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Environmental Sampling Techniques for Herd-Level Surveillance of Bovine Viral Diarrhea Virus

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ENVIRONMENTAL SAMPLING TECHNIQUES FOR HERD-LEVEL SURVEILLANCE OF BOVINE VIRAL DIARRHEA VIRUS

by

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ENVIRONMENTAL SAMPLING TECHNIQUES FOR HERD-LEVEL SURVEILLANCE OF BOVINE VIRAL DIARRHEA VIRUS

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Control of Bovine Viral Diarrhea Virus (BVDV) relies on resource-intensive individual animal sampling to detect and remove persistently infected (PI) cattle. Herd-level surveillance tools would be useful for herds with unknown BVDV status and for monitoring herds with BVDV-free status. The overall objective of this thesis is to explore the viability of BVDV surveillance at a herd-level using samples collected without handling individual animals. The first objective was to determine the feasibility of using stable flies as a sampling tool to detect BVDV. The second objective was to determine the feasibility of using drinking water to detect BVDV. To accomplish the first objective, pools of stable flies were produced with various quantities of BVDV-fed and BVDV-free flies and were harvested 1-3 days after being fed blood from BVDV-PI calves. To accomplish the second objective, drinking water samples from pens with and without BVDV-PI calves were collected, processed through centrifugal filtration, and analyzed using RT-PCR. BVDV was consistently detected 1-day post exposure when ≥ 10% of the pooled flies were exposed to BVDV-PI blood. Polymerase chain reaction analysis of filtration concentrated drinking water samples resulted in RT-PCR amplification in pens with confirmed and suspect PI cases. The results of this research indicate that both stable

fly and drinking water sampling may be useful surveillance tools to monitor herds for BVDV; however, field-based research is needed to validate these techniques.

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CHAPTER I: REVIEW OF THE LITERATURE

History of Bovine Viral Diarrhea Virus

The first report of a newly emerging disease, now known to be caused by Bovine Viral Diarrhea Virus (BVDV), was published in 1946 by Cornell University researchers in a dairy cattle herd in Ithaca, New York.[27] Clinical signs included dehydration, diarrhea, anorexia, abortions, congenital defects, nasal discharge, and severe leukopenia. The disease quickly spread to five surrounding herds with morbidity ranging from 33-88% and mortality ranging from 4-8%. Milk production dropped substantially and abortions occurred 10 days to 3 months post initial infection. Blood transfusions were the treatment of choice but were proven ineffective since the seemingly healthy cows often had more severe leukopenia than the clinically affected animals, suggesting that BVDV infections could also result in subclinical disease.

A similar, but more severe, report of "X Disease" occurred in Saskatchewan, Canada that same year.[4] This outbreak mainly affected young calves and clinical signs included fever, anorexia, watery and bloody diarrhea, skin lesions, and gastric fluid accumulation. In a review article, these Canadian infections were thought to be the first report of mucosal disease (MD).[33] Mucosal disease was given its name in 1953 from an outbreak in Iowa and was thought to be different from BVDV due to the increased severity of gastrointestinal lesions, lower morbidity with high case-fatality rates, and a failure to experimentally transmit disease from affected to naïve animals.[35] In the late 1950s and early 1960s, the discovery of cytopathic BVDV and MD-associated strains led to the development of serum neutralization tests that are still widely used today.[7,48]

Studies using virus neutralization tests to compare BVDV and MD-associated strains led to the conclusion that the viral agents were similar but caused different manifestations of the disease and was later called the bovine viral diarrhea-mucosal disease complex (BVD-MD).[8,16,17,46]

As the effects of BVDV became more widespread, the cattle biologics industry was prompted to mass produce modified-live vaccines.[30] These vaccines caused postvaccinal MD (pvMD) in a small number of animals within herds. Eventually, it was discovered that this pvMD phenomena only occurred in persistently infected (PI) cattle, which lead to the implementation of various eradication and control programs worldwide.[22,36]

Another highly virulent outbreak, termed hemorrhagic syndrome, occurred in North America in the late 1980s and early 1990s.[2] The BVDV strains isolated from this outbreak was grouped separately based on the genotypes from the original strains commonly found in laboratory research and vaccine production.[29] This newly recognized BVDV group was coined BVDV Type 2, while the classical strains were termed BVDV Type 1.[11,37]

Virus Taxonomy

BVDV was originally classified in the family *Togaviridae*.[5] However, the viruses were smaller in size (45 nm) compared to the togaviruses (50-70 nm) and had many differences in viral structure, replication strategy, and gene organization.[50] Therefore, the new family *Flaviviridae* was formed and approved in 1984.

Within the *Flaviviridae* family, there are four genera; *Flavivirus, Hepacivirus, Pegivirus,* and *Pestivirus*. *Bovine Viral Diarrhea Viruses* belong to the *Pestivirus* genus, which are characterized as small enveloped, single-stranded positive sense RNA viruses.[12] There are two distinct BVDV species, BVDV Type 1 and BVDV Type 2, along with Border disease virus and classical swine fever virus (formally hog cholera virus) in the *Pestivirus* genus.[24]

Strains from both BVDV Type 1 and BVDV Type 2 genotypes are also classified as one of two biotypes, cytopathic and noncytopathic. Cytopathic BVDV damages cells in cell culture, does not cause persistent infections, and is frequently used in the production of modified-live vaccines.[31] Noncytopathic BVDV does not damage infected cells, can cause persistent infections, and is more commonly found in nature. BVDV is immunosuppressive, which makes co-infections with other diseases quite common.[32]

Pathogenesis

The time of infection manifests two patterns of the disease. Postnatal acute infections result in animals that shed the virus transiently and the immune system typically clears the infection within two to three weeks. A congenital infection of a noncytopathic strain acquired between days 30 and 125 of gestation produces a persistently infected fetus which will experience lifelong shedding of the virus.[31,34] The immune system of the fetus is not fully developed at this stage of gestation and in turn recognizes the virus as “self.” Therefore, an immune response is not produced, and the calf remains seronegative to BVDV unless infected again after birth with a different

strain. Persistent infections only occur via transplacental transmission of BVDV virus to the calf, either from a transiently infected dam or from a PI dam.

The main source of BVDV transmission comes from direct contact with PI animals since they shed large viral loads into the environment throughout their entire lifespan.[44,47] Other routes of BVDV transmission include saliva, semen, embryo transfer, insects, and fomites such as needles, palpation sleeves, and halters, etc.[1,10,18,23,28,34,45]

Economic Impact and Control Programs

BVDV infections have made a significant economic impact on the livestock industry through the loss of milk production, conception rates, abortions, immune suppression causing co-infections with other pathogens, poor growth, and death of infected animals.[13,51] Actual calculations of direct economic losses in individual herds range from a few hundred to several thousand dollars for costs of treatment, labor, poor growth, and lost animals.[13] At the population level, the economic loss has been estimated to be between $10-40 million per million calvings.[13] An overwhelming majority (87.7%) of cow-calf producers in the United States believe that BVDV is a problem, yet only 4.2% of those surveyed in 2007-2008 had tested any calves for PI in the past three years.[25] Many U.S. beef producers have turned to using BVDV vaccines as a control measure, and while most protect against the effects of BVDV infection, adverse events have been documented.[6,20,25] Other control methods include heightened biosecurity to prevent BVDV from entering herds as well as biocontainment that includes testing and removal of PI animals to minimize challenge.[9,15,19,43]

Diagnostic Tests

There are two forms of detection used for diagnostic testing; immune response detection and pathogen detection. Control programs that limit the use of vaccination have the advantage of using serology to detect immune responses, usually BVDV-specific antibodies, as evidence of infection.[14,38] These programs use a combination of herd-level screening and individual testing to identify and control herds with active BVDV infections. Antibodies against BVDV can cross-react with other species in the *Pestivirus* genus, which allows detection of infection regardless of the specific strain.[38] Also, serum antibody responses to BVDV in sentinel calves can be monitored instead of testing all animals in the herd.[21] Another method for herd-level surveillance is to test for pre-colostral, BVDV-specific, serum antibodies.[41] While this strategy is effective at avoiding maternal antibody and vaccination confounding, these samples are difficult to obtain for many cow-calf producers. The last tool used in immune response diagnostics is testing non-serum samples, such as bulk tank milk.[40]

An estimated 80% of U.S. cattle have been vaccinated with a killed or modified-live vaccine containing a BVDV component.[25,49] Consequently, the prevalence of BVDV seropositive animals is high and the serological testing strategies used in many control programs cannot distinguish between immune responses from the vaccination or infection.[49] Therefore, identifying the presence of the pathogen is useful in vaccinated herds. This is carried out by either protein detection or nucleic acid detection. Immunohistochemistry (IHC) and antigen-capture enzyme-linked immunosorbent assays (ELISAs) are the two most common tests used for protein detection while reverse-

transcriptase polymerase chain reaction (RT-PCR) is used for detection of nucleic acid.[26,39,42] These tests require individual sampling of all at-risk animals, as well as testing a few weeks later to confirm actual PI status and rule out a transient infection. This can become quite costly and labor intensive.

Knowledge Gaps

Many U.S. beef producers do not test for PI animals due to a lack of effective herd-level surveillance tools that are cost effective and simple to fit into their production systems.[49] In order to decrease the effects of BVDV infection worldwide, herd-level diagnostics are needed for the cattle industry to utilize.

Environmental sampling techniques are used in diagnosing many diseases affecting cattle, including manure/soil samples, plant/feed samples, dust/air quality samples, as well as fomites, insects, and water samples. Specifically for BVDV, contact with a PI animal is the most common way for transmission. However, since PI animals shed such a large viral load into the environment, it's logical that the likelihood of transmission to susceptible animals could occur in many environments without direct contact.[49]

Several studies have looked at insects as a path for transmission of BVDV to susceptible animals. In one study, BVDV was detected in stable flies (*Stomoxys calcitrans*), horseflies (*Haematopota pluvialis*), and head flies (*Hydrotaea irritans*) after feeding on a PI animal.[45] Virus was isolated from susceptible animals that had been exposed to those stable flies and horseflies, indicating successful transmission.[45] Another

study isolated BVDV from face flies (*Musca autumnalis*) after feeding on the ocular secretions of a PI animal.[10] The third study detected BVDV in horn flies (*Haematobia irritans*) collected from PI cattle, but all naïve calves remained negative after being exposed to the horn flies found on PI cattle.[3] These studies looked at possible transmission routes using vectors, and this gives us evidence that fly sampling could be useful as a herd-level BVDV surveillance tool. Therefore, the objective of Chapter II of this thesis is to determine the feasibility of using stable flies as a sampling tool to detect BVDV. We hypothesized that BVDV RNA would be detectable in stable flies that had fed on blood from BVDV-PI calves.

It's assumed that BVDV is regularly shed from mucus membranes, yet specific research in this area is scarce. Brief reference to watering facilities as a source of transmission can be found but diagnostic testing of drinking water for BVDV detection has not been extensively researched.[34] Therefore, the objective of Chapter III of this thesis is to determine the feasibility of using drinking water to detect BVDV. We hypothesized that BVDV RNA would be detectable in the drinking water of pens holding PI cattle.

BVDV in general has been researched extensively, yet it is still an important problem in today's livestock industry. In order to diminish the effects of the disease, diagnostic tools need to be developed that can be used at the herd-level and are cost-effective and compatible in U.S. cattle production systems. The aim of the research presented in this thesis is to fill these knowledge gaps and offer diagnostic tools that could be used for BVDV surveillance.

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CHAPTER II: DETECTION OF BOVINE VIRAL DIARRHEA VIRUS IN STABLE FLIES (*STOMOXYS CALCITRANS)* FOLLOWING CONSUMPTION OF BLOOD FROM PERSISTENTLY INFECTED CATTLE

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Running head: Detection of BVDV in stable flies

Bovine Viral Diarrhea Virus (BVDV) control relies on resource-intensive sampling to detect and remove persistently infected (PI) cattle. Herd-level surveillance tools would be useful for herds with unknown BVDV status and for monitoring herds with BVDV-free status. Our objective was to determine the feasibility of using stable flies as a sampling tool to detect BVDV at the herd-level. Stable flies were fed blood from either BVDV-PI or BVDV-free cattle to establish pools of 100 flies with variable quantities of BVDV-fed flies (1%, 10%, 20%, 40%, or 100% in each pool). BVDV-fed flies in these pools were harvested either 1, 2, or 3 days after consuming BVDV-PI blood to determine the impact of time after feeding. Two replicates of a three-day by five-dilution level matrix were produced. BVDV RNA was consistently detected on day 1 when ≥ 10% of the flies in the pool consumed PI blood. On days 2 and 3, positive BVDV RNA detection was variable and became less consistent. These results demonstrate that BVDV RNA can be detected in stable flies after feeding on blood from PI cattle. Further research is needed to assess whether stable fly surveillance of BVDV is useful in field applications.

Bovine viral diarrhea virus (BVDV) is an important, prevalent pathogen of cattle around the world.[5,8] An estimated 91% of U.S. cow/calf operations have at least one seropositive animal as a result of vaccination, maternal antibody, or exposure.[6] Calves infected with BVDV during the first trimester of gestation become persistently infected (PI). These calves shed BVDV for their entire lives and serve as the most important source of new infections in cattle.[7] Thus, effective disease control relies on detecting and removing BVDV-PI cattle from herds.[3] Identifying herds harboring BVDV-PI cattle currently requires resource-intensive, individual animal sampling, followed by individual or pooled testing.[10] True herd-level surveillance strategies for beef herds rely on evidence of seroconversion and is not feasible in vaccinated herds. Consequently, routine surveillance for BVDV is not commonly conducted. Developing methods to classify BVDV status at a herd-level without the need to handle the cattle would remove many obstacles that limit adoption of BVDV control. Previous research has shown that stable flies (*Stomoxys calcitrans*) can harbor the virus for at least 96 hours after feeding on an infected animal and can transmit infections to susceptible animals.[9] The objective of our research was to determine the feasibility of using stable flies as a sampling tool to detect BVDV RNA in cattle herds. We hypothesized that BVDV RNA would be detectable in stable flies that had fed on blood from BVDV-PI calves.

Stable fly pupae were transported from the West Central Research and Extension Center (North Platte, NE) to the Great Plains Veterinary Educational Center (University of Nebraska-Lincoln, Clay Center, NE) and placed in mesh-covered cages at room temperature. Approximately 1000 flies emerged during a two- to five-day period while

being continuously exposed to a sanitary napkin on the top of the cage each day saturated with 20 ml of citrated whole blood from BVDV-PI calves (provided by the Department of Pathobiology, Auburn University, Auburn, AL). Blood samples from several BVDV-PI calves were collected, shipped on ice, and then frozen at -20°C. At the beginning of each experimental replicate, blood was thawed for feeding to the flies and stored at 4°C until the replicate was completed. One aliquot of blood used during each replicate was stored at -80°C for use as a positive control for RT-PCR analysis. Flies were briefly chilled (<5 minutes) to immobilize them and facilitate handling. Unhatched pupae were moved to a separate cage to hatch control (BVDV-free) flies. After the non-eclosed pupae were removed, flies in the BVDV-fed cage were offered blood from BVDV-PI calves for an additional 24 hours. The day after BVDV-fed and BVDV-free fly populations were separated was considered day 1 and both cages received BVDV-free blood for the remainder of the study. Each day from day 1 to 3, enough BVDV-fed and BVDV-free flies were collected to establish the following pools (percentage of BVDV-fed flies out of a 100 total flies per pool): 0%, 1%, 10%, 20%, 40%, 100%. Each pool of 100 flies was suspended in 2 ml of PBS and homogenized using tissue dissociation tubes (gentleMACS M Tubes, Miltenyi Biotec, Auburn, CA). Homogenates were clarified by centrifuging for 5 minutes at 1500 x *g* and 140 µl of the supernatant was used for RNA extraction using a spin-column viral RNA isolation kit per the manufacturer's instructions (QIAamp Viral RNA Mini Kit, Qiagen, Germantown, MD). The process of hatching, feeding, and collecting flies was repeated to obtain two complete replicates of the three day by five dilution level matrix.

Reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out on a real-time PCR thermal cycler and detector (CFX96 Touch Real-Time PCR Detection System, Bio-Rad Laboratories, Hercules, CA) using a kit according to the manufacturer's instructions (OneStep RT-PCR Kit, Qiagen). Each 25 µl of RT-PCR reaction contained 1X reaction buffer, 1 µl of enzyme solution, 400 µM of NTP, 0.4µM each of forward and reverse primers (5'- GGG NAG TCG TCA RTG GTT CG-3'and 5'- GTG CCA TGT ACA GCA GAG WTT TT-3', respectively)[4], 0.1µM of oligonucleotide probe (5'-/56-FAM/CCA YGT GGA CGA GGG CAY GC/3BHQ_1/-3') and 0.005µM of magnesium. The reverse transcription step was carried out at 50°C for 30 min, followed by a 15 min PCR activation step at 95°C, and then 40 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The final step was a 5 min extension at 72°C. Each extracted RNA sample was assayed in duplicate on the same plate. Each experimental feeding trial replicate had a positive control comprised of RNA extracted from the blood from the BVDV-PI calves and multiple negative controls including RNA extracted from the 0% PI flies, RNA extracted from the BVDV-free blood, and nuclease free water. Results were analyzed on a PC with software provided by the manufacturer according to their instruction (CFX Manager version 3.1, Bio-Rad). Relative fluorescence units (RFU) were examined for the negative controls and a single threshold line at 150 RFU was set to classify all negative controls as negative, including the fly pools with BVDV-free flies. Experimental samples failing to exceed the 150 RFU threshold line were classified as negative. A quantification cycle (Cq) value was provided when any sample exceeded the 150 RFU threshold line.

Results showed that BVDV RNA was readily detected by RT-PCR in fly pools with at least 10% exposed flies immediately after their last 24-hour exposure to BVDV-PI blood (Figure 2.1). BVDV RNA was detected in all pools except the 20% pool on Day 2 (Table 2.1). On Day 3, the 100% pool yielded positive results, while the other pools were either not detectable or had only one duplicate classified as positive. No amplification of BVDV RNA was detected in any of the negative controls in either replicate. See Figure 2.1 for representative amplification curves.

We failed to reject our hypothesis that BVDV RNA would be detectable in stable flies after feeding on blood from PI calves. These results are consistent with a previous report from Tarry et al. in which flies allowed to feed directly from a PI calf were able to transmit BVDV to naïve cattle and BVDV remained detectable by virus isolation for 96 hours following feeding on a PI calf.[9] In Tarry et al.'s study, all flies used to isolate BVDV had been exposed to a PI calf; however, in a field situation, the mostly likely scenario would be one in which only a few flies of the total population captured would have fed on any PI cattle present. The prevalence of PI cattle has been estimated to be between 0.5% and 2%, although the number of PI animals within a herd can be variable.[2,11] Therefore, we examined the impact of both diluting BVDV-fed flies with BVDV-free flies and the effect of feeding BVDV-free blood to BVDV-fed flies over the course of three days. While this research provides evidence that BVDV RNA can be detected in stable flies exposed to blood from BVDV-PI cattle, successful use of stable flies as a surveillance tool will depend on validation under field conditions.

Substantial variation across replicates, days, and pools was noted. While many sources of variation could contribute, one notable source could be the feeding status of the flies. Flies in both the BVDV-fed group and the group fed only BVDV-free blood were offered the appropriate type of blood. In the context of this experiment, flies offered the opportunity to feed on blood were assumed to have done so. No attempt was made to confirm the flies that had been offered blood actually consumed it. In the 1% pools, whether or not the particular fly selected for the pool had actually consumed blood was not known and the feeding status of the fly may have impacted our results. In the larger pools, this explanation is less likely as a significant number of flies would have had to forgo blood meals to generate the results seen. Specifically, the results from the 20% pools on days 2 and 3 were anomalous in that those pools appear to have less BVDV RNA amplification than more dilute pools. The reason for these anomalous results is unknown. Overall, the feeding status of the flies included may have impacted the presented results. In any case, any use of stable flies as an effective surveillance tool will depend on identifying and managing sources of variation.

Our results indicate that, generally, both dilution of BVDV-fed flies with BVDV-free flies and time after feeding increased the number of RT-PCR cycles needed to detect BVDV RNA. The dilution of BVDV resulting from lower numbers of BVDV-fed flies can be expected to have a direct effect on the cycle quantification values for the PCR assay. The delay associated with flies tested 1, 2, or 3 days after their last exposure to BVDV-PI blood may have increased cycle quantification values either by degradation of viral RNA or through dilution associated with consuming additional blood from BVDV-

free cattle. Given our results, detecting BVDV RNA in stable flies that have fed on a PI calf may be most probable immediately following the feeding event. Sensitivity of the technique may be better under field conditions if flies can be captured immediately after feeding owing to the fact that a 24-hour delay existed between the final PI blood meal and PCR testing. These findings are consistent with a 2011 study where BVDV was found in horn fly (*Haematobia irritans*) homogenates for up to 48 hours after collection from PI calves.[1]

The evidence from this study confirms that BVDV RNA is detectable in stable flies that have consumed blood from PI cattle and indicates potential utility of stable flies as a surveillance tool for BVDV at a herd level. More research is needed to determine the feasibility of employing this surveillance strategy under field conditions.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. The use of product and company names is necessary to accurately report the methods and results; however, the United States Department of Agriculture (USDA) neither guarantees nor warrants the standard of the products. The use of names by the USDA implies no approval of the product to the exclusion of others that may also be suitable. The USDA is an equal opportunity provider and employer.

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Tables

Table 2.1. Summary RT-PCR quantification cycle data from two replicates of a three-day by five-dilution level matrix of stable fly homogenates.

RT-PCR Results			
Number of exposed flies in a 100-fly pool	Day 1	Day 2	Day 3
0	ND	ND	ND
1	ND	36.7±0.3	37.8†
10	37.5±0.5*	37.1±0.4	39.2†
20	35.9±0.2	ND	ND
40	35.0±0.3	37.4±0.4	38.4†
100	33.4±0.4	36.0±0.3	33.2±0.5
Positive Control	30.1±1.6	-	-
Negative Control	ND	-	-

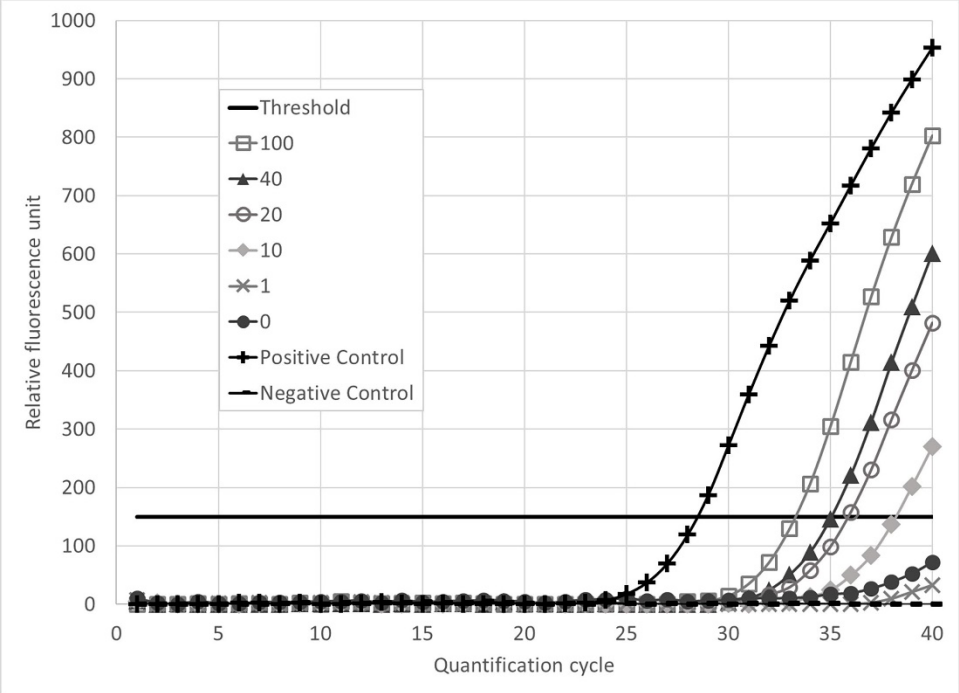
ND = Not Detectable

* Mean Cq value ± SEM.

† These results had only one duplicate that exceeded the RFU threshold, therefore a standard error of the mean could not be calculated.

Figures

Figure 2.1. Representative RT-PCR quantification cycle data on Day 1 from two replicates of a three-day by five-dilution level matrix of stable fly homogenates.



CHAPTER III: INVESTIGATION OF BOVINE VIRAL DIARRHEA VIRUS IN DRINKING WATER SAMPLES FROM PENS WITH AND WITHOUT PERSISTENTLY INFECTED CATTLE

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Detection and removal of persistently infected (PI) cattle is essential for controlling Bovine Viral Diarrhea Virus (BVDV). Current diagnostic techniques rely on resource-intensive individual sampling, and consequently, use of BVDV diagnostics is sparse in U.S. beef production systems. Environmental sampling could be useful as a surveillance tool for monitoring BVDV without the need for handling individual animals. Our objective was to determine the feasibility of using drinking water as a sampling tool for BVDV detection at the herd-level. Drinking water samples from pens with and without PI cattle were collected. Samples were passed through vacuum and centrifugal filtration systems before RNA extraction and RT-PCR was conducted. BVDV RNA was detected in drinking water samples from a pen holding approximately 25 PI cattle. BVDV RNA was also detected in a drinking water sample from a shared water tank with one pen holding approximately 5 suspect PI cattle and another pen holding approximately 200 BVDV-free cattle. BVDV RNA was not detected in any drinking water samples from pens holding BVDV-free cattle. These results conclude that BVDV RNA detection in drinking water samples could be useful as a herd-level sampling technique without the need to handle individual animals. Further research is needed to assess the sensitivity and specificity of our technique in field situations.

Bovine Viral Diarrhea Virus (BVDV) is an economically important *Pestivirus* affecting livestock species throughout the world.[3] Common clinical signs include abortions, stillbirths, congenital defects, decreased milk production, fever, diarrhea, and immune suppression. BVDV can manifest as either a transient infection or as a persistent infection. Transient infections can occur in all ages of cattle, but the immune system usually clears the infection in 2 to 3 weeks.[7] Persistent infections are lifelong and occur from congenital infections in the first four months of gestation. PI animals shed large viral loads into the environment and are the main source of new infections in the herd, so consequently, detecting and removing these PIs are essential for effective control of BVDV.[9]

There are two fundamental principles for detecting BVDV: immune response detection and pathogen detection. Serology testing strategies are used to detect BVDV-specific antibodies in herds of cattle that haven't had an immune response as a result of vaccination. However, an estimated 80% of cattle in the U.S. are vaccinated with a BVDV vaccine, therefore, serology testing to detect an immune response is confounded by the presence of vaccine-induced serum antibody.[6,8] In order to differentiate between infected and vaccinated animals, pathogen detection testing strategies are commonly employed. Specifically, immunohistochemistry (IHC) and enzyme-linked immunosorbent assays (ELISAs) are used in protein detection while reverse-transcriptase polymerase chain reaction (RT-PCR) is useful in detecting viral nucleic acids.[8] Current strategies for PI detection in beef herds include sampling individual animals for individual or pooled

diagnostic testing.[4] In order for U.S. beef producers to adopt BVDV control systems, reliable and economical herd-level surveillance techniques need to be developed.[4]

It is well known that waterborne transmission of pathogens in livestock is common, especially in cases involving other *Pestiviruses*, like classical swine fever virus (formally hog cholera virus).[1,2,5] The objective of our research was to determine the feasibility of using drinking water as a sampling tool for BVDV RNA detection at the herd-level. We hypothesized that BVDV RNA would be detectable in the drinking water of pens holding PI cattle.

To establish proof of concept, we first spiked 250 ml of tap water with 1 ml of blood collected from a PI calf. This spiked mixture was passed through a .22 µm sterile vacuum filtration system (MilliporeSigma™ Stericup Quick Release-GP Sterile Vacuum Filtration System, Thermo Fisher Scientific, Waltham, MA) with a vacuum of 10 inches of Hg for 3 min to clarify the sample. The filtered material was stored at -80°C after 76 ml was extracted and allocated into 15 ml centrifugal filter units of varying membrane sizes (100 KD, 50 KD, 30 KD, 10 KD, and 3 KD) (MilliporeSigma™ Amicon™ Ultra Centrifugal Filter Units, Thermo Fisher Scientific) to determine which size would work best to retain BVDV RNA. Per the manufacturer's instructions, all centrifugal filter units were spun at max speed (3280 x g) and removed from the centrifuge at different times (50 KD at 15 min, 30 and 10 KD at 20 min, 100 KD at 30 min, and 3 KD at 45 min) to reach a final retentate volume of approximately 200 µl. Samples collected for RNA extraction and RT-PCR included tap water, PI blood, spiked virus, .22 µm filtrate, and all retentates and filtrates from the differing centrifugal filter units. RNA extraction was

accomplished by using 140 µl of each sample and a spin-column viral RNA isolation kit per the manufacturer's instructions (QIAamp Viral RNA Mini Kit, Qiagen, Germantown, MD). All samples were extracted in duplicate except for the retentates of the centrifugal filter units since sufficient sample was available to do only one extraction each.

Reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out on a real-time PCR thermal cycler and detector (CFX96 Touch Real-Time PCR Detection System, Bio-Rad Laboratories, Hercules, CA) using a kit according to the manufacturer's instructions (OneStep RT-PCR Kit, Qiagen). Each 25 µl of RT-PCR reaction contained 1X reaction buffer, 1 µl of enzyme solution, 400 µM of NTP, 0.4µM each of forward and reverse primers (5'- GGG NAG TCG TCA RTG GTT CG-3'and 5'- GTG CCA TGT ACA GCA GAG WTT TT-3', respectively)4, 0.1µM of oligonucleotide probe (5'-/56-FAM/CCA YGT GGA CGA GGG CAY GC/3BHQ_1/-3') and 0.005µM of magnesium. The reverse transcription step was carried out at 50°C for 30 min, followed by a 15 min PCR activation step at 95°C, and then 40 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The final step was a 5 min extension at 72°C. Each extracted RNA sample was assayed in duplicate on the same plate.

Drinking water samples from multiple pens were collected at a stocker cattle operation in Missouri before being transported back to the Great Plains Veterinary Educational Center in Clay Center, NE. This stocker cattle operation tests all animals considered to be at high risk for BVDV infections, separates the positive animals, and retests them > 2 weeks later to confirm PI status. If the second test also comes back as positive, those animals are moved to an isolated pen, fed to market weight, and sold

directly to a beef processor. Drinking water samples were collected from different pen types and water sources, including: a confirmed PI pen holding approximately 25 calves separate from any other animals, a pen holding approximately 5 suspect PI cases that were to be retested for PI confirmation that shared a water tank with an adjacent pen holding approximately 200 newly arrived cattle, and several pens with cattle that had been tested and contained no BVDV PIs.

Different sampling techniques were used, including skimming the top of the water to capture the floating mucus from saliva, as well as stirring the water to capture the sunken material. Drinking water samples were stored at 4°C overnight before being processed through the filtration systems. 100 ml of each water sample was passed through individual .22 µm sterile vacuum filtration systems with a vacuum of 10 inches of Hg for 3 min to remove large particles like algae and bacteria. The filtered material was stored at -80°C after 16 ml was extracted and allocated into either the 50 KD centrifugal filter unit (15 ml) or stored at -80°C for RNA extraction and RT-PCR analysis (1 ml). Per the manufacturer's instructions, the centrifugal filter units were spun at max speed (3280 x g) for 15 min. Viral RNA extraction and RT-PCR was also performed as previously described. Blood from a PI calf served as the positive control while tap water served as the negative control.

Results of the proof of concept experiment showed that BVDV RNA in the spiked water sample was detected in all sizes of the centrifugal filter unit retentates (Table 3.1). However, the 50 KD centrifugal filter unit offered an optimal balance between throughput and efficacy since it appeared to concentrate BVDV RNA most effectively

(lowest average Cq value) and took the least amount of time in the centrifuge. Therefore, this size filter was used for all of the field samples.

In the drinking water samples, BVDV RNA was detected in the PI pen samples, the suspect cases pen sample, the spiked water sample, and the positive control (Table 3.2). BVDV RNA was not detected in any of the pens that did not contain PI animals or the negative control (Table 3.2).

We failed to reject our hypothesis that BVDV RNA would be detectable in the drinking water of pens holding PI cattle. This is not surprising since PI animals shed a large viral load into the environment, including water troughs. The skimmed sample from the PI pen yielded more BVDV RNA detected than the stirred PI sample and the spiked virus water. This result shows that skimming the top of the water to pick up any floating mucus from saliva may result in improved diagnostic sensitivity compared to stirring the water to capture any sunken material.

In addition, the sample from the water trough that was shared between a pen holding approximately 5 suspect PI cattle and a pen holding approximately 200 newly arrived cattle was positive. This result shows that the technique could be used to identify pens holding a few infected animals while being diluted out by approximately 200 other animals.

Two 50 KD filtrate samples (PI-Skimmed and PI Free Pen 4) were positive, which could be due to a number of different scenarios. Pipetting error or other contamination in either the extraction or RT-PCR steps could be one possibility for these

results. Also, the 50 KD filter could have let small fragments of BVDV RNA through the membrane. Repeated experiments are necessary to clarify the results found here.

Overall, the results from this study confirms that BVDV RNA is detectable in drinking water from pens with PI cattle present and indicates potential use of drinking water as a sampling tool for BVDV surveillance at a herd-level without needing to handle individual animals. More research is needed to determine the sensitivity and specificity of this surveillance strategy under differing field conditions.

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Tables

Table 3.1. Summary RT-PCR quantification cycle data from the centrifugal filter unit experimental trial.

	RT-PCR Results		
	Average Cq*	Average End RFU†	Centrifuge Time
Positive Control	25.0	1815.7	-
Negative Control	0.0	-3.9	-
Spiked Virus Water	32.0	759.6	-
.22 µm Filtrate	32.8	633.1	-
100 KD Retentate	25.9	1748.4	30 min
100 KD Filtrate	0.0	3.9	
50 KD Retentate	25.2	1735.3	15 min
50 KD Filtrate	0.0	-1.0	
30 KD Retentate	26.0	1823.5	20 min
30 KD Filtrate	0.0	-1.5	
10 KD Retentate	26.0	1479.8	20 min
10 KD Filtrate	0.0	-0.5	
3 KD Retentate	26.4	1554.7	45 min
3 KD Filtrate	0.0	-0.4	

*Average quantification cycle values

†Average relative fluorescence unit values at the end of 40 cycles

Table 3.2. Summary RT-PCR quantification cycle data from the drinking water experimental trial.

	RT-PCR Results			
	Original Sample	.22 µm Filtrate	50 KD Retentate	50 KD Filtrate
PI-Skimmed	28.2*	30.4	25.8	39.8
PI-Stirred	35.0	36.2	31.9	0.0
Suspect PIs	36.6	35.4	35.5	0.0
PI Free Pen 1	0.0	0.0	0.0	0.0
PI Free Pen 2	0.0	0.0	0.0	0.0
PI Free Pen 3	0.0	0.0	0.0	0.0
PI Free Pen 4	0.0	0.0	0.0	30.5
Spiked Virus Water	30.6	34.8	26.2	0.0
Positive Control	28.4	-	-	-
Negative Control	0.0	-	-	-

*Average quantification cycle values

CHAPTER IV: CONCLUSIONS

The first objective of the research in this thesis was to determine the feasibility of using stable flies as a sampling tool to detect BVDV. Several studies have examined the ability of mechanical vectors to transmit the virus to susceptible animals, but none have explored the viability of using insects as a sampling tool for BVDV. In this study, BVDV RNA was detected by RT-PCR in stable fly homogenates after feeding on blood from PI calves. Some variability was found in the results, so further research is needed to determine the viability of utilizing this test in field situations.

The second objective of the research in this thesis was to determine the feasibility of using drinking water as a sampling medium to detect BVDV. BVDV RNA was detected by RT-PCR from drinking water samples in pens with confirmed PI animals as well as in a shared water tank between possible suspect PI calves and newly arrived cattle. This is evidence that drinking water could be useful for BVDV surveillance, and further research is needed to test the sensitivity and specificity of the test in field situations.