

EMERGING AND REEMERGING DISEASES OF TEXAS BEEF CATTLE

A Dissertation

by

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## ABSTRACT

This dissertation focused on three emerging/reemerging diseases posing economic impacts on Texas livestock producers. Bovine trichomoniasis is a regulated disease requiring diagnostic testing; however, current diagnostic protocols are problematic. Major obstacles resulting from variable collection procedures and discrepancies in sample handling and laboratory test accuracy need further investigation. Epizootic hemorrhagic disease primarily affects white-tailed deer; however, it is unclear why clinical disease is rarely exhibited in cattle in the same region, requiring the investigation of seroprevalence in Texas cattle. Bovine viral diarrhea (BVD) causes significant reproductive loss and complicates other diseases through immunosuppression. Although vaccination is the primary method of mitigating fetal infection, a systematic review assessing fetal protection from vaccination is needed.

Methods to examine the collection, shipment, and diagnostics associated with bovine trichomoniasis included (1) testing of infected bulls for sample quality and testing accuracy related to time, collectors, and individual bulls; (2) evaluation of samples with temperature sensors in a controlled environment when shipped by common carrier; and (3) evaluation of a patented polymerase chain reaction (PCR) technique for expedient sample handling and improved diagnostic sensitivity. Methods to evaluate prevalence of epizootic hemorrhagic disease in Texas cattle were based on random blood sera collections from 11 auction markets. Methods to assess safety and efficacy of BVD

vaccines for fetal protection were based on a systematic review of the scientific literature.

There was little variation in bovine trichomoniasis test results due to collector or bull, indicating proper and standardized sample collection protocol increased test accuracy. Shipping samples in temperature-controlled containers to arrive at the laboratory within 24 hours also improved diagnostic accuracy. The newly patented PCR test exhibited 100% diagnostic sensitivity and 99% specificity for field samples from 56 positive and 110 negative bulls for improved test accuracy.

A high seroprevalence of epizootic hemorrhagic disease (70% to 97% depending on different threshold titer positive cutoff values) was seen in Texas auction cattle, but clinical disease is rare.

Much of the scientific literature dealing with BVD supports vaccination for fetal protection but lacks transparency regarding experimental design, creating a potential for bias and making evaluation of these studies difficult.

## DEDICATION

I dedicate this dissertation to my wife Peggy Thigpen Hairgrove. You are my best friend and strongest critic and I have truly been blessed because you have chosen to share your life with me.

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# CHAPTER I

## INTRODUCTION

This dissertation focuses on different aspects of 3 emerging or reemerging diseases affecting Texas cattle producers, bovine trichomoniasis, bovine viral diarrhea virus, and epizootic hemorrhagic disease. An emerging or reemerging disease is defined as “infection that has newly appeared in a population or has existed but is rapidly increasing in incidence or geographic range (Morse, 1995). Understanding the epidemiology of these diseases and developing management protocols must be based on evidenced based information not just past experiences and “common sense.” Cockcroft and Holmes (2003) define evidence-based medicine as “the use of current best evidence in making clinical decisions,” and this includes searching the current literature, not relying on an old textbook. Evidence based decisions must be based on the knowledge and experience of the veterinary practitioner, the needs of the patient (producer and animals), and the best external evidence as expressed in Figure 1.1. Published peer reviewed manuscripts must be critically evaluated for the accuracy and applicability of the information. A representation of the hierarchy of information is depicted in Figure 1.2 with systematic reviews representing the most reliable quality of external evidence while expert opinions and case reports provide the least reliable evidence.



Figure 1.1. Evidence-based medicine is the integration of best research evidence with clinical expertise and patient value (Sackett et al., 2000).

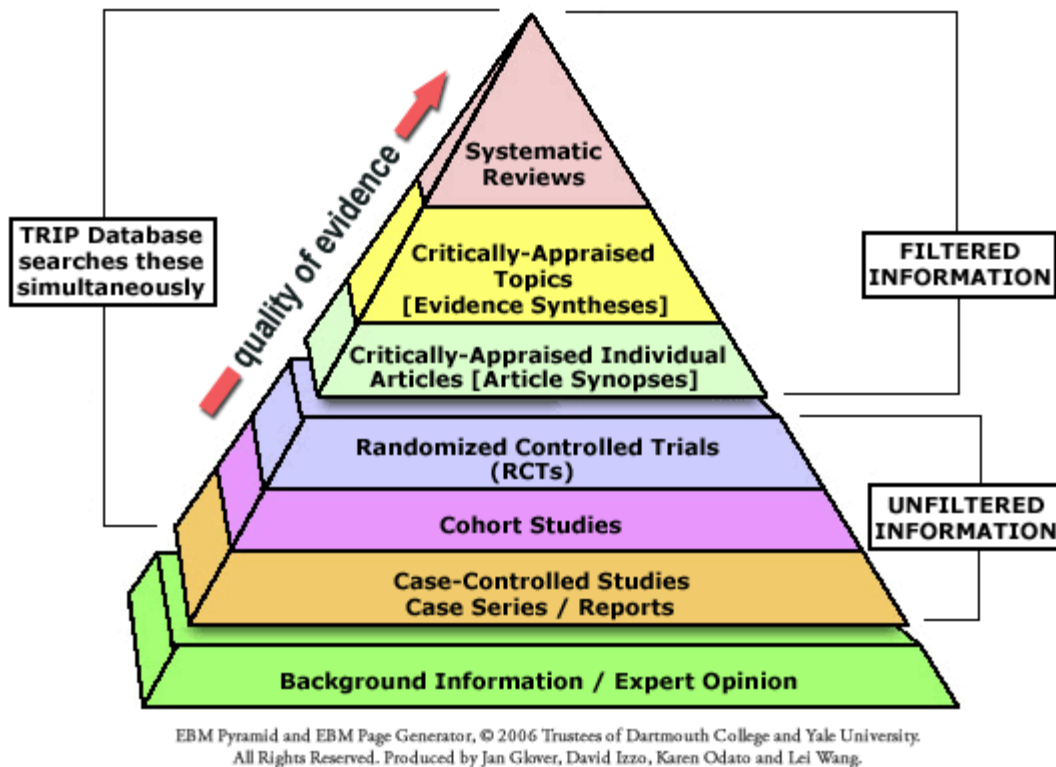


Figure 1.2. Pyramid representing the hierarchy of published evidence (Glover et al., 2006). Reprinted with permission.

### **Bovine Trichomoniasis**

Bovine trichomoniasis probably became rampant in Texas after importation of cattle from the drought stricken west and mid-western states. Agricultural Economist with Texas A&M AgriLife Extension (D. Anderson and T. Hairgrove, unpublished data) has estimated that the annual cost of trichomoniasis to the cow-calf sector is \$100 million or \$25 per cow in Texas herds. Currently bovine trichomoniasis is a regulated disease in Texas, and testing of all breeding bulls is required when they change possession. The producer's cost for testing is \$70-100 per bull. Disease management requires best management practices be based on scientific evidence, not "cowboy logic."

Diagnostic testing for a disease of such a high economic consequence requires collection techniques that are simple and reproducible between collectors, shipping requirements that are uniform regardless of weather, an accurate diagnostic test with high sensitivity/specificity and a rapid laboratory turn-around time.

I serve on the Bovine Trichomoniasis Subcommittee of the United States Animal Health Association, which makes recommendations to regulatory veterinarians concerning diagnostic testing and control measures. Unfortunately, many regulations are based on non-peer reviewed information presented at conferences, often in the abstract form or worse dogma based on opinions; for example, Mukhufhi et al. (2003) reported a significant drop in the sensitivity of the polymerase chain reaction (PCR) when testing for *Tritrichomonas foetus* using samples 30 hours post collection, yet regulatory officials allow samples to arrive at the laboratory 120 hours post collection (Texas Administrative Code, 2010).

I have examined the literature to ascertain the quality of available evidence and designed a study comprised of 3 experiments to investigate methods to improve the diagnostic accuracy related to this costly disease. This study examined the diagnostic protocol for bovine trichomoniasis, examining collection techniques, shipping protocols, and improved laboratory diagnostic methodology. Information gathered in this study resulted in the validation of a recently patented novel RT-qPCR developed by the Texas A&M Veterinary Medical Veterinary Diagnostic Laboratory. Results from this study have practical application and contribute to the body of evidence concerning bovine trichomoniasis and emphasizes the need for more practical research, for example factors

affecting the repeatability of test results within individual bulls over time need to be investigated in depth.

### **Epizootic Hemorrhagic Disease**

The second aspect of this dissertation focuses on epizootic hemorrhagic disease, perceived as a relatively new emerging cattle disease in Texas. The international association for animal health (OIE, 2014) considers this disease to be reportable and its existence could affect trade. There have been recent increases in clinical epizootic hemorrhagic disease in cattle in the mid-western states but there have been no reported outbreaks in Texas. Serologic testing of diagnostic samples procured for a study to investigate late term cattle abortions indicated a high seroprevalence of epizootic hemorrhagic virus in Texas cattle. To begin to understand the potential statewide ramifications of this disease, a study was developed to estimate spatial serologic prevalence using serum collected at auction markets for the purpose of brucellosis testing. Knowing the seroprevalence as well as the spatial and temporal distribution of this virus is essential to making sound decisions regarding cattle movement and trade issues.

### **Bovine Viral Diarrhea**

Bovine viral diarrhea virus infection is a reemerging viral disease that is associated with significant reproductive wastage and immunosuppression. The production of immune-tolerant calves is necessary for maintaining bovine viral diarrhea virus in the cattle population. When a dam is exposed to the non-cytopathic biotype of the virus during the first 125 days of gestation the fetus can become immune tolerant or

persistently infected. If the fetus survives it will shed the virus in all body secretions throughout its life, providing a source of infection to herd mates. Vaccines for bovine viral diarrhea have been available since the 1960s; however, when Perino and Hunsaker (1997) performed a systematic review of the literature to determine the field efficacy of vaccines for bovine respiratory disease they found no reliable reports examining the effects of bovine viral diarrhea vaccines in North American beef cattle. Vaccination is important in the management of this disease in the United States, but there is conflicting data on the efficacy and safety of modified live vs. killed vaccines given to dams for prevention of fetal infection. Much of the literature cited by competing vaccine manufacturers in support of their claims is in the form of case reports or company trials, in which materials and methods are often only partially reported leading to potential risk of bias. Veterinary practitioners and livestock producers are very busy, but they can use systematic reviews based on scientific principles to summarize the existing evidence to aid in management decisions. Publication does not ensure proper experimental design, or that the study reached valid scientific conclusions, but a systematic review enables the reader to objectively evaluate the manuscripts (Sebastien and Vanderweerd, 2012). A systematic review of the scientific literature to determine the results of controlled trials with statistically significant findings concerning the safety and efficacy of bovine viral vaccines for fetal protection was performed.

The 3 studies reported on in this dissertation all focus on emerging/reemerging diseases perceived by the Texas cattle Industry as problematic and needing further investigation. After reviewing and reporting the existing literature, studies were

designed to address targeted problems, thereby providing new evidence that can be applied after critical evaluation.



## CHAPTER II

### BOVINE TRICHOMONIASIS

#### **Literature Review**

*Tritrichomonas foetus* (*T. foetus*) is the parasite responsible for bovine trichomoniasis, which causes a venereal disease resulting in infertility, early fetal death, extended calving intervals, and late term abortions (Rae and Crews, 2006). Kunstler recorded observing the parasite in the vagina of a cow; however, Mazzanti was later credited with its discovery when he described trichomonads in the reproductive of 3 infertile cows (Morgan, 1946; Skirrow and BonDurant, 1988; Rae and Crews, 2006). Bang's 1897 discovery of the causative agent for contagious bovine abortion (later named *Brucella abortus*) overshadowed trichomoniasis research for the next 20 years (Emmerson, 1932).

Interest in bovine trichomoniasis increased in the mid-1920s after 2 independent investigators, Hofengartner and Pfenninger, identified trichomonads in aborted fetuses (Emmerson, 1932; Morgan, 1946). Riedmuller, focusing on abortion, described the infectious nature of the parasite in the fetus in 1928-1929, and proposed the name *Trichomonas foetus* (Emmerson, 1932). Riedmuller evaluated 105 cases of abortion in which he attributed 9 nine cases to *T. foetus* (Rae and Crews, 2006). Emmerson (1932) discovered bovine trichomoniasis in Pennsylvania, the first case reported in the United States.

Increased disease prevalence is associated with natural breeding (Ladds et al., 1973) and infection is uncommon in intensively managed systems that use artificial insemination (Skirrow and BonDurant, 1988). Trichomoniasis is endemic to many areas of the United States (Szonyi et al., 2012; Yao, 2015), with reported beef herd infection rates of 30.4% in Florida (Rae et al., 2004), 15% in California (BonDurant et al., 1990), and 44% in Nevada (Kvasnicka et al., 1989). The disease prevalence in Texas beef herds has not been determined. The negative economic impact of this disease has resulted in 28 states, including Texas, enacting regulatory programs that primarily focus on the testing and slaughter of infected males with minor emphasis on the female's role in disease transmission (Jin et al., 2014).

Taxonomic classification of *T. foetus* has been a topic of debate, but using the most current information based on morphological and molecular data, Cepicka et al. (2010) recommended the classification: Phylum Parabasalia: Class Tritrichomonadae; Family Tritrichomonadae. There are the 3 recognized serotypes of *T. foetus*, var. *manley*, var. *belfast*, and var. *brisbane*; however, genetic variation does not appear to alter host immunity or disease epidemiology with all serotypes being equally pathogenic (BonDurant and Honigberg, 1994; Rae and Crews, 2006). There are reports of serotypes var. *belfast* and var. *brisbane* coexisting as mixed populations in males and females (Dennett et al., 1974; Wosu, 1977).

The life cycle of *T. foetus* is simple with reproduction by longitudinal binary fission (Levine, 1973). This unicellular parasite is aerotolerant, thriving in a microaerophilic environment, but it can adapt to higher levels of oxygen over time

(Lloyd et al., 1987). The parasite lacks mitochondria and thus does not depend on a functioning Krebs Cycle; rather it relies on the cytosolic fraction and hydrogenosomal organelles for energy production (Lloyd et al., 1987; BonDurant and Honigberg, 1994; Silva et al., 2011). *Tritrichomonas foetus* is pleomorphic appearing as pear shaped organisms with a single nucleus approximately 20 X 10 microns in size with a somewhat shorter undulating membrane with 3 to 5 waves, supported by a costa (BonDurant and Honigberg, 1994; Rae and Crews, 2006). The parasite has 3 anterior flagella 11 to 17 microns in length and posterior flagellum, which is an extension of the external margin of the undulating membrane, and is approximately 16 microns in length (BonDurant and Honigberg, 1994). The movement of the undulating membrane produces the characteristic “rolling jerking” movement associated with *T. foetus* (Rae and Crews, 2006).

Unfavorable conditions such as changes in temperature decrease in nutrients, or exposure to drugs prompts the pear shaped trophozoite to internalize its flagella and transform into a spherical shaped cell termed a pseudocyst, which lacks a true cell wall (Mariane et al., 2004; Pereira-Neves et al., 2011). It has long been thought that the pseudocyst was a degenerative form of *T. foetus*, but recent studies have shown that over 50% of parasites recovered from preputial scraping are in the pseudocyst stage, and the condition is reversible (Pereira-Neves et al., 2011). The pseudocyst is a functional form of the parasite that is able to adhere to epithelial cells, to reproduce by budding, and has the ability to produce cytotoxic effects (Mariane et al., 2004; Pereira-Neves et al., 2012).

*Tritrichomonas foetus* infects the lumen and mucosal surfaces of the respiratory, gastrointestinal, or reproductive tract of mammals (Robertson, 1963; Stockdale et al., 2007). *Tritrichomonas foetus* associated with the gastrointestinal tract and nasal cavity of swine and the gastrointestinal tract of felines are both similar morphologically and molecularly to the parasite associated with bovine trichomoniasis causing speculation that *T. foetus* may be a multi-host protozoon spp. (Fitzgerald et al., 1955; Stockdale et al., 2007). Researchers have been successful in establishing infection in cattle with both the feline and porcine trichomonads, but have not demonstrated reproductive loss (Fitzgerald et al., 1955; Stockdale et al., 2007), while others were unable to colonize bovine heifer reproductive tracts by vaginal inoculation with *T. foetus* of porcine origin (*T. suis*) (Cobo et al., 2001). Infection of cattle with *T. foetus* of feline origin resulted in less destruction of the endometrial surface epithelium than did the cattle parasite, suggesting a different parasite host relationship (Stockdale et al., 2007). Some have concluded that on the basis of morphology, ultrastructure, host specificity, immunology, biochemistry, and current molecular biology that *T. foetus* and *T. suis* are a single species (Lun et al., 2005; Slapeta et al., 2010); while others have reported minor differences (Frey and Muller, 2012; Reinmann et al., 2012).

Bulls exhibit no noticeable signs of the disease other than possible early observance of an insignificant purulent preputial discharge that fails to develop into a noticeable pathologic lesion. *Tritrichomonas foetus* is known to localize on the epithelial lining of the penis, prepuce, and distal urethra, and limited observations indicate the organism may even occasionally inhabit the testes, epididymis, ampulla,

prostate and proximal urethra (Morgan, 1947; Ladds et al., 1973). *Tritrichomonas foetus* is believed to cause no change in semen quality (Rae and Crews, 2006); however, recent reports indicate cytotoxicity to the bovine sperm cell as the result of parasite adhesion, phagocytosis of sperm and agglutination with a resulting decrease in progressive motility (Benchimol et al., 2008; Ribeiro et al., 2010).

The most important feature of *T. foetus* infection in the bull is the development of persistent infection resulting in a chronic carrier state (Rae and Crews, 2006). Persistent infection is primarily observed in bulls  $\geq 4$  years of age, believed to be related to increase in depths of epithelial folds of the penis and prepuce (Rae and Crews, 2006; Silva et al., 2011). These deeper epithelial folds sometimes referred to as crypts, were thought to provide the microaerophilic environment necessary for the establishment and maintenance of chronic infections; however, recent research indicates depths in epithelial folds are similar in bulls of all ages (Strickland, 2010). Australian researchers established experimental infections in 12 of 13 bulls ages 3 to 7 years, and could only establish infection in 3 of 19 bulls that were 1 to 2 years of age (Clark et al., 1974). Studies suggest that a reduction in the incidence of infection can be achieved by the exclusive use of young bulls, and their use during a limited breeding season can be used to moderate overall disease transmission (Clark et al., 1974; Christensen et al., 1977). Bulls are believed to have a very limited local immune response, and antigen does not appear to be absorbed from the mucosal surface of the external genitalia (Robertson, 1963).

Age alone does not preclude the possibility of a carrier state as bulls'  $\leq 2$  years old have been identified as carriers (Clark et al., 1974). Several studies have examined differences in breed susceptibility to *T. foetus*, but none are conclusive (Dennett et al., 1974; Rae and Crews, 2006). Venereal transmission of the disease occurs when an infected bull breeds a non-infected cow or a non-infected bull breeds an infected cow (Morgan, 1947). Passive transfer is considered to be a less efficient method of disease transmission, especially when using young bulls (BonDurant and Honigberg, 1994; Rae and Crews, 2006). Abbitt (1980) suggests homosexual activity in bulls could be a mode of parasite transmission, but this has not been investigated.

The parasite colonizes the vagina, uterus and oviduct of the female after coitus with a chronically infected carrier bull or by passive transfer with a previously uninfected bull that has had recent coitus with an infected female (BonDurant, 1997; Skirrow and BonDurant, 1988; Rae and Crews, 2006). There is high rate of passage from infected male to naïve female, with colonization of the entire reproductive tract occurring within 1 to 2 weeks (Clark et al., 1983; Rae and Crews, 2006).

*Tritrichomonas foetus* causes genital inflammation to include vaginitis, cervicitis, and endometritis, which are often undetected by the producer (Rae and Crews, 2006; Cobo and Favetto, 2014). The number of parasites in the vagina peak 14-18 days after natural breeding (Hammond and Bartlett, 1945). The parasite is deposited in the vagina and moves through the cervix, gaining access to the uterus and oviducts due in part to the parasites mobility and to the relaxation and contraction of the uterus due to estrus (BonDurant and Honigberg, 1994). The infection is cleared or minimized in the vaginal

tract fairly rapidly; however, the parasites remained confined to the uterus except during estrus, which complicates culture of female vaginal secretions (Bartlett, 1947). The cytotoxicity produced by *T. foetus* is a result of the parasite using adhesions to bind to the host epithelial cells (Corbeil et al., 1989) and then interacting with those cells to cause the secretion of enzymes to include extracellular proteases and glycosidases (Silva et al., 2011). Through cytolysis, the epithelial junctions are damaged, resulting in remodeling of the surrounding extracellular structure, which enables the parasite to create an environment conducive to survival in various parts of the host (Petropolis et al., 2008; Silva et al., 2011).

Though immune function allows the vagina to clear the initial infection, there is brief re-colonization of the vagina occurring during estrus that is instrumental to the transfer of the parasite to naïve bulls (Bartlett, 1947). *Tritrichomonas foetus* is not believed to interfere with fertilization and the reason for reproductive loss, although not fully understood, is believed to be associated with endometritis (Rae and Crews, 2006); however, recent research has demonstrated in vitro reactions of *T. foetus* with spermatozoa and oocytes which may interfere with fertility (Benchimol et al., 2007; Benchimol et al., 2008). Fetal death is thought to occur most often at approximately 60 days in gestation; however, death of the embryo/fetus can occur at any time during gestation, with 1 report of 33% of the losses occurring during the last trimester (Rhyan et al., 1988). Bartlett (1947) reported that older cows previously infected, upon reinfection, are more likely to develop pyometra, have later term abortions, or carry infection over into next gestation. In less than 5% of cases the fetus is not expelled, resulting in a

retained corpus luteum causing continual secretion of fluid and development of pyometra (BonDurant, 1997).

*Tritrichomonas foetus* remains in the female reproductive tract for a variable period ranging from 3 to 22 months (Bartlett, 1947; Alexander, 1953; Clark et al., 1983). Heifers retained the pathogen in the reproductive tract for 13 to 28 weeks following experimental infection (Skirrow and BonDurant, 1990). Females are infertile for 2 to 5 months following reproductive loss, after which time they usually acquire short-term immunity and conceive (Rae and Crews, 2006). Phagocytosis and vaginal secretion of IgG1 and IgA are important in the removal of the parasite from the uterus (BonDurant and Honigberg, 1994). Vaccination or infection can result in the female producing circulating antibodies, but humoral immunity is not protective (Robertson, 1963).

Although most cows will eliminate the organism at the first or second postpartum estrous cycle, they occasionally retain infection throughout their pregnancy (Rae and Crews, 2006). Cows persistently infected throughout gestation and into the subsequent breeding season are continual sources of herd infection (Morgan, 1944; Skirrow, 1987). The frequency of persistently infected cows is thought to be less than 1%; but the presence of 1 cow in a herd can be disastrous to a disease control program (Rae and Crews, 2006).

Diagnosis of *T. foetus*, while appearing to be straightforward can be challenging. There are 3 distinct stages that represent the important links in the diagnostic chain, which are collection, transport, and laboratory diagnosis. Failure to adhere to stringent protocol during any stage can result in misdiagnosis. There are many factors beyond the



control of the veterinary practitioner collecting field samples, but diagnosis can be improved if collection procedures are well planned and everyone involved understands their role. The current testing methodology is based on the polymerase chain reaction (PCR), which is required by many regulatory agencies and preferred by veterinary practitioners. The PCR test detects the presence of nucleic acid associated with *T. foetus*, so prevention of cross contamination of diagnostic samples is important. Samples need to be protected from light and temperature extremes, with shipment to the appropriate laboratory immediately post collection. Delay in shipment will allow overgrowth of contaminants in the transport media, which can result in production of DNAase and destruction of nucleic acid necessary for diagnosis. Heat has a negative impact on the protozoa and causes a breakdown of nucleic acid resulting in a false negative test (Clavijo et al., 2011; Davidson et al., 2011). Bryan and coworkers (1999) found a negative effect on the viability of *T. foetus* when temperatures were 4.0<sup>0</sup> C for more than 5 days or -20.0<sup>0</sup> C for more than 3 hours. Historically, diagnosis has focused on recovery of the parasite from the genitalia of the male or female. The organism was either identified upon collection or grown in culture in order produce viable organism that could be identified (Bartlett, 1947). Current diagnostic protocols focus on the analysis of preputial smegma, using either culture or PCR testing (Cobo and Favetto, 2014). Intestinal trichomonads sometimes contaminate the preputial area and when observed microscopically they can be mistaken for *T. foetus*, resulting in a false positive diagnosis; however, molecular diagnostics will differentiate the organisms (Cobo et al., 2003; Dufernez et al., 2007; Agnew et al., 2008). Collection methodology in the male

has been evaluated over time; obtaining samples by swabbing the preputial fornix with gauze, lavage of preputial area with saline, scraping with a glass pipette, wire brush, or an inseminating pipette have all been deemed acceptable; however, the use of a disposable large animal-inseminating pipette is considered the most practical (Hammond and Bartlett, 1943; Abbitt and Meyerholz, 1979; BonDurant and Honigberg, 1994; Parker et al., 1999). Current collection methodology involves blindly scraping the preputial cavity, and several investigators have determined that the parasite is more likely to be adhered to the penis than the prepuce (Hammond and Bartlett, 1943; Bartlett, 1947; Parker et al., 2003). Parker et al. (2003) reported that preputial collections from the right side of the bull were 4 times more likely to be positive than samples taken from the left side. Culture media that is overly contaminated with fecal debris and associated bacteria is more likely to result in a false negative result (Clothier et al., 2015).

Modified Diamonds Media (MDM) was considered the media of choice for shipment and culture of trichomonads; however a more recently a commercialized plastic pouch (BioMed in pouch) utilizing a proprietary media has shown to be 6.95 times more likely to be positive as samples tested using MDM (Diamond, 1957; Parker et al., 1999). Most regulatory agencies require use the specialized pouch containing a proprietary media.

Once samples are collected, packing and method of shipment are critical to accurate laboratory diagnosis. Samples should be packed in container that will maintain the pouch near room temperature during shipment, and samples should be shipped for overnight delivery to the laboratory. Investigators have determined that heat above

42.0°C is detrimental to *T. foetus*, resulting in false negative tests by both PCR and culture, and temperatures ranging between 4.0°C and 20.0°C are detrimental to growth of the organism; therefore, failure to control temperature compromises diagnosis (Bryan et al., 1999; Davidson et al., 2011; Clavijo et al., 2011).

Because of the female's role in disease transmission, there is interest in developing reliable diagnostics for use in females. The use of direct microscopic examination of vaginal secretions, measuring vaginal antibodies, or measuring systemic immune response has been examined as a diagnostic protocol for decades (Pierce, 1950). Currently, detection of the organism by culture or PCR is the standard; however, the sensitivity of these methods is lower in the female (Skirrow and BonDurant, 1990). *Tritrichomonas foetus* initially infects the vagina, then travels through the cervix, and ultimately infects the uterus (Morgan, 1947). *Tritrichomonas foetus* is likely to be detected in the vagina only during estrus (Bartlett, 1947; Abbitt and Ball, 1978). The female secretes vaginal IgG<sub>1</sub>, which has been shown to persist in vaginal secretions for 2 months, and IgA, which persists for 6 months post infection (Skirrow and BonDurant, 1990). Diagnosis centered on recovery of the organism is difficult in the female due to the above-mentioned hormonal and immune response resulting in systemic and secretory antibody production, which can eliminate the parasite (Bartlett, 1947; Cobo and Favetto, 2014). Because some studies indicate the PCR is more sensitive and specific than culture many regulatory agencies only approve the PCR for diagnostic testing; however, other investigators have found the tests to be comparable (Mukhufhi et al., 2003; McMillen and Lew, 2006; Ondrak et al., 2010; Guerra et al., 2013). The PCR provides

faster laboratory turnaround time, is able to differentiate non-pathogenic trichomonads, and is often able to detect nucleic acid in culture media after the parasites have died; but the sensitivity of the PCR can decrease rapidly after sample collection due to accumulation of inhibitory compounds or sample contamination (Mukhufhi et al., 2003; Kennedy et al., 2008).

Biosecurity focuses on the precluding the introduction of disease agents into a group of animals while biocontainment refers to the control of the disease once introduced into the group (Dargatz et al., 2002). Because males usually remain persistently infected, avoiding the introduction of infected males is essential to the control of *T. foetus* transmission (Clark et al., 1974). The female often develops limited short-term immunity to *T. foetus*, clearing the infection and rebreeding (Morgan, 1946). Before purchasing replacements, investigating the herd of origin and purchasing from herds with excellent reproductive histories will lower the risk of importing trichomoniasis (Sanderson and Gnad, 2002).

Previous proposed control measures have focused on developing immunity in heifers by allowing them to be bred by infected bulls, thus allowing the heifers to become infected and develop immunity, which results in natural vaccinates entering the cow herd (Ball et al., 1987). Herds with long standing infections have calving rates in the upper 70 to lower 80 range, but failure to remove infected animals and institute management measures still result in significant losses (Clark et al., 1983; Goodger and Skirrow, 1986; Ball et al., 1987).

## Introduction

Bovine trichomoniasis is an emerging disease that is currently regulated by 28 states including Texas (Personal communication TAHC). The disease leaves a large economic footprint on the cattle industry due to fewer weaned calves and a less uniform calf crop because of a longer breeding and calving season. There is also an added financial burden of disease control because of regulatory testing. All bulls of breeding age are required to be tested prior to change ownership or management (Texas Animal Health Commission, 2015) and producers are required to bear the burden of diagnostic testing which ranges from \$70 to \$100 per animal. Rae (1989) conducted a spreadsheet simulation model based on 20 to 40% infection prevalence with *T. foetus* in the bull population, and he estimated a reduction of 14 to 50% in annual calf crop and net return per cow exposed to an infected bull was reduced 5 to 35%. Anderson (2014), extrapolating from data collected from 5 counties in northwest Texas, estimated *T. foetus* infection impacted approximately 20% of Texas beef herds, resulting in approximately 96,000 fewer calves weaned per year and a loss to the cow-calf industry of \$95 million dollars based on 2013 calf prices.

Regulatory disease programs entail structured management and testing protocols that are not necessarily evidenced based, for example regulatory officials permitted samples to be evaluated up to 120 hours after collection (Texas Animal Health Commission, 2015), even though Mukhufhi et al. (2003) showed a decline in tests sensitivity at 30 hours post collection.

The objective of this study was to evaluate the perceived shortcomings of the diagnostic process and their impact on disease diagnosis and management. Processes evaluated included minor variations in sample collection technique, variations in temperature and time during shipment, and laboratory diagnosis. In concert with this study a novel real time polymerase chain reaction test (RT-qPCR) was developed and patented (Patent # 62/033,893, filed August 6, 2014). This novel RT-qPCR test does not require sample incubation, can be accomplished using direct smegma shipped on ice, and produces more sensitive quantitative test results allowing easier interpretation of disease status.

### **Materials and Methods**

Institutional Animal Care and Use Committee –Texas A&M University (AUP 2013-00680 and the Agriculture Care and Use Committee-Texas A&M AgriLife Research (AUP 2014.022A) approved these studies.

#### **Experiment One**

This experiment was designed to investigate variation between individuals collecting preputial scrapings for diagnostic sampling, and to evaluate variation in weekly diagnostic results between bulls. Four bulls testing positive to *T. foetus* on 2 separate occasions were purchased for this study. One bull remained consistently negative for the duration of this experiment and is included in the analysis. The bulls were maintained at the Texas A&M College of Veterinary Medicine Research Facility at College Station, Texas.

Four veterinary students with no prior experience collecting preputial scrapings to diagnose *T. foetus*, along with an experienced veterinary clinician from the Texas A&M University College of Veterinary Medicine were recruited for this experiment. The clinician mentored the participants, provided initial instruction, and also served as a control, collecting scrapings 5 minutes after each student participant's collection.

The experimental design was based on Latin Square requiring each participant to sample a different bull each week; thus allowing each student to examine each individual bull over the course of 4 weeks. To ensure baseline sample collection competency, participants were given instruction in sample collection by the clinician. During the experiment, the student participant first collected smegma by preputial scraping, and the clinician serving as a control performed a follow up scraping on the same bull 5 minutes later so each bull was collected twice weekly with a 5 minute interval between collections. After each individual collection, the smegma was placed in the falcon tube (Thermo Fisher, Scientific, Waltham, MA) containing 1 ml of saline and homogenized; the contents were then equally divided and placed into a Transit Tube and InPouch containing a commercial proprietary growth media (Biomed Diagnostics Inc., P.O. Box 2366, White City, OR 97503). Chute side the samples were immediately placed in an insulated container without ice to provide protection from direct sunlight. The dimensions of the container (manufactured by Polyfoam Packers Corp., Wheeling, IL) were 11 cm wide, 16 cm long, 16 cm deep and 5 cm thick. Immediately after all preputial scrapings were completed, Transit Tubes and InPouch containing the smegma were immediately transported to the Texas A&M Veterinary Medical Diagnostic

Laboratory and incubated at 37.0°C for 48 hours in a Precision incubator (Thermos Scientific, Waltham, MA). Samples were observed microscopically at 10 X magnification daily for 6 days and culture reports noted. The Texas A&M Veterinary Medical Diagnostic Laboratory's standard qPCR was used to analyze InPouch and Transit Tube samples at 48 hours post collection. Microscopic evaluation of InPouch culture was considered the gold standard for this analysis.

Statistical analysis was conducted using Stata (StataCorp, 4905 Lakeway Drive, College Station, TX 77845). Culture and qPCR were evaluated as binomial data positive/negative. McNemar's and Kappa were used to evaluate agreement between InPouch, Transit Tube, and culture. Logistic regression for binary data was used to evaluate difference between collectors, bull or week. Data was considered significant when the p value of  $\leq 0.05$ .

## **Experiment Two**

The purpose of this experiment was to determine temperatures to which diagnostic samples were subjected during shipment by common carrier during temperature extremes. Temperature recording devices, iButtons (iButton, model 1921G, Maximum Integrated Products, Sunnyvale, CA) were used to record temperature changes at 3-minute intervals (480 measurements in 24 hours). Calibrated iButtons were placed in shipping containers and shipped from College Station, TX to 3 locations, Silver City, NM, Corpus Christi, TX, and Amarillo, TX during the summer of 2013. Shipping containers, consisted of 2 un-insulated cardboard boxes measuring 20 cm wide, 24 cm long and 20 cm deep, and 4 insulated styrofoam containers (Polyfoam Packers



Corp., Wheeling, IL) that were 11 cm wide, 16 cm long, 16 cm deep and 5 cm thick. Two insulated containers contained an icepack to provide refrigeration and 2 insulated containers did not contain ice packs. To provide refrigeration in the insulated containers, 1 Black Ice cold pack (Polar Tech Industries, Inc., Genoa IL) measuring 14 cm long and 10 cm wide and 3 cm thick was placed in the in the insulated container. Paper towels were placed between the icepack and the iButton to avoid direct contact.

The samples were packaged in accordance with laboratory instructions accessed at the diagnostic laboratories website (Texas Veterinary Medical Diagnostic Laboratory, 2013), with iButtons sensors replacing the diagnostic samples in the packing process. The common carrier used in this experiment was FedEx and containers were shipped “FedEx Priority Overnight” with instructions for the recipient to ship the container back to College Station, TX using the same carrier and shipping priority. The recipient was instructed not to open the package, and to immediately ship back to the sender.

When packages arrived in College Station, the iButtons were removed and the data extracted and entered into a spreadsheet (Microsoft Excel 2010) for analysis.

Clavijo et al. (2011) reported that when an InPouch containing *T. foetus* was exposed to temperatures of  $\geq 42.0^{\circ}\text{C}$  for 24 hours all qPCR diagnostic testing was negatively affected, resulting in false negative results. Temperatures of  $60.0^{\circ}\text{C}$  have been recorded in non-climate controlled parcel delivery vehicles (Davidson et al., 2011). In an attempt to determine the period of time the diagnostic sample would reach the critical temperature of  $\geq 42.0^{\circ}\text{C}$ , 3 containers identical to those used in the shipping phase of the experiment were used, 1 un-insulated container, 1 container insulated

without an ice pack and 1 container insulated with an ice pack. The containers were packaged as described in the shipping phase, with 2 calibrated iButtons placed in the container to record sample temperature and 1 calibrated iButton placed on the outside to record external temperatures. The iButtons were calibrated to record temperature changes every 10 minutes. The containers were placed in a forage drying furnace (Stabil-Therm, Blue M Electric Company, Blue Island, IL) with a temperature setting of 48.8°C. The samples were left in the drying furnace for 48 hours and then removed and data on the iButtons was entered in an Excel spreadsheet to determine when the threshold temperature was reached.

### **Experiment Three**

This experiment involved a novel RT-qPCR developed by researchers at The Texas A&M Veterinary Medical Diagnostic Laboratory. The novel RT-qPCR reported in this study and the intellectual property acquired with the development of this test, has been patented (Patent # 62/033,893, filed August 6, 2014) and the rights to patent use are in negotiations between The Texas A&M University System and a private cooperation, therefore this paper will focus on the field validation of this novel RT-qPCR and the advantages it affords in collection and shipment.

A total of 166 bulls were sampled to evaluate diagnostic performance of the novel RT-qPCR; 56 positives and 110 negatives hereafter denoted as reference samples. Positive reference samples were identified through routine diagnostic submissions to The Texas A&M Veterinary Medical Diagnostic Laboratory. Owners and veterinarians were contacted after bulls tested positive on the current diagnostic laboratory employed

culture qPCR test and bulls were resampled with the owners' consent. Negative reference samples were identified as having negative culture qPCR results and being from a herd with a good reproductive history. Microscopic examination of culture media is considered the "gold standard" for this experiment and all reference samples were positive by that method before being considered for test validation.

These reference samples were collected by first washing the prepuce area with ~10 ml or more of saline to remove dirt or fecal contamination, followed by scraping of the inside of the prepuce 10 times with a sterile artificial insemination pipette. For some samplings, multiple collections were required to obtain sufficient sample (i.e., at least 500  $\mu$ l smegma). Smegma was collected into a 5 ml polypropylene tube (Thermo Fisher Scientific, Waltham, MA) containing ~1000  $\mu$ l of Phosphate Buffered Saline (PBS). Each sample was mixed to homogeneity using a transfer pipette (Thermo Fisher Scientific, Waltham, MA); approximately 200  $\mu$ l of each sample was reserved in the original tube and the rest of the sample was deposited into the InPouch media (BioMed Diagnostics, White City, OR). The tube containing the smegma was placed in an insulated Styrofoam cooler with ice packs and the InPouch sample was placed in a similar container without ice packs and transferred to the laboratory. All samples were delivered to the laboratory within 8 hours of collection and upon arrival at the laboratory, the smegma sample was refrigerated and the InPouch was incubated at 37.0°C in a Precision incubator for up to 96 hours (at which time the microscopic examination was completed).

For microscopic examination, the 37.0°C incubated reference InPouch samples were observed daily for *T. foetus* following The Texas A&M Veterinary Medical Diagnostic Laboratory employed culture microscopic examination method. This method consisted of 4 readings, each 24 hours apart, within 6 days. The first reading occurred after 24 hours incubation at 37.0°C. InPouches were examined at 10X magnification. The entire bottom edge of the InPouch was scanned, as well as, approximately 2.5 cm up each side. If no motile *T. foetus* were observed, the InPouch was identified as negative for that reading and additional incubation and 3 additional readings at 24 hour intervals were required. If motile *T. foetus* were observed during any of the 4 readings, the InPouch was identified as positive and no further incubation or readings were required.

In order to determine the diagnostic sensitivity of the newly developed molecular test, bulls that tested positive by microscopic culture were subsequently tested by the culture qPCR currently employed by The Texas A&M Veterinary Medical Diagnostic Laboratory, as well as, the novel RT-qPCR under development.

## **Results and Discussion**

### **Experiment One**

Quantitative tests results on the qPCR are reported as Cq values. The larger the quantity of nucleic acid amplified by the qPCR, the lower the Cq value, and conversely the higher the Cq value the less nucleic acid amplified, values of 40 indicating no amplification. There was variation in Cq values between bulls, but each individual bull was fairly consistent testing in a similar quantitative value range.

Quantitative Cq values are converted to qualitative binary data with positive results being determined by a cutoff value of  $<35$ . Bull 77 consistently tested positive and bulls 53 and NT usually tested positive but were occasionally negative, while bull 54 was consistently negative on all tests (Figure 2.1). Interestingly, 2 of 24 InPouch samples (8.3%) and 4 samples of 24 Transit Tube samples (16.6%) were positive by microscopic culture while negative by culture qPCR (Table 2.1). The culture qPCR and microscopic culture results were evaluated as either positive or negative (Cq value  $<35$  reported as positive) as would be reported to practitioners submitting field samples. Even though bull 54 was consistently negative, results obtained for all 4 bulls were analyzed with a P value of  $\leq 0.05$  being considered significant.

InPouch, culture, and Transit Tube were analyzed as dependent variables with bull, week, and collector analyzed as independent variables. There was a consistent difference in bulls ( $P=0.005$ ) when analyzed with all 3 dependent variables. This was not surprising in that 1 bull was consistently negative throughout the 4-week period of the experiment. InPouch and culture results were not influenced by week ( $P=0.696$  and  $0.248$ ) respectively; however, there was a statistically insignificant trend ( $P=0.082$ ) for week to affect Transit Tube. InPouch, culture, and Transit Tube were not affected by collector variation ( $P=0.249$ ,  $0.805$ , and  $0.688$ ) respectively.

Proportions of agreement (McNemar's Test) and Kappa were used to evaluate agreement between culture qPCR of the InPouch and Transit Tube, as well as the microscopic culture. The Transit Tube and InPouch, although not significant did tend to lack show lack of agreement with McNemar's test ( $P=0.083$ ), with a Kappa value of

0.805. The microscopic culture demonstrated agreement both with the Transit Tube and InPouch with McNemar's test ( $P=0.179$  and  $1.0$ ) and Kappa values of  $0.675$  and  $0.723$  respectively. The second collector (veterinarian) tended to obtain more positive tests (94%) compared with the first collector (students) (72%), which is compatible with results reported by Canadian workers where a second collection 5 minutes after the initial collection was more likely to result in a positive test (Parker et al., 1999).

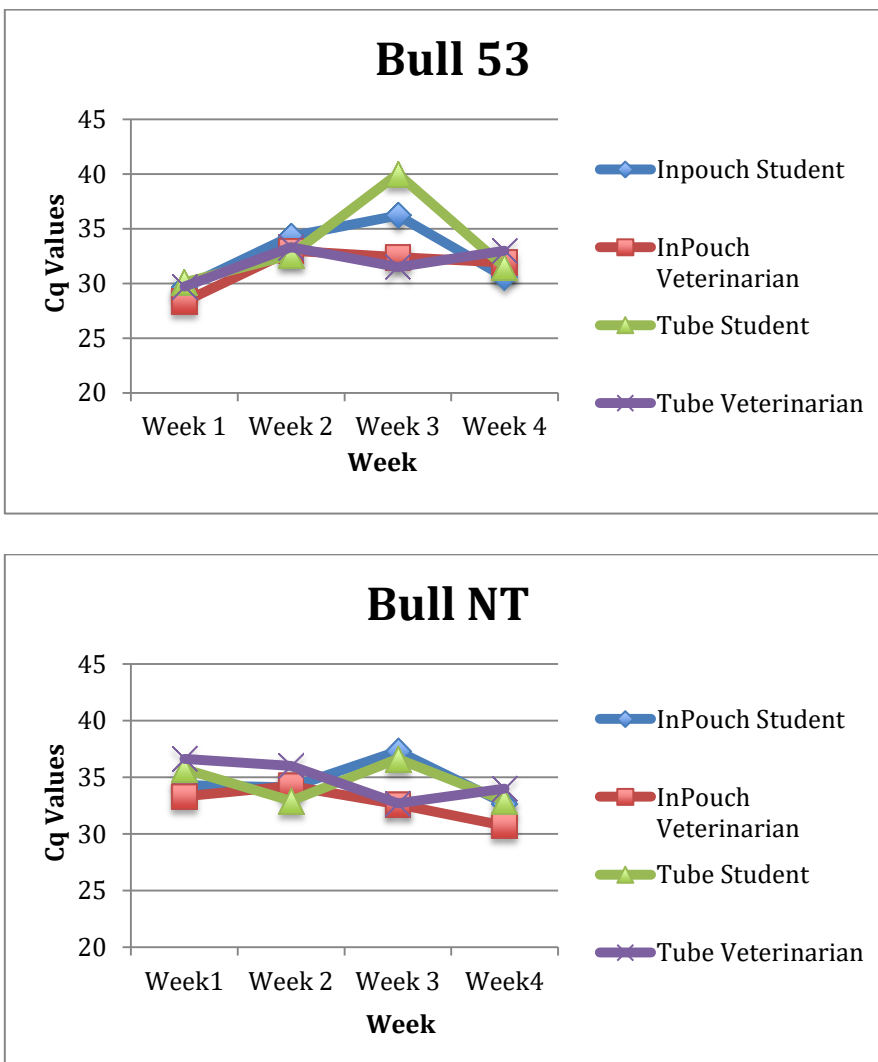


Figure 2.1. Cq values over the 4 weekly collection periods organized by students and veterinarians evaluating InPouch and Tube tests. A Cq value  $>35$  is considered positive.

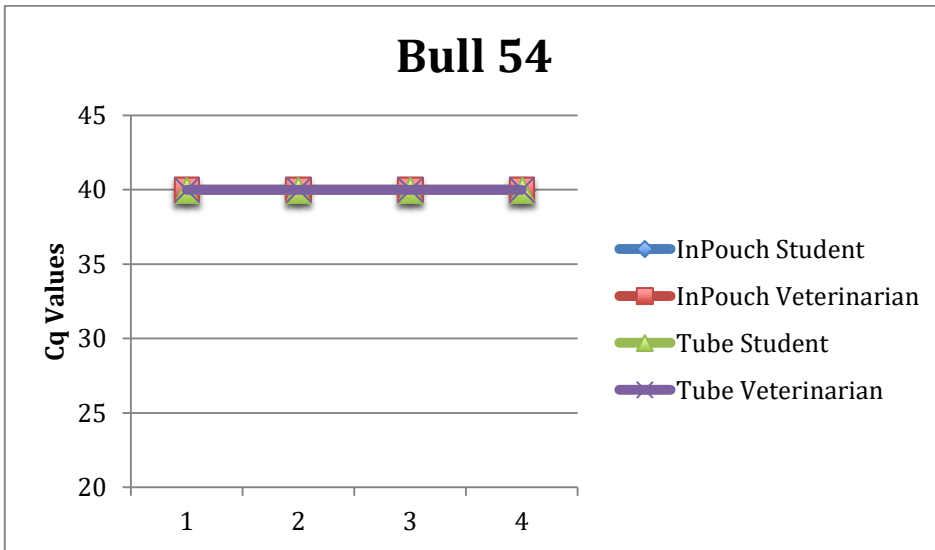
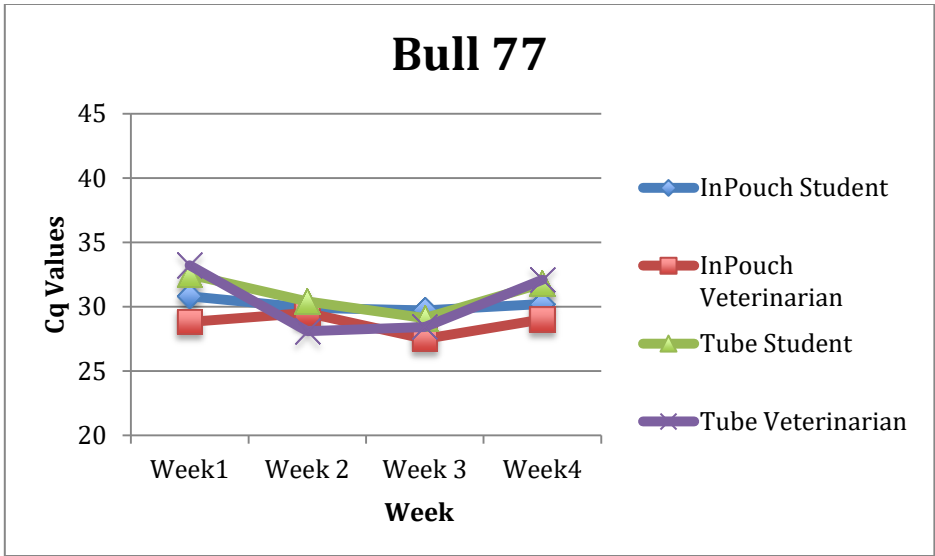


Figure 2.1. Continued.

Table 2.1. Values used in the graphs in Figure 2.1 are taken from this table. Cq values <35 are reported as positive with 35≥ reported as negative. Test values for the 3 tests evaluated, obtained by students and veterinarian from each bull, taken each week are listed below.

<b>Bull ID</b>	<b>Week Sampled</b>	<b>Collector</b>	<b>InPouch (qPCR)</b>	<b>Transit Tube (qPCR)</b>	<b>Culture Microscope</b>
77	1	Student 1	30.8	32.5	Positive
		Veterinarian	28.8	33.2	Positive
	2	Student 4	29.9	30.4	Positive
		Veterinarian	29.5	28.8	Positive
	3	Student 3	29.7	29.1	Positive
		Veterinarian	27.5	28.4	Positive
	4	Student 2	30.2	31.8	Positive
		Veterinarian	29	32.1	Positive
53	1	Student 2	Negative 35.6	Negative 35.5	Negative
		Veterinarian	30.5	32.9	Positive
	2	Student 1	34.3	32.6	Positive
		Veterinarian	33	33.3	Positive
	3	Student 4	Negative 36.2	Negative 40	Positive
		Veterinarian	32.4	31.5	Negative
	4	Student 3	30.6	31.5	Positive
		Veterinarian	31.9	33	Positive
NT	1	Student 4	34.3	Negative 35.7	Negative
		Veterinarian	33.3	Negative 36.6	Positive
	2	Student 3	34.1	32.9	Positive
		Veterinarian	34.2	Negative 36	Positive
	3	Student 2	Negative 37.3	Negative 36.6	Positive
		Veterinarian	32.6	32.7	Positive
	4	Student 1	32.7	32.9	Positive
		Veterinarian	30.7	34	Positive
54	1	Student 3	40	40	Negative
		Veterinarian	40	40	Negative
		Student 2	40	40	Negative
		Veterinarian	40	40	Negative
		Student 1	40	40	Negative
		Veterinarian	40	40	Negative
		Student 4	40	40	Negative
		Veterinarian	40	40	Negative



The results of this experiment indicate that with minimal instruction, an inexperienced individual can collect an acceptable preputial scraping and package it for shipment. There were no differences in diagnostic testing sensitivity as a result of collection technique between student collectors or between students and an experienced veterinary clinician. This experiment was carried out over a 4-week period and it was noted that quantitative test results over time for each individual bull tended to be similar, but there were differences between bulls. One bull consistently recorded a qPCR value  $< 35$  considered the cutoff for a positive test, while 2 bulls were recorded qPCR values on either side of the cutoff of  $< 35$ . All samples were protected from temperature extremes and sunlight and were delivered to the diagnostic laboratory within 1 hour of collection; reducing the confounding effects of sample shipment on the experiment.

## **Experiment Two**

The purpose of the second experiment was to evaluate temperature extremes inside the shipping container during routine ground shipment and to determine time needed to reach the critical temperature of  $42.0^{\circ}\text{C}$  and the duration of time until temperature subsided. Packages were shipped in the summer of 2013 when weather forecast indicated triple digit temperatures for Texas (National Oceanic and Atmospheric Administration, 2016).

Samples were all in transit for 4 days, and the only container to approach  $42.0^{\circ}\text{C}$  in 24 hours was the un-insulated cardboard box sent to Corpus Christi (Figure 2.2), but 5 of the packages sent to New Mexico exceeded  $42.0^{\circ}\text{C}$  during the 96 hour time period.

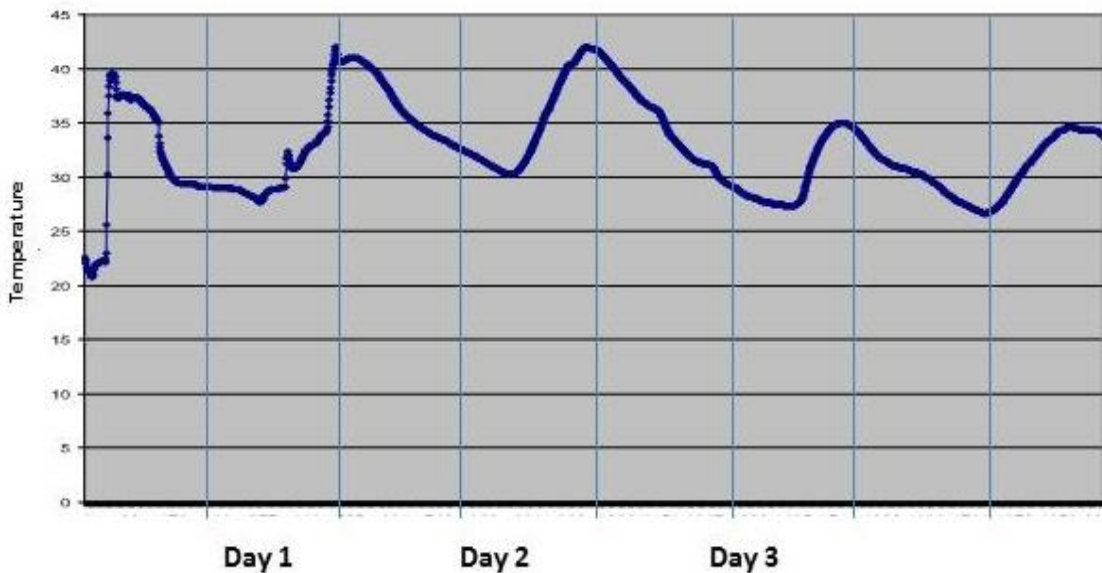


Figure 2.2. The temperatures recorded in the un-insulated container shipped to Corpus Christi, TX. Temperatures were recorded at 3-minute intervals and the threshold temperature of 42.00°C was reached in approximately 24 hours, reinforcing the need to expedite shipping.

A follow-up experiment was conducted using controlled temperature environments. A calibrated iButton was placed on the exterior of each package to ensure recorded external temperature was similar on all packages. The external monitors recorded a consistent 49.4°C (+-.5°C). The iButtons in the cardboard container recorded temperatures in excess of 42.0°C within 1 hour, the insulated container with no ice pack exceeded 42.0°C in 1.5 hours, and the insulated container with ice did not exceed the threshold temperature for 8.5 hours (Figure 2.3).

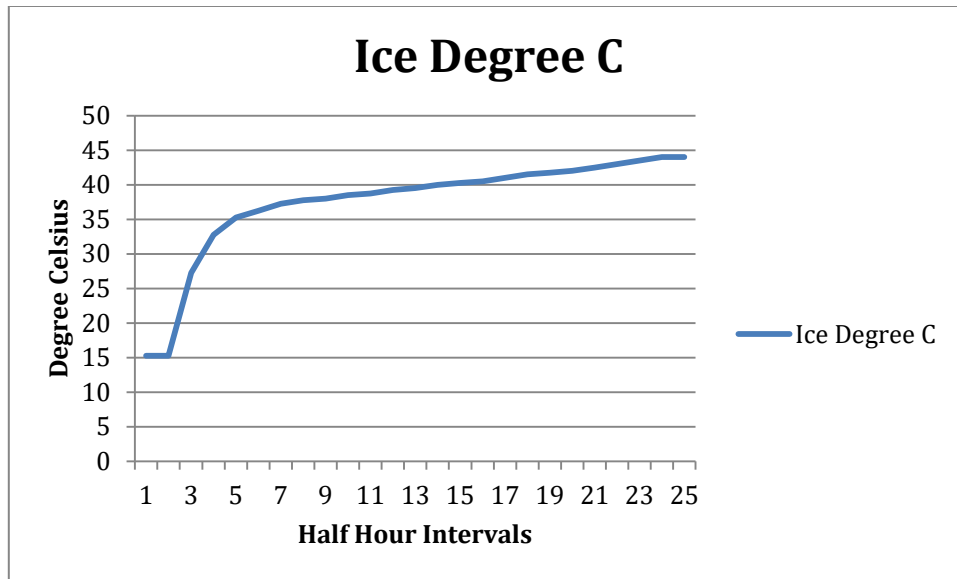


Figure 2.3. Temperatures were recorded every 30 minutes in an insulated container containing the ice pack. The container reached the threshold temperature of 42.00°C in 8 hours and 30 minutes, reinforcing the need to take extra precautions during temperature extremes and expedite shipping.

Packing and shipping samples to the laboratory is a critical link in the diagnostic chain. Clavijo et al. (2011) demonstrated a negative effect on the diagnostic accuracy of microscopic or culture qPCR evaluation when *T. foetus* was subjected to temperatures of 42.0°C for 24 hours, with neither test being interpreted as positive. Some diagnostic laboratories have advocated refrigerating or even freezing samples in culture upon collection and shipping to the laboratory on ice (Oregon Department of Agriculture, 2016). Biomed Diagnostics, the manufacturer of the InPouch recommends maintaining the pouch at 15.0-20.0°C for a maximum time of 48 hours prior to incubation and they state “NEVER refrigerate or freeze the specimen” (Biomed Diagnostics, 2016).

Davidson et al. (2010) reported temperatures in excess of 60.0°C or 140.0°F measured in non-climate controlled parcel delivery vehicles. Experiment 2 focused on the use of a common carrier to ship samples containing iButton temperature sensors in insulated containers, with and without ice packs, and non-insulated containers. Results of this experiment indicate that packing specimens in an insulated container with an ice pack is most effective for mitigating temperature extremes. The ice pack should be wrapped in paper towels or similar insulating material eliminating direct contact with the specimen. Temperature sensors (iButtons) transported in un-insulated or insulated containers without ice packs recorded temperatures of 42.0°C early in shipment, which is detrimental to maintenance of a viable laboratory sample, and even when supplied with ice packs, samples only remained in a safe temperature range for approximately 24 hours. Guaranteed overnight delivery and shipping in an insulated container with an icepack is the preferred method of shipment during summer months.

A more controlled experiment where samples packed identical to the shipping experiment was placed in a forage-drying oven at a constant temperature of 49.4°C for 48 hours. The un-insulated cardboard box and un-insulated box recorded a specimen temperature in excess of 42.0°C within 1 hour and 1.5 hours respectively. The insulated box containing an ice pack took 8.5 hours to reach the same threshold temperature (Figure 2.3). Results of both experiments show the need to expedite shipping so the sample arrives at the laboratory within 24 hours.

### **Experiment Three**

The diagnostic sensitivity and specificity of the novel RT-qPCR (proprietary patented technology negotiations with Texas A&M System and a private cooperation) were calculated using a reference set of Culture microscopic examinations collected from animals in the field that determined 56 bulls to be positive and 110 bulls to be negative. The novel RT-qPCR exhibited 100% diagnostic sensitivity and 99% specificity; the agreement between the novel RT-qPCR and Culture readings was 99% ( $\kappa = 0.99$ ); McNemar's Chi-square test for paired nominal data ( $P\text{-value}=1.00$ , indicating no significant difference between these 2 tests. Specificity of the novel RT-qPCR was also assessed using 543 preputial samples collected from low-risk herds exhibiting good reproductive history that had previously tested negative by the Culture qPCR; these samples were found to be negative by the novel RT-qPCR and the concurrent Culture qPCR testing.

The diagnostic sensitivity and specificity of the Culture qPCR were also calculated using the same reference set of Culture readings. The Culture qPCR exhibited 95% diagnostic sensitivity and 100% specificity; the agreement between the Culture qPCR and Culture readings was 98% ( $\kappa = 0.96$ ); McNemar's Chi-square test for paired nominal data ( $P\text{-value} = 0.25$ ), indicated no significant difference between the 2 tests.

The agreement between the Culture qPCR and novel RT-qPCR was 98% ( $\kappa = 0.95$ ) and McNemar's Chi-square test for paired nominal data ( $p=0.125$ ), indicated no significant difference between the 2 tests. However, the novel RT-qPCR identified 4

additional positive animals and  $C_q$  values were significantly lower for the positive specimens: 13.6-33.5 for Direct Sample RT-qPCR vs. 18.7-37.4 for Culture qPCR ( $p < 0.050$ ). The average  $C_q$  value for the Direct Sample RT-qPCR was  $22.9 \pm 4.5$ , while the average  $C_q$  value for the Culture qPCR was  $26.5 \pm 4.6$ , and a paired t-test indicated significant difference ( $p = 0.0007$ ). The difference between  $C_q$  values (novel RT-qPCR minus Culture qPCR) ranged from (-) 11.0 to (+) 3.4 (Figure 2.4). The lower  $C_q$  range of the Direct Sample RT-qPCR results enabled better data interpretation since all  $C_q$  values were outside of the inconclusive and suspect range.

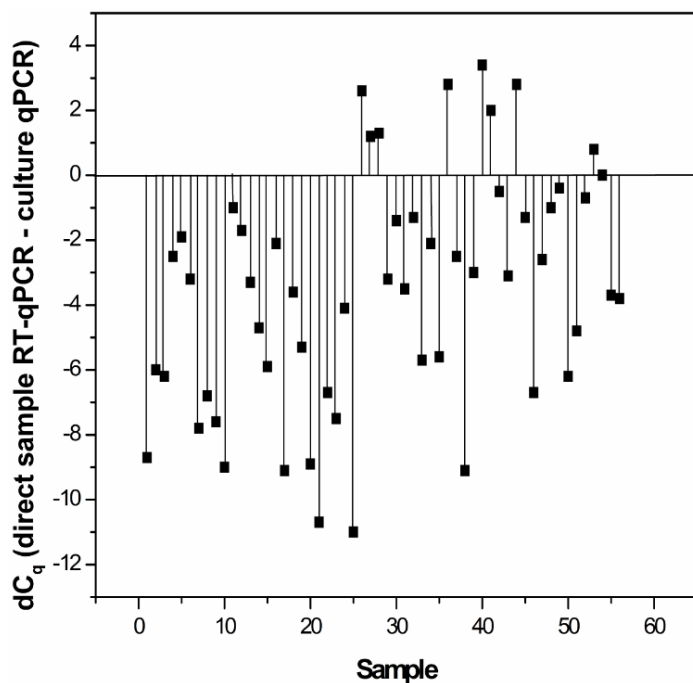


Figure 2.4. Direct sample RT-qPCR and culture qPCR  $C_q$  values difference plot for 56 culture reading positives. The y-axis represents the difference in  $C_q$  value between the 2 PCR tests; negative values indicates that the direct sample RT-qPCR results in lower  $C_q$  values, indicating enhanced detection sensitivity.

This experiment entailed validating a more sensitive novel RT-qPCR that is performed on direct smegma and does not require incubation, eliminating many of the problems encountered with collection and shipment. The *T. foetus* organism survives in a microaerophilic environment and historically this has been a factor in shipment. When transport media is upright in a fixed position, the protozoa is in an environment conducive to growth, but during shipment if the tube does not remain upright, allowing *T. foetus* to be exposed to oxygen the survival and growth of the protozoa can be compromised. The design of the InPouch circumvents this problem limiting oxygen exposure and allowing growth of the organism, but temperature must remain 18-20°C until placed in an incubator.

The increased sensitivity of the novel qPCR eliminates the need to enhance the organism to grow in culture media; therefore, the sample can be promptly placed on ice and shipped to the laboratory. The qPCR currently in use requires 48 hours incubation prior to testing; the new novel RT-qPCR requires no incubation, shortening turnaround time significantly.

The novel RT-qPCR, if accepted by regulatory agencies and diagnostic laboratories, mitigates most of the concerns with diagnostic variability at collection, shipment, and testing at the laboratory. Veterinary practitioners would place the sample on ice immediately after collection, with no need for growth media or incubation. The samples would be shipped on ice to arrive at the laboratory within 24 hours. Once at the laboratory there would be no need for 48-hour incubation and producers could have test results with 48 hours of arrival at the laboratory. The only major drawback is the

inability to identify organisms on culture using direct smegma. Many regulatory agencies only recognize PCR as an official test, but veterinary practitioners may still use culture in their initial herd diagnostic plans.

In summary, this study evaluates the 3 links in the diagnostic chain; collection, shipping, and laboratory testing and contributions of each link to the interpretation and reporting of reliable diagnostic data to assist in effective disease monitoring and control. Bovine trichomoniasis is considered to be a reemerging disease in Texas, as defined by Morse (1995) “as a disease that has existed, but has newly appeared in a population or has existed but is rapidly increasing in incidence in geographic range.” The historic prevalence of bovine trichomoniasis within Texas has been the subject of debate, with much speculation that trichomoniasis became a threat during the last decade when producers imported bulls from drought stricken western states where public grazing is common.

The disease was probably more common than perceived and recent improvements in diagnostic techniques have made it easier to identify infected animals. Development of the InPouch, a culture media and container specifically adapted to trichomonads has contributed significantly to diagnostics; however advanced PCR technology no longer requires additional growth of the protozoa after collection, eliminating the need for culture media. The employment of PCR testing, centering on the detection of nucleic acid has also improved diagnostic testing, eliminating the requirement for live protozoa. Polymerase chain reaction methodology can also differentiate non-pathogenic enteric protozoa that sometimes contaminate the culture



from *T. foetus* lowering the risk of reporting a false positive test. Many regulatory agencies recognize the PCR as the only official test. The perceived superiority of the PCR test has been a blessing and a curse for disease management. It is a common belief among producers, veterinary practitioners, and regulatory officials this test can overcome compromised collection and shipping techniques. They believe that only small quantities of nucleic acid are necessary for the diagnosis regardless of the sample condition. Bacterial overgrowth with subsequent production of deoxyribonuclease (DNase) can degrade the sample, resulting in false negative results. Excess temperature variation can result in trichomonas death and production of DNase (Mukhufhi et al., 2003). Laboratory technique and chance events can result in inconsistent results, for example, in the first experiment using known positive bulls, 8-11% of the test results were culture positive, but incorrectly classified by being negative by PCR.

The economic impact of bovine trichomoniasis is believed to be substantial; Anderson (2014) has estimated that bovine trichomoniasis costs the Texas cow-calf sector \$95 million. There are approximately 4 million cows in Texas, making the impact of this disease to be \$23.75 per cow in Texas herds. Currently, bovine trichomoniasis is a regulated disease in Texas requiring testing of all breeding bulls that change possession. The producer's cost for diagnostic testing is \$70-100 per bull, so it is important that all links in the diagnostic chain, which includes sample collection, sample preparation, and shipment to the laboratory, and diagnostic testing, be as efficient and accurate as possible. Environmental conditions during collection and shipping are not always optimal, nor are diagnostic tests flawless; however, because of the large

economic footprint of this disease, it is imperative that everyone, including, producers, veterinary practitioners, laboratory diagnosticians, and regulatory officials understand and mitigate impediments to the control of this disease.

CHAPTER III  
SEROPREVALENCE OF EPIZOOTIC HEMORRHAGIC DISEASE IN TEXAS  
CATTLE

**Literature Review**

Epizootic hemorrhagic disease is a non-contagious infectious viral disease of wild and domestic ruminants transmitted by biting midges of the genus *Culicoides* (Jones et al., 1977; Savini et al., 2011; Maclachlan et al., 2015). The causative agent epizootic hemorrhagic disease virus is a double stranded RNA virus, belonging to the family *Reoviridae*, genus *Orbivirus* (Aradaib et al., 2005; Savini et al., 2011). *Orbivirus* viruses such as bluetongue virus and African horse sickness virus are similar in morphology and structure and The Animal World Health Association (OIE) lists, epizootic hemorrhagic disease, bluetongue, and African horse sickness as notifiable diseases, and their presence can negatively impact trade (Boyer et al., 2010; Savini et al., 2011). Globally there are 7 recognized serotypes of epizootic hemorrhagic disease virus, (EHDV-1, EHDV-2, EHDV-4, EHDV-5, EHDV-6, EHDV-7, and EHDV-8), with 3 serotypes (EHDV-1, EHDV-2, and EHDV-6) being found in North America (Anthony et al., 2009a; Ruder et al., 2015b).

Epizootic Hemorrhagic Disease is believed to be the causative agent of black tongue, a disease of deer observed by hunters in the southeastern United States since the 1890s (Ruff, 1950). Shultz (1979) describes white-tailed deer (*Odocoileus virginianus*) die offs in Wyoming during 1886 and 1901 where mule deer (*Odocoileus heminous*)

were not affected. Both die off events followed unusually high precipitation, involving sudden death of white-tailed deer, with disease ceasing after frost (Schultz, 1979).

Epizootic hemorrhagic disease virus was first described in the United States by Shope (1955), who determined that a severe 1955 disease outbreak in New Jersey affecting 500-700 deer and a similar outbreak in South Dakota during 1956, were caused by 2 different strains of a similar virus, which had been designated epizootic hemorrhagic disease virus (EHDV) (Shope et al., 1960).

The generic term hemorrhagic disease is defined as a condition caused by either bluetongue virus or epizootic hemorrhagic disease virus (Nettles et al., 1991; Yadin et al., 2008). In 1966, the first case of hemorrhagic disease in Texas deer was diagnosed in a captive white-tailed deer, followed by the discovery of hemorrhagic disease in a bighorn sheep (*Ovis Canadensis*) in 1967 (Robinson et al., 1967; Stair et al., 1968).

Cattle readily seroconvert to enzootic hemorrhagic disease virus without observable clinical lesions and while there is uncertainty, cattle are probably involved in the epidemiology of epizootic hemorrhagic disease virus in white-tailed deer (Gibbs and Lawman, 1977). While not generally considered a significant disease of cattle, there have been sporadic national and international outbreaks for decades (Yadin et al., 2008; Kdemi et al., 2011; Cetre-Sossah et al., 2014; Maclachlan et al., 2015; Hirashima et al., 2015). Cattle infected with epizootic hemorrhagic disease virus amplify the virus and provide infectious virus for vector transmission to more susceptible species (Aradaib et al., 2005).

Since its isolation in North America in 1955, epizootic hemorrhagic disease is considered the most important viral agent affecting white-tailed deer populations in the United States; with sudden death outbreaks occurring in the summer and early fall, coinciding with vector seasonality (Shultz, 1979; Nettles et al., 1991; Hecht, 2010). In North America white-tailed deer are the most commonly affected wildlife species but mule deer (*Odocoileus hemionus*), elk (*Cervus elaphus*), and pronghorn (*Antilocapra americana*) have developed fatal disease (Nettles et al., 1991; Fischer, 2010; Weaver, 2013). Though there are documented reports of epizootic hemorrhagic disease virus in mule deer, massive mule deer die offs attributed to this virus was determined to be an adenoviral infection (Woods et al., 1996; Maclachlan et al., 2015). While epizootic hemorrhagic disease can be catastrophic to white-tailed deer, clinical disease in other wildlife species and domestic ruminants is sporadic and generally subclinical (Nettles et al., 1991; Maclachlan et al., 2015). Favero et al. (2013) reported the only confirmed clinical case of epizootic hemorrhagic disease in wild ruminants outside of North America, occurred in a captive pygmy brocket deer (*Mazama nana*) in South America; however epizootic hemorrhagic disease virus antibodies have been detected in numerous wildlife species (Ruder et al., 2015a).

Epizootic hemorrhagic disease virus was diagnosed in cattle disease outbreaks with lesions suggestive of vesicular disease: in Oregon cattle in 1969, in Tennessee and Colorado cattle in 1972 (House et al., 1998; Boyer et al., 2008), in cattle, bison, and Yak herds in Colorado in 2012-13 (Van Campen et al., 2013); in cattle in Indiana, Illinois, South Dakota, and Nebraska in 2012-13 (Garrett et al., 2015; Stevens et al., 2015). The

average in-herd morbidity for Colorado cattle and bison during the 2012-13 disease outbreaks was 7% (Stevens et al., 2015). In 1955, the year of the deer die offs in New Jersey; hundreds of cases of a cattle vesicular disease were observed in southeastern Pennsylvania and Delaware (Hollister et al., 1956; Shope et al., 1960; Metcalf et al., 1992). Hollister et al. (1956) reported low morbidity and mortality associated with this outbreak referred to as “muzzle disease” which caused erosions in the tongue, dental pad, teats, feet, encrustations of the skin, and gastrointestinal lesions on necropsy.

There are global reports of severe epizootic hemorrhagic disease outbreaks in cattle during the last 50 years such as Ibarake virus, an EHDV-2 serotype in Japan and Korea (Hirashima et al., 2015), and recent cattle disease outbreaks of various serotypes in the France’s Reunion Island (Cetre-Sossah et al., 2014), Israel, Turkey, Morocco, Algeria, Jordan, and portions of North America (Yadin et al., 2008; Kedmi et al., 2011; Maclachlan et al., 2015).

There are statements in the literature that infection of cattle in endemic areas is associated with indirect losses such as decreased milk production, loss of weight and body condition score and poor reproductive performance, (Mohammed et al., 1996; Aradaib et al., 2005) but when traced to the original reference Mohammed and Mellor (1990) the authors only propose those production losses as a possibility. There is limited information on the economic impact of epizootic hemorrhagic disease on the cattle industries. Kedmi et al. (2010) determined a loss of US \$25.50/cow to the Israeli dairy industry as a result of their 2006 epizootic hemorrhagic disease outbreak.

Epizootic hemorrhagic disease virus and bluetongue virus are antigenically different orbivirus that share common vectors and hosts, as well as similar spatial and temporal distributions (Gibbs and Lawman, 1977; McLaughlin, 2003; Hecht, 2010; Savini, 2011; Schoenthal, 2015). Epizootic hemorrhagic disease virus and bluetongue virus produce disease in white-tailed deer that is clinically and pathologically parallel and can only be diagnosed by pathogen identity (Rudder et al., 2015). Epizootic hemorrhagic disease virus and bluetongue virus are transmitted by *Culicoides* midges located in the world's temperate and tropical regions between the latitudes of 40-50°N and 35°S (Arby, 1997; Maclachlan et al., 2015; Schoenthal, 2015). While *Culicoides* midges are associated with the spatial and temporal distribution of epizootic hemorrhagic disease virus and bluetongue virus there is evidence that epizootic hemorrhagic disease virus is shed in oral and fecal contents of infected animals and contact transmission is possible (Gaydos et al., 2002a).

Previous reports listed up to ten serotypes of epizootic hemorrhagic disease virus in the world. Anthony et al. (2009a) determined by molecular sequencing of the outer coat proteins VP2 and VP5 region of the genome and serologic analysis that only 7 serotypes can be defined. Currently, EHDV-1, 2 and 6 are the three serotypes known to be circulating in the US (Fischer, 2010; Ruder et al., 2015b). Shope (1960) reported the strain of epizootic hemorrhagic disease virus isolated in New Jersey was different than the South Dakota isolate. Unfortunately, the South Dakota strain that Shope (1960) reported as different from the New Jersey strain was no longer available for analysis

when Barber et al. (1975) proposed designating the New Jersey strain reported by Shope (1960) as EHDV-1 and a strain isolated in Alberta as EHDV-2.

Epizootic hemorrhagic disease strains EHDV-1 and EHDV-2 have been associated with epidemics in North American white-tailed deer for decades and in 2006 EHDV-6 was first isolated in the United States from a dead white-tailed deer (Allison et al., 2010) and in subsequent years continues to be isolated over a larger geographic area of the United States (Hecht, 2010; Allison et al., 2012). The most common viral ancestor of EHDV-2 may have emerged in North America approximately 100 years ago as a result of host shift or introduction from another continent (Biek, 2007). The first known reports of deer die offs were in the 1800s, which coincide with the time EHDV-2 is proposed to have emerged in North America (Shope et al., 1960; Schultz, 1979; Biek, 2007; Anthony et al., 2009b).

There appears to be no correlation between virulence and epizootic hemorrhagic disease serotype, for example Ibaraki virus, a strain of EHDV-2 is very pathogenic to cattle in Japan and Korea, while EHDV-2 strains in North America usually do not cause clinical disease in cattle (Savini et al., 2011). Western and eastern strains have specific sequences and Anthony (2009b) suggests that western strains are more virulent than eastern strains as most clinical disease has been associated with western strains with the exception of Ibaraki (EHDV-2) causing severe clinical disease in Japan and Korea (Ali, 2012; Hirashima et al., 2015; Maclachlan et al., 2015). Ibaraki is considered an eastern virus, but has a typical western sequence (Anthony et al., 2009b).



Pathogenesis of epizootic hemorrhagic disease infection in cattle is similar to other wild and domestic ruminants (McLaughlin et al., 2003). Initially the virus replicates in the lymph nodes and lymphatic vessels that drain the area of vector inoculation (McLaughlin et al., 2003; Savini et al., 2011; Maclachlan et al., 2015). Epizootic hemorrhagic disease virus is then hematogenously disseminated to secondary sites and replicates in the endothelial cells of tissues such as lung and spleen (Maclachlan et al., 2015). Clinical signs of vascular injury and associated intravascular coagulation include hemorrhage, edema, and tissue necrosis (Tsai et al., 1973; Maclachlan et al., 2015). Virus is associated with the cell fractions of the blood, especially the erythrocytes, resulting in a prolonged viremia, providing a source of continued vector infection, important in the epidemiology of epizootic hemorrhagic disease (Gibbs and Lawman, 1977; Abdy et al., 1999; Savini et al., 2011; Maclachlan et al., 2015).

*Culicoides* biting midges are represented by 1400 species and are a common global vector for about 50 different viruses most of which are animal pathogens (Mellor et al., 2000). *Culicoides* are responsible for transmitting bluetongue virus and epizootic hemorrhagic disease virus. Epizootic hemorrhagic disease virus is most often found between 45° latitude north and 35° latitude south, with viral enzootic stability being associated with more tropical regions (Maclachlan et al., 2015). *Culicoides variipennis sonorensis* is considered the primary vector in United States, but other *Culicoides spp.* is associated with viral transmission in other geographical areas (Foster et al., 1977; Mellor et al., 2000; Stevens et al., 2015). Increased environmental temperatures are more

conducive to viral replication within the *Culicoides spp.* vector (Mellor, 2000; Ruder et al., 2015c). Movement of the vector and/or movement of infected animals are associated with disease spread. Kedmi et al. (2010) determined the 2006 epizootic hemorrhagic disease outbreak in Israeli cattle was not associated with animal movement, rather high altitude wind movement of *Culicoides spp.* vectors.

A nationwide survey measuring morbidity and mortality in wild ungulates was conducted from 1980 to 1989 and found only 0.06% of reported wildlife (10 of 1,608) diagnosed with hemorrhagic disease were of Texas origin and 9 of the reports were from the eastern part of the state indicating disease variation based on geographical distribution (Nettles et al., 1991; Stallknecht et al., 1996). Stallknecht et al. (1996) also noted geographical distribution of epizootic hemorrhagic disease when he serologically evaluated 685 white-tailed deer throughout Texas, with samples being collected over a 5-month period during the winter of 1991-1992. State seroprevalence was 84% but varied with ecological regions, increasing in a westerly direction with 100 % seroprevalence in the northwest Edwards Plateau; considerably higher than the 57% observed in the Gulf Prairie region (Stallknecht et al., 1996). Increase in seroprevalence as a result of exposure increase was associated with decrease in clinical disease speculated to be related to enzootic stability as a result of a near perfect host-virus relationship (Stallknecht et al., 1996; Martinez et al., 1999; Gaydos et al., 2002c). Nettles et al. (1991) noted more deer die offs in temperate regions and deer mortality observed by Shultz (1979) in Wyoming and Pasick et al. (2001) in British Colombia would suggest that the lack of enzootic stability contributes to more clinical disease in

deer. Extensive mortalities have been observed in farmed white-tailed deer moved from temperate to more tropical regions (Fischer, 2010).

A serological survey of auction market cattle in British Columbia and Alberta was conducted in the fall of 1987 following an disease outbreak in the Okanagan valley of British Columbia and indicated a seroprevalence of only 3%, indicating a lack of enzootic stability (Shapiro et al., 1991). Enzootic stability associated with more tropical regions would explain high seroprevalence and associated lack of clinical disease associated with epizootic hemorrhagic disease virus in Kenya, French Guiana, and northern Australia and the extensive disease outbreaks in more temperate regions such as Israel, Morocco, Algeria, Jordan, and frequent epidemics of Ibarake disease in Japan (Weir et al., 1997; Temizel et al., 2009; Toye et al., 2013; Viarouge et al., 2014; Hirashima et al., 2015). Gaydos et al. (2002c) observed differences in innate resistance among epizootic hemorrhagic disease virus in challenged white-tailed deer subspecies; subspecies originating in more temperate climates (*Odocoileus virginianus borealis*) experienced higher mortality than subspecies from subtropical climates (*Odocoileus virginianus texanus*) but humoral immune responses were similar, indicating acquired immune responses were similar (Ruder et al., 2015c). Deer challenged with EHDV-1 or EHDV-2 appear to be protected against clinical disease when later challenged with the same strain, and deer infected with EHDV-2 were protected against clinical disease when exposed to EHDV-1, but deer still developed viremia to the challenged epizootic hemorrhagic disease virus serotype, indicating challenged deer serve as viral amplifying hosts (Shope et al., 1960; Quist et al., 1977; Gaydos et al., 2002b; Hecht, 2010).

There have been sporadic severe global disease outbreaks in domestic ruminants, namely the Ibaraki disease (EHDV-2) outbreaks in Japan, Korea and Taiwan, 1 occurring in the 1960s and the second in Japan in 1997 (Savini et al., 2011). There are studies where cattle were clinically infected with enzootic hemorrhagic disease and subsequently developed viremia, but did not develop clinical disease, suggesting that cattle are involved as a reservoir in the transmission cycle of the virus (Boyer et al., 2008). Although there are numerous publications on the seroprevalence of epizootic hemorrhagic disease in wild cervids, there is limited data on seroprevalence in cattle (Boyer et al., 2008). Cattle are involved in the epidemiology of epizootic hemorrhagic disease, serving as amplifying hosts and because sampling of individual wildlife is usually a single event, cattle are employed as sentinel animals (Aradaib et al., 2005; Boyer et al., 2008).

### **Introduction**

Epizootic hemorrhagic disease virus periodically causes clinical disease in cattle; with clinical disease being associated with the more temperate geographical regions of the United States. News of recent outbreaks in other states caused some Texas producers to question the risk of a clinical disease outbreak in Texas (Stevens et al., 2015). The objective of this study was to estimate the seroprevalence of epizootic hemorrhagic disease in Texas cattle. This study entailed the use of serum samples collected at 11 Texas auction markets for the purpose of brucellosis testing, which is the most practical way to sample cattle over a large geographical area. One limitation to the study is only cattle  $\geq 18$  months were evaluated, so seroprevalence in younger cattle was

not measured. This study only reports on the seroprevalence of epizootic hemorrhagic disease in cattle marketed through the 11 respective auction markets; however, the inference is that market seroprevalence is related to seroprevalence of cattle in the surrounding area. Seroprevalence in cattle was hypothesized to be high as a result of enzootic stability due to the presence of an abundant disease vector *Culicoides spp.* Establishing an estimate of seroprevalence of epizootic hemorrhagic disease virus in the cattle population is necessary to evaluate of the impact of this disease on the Texas beef industry.

### **Materials and Methods**

This study was approved by the Agriculture Animal Care and Use Committee—Texas A&M AgriLife Research (AUP 2014.022A).

#### **Sample Procurement**

Serum samples were obtained from eleven auction markets; all located in cattle dense regions of Texas and are also engaged in first point brucellosis testing (Figure 3.1). The Texas Animal Health Commission-State Federal Laboratory confirms brucellosis test results and the Texas State Veterinarian supplied serum samples. Samples were from breeding cattle  $\geq 18$  months, and to ensure that samples were collected within the vector season and to reduce temporal confounding only samples collected during the month of June 2014 were utilized. Serum samples were transported from the State Federal Laboratory to the Animal Science Department at Texas A&M University by common carrier for next day delivery. The number of samples submitted from each market and the sample size for analysis are depicted in Table 3.1.

Table 3.1. Population samples from each market collected from 11 livestock markets in June, 2014. Sample size was determined in Epi Info version 7.1.5.

<b>Markets North to South</b>	<b>Population Sample From Market</b>	<b>Sample Size to Analyze</b>
1	396	32
2	360	32
3	353	32
4	120	27
5	303	31
6	606	33
7	82	25
8	88	25
9	17	12
10	199	30
11	153	29
Totals	2,677	308

Previous laboratory submissions indicated that the seroprevalence of EHDV-2 to be substantial, therefore true prevalence was estimated at 90%. Epi Info version 7.1.5 was used to determine the market sample size based on a prevalence of 90% (+ of- 10%), a confidence limit of 10% and 95% degree of confidence. Once the market sample size was determined, samples were numbered in sequence and a random generator available on line was used to select samples for testing (Table 3.1). Samples were delivered to the Texas A&M Veterinary Medical Veterinary Diagnostic Laboratory for serologic analysis.

### **Virus Neutralization Test**

OIE (2014) considers virus neutralization (VN) to be the gold standard for identification and quantification of EHDV antibodies and was the diagnostic protocol used in this project. The test is labor intensive and required 3-5 days to complete, but is

considered by the OIE to be superior to other immune response tests to determine prevalence during surveillance, to assist in eradication efforts and to determine the immune status of other individual animals in the environment (OIE, 2014).

The Texas A&M Veterinary Medical Diagnostic Laboratory utilizes the microtiter virus neutralization test for detection of EHDV antibodies. This procedure quantifies antibody level of various serum dilutions against a constant dilution of known virus. Observance of the virus's cytopathic effect on susceptible cell cultures indicates that the sample being tested does not contain detectable neutralizing antibodies against a virus at a certain dilution.

The OIE (2014) Terrestrial Manual describes the procedure: "Approximately 100 TCID<sub>50</sub> (50% cell culture infective dose) of the standard or serial dilution of the un-typed virus is added in 50 µl volumes to test wells of a flat bottomed microtiter plate and mixed with an equal volume of a constant dilution of standard antiserum in tissue culture medium. After one hour incubation at 37.0°C and 5% CO<sub>2</sub> approximately 104 cells are added per well in a volume of 100 µl, and the plates incubated for 3–5 days at 37°C and 5% CO<sub>2</sub>. The test is read using an inverted microscope. Wells are scored for the degree of cytopathic effects (CPE) observed. Those wells that contain cells only or cells and antiserum, should show no CPE. In contrast, wells containing cells and virus should show 75–100% CPE. The unidentified virus is considered to be serologically identical to a standard EHDV serotype if both are neutralized in the test to a similar extent, i.e. 75% and preferably 100% protection of the monolayer is observed."

## Statistical Analysis

Raw data was entered into a spreadsheet (Microsoft Excel 2010) and imported into Stata (StataCorp, 4905 Lakeway Drive, College Station, TX 77845) for analysis. Titers received from the diagnostic laboratory ranged from <20 to >1280 with no titer endpoints. Titers reported as <20 were assigned a value 10, the nearest lower dilution and titers reported as >1280 were assigned a value of 2560, the next higher dilution. There are no published reports on the sensitivity or specificity of the virus neutralization (VN) test for epizootic hemorrhagic disease virus; therefore, sensitivity and specificity are assumed to be 100%. The United States Department of Agriculture, National Animal Disease Center reports titer values cutoffs of  $\geq 10$  as positive (House et al., 1998), and TVMDL reports epizootic hemorrhagic disease virus titers of  $\geq 20$  as positive.

Data was analyzed to determine prevalence associated with all cutoff values reported by the laboratory. Mean titers were converted to  $\log_{base}^2$  to check for normal distribution. Titers were also converted into binary data negative=0 positive=1 and analyzed to determine % positive at each market and region and to determine if titers were similar in frequency across markets.

Mean Titers converted to  $\log_{base}^2$  were used to analyze the difference in mean titers across markets. Markets were also grouped into 5 regions representing the extreme north central Texas, central Texas markets north of Waco, central Texas markets south of Waco, one market east of San Antonio and one market west of San Antonio (see Figure 3.1). The number of markets was small, but data were analyzed to determine if there were differences between regions of the state.



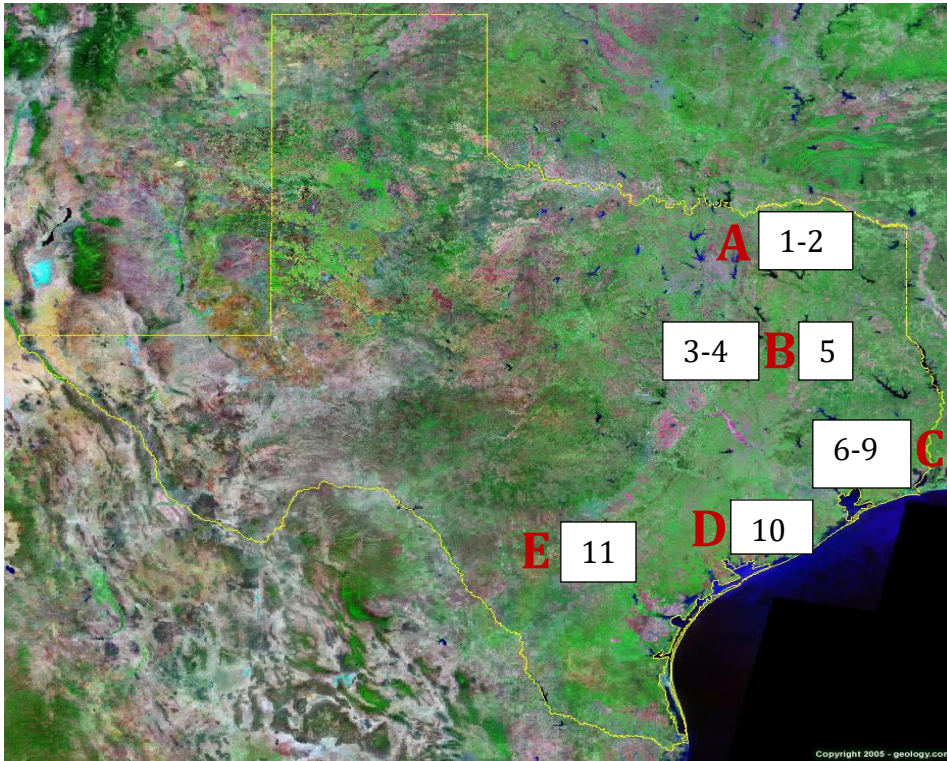


Figure 3.1. Locations of 11 auction markets denoted by numbers 1-11. The letters A-E denote regional grouping of auction markets.

## Results and Discussion

Seroprevalence was measured using the VN test, which quantifies the level of antibody in various serum dilutions against a constant dilution of known virus. The Texas A&M Veterinary Medical Diagnostic Laboratory reports epizootic hemorrhagic disease titers  $\geq 20$  as positive, which is the lowest definitive titer they report. The sensitivity and specificity have not been described for this test, thus to preclude reporting false positive results, the effects of positive cutoff values at each titer dilution were analyzed as binary data for their impact on seroprevalence. Even when serial dilution titers  $\geq 320$  were evaluated as positive, seroprevalence approached 70%; therefore, using

$\geq 20$  positive cutoff with the respective 97.2% seroprevalence is reasonable for this study. Table 3.2 details the seroprevalence of markets when animals were considered positive at each specific cutoff.

A  $X^2$  test was performed on binary data from each market to determine if there were differences in seroprevalence between markets at different titer levels shown in Table 3.3. When titer cutoff values of 20, 40, and 80 were used, and there were no difference in p-values between markets, whereas titers  $>80$  were associated with differences in P-value.

To evaluate differences in mean titers among auction markets, titer dilutions were  $\log^2$  transformed and analyzed as a generalized linear model. Results indicate a variance of 0.09 from the mean between markets ( $P < 0.01$ ), but this does not identify the variation between individual markets. Analyzing mean titers data as grouped by region seem to be of more practical relevance.

Markets were grouped into 5 regions from north to south; the hypotheses that mean titers would increase as the groups took on more southerly distribution. A generalized linear model was used to determine that there was a 0.20 variance from the mean titer values between regions ( $P = 0.01$ ). To determine regional differences, mean titers across markets in different regions were analyzed using pairwise t-tests with Tukey-Kramer adjustment. The lowest mean titers were in the most northern part of the state and the highest mean titers were in the most southern part of the state, with intermediate mean titer in central Texas as depicted in Table 3.4.

Table 3.2. Seroprevalence of EHDV-2 in cattle >18 months of age marketed through 11 Texas markets comparing different positive cutoff values.

<b>Titer Cutoffs Designating Positive (All Markets)</b>	<b>Seroprevalence All Markets</b>
≥20=Positive	97.08%
≥40=Positive	94.81%
≥80=Positive	94.10%
≥160=Positive	87.66%
≥320=Positive	69.16%
≥640=Positive	44.16%
≥1280=Positive	20.13%

Table 3.3. Chi-square analysis on each market for differences across markets.

Titer	Pearson Chi-Square (10)	P-value	Similar Across Markets
≥20=Positive	13.54	0.195	Yes
≥40=Positive	11.94	0.289	Yes
≥80=Positive	17.24	0.069	Yes
≥160=Positive	27.97	0.002	No
≥320=Positive	80.27	0.0	No
≥640=Positive	72.38	0.0	No
≥1280=Positive	56.76	0.0	No

Table 3.4. Pairwise t-test with Tukey-Kramer adjustment to determine mean titers across livestock markets by region.

Regional Clustering	Mean Titers	Ranking
1. Extreme North Texas (2 Markets)	8.28	B
2. Central North Texas (3 Markets)	8.32	AB
3. Central South Texas (4 Markets)	8.48	AB
4. South East Texas (1 Market)	9.34	A
5. South West Texas (1 Market)	8.78	A

Seroprevalence studies have largely focused on epizootic hemorrhagic disease in wild cervids and the impact of the virus in captive and wild deer (Chomel et al., 1994; Stallknecht et al., 1995; Stallknecht et al., 1996; Weaver, 2013). Massive white tailed deer die-offs were noted in the late 1800 and early 1900s in Montana and retrospectively they are believed to be the earliest reports of epizootic hemorrhagic disease (Shultz, 1979). Variability in spatial distribution of morbidity and mortality was reported in nationwide studies of wild ungulates conducted during the 1980s, and those studies found only 0.06% of reported cases (10 of 1,608) of hemorrhagic disease were of Texas origin, with 9 of those cases associated with the eastern part of the state, indicating disease variation based on geographical distribution (Nettles and Stallknecht, 1992; Stallknecht et al., 1996). Stallknecht et al. (1996) also noted geographical distribution of epizootic hemorrhagic disease when he serologically evaluated 685 white-tailed deer throughout Texas, with samples collected over a 5-month period during the winter of 1991-1992. He recorded a state seroprevalence of 84.0% that varied with ecological regions, increasing in a westerly direction with 100 % seroprevalence in the northwest Edwards Plateau; much higher than observed in the Gulf Prairie region. Increase in seroprevalence, as a result of exposure increase was associated with decrease in clinical disease; speculated to be related to enzootic stability as a result of a near perfect host-virus relationship (Stallknecht et al., 1996; Martinez et al., 1999; Gaydos et al., 2002c). Nettles et al. (1991) noted more deer die offs in temperate regions and deer mortality observed by Shultz (1979) in Wyoming and Pasick et al. (2001) in British Colombia, suggesting the lack of enzootic stability contributes to clinical disease in deer. Extensive

mortalities have been observed in farmed white tailed-deer moved from temperate to more tropical regions (Fischer, 2010).

The results of this study focusing on cattle appear validated by previous work cited on wild and captive cervids. It must be stressed that this study only measured seroprevalence of cattle moving through auction markets and the animals may have originated from other geographic areas, but this is the most expedient method to sample different geographical regions of the state, and there is reasonable assumption that most of these cattle originated in proximity to the market.

Even in endemic populations a seroprevalence of 97.2% measured at a titer cutoff of 20, appears high. Cutoff titers at each dilution were analyzed and a similar trend was observed, with approximately 70.0% of the population remaining positive at dilution titer of 320, 4 dilutions above the reported positive titer of 20, indicative of a high seroprevalence of epizootic hemorrhagic disease in cattle moving through Texas auction markets. Differences in seroprevalence observed across markets at cutoffs of 20, 40, and 80 were similar ( $X^2$  P-value>0.05).

Differences across markets and across market clusters were measured comparing mean titers. Analyzing markets using generalized mixed modeling indicated there was difference between markets, but determining which markets were different became problematic. A pairwise t-test with Tukey-Kramer adjustment was considered, but there was concern of introducing a type-1 error because of the large number of markets. Markets were grouped into 5 regions and a pairwise t-test with Kramer adjustment was used to determine mean titer differences between regions (Table 3.4). There was a

difference in mean titers between regions; the highest mean titers were in the southern most regions and the lowest mean titers in the northern most regions with intermediate titers in between.

Clinical disease associated with epizootic hemorrhagic disease virus in cattle is extremely rare in Texas, due to enzootic stability as a result of vector stability; likewise, clinical disease in cervids is more common progressing into northern latitudes due to the lack of enzootic stability associated with erratic populations of *Culicoides spp.* In areas where seroprevalence is high, occurrence of clinical disease is rare because a large percent of the population possess circulating protective antibodies.

The trends in seroprevalence as measured by mean titers observed in this study are consistent with the concept of enzootic stability; with mean titers increasing progressing toward southern latitudes. Cattle also serve as amplifying hosts and with 10.8 million cattle in Texas, one could argue cattle density contribute to enzootic stability, but Texas land mass is 261,797 square miles, while Kansas and Nebraska have combined cattle numbers similar to Texas, their land mass is only 158,687 square miles. Kansas and Nebraska have greater cattle density, fluctuating vector populations and sporadic outbreaks of clinical disease associated with epizootic hemorrhagic disease, indicating that the largest contribution to enzootic stability is consistent exposure to vectors.

As with other vector borne diseases, ranchers should be aware that movement of naive cattle into endemic areas with abundant vectors can result in clinical disease. Nationwide movement of cattle has increased in the last decade as a result of drought.

Moving cattle from temperate to tropical regions could conceivably be problematic, introducing naïve cattle into areas with high pathogen load and abundant vectors. Currently a licensed vaccine is not available, but if required an inactivated product should provide sufficient immunity (McVey and MacLachlan, 2015). Although this study focused on auction market cattle, information gained from this study provides the Texas cattle industry with an indication of epizootic hemorrhagic disease prevalence and its association with the stability of the *Culicoides spp.* vector.

CHAPTER IV  
SYSTEMIC REVIEW OF SAFETY AND EFFICACY OF BOVINE VIRAL  
DIARRHEA VACCINES FOR FETAL PROTECTION

**Introduction**

Bovine viral diarrhea is a complex of clinical presentations that collectively results in significant reproductive wastage, immunosuppression, and substantial economic losses to beef cattle producers (Kleiboeker et al., 2003). Bovine viral diarrhea is a reemerging disease that was first described in 1946 (Olafson et al., 1946) and was characterized by diverse clinical signs including high fever, depression, diarrhea, salivation, and nasal discharge (Deregt, 2005). The causative agent is a single stranded RNA virus designated bovine viral diarrhea virus (BVDV) (Lee and Gillespie, 1957). It was not until the 1970s that the concept of immunotolerance and resulting persistent infection (PI) with bovine viral diarrhea virus was beginning to be understood (Coria and McClurkin, 1978; McClurkin et al., 1979). Subsequently it was determined that bovine viral diarrhea could be caused by 1 of 3 species of virus, BVDV1, BVDV2 and putative species HoBi-like viruses, with only BVDV1 and BVDV2 being present in the United States (Ridpath et al., 2013)

The economic losses associated with the introduction of bovine viral disease virus into a herd of susceptible pregnant cows result from suboptimal reproductive performance caused by infertility, abortion, congenital defects, stillbirths, increased neonatal mortality, prenatal and postnatal growth retardation, deaths from mucosal



disease and early disposal of persistently infected animals (Kelling, 2004; Smith et al., 2013).

Persistent infection is the result of fetal immunotolerance, developed as a consequence of the virus invading the fetus during the first trimester of gestation. Cattle persistently infected with bovine viral diarrhea virus are the major source of disseminated virus within and between livestock operations (Grooms, 2004; Kelling et al., 2005). The typical scenario for a cattle herd exposed to bovine viral diarrhea virus is an initial disease peak followed by low-level chronic reproductive disease in subsequent months and years (Larson et al., 2004). Fulton et al. (2006) reported that approximately 0.5% of cattle entering feedlots are persistently infected, but this may be an underestimation of cattle born persistently infected because many will die prior to weaning and do not enter the stocker or feeder phase

Vaccination is frequently used in the control of bovine viral diarrhea infections (Dargatz et al., 2002; England, 2002). The goal of a vaccination program in a cow-calf operation is to reduce the risk of viral exposure to the fetus through vaccination of the dam (Kelling et al., 2005). The ability of maternal vaccination to provide fetal protection when the dams were challenged experimentally has been reported to range from 25% to 100% for inactivated vaccines and from 58% to 92% for modified-live vaccines (Dean et al., 2003; Grooms, 2004; Larson et al., 2004). A multitude of factors such as stress, nutrition, concurrent disease exposure, and improper vaccine handling can affect immune response, so persistently infected calves and reproductive losses resulting from bovine viral diarrhea virus can occur in vaccinated herds (Kelling, 2004).

There is controversy and confusion concerning the use of modified-live and killed vaccines used in cowherds. While there is a wealth of published literature on the efficacy and safety of bovine viral diarrhoea vaccines, there is also a plethora of methods and metrics which makes it difficult to draw conclusions. A detailed systematic review of the literature was undertaken to evaluate published data on the safety and efficacy of vaccines administered to cows for control of persistently infected calves and to evaluate the bias of the published data (PRISMA, 2016).

Systematic reviews support the concept of Evidence Based Veterinary Medicine by providing veterinary practitioners and cattle producers an objective evaluation of the scientific literature to aid in development of vaccine protocols. This review focused on the use of modified-live, killed, or combination vaccine programs and their impact on vaccine safety and efficacy as related to reproductive efficiency.

## **Material and Methods**

### **Identification of Studies**

Searches in CAB Abstracts (Ovid), Medline (Ovid), and Searchable Proceedings of Animal Conferences (SPAC) were conducted in February 2014. In addition *The Bovine Practitioner* was hand searched from 1980 to February 2014. The focus of the search was on peer-reviewed manuscripts, but conference proceedings were also evaluated. Concepts searched included cattle and (BVDV and vaccine) and pregnancy or fetus or fetal death. Details of the search format are shown in Table 4.1.

During the search, articles were uploaded into RefWorks, (ProQuest, Ann Arbor, MI) and duplicates removed. Manuscripts were screened in 2 phases, first by abstract

and then by full text. Two individuals independently screened each manuscript using the criteria: bovine viral diarrhoea, BVD, vaccine, cattle, and evaluation of results.

Elimination of manuscripts from the study was determined by consensus of the 2 individuals.

### **Eligibility Criteria**

Eligibility criteria included a study design focused on randomized controlled trials, with methods and results fully described in the manuscript. In addition only manuscripts published after 1979 were considered, because prior to that time the concept of fetal immunotolerance to bovine viral disease virus and its contribution to disease pathogenesis was not well known (Coria and McClurkin, 1978; McClurkin et al., 1979). This study only considered manuscripts published in English.

Table 4.1. The standard format for the Cochrane search and is included to allow readers to replicate the search in CAB Abstracts (Ovid), Medline (Ovid), and Searchable Proceedings of Animal Conferences (SPAC).

Cochrane Standards for Search
1. cattle.od
2. (cattle or cow or cows or bovine* or heifer*).ti.ab
3. or/1-2
4. ((virus or viral) adj2 diarrh*).ti,ab,od
5. (1 or 2) and 4
6. Bovine viral diarrhoea virus 2/ or Bovine viral diarrhoea virus 1/
7. bvdv.ti,ab
8. or/5-7
9. exp vaccination/
10. vaccin*.ti,ab.
11. or/9-10
12. exp pregnancy/ or exp fetal death/
13. (pregnan* or fetal* or foetal* or fetus*).ti,ab,od.
14. 12 or 13
15. 8 and 11 and 14
16. limit 15 to English

## **Data Abstraction**

A data collection form was piloted allowing 2 individuals to independently complete the forms using Qualtrics (<http://www.qualtrics.com/>). Each manuscript were reviewed for the following criteria:

- Study design, which included randomized controlled trials, controlled trails and case reports.
- Vaccine safety and how it was measured, by a variety of methods to include, virus isolation and serology using fetal and maternal blood, as well as evaluation of the dam's pregnancy status.
- Vaccine efficacy and how it was measured, by determining the number abortions, persistently infected calves, and serology to access maternal and fetal antibodies with a limited number of studies measuring duration of immunity
- Effect of vaccination on reproductive efficiency, which included conception rates, and losses during gestation and the neonatal period.
- Type of vaccine, which included modified-live, killed or combinations. Timing of vaccination was also assessed; for example, were vaccines given to cows pre-breeding or during pregnancy?
- Year of publication. Only manuscripts published after 1979 were included because the concept of immunotolerance was not fully understood until the late 1970s.
- Country of origin for the study.
- Type of cattle, dairy, beef or dual purpose.

- Vaccine manufacture's involvement in study and tendency for bias as a result of their support.

### **Risk of Bias List Assessment**

Full text screening was accomplished using 2 independent evaluators, which included the author and a graduate student. Additionally, several faculty from the College of Veterinary Medicine and Department of Animal Science at Texas A&M University, as well as subject matter experts from Agricultural Research Service, United States Department of Agriculture, critically appraised each study, allowing a more broad based evaluation. The form, developed in Qualtrics, asked evaluators to rate different aspects of the study: randomization, allocation concealment, blinding of participants, blinding of outcomes assessment, completeness of outcome data, selective reporting, and any other noted bias. Ethical approval and sponsor involvement were also recorded.

At the conclusion of the full text screening, the author and graduate student evaluated the manuscripts to be included in the qualitative synthesis using the PICO model (Table 4.2), the acronym denotes **P**atient or population, **I**ntervention or exposure, **C**omparison or intervention, **O**utcomes to measure or achieve.

Table 4.2. PICO model definitions for clinical questions (University of Illinois, 2016).

<b>P</b>	<b>P</b> atient, <b>P</b> opulation, or <b>P</b> roblem	How would I describe a group of patients similar to mine?
<b>I</b>	<b>I</b> ntervention, <b>P</b> rognostic <b>F</b> actor, or <b>E</b> xposure	Which main intervention, prognostic factor, or exposure am I considering?
<b>C</b>	<b>C</b> omparison or <b>I</b> ntervention (if appropriate)	What is the main alternative to compare with the intervention?
<b>O</b>	<b>O</b> utcome you would like to measure or achieve	What can I hope to accomplish measure, improve, or affect?
	What type of question are you asking?	Diagnosis, Etiology/Harm, Therapy, Prognosis, Prevention
	Type of study you want to find	What would be the best study

## Results

Nine hundred twenty seven records were retrieved from the databases, and after removing 85 duplicates, 2 evaluators screened the remaining 842 records by abstract reading and excluded 529 manuscripts for the following reasons: not specific to cattle, not specific to bovine viral diarrhea vaccine, vaccine safety not addressed, fetal protection not evaluated, or the wrong study type (Figure 4.1). The same 2 independent evaluators screened 313 manuscripts by full text reading and excluded 292 records for reasons noted in Figure 4.1. Subject matter experts, university faculty, and graduate students also assisted with each individual screening 2 to 4 papers; however, the 2 independent evaluators ultimately made all decisions by consensus, resulting in 20 manuscripts being chosen for the final analysis.

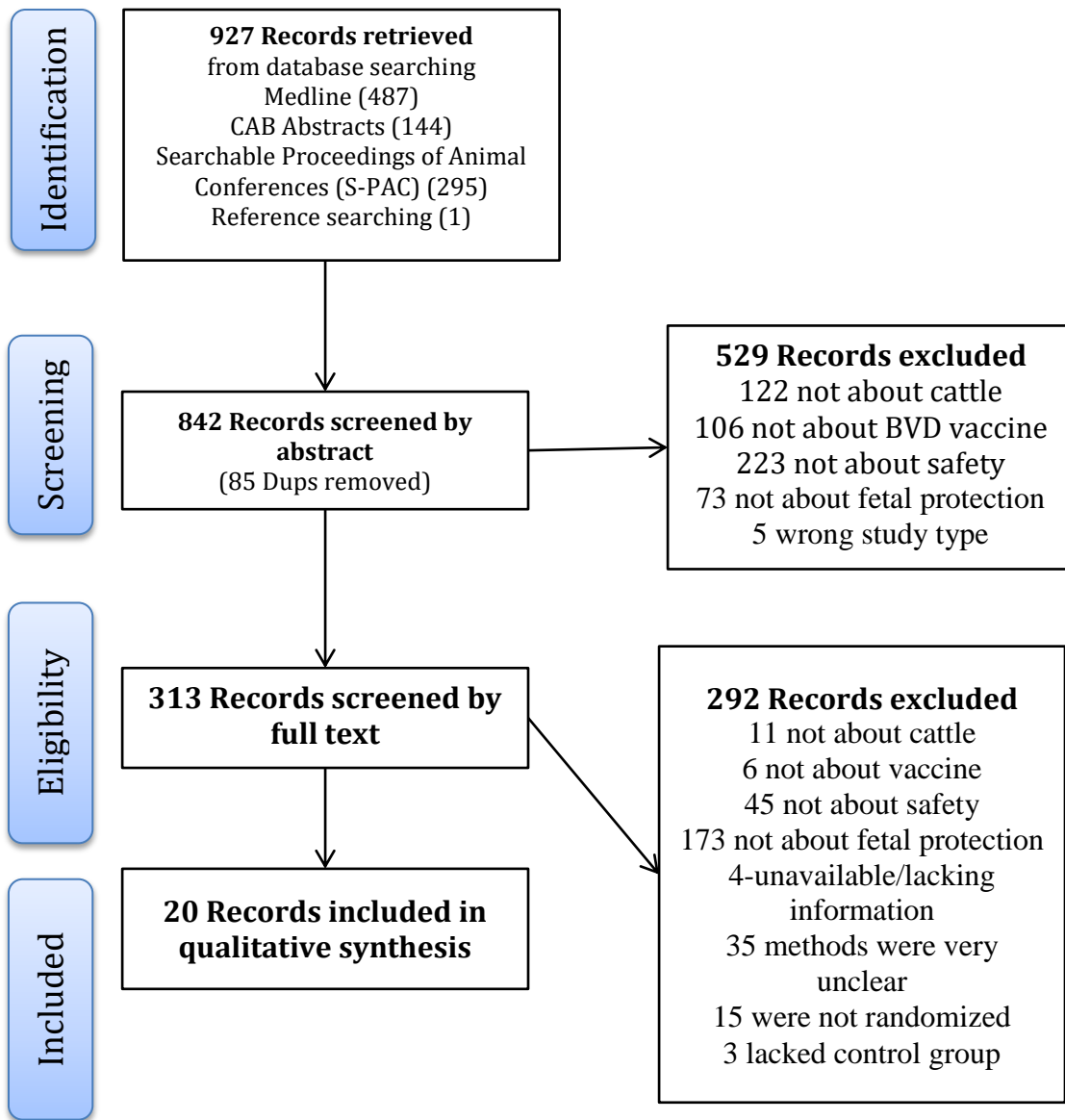


Figure 4.1. PRISMA flow chart depicts the screening and appraisal process. After searching databases to retrieve scientific articles and conference proceedings 927 articles were retrieved and with 85 duplicates removed, the remaining 842 articles were screened by abstract and 313 by full text screening, resulting in 20 articles being included in this review, all randomized control trials.



## **Population and Intervention**

There were 26 separate studies reported in the 20 manuscripts examined. The These manuscripts encompassed 2,118 animals focusing on beef cattle, 1 article with 37 animals dealt with dairy cattle, and 1 study with 30 animals did not define the cattle type. Sixteen of the studies were conducted in the United States and 1 each in Canada, The Netherlands, The United Kingdom, and Germany.

Four of the manuscripts examined killed vaccines (Brownlie et al., 1995; Zimmer et al., 2002; Grooms et al., 2007; Rodning et al., 2010) while the remaining 16 papers focused on modified live products. Six papers described administering a minimum immunizing dose of vaccine (Fairbanks et al., 2004; Ellsworth et al., 2006; Ficken et al., 2006a; Ficken et al., 2006b; Schnackel et al., 2007; Leyh et al., 2011) while the remaining papers used a commercially available product. Brownlie et al. (1995) administered killed products pre-breeding and at breeding, and all other studies addressed vaccines pre-breeding.

Twenty-two studies examined the ability of vaccines to provide fetal protection after challenging pregnant animals. All studies except Brownlie et al. (1995) described using a heterologous strain of virus for challenge. Dean et al. (2003) administered challenge virus by the intravenous route, while 15 studies in 10 manuscripts described intranasal challenge (Brownlie et al., 1995; Zimmer et al., 2002; Grooms et al., 2003; Kovacs et al., 2003; Fairbanks et al., 2004; Ficken et al., 2006a; Ficken et al., 2006b; Schnackel et al., 2007; Xue et al., 2009; Xue et al., 2011). Five studies in 5 manuscripts

used persistently infected animal to disseminate a viral challenge (Ellsworth et al., 2006; Grooms et al., 2007; Rodning et al., 2010; Leyh et al., 2011; Givens et al., 2012).

### **Comparison and Outcomes**

One important clinical concern related to vaccine use is the effects of modified live vaccines on conception when given in close proximity to breeding. The 4 studies that examined the question of vaccine safety pre-breeding in Table 4.3 concluded that modified live vaccines administered as early as 3 days pre-breeding demonstrated no negative effects on conception (Tucker et al., 1989; Campbell and Myers, 1999; Bolton et al., 2004; Walz et al., 2014). Campbell and Myers (1999) reported the majority of the mature cows in their study had been previously vaccinated and the other 3 studies reported a primary vaccination given prior to the pre-breeding immunization regimen, so these cattle were not naïve when they received their pre-breeding vaccination. The label on most modified live vaccines indicate vaccines should be given 28 days prior to breeding (Walz et al., 2014); therefore, nothing in the literature reviewed indicates modified live products would reduce conception when given according to label. It must be noted that all 4 studies reviewed were sponsored totally or in part by vaccine manufacturing entities, and sponsored studies in this review tend to report only positive results.

Vaccine protection in response to challenge was measured by evaluation of harvested fetal tissues in 5 studies (Fairbanks et al., 2006; Grooms et al., 2007; Schnackel et al., 2007; Xue et al., 2009; Leyh et al., 2011). Analysis of the fetal tissues after abortions and measurement of viremia and pre-colostrum seroconversion of calves

at parturition in the remaining 15 studies are detailed in Table 4.4. Eighteen of the papers described the statistical evaluation of their research, while 3 studies did not describe the statistical analysis used in their manuscript. Vaccine manufacturers were listed as authors and sponsored all or portions of the research in all but 2 of the manuscripts (Zimmer et al., 2003; Rodning et al., 2010), and these were the only papers to report negative results after vaccine intervention.

Table 4.3. Studies measuring the effect of vaccine on conception when given in close proximity to breeding. Population includes numbers and type, intervention includes vaccines and time given, and conclusions are based on measurement of pregnancy status.

Studies Measuring Conception Rates			
Manuscript Authors (year)	Population Sample size (cattle type) location	Intervention Groups control experimental (vaccine type)	Conclusions/ Outcome Pregnancy rate
Bolton et al. (2007)	799 (beef) US	con: vax2 45d pb exp: vax2 3d pb <i>vax mlv</i>	con: 341/399 exp: 346/400
Campbell & Myers (1999)	146 (beef) Canada	con: nonvax exp1: vax1 14d pb exp2: vax1 day of breeding <i>vax mlv</i>	con: 43/48 exp 1: 45/49 exp 2: 42/49
Tucker et al. (1989)	84 (beef) US	con: vax1/dewormed 30 days prior to syncro exp: vax1/dewormed 9 days prior to syncro <i>vax mlv</i>	con: 32/43 exp: 29/38
Walz et al. (2014)	60 (beef) US	con 1: inactivated vax1 10d pb exp 1: vax1 10d pb con 2: inactivated vax1 30d pb exp 2: vax1 30d pb <i>vax mlv</i>	con 1: 9/10 exp 1: 14/20 con 2: 10/10 exp 2: 17/20
pb=prebreeding vax: vaccinated			

Table 4.4. Population, interventions, and conclusion—reproductive loss post viral challenge.

Manuscript Authors (year)	Population Sample size (cattle type) location	Intervention Vaccine Groups control experimental ( <i>Vaccine type</i> )	Intervention Challenge (BVDV type)	Intervention Challenge & vax heterologous?	Conclusion/ Outcomes Measure of fetal protection
Brownlie et al. (1995)	30 (not stated) UK	con: unvax exp 1: vax2  exp 2: vax3 <i>vax: killed</i>	intranasal (type 1)	not clear	con: 15/15 VI exp1&2*: 0/15 VI *not reported separately
Cortese et al. (1998)	32 (beef) US	con: unvax exp: vax1 pb <i>vax: mlv</i>	intranasal (type 1)	yes	con: 6/6 PI exp: 2/12 PI
Dean et al. (2003)	40 (beef) US	con: sham injection exp 1: vax1 IM exp 2: vax1 SQ <i>vax: mlv</i>	IV (type 1)	yes	con: 11/11 PI exp 1: 1/11 VI exp 2: 2/13 PI
Ellsworth et al. (2006)	30 (beef) US	con: sham injection x1 exp: vax1 pb mid-level <i>vax: mlv</i>	PI calves (type 2)	yes	con: 9/10 conv exp: 1/20 conv
Fairbanks et al. (2004)	28 (beef) US	con: unvax  exp: vax1 pb mid-level <i>vax mlv</i>	intranasal (type 1)	yes	con: 10/10 conv exp: 0/18 conv
Fairbanks et al. (2004)	27 (beef) US	con: unvax exp: vax1 pb mid-level <i>vax: mlv</i>	intranasal (type 2)	yes	con: 8/8 conv exp: 1/19 conv
Ficken et al. (2006a)	100 (beef) US	con: sham vax exp 1: vax1  exp 2: vax2 <i>vax: type 1 BVD, mid-level virus, mlv</i>	intranasal (type 2)	yes	con: 9/10 PI exp 1: 6/18 PI exp 2: 7/19 PI
Ficken et al. (2006a)	60 (beef) US	con: sham vax exp: vax1 pb <i>vax: type 1 &amp; 2, mlv</i>	intranasal (type 2)	yes	con: 9/9 PI exp: 0/18 PI
Ficken et al. (2006b)	29 (beef) US	con: sham vax exp: vax1 pb <i>vax: type 1 &amp; 2, mlv</i>	intranasal (type 1)	yes	con: 8/9 PI exp: 0/20 PI
Ficken et al. (2006b)	30 (beef) US	con: sham vax exp: vax1 pb <i>vax: type 1 &amp; 2 mid-level, mlv</i>	intranasal (type 2)	yes	con: 10/10 PI exp: 0/19 PI

Table 4.4. Continued.

Manuscript Authors (year)	Population Sample size (cattle type) location	Intervention Vaccine Groups control experimental ( <i>Vaccine type</i> )	Intervention Challenge (BVDV type)	Intervention Challenge & vax heterologous?	Conclusion/ Outcomes Measure of fetal protection
Givens et al. (2012)	29 (beef) US	con: unvax exp: vax1 pb <i>vax mlv</i>	3 PI steers (type 1a, 1b, & 2)	yes	con: 6/6 PI exp: 2/12 PI
Grooms et al. (2007)	60 (beef) US	con: sham vax exp: vax2 pb <i>vax: commercial killed BVD product w/ heat sensitive BHV</i>	4 PI cows 2 (type 1b), 2 (type 2)	yes	con: 14/14 VI exp: 4/15 VI
Kovacs et al. (2003)	19 (dairy) US	con: sham vax exp: vax1 pb <i>vax: type 1, mlv</i>	intranasal (type 1)	yes	con: 8/8 VI exp: 0/11 VI
Kovacs et al. (2003)	18 (dairy) US	con: sham vax exp: vax1 pb <i>vax: type 2, mlv</i>	intranasal (type 2)	yes	con: 7/7 VI exp: 0/11 VI
Leyh et al. (2011)	50 (beef) US	con: sham vax exp 1: vax1 IM exp 2: vax1 SQ <i>vax: type 1 &amp; 2 mid-level, mlv</i>	PI cattle (type 1b)	yes	con: 10/10 VI exp 1: 1/20 VI exp 2: 2/20 VI
Rodning et al. (2010)	70 (beef) US	con: sham vax exp 1: company A vax1 exp 2: company B vax1 exp 3: company C vax1 <i>vax: type 1 &amp; 2, mlv and killed</i>	PI cattle (type 1a, 1b, and 2)	yes	con: 10/10 PI exp 1: 0/19 PI exp 2: 0/18 PI exp 3: 2/18 PI
Schnackel et al. (2007)	35 (beef) US	con: unvax exp: vax1 pb <i>vax: type 1, midlevel, mlv</i>	intranasal (type 2)	yes	con: 10/10 PI exp: 0/25 PI
Schnackel et al. (2007)	35 (beef) US	con: unvax exp: vax1 pb <i>vax: type 1, midlevel, mlv</i>	intranasal (type 1b)	yes	con: 6/6 PI exp: 1/24 PI
Xue et al. (2009)	37 (beef) US	con: unvax	intranasal (type 1)	yes	con: 5/12 PI
		exp: vax1 pb <i>vax mlv</i>			exp: 0/25 PI

Table 4.4. Continued.

Manuscript Authors (year)	Population Sample size (cattle type) location	Intervention Vaccine Groups control experimental ( <i>Vaccine type</i> )	Intervention Challenge (BVDV type)	Intervention Challenge & vax heterologous?	Conclusion/ Outcomes Measure of fetal protection
Xue et al. (2009)	46 (beef) US	con: unvax	intranasal (type 2)	yes	con: 17/18 PI
		exp: vax1 pb <i>vax mlv</i>			exp: 2/28 PI
Xue et al. (2011)	35 (beef) US	con: unvax exp: vax1 pb <i>vax: mlv, type 1a &amp; 2</i>	intranasal (type 1b)	yes	con: 12/12 PI exp: 1/23 PI
Zimmer et al. (2002)	41 (beef) Netherlands	con: unvax	intranasal (3 NCP strains)	yes	con: 12/12 PI
		exp1: company A vax2 (cytopathic)			exp 1: 6/9 VI
		exp 2: company B vax2 (noncytopathic) <i>vax: killed</i>			exp 2: 8/15 VI

vax2: vaccinated twice  
pb: pre-breeding  
mid-level: minimum immunizing dose

## Risk of Bias

Assessment of bias was accomplished using the Cochrane Collaboration's tool for assessing risk of bias (see Table 4.5). The questions and guidelines were very specific, examining the described methods of randomization, allocation concealment, blinding of participants, blinding of outcome assessment, incomplete outcome data, and selective reporting. To ensure the evaluations were objective, 2 manuscripts (Grooms et al., 2007; Givens et al., 2012) were evaluated by 2 individuals, and the remaining papers were evaluated by a minimum of 3 individuals comprised of graduate students and faculty from the Department of Animal Science and faculty from the College of Veterinary Medicine at Texas A&M University.

Table 4.5. Cochrane evaluations for risk of bias with assessments of potential risks for bias for each study reviewed. Instructions for completion found in chapter 8.5 d, Cochrane Handbook (Higgins et al., 2011).

Author (year)	Random sequence generation	Allocation Concealment	Blinding of Participants	Blinding of Outcomes Assessment	Incomplete Outcome Data	Selective Reporting	Other Bias	Funding
Bolton et al. (2007)	Unclear	Unclear	Low	Unclear	Low	Low	Unclear	Yes
Brownlie et al. (1995)	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Unclear	Yes
Campbell & Myers (1999)	High	Unclear	Low	Low	Unclear	Unclear	Unclear	Yes
Cortese et al. (1998)	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Unclear Health	Yes
Dean et al. (2003)	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Unclear	Yes
Ellsworth et al. (2006)	Unclear	Unclear	Low	Low	Unclear	Low	Unclear	Yes
Fairbanks et al. (2004)	Unclear	Unclear	Unclear	Unclear	Unclear	High	Unclear	Yes
Ficken et al. (2006a)	Unclear	Unclear	Unclear	Unclear	Low	Unclear	Unclear	Yes
Ficken et al. (2006b)	Unclear	Unclear	Unclear	Unclear	Low	Low	Unclear	Yes
Givens et al. (2012)	Unclear	Low	Low	Low	Low	Low	Unclear	Yes
Grooms et al. (2007)	Unclear	Unclear	Unclear	Unclear	Low	Low	Unclear	Yes
Kovacs et al. (2003)	Unclear	Unclear	Unclear	Unclear	High	High	Unclear	Yes
Leyh et al. (2011)	Unclear	Unclear	Unclear	Unclear	Low	Low	Unclear	Yes
Rodning et al. (2010)	Unclear	Unclear	Unclear	Unclear	Low	Low	Unclear	No
Schnackel et al. (2007)	Low	Unclear	Unclear	Unclear	High	Low	Unclear	Yes
Tucker et al. (1989)	Unclear	Unclear	Unclear	Unclear	High	Low	Unclear	Yes
Walz et al. (2014)	Low	Low	Unclear	Unclear	Low	Low	Unclear	Yes
Xue et al. (2009)	Unclear	Unclear	Unclear	Unclear	Low	Low	Unclear	Yes
Xue et al. (2011)	Unclear	Unclear	Unclear	Unclear	Low	Low	Unclear	Yes
Zimmer et al. (2002)	Unclear	Unclear	Unclear	Unclear	Low	Low	Unclear	No

Only 3 manuscripts described in detail their methods of randomization, (Campbell and Myers, 1999; Schnackel et al., 2007; Walz et al., 2014). Only 2 manuscripts (Givens et al., 2012; Walz et al., 2014), described blinding participants to allocation of animals. Only 4 papers described the blinding of participants (Campbell and Myers, 1999; Ellsworth et al., 2006; Bolton et al., 2007; Givens et al., 2012), and the same authors with the exception of Bolton et al (2007) describe blinding of outcomes assessment. Eleven papers describe or account for all outcome data, and 15 manuscripts report on all described criteria. Eighteen of the studies indicated the involvement of commercial vaccine manufacturers in the study, either through sponsorship or having employees listed as authors on papers. In 3 studies, statistical analysis was not even mentioned or described (Brownlie et al., 1995; Cortese et al., 1998; Campbell et al., 1999).

## **Discussion**

The role of fetal infection and immunotolerance in the epidemiology of bovine viral diarrhea is established (Newcomer et al., 2015). The employment of sound vaccination programs to prevent fetal infection and thus the viral shedding by immunetolerant animals is essential to control of this disease. In order to make sound management decisions, veterinary practitioners, animal scientists, and cattle producers need access to peer-reviewed articles with detailed descriptions of experimental design, including randomization, as efforts to blind participants, ensuring all outcome data is reported, and any potential bias from private funding or participation declared. Due to the role of bovine viral diarrhea virus as an immunosuppressant and its association with



bovine respiratory disease complex, winter dysentery, and other cattle diseases, unbiased information concerning the safety and effectiveness of vaccine programs is essential.

Only 2 manuscripts reported negative findings concerning safety and efficacy of bovine viral diarrhoea vaccines and those 2 studies that did not have support of the vaccine industry. The materials and methods generally lacked transparency, the methods used in randomization and the protocol to blind participants/investigators was seldom reported, and details of experimental design were many times ambiguous. Often the only part of the paper that was easy to follow was the abstract and conclusion, and it was difficult to surmise how the authors determined their findings. This review does not insinuate that industry involvement in research is always biased, only that publications should be transparent and diligently describe all procedures in sufficient detail to allow critical analysis of the research. Logical recommendations relating to animal health programs require access to all the pieces of the puzzle.

This review did not address reported abortions due to bovine herpes virus following use of multivalent vaccines containing bovine viral diarrhoea, only the safety and efficacy of bovine viral diarrhoea vaccines as related to fetal protection. The included trials were slanted toward modified-live vaccines, with only 4 studies involving killed vaccines (Brownlie et al., 1995; Zimmerman et al., 2002; Grooms et al., 2007; Rodning et al., 2010). While there is much rhetoric concerning the safety and effectiveness of modified live bovine viral diarrhoea vaccines, none of the reviewed manuscripts demonstrated negative effects of a modified-live vaccine on reproduction. One study from the Netherlands (Zimmer et al., 2002) reported on the ineffectiveness of a killed

product after challenge with 3 non-cytopathic strains of virus, and Rodning et al. (2010) evaluated 2 modified live and 1 killed vaccine, while all remaining articles only described positive attributes of the vaccines. Perino and Hunsaker (1997) when evaluating vaccine field efficacy for bovine respiratory disease found that when manuscripts reported a negative or neutral effect of vaccine, the authors were 2.8 times more likely not to have affiliation with manufacturer or developer of the vaccine.

A similar systematic review was recently performed (Newcomer et al., 2015) in order to calculate a more precise vaccine effect estimate via meta-analysis. From 41 references, the authors estimated a 45.0% decrease in abortions and 85.0% decrease in fetal infections attributed to vaccination. The current systematic review is an attempt to add to the evidence about vaccine efficacy by critically appraising many of the same studies to assess the risk of bias. It also provides an understanding of the quality gaps in the literature. Information from both reviews is needed for practicing veterinarians to make vaccination decisions based on evidence.

The ultimate end users of information provided by the scientific literature are practicing veterinarians, livestock producers, and Extension faculty. Transparent reporting of both the positive and negative findings of a research project allows the end-user to objectively evaluate its value. Often journals will not accept manuscripts that report insignificant findings or negative results. It is imperative that authors, editors, reviewers, and all others involved in research and publication transparently report all findings in the scientific literature. There is frustration in the agricultural community that consumer's lack confidence in the science we report, but lack of full disclosure only

contributes to their mistrust. Programs such as the Vetalltrials (2016) that encourage registering clinical trials at their inception and reporting all results are encouraging, but are in their infancy. Registering clinical trials and reporting on all outcomes increases transparency, which can increase consumer confidence in the agricultural scientific community.

With the recent 2014 Presidential executive order (Executive Order-Combating Antibiotic-Resistant Bacteria, 2014) and the subsequent call for new and effective vaccine development in order to reduce the use of antibiotics, hopefully, there will be more public funding available for field evaluation of vaccines used in animal health programs. The application of efficient vaccination programs would logically lower morbidity and mortality associated with the diseases in question; however, it is impossible to make an informed decision without access to transparent research.

## CHAPTER V

### CONCLUSIONS

This dissertation addresses 3 emerging-reemerging cattle diseases that affect the profitability of Texas livestock producers. Bovine trichomoniasis and bovine viral diarrhea virus infections have a negative effect on reproduction, resulting in fewer and lighter calves being marketed each year. Bovine viral diarrhea virus infection is a risk factor for bovine respiratory disease complex and winter dysentery, mainly through the ability of the virus to suppress immune function. Clinical epizootic hemorrhagic disease is not common in Texas cattle, presumably due to enzootic stability; the virus is common and cattle are protected against clinical disease as a result of their previous viral exposure. Epizootic hemorrhagic disease is an OIE reportable disease, and the presence of clinical disease could impact trade.

The study focusing on bovine trichomoniasis is described first. Experiment 1 illustrates that even with limited training; a suitable preputial diagnostic sample can be collected. This study indicated that when sampling the same bull over time, the quantitative results were similar for that bull; however, only 4 bulls were sampled for this experiment. There is a need to examine the variability of samples taken from the same bull over time using a larger number of bulls. The experiment involving temperature fluctuation of samples while they are in transit emphasizes the need to use next day delivery; even in a constant temperature of 107.0°C a sample properly packaged with an ice pack and insulated would not exceed the threshold of 104.0° for 8.5

hours. The less time spent in transit, the less likely temperature extremes will affect diagnostic outcomes. The results of the trichomoniasis study indicate that adoption of the novel RT-qPCR developed during the course of experiments described in this dissertation would reduce costs, by providing a more sensitive test with timelier results, allowing producers to more effectively manage the disease. The newly developed RT-qPCR does not require growth of the protozoa, therefore neither growth media nor incubation are required, allowing the sample to be shipped on ice.

Seroprevalence of epizootic hemorrhagic disease in 11 auction markets was determined to be 97.2%, much higher than anticipated. Veterinary practitioners, animal scientists, and cattle producers need to be aware the disease is endemic in Texas and importing cattle from non-endemic areas can result in the imported animals developing clinical disease. Clinical signs of epizootic hemorrhagic disease in cattle are similar to many vesicular diseases such as foot and mouth disease, so it is important that ALL vesicular diseases are reported to regulatory officials immediately.

Bovine viral diarrhea virus can produce clinical disease associated with the respiratory and gastrointestinal tract, but a large economic impact of this disease is associated with subclinical reproductive diseases, often resulting in the birth of immunotolerant persistently infected animals. Vaccination of breeding females to prevent fetal infection is considered an important management tool addressing persistently infected offspring. Results of a systematic review of safety and efficacy of bovine viral diarrhea vaccines indicate that few manuscripts describe the materials and

methods in enough detail to objectively evaluate results. There are very few studies that report negative results, and most studies are supported by manufactures of biologics.

The public appears to lack faith in science (Cockcroft and Holmes, 2003), and consumers appear skeptical about any scientific evidence related to their food source. Those of us involved in animal agriculture must embrace critical evaluation of the scientific literature, and we must be more concerned with appropriate experimental design and diligent transparent reporting of our all-valid results.

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