Ghorashi SA, Morshedi D, Hajian T, Afshar K. 2007. Detection of bovine viral diarrhea virus in bovine semen using nested-PCR. Iranian J of Biotechnology (IJB) 5(1): 129-139.

24. de Queiroz A, Donoghue MJ, Kim J. 1995. Separate versus combined analysis of phylogenetic evidence. Ann. Rev. Ecol Syst. 26: 657-681.

25. Deng R, Brock KV. 1993. 5 and 3 untranslated regions of pestivirus genome: primary and secondary structure analyses. Nucleic Acids Res 21(8):1949-1957.

26. Driskell EA, Ridpath JF. 2006. A survey of bovine viral diarrhea virus testing in diagnostic laboratories in the United States from 2004 to 2005. J Vet Diagn Invest 18(6):600-605.

27. Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32(5):1792-1797.

28. Ellis JA, Martin K, Robert NG, Haines DM. 1995. Comparison of detection methods for bovine viral diarrhea virus in bovine abortions and neonatal death. J Vet Diagn Invest 7(4):433-436.

29. Felsenstein J. 1978. Cases in which parsimony or compatibility methods will be positively misleading. Systematic Biology 27(4):401-410.

30. Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution:783-791.

31. Fenner F, Maurin J. 1976. The classification and nomenclature of viruses. Arch Virol 51(1):141-149.

32. Flores EF, Ridpath JF, Weiblen R, Vogel FSF, Gil LHVG. 2002. Phylogenetic analysis of Brazilian bovine viral diarrhea virus type 2 (BVDV-2) isolates: evidence for a subgenotype within BVDV-2. Virus research 87(1):51-60.

33. Fulton RW. 2005. Vaccines. Bovine viral diarrhea virus: Diagnosis,management, and control. 1st ed. Ames, Iowa: Blackwell Publishing.

34. Fulton RW, Confer AW, Burge LJ, Perino LJ, d'Offay JM, Payton ME, Mock RE. 1995. Antibody responses by cattle after vaccination with commercial viral vaccines containing bovine herpesvirus-1, bovine viral diarrhea virus, parainfluenza-3 virus, and bovine respiratory syncytial virus immunogens and subsequent revaccination at day 140. Vaccine 13(8):725-733.

35. Fulton RW, Ridpath JF, Saliki JT, Briggs RE, Confer AW, Burge LJ, Purdy CW, Loan RW, Duff GC, Payton ME. 2002. Bovine viral diarrhea virus (BVDV) 1b: predominant BVDV subtype in calves with respiratory disease. Can J Vet Res 66(3):181-190.

36. Fulton RW, Saliki JT, Confer AW, Burge LJ, d'Offay JM, Helman RG, Bolin SR, Ridpath JF, Payton ME. 2000. Bovine viral diarrhea virus cytopathic and noncytopathic biotypes and type 1 and 2 genotypes in diagnostic laboratory accessions: clinical and necropsy samples from cattle. J Vet Diagn Invest 12(1):33-38.

37. Gardner P, Giegerich R. 2004. A comprehensive comparison of comparative RNA structure prediction approaches. BMC bioinformatics 5(1):140.

38. Garnier J, Osguthorpe DJ, Robson B. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J Mol Biol 120(1):97-120.

39. Giangaspero M, Harasawa R, Weber L, Belloli A. 2008. Genoepidemiological evaluation of Bovine viral diarrhea virus 2 species based on secondary structures in the 5'untranslated region. J Vet Med Sci 70(6):571-80.

40. Goens SD. 2002. The evolution of bovine viral diarrhea: a review. Can Vet J 43(12):946-954.

41. Gong Y, Trowbridge R, Macnaughton TB, Westaway EG, Shannon AD, Gowans EJ. 1996. Characterization of RNA synthesis during a one-step growth curve and of the replication mechanism of bovine viral diarrhoea virus. J Gen Virol 77(11):2729-2736.

42. Gadagkar SR, Rosenberg MS, Kumar S. 2005. Inferring species phylogenies from multiple genes: concatenated sequence tree versus consensus gene tree. J Exp Zool B Mol Dev Evol 304(1): 64-74.

43. Gray EW, Nettleton PF. 1987. The ultrastructure of cell cultures infected with border disease and bovine virus diarrhoea viruses. The J Gen Virol 68:2339-2346.

44. Grummer B, Grotha S, Greiser Wilke I. 2004. Bovine Viral Diarrhoea Virus is Internalized by Clathrin dependent Receptor mediated Endocytosis. J Vet Med B Infect Dis Vet Public Health 51(10):427-32.

45. Hietala SK, Crossley BM. 2005. Virus replication. In: Goyal SM, Ridpath JF, editors. Bovine viral diarrhea virus: Diagnosis, management, and control. 1st ed. New Jersey: Blackwell; p 81-89.

46. Hofacker IL, Fontana W, Stadler PF, Bonhoeffer LS, Tacker M, Schuster P. 1994. Fast folding and comparison of RNA secondary structures. Monatshefte fur Chemie/Chemical Monthly 125(2):167-188.

47. Houe H. 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Vet Microbiol 64(2-3):89-107.

48. Huelsenbeck JP, Bull JJ, Cunningham CW. 1996. Combining data in phylogenetic analysis. Trends in Ecology & Evolution 11(4):152-158.

49. Hulst MM, Moormann RJ. 1997. Inhibition of pestivirus infection in cell culture by envelope proteins E (rns) and E2 of classical swine fever virus: E (rns) and E2 interact with different receptors. J Gen Virol 78(11):2779-2787.

50. Isken O, Grassmann CW, Yu H, Behrens SE. 2004. Complex signals in the genomic 3 nontranslated region of bovine viral diarrhea virus coordinate translation and replication of the viral RNA. RNA 10(10):1637-1652.

51. Jackova A, Novackova M, Pelletier NC, Audeval C, Gueneau E, Haffar A, Petit E, Rehby L, Vilcek S. 2008. The extended genetic diversity of BVDV-1: Typing of BVDV isolates from France. Vet Res Commu 32(1): 7-11.
50. Jones DT. 1999. Protein secondary structure prediction based on position-specific scoring matrices1. J Mol Biol 292(2):195-202.

52. Karplus K, Karchin R, Draper J, Casper J, Mandel-Gutfreund Y, Diekhans M, Hughey R. 2003. Combining local-structure, fold-recognition, and new fold methods for protein structure prediction. Proteins 53(6):491-496.

53. Kelling CL, Steffen DJ, Topliff CL, Eskridge KM, Donis RO, Higuchi DS. 2002. Comparative virulence of isolates of bovine viral diarrhea virus type II in experimentally inoculated six-to nine-month-old calves. Am J Vet Res63(10):1379-1384.

54. Kümmerer BM, Tautz N, Becher P, Thiel HJ, Meyers G. 2000. The genetic basis for cytopathogenicity of pestiviruses. Vet Microbiol 77(1-2):117-128.

55. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23(21):2947-2948.

56. Larson RL. 2005. Management systems and control. Bovine viral diarrhea virus: diagnosis, management, and control. 1st ed. Ames, Iowa: Blackwell Publishing.

57. Laude H. 1987. Hog Cholera Virus: art and facts. Ann Rech Vet 18(2):127-138.

58. Lecot S, Belouzard S, Dubuisson J, Rouillθ Y. 2005. Bovine viral diarrhea virus entry is dependent on clathrin-mediated endocytosis. J Virol 79(16):10826-10829.

59. Lee KM, Gillespie JH. 1957. Propagation of virus diarrhea virus of cattle in tissue culture. Am J Vet Res18(69):952-953.

60. Letellier C, Kerkhofs P. 2003. Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhea virus. J Virol Methods 114(1):21-27.

61. Levin JM. 1997. Exploring the limits of nearest neighbour secondary structure prediction. Protein Eng 10(7):771-776.

62. Li QC, Miao LG, Li HT, Liu YH, Zhang GL, Xiao JM. 2010a. Genome sequencing and analysis of the bovine viral diarrhea virus-2 strain JZ05-1 isolated in China. Chinese J Virol (Bing du xue bao) 26(3): 238-243.

63. Li X, Quon G, Lipshitz HD, Morris Q. 2010b. Predicting in vivo binding sites of RNA-binding proteins using mRNA secondary structure. RNA 16(6):1096-1107.

64. Liang D, Sainz IF, Ansari IH, Gil LHVG, Vassilev V, Donis RO. 2003. The envelope glycoprotein E2 is a determinant of cell culture tropism in ruminant pestiviruses. J Gen Virol 84(5):1269-1274.

65. Liess B. 1981. Hog cholera. A world geography of epidemiology and control. London: Academic Press; p 627-650.

66. Lim VI. 1974. Algorithms for prediction of [alpha]-helical and [beta]-structural regions in globular proteins. J Mol Biol 88(4):873-894.

67. Lindenbach BD, Thiel HJ, Rice CM. Knipe DM, Howley PM. Flaviviridae: The viruses and their replication. Fields virology. 2007. Philadelphia Lippincott-Raven.

68. Loneragan GH, Thomson DU, Montgomery DL, Mason GL, Larson RL. 2005. Prevalence, outcome, and health consequences associated with persistent infection with bovine viral diarrhea virus in feedlot cattle. J Am Vet Med Assoc 226(4):595-601.

69. Mahlum CE, Haugerud S, Shivers JL, Rossow KD, Goyal SM, Collins JE, Faaberg KS. 2002. Detection of bovine viral diarrhea virus by TaqMan reverse transcription polymerase chain reaction. J Vet Diagn Invest 14(2):120-125.

70. Mar J, Harlow T, Ragan M. 2005. Bayesian and maximum likelihood phylogenetic analyses of protein sequence data under relative branch-length differences and model violation. BMC Evol Biol 5(1):8.

71. McClurkin AW, Bolin SR, Coria MF. 1985. Isolation of cytopathic and noncytopathic bovine viral diarrhea virus from the spleen of cattle acutely and chronically affected with bovine viral diarrhea. J Am Vet Med Assoc 186(6):568-569.

72. McClurkin AW, Littledike ET, Cutlip RC, Frank GH, Coria MF, Bolin SR. 1984. Production of cattle immunotolerant to bovine viral diarrhea virus. Can J Comp Med 48(2):156-161.

73. McGuffin LJ, Bryson K, Jones DT. 2000. The PSIPRED protein structure prediction server. Bioinformatics 16(4):404-405.

74. Meyers G, Thiel HJ. 1996. Molecular characterization of pestiviruses. Adv Virus Res 47:53-118.

75. Meyling A, Houe H, Jensen AM. 1990. Epidemiology of bovine virus diarrhoea virus. Res Sci Tech 9(1):75-93.

76. Mingala CN, Konnai S, Tajima M, Onuma M, Ohashi K. 2009. Classification of new BVDV isolates from Philippine water buffalo using the viral E2 region. J Basic Microbiol 49(5):495-500.

77. Muñoz-Zanzi CA, Thurmond MC, Hietala SK. 2004. Effect of bovine viral diarrhea virus infection on fertility of dairy heifers. Theriogenology 61(6):1085-1099.

78. Murtaugh F. 1992. Comments on'Parallel algorithms for hierarchical clustering and cluster validity'. IEEE Trans Pattern Anal Mach Intell 14 (10):1056-1057.

79. Nagai M, Hayashi M, Sugita S, Sakoda Y, Mori M, Murakami T, Ozawa T, Yamada N, Akashi H. 2004. Phylogenetic analysis of bovine viral diarrhea viruses using five different genetic regions. Virus research 99(2):103-113.

80. Notredame C, Higgins DG, Heringa J. 2000. T-coffee: a novel method for fast and accurate multiple sequence alignment1. J Mol Biol 302(1):205-217.

81. Odeon AC, Kelling CL, Marshall DJ, Estela ES, Dubovi EJ, Donis RO. 1999. Experimental infection of calves with bovine viral diarrhea virus genotype II (NY-93). J Vet Diagn Invest 11(3):221-228.

82. Oguzoglu TC, Muz D, lmaz Y, Alkan F, Aktα Y, Burgu. 2010. Molecular characterization of Bovine virus diarrhea viruses species 2 (BVDV-2) from cattle in Turkey. Trop Anim Health Prod 42(6):1175-1180.

83. Olafson P, MacCallum AD, Fox FH. 1946. An apparently new transmissible disease of cattle. Cornell Vet 36:205-213.

84. Pace NR, Thomas BC, Woese CR. 1999. Probing RNA structure, funciton, and history by comparative analysis. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; p 113-141.

85. Pankraz A, Thiel HJ, Becher P. 2005. Essential and Nonessential Elements in the 3' Nontranslated Region of Bovine Viral Diarrhea Virus. J Virol 79(14):9119-9127.

86. Pellerin C, Van Den Hurk J, Lecomte J, Tijssen P. 1994. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203(2):260-268.

87. Poole TL, Wang C, Popp RA, Potgieter LND, Siddiqui A, Collett MS. 1995. Pestivirus translation initiation occurs by internal ribosome entry1. Virology 206(1):750-754.

88. Potgieter LND, Brock KV. 1989. Detection of bovine viral diarrhea virus by spot hybridization with probes prepared from cloned cDNA sequences. J Vet Diagn Invest 1(1):29-33.

89. Radwan GS, Brock KV, Hogan JS, Larry Smith K. 1995. Development of a PCR amplification assay as a screening test using bulk milk samples for identifying dairy herds infected with bovine viral diarrhea virus. Vet Microbiol 44(1):77-91.

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

91. Renshaw RW, Ray R, Dubovi EJ. 2000. Comparison of virus isolation and reverse transcription polymerase chain reaction assay for detection of bovine viral diarrhea virus in bulk milk tank samples. J Vet Diagn Invest 12(2):184-186.

92. Ridpath JF. 2010. Bovine viral diarrhea virus: global status. Vet Clin North Amm Food Anim Pract 26(1):105-121.

93. Ridpath JF, Bolin SR. 1995. Delayed onset postvaccinal mucosal disease as a result of genetic recombination between genotype 1 and genotype 2 BVDV. Virology 212(1):259-262.

94. Ridpath JF, Bolin SR. 1998. Differentiation of types 1a, 1b and 2 bovine viral diarrhoea virus (BVDV) by PCR. Mol Cell Probes 12(2):101-106.

95. Ridpath JF, Bolin SR, Dubovlt EJ. 1994. Segregation of bovine viral diarrhea virus into genotypes. Virology 205:66-74.

96. Ridpath JF, Chiang YW, Waldbillig J, Neill JD. 2009. Stability of Bovine viral diarrhea virus antigen in ear punch samples collected from bovine fetuses. J Vet Diagn Invest 21(3):346-349.

97. Ridpath JF, Neill JD, Frey M, Landgraf JG. 2000. Phylogenetic, antigenic and clinical characterization of type 2 BVDV from North America. Vet Microbiol 77(1-2):145-155.

98. Ridpath JF, Neill JD, Peterhans E. 2007. Impact of variation in acute virulence of BVDV1 strains on design of better vaccine efficacy challenge models. Vaccine 25(47):8058-8066.

99. Rijnbrand R, Van der Straaten T, Van Rijn PA, Spaan WJ, Bredenbeek PJ. 1997. Internal entry of ribosomes is directed by the 5'noncoding region of classical swine fever virus and is dependent on the presence of an RNA pseudoknot upstream of the initiation codon. J Virol 71(1):451-457.

100. Risatti GR, Borca MV, Kutish GF, Lu Z, Holinka LG, French RA, Tulman ER, Rock DL. 2005. The E2 glycoprotein of classical swine fever virus is a virulence determinant in swine. J Virol 79(6):3787-3796.

101. Risatti GR, Holinka LG, Fernandez Sainz I, Carrillo C, Lu Z, Borca MV. 2007. N-linked glycosylation status of classical swine fever virus strain Brescia E2 glycoprotein influences virulence in swine. J Virol 81(2):924-933.

102. Roberts KL, Collins JK, Carman J, Blair CD. 1991. Detection of cattle infected with bovine viral diarrhea virus using nucleic acid hybridization. J Vet Diagn Invest 3(1):10-15.

103. Roberts PC, Etchison JR, Bond CW. 1988. A rapid, quantitative assay for titration of bovine virus diarrhoea-mucosal disease virus. Vet Microbiol 18(3-4):209-217.

104. Ronecker S, Zimmer G, Herrler G, Greiser-Wilke I, Grummer B. 2008. Formation of bovine viral diarrhea virus E1-E2 heterodimers is essential for virus entry and depends on charged residues in the transmembrane domains. J Gen Virol 89(9):2114-2121.

105. Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19(12):1572-1574.

106. Rost B. 1996. PHD: Predicting one-dimensional protein structure by profile-based neural networks. Methods Enzymol 266:525-539.

107. Rost B, Yachdav G, Liu J. 2004. The predictprotein server. Nucleic Acids Res 32(suppl 2):W321-326.

108. Rümenapf L, Meyers G, Stark R, Thiel HJ. 1991. Molecular characterization of hog cholera virus. Arch Virol Supplementum 3:7-18.

109. Rümenapf T, Unger G, Strauss JH, Thiel HJ. 1993b. Processing of the envelope glycoproteins of pestiviruses. J Virol 67(6):3288-3294.

110. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4(4):406-425.

111. Saliki JT, Fulton RW, Hull SR, Dubovi EJ. 1997. Microtiter virus isolation and enzyme immunoassays for detection of bovine viral diarrhea virus in cattle serum. J Clin Microbiol 35(4):803-807.

112. Schmitt BJ, Lopez OJ, Ridpath JF, Galeota-Wheeler J, Osorio FA. 1994. Evaluation of PCR for diagnosis of bovine viral diarrhea virus in tissue homogenates. J Vet Diagn Invest 6(1):44-47.

113. Seemann SE, Gorodkin J, Backofen R. 2008. Unifying evolutionary and thermodynamic information for RNA folding of multiple alignments. Nucleic Acids Res 36(20):6355-6362.

114. Seemann SE, Menzel P, Backofen R, Gorodkin J. 2011. The PETfold and PETcofold web servers for intra-and intermolecular structures of multiple RNA sequences. Nucleic Acids Res 39(suppl 2):W107-111.

115. Skinner MA, Racaniello VR, Dunn G, Cooper J, Minor PD, Almond JW. 1989. New model for the secondary structure of the 5'non-coding RNA of poliovirus is supported by

biochemical and genetic data that also show that RNA secondary structure is important in neurovirulence. J Mol Biol 207(2):379-392.

116. Steffens S, Thiel HJ, Behrens SE. 1999. The RNA-dependent RNA polymerases of different members of the family Flaviviridae exhibit similar properties in vitro. J Gen Virol 80(10):2583-2590.

117. Stewart SR, Semler BL. 1998. RNA structure adjacent to the attenuation determinant in the 5 -non-coding region influences poliovirus viability. Nucleic Acids Res 26(23):5318.

118. Tajima M, Dubovi EJ. 2005. Genetic and clinical analyses of bovine viral diarrhea virus isolates from dairy operations in the United States of America. J Vet Diagn Invest 17(1):10-15.

119. Taylor WR. 1988. Pattern matching methods in protein sequence comparison and structure prediction. Protein Engineering 2(2):77-86.

120. Tellinghuisen TL, Paulson MS, Rice CM. 2006. The NS5A protein of bovine viral diarrhea virus contains an essential zinc-binding site similar to that of the hepatitis C virus NS5A protein. J Virol 80(15):7450-7458.

121. Thiel HJ, Collett MS, Gould EA, Heinz FX, Houghton M, Meyers G. 2005. Flaviviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball BA, editors. Virus taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses. San Diego (CA): Elsevier Academic Press; p 981-98.

122. Thomas HS. 2009. The cattle healthy handbook: Preventive care, disease treatments, and emergency procedures for promoting the well-being of your beef or dairy herd. Storey Publishing, LLC.

123. Topliff CL, Chon SK, Donis RO, Eskridge KM, Kelling CL. 2005. In vitro and in vivo translational efficiencies of the 5'untranslated region from eight genotype 2 bovine viral diarrhea virus field isolates. Virology 331(2):349-356.

124. Topliff CL, Kelling CL. 1998. Virulence Markers in the 5'Untranslated Region of Genotype 2 Bovine Viral Diarrhea Virus Isolates. Virology 250(1):164-172.

125. Vilcek S, Greiser-Wilke I, Durkovic B, Obritzhauser W, Deutz A, Kofer J. 2003. Genetic diversity of recent bovine viral diarrhoea viruses from the southeast of Austria (Styria). Vet Microbiol 91(2-3):285-291.

126. Vilcek S, Paton DJ, Durkovic B, Strojny L, Ibata G, Moussa A, Loitsch A, Rossmanith W, Vega S, Scicluna MT. 2001. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch Virol 146(1):99-115.

127. Wang L, Jiang T. 1994. On the complexity of multiple sequence alignment. J Comput Biol 1(4):337-348.

128. Warrilow D, Lott WB, Greive S, Gowans EJ. 2000. Properties of the bovine viral diarrhoea virus replicase in extracts of infected MDBK cells. Arch Virol 145(10):2163-2171.

129. Weiland E, Stark R, Haas B, Rumenapf T, Meyers G, Thiel HJ. 1990. Pestivirus glycoprotein which induces neutralizing antibodies forms part of a disulfide-linked heterodimer. J Virol 64(8):3563-3569.

130. Weinstock D, Bhudevi B, Castro AE. 2001. Single-tube single-enzyme reverse transcriptase PCR assay for detection of bovine viral diarrhea virus in pooled bovine serum. J Clin Microbiol 39(1):343-346.

131. Weiskircher E, Aligo J, Ning G, Konan KV. 2009. Bovine viral diarrhea virus NS4B protein is an integral membrane protein associated with Golgi markers and rearranged host membranes. Virol J 6(1):185.

132. Wiskerchen M, Belzer SK, Collett MS. 1991. Pestivirus gene expression: the first protein product of the bovine viral diarrhea virus large open reading frame, p20, possesses proteolytic activity. J Virol 65(8):4508-4514.

133. Wolfmeyer A, Wolf G, Beer M, Strube W, Hehnen HR, Schmeer N, Kaaden OR. 1997. Genomic (5 UTR) and serological differences among German BVDV field isolates. Arch Virol 142(10):2049-2057.

134. Xia H, Liu L, Wahlberg N, Baule C, Belbκ S. 2007. Molecular phylogenetic analysis of bovine viral diarrhoea virus: a Bayesian approach. Virus research 130(1-2):53-62.

135. Xu J, Mendez E, Caron PR, Lin C, Murcko MA, Collett MS, Rice CM. 1997. Bovine viral diarrhea virus NS3 serine proteinase: polyprotein cleavage sites, cofactor requirements, and molecular model of an enzyme essential for pestivirus replication. J Virol 71(7):5312-5322.

136. Yan L, Zhang S, Pace L, Wilson F, Wan H, Zhang M. 2011. Combination of reverse transcription real-time polymerase chain reaction and antigen capture enzyme-linked immunosorbent assay for the detection of animals persistently infected with Bovine viral diarrhea virus. J Vet Diagn Invest 23(1):16-25.

137. Yu H, Isken O, Grassmann CW, Behrens SE. 2000. A stem-loop motif formed by the immediate 5'terminus of the bovine viral diarrhea virus genome modulates translation as well as replication of the viral RNA. J Virol 74(13):5825-5835.

138. Zuckerman AJ. 1981. Non-Arthropod-Borne Togaviruses. J Clin Pathol 34(9):1070.

139. Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31(13):3406-3415.

140. Zuker M, Stiegler P. 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res 9(1):133-148.

CHAPTER III

COMBINATION OF REVERSE TRANSCRIPTION REAL-TIME POLYMERASE CHAIN REACTION AND ANTIGEN CAPTURE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF ANIMALS PERSISTENTLY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS[1]

3.1 Abstract

Bovine viral diarrhea virus (BVDV) is an economically important pathogen of cattle. A successful control program requires early detection and removal of persistently infected (PI) animals. The objective of the current study was to develop, validate, and apply a cost-effective testing scheme for the detection of BVDV persistent infected animals in exposed herds. Pooled samples were screened by using a real-time reverse transcription polymerase chain reaction (real-time RT-PCR), and individual positives were identified with an antigen capture enzyme-linked immunosorbent assay (ACE). The detection limits of the optimized real-time RT-PCR were 10 and 100 RNA copies per reaction for BVDV-1 and BVDV-2, respectively. The semiquantitative results of real-time RT-PCR and ACE or real-time RT-PCR and immunohistochemistry were moderately correlated. The threshold cycle of real-time RT-PCR performed on pooled samples was significantly correlated with the pool size ($R^2 = 0.993$). The least-cost pool sizes were 50 at a prevalence of 0.25 - 0.5% and 25 at a prevalence of 0.75 - 2.0%. By

[1] Published in the J Vet Diagn Invest. 2011. 23: 16-25

using the combined real-time RT-PCR and ACE procedure, 111 of 27,932 (0.4%) samples tested positive for BVDV. At this prevalence, cost reduction associated with the application of real-time RT-PCR and ACE ranged from 61% to 94%, compared with testing individual samples by ACE, immunohistochemistry, or real-time RT-PCR. Real-time RT-PCR screening also indicated that 92.94% of PI animals were infected with BVDV-1, 3.53% with BVDV-2, and 3.53% with both BVDV-1 and BVDV-2. Analysis of the 5'-untranslated region of 22 isolates revealed the predominance of BVDV-1b followed by BVDV-2a.

3.2 Introduction

Bovine viral diarrhea virus (BVDV) is an economically important pathogen of cattle worldwide (Houe 1999), and, along with Classical swine fever disease virus (CSFV) and Border disease virus, is a member of the Pestivirus genus in the family of Flaviviridae (Lindenbach et al. 2001). The virus has a broad tissue tropism and can infect many cell types of bovine host (Lindenbach et al. 2001). The 2 biotypes of BVDV, cytopathic and noncytopathic, are distinguished according to their effects on cultured cells (Meyers and Thiel 1996). BVDV isolates can be further classified by genotypes, BVDV-1 and BVDV-2, and subgenotypes on the basis of sequence variations in the 5' -untranslated region (UTR) of the viral genome (Ridpath et al. 1994). The BVDV isolates of either genotypes can have a biotype of cytopathic or noncytopathic, and the relationship between genotype and pathogenesis remains to be clearly defined (Fulton et al. 2000). BVDV infection is associated with a diverse array of diseases, including gastrointestinal disorder, respiratory distress, fetal malformation, stillbirth, abortions, and mucosal disease (MD) (Baker 1995). Trans-placental infections of the fetuses between 42

and 125 days of gestation can result in immune-tolerance, and the surviving fetuses become persistently infected. Persistently infected PI animals continually shed a large amount of virus in their body secretions and excretions, thereby serving as a reservoir for BVDV (Baker 1995; Loneragan et al. 2005; McClurkin et al. 1984). Cattle exposed to PI animals have a higher incidence of respiratory diseases and associated risk for treatment (Lonergan et al. 2005). Early detection and subsequent removal of PI animals is essential to a successful disease control program which, however, is often hindered by the high cost associated with laboratory testing.

Many test methods have been developed for the detection of BVDV, including virus isolation (VI), immunohistochemistry (IHC), reverse transcription polymerase chain reaction (RT-PCR), and antigen capture enzyme-linked immunosorbent assay (ACE) (Afshar et al. 1991; Cornish et al. 2005; Driskell and Ridpath 2006; Saliki et al. 1997; Sandvik and Krogsrud 1995; Schmitt et al. 1994). Each test has its own strengths and weaknesses in terms of sensitivity, cost, and turn-around time. Although VI is still perceived as the criterion standard, it is laborious, time-consuming, and unsuitable for whole-herd testing (Renshaw et al. 2000; Saliki et al. 1997). ACE is suitable for screening large numbers of samples; however, the cost for testing individual animals is relatively high (Driskell and Ridpath 2006; Sandvik and Krogsrud 1995). Immunohistochemistry detects viral antigens by staining formalin-fixed, paraffin-embedded skin biopsies specimens, which is time intensive and can only be applied on tissue samples (Cornish et al. 2005). Real-time RT-PCR has been widely accepted in recent years because of its rapid turn-around time and high sensitivity that, in principle, enables the testing of pooled samples (Bhudevi and Weinstock 2003; Hamel et al. 1995; Mahlum et al. 2002).

The objective of the current study was to develop, validate, and apply a cost-effective, reliable, and rapid testing protocol for the detection of BVDV PI animals in exposed herds. To achieve this goal, real-time RT-PCR was optimized to detect extremely low numbers of BVDV in clinical specimens. Pooled samples were screened by real-time RT-PCR, and individual positive samples were then identified by ACE. The validity of the combined real-time RT-PCR and ACE testing scheme was assessed by performing interest and intersample type comparisons. The least-cost pool sizes were determined based on the predicted prevalence.

3.3 Materials and methods

3.3.1 Virus and cell culture

BVDV Singer (type 1) and BVDV 125c (type 2) were obtained from the National Veterinary Services Laboratories (NVSL, Ames, IA). The Madin-Darby bovine kidney cell line free of BVDV was maintained in minimum essential medium supplemented with 5% horse serum (Sigma-Aldrich, St. Louis, MO) in 75-cm tissue culture flasks. After inoculation of Madin-Darby bovine kidney cell line with BVDV, the cultures were incubated at 37°C and observed daily for cytopathic effect. The inoculated cultures that showed 70-80% cytopathic effect were subjected to a freeze-thaw cycle and low-speed centrifugation for 10 min at 1,000 × g. The culture supernatants were collected, divided into aliquots, and then stored at -80°C before use. The virus stocks were titrated in 96-well microtiter plates, and the titers (log 50% tissue culture infective dose [$TCID_{50}$]) were calculated as previously described (Reed and Muench 1938).

3.3.2 Sample collection, preparation, and pooling

Serum samples from 899 animals and skin biopsy (ear notch) samples from 27,033 animals submitted to the Mississippi Veterinary Research and Diagnostic Laboratory (Pearl, Mississippi) between 2006 and 2008 for the diagnosis of bovine viral diarrhea were used in the present study. Approximately 2 ml of non-hemolytic serum was taken from each of the original samples for further testing. The ear notches, approximately 1.25 cm in diameter (0.19 ± 0.02 g), were placed in individual sterile tubes that contained 2 ml 0.1 M phosphate buffered saline (PBS, pH 7.4), supplemented with 0.02% sodium azide as a preservative. After a brief vortexing (10 sec) and 10-min incubation at room temperature, the supernatants of the ear notch-PBS suspensions were collected for further testing. The aliquots of serum and ear notch extracts were refrigerated at 4°C before being tested by real-time RT-PCR and ACE within one week. All ACE-positive as well as 50 ACE-negative ear notches were cut into two halves. One half was fixed in 10% neutral formalin for IHC, whereas the other half, in PBS, was stored at -80°C for later use. To screen a herd for the presence of BVDV PI animals, the samples were pooled by mixing equal volumes (200 µl) of sera or ear notch-PBS extracts from 25 to 50 individual animals of the same herd. The pool sizes (25-50) were chosen based on 2 factors, the number of samples to be tested and the lowest possible diagnostic cost per animal. The prevalence (0.5%) data for the year 2005 generated by testing individual samples were used for cost estimation.

3.3.3 RNA extraction

Two methods were used to extract RNA from different samples. For serum samples, RNA was extracted from either 200 µl of non-hemolytic individual or 200 µl of pooled sample by using a commercially available RNA purification system – Trizol

(Invitrogen Corp., Carlsbad, CA). With ear-notch extracts, cell-cultured BVDV, viral stocks, and bacterial stocks, RNA was isolated from 500 µl of supernatant by using a commercial RNA purification kit – RNeasy® Mini kit (Qiagen, Alameda, CA). RNA extraction was carried out by following the manufacturers' instructions with minor modifications (2 additional washes of RNA pellets or columns). RNA was either resuspended in A or eluted with B in 50 µl of distilled DNase- and RNase-free H_2O. The RNA preparations were stored at -80°C prior to real-time RT-PCR analysis.

3.3.4 Primers and probes

Two sets of primers and probes that target BVDV 5'UTR were derived from previous publications (Bhudevi and Weinstock 2003; Letellier and Kerkhofs 2003). Two additional sets of primers and probes that target the same region were designed by using the Primer Express (Applied Biosystems Inc., Foster City, CA) software. All primers and probes were commercially synthesized (Qiagen Operon Technologies, Alameda, CA). Two common primers for BVDV-1 and BVDV-2, and 2 distinct probes capable of differentiating the 2 genotypes were included in the real-time RT-PCR assay. The 5'-ends of the BVDV-1 and BVDV-2 probes were labeled with fluorescent reporter molecules, 6-carboxyfluorescein (FAM) and cyanine 5 (Cyc5), respectively, whereas the 3'-ends were labeled with the Black Hole Quencher dye (BHQ-1). To clone the 5'-UTR, primers BVDV–LYF 5'–TAGCCATGCCCT TAGTAGGAC–3' and BVDV–LYR 5'–CTCCATGTGCCATGTACAGCA –' were used.

3.3.5 Real-time RT-PCR procedure

One-step real-time RT-PCR was performed by using commercial reagents according to the manufacturer's instructions (Qiagen Operon Technologies, Alameda,

CA). The optimal annealing temperature for a given combination of primers and probes was determined by increasing the temperature from 55°C to 59°C in increments of 1°C. The optimal concentrations of primers and probes were determined by titration between 0.1 µmol and 1 µmol. The optimal $MgCl_2$ concentration was determined by titration between 1.5 mM to 2.5 mM. The final volume of real-time RT-PCR was 25 µl, which contained 8 µl sample RNA and 17 µl of reagent mixture with 2.5 mM $MgCl_2$, 10 mM deoxyribonucleotide triphosphates, 0.2 µmol of forward primer, 0.2 µmol of reverse primer, 0.24 µmol of BVDV-1 probe, 0.96 µmol of BVDV-2 probe, 5µmol random hexamers, 10 U of RNAse inhibitor, and 1 µl of enzymes (Qiagen, Alameda, CA). The reactions were carried out on a programmable thermocycler Smart Cycler II (Cepheid Inc., Sunnyvale, CA) as follows: 48°C for 25 min; 95°C for 10 min; 50 cycles of 95°C for 25 s, 56°C for 25 s, 72°C for 30 s; and final elongation at 72°C for 10 min. To avoid potential contaminations associated with BVDV-contaminated bovine serum, equine serum was used for cell culture and propagation of viruses. Procedures for sample processing, PCR reaction set-up, PCR amplification, and cell culture were performed in separate laboratory sections.

3.3.6 Preparation of BVDV control RNAs

The 5'UTRs of BVDV-1 and BVDV-2 were amplified by PCR and cloned in the TOPO (topoisomerase I) TA (*Taq*-amplified) cloning vector (Invitrogen Corp., Carlsbad, CA) downstream of the T7 promoter to yield plasmids pBVDV-1-5' and pBVDV-2-5', respectively. The 2 plasmids were linearized with restriction enzyme *BamH* I and purified by phenol-chloroform extraction and ethanol precipitation. The control RNA was transcribed from the T7 promoters by using a T7 RNA polymerase according to the

manufacturer's instructions (Applied Biosystems/Ambion, Austin, TX). The transcribed RNA was purified by using a RNA purification kit – Rneasy® Mini kit (Qiagen, Alameda, CA) to remove nonincorporated nucleotides and then quantified by spectrophotometry.

3.3.7 Determination of real-time RT-PCR sensitivity and specificity

The analytical sensitivities were determined by performing real-time RT-PCR assays by using RNA prepared from the following: 1) 10-fold serial dilutions of BVDV-1 and BVDV-2 stocks, 2) pooled negative ear notch samples (n = 25) spiked with serial dilutions of viral stocks, 3) 10-fold serial dilutions of in vitro transcribed BVDV-1 and BVDV-2 5'-UTR RNA, and 4) pooled negative ear samples (n = 25) spiked with serial dilutions of in vitro transcribed RNA. The input amount of viruses and RNA ranged from 0.33 to 6.33 log $TCID_{50}$ or 1 to 8 log RNA copies per reaction. Standard curves were generated by plotting threshold cycle (Ct) values in the logarithmic linear phase of amplification against the log $TCID_{50}$ or log RNA copies. The detection limits of real-time RT-PCR were determined as the last dilution at which all replicate reactions gave positive results.

The specificity was evaluated by performing real-time RT-PCR by using RNA extracted from 10-fold serial dilutions (10^7 – 10 $TCID_{50}$ or colony-forming units) of various bovine pathogens, including *Bovine herpes virus-1* (NVSL), *Bovine respiratory syncytial virus* (NVSL), *Bovine parainfluenza virus-3* (NVSL), *Escherichia coli* (American Type Culture Collection [ATCC] 25922), *Mycobacterium paratuberculosis* (ATCC 19698), *Pastarella multocida* (Mississippi Veterinary Research and Diagnostic Laboratory clinical isolate), *Mannheimia hemolytica* (ATCC 43270), *Arcanobacterium*

pyogenes (ATCC 19411), and *Histophilus somni* (ATCC 700025). The viral and bacterial concentrations ranged from 1 to 8 log $TCID_{50}$ and 1 to 8 log CFU, respectively. The fluorescent signals of real-timeRT-PCR were analyzed for each organism and compared to that of BVDV-1 and BVDV-2.

To assess the effect of sample pooling on the sensitivity of real-time RT-PCR, positive (PCR and ACE) ear-notch extracts (n = 8) were used to artificially contaminate negative ear-notch pools of different sizes. The pools were created by mixing equal volumes (100 µl) of ear notch extracts from 1 positive sample and 11, 24, or 49 negative samples, yielding a final pool size of 12, 25, or 50. Real-time RT-PCR assays were subsequently carried out by using RNA prepared from these pools.

3.3.8 Antigen capture ELISA and IHC

ACE detection of BVDV in sera and ear notches was conducted by using a commercially available test kit per manufacturer's instruction (IDEXX Laboratories, Westbrook, ME). Immunohistochemistry detection of BVDV antigen was conducted as described previously (Cornish et al., 2005; Shin and Acland, 2001). Briefly, the ear notch samples were fixed in 10% neutral formalin for 2-3 weeks, embedded in paraffin, and sectioned at 5 µm. Tissue sections were treated with proteinase K (Qiagen, Alameda, CA), followed by incubation with a primary monoclonal antibody against BVDV (Oklahoma State University, Stillwater, OK), and alkaline-phosphatase reagents (Biopath, Oklahoma City, OK) per manufacturers' instructions. The IHC staining was graded by 2 pathologists by using a 4-point scale system similar to a previously described study (Shin and Acland, 2001). A consensus grade of the 2 pathologists was assigned to each sample. In brief, positive results were interpreted as 1 = faint minimal staining, 2 =

mild staining, 3 = moderate staining, and 4 = marked intense staining. Negative staining was scored as 0.

3.3.9 Testing scheme and cost structural analysis

For cost-effectiveness, the samples were pooled and screened by real-time RT-PCR, and individual samples in positive pools were then tested by ACE. The cost for screening BVDV PI animals was predicted for herd prevalence that ranged from 0.25 to 3.0% as described previously (Muñoz-Zanzi et al. 2000). The costs of the 3 tests, estimated based on the costs of reagents and labor as well as the actual fees of several diagnostic laboratories, were as follows: ACE, $4 – $8 (average $6); IHC, $3 – $7 (average $5); real-time RT-PCR, $25 – $45 (average $35); and VI, $20 – $40 (average $30). The cost was assumed to be the same for a given type of test performed on an individual sample or a pooled sample. The prevalence of BVDV-positive animals in a herd was assumed to be 0.25%, 0.3%, 0.5%, 0.75%, 1.00%, 1.25%, 1.50%, 1.75%, and 2.00%. The costs for individual real-time RT-PCR and ACE tests were assigned as *c* and *d,* respectively. The prevalence was represented by π. The total number of animals in a herd was designated as *N,* the pool size was *k*, and the number of pools was *r*, where $r = N/k$, with assuming *r* is an integer. A positive pool was expected to contain at least 1 positive sample. The probability that a pool would test positive was the binomial probability, $\rho = 1- (1-\pi)^k$. Thus, the cost for pools of *r* was $cr + dr\ [1-(1-\pi)^k]*k$, and the cost for testing each animal was $c/k+d\ [1-(1-\pi)^k]$. The costs for testing individual animals for different pool sizes and prevalence rates were determined. The cost reductions associated with the application of the combined real-time RT-PCR and ACE

testing scheme, compared with testing individual animals by ACE, IHC, and real-time RT-PCR were assessed based on the local prevalence and sample pool size used.

3.3.10 Genetic analysis of the field isolates

The 5'UTR of BVDV isolates were PCR amplified and cloned into a TA cloning vector (Invitrogen Corp., Carlsbad, CA), and sequenced by a commercial sequencing facility (Qiagen Operon Technologies, Alameda, CA). The sequences of 22 local BVDV isolates, 23 reference strains (12 BVDV-1 and 11 BVDV-2), 4 reference *Border disease virus strains*, and 4 reference CSFV strains were compared. The 5'-UTR sequences were aligned by using ClustalX version 2.0 (Larkin et al. 2007). Maximum parsimony (MP) and neighbor-joining analyses were performed by using PAUP* 4.0 Beta as described previously (Wan et al. 2008). Maximum likelihood tree estimation was evaluated by using GARLI version 0.951 (Wan et al. 2008). Tree topologies were confirmed between each of these 3 methods. Bootstrapping support for tree topologies was performed by using the neighbor-joining method implemented in PAUP* 4.0 Beta with 1,000 replicates.

3.3.11 Statistical analysis

The correlations between real-time RT-PCR Ct values and the input numbers of BVDV (log $TCID_{50}$) and between real-time RT-PCR Ct values and the pool sizes (ln dilution factors) was determined by linear regression analysis by using Excel 2000 software (Microsoft Corp., Redmond, WA). The correlations between different BVDV diagnostic tests, including real-time RT-PCR, ACE, and IHC, were determined by using the CORR procedure of SAS 9.1 software (SAS Institute Inc., Cary, NC). The Pearson correlation coefficient (r) was used as a measure of the strength of correlation, where +1

indicated a perfect linear correlation and -1 suggested a perfect inverse (negative) correlation. The criteria for interpreting the strength of correlations were 0.00 to 0.39, no correlation to weak correlation; 0.40 to 0.70, moderate correlation; and 0.7 to 1.0, strong correlation. A p-value less than 0.05 indicated statistical significance.

3.4 Results

3.4.1 Sensitivity and specificity of real-time RT-PCR

Of the multiple primers and probes tested, a previously published set of primers and probes (BVDV-F2/BVDV-PESTR, BVDV-P-1, and BVDV-P-2) (Letellier and Kerkhofs 2003) demonstrated the highest sensitivity and lowest background noise (data not shown). These primers and probes were used throughout the current study to amplify the 5'-UTR of BVDV-1 and BVDV-2. Linear correlations ($R > 0.99$) were found between the Ct value and the log $TCID_{50}$ or log RNA copies. The detection limits of the assay defined based on virus concentrations were 1.33 log $TCID_{50}$ per reaction for BVDV-1 and 2 log $TCID_{50}$ per reaction for BVDV-2. The detection limits on the input RNA copy numbers were 1 log copies per reaction for BVDV-1 and 2 log copies per reaction for BVDV-2 (Figure 3.1A). When in vitro transcribed RNA mixed with negative ear-notch samples, the detection limits were 2 log copies per reaction for BVDV-1 and 3 log copies per reaction for BVDV-2 (Figure 3.1B). When the real-time RT-PCR assays were applied on 3 commonly isolated bovine viral pathogens and 6 bacterial strains, no fluorescent signals were detected, which suggested a satisfactory specificity.

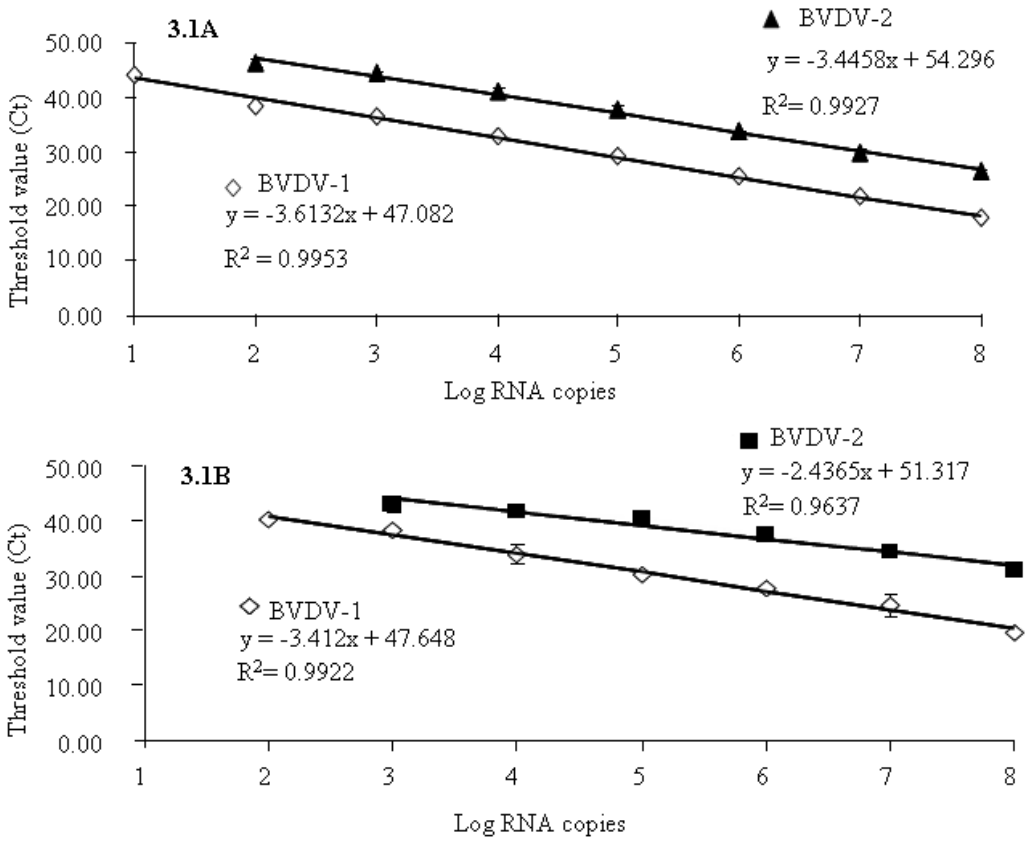

Figure 3.1 Real-time reverse transcription polymerase chain reaction sensitivity and linearity for in vitro transcribed 5 -untranslated region RNA (A) and RNA-spiked ear-notch samples (B)

Notes: Standard curves were obtained by plotting the threshold cycle (Ct) value versus log RNA copies per reaction. Data shown are the average of 3 independent experiments. BVDV= *Bovine viral diarrhea virus*.

3.4.2 Correlation between testing methods

To validate the testing system, the correlations between the semiquantitative results of 3 methods were determined. Thirty-four archived ACE-positive ear notches (from positive pools) with sample-to-positive (S/P) ratios that ranged from 0.4 to 1.4 were subjected to real-time RT-PCR analyses. The real-time RT-PCR Ct values were initially between 34 and 45 for pools that contained those individual positive samples. The pool sizes were between 25 and 50. After 1-year storage at -80°C and several cycles

of freeze-thaw, 4 individual samples tested negative by real-time RT-PCR. For calculation convenience, a Ct value of 50 was assigned to these 4 samples. The Ct values for the 34 archived individual samples ranged from 29 to 50. For IHC confirmation, ear notches were fixed in 10% formalin within a week of their arrival to the diagnostic laboratory and then were processed for microscopic evaluations. Results of the present study indicated that the BVDV antigen was mainly present in the epithelial cells located at the epidermis and follicles. No differences in staining pattern and intensity were observed between infections with genotype 1 and 2 viruses or between infections with a single genotype and both genotypes. Consensus scores (1 to 4) obtained by 2 pathologists were assigned to the positive samples. Pearson correlation coefficients (r) and the probability values (p) showed that the correlations between the semiquantitative results of real-time RT-PCR and ACE or IHC were moderate and significant ($r > 0.4$, $P<0.05$), whereas the correlation between ACE and IHC was weak ($r = 0.338$, $P = 0.051$; Table 3.1). The inverse correlation between Ct values and ACE S/P ratios or IHC grades was in accordance with the fact that Ct value is inversely related with the amount of input virus RNA.

Table 3.1 Strength and significance of correlations between semiquantitative results of different *Bovine viral diarrhea virus*

Test Methods	Pearson correlation coefficients (n = 34)		
	RT-PCR (Ct value)	ACE (S/P ratio)	IHC staining grade
Real-time RT-PCR (Ct value)	r = 1.000	r = -0.432	r = -0.421
	P = 0.000	P = 0.004	P = 0.013
ACE (S/P ratio)	r = - 0.432	r = 1.000	r = 0.338
	P = 0.004	P = 0.000	P = 0.051

Notes: RT-PCR = reverse transcription polymerase chain reaction; Ct = threshold cycle; ACE = antigen capture enzyme-linked immunosorbent assay; S/P ratio = sample-to-positive ratio; IHC = immunohistochemistry. Pearson correlation coefficients (*r)* and the *P* value are indicators of the strength and significance of the correlation between 2 tests. A negative number indicates an inverse correlation between 2 tests. Results from 34 individual samples were subjected to analysis.

3.4.3 Effect of sample pooling on real-time RT-PCR detection of BVDV

Eight PCR-positive ear notches with real-time RT-PCR Ct values that ranged from 30.54 to 34.10 were diluted with equal volumes of varying numbers of negative ear-notch samples to create pools of different sizes. Threshold cycle values for individual positive samples and the corresponding pools at sizes of 12, 25, and 50 are summarized in Table 3.2. The changes in Ct versus the changes in sample pool size are presented in Figure 3.2A. Overall, a linear correlation (R^2 = 0.993) was found between the changes in Ct and Ln pool sizes (Figure 3.2B). At pool sizes of 12 and 25, the average (SD) increases in Ct were 5.02 ± 0.94 and 6.49 ± 1.53 cycles, respectively. At a pool size of 50, sharp increases in Ct values as well as interest variations for 3 of the 8 samples (nos. 2, 5, and 8) were detected (Table 3.2 and Figure 3.2A).

Table 3.2 Real-time RT-PCR Ct values of individual positive samples before and after dilutions with negative samples

Sample ID /Pool	1	2	3	4	5	6	7	8
1×	30.54±0.5	34.10±1.4	33.35±0.3	31.96±1.7	33.85±1.6	32.21±1.5	31.27±0.5	32.77±0.6
12	34.58±0.7	38.60±2.8	37.32±0.5	37.23±0.9	38.62±0.3	37.57±0.3	36.62±0.4	39.68±2.6
25	36.15±1.2	41.92±2.8	39.39±0.9	38.87±1.4	41.29±0.2	38.53±0.7	39.60±1.1	39.98±2.0
50	38.15±2.0	44.64±4.7	40.86±1.4	39.63±1.7	45.13±4.2	39.12±1.4	40.16±1.6	44.62±4.8

Notes: Data shown are average threshold cycle values and standard deviations of 3 independent experiments with replicate reactions in each assay.

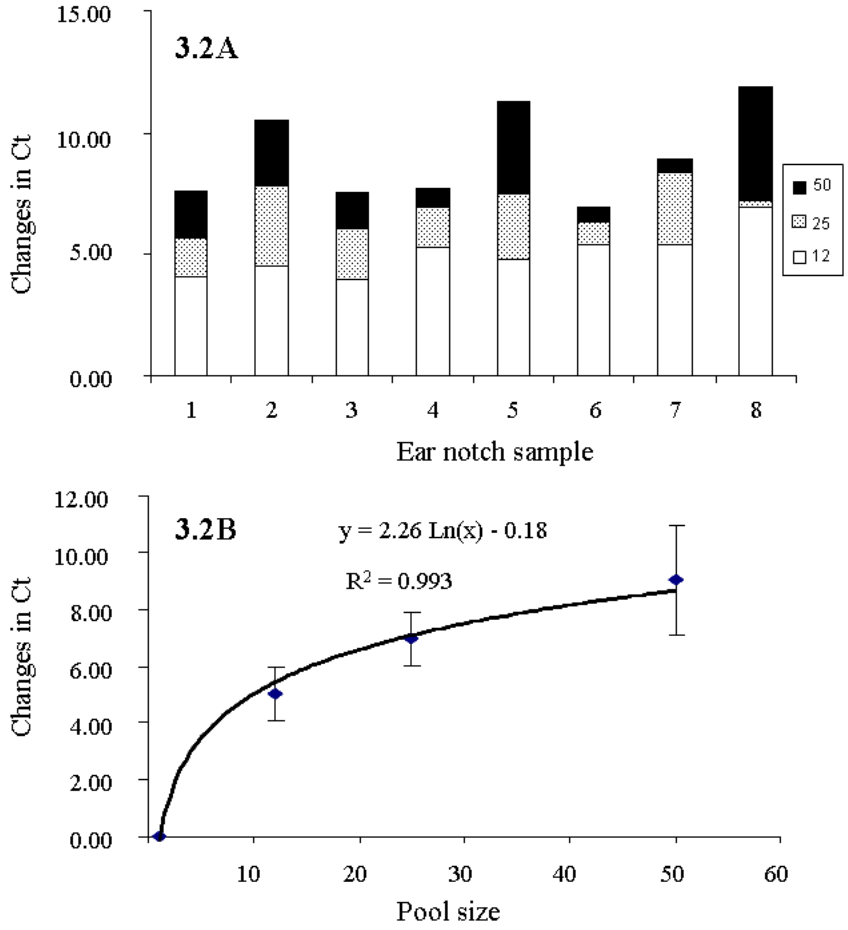

Figure 3.2 Effect of sample pooling on threshold cycle (Ct) values for 8 individual ear-notch samples

Notes: Bars (A) represent the changes in Ct after mixing 1 positive sample with 11, 24, and 49 negative samples to yield pools of 12, 25, and 50. The line graph (B) indicates a linear correlation between the increases in Ct value and Ln pool sizes. Data represent the average of 3 independent experiments.

3.4.4 Prevalence of BVDV

From 2006 to 2008, a total of 27,932 animals were tested for BVDV persistent infection (Table 3.3). A total of 22,732 samples (ear notch or sera from different animals) were subjected to real-time RT-PCR screening of pooled samples, followed by ACE testing of individual samples in positive pools. Approximately 5,300 ear-notch samples were tested directly by ACE. Immunohistochemistry was used to confirm

results when it was necessary. Eighty-five of more than 1,000 pools at sizes that ranged from 25 to 50 tested positive by real-time RT-PCR, and 26 individual ear notches tested positive by direct ACE. The majority of positive pools contained 1 positive sample that was subsequently identified by ACE. Of the 85 positive pools, 3 pools (3.53%) contained 2 positive samples and 1 pool (1.18%) contained 3 positive samples. The average prevalence of BVDV in Mississippi for a 3-year period was 0.40% with annual fluctuations of 0.24% to 0.72%, which was similar to previously reported prevalence of 0.3% to 0.5% in the central south United States (Fulton et al. 2009).

Table 3.3 Prevalence of *Bovine viral diarrhea virus* during a 3-year period

Year	No. of ear notches	No. of sera	No. of total	Prevalence
2006	8,610 (36)	26 (0)	8,636 (36)	0.42
2007	13,001 (31)	357 (1)	13,358 (32)	0.24
2008	5,422 (30)	516 (13)	5,938 (43)	0.72
2009	27,033 (97)	899 (14)	27,932 (111)	0.40

Notes: The total number of samples represents the number of animals being tested. Ear-notch and serum samples were derived from different individual animals. The numbers in parentheses are positive samples.

3.4.5 Cost-effectiveness

The cost effectiveness of the combined real-time RT-PCR and ACE testing scheme was evaluated for different pool sizes and prevalence rates (Figure 3.3). At all pool sizes, the cost per animal associated with real-time RT-PCR/ACE increased as the prevalence increased. The least cost pool size at prevalence of 0.25–0.50% was 50 and at a prevalence of 0.75–2.0% was 25. The second-best pool sizes for different prevalence were as follows: 100, 0.25%; 25, 0.30–0.50%; and 50, 0.75–2.0%. For instance, the cost per animal at a prevalence of 0.5% with sample pool sizes of 25 and 50 were predicted to be $2.11 and $2.03, respectively. For an average local prevalence of 0.40%, the

predicted cost per animal for pools of 25 was $1.97 and for pools of 50 was $1.78. However, the true prevalence for a given farm or herd was unknown, and the cost per animal might have deviated slightly from the predicted dollar amount. At an average prevalence of 0.4%, application of the real-time RT-PCR and ACE screening protocol could reduce the cost per animal at a pool size of 25 by 67%, 61%, 94%, and 93%, compared with testing individual animals by ACE, IHC, real-time RT-PCR, and VI, respectively (Table 3.4).

Table 3.4 Cost reduction associated with the application of combined real-time reverse transcription (RT-PCR) and antigen capture enzyme-linked immunosorbent assay (ACE)

	Cost of individual test per animal		Pool size (animal per pool) Cost reduction ($ per animal)	
	Range ($)	Average ($)	25% (1.97 per	50% (1.78 per
ACE	4-8	6	67.17	70.33
IHC	3-7	5	60.60	64.40
RT-PCR	25-45	35	94.37	94.91
VI	20-40	30	93.43	94.07

Notes: IHC = immunohistochemistry; VI = virus isolation. Cost reduction associated with the application of RT-PCR and ACE was calculated based on the predicted cost per animal at pool sizes of 25 and 50.

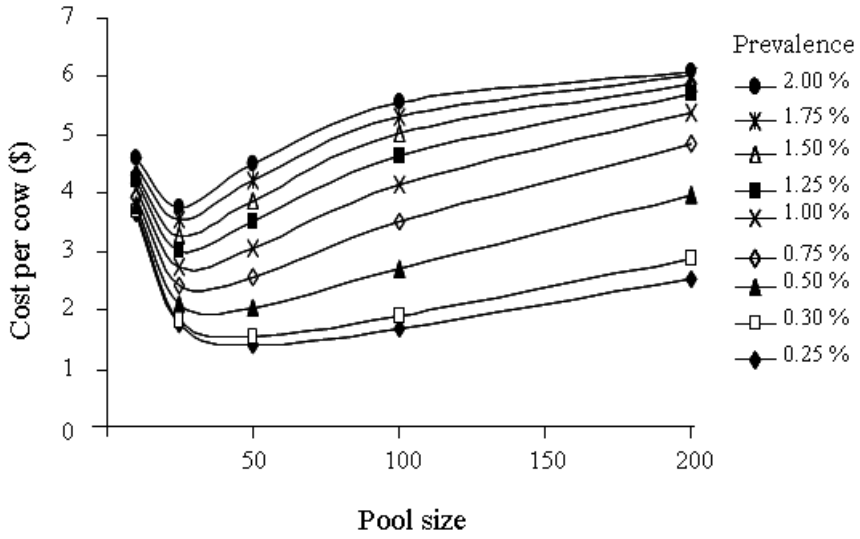

Figure 3.3 Cost structural analysis for the combined reverse transcription polymerase chain reaction and antigen capture enzyme-linked immunosorbent assay testing scheme.

Notes: Cost per animal was calculated for different sample pool sizes at various presumed prevalence. The predicted least cost pool sizes are 25 samples per pool at prevalence from 0.75% to 2.0% and 50 samples per pool at prevalence from 0.25% to 0.5%.

3.4.6 Genetic analysis of BVDV field isolates

Genotyping by real-time RT-PCR showed that 92.94% of PI animals were infected with BVDV-1, 3.53% with BVDV-2, and 3.53% with both genotypes (1 and 2). Analysis of the 5'UTR of 22 field isolates suggested that all 18 BVDV-1 isolates belonged to the BVDV-1b subgenotype, whereas the 4 BVDV-2 isolates fell into the BVDV-2a subgenotype (Figure 3.4). Genetic variations among the isolates of each subgenotype were apparent. Within the BVDV-1b subgenotype, MS7 and MS14 were clustered with the KE strain; MS2 and MS9 were apart from other known strains; MS19 was closely related to the Osloss strain; MS5, MS1, and MS3 were related to the ILLNC strains; and MS6, MS10, MS8, and MS15, along with CP 7 and PAT, constituted another

cluster. The BVDV-2b field isolates formed two distinct clusters with MS13 and MS20 more closely related to BVDV2-AU501, and MS4 and MS12 to BVDV-2-890.

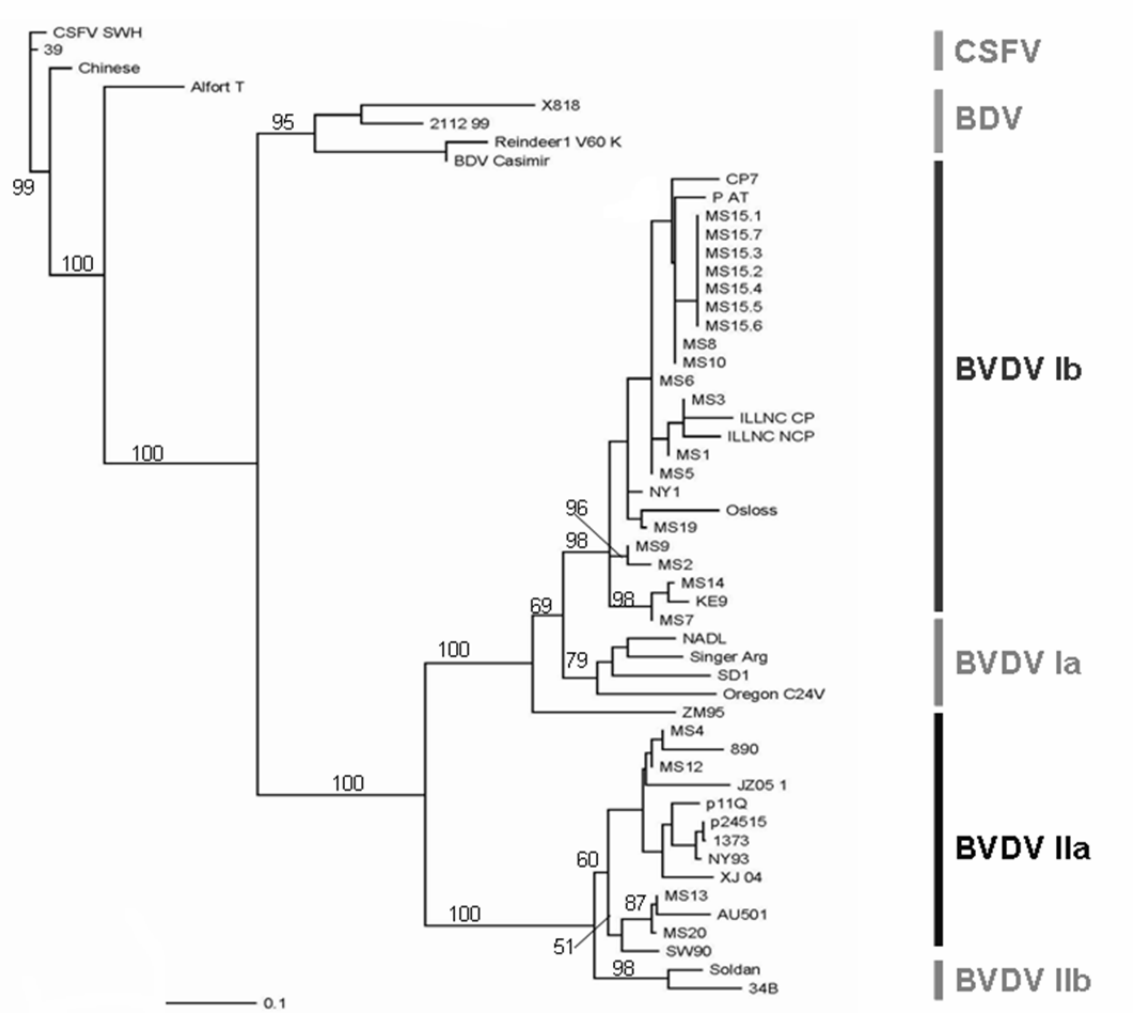

Figure 3.4 Phylogenetic analysis of the 5 -untranslated region (UTR) sequences

Notes: The 5'-untranslated region (UTR) sequences of 22 local Bovine viral diarrhea virus (BVDV) isolates and selected reference strains of BVDV, Border disease virus (BDV), and Classical swine fever virus (CSFV) were used in the phylogenetic analysis. The dendrogram was generated by using the maximum parsimony and neighbor-joining methods. The 22 local BVDV strains belonged to BVDV-1b and BVDV-2a with genetic variations within each subgenotype.

3.5 Discussion

Progress has been made in developing PCR-based assays for detection of BVDV in various clinical specimens (Bhudevi and Weinstock 2003; Hamel et al. 1995; Kennedy et al. 2006; Letellier and Kerkhofs 2003; Mahlum et al. 2002; Schmitt et al. 1994; Weinstock et al. 2001; Young et al. 2006). To validate real-time RT-PCR assay in our author's laboratory, multiple primers and probes that target the 5'-UTR of BVDV were compared, and a previously published combination demonstrated the highest sensitivity and consistency (Letellier and Kerkhofs 2003). By following extensive optimization, the real-time RT-PCR assay was able to detect low numbers of viruses in viral stocks or in virus-spiked ear-notch samples. However, the analytical sensitivity determined based on $TCID_{50}$ titer could be affected by the viability of the stock virus. To overcome this inherent limitation, real-time RT-PCR standard curves were constructed by using serial dilutions of in vitro transcribed 5'-UTR RNA, which again demonstrated high analytical sensitivities for both BVDV-1 and BVDV-2. In the present study, cell-free specimens were tested by real-time RT-PCR, which limited the use of housekeeping genes as an internal positive control. An external control, such as an unrelated RNA virus, could have been used to spike clinical samples before RNA extraction and RT-PCR as described previously (Young et al. 2006). Because of concerns about potential cross-contaminations, this technique was not adopted. The performance of the real-time RT-PCR was monitored via spiking negative specimens with known quantities of viruses or in vitro transcribed RNA.

Intra- and interlaboratory studies have shown that real-time RT-PCR, IHC, and ACE are all suitable for the detection of BVDV PI animals (Cornish et al. 2005; Driskell and Ridpath 2006). Good correlations have been found between the qualitative results of

ACE and IHC or IHC and real-time RT-PCR methods (Cornish et al. 2005; Sandvik 2005; Hilbe et al. 2007). In the current study, the qualitative results of these tests were in 100% agreement for all positive samples, which was in accordance with previous reports (Cornish et al. 2005; Driskell and Ridpath 2006). Further analysis of the quantitative results revealed a moderate, but significant, correlation between Ct value and S/P ratio or Ct value and IHC grade, indicating the usefulness of Ct in estimating BVDV load in specimens. No significant correlation occurred between the ACE S/P ratio and IHC grade, which might result from the limited capability of ACE to quantify viruses or the subjective grading system of IHC. The present study did not focus on the impact of ear-notch size on the quantitative results of real-time RT-PCR or ACE. A previous study showed that the amount of virus detected in ear notch extract was not significantly affected by sample size over a wide weight range (0.75 g and 0.05 g) (Ridpath et al., 2006). Because the ear-notch samples used in the current study weighed approximately 0.2 g (1.25 cm in diameter), the sample size was reasonably assumed to have minimal impact on Ct value or S/P ratio.

It has been suggested that RT-PCR can detect individual positive serum or ear notch samples in pools of 100 or more (Weinstock et al. 2001; Kennedy et al. 2001). The current study determined the validity of real-time RT-PCR for screening ear-notch pools by using multiple individual positives to spike negative pools of various sizes. One interesting observation from the current study was that the real-time RT-PCR Ct values for most PI animals fell in the range of 30 to 34 (approximately 4.7 to 3.6 log RNA copies). Theoretically, mixing a weak-positive ear notch of Ct 34 with 99 negative samples (pool size of 100) would increase the Ct to 41.22, a value considered positive.

However, the high Ct values and wide intertest variations for 3 of the 8 pools at a size of 50 indicated a risk of false-negative results associated with large pool sizes (≥ 50).

The effect of sample pooling on the cost, sensitivity, and specificity of BVDV testing has been predicted by using mathematical models (Muñoz-Zanzi et al. 2000; Muñoz-Zanzi et al. 2006). Cost analysis for the combined real-time RT-PCR and ACE testing scheme indicated that 50, followed by 25, was the most cost-effective pool size at prevalences from 0.25% to 0.50%. A comparison of the Ct values for individual positives in pools of various sizes suggested that pooling 25 samples had the desired minimal impact on real-time RT-PCR results. In addition, a pool size of 25 was conducive to ease of laboratory bench operation and data management. At a pool size of 25, a 60-90% reduction in cost per animal could be achieved by using real-time RT-PCR and ACE, compared with individual sample testing by IHC, ACE or real-time RT-PCR. Because the true prevalence for a given herd is unknown until it is tested, the actual cost per animal and cost reduction may deviate slightly from the calculated dollar amount, depending on the prevalence, the herd size, and the number of animals to be tested (Muñoz-Zanzi et al. 2000; Muñoz-Zanzi et al. 2006).

By using the combined real-time RT-PCR and ACE procedure, more than 27,000 samples were tested in a 3-year period. Analysis of the data showed that BVDV prevalence in Mississippi was relatively low, ranging from 0.24% to 0.72. Although the presence of both BVDV-1 and BVDV-2 in 3 specimens was confirmed by repeated real-time RT-PCR testing, it is unusual to have a dual persistent infection with both genotypes. One possible explanation for this phenomenon is that these PI animals were exposed to a second BVDV virus of a different genotype. Analysis of the 5'UTR of 22 isolates revealed the predominance of BVDV-1b followed by BVDV-2a and the absence

of BVDV-1a, which differed from the previously reported prevalent types in central south United States (BVDV Ib only) or in southwest United States (Ib followed by Ia and IIa) (Fulton et al. 2000; Fulton et al. 2009; Vilcek et al. 2001). Although genetic variations were detected within each subgenotype, 1b or 2a, it was unknown whether these variations were associated with any antigenic changes. Further study in this area will provide useful information for formulating successful vaccination programs.

3.6 References

1. Afshar A, Dulac GC, Dubuc C, Howard TH. 1991. Comparative evaluation of the fluorescent antibody test and microtiter immunoperoxidase assay for detection of bovine viral diarrhea virus from bull semen. Can J Vet Res 55: 91-93.

2. Baker JC. 1995. The clinical manifestations of bovine viral diarrhea infection. Vet Clin North Am Food Anim Pract 11: 425-445.

3. Bhudevi B, Weinstock D. 2003. Detection of bovine viral diarrhea virus in formalin fixed paraffin embedded tissue sections by real time RT-PCR (Taqman). J Virol Meth 109(1): 25-30.

4. Cornish TE, van Olphen AL, Cavender JL, et al. 2005. Comparison of ear notch immunohistochemistry, ear notch antigen-capture ELISA, and buffy coat virus isolation for detection of calves persistently infected with bovine viral diarrhea virus. J Vet Diagn Invest 17:110-117.

5. Driskell EA, Ridpath JF. 2006. A survey of bovine viral diarrhea virus testing in diagnostic laboratories in the united states from 2004 to 2005. J Vet Diagn Invest 18:600-605.

6. Fulton RW, Saliki JT, Confer AW, et al. 2000. Bovine viral diarrhea virus cytopathic and noncytopathic biotypes and type 1 and 2 genotypes in diagnostic laboratory accessions: clinical and necropsy samples from cattle. J Vet Diagn Invest 12:33-38.

7. Fulton RW, Whitley EM, Johnson BJ,et al. 2009. Prevalence of bovine viral diarrhea virus (BVDV) in persistently infected cattle and BVDV subtypes in affected cattle in beef herds in south central United States. Can J Vet Res. 73(4): 283–291.

8. Hamel AL, Wasylyshen MD, Nayar GP. 1995. Rapid detection of bovine viral diarrhea virus by using RNA extracted directly from assorted specimens and a one-tube reverse transcription PCR assay. J Clin Microbiol 33:287-291.

9. Hilbe M, Stalder H, Peterhans E, et al. 2007. Comparison of five diagnostic methods for detecting bovine viral diarrhea virus infection in calves. J Vet Diagn Invest 19:28-34.

10. Houe H. 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Vet Microbiol 64:89-107.

11. Kennedy JA, Mortimer RG, Powers B. 2006. Reverse transcription-polymerase chain reaction on pooled samples to detect bovine viral diarrhea virus by using fresh ear-notch-sample supernatants. J Vet Diagn Invest 18:89-93.

12. Larkin MA, Blackshields G, Brown NP, et al. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948.

13. Lettllier C, Kerkhofs P. 2003. Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhea virus. J Virol Methods 114:21-27.

14. Lindenbach BD, Rice CM. 2001. Flaviviridae: the viruses and their replication. *In:* Fields Virology, ed. Knipe DM, Howley PM, 4th ed., pp. 991-1041. Lippincott Willams & Wilkins, Philadelphia, PA.

15. Loneragan GH, Thomson DU, Montgomery DL, et al. 2005. Prevalence, outcome, and health consequences associated with persistent infection with bovine viral diarrhea virus in feedlot cattle. J Am Vet Med Assoc 226:595-601.

16. Mahlum CE, Haugerud S, Shivers JL, et al. 2002. Detection of bovine viral diarrhea virus by TaqMan reverse transcription polymerase chain reaction. J Vet Diagn Invest 14:120-125.

17. McClurkin AW, Littledike ET, Cutlip RC, et al. 1984. Production of cattle immunotolerant to bovine viral diarrhea virus. Can J Comp Med 48:156-161.

18. Meyers G, Thiel HJ. 1996. Molecular characterization of pestiviruses. Adv Virus Res 47:53-118.

19. Muñoz-Zanzi C, Thurmond M, Hietala S, Johnson W. 2006. Factors affecting sensitivity and specificity of pooled-sample testing for diagnosis of low prevalence infectious. Prev Vet Med 74:309-322.

20. Muñoz-Zanzi CA, Johnson WO, Thurmond MC, Hietala SK. 2000. Pooled-sample testing as a herd-screening tool for detection of bovine viral diarrhea virus persistently infected cattle. J Vet Diagn Invest 12:195-203.

21. Reed LJ, Muench H. 1938. A simple method of estimating fifty percent end point. Am J Hyg 27:493-497.

22. Renshaw RW, Ray R, Dubovi EJ. 2000. Comparison of virus isolation and reverse transcription polymerase chain reaction assay for detection of bovine viral diarrhea virus in bulk milk tank samples. J Vet Diagn Invest 12:184-186.

23. Ridpath JF, Bolin SR, Dubovi EJ. 1994. Segregation of bovine viral diarrhea virus into genotypes. Virology 205:66-74.

24. Ridpath JF, Hessman BE, Neill JD, et al. 2006. Parameters of Ear Notch Samples for BVDV Testing: Stability, Size Requirements and Viral Load. Proc Am Assoc Bov Pract Conf 39:269-270.

25. Saliki JT, Fulton RW, Hull SR, Dubovi EJ. 1997. Microtiter virus isolation and enzyme immunoassays for detection of bovine viral diarrhea virus in cattle serum. J Clin Microbiol 35:803-807.

26. Sandvik T, Krogsrud J. 1995. Evaluation of an antigen-capture ELISA for detection of bovine viral diarrhea virus in cattle blood samples. J Vet Diagn Invest 7:65-71.

27. Sandvik T. 2005. Selection and use of laboratory diagnostic assays in BVD control programmes. Prev Vet Med 72:3-16.

28. Schmitt BJ, Lopez OJ, Ridpath JF, et al. 1994. Evaluation of PCR for diagnosis of bovine viral diarrhea virus in tissue homogenates. J Vet Diagn Invest 6:44-47.

29. Shin T, Acland H. 2001. Tissue distribution of bovine viral diarrhea virus antigens in persistently infected cattle. J Vet Sci 2:81-84.

30. Vilcek S, Paton DJ, Durkovic B, et al. 2001. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch Virol 146:99-115.

31. Wan H, Sorrell EM, Song H, et al. 2008. Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS One 3:e2923.

32. Weinstock D, Bhudevi B, Castro AE. 2001. Single-tube single-enzyme reverse transcriptase PCR assay for detection of bovine viral diarrhea virus in pooled bovine serum. J Clin Microbiol 39:343-346.

33. Young NJ, Thomasa CJ, Collinsa ME, et al. 2006. Real-time RT-PCR detection of bovine viral diarrhoea virus in whole blood using an external RNA reference. J Virol Methods 138:218-222.

CHAPTER IV

DETECTION FAILURE OF BOVINE VIRAL DIARRHEA VIRUS-2 BY DIRECT FLUORESCENT ANTIBODY TEST ON TISSUE SAMPLES FROM ANIMALS WITH SEVERE CLINICAL DISEASE AND FATAL OUTCOME

4.1 Abstract

Two virulent BVDV strains were successfully recovered from a beef breeding cow and a beef calf respectively. Infected animals exhibited severe clinical signs compatible with thrombocytopenia/hemorrhagic syndrome recently reported in North American. FA test performed on fresh tissues failed to detect BVDV antigen in both cases. However, viral RNA was detected by real-time RT-PCR on selected tissues from both animals and genotyped as BVDV-2. Viral antigen was also detected in various cells of oral mucosa, esophagus mucosa, and Rare peyer's patches by IHC test. Analysis of protein motif on E2 glycoprotein revealed motif changes for both M10-3432 and M10-5347 compared to reference strain 125c. However the potential involvement of these changes in diagnostic tests or virus pathogenesis remains unknown. Results from IFA showed sensitivity reduction while cross-neutralization test indicated antigenic changes on both M10-3432 and M10-5347. Phylogenetic analysis of E2 region grouped both Mississippi isolates in BVDV-2a, a subtype containing high virulent strains. M10-3432 was clustered nicely with high virulent strain 890 while M10-5347 was clustered with another high virulent strain CD 87. In conclusion, the results from studying these two cases suggest a need for periodically reevaluation of the efficacy of various diagnostic

tests, especially direct FA-based detection of BVDV when virulent BVDV-2 strains are implicated.

4.2 Introduction

Bovine viral diarrhea virus-1 (BVDV-1) and BVDV-2 along with classical swine fever virus (CSFV) and sheep border disease virus (BDV) consist of the *Pestivirus* genus in the *Flaviviridae* family (Thiel et al. 2005). With a broad tissue tropism, BVDV infections are associated with a wide variety of clinical manifestations in cattle, such as respiratory distresses, diarrhea, reproductive failure, encephalitis, and congenital abnormities (Baker 1995). Most BVDV infections are mild or subclinical, which are commonly associated with reduced production and immunosuppression (Baker 1995; Brownlie et al. 1987; Moerman et al. 1994). Acute BVDV infections usually manifest as diarrhea and/or respiratory distress (Baker 1995; Brownlie et al. 1987; Moerman et al. 1994). When a susceptible pregnant cow becomes infected, transplacental transmission can occur. The consequences of fetal infections are embryonic death, abortion, stillbirth, or congenital abnormities. If the infection occurs in the first trimester of the gestation it usually leads to immune tolerance to BVDV, thus resulting in the birth of a persistently infected (PI) calf (Baker 1995). PI animals have no detectable antibodies against BVDV, but consistently shed the virus in their excretions and secretions, serving as a primary source of BVDV infections. Therefore, prompt identification and subsequent removal of PI animal (s) from exposed herds are critical to control and prevention programs (Baker 1995; Brownlie et al. 1987).

BVDV has two biotypes, CP and NCP based on their growth characters on cell culture (Donis 1995; Kümmerer 2000). The strains isolated from clinical specimens are

usually NCP whereas CP strains are rare and often co-isolated with NCP strains from mucosal diseases (Bolin and Ridpath 1998). The clinical manifestations of mucosal disease are characterized by erosive lesions throughout the digestive tract, profuse diarrhea, and high mortality (Baker 1995; Brownlie et al. 1987; Moerman et al. 1994). Phylogenetic analysis based on the 5' un-translated region (UTR) of BVDV genome has led to the recognition of genotype 1 and 2 (Ridpath et al. 1994). Each BVDV genotype can be further divided into subtypes, such as 1a and 1b, or 2a and 2b. The two genotypes can be also differentiated serologically (Deregt and Prins 1997). BVDV-1 is often associated with mild or subclinical infections while BVDV-2 is frequently isolated from outbreaks of hemorrhagic syndrome in North American. Hemorrhagic syndrome is an acute, severe form of BVDV, which has been documented in dairy herds, beef cattle, and veal calves (Carman et al. 1998; Goens 2002; Moerman et al. 1994; Pellerin et al. 1994; Perdrizet et al. 1987; Ridpath et al. 2006). The condition of the disease is marked by significantly decreased platelet and white blood cell counts (Walz et al. 1999). The common clinical signs observed include high fever, bloody diarrhea, hemorrhages on mucus membrane, and eventually sudden death (Baker 1995; Pellerin et al. 1994; Ridpath et al. 2006). Young calves inoculated with BVDV-2a strain CD 87 or BVDV-2a strain 890 successfully developed the hemorrhagic form of BVD in two independent experiments (Corapi et al. 1989; Walz et al. 1999). Effort in identification of genetic markers for virulence of BVDV-2 strain has resulted in limited success and the underlying mechanism for the emergence of virulent BVDV remains unknown (Pellerin et al. 1994; Ridpath et al. 2006; Topliff and Kelling 1998; Tussen et al. 1996). Prevalence of BVDV-2 with its antigenicity distinct from that of BVDV-1 raised the

concerns about adequate cross-protection of vaccines made of mainly BVDV-1 strain (Dean 1999).

Genetic diversity is the basis for phenotypic variations and pathogenic heterogeneities (Hanmers et al. 200; Donis 1995; Kümmerer et al. 2000). Like other members of the pestiviruses, genetic recombination between different BVDV strains, between BVDV and other pestiviruses, or even between viral genomic RNA and host cell RNA can occur (Kümmerer et al. 2000; Jones et al. 2002; Vilcek et al. 2005). Recombination can result in gene deletion or insertion. High frequency of point mutation generated in the RNA virus replication has been contributed to the lacking proofreading by the viral RNA-dependent-RNA polymerase. It is estimated that such point mutation is readily generated at a frequency of one mutation per virus replication cycle per 10 kb. The high frequency of mutation during virus replication can result in the production of quasispecies, an abundance of genetic variants within a single host (Jones et al. 2002). The E2 gene of BVDV has a hyper variable region that is accountable for the quasispecies variation (Donis 1995). Because E2 protein is a major glycoprotein associated with the induction of host immune response as well as attachment process during virus replication, antigenic changes due to mutations in E2 will have a direct effect on the efficacy of BVDV vaccination, the sensitivity and integrity of serological tests, and the pathogenesis of the virus. Previous reports indicated that antigenic variations had be associated with multiple test failures in detection of BVDV field isolates by various diagnostic tests, such as immunohistochemistry, ELISA, and peroxidase-linked antibody assay (PLA assay) (Elahi et al. 1997; Gripshover et al. 2007).

In this study, we report that direct FA with proper controls failed to detect BVDV antigen on tissue samples in two cases with severe clinical diseases and fatal outcome,

which are compatible to BVDV hemorrhagic syndrome. Two BVD viruses, M10-3432 and M10-5347, were recovered from tissue pool of respective animal. Our goal was to investigate the underlying mechanism in which detection failure of direct FA for BVDV antigen on various tissues by studying the virus distribution in various tissues using different diagnostic tests and by serologically, genetically, and molecularly characterizing the two clinical isolates. The results generated from this study will surely help us better understand how genetic and antigenic diversity among BVDV isolates affect the performance of diagnostic test, especially direct FA test on fresh tissue specimens.

4.3 Materials and methods

4.3.1 Animals

The first animal was an adult beef breeding cow (M10-3432) that was found dead and the carcass was submitted to Mississippi Veterinary Research and Diagnostic Laboratory (MVRDL) for necropsy. Prior to death, the animal developed acute respiratory signs and bloody diarrhea. The death was the 17th fatality in a herd with forty animals within a 3-month period of time. A full necropsy was performed and samples were collected from major organs, including the brain, skin sample from ear, the oral and esophageal mucosa, the small and large intestine, the mesenteric lymph node, the lung, the liver, the spleen, and the kidney. The second animal (M10-5347) was a feedlot calf that exhibited similar clinical signs and died subsequently. The necropsy of the second animal was performed by the attending veterinarian. Only tissue samples of the lung, the mesenteric lymph node, and the small intestine were submitted for laboratory diagnosis.

4.3.2 BVDV reference strains and antisera

BVDV genotype 1 (Singer) and genotype 2 (125c) were obtained from the National Veterinary Services Laboratories (NVSL) and propagated in MDBK cells as described previously (Yan et al. 2011). The reference sera against genotype 1 and 2 were also obtained from NVSL with neutralization titers greater than 16. Anti-BVDV polyclonal antiserum (porcine origin) that was used to produce anti-BVDV FITC labeled FA conjugate was obtained from same manufacture (VMRD, Pullman, WA).

4.3.3 Direct fluorescent antibody assay (FA)

Direct FA was performed to detect BVDV, infectious bovine rhinotracheitis (IBR), bovine parainfluenza type 3 (PI3), bovine respiratory syncytia virus (BRSV), and blue tongue virus (BT) antigens in tissue specimens. Briefly, cryostat frozen sections of fresh tissues were cut at 5 µm and fixed in cold acetone for 10 min at room temperature. Following air drying, the sections were stained with BVDV FA conjugate (VMRD, Pullman, WA) for 30 min at 37^{o}C and counter-stained with Evens blue. The slides with tissue sections were mounted and examined under FA microscope. The results were recorded as positive or negative.

4.3.4 Immunohistochemistry (IHC)

IHC detection of BVDV antigen was conducted as described previously (Yan et al. 2011). Briefly, the tissue specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 µm. Following treatment with proteinase K, the sections were incubated with a monoclonal antibody recognizing both BVDV-1 and 2 for 30 min at 37^{0}C. After 3 washes, the sections were incubated with an anti-murine antibody labeled with alkaline-phosphatase according to the manufacturer's instructions

(Biopath, Oklahoma city). After substrate addition the sections were examined under light microscopy.

4.3.5 Antigen capture enzyme linked immunoabsorbent assay (AC-ELISA)

AC-ELISA was performed to detect BVDV antigen in the ear notch and serum specimens of the beef cow according to the manufacturer's instructions (IDEXX, VT).

4.3.6 Virus isolation

BVDV viruses (M10-3432 and M10-5347) were isolated using Madin-Darby bovine kidney (MDBK) cells as previously described (Yan et al. 2011). Briefly, the tissue samples (except the intestinal tissues) were pooled and homologized in 10 ml Hank's buffered saline solution (HBSS) containing penicillin (100 unit/ml), streptomycin (100 µg/ml), and amphotericin B (2.5µg/ml). The tissue homogenates were clarified by low centrifugation at 1500 g for 10 minute. 1 ml supernatant from each clarified homogenate was used to inoculate MDBK cells at 70-90% confluence in T-25 tissue culture flasks. After 1 hour incubation at 37^{o}C, 5 ml minimum essential medium (MEM) supplemented with 5% horse serum (V/V) and a final concentrations of penicillin (100 unit/ml), streptomycin (100 µg/ml), and amphotericin B (2.5µg/ml) was added to each flask. For negative controls, MDBK cells in T-25 flasks were mock-infected with 1 ml HBSS followed by addition of 5 ml growth medium. The inoculated flasks were incubated at 37^{o}C in 5% CO_2 and inspected daily for 5 days to observe virus induced cytopathogenic effect (CPE). To detect non-cytopathic BVDV, infected MDBK cells were stained directly with BVDV antiserum conjugated with fluorescein isothiocyanate (FITC) and examined under an FA microscopy. To harvest the virus, BVDV-infected MDBK cells were lysed through one cycle of freeze-thaw and clarified by low-speed centrifugation for

10 min at 1,500 g. The supernatants of MDBK cell lysates were collected and stored at -80°C until further testing. The virus isolates were titrated in 96-well plates and the titers ($logTCID_{50}$) were determined as described by Reed and Muench (1938).

4.3.7 Indirect fluorescent antibody assay (IFA)

MDBK cells were grown to confluent monolayers in 96-well tissue culture plates. The cells were inoculated with BVDV at a dose of 100 $TCID_{50}$ per well. BVDV Singer and the 125c strains were included as positive controls. At 48 hours post inoculation, the cells were fixed in a mixed solution of acetone and methanol (equal volume, 50 µl/well) for 10 min at room temperature. The fixed cells were treated with 2-fold serially diluted porcine anti-BVDV antiserum (25 µl/well) for 30 min at 37°C followed by 3 washes in FA buffer. The cells were then incubated with anti-porcine IgG conjugated with FITC (25 µl/well) for 30 min at 37°C. After three times washing the stained cells were examined under an FA microscope.

4.3.8 Serum neutralization assay (SN)

SN assays were performed using a standard microtitration procedure. Briefly, reference sera were 2-fold serially diluted in a 96-well plate followed by addition of various testing viruses in an amount of 100 $TCID_{50}$ per well. After 1 hour incubation MDBK cells (5,000 /well) were seeded. Plate was incubated at 37°C and cytopathogenic effect (CPE) was observed daily for a period of five days. For NCP strains, a direct FA test was used to detect viral antigen. Duplicate wells were included for each serum dilution and the experiments were repeated 3 times. The neutralization titer was calculated based on the endpoint dilution, at which 50% cell protection from virus infection was observed.

4.3.9 RNA extraction

Viral RNA was extracted from cell culture supernatant using the RNeasy mini kit according to the manufacturer's instructions (Qiagen, CA). Total RNA from tissue homogenates samples were extracted by using TRIzon reagent per manufacturer's instructions (Life Technologies, CA). RNA from each sample was eluted in 50 µl of DNase and RNase-free water and stored at -80^{o}C until use.

4.3.10 Reverse transcriptase polymerase chain reaction (RT-PCR)

One-step real-time RT-PCR was performed to detect BVDV in tissue specimens and cell culture supernatants as described previously (Yan et al. 2011). Two pairs of primers were used to amplify the 5'-UTR for sequence determination (Table 4.1). Four pairs of primers were used to amplify the E2 coding region (Table 4.1). Primers and probes used in BVDV diagnosis and subsequent genotyping were described previously (Letellier et al. 2003; Yan at al. 2011). The sensitivity of the real-time RT-PCR in detecting BVDV clinical isolates was determined. In brief, RNA was extracted from 10-fold serial dilutions of each clinical isolate, BVDV-1 reference strain Singer, BVDV-2 reference strain 125c, and *in vitro* transcribed RNA using plasmid containing 5'UTR sequences of BVDV-1 or -2. A series of real-time RT-PCR was performed. Standard curves were generated by plotting threshold cycle values (Ct) in the logarithmic linear phase of amplification against the log RNA copy number. The detection limits were determined as the highest dilution at which all replicate reactions gave positive results.

Table 4.1 Primers of 5'UTR and E2

Targeting Region	Virus ID	Primer ID	Primer Sequences	Tm (°C)
5'UTR	BVDV1	1F	CTC GTA TAC ATA TTG GAC ACTC	58.9
		1R	CTC CAT GTG CCATGTACAGCA	62.57
	BVDV2	2F	GTATACGAGATTAGGTAAAGWAC	57.1
		2R	CTC CAT GTG CCATGTACAGCA	62.57
E2	BVDV1	1E2F1	GAC CAR ATT GGT GGC CTT ATG AGAC	65.4
		1E2R1	CAC YGT GTC CCC TAT CTT GCAT	63.6
		1E2F2	GAA GAC CTC TAC GAC TGT GCCT	64.5
		1E2R2	CAG TGG GAT CAC GCC TRG CTAT	65.5
	BVDV2	2E2F1	GCC ATC AGA GAC TTA ACT AGG	60.6
		2E2R1	GGTG TCA TCT ACA TAC CTG TAC	60.8
		2E2F2	TCY ATG AAT GCA TTC TDG GWGG	60.5
		2E2R2	GTC CAT AGC ACT TTG GTC ATC AG	62.8

Notes: Primers used for amplification of 5 UTR region and E2 coding region by RT-PCR. PCR products were cloned in the TOPO TA Cloning® Vector according to the protocol provided by the manufacturer (Invitrogen, CA) and sequenced from both directions.

4.3.11 Sequencing the 5 UTR and E2 glycoprotein gene

The 5 UTRs were amplified by PCR and cloned into the TOPO TA Cloning® Vector according to the manufacturer's instructions (Invitrogen, CA). Plasmid DNA was extracted using the DNAesy kit and the inserts were sequenced from both directions (Eurofins mwg operon, CA). The E2 glycoprotein gene was amplified using primers listed in Table 4.1. The PCR amplicons with overlapping ends were sequenced from both directions. Multiple sequence alignment was performed and the full length amino acid sequence of the E2 genes (approximately 1,116bp) was deduced.

4.3.12 Phylogenetic analysis

E2 amino acid sequences and 5 UTR nucleotide sequence of the clinical isolates and reference strains were aligned using Muscle program (Edgar 2004). The Genbank

accession numbers of the reference sequences are listed in Table 4.2. Best-fit model of the alignments was selected based on the Akaike information criterion (AIC) score, the best-fit model of nucleotides was selected using the Modeltest3.7 (Posada and Crandal 1998; Posada and Buckley 2004), and the best-fit model of amino acid sequences was selected using the prottest2.4 (Abascal et al. 2005). Phylogenetic trees were constructed using the Bayesian and Maximum Likelihood (ML) methods. With the Bayesian approach, phylogeny was inferred by using the Markov chain Monte Carlo (MCMC) algorithm (Huelsenbeck and Ronquist 2001). With the ML method, phylogeniy was inferred using the Phyml as secribed previously (Hanmers et al. 2001). The reliability of phylogenetic trees were estimated using the bootstrap analysis with 1000 replicates, and the value of greater than 70% (70/100 for ML and 0.7/1.00 for Bayesian) support the phylogenetic position.

Table 4.2 Reference strains used in the phylogenetic tree of E2

Strain name	Accession #	Sources	Strain name	Accession #	Sources
BVDV2_17237	EU747875	Slovakia	BDV_H2121	ADK63187	Germany
BVDV2_Gi_6	AF144612	Germany	BDV_Gifhorn	ADI58614	Denmark
BVDV2_Hokudai	BAJ09450	Japan	CSFV_SWH	AAZ23587	China
BVDV2_8827	ADX31697	USA	CSFV_Alfort	Aab50409	France
BVDV2_8833	ADX31699	USA	BVDV1_Osloss	AAA02769	Belgium
BVDV2_446	ADX31696	USA	BVDV1_VEDEVAC	CAE51342	Hungary
BVDV2_AU501	ABS19605	USA	BVDV1_ILLNC_CP	AAB58571	USA
BVDV2_CD87	AAA98608	Canada	BVDV1_ILLNC_NCP	AAB58572	USA(ND)
BVDV2_XJ_04	ACQ83621	China	BVDV1_ZM95	AAM88294	China
BVDV2_p11Q	AAN78116	Canada	BVDV1_KE9	ABK96992	Germany
BVDV2_Sil_Lke	AAC72815	Canada	BVDV1_M181 22	JN377424	USA
BVDV2_37621	ADX31702	USA	BVDV1_CP7	AAC55984	Germany
BVDV2_IAF_103	ADX31700	Canada	BVDV1_CP7_5A	AAG00378	Germany
BVDV2_793	ADX31701	Indiana	BVDV1_MS15.1	JN377417	USA
BVDV2_NY93	AAM91913	Germany	BVDV1_MS15.2	JN377418	USA
BVDV2_24301	AAC72816	Canada	BVDV1_MS15.3	JN377419	USA
BVDV2_p24515	AAN78117	Ontario	BVDV1_MS15.4	JN377420	USA
BVDV2_1373	AAD38683	USA	BVDV1_MS15.5	JN377421	USA
BVDV2_890/256	AAR24084	Belgium	BVDV1_MS15.6	JN377422	USA
BVDV2_890	AAA82981	USA	BVDV1_MS15.7	JN377423	USA
BVDV2_MS12	JN387139	USA	BVDV1_Singer_Arg	ABP57735	Argentina
BVDV2_Ind141353	AEA08367	India	BVDV1_NADL	AAA42854	USA(IA)
BVDV2_JZ05_1	ACX42224	China	BVDV1_SD1	AAA42860	USA(OH)
BVDV2_M10 5347	JN377415	USA	BVDV1_Oregon_C24V	AAC61755	England
BVDV2_M10 3432	JN377416	USA	BVDV1_8844	ADX31692	USA (IA)
BVDV2_125c	AAC72814	Canada			

Notes: Amino acid sequences of the E2 glycoprotein used in phylogenetic analysis were provided with strain name, GenBank accession number, and the origin of isolation.

4.3.13 Amino acid sequence and protein motif analyses

The E2 glycoprotein amino acid sequences of the isolates were deduced with the help of nucleotide tool and the protein motifs were analyzed using the protein tools provided by the SDS Biology Workbench (http://workbench.sdsc.edu).

4.3.14 Bacterial culture and fecal flotation

Standard aerobic and anaerobic bacterial cultures were setup for the tissue specimens of both animals. Routine fecal flotation procedures were performed to detect parasites in the intestinal contents of both animals.

4.3.15 Serological survey

Sera were collected from 10 animals (8 females and 2 males) on the beef breeding farm where case M10-3432 was originated. The age of the testing animals varied greatly from 3-month to 10-year old (Table 4.6). Serological assays were performed to detect antibodies against Blue tongue virus (BTV), bovine rhinotracheitis virus (IBR), and BVDV. BTV ELISA was performed according to the protocol provided by the manufacturer of the ELISA kit (IDEXX, VT). IBR and BVDV serum neutralization tests were performed as described above.

4.4 Results

4.4.1 Pathology

Gross examination of adult beef breed cow (M10-3432) revealed multifocal shallow erosions and ulcers in the oral cavity predominantly along the soft palate and tongue. Numerous erosions and shallow ulcers were also present along the entire length of the esophagus. The lung contained multiple areas of hemorrhage, and the abomasal mucosa was multifocally reddened (hyperemic). The uterus contains a proximately 5-6 month old bull calf fetus. No significant lesions were observed in the rumen, reticulum, omasum, small intestine, large intestine, liver, kidney, adrenal gland, spleen, and brain. With the beef calf (M10-5347), the lung was congested and multifocal hemorrhage was appreciated. No significant gross lesions were appreciated in mesenteric lymph node and

intestine tissue samples. Histopathologic examination of the tissues from the beef breed cow revealed multifocal ulcerative and lymphocytic stomatitis, multifocal ulcerative esophagitis, moderate eosinophilic and lymphocytic enteritis, multifocal lymphocytic abomasitis, and multifocal moderate to severe nonsuppurative perivascular encephalitis. Due to sample autolysis histopathology examination was not performed on tissue samples from beef calf (M10-5347).

4.4.2 Detection of viral antigen

FA did not detect BVDV or any other major bovine viruses (IBR, PI3, BRSV, BT) in the lung, oral mucosa, esophagus, brain, spleen, small intestine, liver, and kidney tissues of the beef cow (Table 4.3A). FA tests for BRSV, BTV, BVDV, and IBR were also negative for the lung, mesenteric lymph node, and small intestine tissues of the beef calf (Table 4.3B). However, IHC demonstrated the presence of BVDV antigen in the epithelial cells lining the tubuloacinar glands of the oral and esophageal mucosa of the beef cow (Figure 4.1). Positively stained macrophages were also observed in the Peyer's patches (M10-3432). BVDV antigen was not detected in the ear notch or serum samples of the beef cow (M10-3432), indicating that the animal was not persistently infected by BVDV. Due to the compromised histological structure associated with tissue autolysis, IHC could not be successfully performed for the beef calf (M10-5347).

Table 4.3 Detection of BVDV antigen on various tissues by FA, IHC, or ACE

A: M10-3432			
Tissue	IHC	FA	ACE
Lung	-	-	ND
Spleen	-	-	ND
Esophagus	+	-	ND
Oral	+	-	ND
Brain	-	-	ND
Intestine	±	-	ND
Ear Notch	-	ND	-

B: M10-5347			
Tissue	IHC	FA	AC-
Lung	ND	-	ND
MLN	ND	-	ND
Intestine	ND	-	ND

Notes: PP - Peyer' patches; MLN - Mesenteric lymph node; ± - weak positive; ND: Not done. Detection of BVDV antigen in various tissue specimens using fluorescent antibody test assay (FA), immunohistochemistry (IHC), or AC-ELISA (ACE). A.Results for the beef breeding cow (M10-3432). B. Results for beef calf (M10-5347). IHC was not performed for M10-5347 due to compromised morphology associated with tissue autolysis.

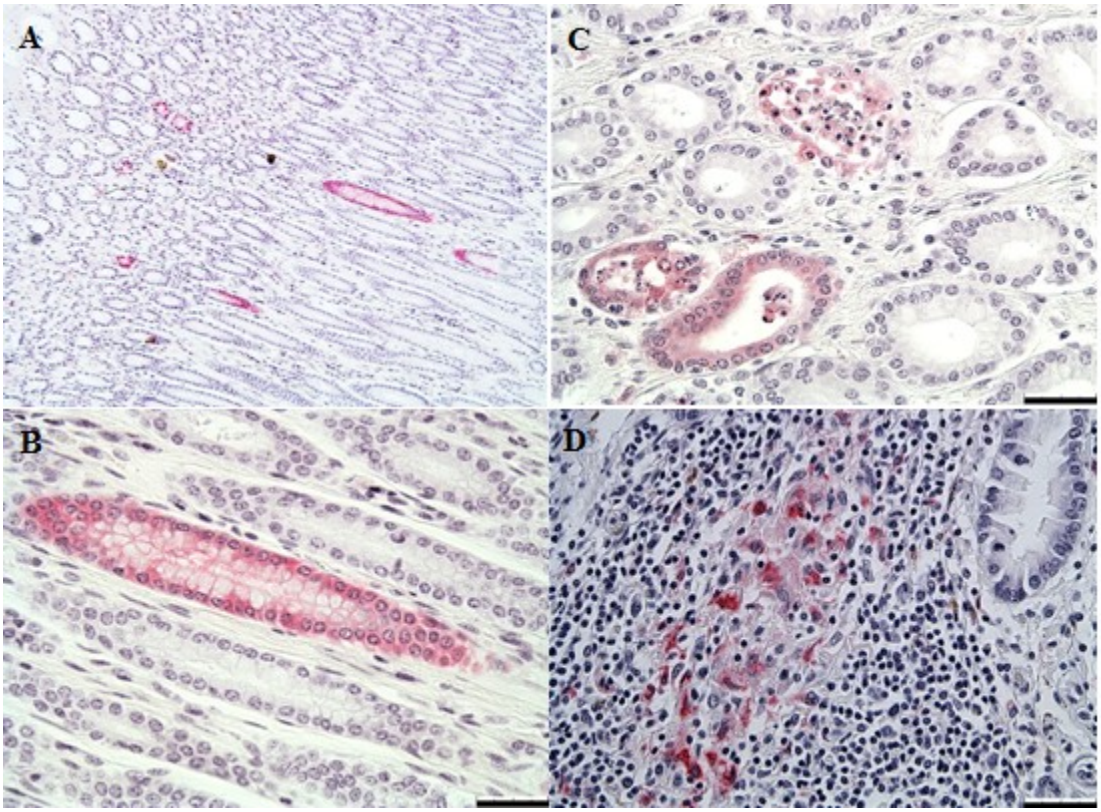

Figure 4.1 BVDV immunohistochemistry staining

Notes: A, oral mucosa. Epithelial cells lining multifocal mucosal glands demonstrated positive labeling for BVDV antigen. Bar=40 µm. B, oral mucosal gland higher magnification. C, small intestine. Multiple intestinal crypt epithelial cells and intraluminal cellular debris demonstrated strong positive labeling for BVDV antigen. Bar=40 µm. D, Small intestine. Scattered macrophages within deep mucosal lymphocytic aggregates demonstrated cytoplasmic BVDV antigen immunoreactivity. Bar=40 µm.

4.4.3 Real-time RT-PCR detection of BVDV isolates

Real-time RT-PCR performed on pooled tissues samples of the lung, oral mucosa, and esophageal mucosa of the beef cow (M10-3432) was positive for BVDV. Real-time RT-PCR based genotyping revealed that the virus belonged to genotype 2. Real-time RT-PCR did not detect the virus in the tissue specimens of the brain, spleen, kidney, and small intestine of M10-3432. Real-time RT-PCR also detected BVDV genotype 2 in the lung tissue of M10-5347. Real-time RT-PCR did not detect the virus in the lymph node

and small intestine tissue specimens of M10-5347. The detection limit for BVDV 125c is 1.33 log RNA copy number per reaction, 2 log RNA copy number for both M10-3432 and M10-5347 (Figure 4.2). Sequence analysis indicated that single-nucleotide mutations occurred in primer and probe binding sites, which could be accountable for the reduced sensitivity of real-time RT-PCR (data not shown). Nonetheless, the real-time RT-PCR assay was still able to detect low numbers of the BVDV clinical isolates.

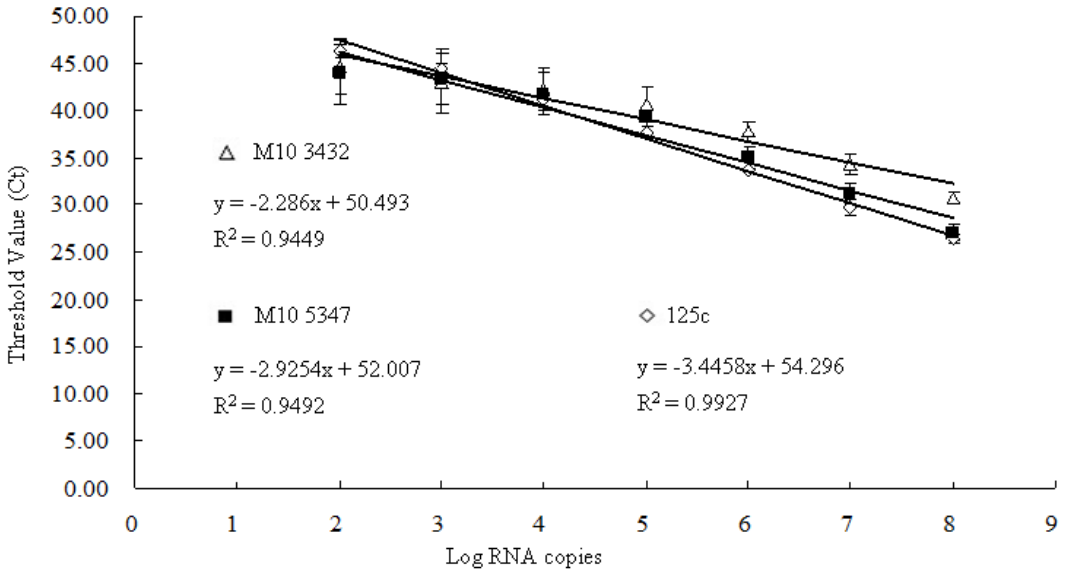

Figure 4.2 Standard curves of reference strain 125c and Mississippi isolates M10-3432 and M10-5347

Notes: Standard curves were constructed based on the Ct values obtained from real-time RT-PCR assays using 10-fold serially diluted, in vitro transcribed 5'-UTR RNA of BVDV 2 reference strain 125c, isolate M103432, and M105347. Data presented are geometric means of three independent experiments with duplicate reactions ± standard deviation. Y axis represents Ct value while X axis stands for RNA copy number.

4.4.4 Virus isolation and phylogenetic characterization

BVDV was isolated from the pooled tissue samples of both animals. Virus isolation was confirmed by real-time RT-PCR and FA detection of BVDV in inoculated

MDBK culture supernatant and/or fixed cells. The isolate derived from the breed cow, designated M10-3432, was NCP isolate. The virus isolated from the beef calf, designate M10-5347, was CP isoalte. Phylogenetic analysis using amino acid sequence of E2 glycoprotein classified both isolates, M10-3432 and M10-5347, as genotype 2a. However, the two isolates were further grouped into two distinct small clusters within 2a cluster. The isolate M10-3432 was closely related to MS12 (BVDV-2) and BVDV-2a 890 with the former being associated with an acute BVDV infection in Mississippi and the latter being a highly virulence strain isolated from yearling heifer in Canada that died of an acute uncomplicated BVDV infection (Bolin et al. 1992). The isolate M10-5347 was closely related to BVDV-2a CD 87, another high virulent BVDV 2a strain recovered from a severe outbreak in dairy herd (Figure 4.3).

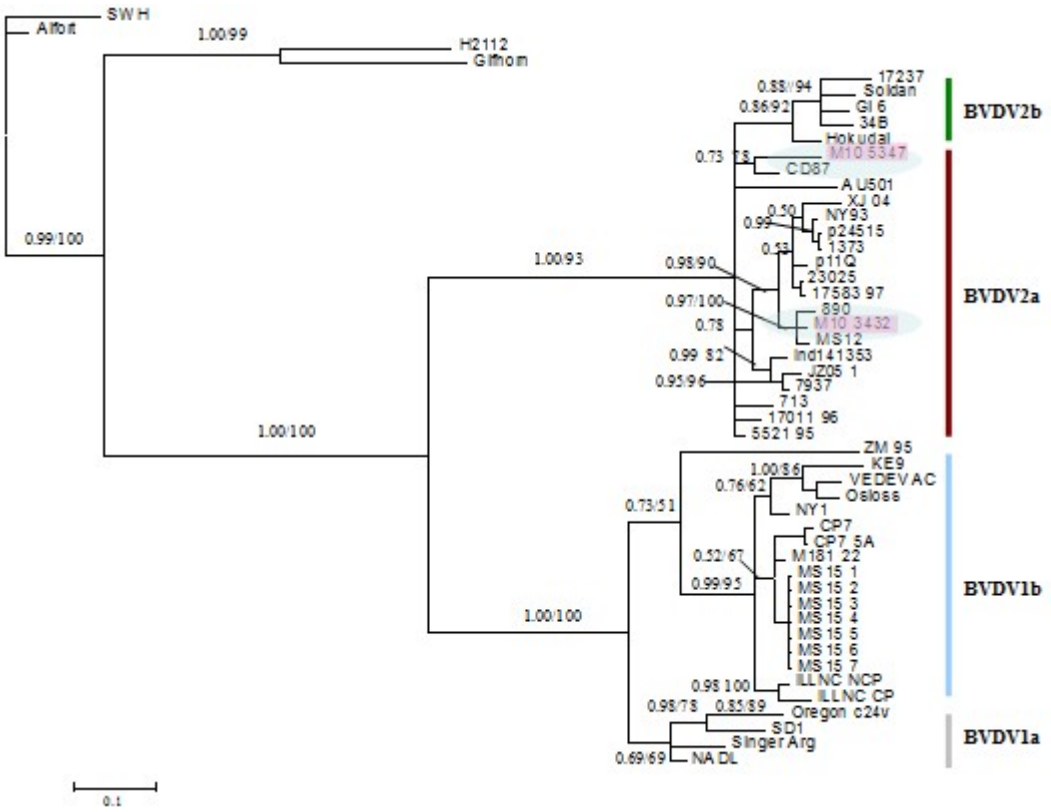

Figure 4.3 Phylogenetic tree inferred from E2 amino acid sequences

Notes: M10-3432 was clustered with high virulence strain 890 while M10-5347 was grouped with another virulence strain CD87 (highlighted in pink and blue). Both isolates were clustered together in BVDV-2a group, a subtype containing many virulent BVDV strains.

4.4.5 IFA sensitivity

Due to failure of FA in detecting BVDV antigen in tissue specimens, IFA was performed to investigate the reactivity of the isolates to the porcine anti-BVDV polyclonal antiserum that was used in the direct FA tests. The results showed that the reference BVDV strains (Singer and 125c) reacted with the antiserum at a dilution of 2,048 x (11 $\log_2$). Compared to the reference strains, a 3-log2 reduction in the reactivity to porcine anti-BVDV serum was observed for M10-5347 and 1 log reduction was found for M10-3432 (Table 4.4). The data suggested that the two clinical BVDV isolates had

reduced reactivity to the BVDV antiserum that is used in production of FA conjugate, which is commercially available to many diagnostic laboratories.

Table 4.4 IFA sensitivity

	2^3	2^4	2^5	2^6	2^7	2^8	2^9	2^10	2^11	2^12	CC	VC
BVDV-1	+	+	+	+	+	+	+	+	+	-	-	+
	+	+	+	+	+	+	+	+	+	-	-	+
BVDV-2	+	+	+	+	+	+	+	+	+	-	-	+
	+	+	+	+	+	+	+	+	+	-	-	+
M10-5347	+	+	+	+	+	+	-	-	-	-	-	+
	+	+	+	+	+	+	-	-	-	-	-	+
M10-3432	+	+	+	+	+	+	+	+	-	-	-	+
	+	+	+	+	+	+	+	+	-	-	-	+

Notes: Indirect FA was performed to determine the reactivity of BVDV isolates to porcine anti-BVDV polyclonal antiserum that was used to produce BVDV FA conjugate. Reactivity was presented as the highest dilution (titer) that was able to detect BVDV antigen on cell culture. Data presented are geometric means of three independent experiments with duplicate reactions. CC: cell control; VC, virus control.

4.4.6 Cross-neutralization activity

Anti-BVDV-1 reference serum (NVSL) effectively neutralized the Singer strain, a reference strain of BVDV-1, with a mean neutralization titer of 149. The anti-BVDV1 serum had no neutralizing activity against 125c, a reference strain of BVDV 2. The reference serum against BVDV-2 effectively neutralized 125c strain with a neutralization titer of 64 while it had very limited neutralization ability against the Singer strain (BVDV-1) with a low titer of 4 (Table 4.5). In contrast to the reference strains, M10-3432 was neutralized by reference sera against both genotype 1 and 2 with a titer of 1024. M10-5347 could also be neutralized by both types of antisera with neutralization titers of

64 and 32 for BVDV-1 and BVDV-2, respectively (Table 4.5). The cross neutralization data indicated that the isolates, although genotypically classified as type 2a, could be effectively neutralized by antisera against both BVDV-1 and BVDV-2. The cross neutralization activity with serum against BVDV-1 virus revealed that the strains had undergone major antigenic variations (Figure 4.4).

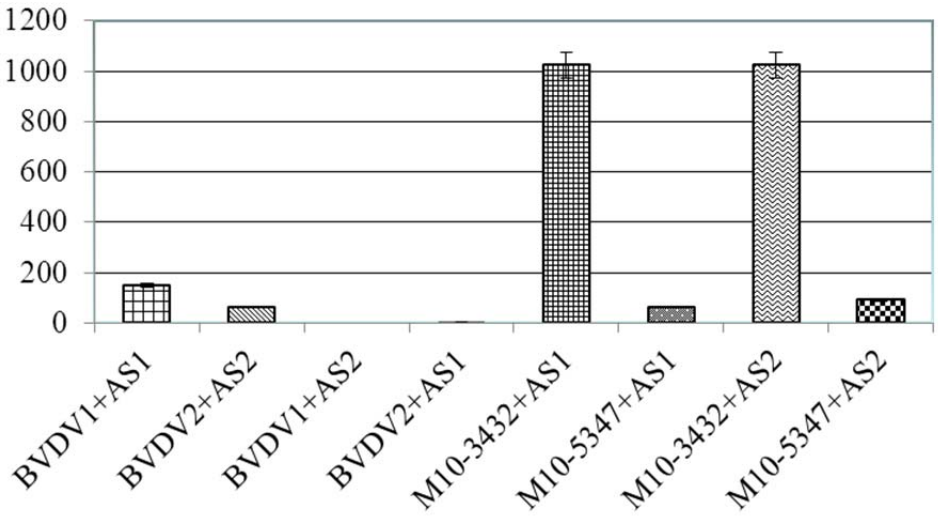

Figure 4.4 Antigenic variation detected by cross-neutralization assay

Notes: Antigenic Variation Detected By Cross-Neutralization Assay. Cross neutralization of BVDV isolates using standard reference antisera. Neutralization tests were performed using serially diluted antisera against genotype 1 and genotype 2. The neutralization titer was determined based on the highest dilution of a serum that was able to neutralize 100 $TCID_{50}$ virus. Data presented are geometric means of three independent experiments with duplicate reactions ± standard deviation. Note: AS1, anti-BVDV 1; AS2, anti-BVDV 2 reference serum; BVDV 1, Singer strain; and BVDV 2, 125c strain.

Table 4.5 Serum neutralization titer

BVDV-1 +AS1	BVDV-1 + AS2	BVDV-2 +AS1	BVDV-2 +AS2	M10-3432 +AS1	M10-3432 +AS2	M10-5347 +AS1	M10-5347 +AS2
149	0	4	64	1024	1024	64	32

Notes: Cross neutralization of BVDV isolates using standard reference antisera. Neutralization tests were performed using serially diluted antisera against genotype 1 and genotype 2. The neutralization titer was determined based on the highest dilution of a serum that was able to neutralize 100 TCID50 virus.

4.4.7 The amino acid sequence of E2 glycoprotein

The E2 glycoprotein consists of 373 amino acid residues with a projected molecular weight of 42.478 KD. A comparison of the E2 amino acid sequences of the two isolates with BVDV-2 reference strain (125c) revealed multiple mutations in the E2 genes of M10-3432 and M10-5347. Five unique mutations, highlighted in pink color, were identified in the E2 protein of M10-5347 (Figure 4.5). These five mutations were located at positions 29, T to A; 102, L to P; 275, M to L; 281, E to V; and 302, V to G. Three unique amino acid changes, highlighted in blue color, were identified in the E2 protein of M10-3432. These three amino acid changes were identified at the following positions: 142, A to V; 164, R to K; and 192, D to A. In addition to above mutations unique to each isolate, additional changes in amino acid sequences of the E2 gene, compared to that of the reference strain 125c, were observed for both isolates (Figure 4.5).

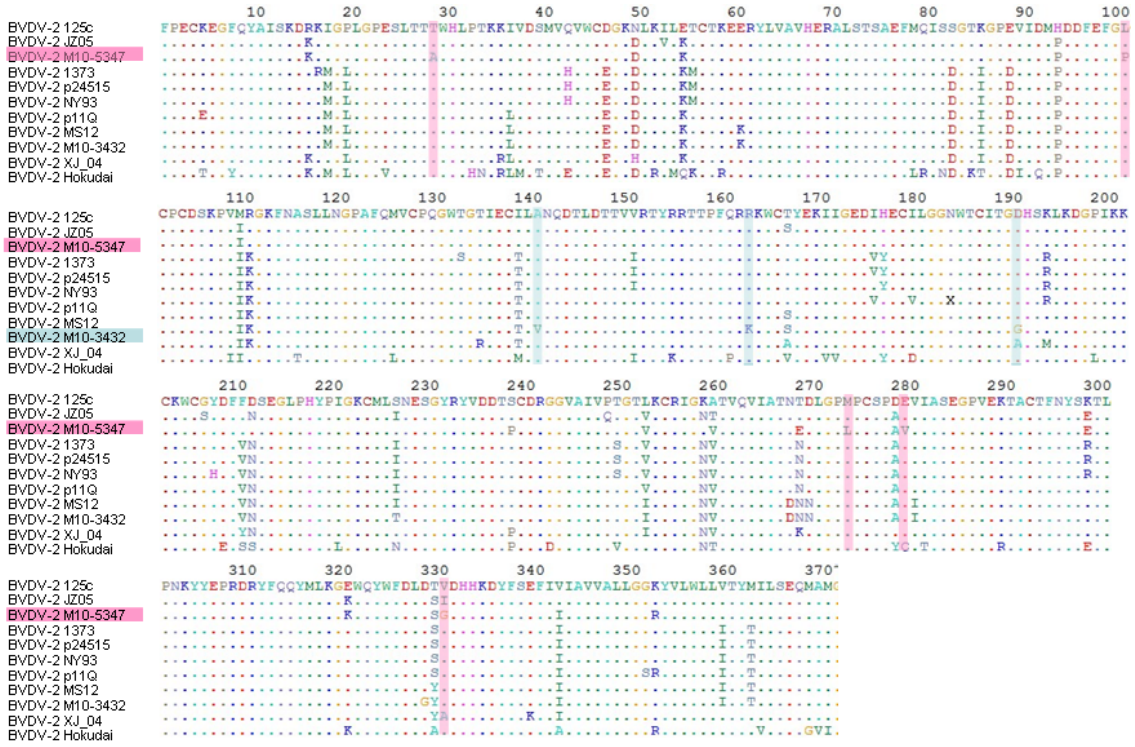

Figure 4.5 E2 amino acid sequence alignments

Notes: Multiple sequence alignment was performed using the ClustalW program of SDS Biological Workbench (http://workbench.sdsc.edu/). Altered amino acid residues aer shown in letters while consensus seuqnce was indicated by dots. Alterations unique to BVDV M10-3432 and M10-5347 are highlighted in pink and blue, respectively.

4.4.8 Protein motifs of the E2 glycoprotein

The protein motifs of the isolates and the reference strain 125c were analyzed. Five major types of protein motifs was identified in the E2 glycoprotein amino acid sequence, including the N-glycosylation site, cAMP- and cGMP-dependent protein kinase phosphorylation site, protein kinase C phosphorylation site, casein kinase II phosphorylation site, and N-myristoylation site. The E2 protein of M10-3432 possessed 5 N-glycosylation sites, which was one site more than that of BVDV 125c. At meantime, the E2 protein of M10-3432 lacked two Casein kinase II phosphorylation sites, compared to BVDV 125c. For M10-5347, its E2 protein missed one casein kinase II

phosphorylation site and one N-myristoylation site when compared to reference strain 125c that had 5 casein kinase II phosphorylation sites and 4 N-myristoylation sites. The comparative results were presented in Table 4.6.

Table 4.6 Protein motifs identified in BVDV E2 protein

Protein Motifs	Location		
	BVDV 125c	BVDV 125c	BVDV 125c
N-glycosylation site (4)	115->119	115->119	115->119
	184->188	184->188	184->188
	228->232	228->232	228->232
	296->300	296->300	296->300
cAMP- and cGMP-	155->159	155->159	155->159
Protein kinase C phosphorylation site (3)	34->37	34->37	34->37
	153->156	153->156	153->156
	252->255	252->255	252->255
Casein kinase II phosphorylation site (5)	58->62	58->62	58->62
	74->78	74->78	74->78
	238->242	238->242	238->242
	267->271	267->271	267->271
	277->281	277->281	277->281
N-myristoylation site (3)	100->106	100->106	100->106
	134->140	134->140	134->140
	182->188	182->188	182->188
	251->257	251->257	251->257

Notes: Protein motifs identified in BVDV E2 protein. Protein motifs identification was carried out by using protein tool on SDS Biological Workbench (http://workbench.sdsc.edu/). BVDV 125c, M10-3432, and M10-5347 were included in the analysis. The type and location of motifs as well as the quantity of the motifs (in parenthesis) in BVDV 125c are presented. The motifs in E2 protein consisted of N-glycosylation site, cAMP- and cGMP-dependent protein kinase phosphorylation site, Protein kinase C phosphorylation site, Casein kinase II phosphorylation site, and N-myristoylation site.

4.4.9 Bacteriology and parasitology

Routine aerobic bacterial culture isolated *Escherichia coli* and *Clostridium perferingens* from the intestine of beef cow (M10-3432). No bacteria were isolated from any other tissues of this animal (M10-3432). Light growth of *E. coli* was isolated from the intestine, lung, and lymph nodes of the beef calf (M10-5347), suggesting either postmortem contamination or antemortem bacteremia. No parasite ova/oocystes were observed in the fecal samples of both animals.

4.4.10 Serological survey

Two weeks after the initial submission of M10-3432, 10 additional serum samples were collected from the breeding farm and tested for serum antibodies against BVDV, BTV, and IBR (BHV-1). For BVDV, 8 animals (65FHC, 2A, 3A, 4A, 72, Bull, Blue 15, and Jersey) were positive with neutralization titers ranging from 4 to 256 and 2 animals (1A and 5A) were negative. For BT, sera from 8 animals (65FHC, 1A, 2A, 4A, 72, Bull, 5A, and Blue15 were positive and 2 animals (3A and Jersey) were negative. For IBR, 8 animals (65FHC, 1A, 2A, 4A, 72, Bull, Blue15, and Jersey) were positive with titers ranging from 4 to 64 and 2 animals (3A and 5A) were negative. The complete results are presented in Table 4.7.

Table 4.7 Serological survey results from selected animals of farm where case M10-3432 was originated

Animal	Age	Sex	BTV-ELISA	IBR-SN		BVDV-SN		Notes
65FHC	10	Cow	+	16	+	64	+	Healthy
1A	5	Cow	+	8	+	<4	-	Healthy
2A	heifer	Cow	+	64	+	8	+	Sick
3A	calf	Bull	-	<4	-	4	+	Healthy
4A	5	Cow	+	4	+	4	+	Healthy
72	6	Cow	+	4	+	32	+	Healthy
Bull	5	Bull	+	4	+	256	+	Healthy
5A	4	Cow	+	<4	-	<4	-	Healthy
Blue15	10	Cow	+	8	+	256	+	Healthy
Jersey	4	Cow	-	32	+	8	+	Healthy

Notes: Serological tests were performed to detect antibodies against BVDV, IBR, and BT. Serum samples were collected from ten animals in the beef breeding farm from which M10-3432 was originated. The age and sex of each animal are presented. Data shown are qualitative results of BTV-ELISA and quantitative results (titer) of serum neutralization (SN) for BVD and IBR.

4.5 Discussion

BVDV is an economically important viral pathogen of cattle. BVDV infection is associated with a wide range of clinical diseases, including respiratory distresses, diarrhea, reproductive losses, or the fatal mucosal disease. The infection often suppresses the host immune system, thereby increasing the susceptibility of the host, especially calves, to other viral and bacterial agents. In this study, we isolated two BVDV strains from two animals with severe diseases and fatal outcome. Clinical manifestations in both cases were compatible to recently described hemorrhagic syndrome. The breeding cow developed extensive lesions along the gastrointestinal mucosa. Beef calf had bloody diarrhea and respiratory stress. Initially, FA test was performed on fresh lung tissue as well as other available tissue samples. With no specific fluorescence observed on examined tissue sections both cases were recorded as FA negative. However, clinical

signs and histologic findings strongly suggested BVDV involvement. Subsequently real-time RT-PCR test was performed on the lung tissues and results were positive for both animals. Although the exact reason for this discrepancy remains unknown we hypothesized that antigenic changes on surface protein of new isolates, especially E2 glycoprotein, may be to blame for the FA detection failure. Results from serological studies indeed showed existing antigenic variations compared to reference strain 125c These antigenic changes, no matter how subtle, will have a direct impact on the efficacy of FA test. Furthermore, high auto-fluorescence associated with lung tissue can certainly complicate FA test.

Both isolates were genotyped by real-time RT-PCR as BVDV-2 and phylogenetic analyses based on E2 glycoprotein amino acid sequence further grouped M10-3432 and M0-5347 into a BVDV-2a cluster containing many virulent strains isolated recently from North American (Figure 4.3). M10-3432 is most closely associated with a highly virulent NCP strain BVDV 890 while M10-5347 is more close to CD87, another high virulent NCP strain. This is our first time to isolate a high virulent CP strain. To rule out the possibility that the beef calf could be a PI animal and the severe clinical signs could be due to mucosal disease we performed multiple tests on all available tissue samples including lung, mesenteric lymph node and small intestine. No virus was detected by PCR on mesenteric lymph node and small intestine except lung tissue. To rule out the possibility of a mixed infection with M10-5374 and an antigenic matching noncytopathic strain, we examined M10-5347 virus stock by limited dilution. No NCP strain was identified by FA test after multiple repeats (data not shown).

Antigenic variation associated with diagnostic test failure has been previously reported (Elahi et al. 1997; Gripshover et al. 2007). To investigate the causes for the

false negative results of FA in diagnosing two clinical BVDV cases, we examined the cross-reactivity between each BVDV isolate and the porcine anti-BVDV serum, which is used to produce FA conjugate by labeling it with FITC (VMRD, WA). The IFA tests did show a reduced reactivity of the isolates with anti-BVDV polyclonal antiserum, compared to the standard reference strains, Singer or 125c (Table 4.4). Cross-neutralization test using reference sera against BVDV-1 and BVDV-2 revealed that standard type specific serum reacted to both isolates with about equal strength, therefore were indistinguishable by the two types of reference sera (Figure 4.4). This phenomenon could not be explained by potential experimental artifacts because both reference strains, Singer (BVDV-1) and 125c (BVDV-2), were neutralized specifically by their respective antisera (Figure 4.4). Together, the reduced reactivity to porcine anti-BVDV antiserum and the cross neutralization activities suggest that the strains had mutated in the genes encoding major antigens, such as the E2 glycoprotein that is known to be able to induce strong immune response.

To determine the genetic basis for the antigenic variations observed in serological tests, E2 amino acid sequences of both isolates were compared with that of BVDV 125c, the reference strain for genotype 2. From the sequence analysis, we did identify multiple mutations that were unique to isolates M10-3432 and M10-5347 respectively. We further analyzed the secondary structures of the E2 proteins which led to identification of five types of protein motifs, including N-glycosylation sites, cAMP- and cGMP-dependent protein kinase phosphorylation sites, protein kinase C phosphorylation sites, casein kinase II phosphorylation sites, and N-myristoylation sites (Abou-Jaoude et al. 2007). Mutations in the E2 gene of the two isolates resulted in the addition or loss of two protein motifs. Compared to the reference strain 125c, M10-3432 had one more N-glycosylation

site and lost two casein kinase II phosphorylation sites. The isolate M10-5347 lost one casein kinase II phosphorylation site as well as one N-myristoylation site. N-glycosylation is known as necessary for the proper folding and trafficking of viral proteins. Alteration or disruption of N-glycosylation sites can affect the survival and transmissibility of many viruses. Changes in glycosylation can also affect interaction with cellular receptors, thereby impacting infectivity (Vigerust and Shepherd 2007). A recent study demonstrated that mutations in N-glycosylation site in the E2 protein of hepatitis C virus enhanced the infectivity of the virus (Bungyoku et al. 2009). N-terminal N-myristoylation is an irreversible lipid anchor protein modification process, catalyzed by the enzyme N-myristoyltransferase (NMT) (Podell and Gribskov 2004; Zha et al. 2000). During this process, a myristate is covalently liked to N-terminal amino acids, such as an exposed glycine residue, of nascent polypeptides (Podell and Gribskov 2004; Zha et al. 2000). N-myristoylation is critically involved in signal transduction, apoptosis, as well as viral pathogenicity. A recent study has demonstrated that myristoylation signal transfer from large to small envelope proteins can abolish the infectivity of hepatitis delta virus (Abou-Jaoudé et al. 2007; Maurer-Stroh et al. 2002). Casein kinase II (CKII) is a ubiquitous and constitutively active serine and threonine (Ser/Thr) protein kinase implicated in the budding of influenza viruses (Hui and Nayak 2002; Tucker et al. 1991) and viral transcription of vesicular stomatitis virus (Gao and Lenard 1995; Mathur and Banerjee 2002). At the present time, it is not known whether alterations in these motifs have any implications for the pathogenicity of BVDV. Given the important biological functions associated with these motifs, further investigation is needed to understand what roles protein motif in E2 glycoprotein play in BVDV pathogenesis, especially N-myristoylation and CKII phosphorylation motifs.

A serological survey of the farm where the beef cow (M10-3432) developed severe clinical signs suggested poor herd immunity as evidenced by the uneven antibody titers against BVDV, IBR, and BTV. Negative animals and animals with antibody titer below protection contributed primarily to the poor population immunity, which could lead to the large number of deaths experienced in the farm. Because persistent infection was ruled out, the lesions along the oral, esophageal, and intestinal mucosa of the beef cow were not the result from a "mucosal disease" that is often caused by a mixed infection of noncytopathic BVDV and cytopathic BVDV in persistently infected animals. Thus, the virulence of the infecting BVDV should be further investigated.

The genetic and antigenic variations of BVDV have a great impact on the diagnosis, prevention, and control of the diseases associated with this virus. It is likely that such variations or diversities will continue to increase due to evolutionary pressures. Prevention and control of BVDV can be complicated by diagnostic and immunization failure due to virus diversity. Nevertheless, close monitoring the genetic mutations and antigenic variations in clinical isolates and frequent evaluation of diagnostic reagents will provide useful information for the improvement of vaccines and diagnostic tests.

4.6 References

1. Abascal F, Zardoya R, Posada D. 2005. ProtTest: selection of best-fit models of protein evolution. Bioinformatics 21 (9):2104-2105.

2. Abou-Jaoudé G, Molina S, Maurel P, and Sureau C. 2007. Myristoylation signal transfer from the large to the middle or the small HBV envelope protein leads to a loss of HDV particles infectivity. Virology 365(1):204-9.

3. Baker JC. 1995. The clinical manifestations of bovine viral diarrhea infection. Vet Clin North Am Food Anim Pract 11(3):425-45.

4. Bolin SR, Ridpath JF. 1998. Prevalence of bovine viral diarrhea virus genotypes and antibody against those viral genotypes in fetal bovine serum. J Vet Diagn Invest 10:135–139.

5. Bolin SR, and Ridpath JF. 1992. Differences in virulence between two noncytopathic bovine viral diarrhea viruses in calves. Am J Vet Res 53 (11):2157-2163.

6. Brownlie J, Clarke MC, Howard CJ, Pocock DH. 1987. Pathogenesis and epidemiology of bovine virus diarrhoea virus infection of cattle. Ann Rech Vet. 18(2):157-66.

7. Bungyoku Y, Shoji I, Makine T, Adachi T, Hayashida K, Nagano-Fujii M, Ide YH, Deng L, Hotta H. 2009. Efficient production of infectious hepatitis C virus with adaptive mutations in cultured hepatoma cells. J Gen Virol. 90:1681-1691.

8. Carman S, van Dreumel T, Ridpath J, Hazlett M, Alves D, Dubovi E, Tremblay R, Bolin S, Godkin A, Anderson N. 1998. Severe acute bovine viral diarrhea in Ontario, 1993-1995. J Vet Diagn Invest 10(1):27-35.

9. Corapi WV, French TW, Dubovi EJ. 1989. Severe thrombocytopenia in young calves experimentally infected with noncytopathic bovine viral diarrhea virus. J. Virol 63 (9): 3934–3943.

10. Dean H, Leyh R. 1999. Cross-protective efficacy of a bovine viral diarrhea virus (BVDV) type 1 vaccine against type 2 challenge. Vaccine 17(9-10):1117-1124

11. Deregt D, Bolin SR, van den Hurk J, Ridpath JF, Gilbert SA. 1998. Mapping of a type 1-specific and a type-common epitope on the E2 (gp53) protein of bovine viral diarrhea virus with neutralization escape mutants. Virus Res 53(1): 81-90.

12. Donis RO. 1995. Molecular biology of bovine viral diarrhea virus and its interactions with the host. Vet Clin North Am Food Anim Pract 11(3):393-423.

13. Edgar RC. 2004. Muscle: multiple sequence alignment with high accuracy and high throughout. Nucl. Acids Res 32(5): 1792-1797.

14. Elahi SM, Harpin S, Cornaglia E, Talbot B, Elazhary Y. 1997. Antigenic variation among bovine viral diarrhea virus (BVDV) strains and the role of different cell fixation methods in immunoassays. Can J Vet Res 61:34-38.

15. Gao Y, Lenard J. 995. Multimerization and transcriptional activation of the phosphoprotein (P) of vesicular stomatitis virus by casein kinase-II. EMBO J 14(6):1240-7.

16. Goens SD. 2002. The evolution of bovine viral diarrhea: a review. Can Vet J 43:946-954.

17. Gripshover EM, Daniel Givens M, Ridpath JF, Brook KV, Whiteley EM, and Sartin EA. 2007. Variation in E^{rns} viral glycoprotein associated with failure of immunohistochemistry and commercial antigen capture ELISA to detect a field strain of bovine viral diarrhea virus. Vet Microbiol 125:11-21.

18. Guindon S, Gascuel O. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52 (5): 696-704.

19. Hamers C, Dehan P, Couvreur B, Letellier C, Kerkhoft P, and Pastoret PP. 2001. Diversity among bovine pestiviruses. Vet J 161:112-122.

20. Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17 (8): 754-755.

21. Hui EK, Nayak DP. 2002. Role of G protein and protein kinase signalling in influenza virus budding in MDCK cells. J Gen Virol 83:3055-66.

22. Jones LR, Zandomeni R, and Weber EL. 2002. Quasispecies in the 5' untranslated genomic region of bovine viral diarrhea virus from a single individual. J Gen Virol 83:2161-2168.

23. Kümmerer BM, Tautz N, Becher P, Thiel H, Meyers G.2000. The genetic basis for cytopathogenicity of pestiviruses. Vet Microbiol. 77(1-2):117-128.

24. Letellier C, Kerkhofs P, Wellemans G, Vanopdenbosch E. 1999. Detection and genotyping of bovine diarrhea virus by reverse transcription-polymerase chain amplification of 5' untranslated region. Vet Microbiol 64:155-167.

25. Mathur M, Banerjee AK. 2002. Novel binding of GTP to the phosphoprotein (P) of vesicular stomatitis virus. Gene Expr 10(4):193-200.

26. Maurer-Stroh S, Eisenhaber B, Eisenhaber F. 2002. N-terminal N-myristoylation of proteins: refinement of the sequence motif and its taxon-specific differences. J Mol Biol 317(4):523-40.

27. Moerman A, Straver PJ, de Jong MC, Quak J, Baanvinger T, van Oirschot JT. 1994. Clinical consequences of a bovine virus diarrhoea virus infection in a dairy herd: a longitudinal study. Vet Q 16(2):115-9.

28. Pellerin C, van den Hurk J, Lecomte J, Tussen P. 1994. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203 (2):260–268.

29. Perdrizet JA, Rebhun WC, Dubovi EJ, Donis RO. 1987. Bovine virus diarrhea–clinical syndromes in dairy herds. Cornell Vet. 77 (1):46–74.

30. Podell S and Gribskov M. 2004. "Predicting N-terminal myristoylation sites in plant proteins", BMC Genomics 5:37.

31. Posada D, Buckley TR. 2004. Model selection and model averaging in phylogenetics: advantage of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst Biol 53(5):793-808.

32. Posada D, Crandall KA. 1998. Model test: testing the model of DNA substitution. Bioinformatics 14 (9):817-818.

33. Reed LJ, Muench H. 1938. A simple method of estimating fifty percent endpoints. Am J Hyg 27:493-497.

34. Ridpath JF, Bolin SR, and Dubovi EJ. 1994. Segregation of bovine viral diarrhea virus into genotypes. Virology 205:66-74.

35. Ridpath JF, Bendfeldt S, Neil JD, and Liebler-Tenorio E. 2006. Lymphocytopathogenic activity in vitro correlates with high virulence in vivo for BVDV type 2 strains: criteria for a third biotype of BVDV. Virus Research 118:62-69.

36. Ridpath JF, Neill JD, Vilcek S, Dubovi EJ, Carman S. 2006. Multiple outbreaks of severe acute BVDV in North America occurring between 1993 and 1995 linked to the same BVDV2 strain. Vet Microbiol 114 (3-4):196-204.

37. Thiel HJ, Collett MS, Gould EA, Heinz FX, Houghton M, Meyers G. 2005. Flaviviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball BA, editors. Virus taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses. San Diego (CA): Elsevier Academic Press. 981-998.

38. Topliff CL and Kelling CL. 1998. Virulence markers in the 5' untranslated region of genotype 2 bovine viral diarrhea virus isolates. Virology 250:164-172.

39. Tucker SP, Penn CR, McCauley JW. 1991. Characterisation of the influenza virus associated protein kinase and its resemblance to casein kinase II. Virus Res 18(2-3):243-261.

40. Tussen P, Pellerin C, Lecomte J, and van den Hurk J. 1996. Immunodominant E2 (gp53) sequences of high virulent bovine viral diarrhea group II viruses indicate a close resemblance to a subgroup of border disease viruses. Virology 217:356-361.

41. Vigerust DJ, Shepherd VL. 2007. Virus glycosylation: role in virulence and immune interactions. Trends Microbiol 15:211-218.

42. Vilcek S, Durkovic B, Kolesarova M, Paton DJ. 2005. Genetic diversity of BVDV: consequences for classification and molecular epidemiology. Prev Vet Med 72:31-35.

43. Walz PH, Bell TG, Steficek BA, Kaiser L, Maes RK, Baker JC. 1999. Experimental model of type II bovine viral diarrhea virus-induced thrombocytopenia in neonatal calves. J Vet Diagn Invest 11:505-514.

44. Yan L, Shuping Zhang, Lanny Pace, Floyd Wilson, Michael Zhang. 2011. Combination of reverse transcriptase real-time polymerase chain reaction (PCR) and antigen capture enzyme-linked immunosorbent assay (ACE) for the detection of bovine viral diarrhea virus (BVDV)-persistently infected animals. J Vet Diagn Invest 23(1): 16-25.

45. Zha J, Weiler S, Oh KJ, Wei MC, Korsmeyer SJ. 2000. Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. Science 290:1761-1765.

CHAPTER V

PHYLOGENETIC ANALYSES OF BOVINE VIRAL DIARRHEA USING THREE REGIONS 5 UTR, E2, AND NS5B

5.1 Abstract

Bovine viral diarrhea virus (BVDV) is a major viral pathogen affecting respiratory and reproductive systems of ruminants. BVDV associated infections cause significant economic losses worldwide. Phylogenetic analysis can be used to estimate the genetic relationships among different strains, cluster known viruses, and genotype new isolates. The hyper virulent strains of BVDV associated with hemorrhagic syndrome discovered in North American in recent years are genotyped as BVDV-2 by phylogenetic analysis of 5'UTR region. The *objective* of this study was to conduct comparative phylogenetic analyses on genetic typing of virulent BVDV isolates using a single region or concatenated regions of 5'UTR, E2, and NS5B at either nucleotides or amino acids level. Our results indicated that although slightly difference was observed, the virulent BVDV isolates were consistently classified into BVDV-2a cluster regardless of date sets used. Furthermore, phylogenetic tree constructed using combined two or more regions gave higher posterior probability and bootstrap value than using single region.

5.2 Introduction

Bovine viral diarrhea virus (BVDV) is a major viral pathogen of cattle. BVDV associated infections cause significant economic losses worldwide. Classical swine fever virus (CSFV), border disease virus (BDV), BVDV, and tentative giraffe-1 virus are

members of the genus *Pestivirus* of the family *Flaviviridae*, which also includes hepatitis C virus and flavivirus (Fauquet 2005). The BVDV genome possesses an ORF which was flanked by 5'UTR (372-385 nucleotides in length) and 3'UTR (229-273 nucleotides in length) (Collett *et al.* 1988a; Collett *et al*. 1988b; Lindenbach *et al.* 2007). The ORF of BVDV encodes N-terminal protease fragment N^{pro}, four structural proteins C, E^{rns}, E1, and E2, and seven nonstructural proteins p7, NS2-3, (NS2), (NS3), NS4A, NS4B, NS5A, and NS5B. 5'UTR, N^{pro}, E2, NS3 and NS5B of BVDV have been used for phylogenetic analysis (Baule et al. 1997; Mingala et al. 2009; Oguzoglu et al. 2010; Pellerin et al.; 1994; Ridpath et al. 2000; Vilcek et al. 2001; Wolfmeyer et al. 1997; Xia et al. 2007). The 5'UTR harbors an internal ribosomal entry site, which is highly conserved among all members within the genus *Pestivirus*. But there are three variable loci, which are able to form a stable and peculiar stem-loop structure for each genotype or species of pestivirus, thus being used extensively for the characterization of BVDV genotypes (Harasawa 1996; Pestova et al. 1998).

BVDV can be classified into two genotypes BVDV-1 and BVDV-2 based on the genetic variations of 5'UTR (Ridpath et al. 1994). BVDV-1 can be further divided into at least 11 subtypes (1a to 1l) based on the phylogenetic analysis of 5'UTR and N^{pro} (Jackova et al. 2008; Vilcek et al. 2001) while BVDV-2 can be further divided into four subtypes BVDV-2a, BVDV-2b, BVDV-2c, and BVDV-2d (Giangaspero et al. 2008). BVDV-1a, 1b and 2a are the major subtypes in North America (Ridpath 2010). A recent study of phylogenetic analysis on BVDV-1b suggested that BVDV-1b could be further clustered into 1b1 – Osloss and 1b2 – CP7 based on the variation of E2. Epidemiological study indicates that BVDV1b2 is the predominant subtype in the United States (Tajima and Dubovi 2005).

E1, E2, and E^{rns} are glycoproteins embedded in viral envelope of BVDV and induce strong immune response during the course of infection (Meyers and Thiel 1996; Mingala et al. 2009; Pellerin et al. 1994; Ridpath et al. 1994; Rümenapf et al. 1993; Tajima and Dubovi 2005). Immunodominant protein E2 is the most variable protein of pestiviruses, which is the location of the majority of neutralization epitopes and is the target protein for serological tests to differentiate pestiviruses.

Phylogenetic analysis of pestivirus has been widely used to genotype the new isolates to understand their evolutionary history. Multiple methods have been used to construct the phylogeny of a species, and two fundamental approaches have been used either based on a single genetic element or concatenating multiple genetic sequences. Several studies have suggested that the phylogenetic tree constructed with concatenated sequences is considered more stable than phylogenetic trees constructed using a single region in mammals and many other species (de Queiroz et al. 1995; Gadagkar et al. 2005; Huelsenbeck et al. 1996; Nei et al. 2001; Yang 1996).

Phylogenetic analysis can be inferred using different methods and most common methods are distance, maximum parsimony, maximum likelihood (ML), and Bayesian, in which each methodology has its own uniqueness. Maximum likelihood outputs the highest probability or likelihood tree by evaluating the differences of tree topologies and employing all sequence information. Maximum likelihood has a lower variance than other methods, but is time and memory consuming. The result of maximum likelihood is dependent on the model of evolution (Aldrich 1997; Cam 1990). Bayesian inference is closely related to maximum likelihood, but allows the implementation of more complex, parameter-rich evolution models than maximum likelihood. The prior probability distribution of the possible trees was assumed in the Bayesian analysis. Markov chain

Monte Carlo (MCMC) algorithm was used to estimate posterior probabilities in the Bayesian approach, and posterior probability indicates the reliability of inference (Mar et al. 2005; Ronquist and Huelsenbeck 2003). Although 5'UTR, E2, and NS5B have been used in the phylogenetic analysis of BVDV, there is no comprehensive comparison among these three genes and between nucleotides and amino acids. The objective of this study is to conduct comparative phylogenetic analyses on virulent BVDV isolates using single or combined regions (5'UTR, E2, and NS5B) and compare the results achieved using nucleotide dataset to the results generated from amino acids dataset.

5.3 Materials and methods

5.3.1 Reference strains

Twelve BVDV-1, eleven BVDV-2, two BDV, and two CSFV reference strains were used in the phylogenetic analyses (Table 5.1). Three regions (5'UTR, E2 and NS5B) of BVDV references employed in the phylogenetic analyses were downloaded from GenBank. Three isolates MS12, M10-3432 and M10-5347 were isolated from Mississippi and M10-3432 and M10-5347 were described in the Chapter IV.

Table 5.1 Strains used in the phylogenetic analysis

Strain name	Accession #	Sources	Strain name	Accession #	Sources
CSFV_SWH	DQ127910	China	BVDV1_Singer	DQ088995	Argentina
CSFV_ Alfort	U90951	France	BVDV1_CP7_5A	AF220247	Germany
BDV_H2121	GU270877	Germany	BVDV2_M10_5347	JN377415	USA (MS)
BDV_Gifhorn	GQ902940	Denmark	BVDV2_MS12	GU395545	USA (MS)
BVDV1_Osloss	M96687	Belgium	BVDV2_M10_3432	JN377416	USA (MS)
BVDV1_ZM95	AF26381	China	BVDV2_JZ05_1	GQ888686	China (CP)
BVDV1_VEDEVAC	AJ585412	Hungary	BVDV2_Hokudai	AB567658	Japan
BVDV1_ILLNC_CP	U86599	USA (ND)	BVDV2_p24515	AY149216	Canada(HVNCP)
BVDV1_ILLNC_NCP	U86600	USA(ND)	BVDV2_1373	AF145967	USA(IA)
BVDV1_Oregon_c24v	AF091605	England	BVDV2_NY93	AF502399	Germany
BVDV1_SD1	M96751	USA OH)	BVDV2_p11Q	AY149215	Canada (LVNCP)
BVDV1_CP7	U63479	Germany	BVDV2_XJ_04	FJ527854	China
BVDV1_KE9	EF101530	Germany	BVDV2_890	AAA82981	USA(IA)
BVDV1_NADL	M31182	USA(IA)			

Notes: Two border disease viruses, two classical swine fever viruses, twelve BVDV-1, and eleven BVDV-2 were downloaded from GenBank. Two BVDV-2 isolates M10-3432 and M10-5347 were isolated from Mississippi and described in the chapter IV.

5.3.2 Primers

5'UTR and E2 of M10-3432, M10-5347, and MS12 were amplified and sequenced in Chapter IV. Two sets of primers were designed to target NS5B region, and they are listed in table 5.2. All primers were commercially synthesized and purchased from Eurofin Operon MWG **(**Huntsville, AL**).**

Table 5.2 Primers of NS5B of BVDV-2

Primers	Sequences	Tm (^{o}C)
F1	GA CTT ATA CCA CCT AAT TGG CAG	61.0
R1	GTC CTG GAA TGA ATC CCA TTC	60.6
F2	GAA GCC GGA GAG TTC GTT GAT	62.6
R2	CCA ACA GAG TGA CAC CAG TGT	62.6

Notes: Two sets of primers were designed to target NS5B.

5.3.3 RNA extraction

Viral RNA was extracted from cell culture supernatant using RNA easy kit (Qiagen, Valencia, CA) based on the instructions provided by the manufacturer. The detailed procedures have been described in the Chapter III.

5.3.4 Reverse transcriptase – polymerase chain reaction (RT-PCR) and sequencing

Two-step RT-PCR was performed using commercial reagents (Qiagen, Valencia, CA) according to the manufacturer's instructions. PCR products of the NS5B region of M10-5347, M10-33432 and MS12 were sequenced by Eurofin Operon MWG (Huntsville, AL), and the available sequences of these three strains were 1941, 1959 and 1954 nucleotides in length, respectively.

5.3.5 Phylogenetic analysis

Three regions of 5'UTR, structural protein E2, and nonstructural protein NS5B of BVDV were employed in the phylogenetic analyses based on either nucleotides or amino acids. For nucleotides, there are three single data sets 5'UTR, E2, NS5B and three combined data sets 5'UTR+E2, E2+NS5B and 5'UTR+E2+NS5B. For amino acids, there are two single data sets E2 and NS5B and one combined data set E2+NS5B. Both nucleotides and amino acids sequences were aligned using Muscle 3.8.31 (Edgar 2004). Sequence alignments were employed to estimate the best-fit model for each data set, which were performed using the Modeltest 3.7 for nucleotides (Posada and Buckley 2004; Posada and Crandall 1998), and the Prottest 2.4 for amino acids (Posada and Buckley 2004) based on Akaike Information Criterion. Phylogenetic analysis was performed by using Bayesian and maximum likelihood approaches (Mar et al. 2005; Ronquist et al. 2003; Yang 1996). Bayesian inference estimates the posterior probability

of phylogenetic trees based on the prior parameters and the likelihood of data, and the posterior probability supports the branches of phylogenetic tree. The phylogenetic tree and model parameters values were sampled by using Markov chain Monte Carlo sampler (Ronquist et al. 2003). Bayesian inference program – MrBayes was used to infer phylogeny and the average standard deviation of split frequencies less than 0.01 will achieve a stable tree. MrBayes was performed 1,000,000 generations and sampled the Markov chain every 1000 generations. The first 25% of trees were discarded as "burn-in" (Huelsenbeck et al. 1996). Maximum likelihood (ML) outputs the highest likelihood phylogenetic tree by using all sequence information, and evaluates the different tree topologies (Aldrich 1997; Cam 1990; Yang 1996). Phyml was used to infer phylogeny and the reliability of phylogenetic trees was estimated using the bootstrap analysis with 1000 replicates. A bootstrap value greater than 70% (0.7 for posterior probability) indicated strong support for a branch of the tree (Guindon and Gascuel 2003).

5.4 Results

5.4.1 Model selection

Model tests indicated that GTR+I+G is the best-fit model of nucleotide dataset. For amino acids, JTT+G+F is the best-fit model of E2 and NS5B, and JTT+I+G+F is the best-fit model of combined set data of E2+NS5B.

5.4.2 Phylogenetic analyses of BVDV using a single or concatenated regions of 5'UTR, E2, and NS5B

All phylogenetic analyses of a single gene or concatenated regions clearly classified BVDV into the corresponding groups BVDV-2a, BVDV-1a, 1b1 and 1b2, which were supported by posterior probability and bootstrap value. Among twelve

BVDV-1 isolates, Singer, NADL, SD1 and Oregon were classified into BVDV-1a, VEDEVAC and KE9 were classified into the same branch with BVDV-1b1 – Osloss (Tajima et al. 2001). Among eleven BVDV-2 strains (isolates), all were classified into BVDV-2a except Hokudai.

5.4.3 Phylogenetic analysis of 5'UTR

Using phylogenetic analysis of 5'UTR, BVDV isolates were classified into subgroups BVDV-2a, BVDV-1a, 1b1 and 1b2, which were supported by posterior probability and bootstrap value (Figure 5.1).

All eleven BVDV2 isolates except Hokudai, were classified into BVDV-2a. M10-3432, NCP high virulence isolate, was clustered into the same branch with MS12 (isolated from the ear notch of a diseased cow in Mississippi, USA) and 890 (a noncytopathic, high-virulence strain, isolated from a yearling heifer in Iowa, USA) (Bolin and Ridpath 1992). However, CP high virulence isolate M10-5347 was clustered in different subgroup from MS12. ILLNC CP and ILLNC NCP isolated from mucosal disease were classified into the same branch with BVDV-1b2 CP7 (Tajima et al. 2001) and CP7-5A supported by high posterior probability and bootstrap value (Figure 5.1).

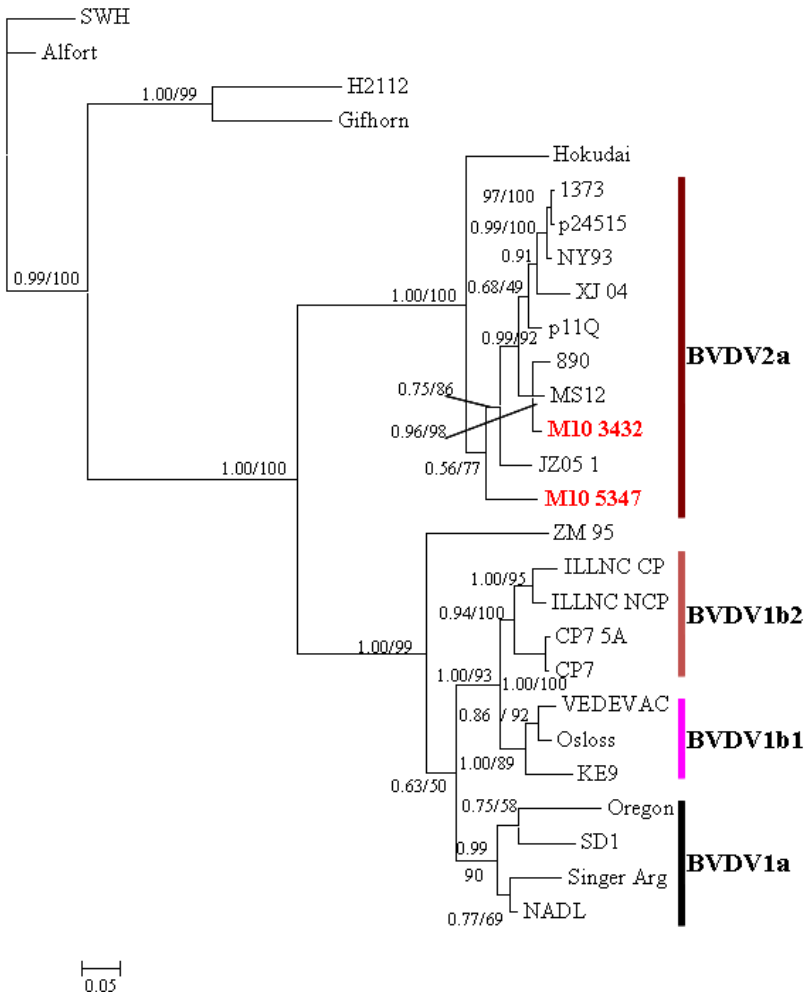

Figure 5.1 Phylogenetic tree of 5 UTR

Notes: The 5'UTR sequences were aligned using Muscle (Edgar 2004), and the best-fit model was selected using Modeltest3.7 (Posada and Buckley 2004; Posada and Crandall 1998). Phylogenetic tree of 5'UTR was constructed using Mr.Bayes (Huelsenbeck et al. 1996) and Phyml (Guindon and Gascuel 2003).

5.4.4 Phylogenetic analysis of E2 based on nucleotides and amino acids

Both phylogenetic tree of nucleotides and amino acids of E2 clearly classified BVDV into the corresponding groups BVDV-2a, BVDV-1a, 1b1 and 1b2, which were supported by posterior probability and bootstrap value (Figures 5.2A and 5.2B). In both phylogenetic trees of E2, twelve BVDV-2 isolates except Hokudai were classified into BVDV-2a, NCP high virulence isolate M10-3432 was clustered into the same branch

with MS12 (isolated from the ear notch of a cow in Mississippi, USA) and 890 (a noncytopathic, high-virulence strain, isolated from a yearling heifer in Iowa, USA) (Bolin and Ridpath 1992). M10-5347, a CP isolate high virulence isolate, was clustered into the same branch with JZ05_1 CP low virulence strain isolated from China (Li et al. 2010). ILLNC CP and ILLNC NCP isolated from mucosal disease were classified into the same branch with BVDV-1b2 CP7 (Tajima et al. 2001) and CP7-5A (Figure 5.2). However, ILLNC CP and ILLNC NCP were classified into the same branch with BVDV-1b1 – Osloss (Tajima et al. 2001) in the amino acids of E2 (Figure 5.3).

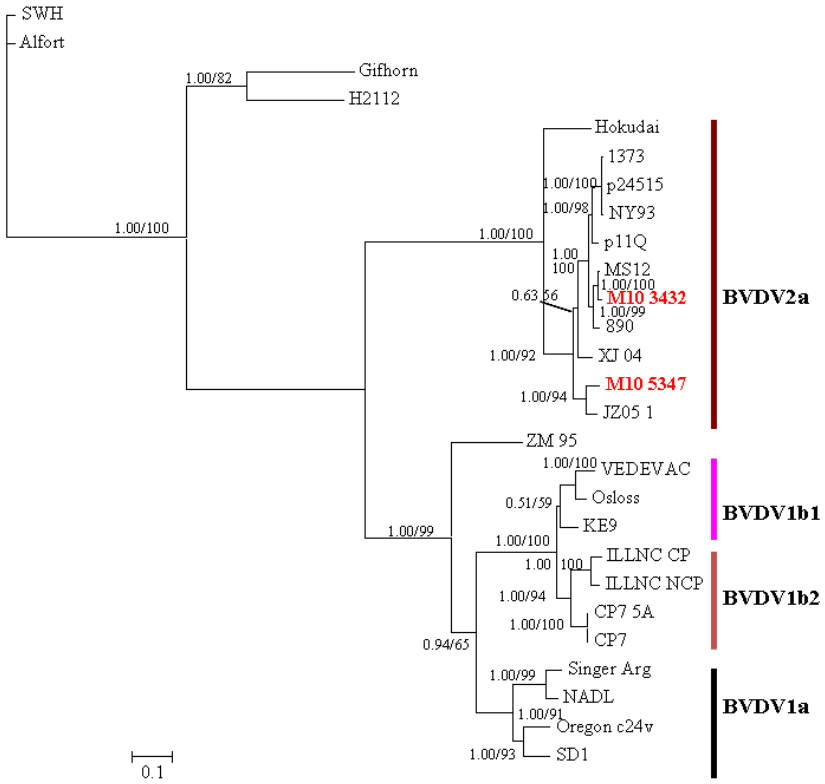

Figure 5.2 Nucleotides tree of E2

Notes: The nucleotides sequences of E2 were aligned using Muscle (Edgar 2004), and the best-fit model was selected using Modeltest3.7 (Posada and Buckley 2004; Posada and Crandall 1998). Phylogenetic tree was constructed using Mr.Bayes (Huelsenbeck et al. 1996) and Phyml (Guindon and Gascuel 2003).

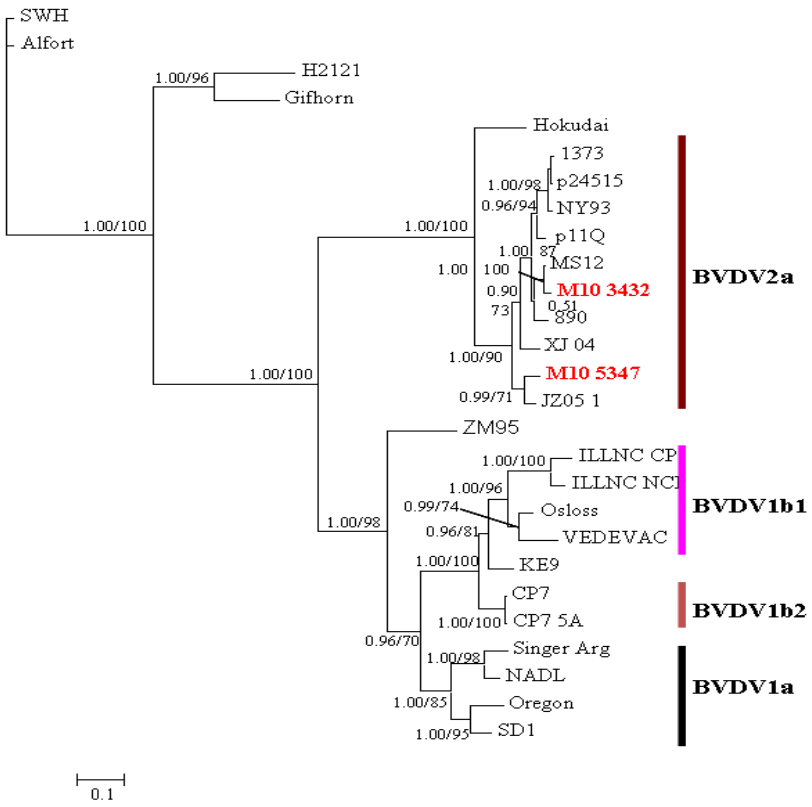

Figure 5.3 Amino acids tree of E2

Notes: The amino acids sequences of E2 were aligned using Muscle (Edgar 2004), and the best-fit model was selected using Prottest 2.4 (Posada and Buckley 2004). Phylogenetic tree was constructed using Mr.Bayes (Huelsenbeck et al. 1996) and Phyml (Guindon and Gascuel 2003).

5.4.5 Phylogenetic analysis of NS5B based on nucleotides and amino acids

Both phylogenetic tree of nucleotides and amino acids of E2 clearly classified BVDV into the corresponding groups BVDV-2a, BVDV-1a, 1b1 and 1b2, which were supported by posterior probability and bootstrap value (Figures 5.4and 5.5).

Similarly with phylogenetic trees of 5'UTR and E2, twelve BVDV-2 isolates except Hokudai were classified into BVDV-2a, NCP high virulence isolate M10-3432 was clustered into the same branch with MS12 (isolated from the ear notch of a cow in Mississippi, USA) and 890 (a noncytopathic, high-virulence strain, isolated from a

yearling heifer in Iowa, USA) (Bolin and Ridpath 1992). Similarly with the phylogenetic tree of 5'UTR, CP isolate M10-5347 was clustered into a single branch without JZ05_1. Among the BVDV-1 group, tree topology of NS5B is exactly the same with the tree topology of E2 in both nucleotides and amino acids trees (Figures 5.4 and 5.5).

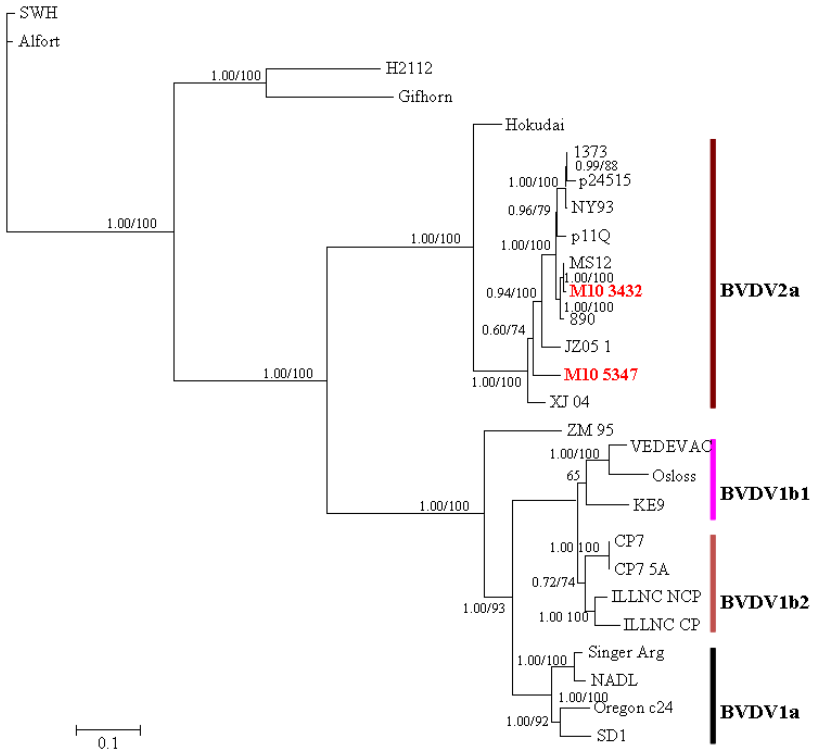

Figure 5.4 Nucleotides tree of NS5B

Notes: The nucleotides sequences of NS5B were aligned using Muscle (Edgar 2004), and the best-fit model was selected using Modeltest3.7 (Posada and Buckley 2004; Posada and Crandall 1998). Phylogenetic tree was constructed using Mr.Bayes (Huelsenbeck et al. 1996) and Phyml (Guindon and Gascuel 2003).

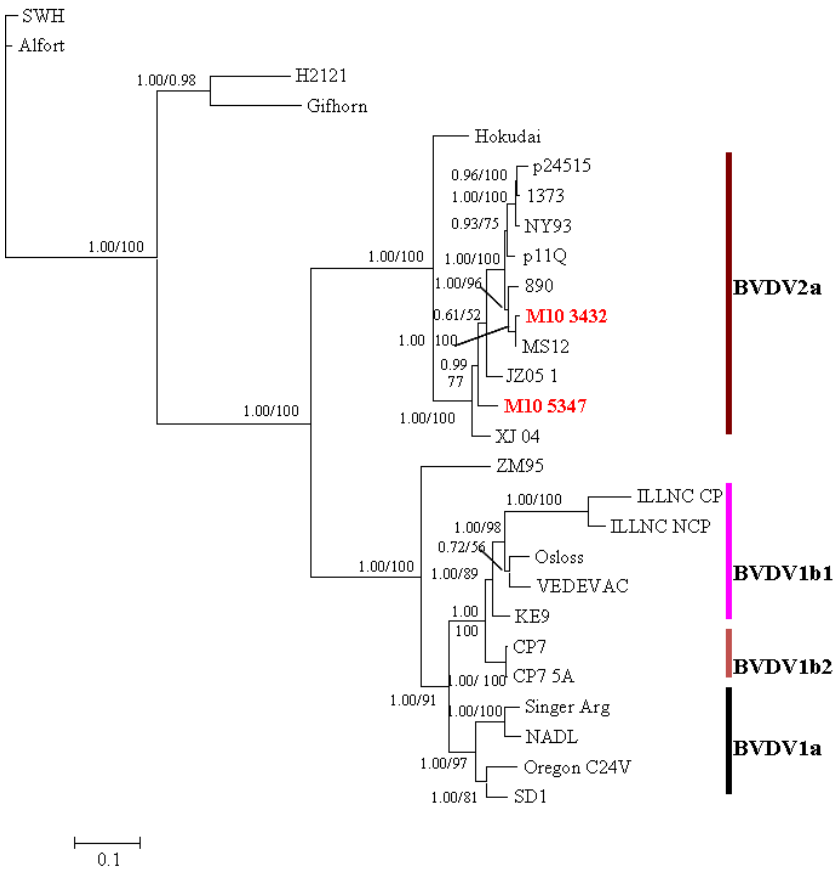

Figure 5.5 Amino acids tree of NS5B

Notes: The amino acids sequences of NS5B were aligned using Muscle (Edgar 2004), and the best-fit model was selected using Prottest 2.4 (Posada and Buckley 2004). Phylogenetic tree was constructed using Mr.Bayes (Huelsenbeck et al. 1996) and Phyml (Guindon and Gascuel 2003).

5.4.6 Phylogenetic analysis of combined E2 with NS5B (E2+NS5B)

Both phylogenetic trees of nucleotides and amino acids sequences of E2+NS5B clearly classify BVDV strains into the corresponding subtypes BVDV-1a, 1b1, 1b2 and 2a (Figure 5.6). Similarly with other described trees, twelve BVDV-2 isolates except Hokudai were classified into BVDV-2a, M10-3432 was clustered into the same branch with MS12 and 890 (Bolin and Ridpath 1992) in the both nucleotides and amino acids trees. M10-5347 was classified into the same branch with JZ05_1 in the nucleotides tree

of E2+NS5B, but M10-5347 did not grouped with JZ05_1 in the amino acids tree of E2+NS5B. Among the BVDV-1 group, tree topology of NS5B was exactly the same with the tree topology of E2 and NS5B in both nucleotides and amino acids trees (Figures 5.6 and 5.7).

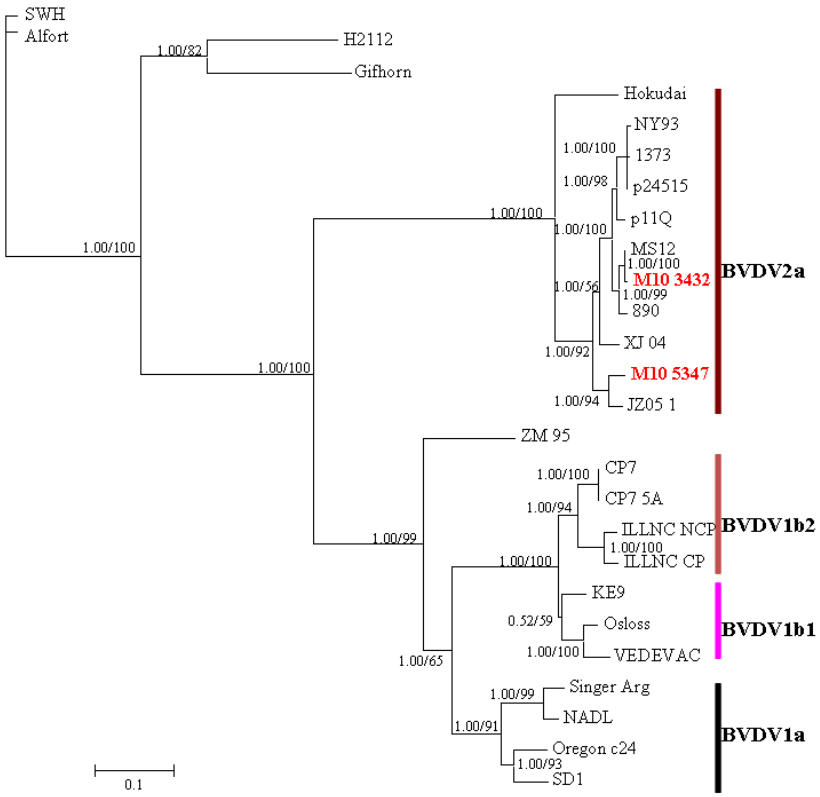

Figure 5.6 Nucleotides tree of E2+NS5B

Notes: The nucleotides sequences of E2+NS5B were concatenated from the nucleotides sequences of E2 and NS5B. The nucleotides sequences of E2 and NS5B were aligned using Muscle (Edgar 2004), and the best-fit model was selected using Modeltest3.7 (Posada and Buckley 2004; Posada and Crandall 1998). Phylogenetic tree was constructed using Mr.Bayes (Huelsenbeck et al. 1996) and Phyml (Guindon and Gascuel 2003).

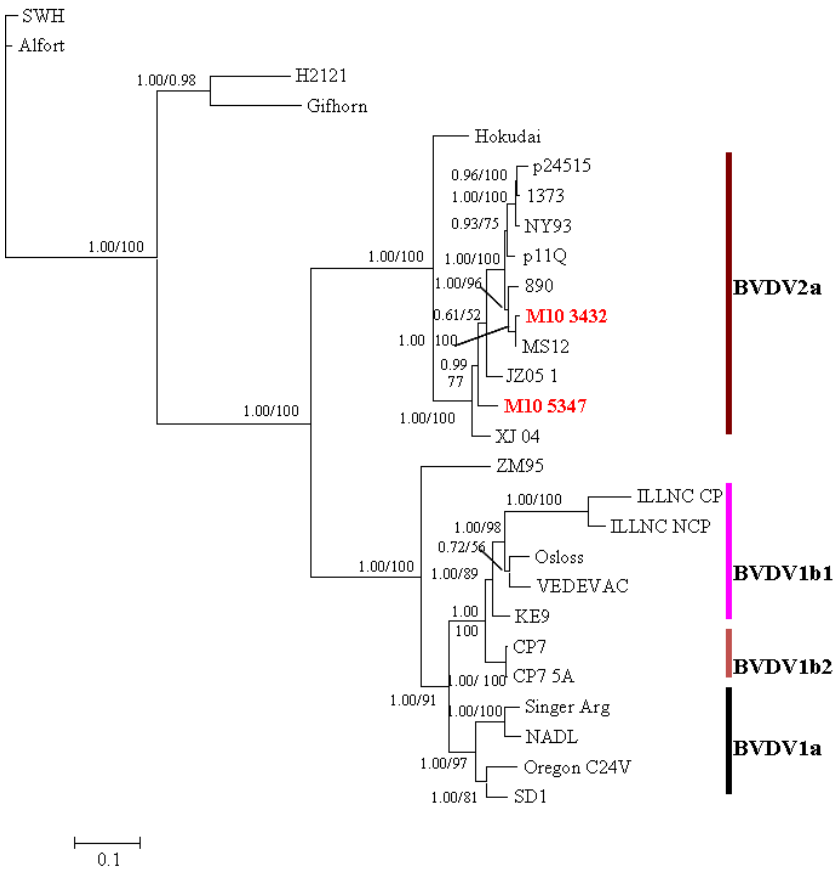

Figure 5.7 Amino acids tree of E2+NS5B

Notes: The amino acids sequences of E2+NS5B were concatenated from the amino acids sequences of E2 and NS5B. The amino acids sequences were aligned using Muscle (Edgar 2004), and the best-fit model was selected using Prottest 2.4 (Posada and Buckley 2004). Phylogenetic tree was constructed using Mr.Bayes (Huelsenbeck et al. 1996) and Phyml (Guindon and Gascuel 2003).

5.4.7 Phylogenteic analysis of concatenated genes 5 UTR+E2 and 5 UTR+E2+NS5B

Both phylogenetic trees of 5'UTR+E2 and 5'UTR+E2+NS5B clearly classify BVDV strains into the corresponding subtypes BVDV-1a, 1b1, 1b2 and 2a (Figures 5.8 and 5.9). Based on the concatenate 5'UTR+ E2 and 5'UTR+E2+NS5B, the tree topologies of these two datasets outputted were exactly the same. All taxa were classified into the corresponding subtypes BVDV-1a, 1b1, 1b2 and 2a. M10-3432 was classified into the

same group as MS12 and 890 (Bolin and Ridpath 1992), supported by a posterior probability of 1.00 and a bootstrap value greater than 96 in the phylogenetic tree of both groups data. M10-5347 was classified into the same group with JZ05_1, supported by a posterior probability of 1.00 and a bootstrap value greater than 80. Among the BVDV-1 genotype, tree topology of 5'UTR+E2 and 5'UTR+E2+NS5B were similar with the other tree topology constructed from nucleotides sequences (Figures 5.8 and 5.9). However, all branches of the phylogenetic tree 5'UTR+E2 supported with the posterior probability 0.97 or higher and the bootstrap value 70 or higher (Figure 5.8). All branches of the phylogenetic tree of 5'UTR+E2+NS5B supported with posterior probability 1.00 and bootstrap value 77 or higher (Figure 5.9).

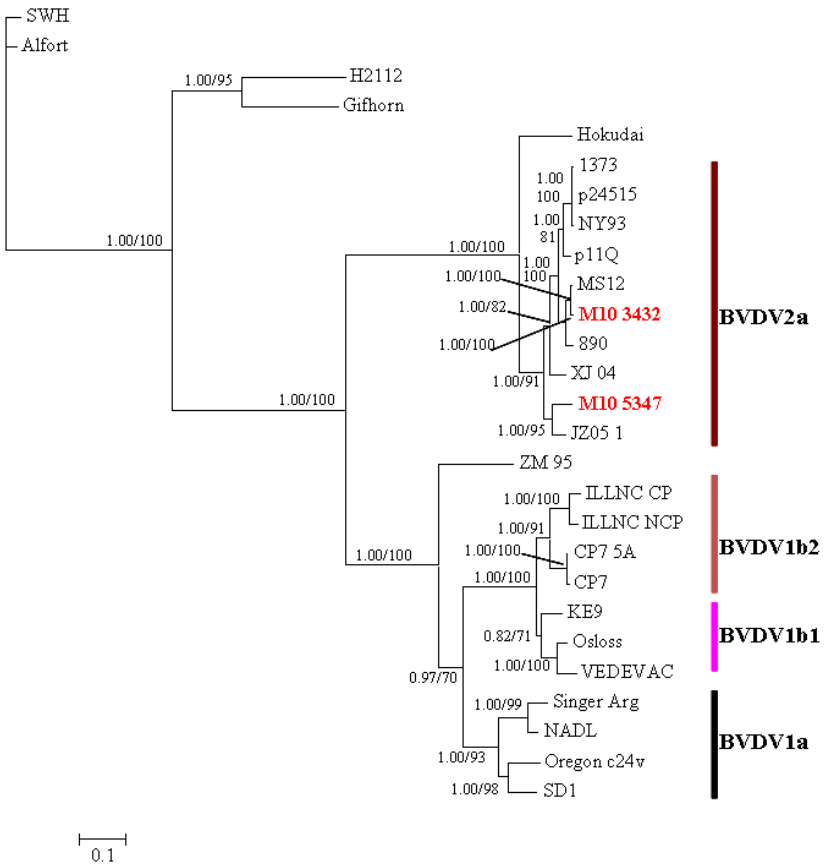

Figure 5.8 Phylogenetic tree of 5 UTR+E2

Notes: The nucleotides sequences of 5'UTR+E2 were concatenated from the nucleotides sequences of 5'UTR and E2. The nucleotides sequences of 5'UTR+E2 were aligned using Muscle (Edgar 2004), and the best-fit model was selected using Modeltest3.7 (Posada and Buckley 2004; Posada and Crandall 1998). Phylogenetic tree was constructed using Mr.Bayes (Huelsenbeck et al. 1996) and Phyml (Guindon and Gascuel 2003).

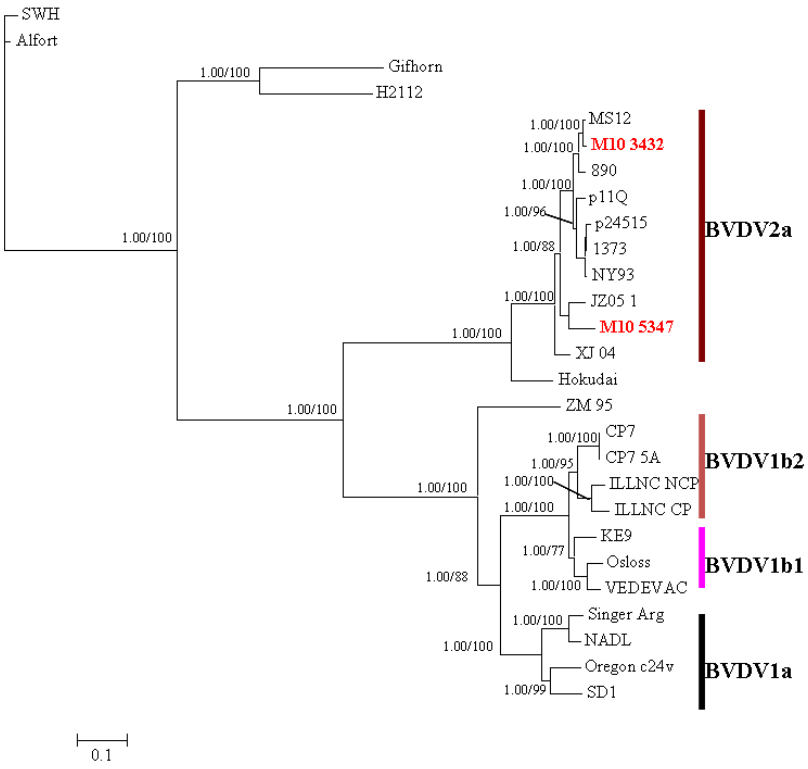

Figure 5.9 Phylogenetic tree of 5 UTR+E2+NS5B

Notes: The nucleotides sequences of 5'UTR+E2+NS5B were concatenated from the nucleotides sequences of 5'UTR, E2, and NS5B. The nucleotides sequences of 5'UTR+E2+NS5B were aligned using Muscle (Edgar 2004), and the best-fit model was selected using Modeltest3.7 (Posada and Buckley 2004; Posada and Crandall 1998). Phylogenetic tree was constructed using Mr.Bayes (Huelsenbeck et al. 1996) and Phyml (Guindon and Gascuel 2003).

5.5 Discussion

Phylogenetic analysis is an important approach to classify the new isolates of pestivirus. Results suggested that phylogenetic trees constructed using concatenated two or more regions have higher posterior probability and bootstrap value than phylogenetic trees constructed using a single region. Phylogenetic analysis also indicated that there are differences in clustering BVDV isolates using different regions of BVDV genome. There were also differences between nucleotide-based phylogenetic trees and amino acids-based.

Phylogenetic analyses of both nucleotides and amino acids and both a single region or combined regions indicated that Mississippi isolates M10-3432 was clustered into the same branch with the high-virulence, NCP strain 890 from a yearling calf in Iowa (Bolin and Ridpath 1992) and Mississippi isolates MS12, isolated from the ear notch of a cow in Mississippi. Based on amino acid sequence, BVDV-1 strains ILLNC CP and ILLNC NCP isolated from mucosal disease were closely related with BVDV-1b1 – Osloss; however, these two isolates were closely related with BVDV-1b2-CP7 and CP7-5A based on nucleotides analysis. ILLNC CP and ILLNC NCP were clustered into BVDV-1b based on 5'UTR, E2, and NS5B that is consistent with the results reported by Nagai et al. (2004) based on 5'UTR, N^{pro}, E2, and NS5B-3'UTR. However, ILLNC CP and ILLNC NCP can be classified into 1a if the NS3 region was used (Xia 2007). Genetic variations has been reported in NS3 region due to recombination between a pair of viruses 190 CP and 190 NCP isolated from mucosal disease (Becher et al. 1999; Nagai et al. 2004).

Mississippi isolates M10-5347 was clustered in the same branch with the low-virulence, CP strain JZ05_1 in the phylogenetic trees constructed using nucleotides of concatenated two or three genes and using amino acids of E2. However, M10-5347 was classified into the separate branch in the phylogenetic trees of 5'UTR and amino acids of NS5B and E2+NS5B. However, there is slightly different from Tajima and Dubovi's (2005) findings that phylogenetic tree of BVDV isolates generated using the E2 region is similar with the one generated using 5' untranslated region.

Phylogenetic tree of combined three regions 5'UTR, E2, NS5B has the exact same topology with combined two genes 5'UTR, E2. Tree topology constructed using two or more regions had higher posterior probability and bootstrap value than constructed from a

single gene. Our findings are consistent with the study of Xia et al. (2007) based on five regions (5 UTR, N^{pro}, E2a, E2b, and NS3). The authors suggest that a combined approach increases the confident placement of new strains in viral classification. Our results found that the tree topologies of two or three region are more consistent than a single region. However, the virulence of BVDV was based on clinical description as well as laboratory data in published data. The consistency of virulence determination by different laboratories and clinicians may not be the same. Therefore, a standard method a criteria to determine the virulence of BVDV is needed to help us to understand the relationship between genotypes and virulence.

The genotype method proposed in this study were solely based on the phylogenetic analysis of three regions 5'UTR, E2, and NS5B. Previous study demonstrated that RNA recombination events occurred in BVDV. It is likely the RNA recombination may affect the tree topology of phylogenetic trees thus the genotypic results. Due to lack of complete genomic sequences of Mississippi isolates and most isolates in the public database, RNA recombination were not conducted in this study. Further study will be needed to assess the presence of RNA recombination events in BVDV and, if yes, their potential impacts on the genotypic analysis of BVDV.

5.6 References

1. Albascal F, Zardoya R, Posada D. 2005. ProtTest: selection of best-fit models of protein evolution. Bioinformatics 21(9):2104-2105.

2. Aldrich J. 1997. RA Fisher and the making of maximum likelihood 1912-1922. Statistical Science 12: 162-176.

3. Baule C, Van Vuuren M, Lowings JP, Belak S. 1997. Genetic heterogeneity of bovine viral diarrhoea viruses isolated in Southern Africa1. Virus research 52(2): 205-220.

4. Becher P, Orlich M, Konig M, Thiel HJ. 1999. Nonhomologous RNA recombination in bovine viral diarrhea virus: molecular characterization of a variety of subgenomic RNAs isolated during an outbreak of fatal mucosal disease. J Virol 73:5646-5653

5. Becher P, Orlich M, Kosmidou A, Konig M, Baroth M, Thiel HJ. 1999. Genetic diversity of pestiviruses: identification of novel groups and implications for classification. Virology 262: 64-71.

6. Bolin SR, Ridpath JF. 1992. Differences in virulence between two noncytopathic bovine viral diarrhea viruses in calves. Am J Vet Res 53(11): 2157-2163.

7. Cam LL. 1990. Maximum likelihood: an introduction. Int Stat Rev 58(2): 153-171.

8. Collett MS, Anderson DK, Retzel E. 1988a. Comparisons of the pestivirus bovine viral diarrhoea virus with members of the flaviviridae. J Gen Virol 69(10): 2637-2643.

9. Collett MS, Larson R, Belzer SK, Retzel E. 1988b. Proteins encoded by bovine viral diarrhea virus: the genomic organization of a pestivirus. Virology 165 (1): 200-208.

10. de Queiroz A, Donoghue MJ, Kim J. 1995. Separate versus combined analysis of phylogenetic evidence. Ann. Rev. Ecol Syst. 26: 657-681.

11. Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32(5): 1792-1797.

12. Fauquet C. 2005. Virus taxonomy: classification and nomenclature of viruses: eighth report of the International Committee on the Taxonomy of Viruses. Academic Press.

13. Gadagkar SR, Rosenberg MS, Kumar S. 2005. Inferring species phylogenies from multiple genes: concatenated sequence tree versus consensus gene tree. J Exp Zool B Mol Dev Evol 304(1): 64-74.

14. Giangaspero M, Harasawa R, Weber L, Belloli A. 2008. Genoepidemiological evaluation of Bovine viral diarrhea virus 2 species based on secondary structures in the 5'untranslated region. J Vet Med Sci 70(6): 571-580.

15. Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52(5): 696-704.

16. Harasawa R. 1996. Phylogenetic analysis of pestivirus based on the 5'-untranslated region. Acta Virol 40:49-54.

17. Huelsenbeck JP, Bull JJ, Cunningham CW. 1996. Combining data in phylogenetic analysis. Trends in Ecology & Evolution 11(4): 152-158.

18. Jackova A, Novackova M, Pelletier NC, Audeval C, Gueneau E, Haffar A, Petit E, Rehby L, Vilcek S. 2008. The extended genetic diversity of BVDV-1: Typing of BVDV isolates from France. Vet Res Commu 32(1): 7-11.

19. Li QC, Miao LG, Li HT, Liu YH, Zhang GL, Xiao JM. 2010. Genome sequencing and analysis of the bovine viral diarrhea virus-2 strain JZ05-1 isolated in China. Chinese J Virol (Bing du xue bao) 26(3): 238-243.

20. Lindenbach BD, Thiel HJ, Rice CM. 2007. Flaviviridae: The viruses and their replication. In Fields virology 5th ed. Knipe DM and Howley P.M. ed, Lippincott Williams & Wilkins, Ambler, PA, 1101-1152.

21. Mar J, Harlow T, Ragan M. 2005. Bayesian and maximum likelihood phylogenetic analyses of protein sequence data under relative branch-length differences and model violation. BMC Evol Biol 5(1): 8.

22. Meyers G, Thiel HJ. 1996. Molecular characterization of pestiviruses. Adv Virus Res 47:53-118.

23. Mingala CN, Konnai S, Tajima M, Onuma M, Ohashi K. 2009. Classification of new BVDV isolates from Philippine water buffalo using the viral E2 region. J Basic Microbiol 49(5): 495-500.

24. Nagai M, Hayashi M, Sugita S, Sakoda Y, Mori M, Murakami T, Ozawa T, Yamada N, Akashi H. Phylogenetic analysis of bovine viral diarrhea viruses using five different genetic regions. Virus research. 2004. 99(2): 103-113

25. Nei M, Xu P, Glazko G. 2001. Estimation of divergence times from multiprotein sequences for a few mammalian species and several distantly related organisms. Proc Natl Acad Sci 98(5): 2497-2502.

26. Oguzoglu TC, Muz D, Yilmaz V, Alkan F, Akca Y, Burgu I. 2010. Molecular characterization of Bovine virus diarrhea viruses species 2 (BVDV-2) from cattle in Turkey. Trop Anim Health Prod 42(6): 1175-1180.

27. Pellerin C, Van Den Hurk J, Lecomte J, Tijssen P. 1994. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203(2): 260-268.

28. Pestova TV, Shatsky IN, Fletcher SP, Jackson RJ, Hellen CUT. 1998. A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initation of hepatitis C and classical swine fever virus RNAs. Genes Dev 12(1): 67-83.

29. Posada D, Buckley TR. 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Systematic Biology 53(5): 793-808.

30. Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14(9): 817-818.

31. Ridpath JF. 2010. Bovine viral diarrhea virus: global status. Vet Clin North Am Food Anim Pract 26(1): 105-121.

32. Ridpath JF, Bolin SR, Dubovlt EJ. 1994. Segregation of bovine viral diarrhea virus into genotypes. Virology 205: 66-74.

33. Ridpath JF, Neill JD, Frey M, Landgraf JG. 2000. Phylogenetic, antigenic and clinical characterization of type 2 BVDV from North America. Vet Microbiol 77(1-2): 145-155.

34. Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19(12): 1572-1574.

35. Rümenapf T, Unger G, Strauss JH, Thiel HJ. 1993. Processing of the envelope glycoproteins of pestiviruses. J Virol 67(6): 3288-3294.

36. Tajima M, Dubovi EJ. 2005. Genetic and clinical analyses of bovine viral diarrhea virus isolates from dairy operations in the United States of America. J Vet Diagn Invest 17(1)1: 10-15.

37. Tajima M, Frey HR, Yamato O, Maede Y, Moennig V, Scholz H, Greiser-Wilke I. 2001. Prevalence of genotypes 1 and 2 of bovine viral diarrhea virus in Lower Saxony, Germany.Virus Res 76(1): 31-42.

38. Vilcek S, Paton DJ, Durkovic B, Strojny L, Ibata G, Moussa A, Loitsch A, Rossmanith W, Vega S, Scicluna MT. 2001. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch Virol 146(1): 99-115.

39. Wolfmeyer A, Wolf G, Beer M, Strube W, Hehnen HR, Schmeer N, Kaaden OR. 1997. Genomic (5 UTR) and serological differences among German BVDV field isolates. Arch Virol 142(10): 2049-2057.

40. Xia H, Liu L, Wahlberg N, Baule C, Belák S. 2007. Molecular phylogenetic analysis of bovine viral diarrhoea virus: a Bayesian approach. Virus Res 130(1-2): 53-62.

41. Yang Z. 1996. Maximum-likelihood models for combined analyses of multiple sequence data. J Mol Evol 42(5): 587-596.

CHAPTER VI

CONCLUSIONS

In this dissertation, we have developed a BVDV testing scheme for the detection of persistently infected animals and characterized two virulent BVDV isolates from Mississippi herds which failed to be detected using the conventional fluorescent antibody test. We further used various computational methods to generate phylogenetic trees of BVDV based on sequences of 5'UTR, E2, and NS5B regions and compare the advantages and disadvantage of each method.

In the first part, a cost-effective testing scheme by combining real-time RT-PCR and ACE was developed for the detection of *bovine viral diarrhea virus* persistently infected animals in exposed herds. In this testing scheme, pooled samples were screened using real-time RT-PCR, and individual positives were identified from the positive pools using ACE. The pool size was estimated based on BVDV prevalence, and the prevalence of BVDV in Mississippi is 0.4% in a three year period from 2006 to 2008. The least-cost pool sizes were 50 at a prevalence of 0.25 – 0.5% and 25 at a prevalence of 0.75 – 2.0%. Also we found the semiquantitative results of real-time RT-PCR and ACE or real-time RT-PCR and immunohistochemistry were moderately correlated. Real-time RT-PCR screening also indicated that 92.94% of PI animals were infected with BVDV-1, 3.53% with BVDV-2, and 3.53% with both BVDV-1 and BVDV-2. Phylogenetic analysis of the 5'-untranslated region of 22 isolates revealed the predominance of BVDV-1b followed by BVDV-2a.

In the second part, two virulent BVDV isolates M10-3432 and M10-5347 were successfully recovered from an adult beef breeding cow and feedlot calf, respectively. Both isolates were genotyped as BVDV-2 using E2 region. FA failed to detect the presence of BVDV antigen from fresh tissues of M10-3432 and M10-5347, while cross-neutralization test on isolated virus from these two cases indicated antigenic changes on both M10-3432 and M10-5347. Five unique amino acids from M10-5347 and three from M10-3432 in E2 region were different from reference strain 125c. Protein motif analysis of E2 glycoprotein revealed motif changes for both M10-3432 and M10-5347 when compared to the reference strain 125c. Phylogenetic analysis grouped both Mississippi isolates in BVDV-2a, a subtype containing high virulent strains. M10-3432 was clustered with high virulent strain 890 while M10-5347 was clustered with another high virulent strain CD87.

In the third part, we compared the phylogenetic analyses of BVDV isolates using a single region or concatenated regions of 5'UTR, E2, and NS5B at either nucleotides or amino acids level. Although slightly difference was observed, the virulent BVDV isolates are consistently classified into BVDV-2a cluster regardless of the region used for analysis. Furthermore, phylogenetic tree constructed using combined two or more regions were supported with higher posterior probability and bootstrap value than phylogenetic trees constructed using a single region.

In summary, we provide an effective testing scheme, the combined real-time RT-PCR and ACE, to detect persistently infected animals to prevent the spread of the disease. We also characterize two clinical Mississippi isolates as well as its relationship with other known isolates/strains to understand the pathogenesis of the disease in Mississippi. We suggest that using multiple regions of BVDV is a better way to generate a phylogenetic

tree for typing and grouping. The information from this dissertation will improve the diagnosis, characterization and prevention of BVDV.