CLINICAL AND IMMUNOLOGIC RESPONSES OF CATTLE TO VACCINAL AND NATURAL BOVINE VIRUS DIARRHEA VIRUS (BVDV)

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Western College of Veterinary Medicine
University of Saskatchewan
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by

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SUMMARY OF DISSERTATION

Submitted in partial fulfillment

Of the requirements of the

DEGREE OF DOCTOR OF PHILOSPHY

By

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CLINICAL AND IMMUNOLOGIC RESPONSES OF CATTLE TO VACCINAL AND NATURAL BOVINE VIRUS DIARRHEA VIRUS (BVDV)

Since its discovery a little over fifty years ago, bovine viral diarrhea virus had been identified as one of the top five economically important viral diseases of cattle. The virus continues to cause endemic infections, with hidden economic losses from reproductive diseases and calf problems, as well as severe acute infections with high death loss. A series of studies were performed to learn more about the responses of cattle to BVDV vaccines.

Safety Studies

Cattle had little or no transmission of BVDV following vaccination with one of two modified live vaccines containing the cytopathic NADL isolate of the virus. These results suggest that the risk from vaccinating animals in close proximity to pregnant animals is low.

The second safety study suggested that administration of vaccines in combinations to calves could stimulate virus-specific antibody responses and did not cause untoward effects on production parameters.

Efficacy Studies

Calf Studies

These studies have shown that an inactivated and a MLV BVDV type I vaccine can protect young calves from a virulent challenge with type II BVDV. The BVDV isolate used in this study produced severe clinical disease that necessitated euthanasia of all control calves. In contrast to the inactivated vaccines, calves that received the modified live vaccine were less affected by this virulent BVDV than were susceptible unvaccinated calves. High levels of maternal antibody against type II BVDV afforded protection against the severe challenge as well.

Mature Cattle Studies

The first was a study of BVDV antibody concentrations in a herd for 18 months after vaccination with a modified live BVDV vaccine. The data from that study suggested that modified live BVDV vaccines could stimulate a strong immune response in sero-negative cows that were still detectable eighteen months after vaccination. The study showed that these antibodies could neutralize antigenically disparate isolates of BVDV for the same 18 months.

The second challenge used the BJ challenge virus (isolate that was different antigenically from the vaccine's NADL BVDV). The modified live vaccine provided efficacy against a type I BVDV fetal challenge with a protection rate of 83%.

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CLINICAL AND IMMUNOLOGIC RESPONSES OF CATTLE TO VACCINAL AND NATURAL BOVINE VIRAL DIARRHEA VIRUS (BVDV)

Abstract

This research had two objectives. First, to determine if BVDV vaccines containing type I BVDV were safe and efficacious against a type II BVDV challenge in calves. Second, to determine if modified live BVDV vaccines could stimulate a greater degree of protection against BVDV reproductive syndromes as well as a longer duration of cross neutralizing antibodies than the current inactivated vaccines.

One to nine week old calves were administered modified live and/or inactivated BVDV containing vaccines on various schedules. The calves were monitored for adverse effects and followed serologically for 12 weeks. BVDV challenge studies were performed in calves vaccinated with either a modified live or inactivated vaccine beginning on day 14 of age. BVDV sero-negative and sero-positive calves were included in the study.

Studies were performed in BVDV sero-negative cows. Safety was determined by assessing the risk of BVDV transmission of vaccinal virus. Duration of cross neutralizing antibodies stimulated by a single modified live BVDV vaccine was determined. Finally, the ability of a modified live BVDV vaccine to provide fetal protection was assessed.

Results

Neither the modified live BVDV vaccines nor the inactivated BVDV vaccine used in these studies caused adverse reactions in the young calves. Calves with BVDV maternal antibody less than 1:64 by virus neutralization testing responded to BVDV vaccination. Calves vaccinated with modified live BVDV vaccine or with high maternal antibody against BVDV were protected from a type II BVDV challenge. Inactivated BVDV vaccine conferred only partial protection against the challenge.

Cows vaccinated with one of two modified live, NADL-containing, BVDV vaccines did not transmit measurable BVDV to contact control animals. One of these vaccines, following a single dose, produced antibodies that neutralized 12 isolates of BVDV that were still detectable 18 months after vaccination. This vaccine conferred 82% fetal protection against a challenge of virulent BVDV.

This research serves to define the responses and protection produced by modified live vaccines and further demonstrates that modified live BVDV vaccines can stimulate a higher degree of protection than an inactivated BVDV vaccine.

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As with any major undertaking, there are many people to whom credit is due for support, guidance and assistance. I would first like to thank my employers of SmithKline Beecham Animal Health and Pfizer Animal Health for giving me the time and financial support for my classes and my research. My supervisors at these two companies, Drs. Kurt Wohlgemuth and William Swafford, have been a source of encouragement, support and advice. They made the process of getting my Ph.D., while working full-time, a less difficult task. A heartfelt thank you is expressed to them.

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My co-researchers on several of my projects, Drs. Steve Bolin and Kenny Brock, have been constant sources of support, advice and examples of dedicated scientists. I have truly been blessed to be allowed to do research with these leaders in their field.

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DEDICATION

To my wife, Debra, and my two children, Cassandra and Joseph, this thesis is lovingly dedicated. They have endured long hours of shared time as I wrote this and allowed me the extra time and travel to do all of the research and classwork needed to complete my Ph.D. Without their love, support, understanding and patience, it would not have been possible.

I would also like to dedicate this work to my parents, Walter and Doris Cortese. Through the years, they have taught me to never accept mediocrity and to constantly strive to reach my full potential. They have been a source of unwavering love and inspiration.

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American Veterinary Medical Association	AVMA
Analysis of variance	ANOVA
Bovine respiratory syncytial virus	BRSV
Bovine turbinate cells	BTU
Bovine viral diarrhea virus	BVDV
Bovine herpesvirus 1	BHV-1
Cell culture infectious dose	CCID
Cell Mediated Immune	CMI
Cluster of differentiation	CD
Cytopathic	CP
Cytopathic effect	CPE
Embryonic bovine turbinate cells	EBT
Enzyme linked immunosorbent assay	ELISA
Failure of passive transfer	FPT
Glycoprotein	Gp
Hog cholera virus	HCV
Immunoglobin	Ig
Infectious bovine rhinotracheitis virus	IBRV
International Committee on Nomenclature of Viruses	ICTV
Major histocompatability site	MHC
Maternal colostrum	MC
Microliter	µl
Modified live vaccine	MLV
Monoclonal antibody	Mab
Mucosal Disease	MD
National Animal Disease Laboratory	NADL
Noncytopathic	NCP
Nonstructural	Ns
Nucleotides	Nts
Number	N
Open reading frame	ORF
Parainfluenza 3	PI3
Persistently infected	PI

Polymerase chain reaction	PCR
Reverse transcription	RT
Sero-negative	S(-)
Sero-positive	S(+)
Tissue culture infectious dose	TCID
United States Department of Agriculture	USDA
Untranslated regions	UTR
Virus neutralization	VN

1. INTRODUCTION AND BACKGROUND OF BOVINE VIRUS DIARRHEA VIRUS

1.1 History of Bovine Viral Diarrhea Virus

A new disease was first diagnosed in 1946 by veterinarians in the ambulatory staff at Cornell University. This new disease of cattle was characterized by depression, dehydration, high fevers, nasal discharge and oral ulceration. Diarrhea was the most consistent sign. Because it was postulated that this new disease was caused by a virus, the agent received its name of bovine viral diarrhea virus (BVDV).[1] The marked decreases in white blood cell counts, or leukopenia, caused by BVDV, was also first demonstrated in these infected herds. The outbreaks continued in the surrounding farms and the university clinicians tried to control this new disease.[2] By the end of 1946, BVDV infections were seen across the United States.[3] It was from this beginning that BVDV was first diagnosed as a new disease-causing virus. The actual virus was first isolated by Olafson and others during these initial outbreaks and several New York isolates were obtained. These findings and the fact that the virus was distinct from rinderpest were reported in 1947.[4] A new disease with similar characteristics to this mucosal disease named "Schleimhautkrankheit" (later proven to be BVDV

was first reported from Germany in 1959.[5,6,7]

Mucosal Disease (MD) was first diagnosed in 1953 in the United States and Canada. It was initially thought that MD and BVDV were two distinctly different diseases. This was in part due to the fact that early non-mucosal disease isolates were noncytopathic when grown in cell culture; whereas, early mucosal disease isolates were cytopathic in cell culture. Two cytopathic isolates were found; one in Oregon (Oregon C24V) and the second, a European isolate (Lamspringer/375).[8] These isolates were studied extensively and led to subsequent research that would show they were different isolates of the same virus.[9] This was shown by both virus neutralization and cattle protection tests by Gillespie and others.[10] Other work showed that all early isolates from Iowa, Indiana, New York and Oregon consistently cross protected calves and decreased the severity of clinical signs.[11] More importantly, it was found that the virus persisted in some cattle for long periods of time after they became severely ill, a characteristic shared with hog cholera.[11] In 1967, at a meeting of the AVMA Bovine Respiratory Symposium, it was agreed that BVDV and MD were caused by the same etiologic agent and the name of BVD-MD was accepted.[12] It was characterized as an enveloped, single-stranded RNA virus. Since that time the virus has been widely studied and the various disease syndromes have been well defined.

1.2 Virus Classification and Characteristics

1.2.1 Overview

Initially, the 1976 International Committee on Nomenclature of Viruses (ICTV) classified BVDV in the *Togaviridae* family in the genus of *Pestivirus*. The virus was moved to the *Flaviviridae* family in 1991 by the ICTV. The other closely related pestiviruses, hog cholera virus and border disease virus, were also reassigned to *Flaviviridae* family. BVDV isolates show some similarities to the human hepatitis C viruses, which are also in the *Flaviviridae* family. As is true with all pestiviruses, BVDV is an RNA virus that is highly mutable.[13]

1.2.2 Genomic Structure

The BVD virus is a single positive-stranded RNA virus. The virion consists of a central core of RNA surrounded by the p14/C capsid protein. A lipid membrane surrounds this capsid protein and is anchored by gp25/E1 and gp53/E2.[14,15] The positive strand was shown in 1966 when pure viral RNA caused infection and disease.[16] Molecular cloning of the virus (first reported in 1987) has provided most of the genomic information that is available.[17]

The BVDV genome consists of a single large open reading frame (ORF) bounded by 5' and 3' untranslated regions (UTR). There are several small ORFs (<30 nucleotides) but they are not translated. The basic size of the BVDV RNA is approximately 12300 nucleotides (nts) with the primary ORF encoding 3898 amino acids.[18,19] The base composition is 32%A, 22%T, 26%G and 20%C.[17] There are 14 potential glycosolation sites in the region encoding structural

polypeptides. Eleven of these sites are conserved among BVDV isolates and 8 are shared with swine pestiviruses.[20] The cytopathogenic isolates have considerable variability in the size of the RNA with most variation occurring in the ORF, the virus may reach sizes of 16000 nts.[17] The 5' terminus is uncapped although the exact nature of this end is not yet known. The 5' is a 385 nucleotide sequence that is fairly conserved among the various isolates.[20] The 5' UTR has a complex loop that contains a pseudoknot.[17] This area is important for ribosome insertion and RNA translation.[17,21] It contains some important variants that are responsible for genotypic differences. This will be discussed more in depth in section 1.2.4.

The 3' UTR is composed of 229 nucleotides.[20] It does not end with a 3' poly A tail but with 3-6 cytidine residues as all other pestiviruses do.[19,22] This lack of a homopolymeric tail is unique to the *Pestivirus* genus of *Flaviviridae*.[23] Its function is not yet known. However, it must be involved in positive RNA replication and possibly in RNA stability.[20]

1.2.3 Biotypes-Cytopathic Versus Noncytopathic Isolates

The cytopathic (CP)/noncytopathic (NCP) differentiation is solely laboratory determined. Cytopathic isolates will, when grown on a cell culture, cause cell damage that ends in cell death, whereas a NCP isolate does not.[24] This cytopathic effect does not relate to the virulence of the isolate.[25] Noncytopathicity is the natural state of the virus and approximately 95% of field isolates will be noncytopathic.[26] It is believed that all cytopathic isolates arise as mutations of a noncytopathic isolate via recombinations or insertions in the genome.[27,28]

Cytopathicity leads to expression of the p80/NS3 protein which is found only and in all cytopathic isolates.[29,30] This protein has not been found in cells infected with hog cholera virus or border disease virus although there are reports of finding it in some outbreaks of atypical border disease.[31] These outbreaks may be attributable to BVDV virus rather than atypical border disease virus isolates.[32]

1.2.4 Genotypes-Type I Versus Type II

The "genotype I versus genotype II" designation of BVDV isolates is based on differences in the 5' untranslated region of the viral genome. Within a BVDV genotype, there is a 78-88% sequence homology along the entire genome with higher homology seen among the more highly conserved regions such as the 5' UTR. Between the two genotypes of BVDV the 5' UTR sequence homology drops to 75% as compared to over 90% within a genotype.[33,34] It is now thought that the pestiviruses can be split into four distinct groups that each share approximately 65% homology with each other (Figure 1.1).[20] These include type I BVDV, type II BVDV, border disease virus in sheep, and hog cholera virus. The various *Pestivirus* groups can cause cross infections and potential disease in any of the domestic and wild ruminants and swine.[32,35,36,37] The type I versus type II designation does not correlate to virulence. The only BVDV group-specific disease syndrome is the "thrombocytopenic form", which has only been reported with several type II isolates.[38] Depending on the isolate, there can be severe death loss with either group.

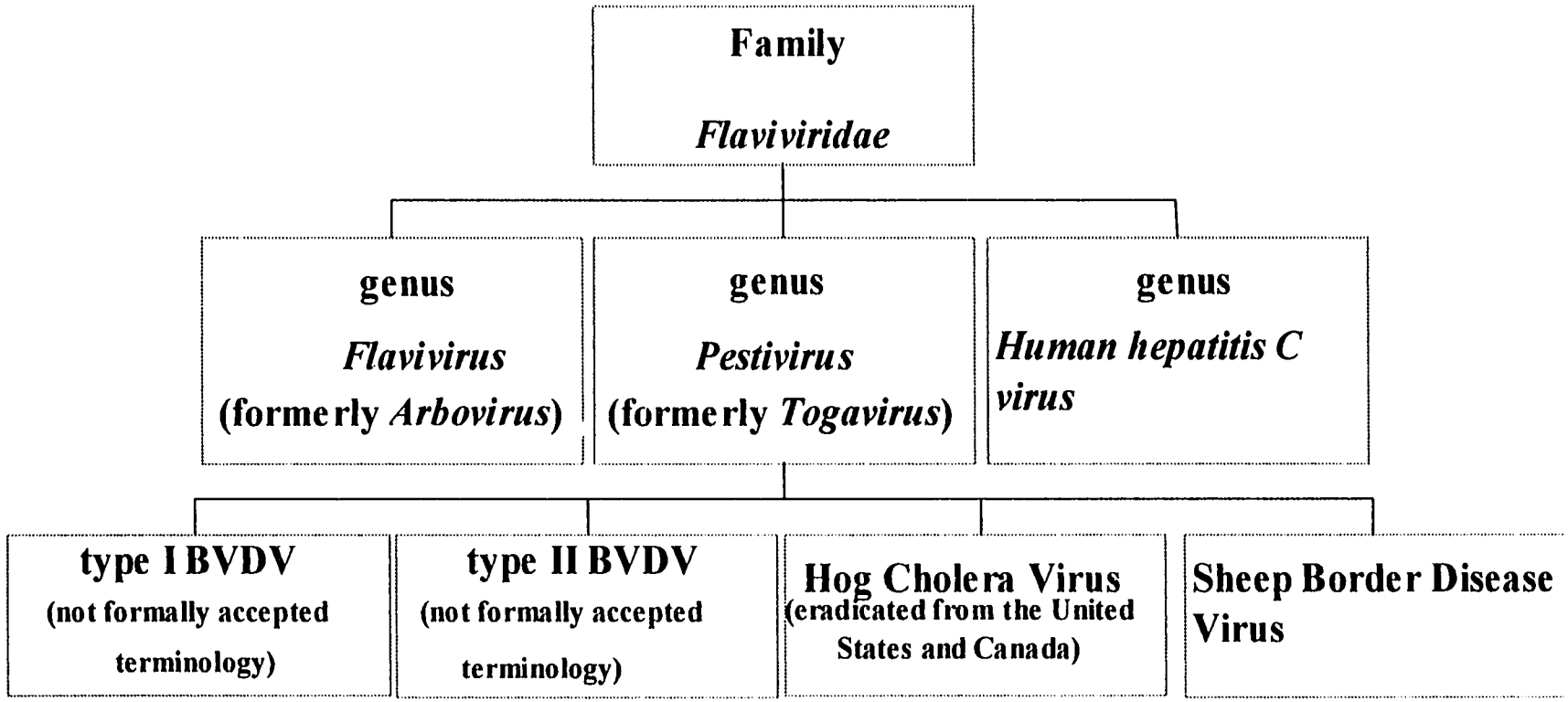

Figure 1.1. Taxonomic structure of the viral family *Flaviviridae*

1.2.5 Phenotypes-Surface Variation

Comparative nucleotide sequencing has shown that several amino acid regions of the gp53/E2 are the most variable of the surface antigens. Since gp53/E2 is the primary target of neutralizing antibodies, monoclonal and polyclonal studies of the variability of this site have been performed.[39,40] These studies have shown that considerable antigenic variability exists among the various isolates of BVDV and it has been suggested that BVDV isolates be grouped according to monoclonal antibody binding patterns.[41,42,43] This antigenic variability has been determined to be important for length and level of protection afforded by vaccination programs.[40,44]

1.2.6 Proteins Encoded by BVDV

The BVDV genome encodes for both structural and nonstructural proteins. The structural proteins make up the virion whereas the nonstructural proteins make up the viral genome. The noncytopathic BVDV encode 10 viral proteins (4 structural and 6 nonstructural); the cytopathic BVDV isolates will add up to 3 more nonstructural proteins as shown in Figure 1.2.[14,15]

The viral proteins are still being completely identified. Cytopathic isolates will express a p80/NS3 protein and, occasionally, other polypeptides of varying sizes.[20] Infected cells may also show some additional peptides or proteins.

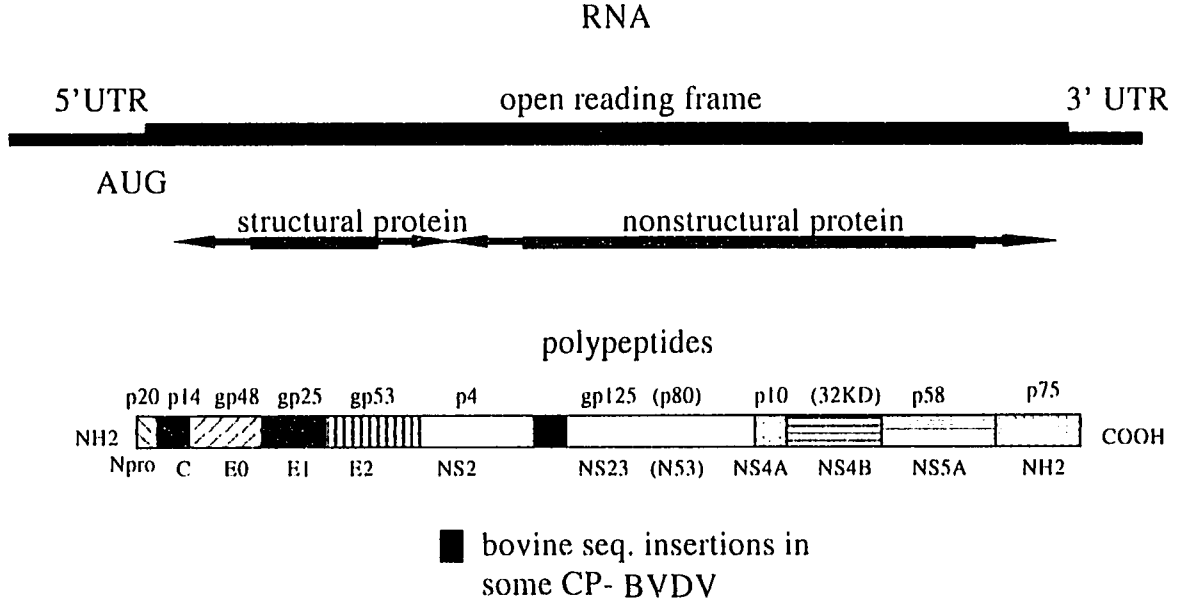

Figure 1.2. Protein map of the BVD virus, the 32-kilodalton protein has not been identified. Note the lack of either a 5' cap or 3' tail.

1.2.6.1 p20/Npro

This is the first product translated by the ORF and has 168 amino acids. It is a cis-acting protease that cleaves intramolecularly at its C-terminus. This papain-like protease is important in the generation of the N-terminus. Other viral replication functions are suspected but have not yet been identified.[20] The other *Flaviviridae* do not have a counterpart to this protein but similar proteins are found in foot-and-mouth disease virus, equine arteritis virus, and a plant potyvirus.[45,46,47]

1.2.6.2 p14/C

The p14/C is a 102 amino acid protein that migrates as a 14 KD polypeptide. This protein is well conserved among all pestiviruses and its N terminus is generated by the action of the p20/Npro. It is found in the infected cell's cytoplasm and functions to package the genomic RNA material and provides information for the manufacture of the enveloped virion.[20] Cattle recovering from BVDV infections do not make antibodies to this protein.[29]

1.2.6.3 gp48/EOms

This is a 227 amino acid glycoprotein whose function is not completely known. It is believed to be loosely attached to the envelope and is well conserved among the pestiviruses.[20] Although this protein can induce high levels of antibodies; these antibodies have very low virus neutralizing ability.[48,49]

1.2.6.4 gp25/E1

This is a 195 amino acid polypeptide that is involved in anchoring the protein to the membrane and initiating the translocation of the adjacent protein. In virions, it is covalently linked to gp53/E2.[20] Convalescent sera do not contain appreciable levels of antibodies to this protein.[29]

1.2.6.5 gp53/E2

GP53 is a 375-400 amino acid glycoprotein. It appears that there are two forms of gp53/E2 found in infected cells due to differences in the C-terminus tail length. GP53/E2 is usually found in the virion envelope as a homodimer or as a heterodimer with gp25. One of three hypervariable regions is found in this sequence and it is the major target of neutralizing antibodies.[20] It is extremely antigenic and this hypervariability may be due to immunologic selective pressure.[29,40,50,51,52]

1.2.6.6 p125/NS23

The two termini of this polypeptide are undetermined. This region has homology with other *Flaviviridae*. As with other *Flaviviridae* it has two distinct regions, the N-terminus (p54/NS2) and C-terminus (p80/NS3).[20]

These two regions further separate into four distinct areas: a hydrophobic domain at the N-terminus which aids in holding viral replication close to the membrane; a zinc finger that binds to RNA; a protease which generates the cleavage of polypeptides for manufacture of nonstructural proteins; and a helicase near the C-terminus which binds and catalyzes ATP dependent RNA.[18]

Insertion of host genome is found in the p125/NS23 polyprotein of some cytopathic BVDV isolates. It is obvious that this protein is directly or indirectly involved in cell damage since the byproduct p80/NS3 is only expressed by cytopathic isolates.

Sera from convalescent cattle, or cattle vaccinated with modified live BVDV vaccines, develop high levels of virus neutralizing antibodies to this protein. These antibodies will also cross react with hog cholera and border disease p125/NS23. Cattle vaccinated with inactivated vaccine make negligible antibody to this protein.[20]

1.2.6.7 p54/NS2

This protein is found exclusively in cytopathic BVDV isolates, however, not all cytopathic BVDV isolates have this nonstructural polypeptide. It is a by-product of protease activity by p125/NS23 and has a variable weight due to inclusions of either foreign sequences or additional rearrangements.[20] It too has a zinc finger, is a poor antigen, and does not induce antibodies in infected cattle.[50,53]

1.2.6.8 p80/NS3

This polypeptide is considered to be the cytopathic marker and is composed of the helicase and protease of p125/NS23. It is homologous to the *Flaviviridae* NS3 and is considered to be directly or indirectly involved in cell damage.[20]

It is the most conserved protein in the genus *Pestivirus*, is stable and stimulates a strong immune response.[50,53]

1.2.6.9 p10/NS4A

Little is known about this protein. It is conserved among pestiviruses and may have an important role in viral replication.[20]

1.2.6.10 p32/NS4B

Little is known on this protein as well. It accumulates in high levels in late infection of cells by CP BVDV. It is highly conserved and stimulates little immunity.[20]

1.2.6.11 p58/NS5A

This protein is the by-product of cleavage of a precursor protein p133. Although not well understood, it is one of two proteins with RNA polymerase activity. It is fairly stable in the infected cell and is variable among the different pestiviruses.[20] Infection does not stimulate humoral immune responses.[50, 53]

1.2.6.12 p75/NS5B

P75/NS5B is the other half of the cleavage of protein p133. It is the second RNA polymerase activity protein, is not well characterized and does not stimulate antibodies.[20,50,53]

1.3 Immune Responses to BVDV Infections

Immune responses to bovine viral diarrhea virus infection take place in two steps. After initial infection in susceptible animals, there is an immunosuppression.

This immunosuppression may very from mild to severe, depending on the BVDV isolate.

Immunosuppression is usually followed by protective immune responses as the animal recovers from the infection and as the virus is cleared from the body.

1.3.1 Immunosuppression

One of the most dramatic and controversial outcomes of BVDV infection is immune suppression.[54,55,56] Many diseases have been shown to worsen when there is a concurrent BVDV infection.[57,58,59] However, it is the nature of the immune suppression that is contested. It has been well documented that following exposure and infection, susceptible animals will display a marked leukopenia.[13,38,60,61] This decrease in numbers and percentages is reflected in most leukocyte sets. The predominant decreases are seen in neutrophils and lymphocytes, both B and T (both CD8+ and CD4+) cells.[62,63] This leukopenia is consistent enough that it is one of the measurements of BVDV vaccine efficacy against challenge used by the USDA.[64] There are little or no decreases seen in null cells or monocytes.[62] Although the decrease in white blood cells may be due to trafficking to the site of infection, BVDV does has an affinity for cells of the immune system. In particular, BVDV will infect and replicate in macrophages and lymphocytes. *In vitro* destruction of the cells has been demonstrated.[60,65,66,67] Furthermore, the function of many of these cells is depressed. Many neutrophil functions are affected and there is impaired iodination reaction;[68] decreased migration; and neutrophil-mediated, antibody-dependent, cell-mediated

cytotoxicity.[69] Depressed neutrophil function may last as long as three weeks after infection with the virus.[68] Lymphocyte responses are impaired as well. Lymphocyte blastogenesis, interleukin 2 release, and interferon release are diminished and the antiviral effectiveness of interferon is also decreased.[70,71,72,73] Monocytes have decreased responses to lymphokines as well.[74]

1.3.2 Humoral Immune Responses to Infection

Although initially immunosuppressed, the immune system develops a high virus neutralizing antibody level and long lasting immune response to BVDV infections. The fetus becomes immunocompetent to BVDV by 180 days of gestation and some are able to respond to BVDV infections as early as 129 days of gestation.[74] Neutralization of the virus is through IgG. There are immune responses to most structural and nonstructural proteins although there are vast differences in the level of detectable immune responses to the various proteins as discussed previously (section 1.2).[53] Both B and T cell responses are stimulated by infection. The T cell responses are primarily CD4+.[53,75,76]

The humoral (antibody) immune responses are the best defined of the immune responses to BVDV infection and it is felt that these are the primary mechanisms of protection.[77,78] Studies have shown that maternal BVDV neutralizing antibodies will provide protection against severe infections.[79] Most neutralizing antibodies are directed to the gp53/E2 epitopes. Three antigenic domains and 9 antigenic sites for virus neutralization have been identified.[39,41,80] There are discontinuous areas of the epitopes making conformation an important

factor in eliciting an effective antibody response.[81] Although there are several conserved regions of gp53/E2, it is a highly variable protein giving rise to many differences among isolates.[82,83] There are from four to six groups of BVDV isolates as determined by monoclonal antibody mapping of the gp53/E2.[41,49] Type I and type II genotypes show distinctly different gp53/E2 antigenic patterns.[27,33,34]

When p80/NS3 is expressed, there is a strong humoral response elicited. These antibodies do not appear to have virus-neutralizing ability.[14,50] According to monoclonal antibody mapping, there are four p80/NS3 antigenic domains.[83]

1.3.3 Cell Mediated Immune Responses to Infections

The involvement of local and cell mediated immunity, in clearing BVDV infection, is not clear. Few studies have been done either measuring cell mediated immunity or comparing the cell mediated immune responses to B cell responses.[75] However, cell mediated immunity may be an important component of protection. Several recent studies have not shown a correlation between virus neutralizing antibodies and protection.[84,85,86] The first study also failed to show a correlation between cell mediated responses and protection. Lymphocyte depletion studies have shown primarily CD4+ involvement with little CD8+ response to BVDV infections. This may indicate that there is a MHC II restricted cytotoxic T-cell activity.[87] It is uncertain what proteins are necessary for a cell mediated immune response but the major envelope glycoprotein may not be a primary T cell antigen.[88] Since the proteins responsible for cell mediated immunity have not been

identified, it may be that either infection or modified live BVDV vaccination are necessary for cell mediated immunity to be established.[75]

1.4 Animal Infection and BVDV Replication

The BVDV virus attaches to the cell wall and then undergoes endocytosis to enter the cell. After entry, the viral genome is delivered via an acid dependent step.[89] It will replicate only in cells from members of the family Artyodactyla, with maximum replication in cattle and goat derived cells.[35] All replication takes place in the cytoplasm of the cell and peak release of virions occurs between 12-24 hours after infection occurs.[90] Each cell will be used to manufacture from 100-1000 new virions.[19] The first virions are released via exocytosis as early as 10 hours after infection.[19]

1.5 Disease Syndromes

1.5.1 Subclinical Infections

The majority of BVDV infections are of a subclinical nature with little apparent clinical disease.[91,92] The severity of the disease is determined by the virulence of the isolate and the susceptibility of the host. The infection may be completely inapparent as is often seen in adult cattle[93] or it may cause a severe disease that may approach the severity of disease associated with mucosal disease.[94,95,96,97] The one constant that appears with these infections is an immune suppression. As previously stated, the severity and duration of the immune

suppression appears to relate to the isolate infecting the animal.[98] In most infections, if the animal is unexposed to other disease agents, it will recover. However, if there is another disease agent present the morbidity and mortality rates can be greatly elevated.[57,58,59] Most endemic BVDV herds have the virus circulating in a subclinical manner and exhibit only reproduction syndromes (see below).

1.5.2 Respiratory Tract Infections

The respiratory form of BVDV infection appears, clinically, much like bovine herpesvirus 1 (BHV-1). The trachea is the primary site of lesions. Oral and tracheal ulcers may be seen. Reddening of the nares is often present. Pneumonia may be found in the anterior lung lobes but the lesions are usually due to secondary pathogens.[98,99,100]

1.5.3 Digestive Tract Infections

Although severe diarrhea is usually associated with mucosal disease, many of the severe cases of acute disease involve the digestive tract. Diarrhea and nonresponsive fever (>104°F) are the most consistent signs with some particular type II challenge viruses. The diarrhea in both calves and cows may be bloody. Digestive tract ulcers, particularly of the Peyer's patches, are common.[84,96,97,98]

1.5.4 Thrombocytopenic Syndrome

The most recently described BVDV syndrome is the thrombocytopenic form. It has also been called the bleeder or hemorrhagic syndrome. In this syndrome, the BVD virus is associated with platelets and is found in

megakaryocytes in the bone marrow. There is an accompanying decrease in the number of circulating thrombocytes. These animals may start with a mild diarrhea or anorexia with a slight fever. Bleeding into the conjunctiva may be the first sign of this form of BVDV. If treated, the calf will often bleed from the injection site for several hours or develop large hematomas. Hemorrhages are often found in or on the intestinal cavity or internal organs when post mortem examinations are performed. This form is caused by several noncytopathic type II isolates in acutely infected animals that are not persistently infected. Originally thought to be primarily a disease of the Holstein calf, it has now been seen in adult dairy and beef cattle as well.[25,101]

1.5.5 Reproductive Infections

Infection with CP and NCP isolates differ in the non-immune pregnant cow and the reproductive syndrome.[102,103] If a non-immune cow is exposed to a NCP isolate while in the first trimester of gestation, early embryonic death, abortion, mummification or persistently infected (PI) calves can result.[104,105,106] If exposure occurs during the second trimester, birth defects, primarily involving nervous tissue, are found, an occasional persistent infection may occur.[91,107] Infection during the last trimester usually has no effect on the fetus and the calf will be born with antibodies against BVDV.[74,91,108] Rarely, there is an overwhelming exposure which causes a late abortion (Figure 1.3). Persistently infected cows can have all of the above fetal outcomes except that a persistently infected cow cannot have a normal calf.[109] Infections with cytopathic isolates cannot give rise to persistently

infected calves but can cause the other reproductive problems.[110] Fetal infections can occur in a high number of susceptible pregnant cattle even in the absence of any clinical disease.

1.5.5.1 Persistent Infection

When BVDV infection occurs before the immune system has fully developed, the fetus recognizes that particular isolate of BVDV as self and never mounts an immune response against that BVD virus. Such calves do mount immune responses to heterotypic BVDV antigen.[111,112,113] Persistently infected calves can be normal at birth or they can be born weak and die. Persistently infected cattle are immunologically wea, have decreased immune function,[114] and have low survivorship. However, some persistently infected animals can appear normal. A low percentage can reach adulthood, breed, and have persistently infected calves. Persistently infected cattle are a major source of viral shedding to the rest of the herd. The current persistent infection rate in the United States among cattle under one year of age is estimated at 1 1/2 - 2%.[115] In some herds, 10-50% of the calves may be carriers. Once an animal is persistently infected, nothing can eliminate the virus or stop its shed while the animal is alive.[99] Recent data has shown that the amount of viral shedding will vary by age, genetics and stress on the animal.[112]

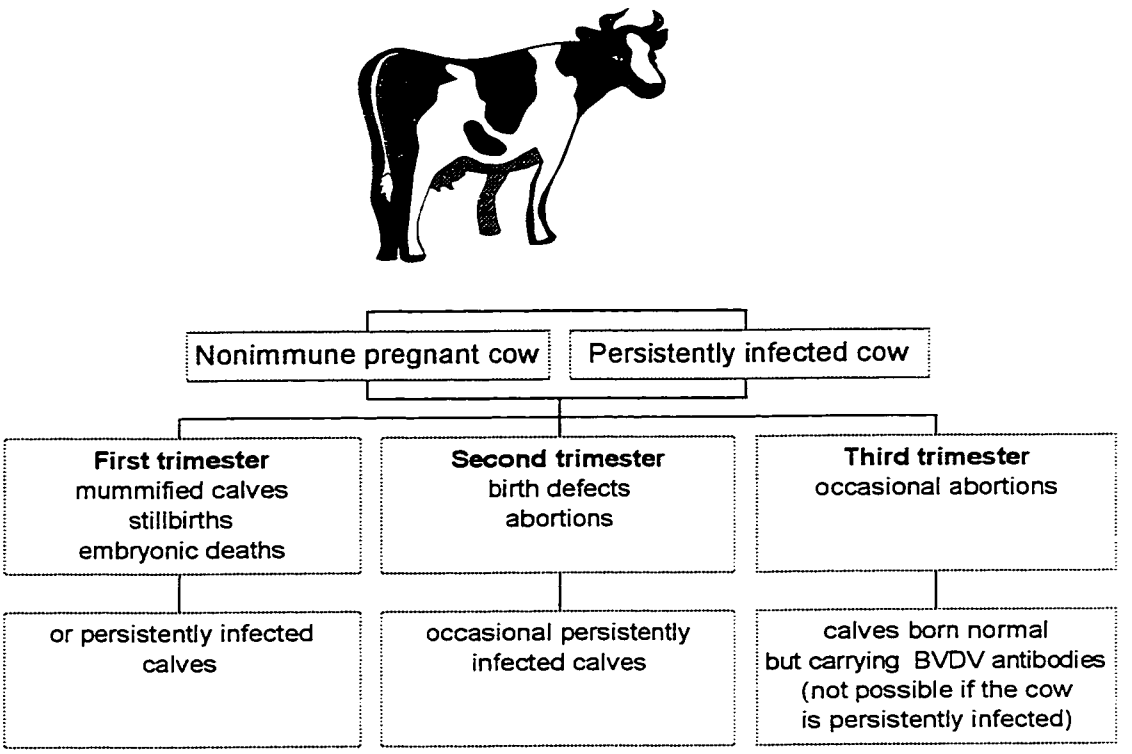

Figure 1.3. Effect of noncytopathic BVDV isolates on the developing fetus.

1.5.6 Mucosal Disease

The exposure of a persistently infected animal to a cytopathic isolate of BVDV can have three possible outcomes (figure 1.4). Mucosal disease is one of the potential results of CP infection in a PI animal. In order for mucosal disease to occur, a specific set of circumstances is required. First, the animal must be persistently infected. Then, the animal must be exposed to another BVD virus that is a cytopathic isolate.[111,112] In mucosal disease cases, usually both isolates can be isolated. Furthermore, in order to consistently cause mucosal disease, new research indicates that this infecting isolate must be closely related to the noncytopathic isolate causing the persistent infection.[117, 118,119] More antigenically disparate cytopathic BVDV isolates (from the PI strain) can cause this fatal disease but not as consistently.[117] Exposure may be from additions to the herd or from the virus in a persistently infected animal spontaneously mutating to a cytopathic isolate. Clinically, there is usually explosive diarrhea and ulcers through the digestive tract. Mortality rates in cattle with mucosal disease are 95% to 100%.[99]

1.5.7 Chronic Mucosal Disease

This form of BVDV also requires persistent infection as a prerequisite. The animal needs to be exposed to a cytopathic isolate. It appears that if the isolate is intermediate in its antigenic relationship to the persistently infecting isolate, chronic mucosal disease may occur.[117] A three to five month incubation period results which allows a recombination of the two viruses. The CP isolate that is formed is antigenically different from the initial NCP or CP isolates.[120] Symptoms

in these animals may begin with a lameness involving multiple feet or with a mild, non-responsive diarrhea. The course of the disease is normally from one to two months, but may last up to 18 months after infection, the mortality rate is also very high with this syndrome (>95%).[98]

1.6 Diagnosis

Diagnosis of BVDV can be simple or difficult depending on the syndrome under investigation. Knowledge of the pathogenesis of the disease is important to determine the timing of sampling, samples to be taken, and interpretation of the results.[121,122] The subclinical infections can be the hardest to diagnose and over interpretation is of concern. Laboratory and isolate variation serve to complicate a clinician's ability to correctly diagnose BVDV infections.[123] The diagnosis of subclinical BVDV that is causing herd reproductive problems can be frustrating. The history will often aid in a presumptive diagnosis of a BVDV infection. The most common history is that the herd has been experiencing a slow increase in reproductive problems manifested as early embryonic death with a few mummified calves and/or abortions.[99] In some herds, the first signs are higher than expected numbers of weak and stunted calves.[99] In other herds, increased calf morbidity and mortality may be the primary complaint.[99]

1.6.1 Virus Isolation

Virus isolation is still considered to be the diagnostic test of choice for many BVDV infections.[122,123,124,125] It is the only way to accurately diagnose persistently infected animals.[121] In order to definitively diagnose a PI animal, the

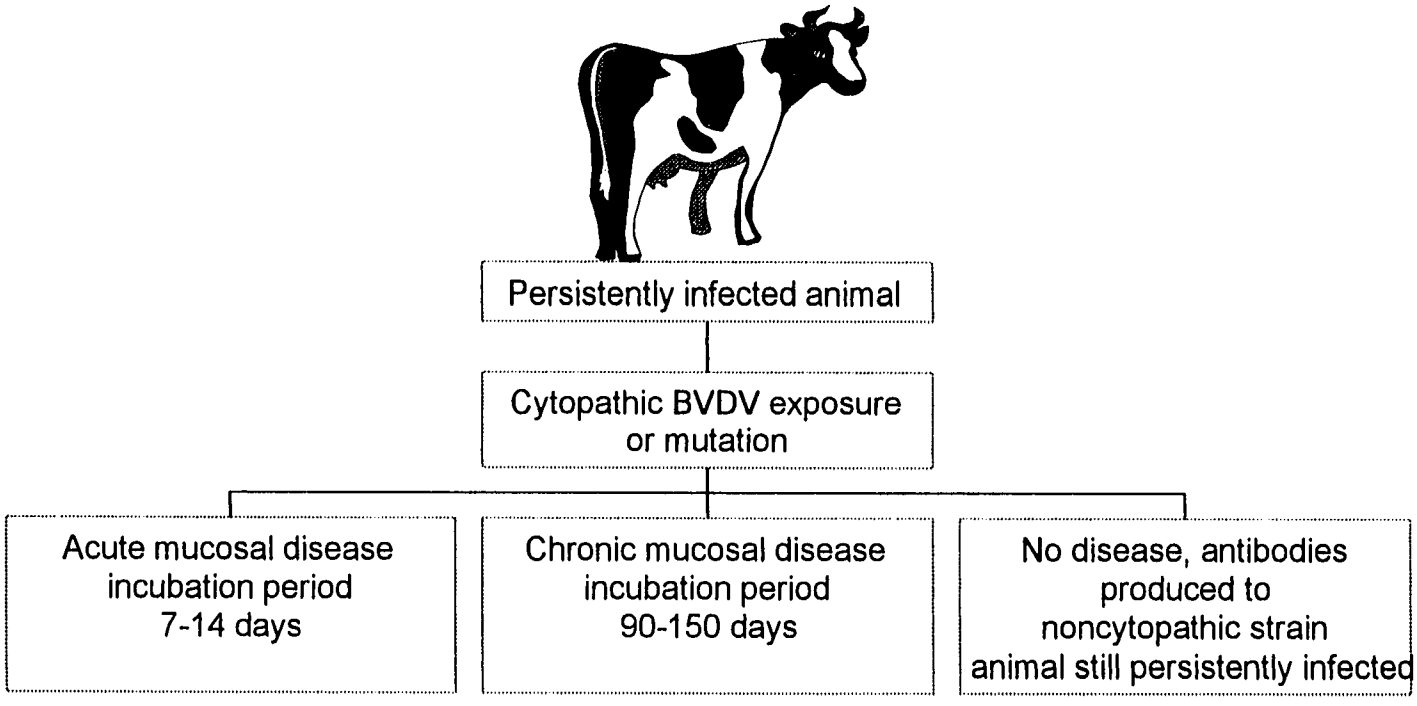

Figure 1.4. Possible outcomes in persistently infected calves after exposure to a cytopathic isolate of BVDV.

virus must be isolated twice from buffy coat or serum with a minimum of four weeks between the two samples.[121] Serum and/or whole blood samples from persistently infected calves will usually yield positive virus isolation results. Calves undergoing mucosal disease, chronic BVD, or acute infections should test positive for BVDV if tested during the correct stage of the infection. Most acutely infected animals are virus isolation positive for 3-10 days after exposure. Mononuclear cells from the buffy coat, obtained from whole blood, are the preferred samples.[121] Noncytopathic isolates are identified in cell culture by detecting viral antigen via immunoflouresence or immunoenzyme staining.[123,126] Other samples that may be submitted for virus isolation include nasal swabs, semen, tissues (particularly lymphoid tissue) and ulcerative areas.[127,128]

If a large number of samples are to be tested, such as in herd eradication programs or PI screening, the microplate virus isolation technique may be used. The samples are incubated in microplate wells and viral antigen is detected using immunoperoxidase labeled conjugates.[82,126,128] This method is usually quicker and less costly than traditional virus isolation techniques.

1.6.2 Antigen Detection

1.6.2.1 Immunohistochemistry

Several immunohistochemical techniques are available for antigen detection from fresh or frozen tissue samples. The two most commonly employed methods of antigen detection are immunoflouresence and immunoperoxidase staining.[123] There have been numerous debates on the accuracy of fluorescent antibody

staining and it appears that immunoperoxidase staining, when available, is more accurate. This test can also be performed on fixed tissues.[129,130] With either test, the most important factor in diagnostic accuracy is the antibody(s) chosen for the detection of the antigen since BVDV antigen variability can affect the results if improper antibodies are chosen. Studies have shown that most tissues have BVDV antigen following acute infection with BVDV when tested using immunoperoxidase staining techniques. New techniques, such as capture ELISAs and flow cytometry for detection of antigen, are generally used in research projects but may become commercially available.

1.6.2.2 Nucleic Acid Hybridization Probes

BVDV hybridization probes were initially used in research to study the genomic variability of BVDV isolates.[121] Probes have been made from both the p80/125 protein region as well as the 5' UTR. Probes from the 5' UTR have detected the widest range of BVDV isolates and have the highest level of detection. Detection ranges from 60% to 100% of various BVDV isolates.[127] To date, the use of hybridization probes is not common in diagnostic laboratories.

1.6.2.3 PCR Amplification

Polymerase chain reaction (PCR) amplification utilizes DNA oligonucleotides to bind to corresponding target sequences. These sequences must have a high degree of specificity for the infectious agent being detected. In PCR amplification, a reverse transcription is followed by the PCR (RT-PCR). RT-PCR can detect from 10^1 to 10^3 fold less virus than virus isolation.[45,132,133] Furthermore,

using PCR amplification, BVDV can be detected for 12-14 days after infection. These two factors should make PCR detection at least as sensitive as virus isolation. PCR tests have been developed for detection of virus in serum, milk, and both fixed and fresh tissues.[121] This method of virus detection will, most likely, become available at most diagnostic laboratories.

1.6.3 Serology

Serology can be difficult to interpret and often leads to over diagnosis of BVDV problems in a herd. Virus neutralization (VN) is the most common serologic test performed for determination of BVDV antibody levels. There is no standard reference isolate used by the various diagnostic laboratories so it is important to send paired samples to the same laboratory.[122] This lack of standardization may explain why different results on the same sample are reported when sent to different laboratories.[34,40,41] The antigenic variability of the infecting isolates leads to differences in detected serologic responses following infection.[34,40,41] Often by the time a reproductive problem is diagnosed, the exposure has occurred weeks or months previously and no changes in titer are detected by paired sampling.

The antibody level of a single sample titer can be useful to determine if more investigation is needed. This method is useful in unvaccinated herds, precolostral samples from calves, or samples from calves, (prevaccination but after colostral antibodies have disappeared). Presence of BVDV VN antibody in these samples indicates exposure to the virus.[121] It is difficult to interpret BVDV titers

from single samples in vaccinated herds. Usually a four-fold to eight-fold increase in titer in paired samples, is used to positively diagnose BVDV exposures. Acute samples should be frozen and submitted at the same time as the convalescent sample to avoid differences due to laboratory techniques.[121]

BVDV ELISA antibody testing has been developed but due to the extensive purification and preparation of the antigen that is required, it has not gained widespread use.[121]

1.7 Prevention and Control

In order to limit the risk of a BVDV infection occurring, a control program must contain two components. The first is to limit exposure of cattle to the BVDV virus. The second, and most controversial, involves implementation of a BVDV vaccination program.

1.7.1 Biosecurity

Many BVDV problems have arisen due to poor biosecurity on the farm. Most procedures that can be instituted are not unique to BVDV, but should be considered for general disease control. Direct contact with infected animals is the most important method of transmission.[13] Aerosol transmission is not an effective means of BVDV transmission.[119,134] Isolation of new arrivals or cattle returning from shows for two weeks will decrease the likelihood of an acutely infected animal shedding the virus to the rest of the herd. Allowing only employees to facilities where animals are housed will also help, although mechanical transmission

is considered low in BVDV infections. Also, changing of needles between animals and maintaining fly control may help in limiting transmission. Transmission in this manner has been shown, but again, it is one that is less likely.[135,136]

PI cattle are the most difficult to detect visually and often are the most important control point in BVDV biosecurity. It is thought that the most common sources of BVDV spread are the PI cattle and these animals are often outwardly normal in appearance.[115] PI cattle will shed large amounts of BVDV in all of their secretions.[115] Isolation of these animals for several weeks will not have any impact on their shed of the virus. To limit risk from persistently infected animals, a virus isolation test can be performed on any new additions to the herd, including breeding bulls. The animals should be kept in quarantine while awaiting the results of the tests. If positive results are obtained, the animals can be culled or retested to confirm the PI diagnosis before culling the suspect animals.

1.7 2 Vaccination

Confusion exists regarding vaccination programs because of the increasing number of BVDV problem-herds diagnosed and the myriad of BVDV vaccines available. The knowledge concerning the ability of different vaccines to protect against BVDV infection is increasing rapidly. Currently, all commercially available modified live and most inactivated BVDV vaccines contain type I isolates. Recent studies have shown that VN antibodies stimulated by both inactivated and modified live BVDV vaccines can neutralize a wide range of type I and type II BVDV isolates.[40,50] The duration of immunity afforded by the inactivated vaccines is

dependent on the antigenic similarity between the vaccine isolate and the wild virus to which the cow is exposed. The duration of cross neutralization among isolates with only a few common proteins is less than a year.[50] Natural exposure can stimulate neutralizing antibodies that cross-neutralize antigenically dissimilar isolates for long periods of time.[106]

Clinical reports have shown the ability of both MLV and inactivated vaccines to prevent acute disease.[95] Studies are being performed now to show the ability of the vaccines to stimulate protection against acute infection. Recent work has shown that a modified live BVDV vaccine can protect young calves against a virulent type II BVDV challenge.[84] Calves with high maternal antibody were also protected from a type II challenge.[79] Vaccination of calves with maternal antibody is a source of debate. Studies have demonstrated the ability of MLV vaccines to stimulate immune responses when maternal BVDV VN antibody levels were below 1:32.[137,138] Nothing can currently be done to prevent mucosal disease or chronic BVDV with vaccination or management except to minimize exposure.

Protection of the fetus is difficult to obtain through vaccination. It takes few viral particles to cross the placenta and cause fetal infection.[139] Several reproductive BVDV studies have been performed. Most have used inactivated BVDV vaccines although one has used a combination of an inactivated followed by a modified live BVDV vaccine. Birth of PI calves was the outcome assessed and the level of protection was variable by study.[140,141,142,143]

There are advantages and disadvantages to both the inactivated and modified live vaccines. Advantages of inactivated vaccines include; no possibility of reversion to virulence or genetic recombinations; no contamination with adventitious agents; no shedding of virus to contact animals; no induction of immune suppression; or mucosal disease unless inactivation is incomplete. Disadvantages include: greater cost; two initial doses required; longer time until peak immunity is achieved; shorter duration of immunity; and potential adverse reactions.

Advantages and disadvantages of modified live BVDV vaccines are the opposite of those indicated for inactivated vaccines. There are two specific concerns with the use of modified live vaccines. Several studies have shown that some modified live BVDV vaccines have caused transient immune suppression. Current vaccines must show that they do not cause any immune suppression as determined by decreases in white blood cell counts or decreased humoral responses to other antigens. The second concern is that a modified live vaccine, when administered to a persistently infected calf, may cause mucosal disease. Although this effect has not been recreated experimentally, it has been reported occasionally following vaccination and would eleiminate the persistently infected animal.

When designing a vaccination program for BVDV, several factors must be considered: the age of the animal; syndrome to be protected against; time until expected exposure; BVDV immune status of the herd; and management of the

herd. One of the more common vaccination programs includes starting young stock with modified live vaccines, then switching to inactivated vaccines in adults.[144] Regardless of which vaccine is used, the label should be followed to obtain maximum benefits from the vaccine used.

1.7.3 Herd BVDV Eradication

One option that is being used with increased frequency is herd BVDV eradication. In small herds, closed herds, and/or herds selling breeding stock, a program of virus isolation, culling and annual vaccination is an attractive option for handling BVDV problems. Eradication can be accomplished via two different options: testing the entire herd; or testing the young stock followed by subsequent testing of any cattle related to the PI calves. In herd eradication programs, it is important to continue testing for PI animals in newborn calves for eight months after instituting the program to find any PI calves that were *in utero* when the program was started. As cowside or in clinic tests become available, this option may gain even more popularity.[145]

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2. ASSESSMENT OF TRANSMISSION OF BOVINE VIRAL DIARRHEA VIRUS (BVDV) FOLLOWING ADMINISTRATION OF ATTENUATED VACCINES TO BVDV SERO-NEGATIVE CATTLE

2.1 Abstract

Two studies were conducted to determine if bovine viral diarrhea virus (BVDV) of vaccine origin was transmitted to co-mingled cattle from cattle vaccinated with two different modified live BVDV vaccines that contained the National Animal Disease Laboratory strain (NADL) of BVDV. In study one, ten 500 pound calves were immunosuppressed with dexamethasone for five days. The calves were vaccinated with one of two different vaccines containing a modified live BVDV NADL fraction on the third day of dexamethasone treatment. Nasal swabs and whole blood samples were obtained from 10 vaccinated, 11 co-mingled non-vaccinated calves and 4 non-vaccinated pregnant cows on a weekly basis for virus isolation.

In a second study, 18 BVDV sero-negative adult Angus cows were vaccinated via intramuscular injection with a modified live BVDV (NADL strain) vaccine and co-mingled with 5 sentinel sero-negative cows. Serum was collected from the cows 105 days prior to vaccination, on the day of vaccination, and six weeks, twelve weeks and six months after vaccination. Virus neutralizing antibody tests were

performed using the NADL strain of BVDV as the reference strain.

During the study period, there was no evidence of significant viral transmission by the vaccinated animals to the co-mingled controls. The control co-mingled pregnant cows had no evidence of transmission and gave birth to normal calves that were free of BVDV.

2.2 Introduction

Vaccination with modified live bovine viral diarrhea virus (BVDV) and bovine herpesvirus 1 (BHV-1) vaccines of cattle in close proximity or co-mingled with pregnant herd mates is a common management practice in many cow-calf and dairy facilities. This practice has raised the question of the risk of viral transmission of the BVDV and BHV-1 vaccine virus and the potential for adverse effects on pregnancy if such transmission occurs.[1] The recent outbreaks of severe acute BVDV infections in adult animals have increased the usage of modified live vaccines in situations that would allow contact between recently vaccinated and pregnant animals.

The lack of BHV-1 transmission following modified live vaccination in immunosuppressed animals has been communicated.[2] However, transmission of the BVDV fraction of these modified live vaccines has not been examined. The present studies were designed to increase the likelihood for post vaccination transmission by utilizing BVDV sero-negative animals. In study one, cattle were immunosuppressed for 2 days prior to, and 3 days after vaccination, and the

amount of virus transmission was determined in contact animals for 30 days after vaccination. In a second study, cattle were monitored for 6 months after vaccination to determine if antibody responses would occur after an extended period in the contact animals.

2.3 Materials and Methods

2.3.1 Animals and Housing

Study I--The study group consisted of twenty-one (21) 500-600 pound crossbred beef calves. Four cows, pregnant for 40-60 days, were also included in the study group. Twenty calves were sero-negative for BVDV specific neutralizing antibodies (1:5 or less) and BVDV negative by virus isolation at the beginning of the study. Three of the four cows were both virus free and sero-negative at the beginning of the study. One cow and one calf were sero-positive (1:10 and 1:20 respectively) but were BVDV isolation negative at the beginning of the study. The animals were divided into 2 groups. Group 1 consisted of 10 sero-negative calves and 2 cows. Group 2 consisted of 10 sero-negative calves, 1 sero-positive calf and 2 cows. Each group was housed in a separate, isolated group pen of equal size. Each pen had only 1 waterer and a common feed bunk to ensure high contact among the group.

Study II--The study group consisted of 23 mature purebred Angus cows (3-8 years old). The animals were housed in an area where there were no other farms for

several miles and the herd had no additions for at least six years except for breeding bull that was purchased three years previous to initiation of the study. The animals were maintained on approximately 20-acre pastures that were rotational grazed and fed a supplement of hay. They shared a common water trough and trace mineral salt blocks. The herd had not received any vaccines in the past six years and there was no history of BVDV vaccination in the herd at any time.

2.3.2 Study Design

Study I--Prior to the initiation of the study, the calves and cows were tested to ensure that cattle were BVDV isolation negative. In addition, the concentration of BVDV neutralizing (VN) antibody was determined. The cows were confirmed to be pregnant by rectal palpation. For the first 5 days of the trial, 5 of the calves in each group were given an immunosuppressive dose of dexamethasone (40 mg/animal) intramuscularly in a single injection per day (days -2, -1, 0, 1, 2). On day 0 (day three of dexamethasone treatment) of the study the 5 dexamethasone treated calves in group 1 were given vaccine 1[a] via intramuscular injection containing modified live BVDV and the 5 dexamethasone treated calves in group 2 were vaccinated in the muscle with vaccine 2[b] also containing a modified live BVDV. Both vaccines contained the NADL strain of BVDV. The NADL strain is

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[a] Bovishield 4® Pfizer Animal Health modified live virus vaccine containing BVDV, IBRV, PI3V, and BRSV.

a "type I", cytopathic strain of BVDV. The viability of BVDV virus in the vaccines was confirmed on the day of administration by virus isolation following inoculation of cell cultures with reconstituted vaccine. The cultures were monitored for cytopathic effect typical of BVDV infection.

On days 0, 7, 14, 21 and 28 after vaccination, blood samples were obtained for BVDV isolation and VN antibody assessment. Nasal swabs were also collected from all cattle on the same days for BVDV isolation. The pregnant cows were palpated at the end of the trial (day 30) to ensure fetal viability and that normal growth had occurred. These cows were isolated from other cattle until parturition, at which time the calves were tested for exposure to BVDV by virus isolation from buffy coat samples and by presence of precolostral BVDV VN antibody. All cattle were monitored daily throughout the trial for any signs of disease.

Study II--The cows were tested twice before the administration of the vaccine for pre-existing BVDV VN antibody titers[3] and virus isolation from buffy coat samples[4] using standard laboratory procedures.[c] Prior to the day of vaccination, numbers 1-23 were put into a hat and 5 were randomly drawn. These numbers indicated which animals through the chute would be the control-unvaccinated

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[b] Resvac 4®/Somubac™ Pfizer Animal Health modified live virus vaccine and bacterin containing BVD, IBR, PI3 and BRSV and *Hemophilus somnus* bacterin

cattle. For example, if the number 5 were drawn, the fifth cow through the chute was left as a control. The cattle were vaccinated using a combination modified live viral vaccine that contained the NADL strain of BVDV[d] and a separate five serovar Leptospira vaccine.[e] Serum was drawn 6 weeks, 3 months and 6 months after vaccination for determination of BVDV VN antibodies. A rise in the VN titer of four fold magnitude or greater was considered indicative of a recent exposure (sero-conversion).[5]

2.3.3 Laboratory Techniques

Study I--Serum was tested to determine VN antibody concentrations (using NADL BVDV as the reference BVDV strain) using published techniques.[5,6] Virus isolation was attempted from white blood cells and nasal swabs using bovine turbinate cell cultures and a microtiter plate isolation technique.[7] Bovine turbinate cells were used for the incubation of the samples. Cultures were examined daily for 5 days for evidence of cytopathic effect (CPE) for 2 passages. Virus isolation, as determined by CPE, was confirmed by immunoperoxidase staining, using an anti-BVDV monoclonal antibody followed by a peroxidase labeled goat anti-mouse IgG antibody.[8,9]

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[c] Initial BVDV titer and isolation testing performed by The Animal Disease Research and Diagnostic laboratory, South Dakota State University

[d] Resvac 4® Pfizer Animal Health modified live virus vaccine and bacterin containing BVD, IBR, PI3 and BRSV

Study II--Initial screening of the serum prior to vaccination for BVDV VN antibodies utilized the Singer strain of BVDV (cytopathic, "type I") as the reference strain. Virus isolation was performed in duplicate at each of the first two time points to determine the presence of any persistently infected cattle. After vaccination, BVDV VN antibody concentrations were assessed using the NADL strain of BVDV. Since the reference strain was the same strain present in the vaccine, it increased the chance of detecting exposure of the controls.[f]

2.4 Results

Study I--The results of the virus isolation and VN antibody tests are shown in Table 2.1 and Table 2.2 (The first five calves in each table were the dexamethasone treated, vaccinated calves). During the study period, BVDV was not isolated from nasal swabs obtained from any of the contact control cattle. BVDV was isolated from the serum and white blood cell samples from 2 of the vaccinated calves on day 7, one from each group. All vaccinated calves except one (calf 1, three fold) had a minimum of four fold increases in BVDV VN antibody levels following vaccination. In contrast, by day 28 of the study, there was no sero-conversion in 11/11 of the non-vaccinated calves (Table 2.1). There were no abortions or other apparent adverse effects in the sentinel cattle. Calves born from the pregnant cows were BVD virus isolation negative and VN antibody negative at birth.

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[e] Leptoferm-5® Pfizer animal Health containing *Leptospira interrogans* serovars cannicola, pomona, hardjo, grippotyphosa and icterohemorrhagiae

One calf (dexamathasone treated) from group 2 died on day 5 of the trial. Necropsy and histopathology results indicated that death was due to peritonitis secondary to a perforating abomasal ulcer. No other calves showed any signs of disease or adverse reactions due to the administration of the vaccines while they were dexamethasone suppressed.

Study II--Two of the twenty-three cattle had low concentrations of BVDV specific antibodies when serum was tested with a reference Singer strain (Table 2.3). Four samples, from the first sampling date, arrived frozen and hemolyzed due to the low ambient temperature on the day of collection. Virus neutralizing antibody tests could not be performed on these samples. The herd was virus isolation negative following testing of samples collected on 2 separate days. BVDV VN antibody concentrations suggested no recent exposure to the virus (>90% sero-negative). One cow became unmanageable after the third sampling and was not sampled after this time. All vaccinated cattle sero-converted after vaccination (4 fold or above) except one (2Y-8OR) that had no sero-conversion. The majority of cows had eight fold or greater increases in VN antibody concentrations. None of the contact cows had any detectable BVDV antibody during the six-month period after vaccination of their herd mates (Table 2.3).

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[f] National Animal Disease Center, USDA, Ames, Iowa

Table 2.1 Virus Isolation and BVDV Neutralizing Antibody Results from Study I, Group 1

Animal #	Day 0*	Day 7	Day 14	Day 21	Day 28
1**	1:5 VI-	1:5 VI-	1:5 VI-	1:10 VI-	1:20 VI-
2**	<1:5 VI-	<1:5 VI-	1:5 VI-	1:80 VI-	1:160 VI-
3**	<1:5 VI-	<1:5 VI+	<1:5 VI-	≥1:40 VI-	≥1:40 VI-
4**	1:5 VI-	<1:5 VI-	<1:5 VI-	≥1:160 VI-	≥1:160 VI-
5**	<1:5 VI-	<1:5 VI-	<1:5 VI-	1:5 VI-	1:80 VI-
6	<1:5 VI-	<1:5 VI-	<1:5 VI-	1:5 VI-	1:10 VI-
7	<1:5 VI-	<1:5 VI-	1:5 VI-	<1:5 VI-	<1:5 VI-
8	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-
9	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-
10	1:20 VI-	1:10 VI-	1:10 VI-	1:5 VI-	1:5 VI-
Preg 1	1:10 VI-	1:40 VI-	1:40 VI-	1:40 VI-	1:40 VI-
Preg 2	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-

Results from group 1 calves on 0, 7, 14, 21, and 28 days after vaccination. Calf #3 was BVDV virus isolation positive using both buffy coat and serum samples on day 7 after vaccination. All nasal swabs were BVDV virus isolation negative. * day 0 is the day of vaccination and day three of dexamethasone administration. ** Calves 1-5 are the vaccinates

Table 2.2
Virus Isolation and BVDV Neutralizing Antibody Results from Study I, Group 2

Animal #	Day 0*	Day 7	Day 14	Day 21	Day 28
11**	<1:5 VI-	<1:5 VI-	<1:5 VI-	1:10 VI-	1:80 VI-
12**	<1:5 VI-	died day 5			
13**	<1:5 VI-	<1:5 VI+	1:20 VI-	1:80 VI-	1:320 VI-
14**	<1:5 VI-	<1:5 VI-	<1:5 VI-	1:20 VI-	1:80 VI-
15**	<1:5 VI-	<1:5 VI-	<1:5 VI-	1:10 VI-	1:40 VI-
16	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-
17	1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-
18	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-
19	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-
20	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-
21	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-
Preg 3	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-
Preg 4	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-	<1:5 VI-

Results from group 2 calves on 0, 7, 14, 21, and 28 days after vaccination. Calf #13 was BVDV virus isolation positive using both buffy coat and serum samples on day 7 after vaccination. All nasal swabs were BVDV virus isolation negative.
* day 0 is the day of vaccination and day three of dexamethasone administration.
** Calves 11-15 are the vaccinates

Table 2.3 Virus Isolation and BVDV Neutralizing Antibody Results from Study II

Animal #	Day –100	Day 0*	Day 42	Day 90	Day 180
24R 8Y	0 VI-	0 VI-	0	0	0
3R 2Y	NA*** VI-	0 VI-	0	0	0
23R 13Y	0 VI-	0 VI-	0	^	^
43OR 15Y	0 VI-	0 VI-	0	0	0
6R 5Y	0 VI-	0 VI-	0	0	0
8OR 2Y** †	0 VI-	0 VI-	0	2	0
11R 4Y**	0 VI-	0 VI-	512	128	256
21R 23Y**	64 VI-	16 VI-	>4096	256	64
21Y 1OR**	0 VI-	0 VI-	1024	256	256
14R 4Y**	0 NA	0 NA	256	256	128
7Y 1Y**	0 VI-	0 VI-	256	128	256
5R 48OR**	0 VI-	0 VI-	512	256	512
9R 23Y**	NA VI-	0 VI-	2048	512	512
1R 24Y**	NA VI-	16 VI-	>4096	512	512
4R 18Y**	0 VI-	0 VI-	256	256	128
12R 47OR**	0 VI-	0 VI-	1024	128	256
13R 38OR**	NA VI-	0 VI-	1024	128	256
25R 6Y**	0 VI-	0 VI-	1024	256	256
20Y 21Y**	0 VI-	0 VI-	128	128	64
10R 21Y**	0 VI-	0 VI-	1024	256	256
41R 2R**	0 NA	0 VI-	1024	256	512
3 y 11Y**	0 NA	0 VI-	1024	512	1024
7R 5Y**	0 VI-	0 VI-	1024	512	512

Results from Study 2 cattle on -100, 0, 42, 90 and 180 days after vaccination.
BVDV virus isolation was performed on buffy coat.
*day 0 is the day of vaccination.
** Cows that were vaccinated.
*** Not available is due to hemolysis from samples freezing on the day of collection.
^ Cow became unmanageable and was not available for testing.

2.5 Discussion

The increased usage of modified live BVDV vaccines in high-risk herds has raised the question of potential transmission of BVDV from vaccinated cattle to pregnant herd mates. A recent USDA bulletin has alluded to the potential risk of transmission of the modified live BVD vaccines[1] and recommended against using these vaccines in animals if they are in contact with pregnant cattle. However, vaccination of cattle in direct contact with pregnant animals is not a contraindication of label instructions. The suggestion to use a modified live BVDV vaccine at least once after six months of age[10] has also caused concern since vaccinated cattle are often in close proximity to pregnant cows. This is particularly true in dairy herds where the vaccinated cows or heifers are commonly housed in the same barn with pregnant cows.

These studies were designed to detect BVDV transmission to susceptible contact animals from the vaccinates as determined by sero-conversion and/or abortion. The possibility of viral transmission was increased in three ways. In Study I, the number of calves in each group was sufficient to have detected viral shed by approximately 10% or more of the vaccinated calves. In order to increase the likelihood of these calves shedding virus, vaccinated calves did not have BVD virus neutralizing antibodies. Furthermore, these cattle were immunosuppressed during the vaccination period with high doses of dexamethasone. Dexamethasone is a potent adrenocorticosteroid[11] and its immunosuppressive effects are well known.[12] Corticosteroids are known to cause and exacerbate gastric ulcers and

may have been responsible for the abomasal ulcer and death in one of the calves.[11] This study assumed that dexamethasone suppression would not adversely affect BVDV replication and transmission in the vaccinated cattle since dexamethasone can enhance the virulence of BVDV field isolates.[13] Furthermore, an earlier study suggested that ACTH and subsequent cortisol release caused higher replication of modified live BVDV vaccines.[14] In addition, in Study II, cattle were monitored for 6 months after vaccination. The long duration increased the likelihood of detecting a low amount of viral transmission and provided a long period to detect sero-conversion. The high ratio of vaccinates to non-vaccinates also increased the likelihood of contact and potential transmission of the virus.

Two vaccinated calves were found BVDV positive by isolation of the virus from serum and buffy coats on day 7 after vaccination. This confirms previous studies documenting that modified live BVDV vaccines can replicate and cause a viremia in sero-negative animals.[15] It is also an indication that the vaccines were handled correctly and contained viable virus. The majority of vaccinated animals were virus isolation negative, it is possible that many of the days, when vaccinated animals were viremic, the virus was not detected since virus isolation was not performed daily. Previous studies indicate that field isolates of BVDV are transmitted in nasal secretions for only two weeks after a primary infection.[16] The lack of viral isolation from the nasal swabs is evidence that BVDV of vaccine origin did not replicate in or was not shed into the nasal passages. Transmission of

wild-type BVDV from nasal secretions has been shown[17] and is thought to be an important mechanism of horizontal transfer.

None of the vaccinated calves (other than the calf that died from an abomasal ulcer) showed any adverse effects following vaccination concurrent with the severe iatrogenic stress of corticosteroid administration. This supports previous findings that stressed calves that received multiple doses of vaccine 1 were not adversely affected.[18]

Due to the lack of precision of the VN test, sero-conversion of a four-fold magnitude or greater is generally considered indicative of a recent exposure. Some authors state that an eight fold increase in antibody concentration is needed to determine that a BVDV infection and subsequent sero-conversion has occurred.[19] None of the unvaccinated control cattle sero-converted by either criteria increase in titer and only three showed any increase in antibody concentration after vaccination occurred. In contrast 24/27 vaccinated cattle sero-converted after the modified live vaccine was administered. The lack of sero-conversion in the unvaccinated co-mingled cattle, using the standard of a four fold increase, indicated either there was a lack of viral transmission or a level of transmission insufficient to cause infection and subsequent sero-conversion in the control cattle. None of the 4 exposed pregnant cows in study I aborted, had embryonic deaths, had mummified calves or gave birth to BVDV persistently infected calves. The calves were also born seronegaive to BVDV. In study II, all cattle were pregnant at the fall herd check, approximately 5 months after

vaccination. One "vaccinated" cow from study II did not sero-convert. Either she was inadvertently not vaccinated, or failed to respond to vaccination during the test period.

This study shows that in BVDV sero-negative animals there is little transmission of BVDV from animals vaccinated with one of two different modified live vaccines, both containing the cytopathic NADL strain of the virus. These results suggest that the risk of vaccinating animals in close proximity to pregnant animals is low. Vaccination programs that include these two modified live BVDV vaccines in this situation is unlikely to cause any problems attributable to the transmission of BVD vaccine virus.

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3. SPECIFICITY OF NEUTRALIZING ANTIBODIES INDUCED IN HEALTHY CATTLE BY ATTENUATED BVDV VACCINATION FOR 18 MONTHS FOLLOWING VACCINATION

3.1 Abstract

The objective of this study was to determine if the antibodies stimulated by a single dose of a modified live BVDV vaccine in sero-negative cattle persists for 18 months after vaccination. Twenty-three mature registered Angus cows, BVDV-virus and viral neutralization antibody negative, were randomly assigned to either control (unvaccinated) or test (vaccinated) groups.

Eighteen BVDV sero-negative adult Angus cattle were vaccinated via intramuscular injection with a modified live BVDV (NADL strain) vaccine and co-mingled with 5 sentinel sero-negative cows. Serum was collected from the cows prior to vaccination, on the day of vaccination, and six weeks, three months, six months, nine months, twelve months, and eighteen months after vaccination. Virus neutralizing (VN) antibody tests were performed at each time point with a panel of 12 strains of BVDV. Based on reactivity with monoclonal antibodies, with the exception of the NADL strain, these strains were identified as heterologous to the vaccine strain. Vaccinated cattle demonstrated viral neutralizing antibody against all strains. Antibodies were detected for 18 months after vaccination although

the titer of antibody to some of the strains was low. Unvaccinated cattle remained sero-negative throughout the study.

These data suggest that a modified live BVDV vaccine (NADL strain derived) can stimulate an antibody response in sero-negative cows that is detectable by VN antibody eighteen months after vaccination and that these antibodies were able to neutralize 11 antigenically diverse strains of BVDV.

3.2 Introduction

The first modified live BVDV vaccines were licensed in the United States in 1959 and contained a cytopathic strain.[1] The currently licensed modified live vaccines contain one of three type I cytopathic strains: NADL; Singer; or Oregon C24V.[2] Other than licensing data, there has been little recently published research on these vaccines. In the past several years, BVDV infection has resurfaced as a major problem for the cattle industry in North America. High morbidity and mortality have been associated with recent outbreaks of acute BVDV induced disease.[3,4,5] This has resulted in an increased awareness of acute BVDV infections and more diagnoses of BVDV infections in cattle with various clinical syndromes.[6,7]

Currently, BVDV is classified as a pestivirus in the Flaviviridae family. The virus contains a single strand of positive sense RNA.[8] It is well recognized that there are many strains of BVDV.[9] There are two biotypes of BVDV, cytopathic and noncytopathic.[10] There is also considerable antigenic variability of

the surface proteins of different BVDV isolates; therefore, BVDV isolates can also be classified phenotypically by reactivity with monoclonal antibodies.[11,12]

The designation of BVDV as type I or type II is based on genotypic differences in the 5' untranslated region of the viral genome.[9] There is approximately 60% homology between the sequences in this region of type I and type II isolates of BVDV.[9] Earlier studies have shown that the antibodies produced by cattle in response to vaccination with type I isolates can cross neutralize genetically and antigenically disparate BVDV isolates *in vitro*.[13,14] The duration of cross neutralizing antibodies induced following inactivated vaccines may be as short as four months.[15] Infection with wild virus stimulates production of antibodies which remain at high levels for over a year,[16] however, the duration of neutralizing antibodies stimulated by modified live vaccines has not been determined. The purpose of this study was to determine the titer of cross neutralizing antibodies stimulated by a single vaccination of a modified live vaccine and to follow antibody levels for 18 months after vaccination.

3.3 Materials and Methods

The study group consisted of 23 mature purebred Angus cows (3-8 years old). Each of the cows had an identification tag placed in each ear and both numbers were recorded. If a tag was lost between sampling dates, it was replaced with a new tag and the changes in the identification number were recorded to maintain individual identification. In this manner, it was possible to ensure

individual identification since there was always at least one tag in every cow each test date. The cattle were kept in an area where there were no other farms for several miles. There were no additions to the herd for at least six years prior to this study except for a breeding bull that was purchased three years before the study began. The cattle were maintained on approximately twenty-acre pastures that were rotationally grazed. They were supplemented with hay and trace mineral salt blocks. The cattle shared a common water trough. The herd had not been vaccinated in the past six years and there was no history of BVDV vaccination in the herd at any time.

3.4 Study Design

The cows were tested twice before the administration of the vaccine for neutralizing antibody against BVDV (VN) and for persistent infection using standard laboratory procedures.[a] The five nonvaccinated control cattle were randomly selected by placing numbers (1-23) into a hat and drawing five. These numbers indicated which animals through the chute would be the control unvaccinated cattle. The cattle were vaccinated using a four component, modified live viral vaccine that contained the NADL strain of BVDV[b]. They were also

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[a] Initial BVDV titer and isolation testing performed by the Animal Disease Research and Diagnostic laboratory, South Dakota State University

[b] Resvac 4®, Pfizer Animal Health®, modified live virus vaccine containing BVDV, IBRV, PI3V and BRSV.

administered a separate five serovar, Leptospira vaccine.[c] Sera were obtained 6 weeks, 3 months, 6 months, 9 months, 12 months, and 18 months after vaccination to determine the BVDV neutralizing antibody titers. The titers of neutralizing antibodies were determined against the following strains of BVDV: Zim; Short; 28508; 125-C; 5912-C; TGAC; NADL; 16425-C; 296-C; 249; 890; and 312 (table 3.1). These BVDV strains were chosen because results of binding studies with 55 monoclonal antibodies indicated they were antigenically different from the vaccine strain.[11,12]

Initial testing of the serum prior to vaccination for viral neutralizing antibodies utilized the Singer strain of BVDV (cytopathic, "type I") as the reference strain. Virus isolation was performed in duplicate at 90 days prior to vaccination and on the day of vaccination to determine if persistent infection with BVDV was present in any of the cattle.[16]

Sera samples were tested using a single row of two-fold dilutions starting at 1:2 and endpointed at $\geq$ 1:4096. Whenever a VN titer was below 1:16, the VN test was repeated five times and the mean titer of the five was recorded. Samples were obtained, centrifuged and maintained cooled until testing was done. All samples for a given date were tested together.

3.5 RESULTS

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[c] Leptoferm 5®, Pfizer Animal Health, containing *Leptospira interrogans* serovars cannicola, pomona, hardjo, grippotyphosa and icterohemorrhagiae

Three cattle had low titers of BVDV specific antibodies (against the Singer strain of BVDV) before vaccination (table 3.2). The remaining cows were sero-negative. No cattle persistently infected with BVDV were identified by virus isolation. One control cow became unmanageable after the third sampling and was not tested after this time and one vaccinated cow died between the 12 month and 18-month sampling dates. All vaccinated cattle sero-converted after vaccination (4 fold or above), except one animal (2Y-8R) in which there was no evidence for sero- conversion to the vaccine strain (NADL) (Figure 3.1). The majority of cows had eight fold or greater increases in VN antibody titers. None of the unvaccinated cows had antibody titers (table 3.2, shown as geometric mean titers). Antibodies from all vaccinated cows, with the exception of 2Y-8OR, neutralized all twelve BVDV strains, at varying levels, for 18 months after vaccination (table 3.3).

3.6 Discussion

The increased usage of modified live BVD vaccines in high risk herds has raised the question of the duration of the persistence of neutralizing antibodies following the use of these vaccines. The fact that many cattle are revaccinated after the maximum one year interval makes this answer of practical importance. Although the cattle in this study were not challenged with BVDV, the titers of cross neutralizing antibodies detected in the sera at one year after vaccination

Table 3.1 Identification of BVDV Strains for which VN Titers were determined

Strain Identification	Cytopathic	Noncyto-pathic	Type I BVDV	Type II BVDV
Zim		X		X
Short		X		X
28508		X		X
125-C	X			X
5912-C	X			X
TGAC	X		X	
NADL	X		X	
16425-C	X			X
296-C	X			X
249		X		X
890		X		X
312		X	X	

The strains are identified by either type I or type II designation and whether they are cytopathic or noncytopathic in cell culture.

Table 3.2 Initial BVDV VN Titer and Virus Isolation Results

Cow Number	Group assignment*	VN Level Day -90	VN level day 0**	Virus isolation Day –90	Virus isolation day 0
24R, 8Y	Sentinel	Neg	Neg	Toxic	Neg
3R, 2Y	Sentinel	Neg	Neg	Neg	Neg
23R, 13Y	Sentinel	Neg	Neg	Neg	Neg
43R, 15Y	Sentinel	Neg	Neg	Neg	Neg
6R, 5Y	Sentinel	Neg	Neg	Neg	Neg
11R, 4Y	Vaccinate	Neg	Neg	Neg	Neg
21R, 23Y	Vaccinate	1:64	1:16	Neg	Neg
2R, 41R	Vaccinate	Neg	Neg	Toxic	Neg
14R, 4Y	Vaccinate	Neg	Neg	Toxic	Neg
7Y, 1Y	Vaccinate	Neg	Neg	Neg	Neg
8R, 2Y	Vaccinate	Toxic	Neg	Neg	Neg
9R, 23Y	Vaccinate	Toxic	Neg	Neg	Neg
1R, 24Y	Vaccinate	Toxic	1:16	Neg	Neg
4R, 18Y	Vaccinate	Neg	Neg	Neg	Neg
50R, 22Y	Vaccinate	Neg	Neg	Neg	Neg
12R, 47R	Vaccinate	Neg	Neg	Neg	Neg
13R, 38R	Vaccinate	Toxic	Neg	Neg	Neg
25R, 6Y	Vaccinate	Neg	Neg	Neg	Neg
20Y, 21Y	Vaccinate	Neg	Neg	Neg	Neg
10R, 21Y	Vaccinate	Neg	Neg	Neg	Neg
7R, 5Y	Vaccinate	Neg	Neg	Neg	Neg
3Y, 11Y	Vaccinate	Neg	Neg	Toxic	Neg
5R, 48R	Vaccinate	Neg	Neg	Neg	Neg

Initial BVDV VN titer and virus isolation results were determined twice before the vaccination administration at 90 days before vaccination and on the day of vaccination.

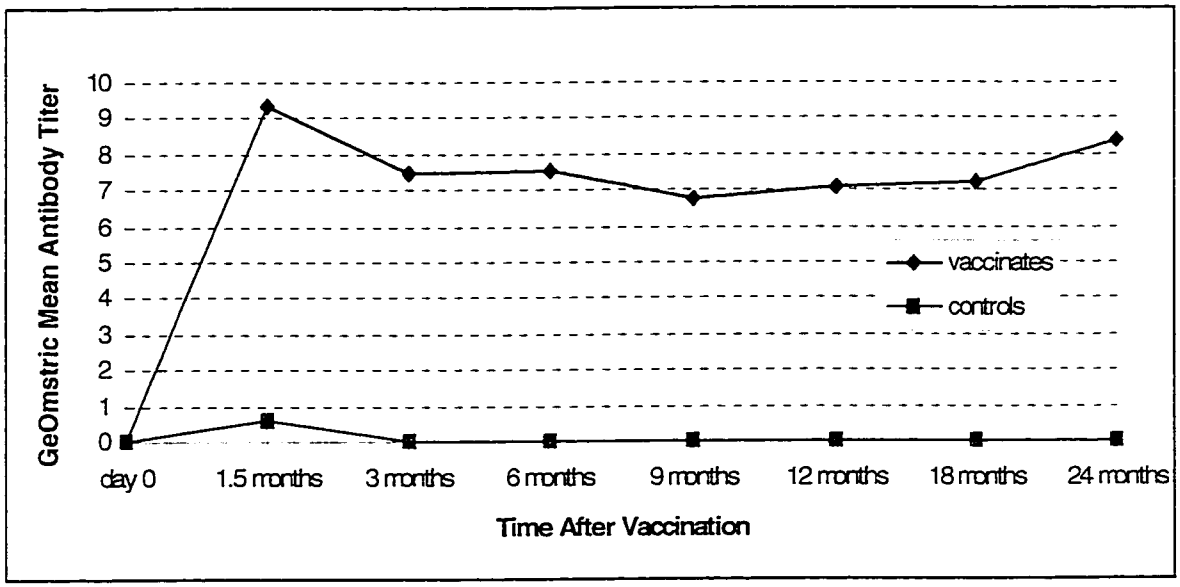

Figure 3.1 Log transformed geometric mean antibody titers to the NADL strain (in the vaccine) over the study period in both the control and vaccinated cattle are shown. The lack of anticipated antibody decay is seen.

Table 3.3 Log Transformed Geometric Mean/Range Antibody Titer after Vaccination

Strain	1.5 months	3 months	6 months	9 months	12 months	18 months
NADL*	9.3 (0-12)	7.5 (0-10)	7.6 (1-9)	6.7 (0-10)	7.1 (0-10)	7.2 (0-10)
Zimmer-man	2.8 (0-7)	4.6 (0-7)	3.1 (0-5)	3.6 (0-7)	4.7 (0-8)	5.7 (0-8)
Short	3.9 (0-7)	4.7 (0-7)	4.3 (0-6)	4.1 (0-7)	5.2 (0-7)	4.5 (3-9)
28508	4.7 (0-8)	5.1 (0-9)	3.9 (0-6)	4.3 (0-7)	5.1 (0-8)	4.7 (0-7)
125-C	4.1 (0-7)	3.6 (0-6)	3.8 (0-6)	3.8 (0-6)	4.7 (0-8)	5 (0-9)
5912-C	3.3 (0-6)	5.2 (0-9)	5.1 (0-7)	4.9 (0-9)	5.9 (0-10)	5.8 (0-9)
TGAC	4.7 (0-8)	6.5 (0-10)	7 (1-9)	6.6 (0-9)	6.7 (0-10)	4.9 (0-7)
16425-C	3.6 (0-7)	3.7 (0-6)	3.9 (0-6)	3.9 (0-8)	3.7 (0-8)	3.1 (0-7)
296-C	2.6 (0-6)	3 (0-5)	3.6 (0-6)	3 (0-5)	3.7 (0-8)	5.1 (0-9)
249	3.3 (0-6)	4.2 (0-7)	3.8 (0-6)	3.2 (0-7)	4.6 (0-9)	3.8 (0-7)
890	3.2 (0-7)	3.4 (0-7)	3.1 (0-5)	3.3 (0-6)	3.9 (0-9)	4.5 (0-8)
312	7.2 (0-11)	6.9 (0-10)	7.1 (1-9)	5.9 (0-8)	6.2 (0-10)	7.1 (0-10)

*contained in the vaccine

Log transformed geometric mean and the range of antibody titers to the various strains over the study period are shown. The cow that apparently did not respond to vaccination is included with the vaccinated cattle.

suggests some degree of protection. Protective antibody titers persisted in many of these cows for eighteen months after vaccination. The presumption of the magnitude required for protection is based on evidence that similar titers of passively acquired antibodies against BVDV are protective.[17] Similarly, the vaccine used in this study conferred protection against a severe type II BVDV challenge in animals in which it induced only low concentrations of serum antibodies.[17] Due to lack of precision of the VN test, sero-conversion of a four fold magnitude or greater is generally considered indicative of a recent exposure.[18] None of the control cattle had detectable antibodies against the vaccination strain and only low (less than 1:16) and there was no consistency among the titers to different isolates. The lack of sero-conversion in the unvaccinated co-mingled cattle indicated there was an absence of viral shedding from vaccinates or natural exposure to BVDV virus; and/or that the level of exposure, which occurred, was insufficient to cause infection and subsequent sero-conversion.

All cattle were pregnant at the fall herd check (approximately 6 months after vaccination). One cow in the vaccinated group did not sero-convert; the most likely explanation is that the cow did not receive the vaccination. However, this animal may have failed to respond to BVDV antigens and therefore was included in the vaccine group for all analysis.

This study supports early work that has shown that both modified live and inactivated BVDV type I vaccines stimulate production of VN antibodies that can neutralize various, antigenically heterologous, BVDV isolates including Type II

isolates. This study extends the previous findings and demonstrates that these antibodies remain fairly constant over many months. The half life of IgG in cattle is 21 days.[19] Since there was no evidence of natural exposure to BVDV during the study period, the persistence of the BVDV antibody may be due to continuing presence of BVDV antigens associated with antigen presenting cells in lymphoid tissues. The persistence of antigen in lymphoid tissue has been proposed as with other viruses.[20] Alternatively, any immune system stimulation may cause renewed prolonged production of BVDV VN antibodies. The prolonged elevation of antibody suggests that protection would be expected in excess of 12 months in contrast to earlier work in which cross neutralizing VN antibodies following inactivated BVDV vaccines lasted only four months. The concentration of some of the cross neutralizing antibodies in individual animals began to drop below 1:16 between the 1 year to 18 month sampling times. From previous studies, levels less than 1/16 may indicate lack of protection against a BVDV infection.[16]

These data suggest that modified live BVDV vaccines can stimulate a VN antibody response in sero-negative cows that is detectable eighteen months after vaccination. Although this study did not involve a challenge, the magnitude of the titers detected suggests that protection against clinical BVD caused by the various strains of BVDV may last until at least a year after a single vaccination with a modified live, NADL BVDV vaccine.

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4. PROTECTION AGAINST TYPE I BOVINE VIRAL DIARRHEA VIRUS FETAL CHALLENGE BY A TYPE I MODIFIED LIVE BVDV VACCINE

4.1 Abstract

This study was performed to determine the efficacy of a vaccine containing modified live bovine virus diarrhea (BVDV) type I in protecting cows from *in utero* infection and disease of the fetus with an isolate of a virulent heterologous BVDV type I isolate.

Eighteen BVDV sero-negative and virus isolation negative, yearling beef heifers were selected for this study. Cattle were randomly assigned to either control (unvaccinated, no = 6) or BVDV modified live vaccinated (no = 12) groups. These 12 heifers were administered a vaccine containing a modified live BVDV comprised of a type I cytopathic (NADL) strain. All 18 heifers were naturally bred and then challenged, intranasally, between 70-75 days of gestation, with a BVDV type I isolate. The pregnancies were monitored and the persistent infection status of the offspring was determined after calving. Antibody levels of the vaccinated and control heifers were also monitored.. All six of the calves born from the control heifers had BVDV isolated on multiple occasions and were diagnosed as persistently infected. In comparison, only 2 of the calves

from 12 vaccinated cows were virus isolation positive and determined to be persistently infected. One vaccinated cow aborted but the fetus was determined not to be persistently infected and there was no evidence that the abortion was due to BVDV infection.

These data indicate that a single dose of a modified live, NADL-derived, BVDV vaccine will confer about 83% fetal protection against a heterologous BVDV challenge.

4.2 Introduction

The prevention and control of bovine viral diarrhea virus (BVDV) centers around the elimination of persistently infected cattle. The identification and removal of persistently infected animals and continued vaccination to prevent fetal infection and subsequent persistent infection are the basis for effective control measures. Persistent infections occur following *in utero* infection of the fetus up to approximately 125 days of gestation with a noncytopathic strain of BVDV.[1] The mechanism of transplacental transfer of BVDV is unknown, however, small amounts of virus in the bloodstream of the dam appear sufficient to cross the placenta resulting in fetal immunotolerant to BVDV.[2] Protection of the dam from clinical BVDV may or may not correlate with protection of the fetus from persistent infection if viremia of the dam occurs. In order to break the cycle of *in utero* infection and persistent infection, it is essential that vaccination provide fetal protection.

Several studies have been performed to assess the ability of vaccines to protect the fetus against either a natural or experimental challenge. When analyzed, the majority of inactivated vaccines failed to provide adequate fetal protection[3,4,5,6,7] with the exception of one newly licensed vaccine.[8] With the latter vaccine, the lack of virus isolation from offspring of vaccinated animals suggested good protection but the challenge of unvaccinated animals only resulted in approximately 50% persistent infections. There is only one published report on the effectiveness of modified-live BVDV vaccines to protect the fetus. In that study, there appeared to be good protection but no controls were included for comparison and evaluation of the challenge.[9] To date, vaccines licensed in the United States have not been required to provide fetal protection. The purpose of this study was to determine the ability of a modified-live vaccine to stimulate responses that could protect the fetus from infection with a heterologous, type I, noncytopathic isolate of BVDV.

4.3 Materials and Methods

4.3.1 Animals

Thirty-two open heifers and cows were initially used in the study. Ten heifers were kept as unvaccinated controls. All animals were sero-negative to BVDV as determined by virus neutralization using using the noncytopathic BJ and cytopathic NADL strains of BVDV as reference strains. The cattle were also determined to be BVDV isolation negative.

4.3.2 Vaccine

Twenty-two animals were vaccinated with a modified-live vaccine[a] according to manufacturer's recommendations. The vaccine contained a cytopathic NADL BVDV vaccine strain. Vaccination was performed by administering a single 2 ml dose intramuscularly in the neck.

4.3.3 Breeding

Thirty days following vaccination, the heifers were given Lutalyse® and exposed to 10 bulls for a period of 7 days. The bulls were BVDV-negative by virus isolation and were seronegative for BVDV antibody. Animals were examined using ultrasound at day 35 following the first day of exposure to the bulls to determine pregnancy status.

4.3.4 Virus Challenge

BVDV challenge was administered by intrasnasal instillation of 5 ml of infected cell culture supernatant using a DeVilbiss® aresolizer at 70 to 75 days of gestation. The challenge stock virus was from a single lot containing 10^5 $CCID_{50}$ of BJ noncytopathic BVDV/ml. The BJ isolate of BVDV was previously isolated from a persistently infected animal.[10]

4.3.5 Gestation

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[a] Resvac® 4, Pfizer Animal Health, Exton, PA.

Following breeding, all animals were tested by ultrasound at weekly intervals for 4 weeks to determine fetal viability by observation of fetal heartbeat. All pregnant animals were allowed to calve normally.

4.3.6 Sampling

Blood was collected for BVDV serology from all heifers at the time of vaccination, at 30 days post-vaccination, at the time of challenge, and at 30 days post-infection. Following BVDV challenge, EDTA whole blood samples were collected each day until 10 days post-infection. The buffy coat was tested for presence of virus. Following the birth of calves, whole blood, serum and nasal swabs were collected from the calves. This was repeated at monthly intervals until the calves were 7 months of age.

4.3.7 Virus Isolation and Serology

Virus isolation was attempted for 2 passages by innoculating the samples and subsequently transferring the supernatant onto primary bovine turbinate (BTU) cells cultured in 96-well microtiter plates. BTU cells were grown in DMEM supplemented with 10% horse serum. Following 3 days of incubation, viral antigen was detected by immunoperoxidase staining using an anti-BVDV monoclonal antibody after each passage.[11] Serum virus neutralization was performed in microtiter plates as previously described.[12] Neutralization titers were determined using 2 BVDV reference strains, cytopathic NADL and noncytopathic BJ virus.

4.3.8 Monoclonal Antibody Analysis

Virus isolated from persistently infected calves and the BJ challenge virus were compared for patterns of monoclonal antibody (Mab) binding. The 29 Mabs used possessed viral neutralizing activity and reacted with the BVDV E2 polypeptide (gp53). Mabs were prepared and characterized as described previously.[13] Mab binding was assessed by indirect immunoperoxidase staining of infected cell monolayers.[14]

4.3.9 Nucleotide Sequencing

Nucleotide sequencing was done using polymerase chain reaction-based tests (PCR), based on the 5' untranslated region (5' UTR).[15] To generate viral template, total RNA from BVDV-infected BTU cells was prepared by acid guanidinium thiocyanate/phenol/chloroform extraction as adapted for cell culture.[16] This procedure was modified by using 5/1 phenol/chloroform (pH 4.7) instead of water-saturated phenol. Total RNA was harvested from BTU cells 48 hours after infection with BVDV. Reverse transcription and PCR amplification were performed as described previously.[17]

4.4 Results

Following the limited breeding period of 7 days, 6 of the unvaccinated control group and 12 vaccinated heifers were found to be pregnant at 60 days post-exposure to bulls. All pregnant heifers were challenged on the same day (approximately 75 days of gestation) as previously described. All control heifers remained BVDV sero-negative until after challenge. Virus was isolated from the

white blood cells from 4 of the 6 control heifers after challenge. Heifers #304, #393, and #309 were positive by virus isolation from buffy coat samples on day 6 after infection and heifer #356 was positive on days 7 and 8. BVDV was not detected in vaccinates on any sampling day. Ultrasound examination revealed that all fetuses were viable during the period of examination (4 weeks post-challenge). One heifer (#306) aborted at approximately 200 days of gestation. Tissues (spleen and thymus) collected from the fetus were negative for BVDV by virus isolation. Due to the poor condition of the heifer, the heifer was euthanized and necropsied. The gross and histopathological diagnosis was parasitic enteritis caused by oesphagostumum.

Serological results using the reference strains BJ and NADL were determined from samples collected on the following days; pre-vaccination, 30 days post-vaccination, day of virus challenge, and 30 days post-challenge (Table 4.1). Heifer #220, in the non-vaccinated control group, had a low initial titer but was sero-negative prior to challenge (Table 4.1). Response of heifer #220 to challenge was similar to other non-vaccinated heifers.

With the exception of the 1 abortion (heifer #306), all calves were normal.at birth. Calves were weaned at approximately 2 to 3 months of age. BVDV status was determined by monthly attempts to isolate BVDV from nasal swabs, serum, and white blood cells over a period of 7 months beginning as soon as possible following birth. Virus isolation results from samples collected

Table 4.1. Serum neutralization titers of dams (vaccinated and non-vaccinated) and the corresponding calf virus isolation results.

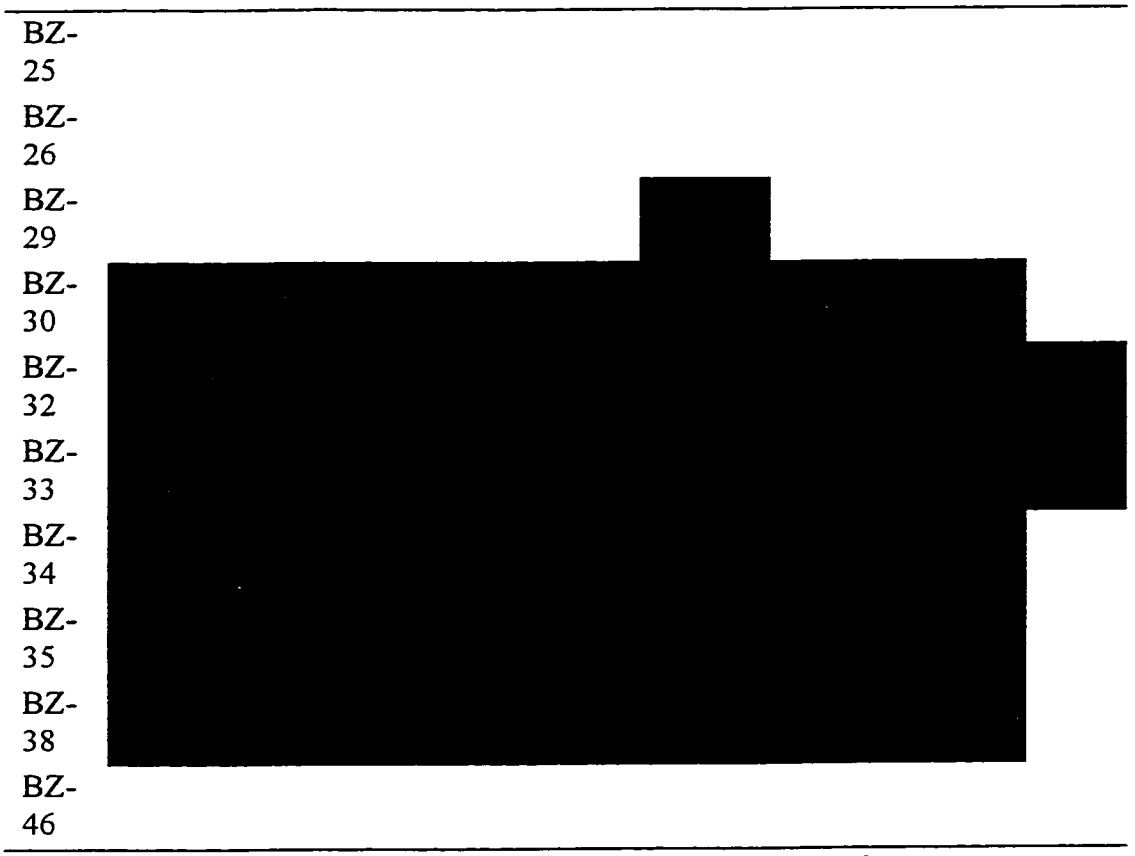
ID	Vac	Pre-vaccination		30 days Post-vaccination		Challenge day 0 (day 75 gestation)		30 days Post-challenge		Calf VI status
		BJ	NADL	BJ	NADL	BJ	NADL	BJ	NADL	
220	No	<1:5	1:10	<1:5	<1:5	1:20	<1:5	>1:640	1:10	Positive
304	No	<1:5	<1:5	<1:5	<1:5	<1:5	<1:5	>1:640	1:40	Positive
347	No	<1:5	<1:5	<1:5	<1:5	<1:5	<1:5	>1:640	1:80	Positive
393	No	<1:5	<1:5	<1:5	<1:5	<1:5	<1:5	>1:640	1:80	Positive
356	No	<1:5	<1:5	<1:5	<1:5	<1:5	<1:5	>1:640	1:40	Positive
367	No	<1:5	<1:5	<1:5	<1:5	<1:5	<1:5	>1:640	1:40	Positive
7	Yes	<1:5	<1:5	1:5	<1:5	1:5	<1:5	>1:640	1:320	Positive
380	Yes	<1:5	<1:5	1:80	1:40	1:40	1:20	>1:640	1:320	Negative

23	Yes	<1:5	<1:5	1:160	1:80	1:80	1:20	>1:640	>1:640	Negative
745	Yes	<1:5	<1:5	1:40	1:10	1:20	1:5	>1:640	1:160	Positive
811	Yes	<1:5	<1:5	1:80	1:80	1:40	1:20	>1:640	>1:640	Negative
908	Yes	<1:5	<1:5	1:80	1:40	1:40	1:20	>1:640	>1:640	Negative
4	Yes	<1:5	<1:5	1:160	1:80	1:40	1:10	>1:640	>1:640	Negative
129	Yes	<1:5	<1:5	1:40	1:40	1:20	1:10	>1:640	>1:640	Negative
80	Yes	<1:5	<1:5	1:80	1:40	>1:640	>1:640	1:320	1:320	Negative
85	Yes	<1:5	<1:5	1:10	1:10	1:160	1:5	>1:640	1:160	Negative
46	Yes	<1:5	<1:5	1:40	1:40	1:5	1:20	>1:640	>1:640	Negative
306	Yes	<1:5	<1:5	1:160	1:80	1:80	1:80	>1:640	>1:640	Negative

from the calves are presented in Table 4.1. Six persistently infected calves were born to the 6 non-vaccinated controls as determined by multiple positive virus isolations from nasal swabs, serum, and white blood cells. From the 12 vaccinated heifers, 2 calves were determined to be persistently infected with BVDV. Results of monoclonal antibody (Mab) binding with a panel of E2 specific anti-BVDV Mab indicated that 4 out of 6 persistently infected calves born to non-vaccinated animals had binding patterns identical to the challenge BJ virus inoculum (Table 4.2). The 2 remaining persistently infected calves from unvaccinated heifers had different binding Mab patterns. Persistently infected calf, P9, had reactivity to 1 additional Mab (BZ-29) when compared with the BJ ch allenge virus (Table 4.2) Mab binding patterns for persistently infected calf P17 born to non-vaccinated heifer #220 suggesteded a virus that differed from the BJ challenge virus. Nucleotide sequencing of the 5' UTR region revealed that the 2 calves from vaccinated dams and 5 out of 6 calves from non-vaccinated dams had 100 % sequence homology extending from nucleotides 107 to 390 of the BVDV SD-1 genome[16] (Table 4.3). The 5' UTR nucleotide sequence of calf P17 was characteristic of a type II BVDV isolate which confirmed the type II BVDV pattern of Mab binding.

Table 4.2 Monoclonal antibody binding patterns of viruses isolated from persistently infected calves

Mab	BJ	vaccinated			Non-vaccinated antibody negative				
		P8	P15	P1	P5	P9	P10	P13	P17
CA-1									
N-2									
CA-3									
CA-34									
CA-36									
CA-39									
CA-72									
CA-78									
CA-80									
CA-82									
BZ-2									
BZ-4									
BZ-15									
BZ-19									
BZ-23									
BZ-24									



Monoclonal antibody binding patterns of viruses isolated from persistently infected calves born to vaccinated (calves P8 and P15) and non-vaccinated dams (calves P1, P5, P9, P10, P13, P17) compared with the BJ challenge inoculum. Monoclonal antibodies were reactive with the E2 BVDV glycoprotein

Table 4.3 Map of aligned 5' untranslated region (5' UTR) nucleotide sequence of the virus isolated from the persistently infected calves born to vaccinated dams (calves P8 and P15) and non-vaccinated dams (calves P5 and P17). Calf P17 was classified as a type II BVDV.

		10	20	30	40	50	
BJVIRUS	1	---CATGCCC	ATAGTAGGAC	TAGCAAAATA	AGGGGGGTAG	CAACAGTGGT	50
P8	1	-TC*******	**********	**********	**********	**********	50
P15	1	--C*******	**********	**********	**********	**********	50
P17	1	TTC*******	**********	******G*GG	*****AC***	*GGT**CA**	50
P5	1	---*******	**********	**********	**********	**********	50
		60	**70**	**80**	**90**	**100**	
BJVIRUS	51	GAGTTCGTTG	GATGGCTGAA	GCCCTGAGTA	CAGGGTAGTC	GTCAGTGGTT	100
P8	51	**********	**********	**********	**********	**********	100
P15	51	**********	**********	**********	**********	**********	100
P17	51	******A***	******C***	T*********	*****A****	****A*****	100
P5	51	**********	**********	**********	**********	**********	100
		110	**120**	**130**	**140**	**150**	
BJVIRUS	101	CGACGCTTTA	GAGGATAAGC	CTCGAGATGC	CACGTGGACG	AGGGCATGCC	150
P8A	101	**********	**********	**********	**********	**********	150
P15A	101	**********	**********	**********	**********	**********	150
P17	101	****A**CC*	TCA*TCG**G	AGTCTCGA*A	TG*CAT*TG*	*C*AGGGCTT	150
P5	101	**********	**********	**********	**********	**********	150
		160	**170**	**180**	**190**	**200**	
BJVIRUS	151	CACAGCACAT	CTTAACCTGG	ACGGGGGTCG	TTCAGGTGAA	AACGGTCTAA	200
P8	151	**********	**********	**********	**********	**********	200
P15	151	**********	**********	**********	**********	**********	200
P17	151	GC*CA*GGCA	*A*CTTAACC	TAT*T**GG*	**GCATG*GT	G*AA*CACC*	200
P5	151	**********	**********	**********	**********	**********	200
		210	**220**	**230**	**240**	**250**	
BJVIRUS	201	CCAACCGCTA	CGAATACAGC	CTGATAGGGT	GCTGCAGAGG	CCCACTGCAT	250
P8	201	**********	**********	**********	**********	**********	250
P15	201	**********	**********	**********	**********	**********	250
P17	201	TTCGTG*TGT	TATGG***CA	GCCTG*TA*G	*TGTAGC**A	GA*CTGCT**	250

P5	201	**********	**********	**********	**********	**********	250
		260	**270**	**280**	**290**	**300**	
BJVIRUS	251	TGCTACTAAA	AATCTCTGCT	GTACATGGCA	CATGGGA...	....	300
P8	251	**********	**********	**********	*******A..	....	300
P15	251	**********	**********	**********	*******A..	....	300
P17	251	*C*GCTAGT*	**AACTCTGC	TGTACAT*GC	ACAT**GA..	....	300
P5	251	**********	**********	**********	*******...	....	300

4.5 Discussion

The purpose of this study was to determine the effectiveness of BVDV vaccination in preventing *in utero* transmission following intranasal challenge. The finding that 6 of 6 non-vaccinated heifers (100%) gave birth to persistently infected calves indicated that the intranasal challenge at 70 to 75 days of gestation was sufficient to provide an adequate challenge.

In this study, whole blood and clotted blood samples were collected as soon as possible following birth for virus isolation. However, the majority of samples weren't taken until approximately 4 to 12 hours after the ingestion of colostrum. Due to this delay, the ability to identify persistently infected animals required multiple sampling over a period of 7 months to ensure that calves identified as persistently infected were indeed persistently infected. Previous reports have documented the potential for false-negative virus isolation due to colostral antibody in calves less than 2 to 3 months of age.[10,17] In addition, the co-habitation of normal calves and persistently infected calves provided the potential for acute infections to occur during the sampling period. Therefore, multiple samplings were tested to make an accurate determination of the persistent infection status. Multiple positive virus isolations (2 positive samples 30 days apart) are also necessary to rule out transient infections in sero-negative animals..

One unexpected occurrence in this study was the identification of persistently infected calf (P17) from a non-vaccinated dam that was persistently infected with a type II BVDV. The data from the Mab binding and the 5' UTR

nucleotide sequence comparisons indicates that this virus was unrelated to the type I, BJ challenge virus.This suggests that the dam was exposed to a type II virus in early gestation from another persistently infected animal housed in the same facility. To date, the source animal has not been determined. In addition, questions arise as to the possibility of exposure of other animals in the study. Results of virus characterization and similarities with the challenge virus with the other BVDVs isolated suggest that this may have been an isolated infection without widespread exposure of the experimental group to a BVDV type II virus. Another possibility was that the exposure to type II BVDV occurred after challenge and the other animals were protected from the subsequent challenge. In addition, it is possible that the type II BVDV may have displaced the initial type I BJ virus as a minor quasispecies population in the persistently infected fetus.

The shifts in the E2 Mab binding patterns may explain the lack of protection in the 2 vaccinated dams (Table 4.2). Both viruses from the 2 vaccinated dams lost the ability to bind with the Mab CA-72, (Table 4.2). The loss of binding indicated the lack of an epitope in the isolated virus. These isolates may represent minor quasispecies that have the ability to escape the immunity stimulated from the modified live vaccination. *In vitro* studies have demonstrated that escape mutants of BVDV occur rapidly in the presence of Mab against the E2 glycoprotein, which possesses the major neutralizing epitopes for BVDV.[18]

From the results of this study, the modified live vaccine provided a reasonable level of efficacy against a type I BVDV fetal challenge with a

protection rate of 83% (2 persistently infected calves out of 12 vaccinated dams). The BJ challenge virus used in this study was chosen to represent a type I BVDV isolate that was different, antigenically, from the vaccine NADL BVDV. The characterization of this virus has previously been reported by Brock et al.[10] In addition, virus neutralization titers in convalescent antisera, using NADL and BJ virus as reference strains, were different.[10] One indication of the effectiveness of vaccination was the anamnestic serological response of the vaccinated animals following experimental challenge (Table 4.1). The vaccinated animals responded to both BJ and NADL strains at 30 days post-infection (Table 4.1). A second indication of the effectiveness of the vaccine is the fact that none of the vaccinated cattle had BVDV isolated from them after challenge. This may be the mechanism in which transplacental infections are prevented. One explanation for the *in utero* infections in the 2 vaccinated heifers may be the failure of an adequate response to vaccination. The 2 vaccinated dams (#7 and #745) that gave birth to the persistently infected calves had low BVDV antibody titers to BJ and NADL BVDV 30 days following vaccination (Table 4.1). It is unclear why the level of antibody or cell-mediated immunity stimulated by vaccination was inadequate to prevent transplacental transfer of BVDV in these 2 vaccinated animals. However, this failure could be due to host response as well as vaccine failure. If fetal challenge is a requirement of vaccine efficacy, further studies must be done to determine the factors involved in providing protection of the fetus as well as the mechanism of transplacental transfer of BVDV to the fetus.

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5. RESPONSES TO VIRAL VACCINES IN YOUNG DAIRY CALVES

5.1 Abstract

A trial was performed to assess the safety and clinical efficacy of several vaccination programs in young dairy calves. Two hundred and twelve week-old Holstein bull calves were stratified upon arrival according to incoming sodium sulfite analysis ranges of serum immunoglobulin. Each group included over 20 calves with specified ranges of serum immunoglobulin concentrations, and each group was randomly assigned to a vaccine protocol. Calves were assessed for weight gain, morbidity, and mortality during a sixteen week feeding program. Serum was collected and analyzed for titers of virus neutralizing (VN) antibodies to bovine respiratory syncytial virus (BRSV), bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (infectious bovine rhinotracheitis) (BHV-1) on a weekly basis from arrival through eleven weeks after arrival. Necropsies were performed on each fatality and cause of death was established when possible.

There was low morbidity and mortality (7.55% mortality rate) in the calves. Antibody titers to BHV-1, BVDV and BRSV had expected decay rates (approximately 3 weeks) in the control calves. Since there were no increases in antibody titers in

the controls to these viral agents through eleven weeks after arrival, it was assumed that no exposure to these agents occurred. Calves that received vaccines containing modified live viruses either had a sustained level or delayed antibody decreases or increases in antibody titers after vaccination. There were no adverse reactions or disease problems attributed to vaccine administration.

The results demonstrate that vaccination of one to nine week old calves with combination modified live vaccines was safe. Blockage of humoral responses, by maternal antibody, to modified live vaccines, as measured by virus neutralizing antibodies, was not complete.

5.2 Introduction

The emergence of the thrombocytopenic form of bovine viral diarrhea virus BVDV, caused by "type II" BVDV isolates, in veal and dairy beef operations[1] and the difficulty in establishing consistent protection by inactivated viral vaccines[2] has led to widespread use of modified live viral (MLV) vaccines in young dairy beef and veal calves.[3] The handling and shipping of these calves, along with multiple source origins, ensure that over time most veal and dairy beef facilities will have groups of calves that are exposed to the primary viral agents responsible for respiratory and gastrointestinal diseases of cattle. The stress associated with shipping of calves has been well documented[4,5,6,7,8] and can have detrimental effects on the immune response at a time when these young animals are exposed to various disease causing agents.

Although vaccines are being used with increased frequency in young calves, there is relatively little data from controlled studies that address the issues of safety and efficacy in this age group of cattle. Therefore, the purpose of this study was to compare the effect of different vaccine regimens using combination viral vaccines on performance parameters, morbidity, mortality, weight gains, serologic responses and adverse reactions in veal calves.

5.3 Materials and methods

5.3.1 Animals

Two hundred and twelve Holstein bull calves were purchased form multiple sale barns and placed in a veal research facility. The calves were between 3 and 5 days of age with a mean weight 45.4 kg (range 35.9-53.2 kg). Two calves died before the initiation of the trial and were excluded from all data sets.

Upon arrival, serum was obtained from all calves to estimate serum IgG concentration using the sodium sulfite test.[9] The calves were stratified according to the results of the incoming sodium sulfite analysis so that each group had equal numbers of calves in each of the following 3 groups: score of 1 or 2 (approximately <800 mg IgG/dl); score of 3 (approximately 800-1600 mg IgG/dl); or score of 4 (approximately >1600 mg IgG/dl). The calves were further stratified by weight and randomly assigned to one of nine different treatment groups. This ensured that each group had relatively even numbers of light and heavy weight calves.

5.3.2 Treatments

The calves were assigned to nine groups and were put into the housing stalls (Table 5.1).

5.3.3 Housing

The calves were housed in a single 35 x 18 meter veal barn that contained 212 elevated wood stalls. The stalls were arranged in 2 groups of 2 rows each. Calves were tethered in individual stalls with a floor space of 0.6 x 1.8 meters and a divider 0.6 meters in length between the calves. The calves from each group were distributed in stalls throughout all the rows to eliminate the effects of housing factors on the outcome of the trial.

5.3.4 Nutrition

All calves were fed for veal production, receiving only milk replacer (dry feed mixed with water and fed at body temperature), consisting of 22.73 kg of a starter milk replacer,[a] 22.73 kg of a high protein starter milk replacer,[b] and 200 kg of a finisher milk replacer[c] mixed for a total of 245.45 kg of powdered milk replacer. The calves were fed twice daily (approximately 04:00 and 16:00 hours) and water was provided at

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[a] Super Starter, American Feed and Livestock Co., Inc./Supreme Veal
[b] High Pro Starter, American Feed and Livestock Co., Inc. /Supreme Veal
[c] Supreme Finisher, American Feed and Livestock Co., Inc./Supreme Veal

TABLE 5.1 Vaccination Schedule for Each Group of Veal Calves beginning on the Day of Arrival

Group No.	Number of calves	Day 2 Vaccine(s)*	Day 7 Vaccine(s)*	Day 35 Vaccine(s)*
1	23	A	b,c	c
2	23	A	b,d	d
3	23	A	b,c	d
4	23	A	b,d	c
5	24	A	c	c
6	24		b,c	c
7	24		c	c
8	22	A	b,c	c
9	24	Control	Control	Control

*Day 0-2 are the days of arrival, Days 7 and 35 are days after arrival.
Vaccine a: Intranasal BHV-1- PI3V (TSV-2®, Pfizer Animal Health, Exton, Pennsylvania)
Vaccine b: Oral modified live rota-coronavirus vaccine (CALF-GUARD®, Pfizer Animal Health, Exton, Pennsylvania)
Vaccine c: Inactivated BVDV, temperature sensitive BHV-1-PI3V and a modified live BRSV (CattleMaster 4®, Pfizer Animal Health, Exton, Pennsylvania) , intramuscular injection
Vaccine d: Modified live BVDV, BHV-1, P13V, BRSV (BoviShield 4®, Pfizer Animal Health, Exton, Pennsylvania), intramuscular injection

10:00 hours. Feed levels were increased during the sixteen weeks to maintain feeding of milk replacer at a level of 14% of body weight daily.

5.3.5 Data Collected

All 210 calves were monitored for sixteen weeks. Calf weights were recorded on day 1 of the study and immediately prior to sold at the conclusion of the trial. Morbidity, mortality and treatment days were recorded. On arrival (day 0), sodium sulfite tests were performed on every calf, and on day 0 and for each of the next eleven weeks, serum was collected for assessment of antibody titers against BRSV, BVDV and BHV-1 from groups 2,3,4,8 and 9. Groups 1, 5, 6 and 7 were not sampled since the vaccine protocols were the same as other sampled groups. Necropsies were performed by the clinical staff and students from the University of Wisconsin, College of Veterinary Medicine on all fatalities. This was done to determine if pneumonia was present based on gross pathologic changes. No histopathology was performed.

All calves were monitored for swelling at the injection sites, anorexia, depression or fever following vaccination and throughout the study by blinded monitors. Any swelling was considered abnormal.

5.3.6 Laboratory Techniques

The incoming IgG concentrations were estimated using the sodium sulfite precipitation test. One tenth of a ml of serum was added to each of the three concentrations of sodium sulfite (14%, 16% and 18%) and incubated for an hour. Samples were visually examined for precipitation at the various concentrations and

calves were assigned a score of 1-4. A "one" designated a total failure of passive transfer (no visual precipitation at 18% sodium sulfate concentration), "2" indicated a marked failure of passive transfer (precipitation at 18% but not at 16%), a "3" indicated a partial failure of passive transfer (precipitation at 18 % and 16% but not a 14%) and a "4" indicated high passive transfer (precipitation at 18%, 16% and 14% concentration).[9]

The determination of the serum antibody titers to the various respiratory viruses was performed by the Colorado Veterinary Diagnostic Laboratory, Colorado State University, Ft. Collins, CO. Serum was collected weekly and frozen until the end of the trial when serum antibodies were determined. Neutralizing antibody titers to BRSV, BVDV and BHV-1 were determined using the laboratory's standard protocol for each virus. Titers were not determined beyond 1:1024 for BVDV and 1:256 for BHV-1. The lowest titer determined for BVDV was $\leq$1:8. BHV-1 and BRSV titers were determined to levels of <1:2. If values were less than the lowest determination, 0 was the assigned value. If the lowest test result was less than or equal to, then the actual lowest titer was used. Wells in which there was non-specific cytotoxicity were identified by a "c" on the final data form. The laboratory determined this when cytotoxicity was seen in a well in which there were valid neutralization results in the adjacent wells.

5.3.7 Data Analysis

If a data value was missing between two known points a value was assigned as follows: If the known values were the same at time points on each side

of the missing value then the same value was added for the missing value. If the points had a one dilution difference (i.e from 1:8 to 1:16) at the two known time points then the lower of the two values was assigned to the missing value. If there was a two dilution (i.e. 1:8 to 1:32) increase between the two known points then the missing value was the middle titer between the two known time point values. If a calf had more than one consecutive missing value or if the difference between the two known time point values was greater than a two fold change, the calf was eliminated from that data set to eliminate skewing in data from missing values.[10] Calves with no detectable antibodies at the lowest dilution tested, for each virus, were assigned a value of 0 for all calculations. The reciprocal antibody concentrations were log 2 transformed and analyzed using a repeated measures analysis of the variance model (ANOVA). Individual time point means were analyzed using a simple t test. Least square means were calculated from the ANOVA and then backtransformed to yield the geometric mean antibody titer (GMAT) for each virus per group. The sick days, treatment days and weight gains were analyzed using an ANOVA method. Mortality rate analysis was performed using a Fisher's Exact test (SAS Institute, Inc. Cary, NC). The 5% level of significance was used to assess statistical differences.

5.4 Results

5.4.1 IgG Concentrations

All calves were tested on arrival (day 0) with the sodium sulfite test to estimate the incoming IgG levels. Only 111/210 calves had a high degree of passive transfer with a sodium sulfate score of 4 (Table 5.2). The remaining 99 calves had some degree of failure of passive transfer according to the results of the sodium sulfite test (scores of 1-3; Table 5.2). There was a trend (but no statistical difference) towards higher weight gains and lower mean number of sick days and days of treatment in the calves with higher IgG concentrations (Table 5.3). The impact of vaccination on specific virus-neutralizing antibodies trended towards greater increases in calves with lower incoming IgG antibody concentrations; however, there were no statistical differences. Approximate corresponding serum immunoglobin concentrations to the sodium sulfite scoring are shown (Table 5.3).

5.4.2 Serologic Data

5.4.2.1 Bovine Herpesvirus-1

Thirty-two calves, of the 115 tested, entered the facility with no detectable antibodies to BHV-1. BHV-1 specific maternal antibodies decreased over time in the control group (Table 5.4). At the initiation of the trial the mean antibody titer was approximately 1:16 ($\log_2$ =4) and decreased to 1:4 ($\log_2$ =2) by week ten. The other groups of calves had increased BHV-1 specific antibodies following the first and second vaccinations with vaccines containing either a temperature sensitive or modified live BHV-1 (Figure 5.1). Significant differences were seen between controls and group 4 at week 11 (p=.0001), and group 8, week 9 (p=.0110), week 10 (p=.0206) and week 11 (p=.0020).

TABLE 5.2 Numbers of Calves in Each Group* and Incoming IgG Level as Determined by Sodium Sulfite Testing

SCORE	4	3	2	1
Group 1	13	8	1	1
Group 2	12	8	3	0
Group 3	12	9	2	0
Group 4	12	9	2	0
Group 5	12	10	2	0
Group 6	12	9	3	0
Group 7	13	8	2	1
Group 8	12	8	3	0
Group 9	13	7	2	2
Total Calves	111	75	20	4

* refer to table 1 for group designations

TABLE 5.3 Total Number of Calves and Incoming IgG Level as Determined by Sodium Sulfite Testing[9]

Incoming IgG Level	Approximate Corresponding IgG Level	Mean Weight Gain	Mean Sick Days	Mean Treatment Day
4	>1600mg/dl	145.68 kg	0.94	1.54
3	1200-1600mg/dl	143.5 kg	1.17	1.78
2	800-1200mg/dl	138.53 kg	1.8	1.68
1	<800mg/dl	126.02 kg	1.25	2.88

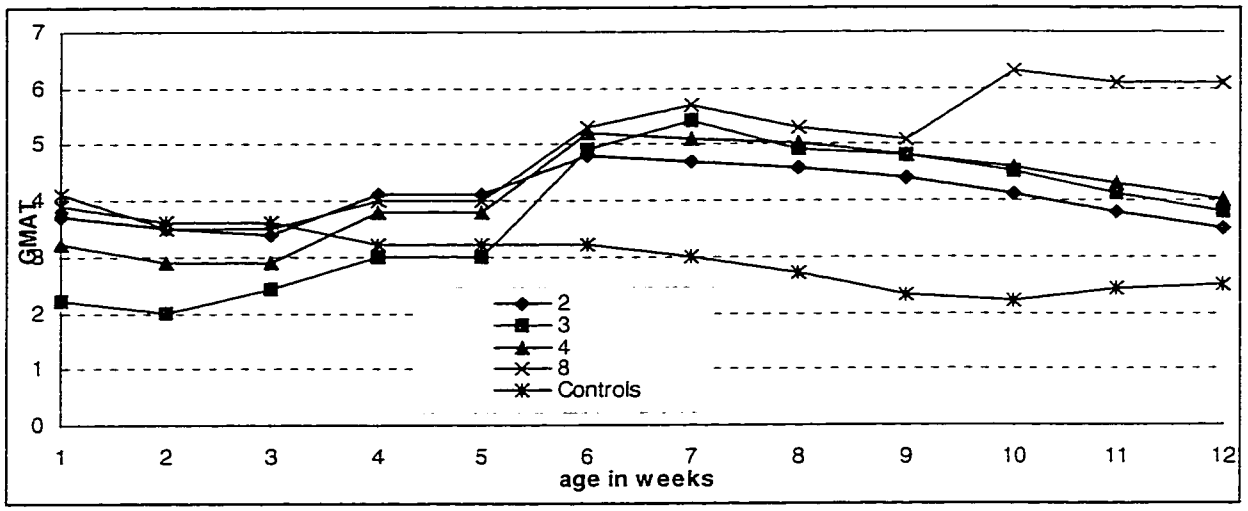

Figure 5.1 BHV-1 VN GMAT per Group Over Time
* refer to table 1 for group designations
a. GMAT= geometric mean antibody titer. The reciprocal of the original antibody level is converted to the log 2 and means were then derived.
b. Refers to days of vaccination. The first vaccines were given 2 days and 5 days after arrival. Day 35 is the day of second intramuscular vaccination. Day 0 antibody levels are prevaccination samples.
c. group 8 received an additional vaccine on day 63.

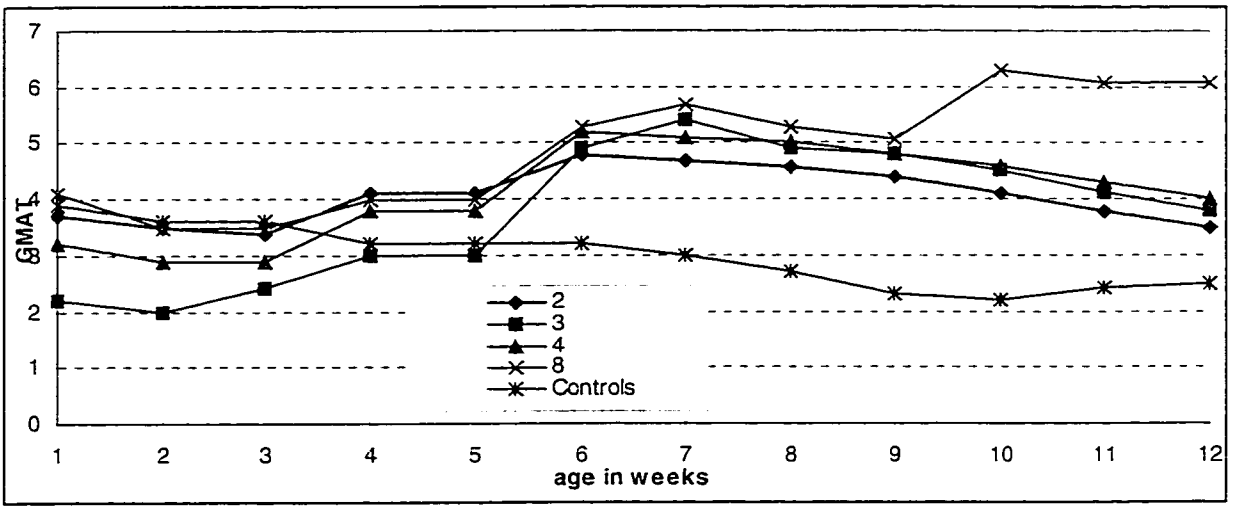

Figure 5.2 BVDV VN GMAT per Group Over Time

* refer to table 1 for group designations

a. GMAT= geometric mean antibody titer. The reciprocal of the original antibody level is converted to the log 2 and means were then derived.

b. Refers to days of vaccination. The first vaccines were given 2 days and 5 days after arrival. Day 35 is the day of second intramuscular vaccination. Day 0 antibody levels are prevaccination samples.

c. group 8 received an additional vaccine on day 63.

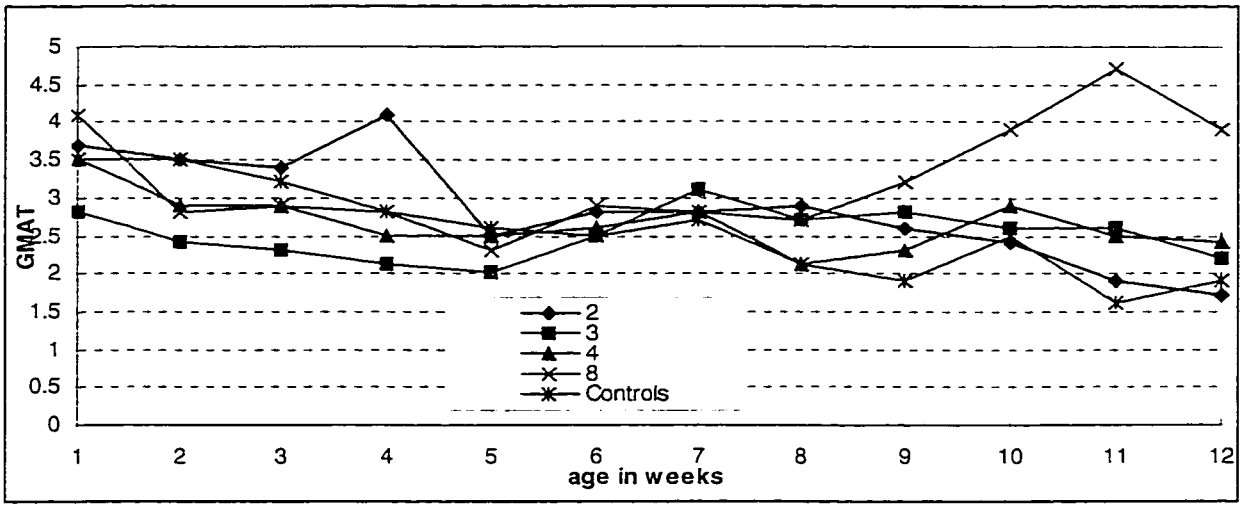

Figure 5.3 BRSV VN GMAT per Group Over Time

* refer to table 1 for group designations

a. GMAT= geometric mean antibody titer. The reciprocal of the original antibody level is converted to the log 2 and means were then derived.

b. Refers to days of vaccination. The first vaccines were given 2 days and 5 days after arrival. Day 35 is the day of second intramuscular vaccination. Day 0 antibody levels are prevaccination samples.

c. group 8 received an additional vaccine on day 63.

5.4.2.2 Bovine Viral Diarrhea Virus

Thirty-one of the calves tested had no detectable antibodies to BVDV. Approximately 1/2 of these calves (18/32) were also BHV-1 sero-negative. More than half (26/45) of the calves that were BVDV/BHV-1 sero-negative had a score of 4 on the incoming sodium sulfate test. The control group and group 2 had a slight, but insignificant, decrease in BVDV specific antibody concentration throughout the study period. Group 8 started with the highest level of maternal antibody and had the highest level of antibody on day 63 after vaccination; groups 3, 4, and 8 had increases in antibody titers in response to vaccination (Figure 5.2). Significant differences between the controls and group 4 were seen on week 2 ($p=.0039$) and week 4 ($p=.0449$) and group 8 on week 11 ($p=.0400$).

5.4.2.3 Bovine Respiratory Syncytial Virus

Only 4 calves had no detectable antibodies to BRSV upon arrival to the facility. The control calves had a decrease in BRSV specific antibodies over the test period. The vaccinated calves in each group had little or no increase in BRSV specific antibodies to the first dose of modified live BRSV; however, each group had increased antibody titers following each vaccination after the initial dose of vaccine (Figure 5.3). Significant differences were seen between controls and group 8 on week 8 ($p=.0519$), week 9 ($p=.0277$), week 10 (.0010) and week 11 ($p=.0200$).

5.4.5 Clinical Responses

The mean sick days, days of treatment, mortality and weight gains per group are shown in Table 5.4. Statistical analysis indicated no differences in any parameters between the control and treatment groups or between treated groups. Sixteen calves died giving an overall mortality rate of 7.55%. Three calves died from congenital heart diseases late in the feeding period. Of the animals dying from contagious disease problems, enteritis, compatible with Clostridial infections, was the most common cause of death. One animal died with pneumonic lesions (calf 194/group 6). There were no clinical signs of BVDV infection seen in the barn.

5.5 Discussion

It has been demonstrated that maternal antibody specific for BRSV and BVDV can decrease severity of disease following infection.[11,12] However, partial or complete failure of passive transfer (FPT) is not unusual in dairy calves. A recent study by the National Animal Health Monitoring Service (USDA) found that approximately 41% of the dairy heifers across the United States had some degree of FPT with IgG levels below 1000 mg/dl.[13] This may have been due to the quantity and/or quality of the colostrum these calves received, or to the timing of the administration of the colostrum. These results of this study indicate that calves can have normal passive transfer and yet be partially or totally deficient in antibodies to individual pathogens (i.e. BVDV sero-

TABLE 5.4 Treatment Group* Clinical Parameter Means and Mortality Rate

Treatment	Mortality /number in group	Weight Gain	Sick Days	Treated Days
1+8	3/45	301.85	1.63	3.19
2	2/23	309.16	1.49	2.71
3	0	317.16	1.41	2.86
4	1/23	303.31	1.48	3.27
5	1/24	297.46	0.86	2.04
6	2/24	297.81	0.92	1.40
7	3/24	308.02	1.24	2.20
9	1/24	304.29	1.09	1.96

* refer to table 1 for group designations

negative). Ingestion of colostrum with low or no virus-specific antibodies could allow severe disease during an outbreak.[14] This phenomenon should be considered in assessing outbreaks of disease in young calves with apparent normal concentrations of passive IgG and when considering vaccination.

The low number of BRSV sero-negative calves was in agreement with previous studies indicating that most cattle are BRSV sero- positive and, therefore, will transfer BRSV-specific antibodies in the colostrum.[16]

Neutralizing antibodies to BHV-1 and BRSV decayed at the expected rates in the unvaccinated control calves. Barring infection, the established half-life of IgG antibodies in "normal" calves is 21 days.[16,17] There was no increase in titers BHV-1 or BRSV specific antibody in control calves during the trial period, suggesting that there was no natural exposure to these viruses in the facility. In contrast, titers of BVDV-specific antibodies remained fairly constant throughout the study in the control calves, as well as in most groups of vaccinated calves. The reason for this was not apparent. The lack of expected decay of the BVDV antibody in the control calves may have been an indication of antigenic stimulation and low level exposure to the BVD virus. A previous study demonstrated exposure to both BVDV and BHV-1 by sero-conversion within the population of the barn (unpublished observations).

The neonatal calf is capable of mounting an immune response to antigenic stimulation, but the amount of antibody production and rate of the immune response may be slower than in older cattle.[18] In these calves, there was generally

a 1-2 fold increase in antibody titers rather than the four fold increases found in older animals in response to similar antigenic stimulation.[19] It has been reported that changes in titers often do not occur when vaccines are administered to young calves, and that maternal antibodies interfere with vaccination responses.[2] Recent studies have shown that modified live BRSV, PI3V and BHV-1 stimulated B cell memory responses that were demonstrable on the basis of anamnestic responses to subsequent vaccination or challenge.[20,21,22] Moreover, a recent study demonstrated the ability of a temperature sensitive BHV-1 and a modified live BRSV vaccine to stimulate BRSV and BHV-1 antigen specific T cells in ten day old calves with high maternal antibody against these viruses.[23]

Results of this study documented increased antibody titers or delayed decay rates in vaccinated calves; however, the changes were not statistically significant. This study, when combined with previous studies, suggest that young calves may be actively immunized by modified live BHV-1 and BRSV despite the presence of maternal antibodies. Without a demonstrated challenge, efficacy comparisons between the vaccine groups were not possible.

Vaccinated calves in this study had increases in BHV-1 specific antibody following the first and second BHV-1 vaccination. The response was seen in these groups by the day of second intramuscular vaccination (day 35) and by one week following the second dose of vaccine. All groups, with the exception of group 2, had week 11 antibody levels that were higher than the maternal antibody levels determined at the onset of the trial. This is contrary to the results of previous

studies in which maternal antibody blocked serologic responses to vaccination.[16,24] This may be due to differences in the vaccines, calves, or the higher number of calves may have allowed the detection of responses.

The groups that had the most pronounced antibody responses to vaccination with BVDV were those that had the highest number of sero-negative calves. Based on the assessment of individual calves, when BVDV-specific antibody titers were lower than 1:64, most calves responded to vaccination with increases in BVDV-specific antibodies (data not shown). This is consistent with what has been reported in the literature.[25]

Calves that received modified live BRSV had no change in antibody titers after the primary vaccination, but had increased (although not statistically significant) BRSV-specific antibodies subsequent to each successive dose. This pattern is similar to the antibody responses following the administration of this and similar vaccines to older cattle[26,27,28] and is compatible with a memory response or anamnestic response following a priming vaccination.

Initial administration of intramuscular viral vaccine was delayed until day 7 of the trial for two reasons. First, the calves were allowed to adjust to the new environment and feeding schedule. This permitted the probable increased corticosteroid concentrations resulting from the stress of transport to return to baseline. Secondly, it assured that all calves were over 5 days of age. Studies in calves have shown that peripheral T lymphocytes are compartmentalized or lysed by the corticosteroids released during the calving process.[17,29,30] This effect is

attenuated by 5 to 7 days of age. The second dose of vaccine was administered at approximately six weeks of age (four weeks after the first vaccination). This timing was based on data indicating that this interval yielded optimum immune and clinical responses in veal and dairy beef calves (unpublished data; McGuirk, Cortese, Shields)[17] and should maximize the potential for memory immune responses.[31]

The lack of significant differences in weight gain, morbidity, mortality and treatment days between the treated and control animals indicated that vaccination of young calves did not cause disease, nor have adverse effects on the production parameters measured. This is in contrast to an earlier report[32] documenting systemic, fatal BHV-1 infections following vaccination of neonatal calves with a modified live vaccine. In that study, the calves were vaccinated at three days of age or less. The older age of the calves vaccinated in this study or differences in the two vaccines may account for the differences between the two studies.

In this study, the necropsy results indicated a surprising lack of pneumonia, considering the mortality rate. Only one calf had significant pulmonary pathologic changes. From previous experience in this facility, and with calves raised in similar environments, higher morbidity and mortality (7%) rates due to pneumonia were anticipated. In this instance, a lack of respiratory viral challenge, as indicated by the serologic findings in control calves, probably accounted for a decreased incidence of respiratory disease.

In conclusion, the results demonstrated that administration of vaccines in various combinations to young, often sero-positive, calves can stimulate virus-specific antibody responses without having any untoward effects on production parameters. Although, as in many field trials, there was insufficient challenge to stringently assess the efficacy of the vaccines in protecting calves from viral infection, these results suggest that mixed viral vaccines can be used safely in combination with other immunogens in early vaccination programs for calves. In order to adequately assess the half-life loss of antibodies and its effect on masking immune responses, specific radiolabeled antibody studies may be necessary.

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6. CLINICAL RESPONSES TO VIRULENT BOVINE VIRAL DIARRHEA VIRUS-TYPE II CHALLENGE IN YOUNG CALVES FOLLOWING VACCINATION WITH AN INACTIVATED BVDV TYPE I

6.1 Abstract

This study was designed to determine the efficacy of a vaccine containing inactivated bovine viral diarrhea virus (BVDV) type I in protecting calves from infection and disease due to a virulent isolate of BVDV type II.

Fifteen neonatal Holstein and Holstein-cross calves were obtained from local dairies. The calves tested BVDV type II sero-negative after colostrum ingestion. Calves were then randomly assigned to either control (unvaccinated, n=6) or test groups (vaccinated, 1 dose; n=3, two doses; n=6). At 10-14 days of age, 9 calves were administered a combination vaccine containing inactivated BVDV (type I cytopathic and noncytopathic isolates), six calves were administered a second dose 21 days later. All calves were intranasally challenged approximately 21 days after the final dose of vaccine with a virulent BVDV type II isolate. The following parameters were assessed: clinical scores, rectal temperatures, complete blood counts, antibody responses and virus isolation was performed for 14 days after infection. Sero-negative unvaccinated calves developed severe disease and all control animals required euthanasia. Vaccination

of sero-negative calves with a single dose of the inactivated vaccine resulted in a slight disease sparing effect and only one animal was euthanized. Administration of two doses of the inactivated vaccine had a significant disease sparing effect and none of these calves were euthanized. Each group had variably severe signs of BVDV infection and virus was isolated from every calf after challenge. The protective immune mechanism was not identified.

These data support the use of two doses of this inactivated BVDV-type I vaccine to protect susceptible young calves from mortality due to virulent BVDV-type II infections. Protection from infection was not complete and viral replication and some signs of disease were seen.

6.2 Introduction

Over the past several years, bovine viral diarrhea virus (BVDV) infections have become a resurgent problem for the cattle industry in North America. There have been outbreaks with high morbidity and mortality from acute BVDV infections[1,2] in addition to the well recognized syndromes associated with persistent infection.[3] This has resulted in an increased awareness of acute BVDV infections and more diagnoses of BVDV infections in cattle with various clinical syndromes.[4,5]

BVDV is a single, positive stranded, highly mutable RNA virus in the pestivirus group of the Flaviviridae family.[6] Based on growth characteristics in

culture, there are two recognized biotypes of BVDV; cytopathic and noncytopathic. While replication of cytopathic strains results in death of target cells, noncytopathic strains replicate without apparent damage to infected cells.[7] The noncytopathic biotype is the natural state of the virus and comprises the majority of field isolates (>95%).[8] Cytopathic BVDV isolates arise from mutation of noncytopathic strains and tend to be antigenically closely related to the noncytopathic biotype from which they arise. There is considerable antigenic variability of the surface proteins among different BVDV isolates; allowing phenotypic differentiation as demonstrated by the use of monoclonal antibodies.[9,10]

Genotypic differences in the 5' untranslated region of the viral genome are the basis for the designation of BVDVs as either type I or type II. There is approximately 60% homology between the sequences in this region of type I and type II isolates of BVDV. Previously, it has been demonstrated that the antibodies produced by cattle in response to vaccination with type I isolates can cross neutralize genetically and antigenically disparate BVDV isolates *in vitro*.[11] There are no published articles documenting the ability of inactivated vaccines to confer protection from a virulent BVDV type II challenge *in vivo*.

The emergence of the thrombocytopenic form of acute BVDV, caused primarily by type II isolates of the virus, and the other severe forms of the disease with high mortality rates, have led to questions concerning the ability of vaccines to cross protect against the many different strains of BVDV.[12] This is compounded by the apparent inability to achieve consistent protection by the use

of inactivated vaccines.[13] The efficacy of vaccines containing inactivated BVDV has not been assessed in controlled challenge experiments using a highly virulent type II strain of BVDV. All licensed BVDV vaccines were approved using a BVDV type I isolate of relatively low virulence as the challenge virus. The objective of this study was to determine if one or two doses of a vaccine containing inactivated BVDV type I could protect young calves from disease following infection with a virulent BVDV type II.

6.3 Materials and Methods

6.3.1 Calves and Experimental Design

Fifteen neonatal Holstein and Holstein-cross calves were obtained from local dairies. Serum from all calves was tested within 7 days after birth for BVDV-specific antibodies, and BVDV isolation was performed to detect and eliminate any calves that were persistently infected with BVDV. Calves were sero-negative for BVDV type I on the basis of ELISA and BVDV type II on the basis of a virus neutralization assay. Calves were then assigned to one of the following groups: 3 sero-negative calves received a single dose of vaccine containing inactivated BVDV type I isolates on days 10-14 after birth; 6 sero-negative calves received a single dose of vaccine containing inactivated BVDV strains on days 10-14 after birth and a second dose on day 21 after the initial dose; and 6 were sero-negative unvaccinated controls. Calves were observed twice daily after vaccination for any adverse reactions.

Approximately 3 weeks after vaccination, all calves were infected intranasally with a BVDV type II isolate. Control calves were challenged at the same age as the calves receiving the single dose vaccine (approximately day 35 of age). After infection, rectal temperatures and clinical scores were recorded twice daily. Clinical scores were assigned according to a predetermined scoring system by a blinded scorer (Table 6.1). These included parameters assessing: appetite; degree of depression; and signs of respiratory and gastrointestinal disease. Serum and whole unclotted blood were obtained prior to vaccination, on the day of vaccination and on days 0 (day of challenge) 1, 3, 5, 7 and 10 days after challenge. The calves were fed a commercial milk replacer twice a day. A calf starter ration, alfalfa hay, and water were available free choice.

Calves that had at least 3 of the 4 following signs for more than 2 consecutive days were euthanized by barbiturate overdose: rectal temperatures $\geq$40.6° C, watery diarrhea, total white blood cell counts <2000 ml, or marked depression. A complete necropsy was performed on all calves that were euthanized or died. The distribution of BVDV replication was assessed using immunohistochemistry. The detailed results of pathologic examinations are presented in another report.[14]

6.3.2 Vaccines

Table 6.1 Clinical Scoring Method

A. Depression	0	none
	1	mild anorexia or listlessness
	2	moderate depression, slow to rise, anorexic
	3	recumbent
	4	death
B. Hemorrhage	0	none present
	1	few, petechiae of mucous membranes and/or sclera
	2	moderate, severe petechiation or hematomas present
	3	large hematomas
	4	bloody diarrhea and/or epistaxis
C. Respiratory signs	0	none
	1	clear nasal discharges and/or slight cough, no treatment required
	2	mucopurulent discharge and/or severe cough, slight increase in lung sounds, requires treatment
	3	severe pneumonia
D. Diarrhea	0	none
	1	mild. slight, less than 5% dehydration, no treatment required
	2	moderate dehydration (%-10%) oral treatment required
	3	severe dehydration, profuse dehydration $>10\%$, intravenous fluids required

A vaccine[a] containing inactivated BVDV-type I (cytopathic National Animal Disease Center (NADC) isolate 6309 and noncytopathic NADC isolate 5960), and modified live BHV-1, PI-3Vand BRSV was administered as either a single dose or as two doses in the quadriceps muscle.

6.3.3 Bovine Viral Diarrhea Virus

Noncytopathic BVDV isolate #24515 was obtained from the tissues of an aborted fetus from a herd that had experienced gastrointestinal and respiratory disease and death in calves and adult cows during a BVDV outbreak in Ontario. This isolate was classified as BVDV type II on the basis of antigenic and genetic analyses.[15] The virus was propagated in low passage (<10) cultures of embryonic bovine turbinate (EBT) cells and virus passages 7 and 8 were used for calf infections. Calves received 5 x 10^5 $TCID_{50}$ of virus in 5 ml of tissue culture fluid (2.5 ml/nostril).

6.3.4 Virus Isolation

Jugular blood was collected in heparinized tubes. Two milliliters were mixed with 13 ml Tris-ammonium chloride buffer to lyse the red blood cells. The remaining leukocytes were washed once in sterile phosphate buffered saline, resuspended in 1 ml of modified Eagles medium and stored frozen at -70 C. Two hundred microliter aliquots of thawed mixed samples were cultured with EBT cells in 24 well tissue culture plates for 7 days. Cultures were freeze-thawed once and

[a] CattleMaster 4®, Pfizer Animal Health, Exton, PA

75 µl of supernatant was cultured with approximately 10^4 MDBK cells in 100 µl (in duplicate) in 96 well tissue culture plates. After 4 days, medium was removed, the cell monolayers were fixed in acetone and stained using a BVDV-specific monoclonal antibody, 15C5[16] utilizing an immunoperoxidase technique.[17] Each 96 well plate contained wells that were inoculated with 25 µl of serum from a BVDV-persistently infected calf (positive control) and uninoculated cells (negative control). Virus isolation was performed using blood samples from all calves before inclusion in the study, on days 0, 7 and on day 14 in all surviving calves.

6.3.5 Complete Blood Cell Counts (CBC)

Complete blood counts were performed by the Clinical Pathology Laboratory at the Western College of Veterinary Medicine on whole blood collected via jugular venipuncture into vacutainer tubes containing sodium EDTA. An automated cell counter[b] and visual assessment of blood smears were used in the analyses. Manual platelet counts were performed on samples with low platelet numbers. Granulocyte counts were determined by adding neutrophil, basophil and eosinophil counts on each of the days.

6.3.6 ELISA Assay

An ELISA to detect antibodies to BVDV was performed as previously described[18] except that a solubilized antigen was used. Briefly, the NADL isolate of BVDV was grown in porcine kidney cells which were lysed and a soluble

[b] Baker System 9000 Haematology Series Cell Counter, Seronon Baker Diagnostics, Allentown, PA

antigen was prepared.[19] Control antigen was prepared similarly using uninfected porcine kidney cells. ELISAs were performed on serum obtained prior to vaccination (day -14) and on days 0, 7, and 14 after infection.

6.3.7 Virus Neutralization (VN) Assay

A standard plaque reduction assay[20] was performed to quantitate BVDV-neutralizing antibodies. Briefly, a standard amount (50-100 $TCID_{50}$) of either of 2 cytopathic isolates, NADL (type I) or 125 (type II) was incubated with dilutions of sera prior to infection of EBT cells in microtiter culture plates. Culture plates were incubated for 7 days prior to visual assessment of virus-induced cytopathic effect and determination of the titer of VN antibodies. VN titers were determined in a single set of assays performed with stored (frozen -20 C) sera at the conclusion of the trial.[21]

6.3.8 Statistical Analysis

The BVDV antibody and white blood cell parameters (including flow cytometric data) were log transformed and the least square means were determined. For results presented as graphs and tables, these means were then log transformed back to normal values. General linear model procedures, testing of hypotheses for mixed model analysis of variance, were used to test the significance of results. The response variables were analyzed with a general linear repeated measure model which partitions the total sum of squares into sources defined in the following model,

$$U_{ijk} = m + a_i + g_{j(i)} + l_k + al_{jk} + e_{ijk} \qquad (6.1)$$

where:

U_{ijk} = response variable

m = overall constant

a_i = fixed effect of ith treatment

$g_{j(i)}$ = random effect of jth animal within the ith treatment

l_k = fixed effect of kth day of study

al_{jk} = fixed interaction effect of treatment by day of study

e_{ijkl} = random residual error

Prior to analysis, the white blood cell data was transformed to the natural log scale. The resulting estimates of least squares means for each treatment from these analyses were then back-transformed to the original scale. Likewise, both ELISA and VN antibody data were transformed to the natural log scale after

adding one [1] to the antibody titer count. Using geometric means diminished the impact of individual calves. The effect of the individual animal, treatment over all days, effect of time only and then effect of treatment by day were evaluated). The latter was used to assess overall significance and then differences among individual days and groups were assessed for significance using a simple t test. A p value of ≤ 0.05 was considered significant. The least square means of the calves' temperatures were also determined and the linear model procedure was used. Differences in the number of calves that required euthanasia were determined by utilizing Fisher's exact tests for significance. Differences in clinical scores were not statistically analyzed due to the categorical nature of these measurements.

6.4 Results

6.4.1 Clinical Signs

No adverse clinical signs were noted in the young calves that received the vaccine after either administration. Following infection, clinical disease was most severe in the unvaccinated control calves. All six of these calves developed marked depression, pyrexia and watery diarrhea. The total mean clinical score in the control calves was 30. Unvaccinated calves consistently had a biphasic fever with an unremitting temperature elevation as high as 41.8°C. Severe diarrhea and other clinical signs tended to coincide with the second elevation (Figure 6.1). Pyretic calves were often anorexic. All six control calves progressed to extreme depression and pyrexia. These calves also exhibited variable degrees of respiratory

disease and/or digestive system disease (pneumonia and diarrhea) and were euthanized from day 10 to day 12 after challenge.

Vaccinated calves showed variable signs of clinical disease, as well, and biphasic fever peaks. The calves that received a single dose of vaccine had a significantly (controls; $p \leq 0.004$, 2 dose, $p \leq 0.02$) higher temperature elevation by day 2 after challenge than the other 2 groups. They maintained the significantly higher temperature elevation until late in the study (day 3: controls and 2 dose, $p \leq 0.03$, day 4: controls; $p \leq 0.019$, 2 dose; $p \leq 0.02$, day 5: controls; $p \leq 0.007$, 2 dose; $p \leq 0.001$, day 7: controls; $p \leq 0.023$, 2 dose; $p \leq 0.002$, day 8: 2 dose; $p \leq 0.007$, day 9: 2 dose; $p \leq 0.005$ and day 10: controls; $p \leq 0.05$, 2 dose; $p \leq 0.0001$). This group had a total mean clinical score of 20.1. The calves that were vaccinated twice had significantly ($p \leq 0.02$) lower temperatures than the controls starting on day 5 after challenge and these differences were seen again at the time of the second temperature elevation (day 8; $p \leq 0.059$, day 9; $p \leq 0.0001$, and day 10; $p \leq 0.0001$). This group had a total mean clinical score of 15.2.

In both calves receiving one vaccination and controls, clinical signs appeared earlier with signs beginning as early as two days after challenge. Calves given 2 vaccinations did not have clinical signs until day 6 after challenge. In all groups, the most severe signs were seen by day 8 after challenge.

6.4.2 Clinical Outcome

All 6 control calves were euthanized due to severe disease 10-12 days after infection. One of three calves that received a single vaccination required

All 6 control calves were euthanized due to severe disease 10-12 days after infection. One of three calves that received a single vaccination required euthanasia. There were highly significant differences in the number of calves requiring euthanasia between the controls and calves that received 2 doses (1 dose, $p = 0.083$; two dose, $p = 0.002$).

6.4.3 Virus isolation

All calves were BVDV virus isolation negative at the initiation of the study. Following viral challenge, all calves were viremic on 1 or more days.

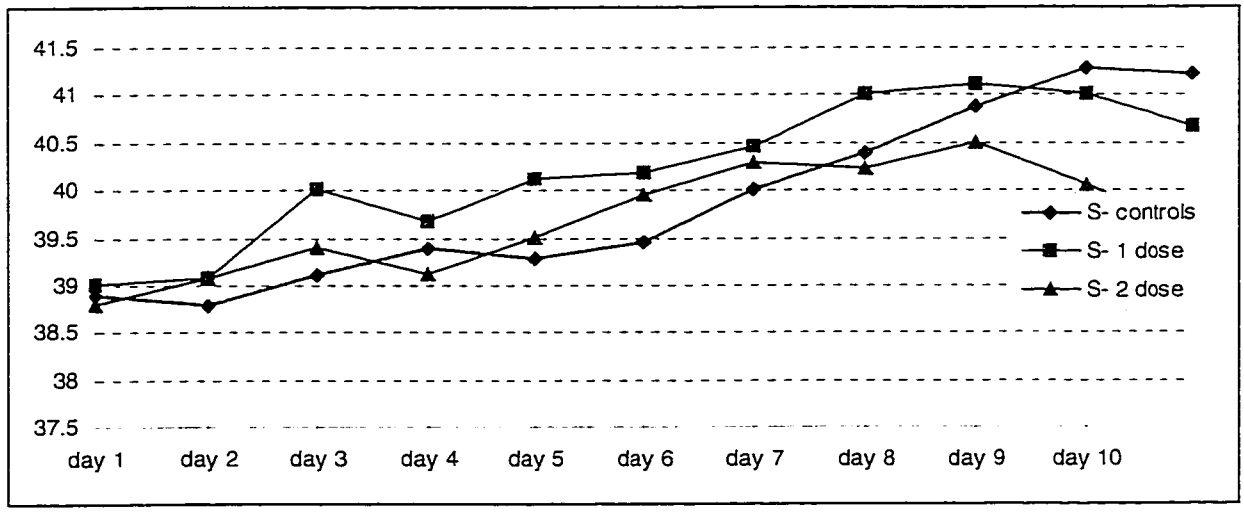
6.4.4 Complete White Blood Cell Counts

All groups of calves had decreases in white blood cell counts (Table 6.2). This decrease started within a day after challenge and was at its lowest by either day 3 or day 5 after challenge, except for the control calves. The WBC counts continued to decrease throughout the study in the latter calves. By the end of the study period, the two dose vaccinated group had reached or surpassed the baseline (prechallenge) values. Unvaccinated calves had marked decreases in their WBC counts when compared to other groups late in the study.

6.4.5 Complete Blood Counts and Differential White Cell Counts

Paralleling changes in the total WBC counts there were marked decreases in lymphocyte counts in the control cattle. The decrease began to occur the day after challenge and continued throughout the study (Table 6.3). There were significant differences in thrombocytes found by day 10 after challenge (Table 6.4) between the controls and the vaccinates. Marked thrombocytopenia (less than

Figure 6.1. Mean Daily Temperature per Group after Type II BVDV Challenge (°C)



less than 50,000) was reflected in haemorrhages found in two of these control calves. Decreases in monocytes were most notable in the controls and 1 dose vaccinates, with significant differences between these groups and the 2 dose group being detectable on day 10 after challenge (Table 6.5). Similarly, significant differences in circulating granulocytes were found between unvaccinated calves and the vaccinated calves (Table 6.6).

6.4.6 BVDV-Specific Antibody Analyses

No BVDV-specific antibodies were detected using ELISA, either before vaccination, or on the day of challenge. Several of the calves that were sero-negative for BVDV specific antibodies based on ELISA, had low concentrations of antibody that neutralized BVDV type I (equal to or less than 1:36) prior to vaccination or challenge (Table 6.7). There were no significant differences in type I or Type II BVDV neutralizing antibody titers between the groups before or after vaccination. By day 10 after challenge, when 5/6 controls were euthanized, calves receiving two vaccines had developed VN antibodies to both type I and type II BVDV that were significantly ($p \leq 0.05$) higher than the calves that had received a single dose of vaccine.

6.5 Discussion

These studies have shown that an inactivated BVDV type I vaccine can provide some protection in young calves from a virulent challenge with type II

BVDV. The BVDV type II isolate used in this study produced severe clinical disease that necessitated euthanasia of all control calves. Although calves that had received the recommended two doses of vaccine exhibited illness, they did not become as severely ill and were not euthanized. The challenge dose used in this study was

approximately 1/2 that used in previous studies with other BVDV type I and II strains.[22,23] The unprotected calves in this study became more severely ill but did not generally have the suffuse hemorrhages that have come to be associated with BVDV type II infections in young calves.[12] These findings underscore the variability in clinical signs and severity of disease seen with various BVDV isolates.

Calves that received one dose of vaccine developed disease that was nearly as severe as the unvaccinated controls. A single dose is not recommended for any inactivated BVDV vaccine. However, as a pilot study, 3 calves were vaccinated only once and challenged, since in many management situations, young calves would be exposed to BVDV and other pathogens before a booster dose could be administered. These results indicate, this would not allow proper immunity to be stimulated prior to exposure. Moreover, these results suggest that in such

Table 6.2 Geometric Mean White Blood Cell Counts

Group	day 0 (day of challenge)	Day 1	day 3	day 5	day 7	day 10
Controls	9020 (6400-14,000	9280 (6000-13900)	4810 (4300-5500)	5750 (3400-12000)	5300 (3400-9500)[a]	2610 (1400-4500)[a,b,c]
1 dose inact. Vacc.	8580 (7.2-10.7)	9094	5455	7006	8607[a]	4394[a,c]
2 dose inact. Vacc.	9306 (7.5-11,000)	9463	4519	5091	6241	8600[b,c]

Differences between groups with similar superscripts are significant at $p \leq .05$

Table 6.3 Geometric Mean Lymphocyte Counts

Group	day 0 (day of challenge)	day 1	day 3	day 5	day 7	day 10
S- controls	5530 (2880-7980)	4610 (3572-5997)	3190 (2340-4223)	3390 (2448-7200)	2480 (1666-4465)	1880 (1190-2904)[a,b]
S- 1 dose inact. Vacc.	4831 (3888-6206)	4625	3280	3655	3306	2996[a,c]
S- 2 dose inact. Vacc.	5040 (4071-6232)	5174	3453	3412	3147	5460[b,c]

Differences between groups with similar superscripts are significant at $p \leq .05$

Table 6.4 Geometric Mean Platelet Counts

Group	day 0 (day of challenge)	day 1	day 3	day 5	day 7	day 10
Controls	649170 (618000-722000)[a]	616270 (568000-704000)	436530 (361000-584000)	406360 (343000-503000)[a]	331250 (229000-419000)	137770 (TFTC*-174000) [a,b]
1 dose inact. Vacc.	855382 (794000-923000)[a,b]	832097[a]	593536a	610894[a,b]	389526	174897[a,c]
2 dose inact. Vacc.	491443 (385000-580000)[a,b]	497232[a]	405633a	411454[b]	337394	356442[b,c]

Differences between groups with similar superscripts are significant at $p \leq .05$

Table 6.5 Geometric Mean Monocyte Counts

Group	day 0 (day of challenge)	day 1	day 3	day 5	Day 7	day 10
Controls	530 (402-768)[a]	520 (345-616)[a]	250 (86-357)[a]	440 (174-1440)[a]	210 (95-350)	70 (22-616)[a]
1 dose inact. vacc.	484 (328-648)	595	151	453[b]	226	150[b]
2 dose inact. vacc.	245 (109-523)[a]	297[a]	96[a]	192[a,b]	186	646[a,b]

Differences between groups with similar superscripts are significant at $p \leq .05$

Table 6.6 Geometric Mean Granulocyte Counts

Group	day 0 (day of challenge)	day 1	day 3	day 5	Day 7	day 10
Controls	2800 (1809-5320)	4030 (1560-6095)	1200 (510-1890)	1770 (714-3360)	2200 (552-4910)[a]	590 (168-1530)[a]
1 dose inact. vacc.	3230 (836-3957)	3780 (1479-5952)	1800 (576-4018)	2630 (574-3320)	5029 (444-3120)[a,b]	1170 (676-3483)
2 dose inact. vacc.	2720 (2496-6600)	3330 (2175-7638	880 (585-2028)	1320 (1104-3870)	2250 (1377-8173)[b]	2240 (1134-3045)[a]

Differences between groups with similar superscripts are significant at $p \leq .05$

Table 6.7 Mean Antibody Level

	Prevaccination			day of challenge		10 days after challenge	
	Type I ELISA*	Type I VN	Type II VN	Type I VN	Type II VN	Type I VN	Type II VN
Controls	0	12 (0-36)[a]	0	.12 (0-36)	0	no calves**	no calves**
1 dose	0	13.7[b]	0	4.1	0	5.3[a]	1.7[a]
2 dose	0	1.7[a,b]	0	7.9	0	98.4[a]	124.1[a]

Differences between groups with similar superscripts are significant at $p \leq .05$

*Statistical evaluation not performed

**all calves except one had been euthanized by this sampling

situations, the use of vaccines\s that are effective after one dose, such as modified live BVDV vaccines, is warranted.

The inactivated vaccine used in this study contained a cytopathic and noncytopathic isolate of BVDV type I. There have been concerns regarding the ability of vaccines that contain BVDV type I isolates to cross-immunize for BVDV type II isolates. This inactivated vaccine did not confer complete protection from clinical disease to young sero-negative calves, but certainly decreased severity. The pyrexia and decrease in circulating white blood cells early after infection indicated that the challenge virus had probably replicated in the calves. This was verified by the fact that all calves developed viremia and were not spared from moderate to severe disease; although none became as ill as the unprotected control calves. Taken together, these results suggest that systemic viral replication is an essential pathogenic feature of severe disease following infection with BVDV, and, relatedly, that control of viremia appears to be an important factor if complete protection is to be conferred by active immunity.

The protective mechanism stimulated by vaccination in these calves was not apparent. All surviving calves had high concentrations of BVDV-specific neutralizing antibody to BVDV types I and II by day 10 after infection. However, on the day of challenge, there was no measurable serum antibody response to either virus type in most of the vaccinated calves that were protected. It is possible that vaccination stimulated memory B cells that responded rapidly with

BVDV-specific antibody production early after infection; unfortunately, we did not assess that possibility in this study.

There is considerable debate about the ability of the immune system of young animals to respond to vaccination.[24] As in our previous study with modified live BVDV vaccines in young calves,[25] the decrease in severity of clinical signs engendered by the administration of the 2 doses of the inactivated vaccine to 10-14 day old sero-negative calves shows that young calves can mount a clinically relevant immune response to commercially available vaccines.

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7. CLINICAL AND IMMUNOLOGIC RESPONSES IN VACCINATED AND UNVACCINATED CALVES FOLLOWING INFECTION WITH A VIRULENT BOVINE VIRAL DIARRHEA VIRUS-TYPE II

7.1 Abstract

This study was designed to determine the efficacy of a vaccine containing modified live bovine virus diarrhea virus (BVDV) type I in protecting calves from infection with an isolate of a virulent BVDV type II and to determine which immune responses correlate with protection.

Twenty-eight neonatal Holstein and Holstein-cross calves received maternal colostrum or were fed pooled colostrum and assigned either to BVDV sero-positive (n=16) or sero-negative groups (n=12) based on BVDV-specific serum antibody concentrations as determined by ELISA. Calves were then randomly assigned to either control (unvaccinated) or test groups (vaccinated).

On days 10-14 of age, test groups were administered a combination vaccine containing a modified live BVDV type I. All calves were challenged intranasally approximately 21 days after vaccination with a virulent BVDV-type II. Clinical and immunologic parameters, including clinical scores, rectal temperatures, complete blood counts with lymphocyte subset analysis, antibody responses, and cell-mediated immune responses, were monitored for 10 days after infection.

Sero-negative unvaccinated calves developed severe disease and required euthanasia. Vaccination of sero-negative calves with modified live BVDV had a significant disease sparing effect as did passively transferred BVDV ELISA specific colostral antibodies. There were no significant clinical differences between vaccinated and unvaccinated BVDV type II sero-positive calves following viral challenge. The protective immune mechanism was not identified.

These data support the use of a single dose of modified live BVDV type I to protect susceptible young calves from virulent BVDV type II infections.

7.2 Introduction

In the past several years, BVDV infections have resurfaced as a major problem for the cattle industry in North America. The problems have included high morbidity and mortality from acute BVDV infection,[1,2] in addition to the well-recognized syndromes associated with persistent infection.[3] This has resulted in an increased awareness of acute BVDV infections and more diagnoses of BVDV infection in cattle with various clinical syndromes.[4,5]

BVDV is classified as a pestivirus in the Flaviviridae family. It is a single positive-stranded RNA virus and is highly mutable.[6] Currently, there are two recognized biotypes of BVDV; cytopathic and noncytopathic. This is a laboratory differentiation based on the characteristics of the two types when grown in tissue culture. Replication of cytopathic biotypes results in death of target cells, whereas non-cytopathic biotypes reproduce without apparent damage to infected cells.[7]

The majority of BVDV field isolates are non-cytopathic (approximately 95%) and the non-cytopathic biotype is the natural state of the virus.[8] It is believed that all cytopathic BVDV strains arise from mutation of non-cytopathic isolates. The cytopathic BVDV isolates tend to be antigenically close to the noncytopathic isolates from which they arise. There is considerable antigenic variability of the surface proteins of different BVDV isolates; therefore BVDV isolates can also be classified phenotypically by the use of monoclonal antibodies.[9,10]

The designation of BVDVs as type I or type II is based on genotypic differences in the 5' untranslated region of the viral genome. There is approximately 60% homology between the sequences in this region of type I and type II isolates of BVDV. All current commercially available modified live BVDV vaccines contain type I, cytopathic isolates. Earlier studies have shown that the antibodies produced by cattle in response to vaccination with type I isolates can cross neutralize genetically and antigenically disparate BVDV isolates including type II isolates *in vitro*.[11] There is no documentation of the ability of such vaccines to confer protection from a virulent BVDV type II challenge *in vivo*.

The thrombocytopenic form of acute BVDV, caused primarily by type II isolates of the virus, has emerged as a major problem in veal and dairy beef operations.[12] The apparent inability to achieve consistent protection by the use of inactivated vaccines[13] has led to widespread use of modified live viral (MLV) vaccines[14] in young dairy beef and veal calves. These calves are stressed by

shipping, handling and mixing. They have variable and often low levels of passive colostral immunity.[14] Thus, they are highly susceptible to infection with various pathogens including BVDV. Clinical impressions have supported the hypothesis that protection from BVDV infection occurs following the administration of MLV BVDV vaccines to young calves reared in these management situations; however, the efficacy of vaccines containing modified live or inactivated BVDV has not been assessed in controlled type II BVDV challenge experiments. The objective of this study was twofold: 1) to determine if a single dose of a vaccine containing modified live BVDV type I could protect young calves from disease following infection with a virulent BVDV type II isolate and 2) to determine which *in vitro* measurement of the immune response to BVDV might correlate with protection from clinical disease.

7.3 Materials and Methods

7.3.1 Calves and Experimental Design

Twenty-eight neonatal Holstein and Holstein cross calves were obtained from local dairies. Fourteen calves were obtained within 12 hours of birth and were assigned to either BVDV sero-positive{S(+)} or sero-negative{S(-)} groups. Calves assigned to the sero-positive groups received 2.5 liters of spray-dried reconstituted pooled colostrum with high concentrations of BVDV-specific

antibodies.[a] Calves assigned to sero-negative groups received pooled colostrum that tested negative for BVDV-specific type I ELISA antibodies and type II VN antibodies. Colostrum was administered in 2 divided doses within the first 18 hours after birth (Table 7.1). Serum from all calves were tested within 7 days after birth for BVDV-specific antibodies, and BVDV isolation was performed on the buffy coat to detect any calves that were persistently infected with BVDV and were classified as S(+) (4 calves) and S- (10 calves). Fourteen additional calves that were fed conventional maternal colostrum were obtained at 10 days of age and classified to either the S(+) (n=11) or S(-) (n=2) groups. If the calves were both BVDV type I ELISA and BVDV type II virus neutralization antibody negative, they were classified to the S(-) groups. Total immunoglobin class G (IgG) levels determined via radioimmuno diffusion assay.

Calves were then assigned to the following groups: S(-) vaccinates (n= 6) received a single dose of vaccine containing modified live BVDV-type I[b] on days 10-14 after birth; S (+) vaccinates (n= 9) received a single dose of vaccine containing modified live BVDV on days 10-14 after birth; S(-) controls (n=6) that were sero-negative and unvaccinated; and S(+) controls (n= 7) that were sero-positive and

[a] Headstart, R, Saskatoon Colostrum Company, Saskatoon, Saskatchewan, Canada
[b]Resvac, 4, Pfizer Animal Health, Exton, PA

Table 7. 1 Source of passive antibody, BVDV passive antibody level and calf group assignment

calf #	Colostrum	ELISA titer		Neutralizing antibody titer*	
	Source	Singer (type 1)	125 (type II)	Singer (type I)	Vaccine
1	MC-	0	<6	6	MLV
2	PC-	0	<6	<6	MLV
3	PC-	0	<6	<6	MLV
4	PC-	3	<6	18	MLV
5	PC-	0	<6	<6	MLV
6	PC-	0	<6	6	MLV
7	MC+	0	18	162	MLV
8	MC+	32	≥324	≥324	MLV
9	MC+	34	162	≥324	MLV
10	MC+	7	≥324	≥324	MLV
11	MC+	35	162	≥324	MLV
12	MC+	47	≥324	≥324	MLV
13	MC+	13	36	≥324	MLV
14	PC+	53	≥324	≥324	MLV
15	PC+	45	>324	>324	MLV
16	MC-	0	36	<6	none
17	PC-	0	18	<6	none
18	PC-	0	18	<6	none
19	PC-	0	<6	<6	none
20	PC-	0	<6	<6	none
21	PC-	1	<6	<6	none
22	MC+	3	162	54	none
23	MC+	13	54	≥324	none
24	MC+	25	54	≥324	none
25	MC+	76	≥324	≥324	none
26	MC+	34	≥324	≥324	none
27	PC+	22	≥324	162	none
28	PC+	46	>324	>324	none

MC+ maternal colostrum containing BVDV ELISA antibody
MC- maternal colostrum containing no BVDV ELISA antibody
PC+ pooled colostrum containing BVDV ELISA antibody
PC- pooled colostrum containing no BVDV ELISA antibody
Colostrum was tested before feeding and sera from the calves were tested on day 7 of age. This table shows the serum results from the calves.
*The virus neutralizing antibody titers were determined in a single set of assay performed after challenge when all serum samples had been collected. Titer was not measured past 1:324 dilution.

unvaccinated. Calves were observed twice daily after vaccination for. all calves any adverse local or systemic reactions

On approximately day 35 after birth (18 days after vaccination), were infected intranasally with a BVDV type II isolate. After infection, rectal temperatures and clinical scores were recorded twice daily. Clinical scores were assigned according to a predetermined scoring system by a blinded scorer (Table7.2). These parameters included appetite; degree of depression; signs of respiratory disease; and gastrointestinal disease. Serum and whole unclotted blood (sodium EDTA and heparin samples[c]) were obtained prior to vaccination, on day of vaccination and on days 0 (day of challenge) 1, 3, 5, 7, and 10 days after challenge. The calves were fed a commercial milk replacer twice a day. A calf starter ration, alfalfa hay and water were available free choice.

Calves that had at least 3 of the 4 following clinical parameters for more than 2 consecutive days were euthanized by barbiturate overdose: rectal temperatures $\geq 40.6^{\circ}C$, watery diarrhea, total white blood cell counts <2000ml or marked depression. A complete necropsy was performed on any calves that were euthanized. Results of pathological examinations are presented in another report.[15]

[c] Vacutainer system, Becton-Dickinson and company, Rutherford, NJ

Table 7.2. Clinical Scoring Method

A. Depression	0	none
	1	mild anorexia or listlessness
	2.	moderate depression, slow to rise, anorexic
	3.	recumbent
	4	death
B. Hemorrhage	0	none present
	1	few, petechiae on mucous membranes and/or sclera
	2	moderate, severe petechiation or hematomas (greater than 1 centimeter) present
	3	large hematomas
	4.	bloody diarrhea and/or epistaxis
C. Respiratory signs	0	none
	1	clear nasal discharges and/or slight cough, no treatment required
	2	mucopurulent discharge and/or severe cough, slight increase in lung sounds, requires treatment
	3	severe pneumonia
D. Diarrhea	0	none
	1	mild. slight, less than 5% dehydration, no treatment required
	2	moderate dehydration (%-10%) oral electrolyte treatment and or antibiotic treatment required
	3	severe dehydration, profuse dehydration >10%. intravenous fluids required

7.3.2 Vaccines

A vaccine[b] containing modified live BVDV-type I (NADL isolate, plaque expanded), BHV-1, PI-3V and BRSV was administered as a single dose in the right quadriceps muscles.

7.3.3 Bovine Viral Diarrhea Virus

Noncytopathic BVDV isolate #24515 was obtained from the tissues of a fetus from a herd in Ontario, Canada that had experienced a severe type II outbreak and death of calves and cows. This isolate was classified as BVDV type II on the basis of antigenic and genetic analyses.[d] The virus was propagated in low passage (<10) cultures of embryonic bovine turbinate (EBT) cells. These cells were obtained locally from a BVDV negative fetus and routinely monitored for the presence of BVDV. Virus passages 7 and 8 were used for calf infections. All cell lines used in the study were tested and determined to be free of noncytopathic BVDV by the virus isolation techniques listed in 7.3.4. Calves received 5×10^5 $TCID_{50}$ of virus in 5 ml of tissue culture fluid (2.5 ml/nostril).

7.3.4 Virus Isolation

Jugular blood was collected in heparinized tubes. Two ml were mixed with 13 ml .05N Tris-ammonium chloride buffer to lyse the red blood cells and the mixture was centrifuged for five minutes at 400 x g. The resulting pellet of leukocytes was washed once in sterile phosphate buffered saline, resuspended in 1

ml of modified Eagles medium, and stored frozen at -70 C. Two hundred microliter aliquots of thawed mixed samples were cultured with EBT cells in 24 well tissue culture plates for 7 days. Cultures were freeze-thawed once and 75 µl of supernatant were cultured in duplicate with approximately 10^4 MDBK[g] cells in 100 µl in 96 well tissue culture plates. Four days after incubation, medium was removed; the cell monolayers were fixed in acetone, and stained using a BVDV-specific monoclonal antibody, 15C5[9] utilizing an immunoperoxidase technique.[17]

Each 96 well plate contained wells that were inoculated with 25 ml of serum from a BVDV-persistently infected calf (positive control) and uninoculated cells (negative control). Virus isolation was performed using blood samples from all calves before inclusion in the study, and on days 0, 7, and 14 of challenge in all surviving calves.

7.3.5 Complete Blood Cell Counts(CBC)

Blood was collected via jugular venipuncture into vacutainer tubes containing sodium EDTA. An automated cell counter[e,f] and visual assessment of blood smears were used in the analyses. Manual platelet counts were performed on samples with low platelet numbers. Granulocyte counts were determined by adding neutrophil, basophil and eosinophil counts on each of the days.

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[d] Carman S, van Dreumel T, Trembley R. et al: Severe acute bovine virus diarrhea(BVD) in Ontario in 1993. Proc 37th Annual Mtg. Of Am Assoc Vet Lab Diag Grand Rapids, MI, 24-25. 1994

[e] American Type Culture Collection, Betheseda, Maryland

[f] Baker System 9000 Hematology Series Cell Counter, Seronon Baker Diagnostics, Allenton, PA

7.3.6 Flow cytometry

Flow cytometric analysis of leukocyte subsets was performed as previously described,[18] except that leukocytes were obtained from whole jugular blood following lysis of red blood cells with .05N Tris-ammonium chloride buffer.[19,20] The following monoclonal antibodies[21] were used to identify subsets of bovine leukocytes: ILA-11 (BoT4); ILA-17 (BoT8); ILA-29 (gamma delta T lymphocytes); and a cocktail of BAQ155A and BAQ44B (B lymphocytes) Leukocyte subpopulation analyses were performed on days 0, 1, 7 and 10 and on some of the calves, on days 3 and 5.

7.3.7 ELISA

An ELISA to detect antibodies to BVDV was performed as previously described[22] except that a solubilized antigen was used. Briefly, the NADL isolate of BVDV was grown in porcine kidney cells which were lysed and a soluble antigen was prepared.[23] Control antigen was prepared similarly using uninfected porcine kidney cells. ELISAs were performed on colostrum samples and on serum obtained from all calves prior to vaccination (day -14).

7.3.8 Virus Neutralization (VN) Assay

A standard plaque reduction assay[24] was performed to quantify BVDV neutralizing antibodies. Briefly, a standard amount (50-100 $TCID_{50}$) of either cytopathic NADL (type I) or 125 (type II) was incubated with dilutions of sera prior to infection of EBT cells in microtiter culture plates. Culture plates were

incubated for 7 days prior to visual assessment of virus-induced cytopathic effect and determination of the titer of VN antibodies.[25] VN antibody concentrations were determined in a single set of assays performed with stored (frozen -20 C) sera at the conclusion of the trial.

7.3.9 Lymphocyte Blastogenesis

Lymphocyte blastogenesis was performed essentially as previously described[26] except that BVDV was used as the antigen. BVDV, type I (NADL) and type II (24515), were grown in MDBK cells (NADL) or EBT cells (24515) for 7 days. The cells were freeze-thawed and the virus was heat inactivated (1 hour, 56 C). Uninfected cells treated in the same way were used as control antigen. One hundred microliters of a 1/100 dilution of virus infected or uninfected cells were added to an equal volume of mononuclear leukocytes and cultures were incubated for 7 days prior to the determination of tritiated thymidine incorporation. Cultures were performed in triplicate. The mean number of counts per minute (cpm) was used to determine differences in counts per minute (cpm in BVDV-stimulated wells minus cpm in control antigen-stimulated wells) and stimulation indices (cpm in BVDV-stimulated wells /cpm control antigen stimulated wells). Lymphocyte proliferative responses to the BVDV-type I antigen were determined on day of vaccination, and days 0 and 7 after challenge. Lymphocyte blastogenesis, to the BVDV-type II antigen, was determined on day 7 after challenge.

7.3.10 Quantitation of Interferon Gamma

A previously described ELISA[26] was used to determine the concentration of interferon gamma in supernatants from the cultures established for lymphocyte blastogenesis assays. Supernatants were harvested on day 7 after the initiation of culture.

7.3.11 Cell Mediated Cytotoxicity

Genetically unrestricted cell-mediated cytotoxicity was assessed using blood leukocytes collected 7 days after BVDV infection as previously described for bovine herpesvirus-1, except that BVDV was used to infect target cells[27,28]. MBDK cells were labeled with ^{51}Cr for approximately 18 hours prior to infection with either BVDV type I (NADL isolate) or type II (24515 isolate) at a multiplicity of infection of approximately 1. Leukocytes from calves were incubated with labeled, infected and uninfected (control) MDBK cells for 18 hours prior to the measurement of ^{51}Cr release. Specific release was calculated as follows: (cpm in wells contained infected or uninfected target cells + leukocytes) - (cpm in wells containing infected or uninfected target cells alone) / total release. Spontaneous release of ^{51}Cr from BVDV-infected and uninfected cells did not exceed 25% of total release.

7.3.12 Statistical Analysis

The BVDV antibody and white blood cell parameters (including flow cytometric data) were log transformed and the least square means were determined. For results presented as graphs and tables, these means were then log

transformed back to normal values. General linear model procedures, testing of hypotheses for mixed model analysis of variance, were used to test the significance of results. The response variables were analyzed with a general linear repeated measure model which partitions the total sum of squares into sources defined in the following model,

$$U_{ijk} = m + a_i + g_{j(i)} + l_k + al_{jk} + e_{ijk} \qquad (7.1)$$

where:

U_{ijk} = response variable

m = overall constant

a_i = fixed effect of ith treatment

$g_{j(i)}$ = random effect of jth animal within the ith treatment

l_k = fixed effect of kth day of study

al_{jk} = fixed interaction effect of treatment by day of study

e_{ijkl} = random residual error

Prior to each analysis, the white blood cell data was transformed to the natural log scale. The resulting estimates of least squares means for each treatment from these analyses were then back-transformed to the original scale. Likewise, both ELISA and VN antibody data was transformed to the natural log scale after adding one (1) to the antibody titer count. The effect of the individual animal, treatment over all days, effect of time only, and then effect of treatment by day were evaluated uisng a t test. The latter was used to assess overall significance and then differences among individual days and groups were assessed for significance. A p value of ≤ 0.05 was considered significant. The least square means of the calves' temperatures were also determined and the linear model procedure was used. Fisher's exact tests for significance determined differences in the numbers of calves requiring euthanasia. Differences in clinical scores were not statistically analyzed due to the categorical nature of these measurements.

7.4 Results

7.4.1 Clinical Signs

No vaccine-associated adverse reactions were noted in the young calves that received the vaccine within the first 2 weeks after birth. Following infection, clinical disease was most severe in the unvaccinated S(-) control calves. All 6 of these calves developed marked depression, pyrexia and watery diarrhea. The

overall mean clinical score in S(-) control calves was 30. Unvaccinated S(-) calves consistently had a biphasic fever, with a high.unremitting temperature elevation (as high as 41.8^{o}C) that persisted until the calves were euthanized. Watery diarrhea and anorexia developed in association with the second temperature elevation (Figure 7.1). Disease in all of these calves progressed with signs such as extreme depression, pyrexia, respiratory, and/or digestive system signs (pneumonia and diarrhea) and was euthanized from day 10 to day 12 after challenge.

In contrast, the S(-) calves that had received 1 dose of modified live BVDV had only mild clinical disease, as indicated by a mean overall clinical score of 2.08. These calves had lower clinical scores, and significantly ($p< 0.001$) lower rectal temperatures throughout the study when compared to the unvaccinated S(-) calves, with significant differences seen on day 3 and continuing through the duration of this study period ($p\leq 0.05$). As well, in this vaccinated group, the mean rectal temperatures returned to normal limits within 2 days after the second elevation and remained within the normal diurnal ranges during the remainder of the study.

Passively-acquired, BVDV-specific antibodies also had a dramatic disease sparing effect. The S(+) calves displayed markedly less clinical signs than unvaccinated S(-) calves. Unvaccinated S(+) calves had a group mean clinical score of 3.67 and S(+) calves that received modified live BVDV had a group mean clinical score of 7.5 (4.7 excluding the calf that died). One S(+) vaccinated calf, developed severe clinical disease and required euthanasia. The S(+) (vaccinated

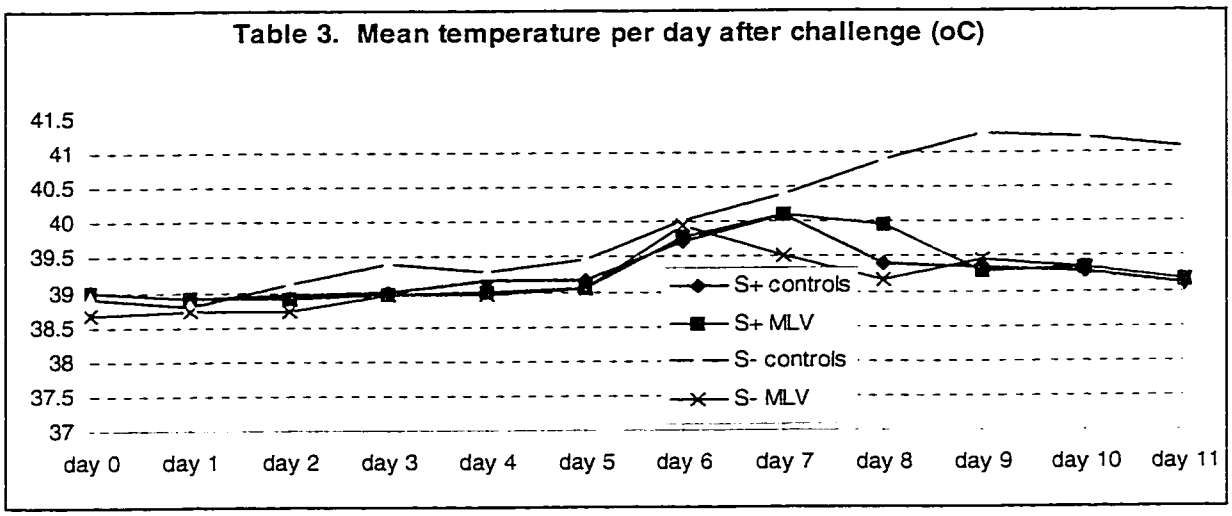

Figure 7.1 Mean daily temperature in sero-negative and sero-positive vaccinated and unvaccinated calves after challenge with type II BVDV (°C)

and unvaccinated) calves had significantly lower temperatures when compared to the S(-) controls on day 3 ($p \leq .05$). The vaccinated calves' temperatures remained significantly lower throughout the remainder of the study period and by day 7 the unvaccinated S(+) calves had significantly lower rectal temperatures than the control calves for the remainder of the study ($p \leq .05$).

The significant differences in rectal temperatures, between S(+) unvaccinated calves and S(+) calves that received a single does of modified live vaccine, were seen on days 5-7 ($p \leq .05$). The unvaccinated S(+) group had a higher mean temperature. However, these S(+) groups did have approximately a 0.5^{o} F degree higher peak fever than the S(-) calves that received modified live vaccine. This difference was significant on day 7 ($p \leq .05$). On day 8, calf #30 influenced the group mean temperature of the S(+) vaccinated calves and it was significantly ($p<0.0002$) higher than the group of S(-) calves that received modified live vaccine. At all other observations throughout the study there were no significant temperature differences among the S(-) and S(+) groups that received modified live vaccine and S(+) unvaccinated calves.

7.4.2 Clinical Outcome

All 6 S(-) control calves were euthanized due to severe disease 10-12 days after infection. One S(+) vaccinated calf required euthanasia. There were highly significant differences in the number of calves requiring euthanasia between the unvaccinated S(-) calves and S(-) calves that received MLV vaccine ($p < 0.002$), S(+) calves that received MLV vaccine ($p<0.002$), and the S(+) unvaccinated

calves $p<0.001$). However, there were no significant differences in the numbers of animals that required euthanasia between unvaccinated S(+) calves and vaccinated S(-) and S(+) calves ($p=0.47$).

7.4.3 Virus Isolation

All calves were BVDV virus isolation negative on the basis of examination of buffy coat samples at the initiation of the study. All S(-) unvaccinated calves were viremic (buffy coat samples) for 2 or more days following infection. Viremia was first detected by day 3 after challenge (2/6 calves) and all calves were viremic by day 5 after challenge. All euthanized calves were BVDV positive on virus isolation. In contrast, none of the S(+) unvaccinated or vaccinated calves (with the exception of one sero-positive vaccinated calf), or S(-) calves that received modified live BVDV were viremic on any day after infection.

7.4.4 Complete White Blood Cell Counts

All four groups of calves had decreases in white blood cell counts (Table 7.3). In most groups this decrease was mild and started within a day after challenge and was at it lowest by either day 3 or day 5 after challenge, except for the S(-) control calves. The WBC counts continued to decrease throughout the study in the latter calves. By the end of the study period, all groups, except the S(-) unvaccinated controls, had reached or surpassed the baseline (prechallenge) values. Sero-negative unvaccinated calves had marked decreases in their WBC counts when compared to other groups. By day 3 after infection, there were significantly less circulating WBCs in the S(-) unvaccinated controls than the other

three groups ($p \leq .04$). Again, on days 7 and 10, all groups had significantly higher WBC counts than the S(-) controls ($p \leq .04$). There were no significant differences between the S(+) groups or the S(-) vaccinated group except on day 1 when the S(+) vaccinates had a higher number of total WBCs than did the S(-) vaccinates ($p \leq .05$).

7.4.5 Complete Blood Counts and Differential White Cell Counts

Paralleling changes in the total WBC counts, there were marked decreases in lymphocyte counts in the S(-) control cattle. The decrease began to occur the day after challenge and continued throughout the study. The differences were not significant until day 7 after challenge (days 7 and 10; $p \leq .002$), when compared to the other three groups (Table 7.4). Similarly, significant differences in circulating granulocytes were found between the S(-) unvaccinated calves and the S(+) unvaccinated calves and the S(+) and S(-) calves that received MLV vaccine (table 7.5). Decreases in monocytes were most notable in the S(-) unvaccinated controls, with significant differences ($p \leq .02$) between this group and the other groups being detectable beginning on day 10 after challenge (Table 7.6).

Interestingly, the S(+) unvaccinated calves had a large decrease in monocytes on day 1 after challenge. Significant decreases in thrombocytes were reflected in hemorrhages found on the serosal surfaces of the digestive tracts in

several of these S(-) control calves and significant differences were found by day 7 after challenge (Table 7.7) between the S(-) controls and the other three groups ($p \leq .0002$)

7.4.6 Flow cytometry

There were significant decreases in BoT8+ ($P \leq .02$), gamma delta T cells ($p \leq .01$), and B cells ($p \leq .0007$) in the S(-) control group by day 7 and day 10 as compared to S(+) unvaccinated calves and S(+) and S(-) calves that received MLV vaccine. There was also a trend toward decreases in BoT4+ cells in the S(-) controls, but these changes were not significant (Tables 7.8-7.11).

7.4.7 BVDV-Specific Antibody Analyses

No BVDV-specific antibodies were detected using ELISA, either before vaccination, or on the day of challenge, in calves that were BVDV antibody negative. All calves had total IgG levels above 1000 mg/dl. Five of the calves that were sero-negative for BVDV specific antibodies based on ELISA results, two vaccinated and three unvaccinated, had low concentrations of antibody that neutralized BVDV type I equal to or less than 1:18 prior to vaccination or challenge. For the purposes of

Table 7.3 Geometric Mean White Blood Cell Counts

Group	Day 0 (day of challenge)	day 1	Day 3	Day 5	day 7	day 10
S+ controls	9858 (6000-15300)	10030 (7400-13600)	8910 (5800-14300)[a]	5570 (3400-6800)	9870 (5300-16500)[a]	7150 (5900-8500)[a]
S+ MLV vacc.	9940 (6000-15300)	10170 (4500-15600)[a]	7570 (4700-12500)[b]	5920 (3900-9800)	8610 (4700-14800)[b]	8210 (2100-18600)[b]
S- controls	9020 (6400-14,000	9280 (6000-13900)	4810 (4300-5500)[a,b,c]	5750 (3400-12000)	5300 (3400-9500)[a,b,c]	2610 (1400-4500)[a,b,c]
S- MLV vacc.	8580 (6800-10800)	7750 (6400-14000)[a]	6730 (4000-11800)[c]	6220 (4600-10400)	7480 (6100-11800)[c]	6160 (5300-6800)[c]

Differences between groups with similar superscripts are significant at $p \leq .05$

Table 7.4. Geometric Mean Lymphocyte Counts

Group	Day 0 (day of challenge)	day 1	day 3	Day 5	day 7	day 10
S+ controls	5890 (4672-7373)	5690 (5180-6552)	4530 (2508-8723)[a]	3360 (2618-5624)	4020 (2669-4992)[a]	5200 (3953-6586)[a]
S+ MLV vacc.	5720 (3267-9130)	4680 (2610-8736)	4110 (2820-5546)	3650 (2967-5456)	4180 (2585-7178)[b]	5240 (1743-10281)[b]
S- controls	5530 (2880-7980)	4610 (3572-5997)	3190 (2340-4223)[a]	3390 (2448-7200)	2480 (1666-4465)[a,b,c]	1880 (1190-2904)[a,b,c]
S- MLV vacc.	4660 (3400-7176)	4980 (4020-8400)	3780 (2214-6586)	3630 (2484-5928)	3870 (2926-5310)[c]	4340 (3120-5092)[c]

Differences between groups with similar superscripts are significant at p≤.

Table 7.5 Geometric Mean Granulocyte Counts

Group	Day 0 (day of challenge)	day 1	Day 3	day 5	day 7	day 10
S+ controls	2990 (1932-7956)	3820 (1975-7452)	3870 (2320-5291)[a]	1650 (510-3726)	4160 (495-12375)[a]	1320 (900-2500)[a]
S+ MLV vacc.	3160 (1210-8228)	4630 (1485-8736)[a]	2750 (1260-5782)[b]	1370 (310-5684)	3800 (1843-9675)	1820 (357-9858)[b]
S- controls	2800 (1809-5320)	4030 (1560-6095)	1200 (510-1890)[a,b,c]	1770 (714-3360)	2200 (552-4910)[a]	590 (168-1530)[a,b,c]
S- MLV vacc.	2730 (1680-4320)	2240 (1491-5040)[a]	2290 (1394-5546)[c]	2080 (1196-4512)	2820 (945-5782)	1250 (689-2280)[c]

Differences between groups with similar superscripts are significant at $p \leq .05$

Table 7.6 Geometric Mean Monocyte Counts

Group	Day 0 (day of challenge)	day 1	day 3	Day 5	day 7	Day 10
S+ controls	630 (276-612)	240 (138-504)[a,b]	310 (152-672)	330 (148-408)	390 (154-785)	380 (118-1020)[a,b]
S+ MLV vacc.	390 (87-660)	440 (156-1180)	380 (93-875)	380 (110-882)	330 (109-810)	820 (231-2418)[a,c,d]
S- controls	530 (402-768)	520 (345-616)[a]	250 (86-357)	440 (174-1440)	210 (95-350)	70 (22-616)[b,c,e]
S- MLV vacc.	450 (272-952)	710 (372-902)[b]	390 (40-1298)	380 (312-672)	400 (183-819)	290 (177-600)[d,e]

Differences between groups with similar superscripts are significant at $p \leq .05$

Table 7.7 Geometric Mean Platelet Counts

Group	day 0 (day of challenge)	day 1	Day 3	day 5	Day 7	day 10
S+ controls	593190 (360000-933000)	659760 (381000-998000)	580940 (450000-830000)[a]	na	534310 (374000-914000)[a]	579460 (491000-928000)[a]
S+ MLV vacc.	649170 (276000-1210000)	437540 (184000-885000)	490380 (260000-1020000)	na	450070 (346000-777000)[b]	476040 (152000-1040000)[b]
S- controls	649170 (618000-722000)	616270 (568000-704000)	436530 (361000-584000)[a]	406360 (343000-503000)	331250 (229000-419000)[a,b]	137770 (TFTC*-174000)[a,b,c]
S- MLV vacc.	574240 (425000-664000)	609180 (505000-713000)	526020 (410000-584000)	490330 (460000-544000)	428300 (348000-576000)	464440 (227000-555000)[c]

Differences between groups with similar superscripts are significant at $p \leq .05$

*Too few to count

na.- not available

analysis, these calves were left in the S(-) groups due to their ELISA and/or VN type II sero-negative status. Only 1 ELISA negative calf {S(+) vaccinated calf, 1:18} had virus neutralizing antibody against type II BVDV prior to challenge, even though ELISA sero-negative, and was reassigned to the S(+) vaccinated group, prior to analysis. As would be expected, there were significant differences between the S(+) and S(-) groups mean antibody concentrations (ELISA, type I VN and type II VN) before vaccination (Table 7.12).

There were no significant differences in type I or type II BVDV VN antibody titers between the sero-negative groups before or after vaccination. By day 10 after challenge, when 5/6 sero-negative controls were euthanized, the S(-) vaccinated calves had developed VN antibodies to both type I and type II BVDV equal to or greater than 1:324. The S(-) calves that were vaccinated with 1 dose of MLV BVDV had significantly ($p \leq .05$) lowerer VN antibody titers than either of the S(+) groups by day 10 after challenge. All S(+) calves that received the spray dried colostrum product and calves that received maternal colostrum had serum antibodies that neutralized both BVDV type I and type II before vaccination.

There were no significant temporal or intergroup differences in antibodies that neutralized either BVDV type in the sero-positive groups of calves throughout the study.

Table 7.8 Geometric Mean BoT4 Cell Counts

Group	Day 0 (day of challenge)	Day 1	Day 3	day 5	day 7	day 10
S+ controls	1828 (873-3175)	Na	1102 (496-2244)	704 (280-1855)	1258 (473-2172)	1121 (162-2304)
S+ MLV vacc.	2129 (1507-3920)	Na	865 (272-2279)	730 (403-2695)	1364 (736-3611)	1647 (728-3372)
S- controls	1545 (1178-2439)	Na	Na	na	407 (176-830)	491 (367-702)
S- MLV vacc.	1260 (726-2899)	Na	Na	na	780 (549-1272)	877 (541-2387)

na- not available

Table 7.9 Geometric Mean BoT8 Counts

Group	day 0 (day of challenge)	Day 1	day 3	day 5	day 7	day 10
S+ controls	920 (332-1375)	Na	539 (216-1559)	306 (72-904)	764 (351-1321)[a]	907 (582-1981)[a]
S+ MLV vacc.	1214 (780-2178)[a]	Na	548 (228-893)	423 (210-990)	872 (298-33225)[b,c]	1081 (248-2205)[b]
S- controls	1024 (726-1252)	Na	Na	Na	169 (82-367)[a,b,d]	408 (328-638)[a,b,c]
S- MLV vacc.	771 (573-1242)[a]	Na	Na	Na	517 (246-898)[c,d]	831 (570-1158)[c]

Differences between groups with similar superscripts are significant at $p \leq .05$
na- not available

Table 7.10 Geometric Mean B cell Counts

Group	day 0 (day of challenge)	day 1	day 3	day 5	day 7	day 10
S+ controls	606 (326-1019)	Na	675 (418-1296)	445 (132-891)	979 (631-2627)[a,b]	602 (289-1106)[a,b]
S+ MLV vacc.	649 (244-1735)	Na	697 (382-1649)	539 (304-1088)	683 (325-1894)[c,d]	834 (353-1688)[c,d]
S- controls	666 (375-1439)	Na	na	Na	205 (95-523)[a,c]	224 (80-437)[a,c]
S- MLV vacc.	428 (348-713)	Na	na	Na	295 (187-866)[b,d]	330 (164-677)[b,d]

Differences between groups with similar superscripts are significant at $p \leq .05$
na- not available

Table 7.11 Geometric Mean Gamma Delta Cells Counts

Group	Day 0 (day of challenge)	Day 1	day 3	day 5	day 7	day 10
S+ controls	1268 (583-1889)[a]	Na	1440 (862-2417)	1046 (395-2325)	1456 (763-4208)[a,b]	1277 (706-2491)a,b
S+ MLV vacc.	918 (173-2427)[b]	Na	951 (248-2948)	1058 (474-1709)	1277 (282-4528)[c,d]	1088 (417-3360)c
S- controls	1267 (758-2416)[c]	Na	na	Na	544 (300-973)[a,c]	359 (215-500)a,c,d
S- MLV vacc.	449 (37-1394)[a,b,c]	Na	na	Na	406 (160-877)[b,d]	720 (396-1058)b,d

Differences between groups with similar superscripts are significant at $p \leq .05$
na- not available

Table 7.12. Geometric Mean Serum Antibody Concentration

	Prevaccination			Day of challenge		10 days after challenge	
	Type I ELISA*	Type I VN	Type II VN	Type I VN	Type II VN	Type I VN	Type II VN
S+ controls	33.3	285 (54-324)[a,b]	224 (54-≥324)[a,b]	282 (108-324)[a]	7.2 (0-128)[a,b]	258 (54-324)[a]	224 (36-324)[a]
S+ MLV	29.1	306 (162-324)[c,d]	224 (18-≥324)[c,d]	283 (36-324)[b]	6.6 (0-128)[c,d]	237(36-324)[b]	224 (108-324)[b]
S- controls	0	12 (0-36)[a,b]	0[a,c]	.12 (0-36)[a,b]	0[a,c]	no calves**	no calves**
S- MLV	0	5 (0-18)[c,d]	0[b,d]	15(0-66)	1 (0-6)[b,d]	149 (12-324)[a,b]	154 (18-324)[a,b]

Differences between groups with similar superscripts are significant at $p \leq .05$

*Statistical evaluation not performed

**all calves except one had been euthanized by this sample day

Table 7.13 Cell Mediated Immune Responses in vaccinated and unvaccinated calves as determined by antigen specific lymphocyte blastogenesis, interon release and MHC nonrestrictive cytotoxicity

	Mean blastogenesis* 7 days after challenge		Mean interferon concentration 7 days after challenge**	Mean cytotoxicity 7 days after challenge***	
	BVDV I	BVDV II	BVDV II	BVDV I	BVDV II
S+ controls	1642.07 (0-8154)	na	7.39 (0-10.62)	0	14.24 (0-37.8)
S+ MLV	1616.9 (112-4424)	na	2.86 (0-10.34)	1.16 (0-5.1)	17.22 (0-41.3)
S- Controls	707.8 (0-1599)	39.8 (0-107)	3.12 (0-12.2)	1.15 (0-4.0)	8.05 (2-16.2)
S- MLV	488.8 (0-894)	156.6 (0-427)	6.86 (.35-16.4)	1.85 (0-5.1)	11.61(3.5-22.4)

*Changes in counts per minute
**pg/ml in leukocyte cultures
***percent specific cytotoxicity

7.4.8 Lymphocyte Blastogenesis

Although BVDV-specific lymphocyte proliferative responses were detected in all but one of the calves tested by day 7 after challenge, there were no significant differences between calves that received MLV vaccine and unvaccinated S(-) calves (table 7.13).

7.4.9 Gamma Interferon Concentrations

Low concentrations of interferon gamma were detected in supernatants from leukocyte cultures derived from approximately 50% of the calves tested. There were no significant differences between vaccinated and unvaccinated calves (table 7.13).

7.4.10 Cell Mediated Cytotoxicity

Cytotoxicity of BVDV-infected cells was mediated by leukocytes from some vaccinated and unvaccinated calves in both S(+) (n=7) and S(-) (n=7) groups. There were no significant differences between vaccinated and unvaccinated, or between S(+) and S(-) calves (table 7.13).

7.5 Discussion

These studies have shown that a MLV BVDV type I vaccine can protect young calves from a challenge with virulent type II BVDV. The BVDV type II isolate used in this study produced severe clinical disease that necessitated euthanasia of all control calves. The challenge dose used in this study was approximately 1/2 that used in previous studies with other BVDV type I and II

strains.[29,30] The unprotected calves became more severely ill, however they did not generally have the suffuse hemorrhages that have come to be associated with BVDV type II infections in young calves.[12] This isolate caused severe decreases in platelets and some hemorrhages. Most calves were euthanized before their platelet numbers decreased below 50,000, which may have been the reason why hemorrhages were not seen in all the calves. The calves that exhibited hemorrhages had platelet counts of "too few to count: (TFTC). The lack of hemorrhages may also be due to variability in the potential for BVDV type II isolates to cause hemorrhages. These findings underscore the variability in clinical signs and severity of disease resulting from infection with various BVDV type II isolates.

The modified live vaccine used in this study contained a cytopathic isolate of type I BVDV (NADL). There have been concerns regarding the ability of vaccines that contain BVDV type I isolates to cross-immunize against BVDV type II isolates. This modified live vaccine conferred virtually complete protection from clinical disease to young sero-negative calves. This study documents for the first time that heterotypic immunity can be achieved by the use of MLV vaccines containing BVDV type I isolates. The slight pyrexia and decrease in circulating white blood cells early after infection indicated that the challenge virus had probably replicated to a limited extent in the calves, most likely locally in tissues of the digestive or respiratory tract. The calves that received MLV vaccine did not develop detectable viremia and were spared the

unprotected control calves. Vaccinated and unvaccinated calves that had passive transfer of BVDV specific antibodies had only slight clinical disease and no viremia. These findings are consistent with previous studies in which calves that received variable protective concentrations of maternal antibodies had mild fevers and decreased duration and degree of viremia after challenge with another BVDV type II isolates.[29,31] Taken together, these results suggest that systemic viral replication is an essential pathogenic feature of severe disease following infection with BVDV, and, relatedly that control of viremia appears to be an important component of the protection conferred by active or passive immunity. The clinical response of the sero-positive vaccinated calf that required euthanasia following BVDV infection, exemplifies the individual variation in response to infection. This emphasizes that, in a minority of apparently protected animals, severe disease will occur regardless of vaccination or presence of antibodies.

There is considerable debate about the ability of the immune system of young animals to respond to vaccination.[32] The prevention of severe clinical disease by the administration of modified live vaccine to 10-14 day old sero-negative calves shows that the young bovine immune system can be effectively immunized. Moreover, our results validate that these vaccines can be used effectively in young animals with failure of passive transfer.

The effect of circulating antibody, either colostral or autologous, on the ability of modified live vaccines to stimulate the immune system, has also been

widely debated. It has long been held that antibody will block the induction of immune responses.[33] However, it has been shown that B and T cell responses can occur in the absence of measurable increases in serum antibody following vaccine administration.[34,35,36,37] One of the objectives of this study was to address this basic issue in a relevant challenge model (neonatal acute BVDV infection). As in previous studies,[27] BVDV-specific colostral antibodies had a dramatic disease sparing effect on infection of young calves with a highly virulent BVDV type II isolate, however, there was no apparent clinical benefit to vaccinating young sero-positive calves with residual maternal antibody (against type II BVDV). Further studies are necessary to address the issue of priming the immune system by challenging neonatally vaccinated, sero-positive calves after maternal antibodies have decayed.

The protective mechanism stimulated by vaccination in these calves was not apparent. Differences in some immunologic parameters were detected between vaccinated and unvaccinated calves before and after challenge. However, none appeared to be consistently related to protection when groups were compared. Protected calves had slight or no alterations in circulating lymphocyte subsets following infection that could be associated with a protective response. All surviving calves had high concentrations of BVDV-specific neutralizing antibody to BVDV types I and II by day 10 after infection. However, on the day of challenge, there was no measurable serum antibody response to either virus type in most of the S(-) vaccinated calves that were protected. Six of the calves that were

sero-negative prior to vaccination on the basis of ELISA had low concentrations of VN antibodies. Of these calves, only the unvaccinated calves required euthanasia, demonstrating the ineffectiveness of low concentrations of VN antibodies in conferring protection. This confirmed previous studies indicating that intermediate titers (1:64) are necessary to modulate disease severity.[27] It is possible that vaccination stimulated memory B cells that responded rapidly with BVDV-specific antibody production early after infection. We did not assess that possibility in this study. There are few previous reports concerning BVDV-specific cell mediated immunity in cattle.[35] As in a previous study of sero-positive cattle,[36] we detected low proliferative responses to BVDV antigens in mononuclear leukocyte cultures from vaccinated and unvaccinated calves following infection. Moreover, we detected previously unreported interferon gamma release in BVDV-stimulated leukocyte cultures, as well as cell-mediated cytotoxicity of BVDV infected cells from vaccinated and unvaccinated calves after challenge. None of these recognized indices of cell-mediated immunity correlated with the protection from disease.

The protection stimulated by vaccination may have been a mucosal antibody or cellular response, or a systemic cell-mediated immune response that we were unable to measure. In preliminary studies on 3 surviving calves, no MHC class I-restricted BVDV-specific cytotoxic (CD8+) T cells were detected in the blood (J. Ellis, unpublished data), suggesting that this may not be the protective mechanism. Although the particular protective immune mechanism conferred by

vaccination was not clear, it most likely mediates control of viral replication early in infection. This was demonstrated by the fact that calves that received the MLV vaccine containing BVDV type I (as well as passively protected calves), were significantly less affected by this virulent BVDV type II than were susceptible unvaccinated calves.

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8. GENERAL DISCUSSION

8.1 INTRODUCTION

Since its discovery a little over fifty years ago, bovine viral diarrhea virus had been identified as one of the top five economically important viral diseases of cattle worldwide.[1] The virus continues to cause both endemic infections, with low level, hidden, economic losses from reproductive diseases and calf problems as well as severe acute infections with high death loss.[2,3] It is the latter that has caused renewed interest in this viral disease in the past several years.

Outbreaks with both type I and type II BVDV isolates have been seen. In the cases reported in the lay literature and discussed in the professional journals, mortality rates in adult cattle have approached 60% and calf death losses may approach 100%.[4,5] While the outbreaks in Ontario and Quebec have been associated with type II BVDV isolates,[6] both type I and type II BVDV have been isolated from various severe cases in Pennsylvania and Wisconsin.

The multitude of recent articles concerning BVDV infections has heightened veterinarians' awareness and caused an increase in testing of cattle for BVDV. Coupled with new and/or improved diagnostics, BVDV infections are being diagnosed with greater frequency.[7] The adoption of the quicker and less expensive microplate virus isolation technique[8,9] has opened ways to control

the spread of this disease through testing and culling. BVDV herd eradication programs have become viable options for some cattlemen and are being utilized with increasing frequency.

With the advent of polymerase chain reaction methods, gene sequencing and monoclonal antibody mapping of the gp53 protein, it became apparent that BVDV was not a single virus but two groups of related viruses.[10,11] Added to this was the knowledge that the virus is a highly mutable RNA virus.[12] Each of the various BVDV isolates can cause varying degrees of immunosuppression. This has led to questions concerning the role of BVDV in many different disease syndromes. Important questions have been raised concerning the ability of current vaccines to control the different aspects of this disease.

Although the first modified live BVDV vaccines were licensed in 1959,[13] there has been little advancement in the knowledge of these vaccines. Certainly, manufacturing and safety have improved dramatically as research was applied to new manufacturing techniques. These improvements have enhanced virus growth, purity of the vaccine strains, and better stability of the virus in the finished product.[14]

With the introduction of inactivated BVDV vaccines, BVDV vaccine usage increased as pregnant cows could be safely vaccinated. Since the introduction and initial research with these inactivated vaccines, little vaccine trial work has been done to assess the efficacy and safety of the modified live BVDV vaccines. They

have tended to be forgotten as the "newer" technology, inactivated vaccines became available.

In recent years, the amount of new information on the modified live vaccines has dwindled to only a few papers. This has come at a time when vaccine efficacy and safety have been questioned. Vaccine concerns have included the ability of the vaccines to protect against antigenically distinct strains of the virus, particularly the type divergent from the vaccinal strain. The ability of modified live vaccines to stimulate the immune system enough to afford protection to the developing fetus has also been questioned. Fetal protection has been proposed as the best method to test BVDV vaccine efficacy. The question of the duration of protection stimulated by the modified live BVDV vaccines has also never been assessed.

There are also several questions regarding safety with the modified live BVDV vaccines. The safety of the vaccines in young calves has been questioned since there have been reports of very young calves with unwanted and dangerous responses to modified live vaccines. Vaccinating unbred cattle that are in close proximity to pregnant cattle, with modified live BVDV vaccines is another issue. The risk of transmission of the virus and subsequent potential fetal effects of the vaccine virus has long been a serious concern.

These studies were designed to take the current information on BVDV and apply it to answer some of these valid questions and concerns for the veterinarian

who deals with BVDV problems every day. This practical knowledge is a natural extension of our current understanding of this virus.

8.2 Safety Studies

Before efficacy studies could be started, safety studies were needed to provide background information for the efficacy studies, as well as to answer some pertinent questions. Background information was necessary in order to develop study designs with the appropriate ages at vaccination and proper housing plans. If the modified live BVDV had shed BVDV after administration then the control calves and cows could not have been housed together. These experiments were the first to be performed in this research.

This first study showed that in BVDV sero-negative animals there is little or no transmission of BVDV from animals vaccinated with one of two different modified live vaccines, both containing the cytopathic NADL isolate of the virus. These results suggest that the potential risk from vaccinating animals in close proximity to pregnant animals is low and vaccination programs that include these two modified live BVDV vaccines in this situation would most likely not cause any problems attributable to the transmission of BVD vaccine virus. These results allowed us to do our efficacy studies with our controls and vaccinated cattle in immediate contact with each other without fear of the vaccine conferring some degree of protection to our control unvaccinated cattle. There was no indication of transmission of vaccine origin BVDV in any of our experiments.

The second safety study suggested that administration of vaccines in various combinations to young, often sero-positive calves could stimulate virus-specific antibody responses and did not cause any untoward effects on production parameters. As in many field trials, there was insufficient challenge to assess the efficacy of the vaccines in protecting calves from viral infection. These results indicated that mixed viral vaccines could be used safely in combination with other immunogens in early vaccination programs for calves. This study provided the information for the efficacy studies in the young calves by removing fears of adverse effects of vaccination in the young calves.

8.3 Efficacy Studies

With these studies finished, the efficacy studies began. All four efficacy studies involved type II BVDV and/or type I BVDV strains antigentically divergent from the vaccinal BVDV strain. Three of the 4 studies also involved a BVDV challenge via intranasal administration of the virus. Two of the studies were performed in calves and 2 in mature cattle.

8.3.1 Calf Studies

These studies have shown that an inactivated BVDV type I vaccine can protect young calves from a virulent challenge with type II BVDV. The BVDV type II isolate used in this study produced severe clinical disease that necessitated euthanasia of all control calves. Although calves that had received the

recommended two doses of inactivated vaccine exhibited illness, they did not become as severely ill and were not euthanized.

In contrast to the inactivated vaccines, calves that received the MLV vaccine containing BVDV type I were less affected by this virulent BVDV type II than were susceptible unvaccinated calves. High levels of maternal antibody against type II BVDV afforded protection against the severe challenge as well. The protective mechanism was not determined.

8.3.2 Mature Cattle Studies

Two efficacy studies were also done in mature cattle. The first was a serologic study of BVDV antibody concentrations in a herd for 18 months after vaccination with a modified live BVDV vaccine. The data from that study suggested that modified live BVDV vaccines could stimulate a strong immune response in sero-negative cows that is still detectable eighteen months after vaccination. The study further showed that these antibodies could neutralize antigenically disparate isolates of BVDV for the same 18 months after vaccination. Although this study did not involve a challenge, the concentration and duration of VN antibodies detected suggested that protection against clinical BVD caused by the various strains of BVDV might last at least a year. This duration of antibodies was determined after a single vaccination with a modified live NADL BVDV vaccine was administered to mature sero-negative cattle.

The second was a challenge study using a diverse type I BVDV isolate. The BJ challenge virus used in this study was chosen to represent a type I BVDV

isolate that was different antigenically from the vaccine's NADL BVDV.[15] The modified live vaccine provided a reasonable level of efficacy against a type I BVDV fetal challenge with a protection rate of 83% (2 persistently infected calves out of 12 vaccinated dams).

8.4 Summary

In conclusion, the research results contained in this dissertation have provided the following new pieces of information concerning BVDV and BVDV vaccines.

1. Modified live and inactivated vaccines are safe in calves over 5 days of age.

2. There is little likelihood that modified live NADL BVDV vaccines shed transmissible virus as determined by serologic responses in co-mingled, sero-negative control cattle.

3. Vaccination with a modified live vaccine, or maternal antibody against BVDV, afford a greater degree of protection to calves than administration of an inactivated type I BVDV vaccine when challenged with a type II BVDV.

4. Vaccination of young calves with maternal antibody did not interfere with protection conferred by the maternal antibody. Calves with maternal antibody below 1:64 had a humoral response to BVDV vaccination.

5. A single dose of modified live BVDV vaccine administered to mature sero-negative cattle can stimulate a serologic response that has the ability to neutralize divergent strains of BVDV for 18 months after an initial vaccination.

6. Fetal protection is more difficult to obtain and is a better measure of BVDV vaccine efficacy. The modified live BVDV vaccine provided a higher degree of protection against a fetal infection than what has been reported for inactivated vaccines.

7. Viremia is associated with severe disease in infected cattle.

Although these studies answered some basic and practical questions about BVDV vaccines and immune responses, as with any study, there were more questions raised as the research was finished. Studies are needed to further define the nature and limitations of the protective immune responses elicited by cattle in response to BVDV infection.

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