THESIS

PREVALENCE AND RISK FACTORS ASSOCIATED WITH BLUETONGUE VIRUS AMONG COLORADO SHEEP FLOCKS

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ABSTRACT OF THESIS

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During the summer of 2007, researchers from Colorado State University undertook a study to measure the prevalence of and identify risk factors associated with Bluetongue Virus (BTV) infection among Colorado sheep flocks. A total of 2,544 serum and whole blood samples were obtained from 1,058 ewes, 992 lambs, and 494 rams located on 108 sheep farms throughout Colorado. Flocks were recruited by the use of a questionnaire and flocks were tested for the presence of BTV antibodies utilizing cELISA, viral RNA utilizing nested RT-PCR, and the presence of clinical disease indicative of BTV based on the criteria of three or more clinical signs present in at least five animals.

Flock level seroprevalence was 28.70% (95% CI, 20.41% to 38.20%), viral RNA was detected in 22.22% (95% CI, 14.79% to 31.24%) of flocks and clinical disease was observed in 19.44% (95% CI, 12.46% to 28.17%) of flocks tested. Animal level seroprevalence within positive flocks ranged from 7.6% to 83 % with a mean of 27.09% (95%CI, 23.87% to 30.51%), viral RNA prevalence within positive flocks ranged from 4.8% to 48% with a mean of 25.62% (95%CI, 21.93% to 29.59%), and clinical disease

within positive flocks ranged from 16.7% to 41.7% with a mean of 24.24% (95% CI, 20.38% to 28.43%). Animal and flock level seroprevalence was higher among the adult population whereas prevalence of viral RNA and clinical disease was higher among the lambs. Positive flocks were distributed heterogeneously throughout the state and all but three flocks that demonstrated clinical disease were identical to those with detectable viral RNA; therefore, there was a significant correlation between the detection of viral RNA and observation of clinical disease among flocks.

The two most significant clinical signs associated with detection of viral RNA were weight loss (OR, 12.366, 95% CI, 2.057-74.343) and oral ulcerations (OR, 11.756, 95% CI, 1.061-130.243). Significant risk factors associated with viral RNA detection included primary purpose of the flock being commercial (OR 3.60, 95% CI , 0.85-15.18) and administration of BTV modified live vaccination (OR, 15.95, 95%CI, 4.51-56.35). Higher maximum temperature at the time of visit, closer proximity to water, and increased cumulative precipitation over a period of thirty-five days previous to the time of flock visit were identified as environmental confounders of these estimates. Risk factors associated with seropositive flocks included administration of BTV modified live vaccine compared to animals not receiving vaccine (OR, 9.360, 95%CI, 3.046-28.764). Environmental confounders of this relationship were closer proximity to water sources and lower elevations. Risk factors significantly associated with the odds of developing clinical disease also included vaccination (OR, 8.336, 95%CI 2.486-27.959) while higher maximum temperatures at the time of flock visit and closer proximity to water confounded this relationship.

These data provide supporting evidence that BTV affects a substantial number of Colorado sheep flocks. Seroprevalence is higher among the adult population whereas the prevalence of viral RNA and clinical disease was higher among the lambs. Higher seroprevalence among adults may be due to acquired immunity secondary to recurrent natural or vaccine exposure to BTV, whereas higher prevalence of viral RNA and clinical disease in lambs may be due to their naïve immunologic status and first exposure to BTV. The two clinical signs, oral ulcerations and weight loss, that were found to be significantly associated with clinical disease represent, respectively, acute and chronic stages of disease. Acute disease is characterized by oral ulcerations secondary to the primary pathogenesis of vasculitis and is followed in the chronic stage by weight loss due to lack of feed intake.

Vaccination was a common predictor that increased the likelihood of a flock having seropositive animals, viral RNA, and clinical signs of disease. The associations between vaccination and both seropositive animals and viral RNA are not surprising because the vaccine was a modified live formulation, and its primary purpose was to initiate an immune response with the production of BTV specific antibodies; however, it was surprising to identify such a strong association with clinical signs of disease. Commercial flocks may be more likely to utilize the BTV vaccine to protect their animals from potential economic loss from disease than are club and show market producers. In addition, commercial flocks are less likely to move animals across state lines and are thus less concerned with the limitations of interstate transport of seropositive animals resulting from the use of the BTV vaccine. In comparison, club and show producers must work to

maintain BTV seronegative status within their animals in order to transport them to respective ram sales or markets.

Proximity to water was a common environmental confounding variable. Animals inhabiting areas closer to water sources had an increased likelihood of developing viral RNA and antibody titers most likely due to increased exposure to *Culicoides spp*., which thrive in moist, warm environments in lower elevations.

The findings of this project have offered a foundation to understand BTV prevalence within Colorado sheep flocks. The risk factors identified through statistical modeling of both husbandry practices and environmental parameters warrant further investigation. Although this study raises many questions regarding vaccination, specifically modified live vaccines, causality has not been demonstrated. Therefore, future investigations should focus on vaccination in addition to understanding infection rates among all ruminant species and their respective vectors (*Culicoides spp*.) in order to further understand the ecology of this arbovirus within Colorado.

ACKNOWLEDGEMENTS

The project that developed into this thesis was initiated by the collaboration of Colorado State University's Diagnostic Lab, the United States Department of Agriculture, and a local producer's sheep that were demonstrating clinical signs of Bluetongue. It later grew into a movement during the summer of 2007, which encompassed the Colorado Woolgrowers, Center for Epidemiology and Animal Health, College Research Council, American Association of Small Ruminant Practitioners, Colorado State University's Veterinary Teaching Hospital, the 4-H Youth Development Organization, local veterinarians, local producers, fairs, and small ruminant shows. To all of these organizations, groups, and individuals; I am extremely grateful for the personal and professional development you have provided me.

Of all the things I learned during this project, the concept of teamwork resounded in my bones. Much like a flock of sheep, this epidemiological investigation only succeeded due to the fortitude of the leaders that taught me and the groups that came together in order to make a difference for the sheep. Within the remaining narrative, a few special people will be acknowledged in good humor for what has now become the initial story of my research career. Thank you to all and it has been a great ride.

During my interview at Colorado State University, I was told three things: it really does not snow much here; biking around Horsetooth is a breeze; and the beer is

really good. Within the first year, it snowed three feet during one December evening, biking Horsetooth almost made me pass out, and well---the beer is really good. Remembering the microbiology residency now brings back such great memories. With my first encounter of Dr. Hana Van Campen, I knew two things: she would make me read and report back on Jane Goodall's book: In the Shadow of Man, and she hated pathologists. Now I find myself living by Dr. Goodall's words on a daily basis: "Only if we understand can we care. Only if we care will we help. Only if we help shall they be saved." This is the start of the love I found for sheep, epidemiology, Bluetongue virus, and yes, even pathologists.

Within four months of the residency, Hana asked me to accompany her to investigate a flock of sheep suspected of having Bluetongue. This initial visit led me across the state of Colorado, through the plains of Wyoming, and would eventually lead me across the country to the central valley of California. For this opportunity, I am forever grateful. With my interest sparked by the oral ulcerations, coronitis, and unresolved answers surrounding the true occurrence or risk factors associated with Bluetongue among sheep, I set out to find someone who would be willing to guide me through this project. The remarkable person and leader was Dr. Ashley Hill. Within our first meeting, I think she knew she had her hands full with my impromptu visit (later being the norm), nervous rambling, and abuse of the epidemiologic language. However, she took me on. Humble thanks and appreciation to Ashley who has always believed in me, shaped my research career, and maintained the patience it takes to put up with me.

This patience was also supremely exhibited by the other Bluetongue team members and individuals comprising my thesis committee: Drs. Rob Callen, Dave Van

Metre and Dick Bowen. Rob supported and nurtured my research and writing skills even when I know they were most exhausting. Dave was always an advocate for my teaching and small ruminant knowledge, and Dick in combination with his wife, Dr. Joan Bowen, gave me gifts that cannot be repaid. Through them I found the balance of structured and focused research while maintaining an understanding of the industry and animals I was trying to help. Plus, Joan makes some killer chocolate chip cookies!

So while we are on the topic of eating, I cannot forgo mentioning the BBQ chicken Sir Dr. Anthony Knight burnt to a crisp on a warm evening during a summer of Ram and Bluetongue testing. While we almost caught the Holiday Inn complex on fire, memories from Tony are forever etched in my brain in addition to the quote: "enough, collect him in the shorts." Some things should not be explained, but sincere thanks to Tony for enhancing my knowledge of small ruminant medicine and helping me collect samples. Through this journey, I also met some of the GREATS in small ruminant medicine: Dr. Cleon Kimberling and Geri Parsons. Without them, the project could not have happened and I would not know some of the finest Colorado Woolgrowers or sheep producers in the country.

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Now that I am moving on to pursue my PhD at the University of California, Davis, I know this move was only made possible by the support of the people mentioned above. I am sincerely thankful to all of you and my fascination with this initial outbreak has inspired me to understand, care, help, and hopefully save.

For the sheep,

and those who care for them.

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Literature Review

Bluetongue Virus, Epidemiology, and current prevention and control methods

1.1 Introduction

Bluetongue virus (BTV) is the causative agent of an economically important arbovirus belonging to the family Reoviridae, genus *Orbivirus*. Twenty-four serotypes of BTV are recognized globally, five of which are considered endemic within the United States (2, 10, 11,13,17). The virus is transmitted by *Culicoides* spp. biting midges, which feed on a large range of hosts including both wild and domestic ruminant species.[1] Within the United States, domestic sheep and wild deer populations have been most significantly affected, with morbidity rates as high as 70% and mortality rates as high as 50% in naïve sheep populations. Annual losses to the US livestock industry have been estimated as high as $144 million dollars due to treatment costs, decreased production, and non-tariff trade restrictions on BTV positive animals and animal germplasm following BTV infection..[2]

All ruminants are susceptible to BTV, but sheep are most severely affected. The primary clinical sign of BTV infection is hemorrhage and ulceration of the mucous membranes in the upper portion of the gastrointestinal tract, including the oral cavity and esophagus. Other signs such as coronitis, laminitis, facial edema, and transient infertility are seen in sheep. Cattle rarely demonstrate clinical disease.[3]

Hematophagous *Culicoides* are biological vectors of the virus and serve as the primary means of transmission. Because this virus is not contagious through direct contact, much of the global distribution of the virus is representative of competent *Culicoides* vectors and environmental ecosystems that support them; these ecosystems are located within the latitude boundaries of 53°N and 34°S.[4] The virus has co-evolved

with different species of the insect vector, resulting in ecologically distinct viral serotype and insect-vector relationships (topotypes) throughout the world, but this distribution is beginning to change in part due to climate change.[5] The direct economic losses due to disease are minimal compared to losses experienced as a result of non-tariff trade and animal movement restrictions. These restrictions have been modified but not completely resolved over the past 30 years in recognition of the facts that ruminants are infected for a period of 60 days or less. *Culicoides* are the primary source of viral transmission and not a consequence of direct horizontal transmission between infected animals.[1]

The recent invasion of the southeastern United States by at least seven previously exotic serotypes of BTV as well as the rapid spread of 6 serotypes of BTV throughout both northern and southern Europe since 1998 has confirmed the importance of ongoing BTV surveillance.[6] The emergence of new species of *Culicoides* insects in Europe, which include Holarctic species that are typically identified within the Mediterranean basin and North America (*Culicoides obsoletus* and *C. chiopterus*) demonstrate the importance of global and national BTV surveillance.[7] Recent invasion of the US by BTV serotypes and strains from adjacent ecosystems in the Caribbean Basin and Central America suggest it is increasingly likely the US will experience future invasions and currently exotic BTV serotypes may become enzootic. The United States must also be concerned about invasion from distant locations including northern Europe where a highly virulent strain of BTV serotype 8 (BTV 8) is currently causing a massive pandemic. European BTV 8 is especially disconcerting because of the high incidence of clinical disease and suspected vertical virus transmission in cattle .[6]

Bluetongue virus has been identified on all continents excluding Antarctica. Current literature suggests that changing environmental parameters are resulting in new habitats for *Culicoides spp*. and in changes to the unique regional distribution of distinct BTV strains (virus topotypes). [8] BTV activity, defined by the Office International des Epizooties's (OIE) Terrestrial Manual, is predominantly found within the latitude boundaries of 53°N and 34°S. Three classifications of BTV status have been defined that affect transportation and free trade of ruminants. These include BTV free zones, BTV seasonally free zones, and BTV infected zones. Colorado is considered a BTV seasonally free zone because *Culicoides* are not active during the cold winter months. Because of this status, interstate transport requirements for sheep originating in Colorado include a negative BTV antibody test prior to exportation to BTV free states. A 60 day quarantine within a BTV free zone is also required before international exportation. Interstate and international trade restrictions associated with BTV continue to be one of many economic concerns impacting Colorado's sheep industry during seasonal ram sales and shows.[9] The current prevalence of BTV infections and disease in Colorado flocks is not known.

1.2 Global Distribution

Bluetongue (BT) disease was first identified in South African Merino sheep in 1902 and classified as malarial catarrhal fever. The disease was first named "bluetongue" in 1905 and expanded beyond the continent of Africa by 1943 when an epidemic occurred in Cyprus.[10] The first case within the United States was documented as a bluetongue-like illness of sheep in 1948 but the virus was not isolated until 1953 in

California from a condition described as "soremuzzle". [11,12] During the latter part of the 1950's, epidemics spread throughout Portugal and Spain resulting in devastating case mortality rate ranging from 70-80%. This outbreak caused concern among sheep rearing areas in Europe and Australia.[13]

International movement of animals and germplasm became a concern during the 1960's and 1970's.[14] Most of the economic loss due to BTV was a result of trade restrictions rather than the actual disease process within production animals, especially sheep and cattle. Appropriate trade restrictions in the 1970s prevented movement of animals with positive BTV antibody titers. These restrictions were put into place to limit exposure of naïve populations and resulted in significant global economic losses of approximately three billion dollars per year. [15,16]

Throughout the 1980's research efforts began to challenge earlier thoughts concerning epidemiology, import-export restrictions, and control measures related to BTV. [17] In the 1980's, BTV was recognized as an enzootic disease in areas that lie between the latitude boundaries of 53°N and 40°S, which included almost all continents including the Americas, Africa, Australia, and Asia.[14,18] The current global distribution has expanded to the latitude of 34°S and graphical representation of BTV serotypes and *Culicoides spp*. are summarized in Table 1 and Figure 1.[19]

Since 1998, the northern latitude boundary appears to be expanding. Eight distinct BTV strains from six different serotypes (1,2,4,8,9,16) have been isolated in southern Europe and an outbreak of BTV serotype 8 in livestock has occurred in northern Europe. [20] The first case of BTV-8 in Northern Europe was reported in the Netherlands

during August, 2006. Over the last three vector seasons (July-November), BTV 8 has been isolated in Belgium, Germany, Luxemburg, Denmark, Switzerland, the Czech Republic, and the UK. Recent activity of BTV 8 within northern Europe has resulted in a total of 2297 cases during 2006, and 40931 cases following the re-emergence throughout 2007. New case reports continued in July, 2008 starting with a sheep located in Germany, in spite of the initiation of a vaccine campaign that began May, 2008.[6] The evolution of BTV ecology in Europe is demonstrating the importance of environmental, vector, viral, and host relationships with respect to this and other arboviral diseases. [20]

Unique features of the European BTV 8 outbreaks have included the change in current vector distribution, identification of new vector species, increased incidence of clinical disease, and suspected vertical transmission among cattle populations. [7,21] Wind patterns in conjunction with warmer climatic conditions that support *Culicoides* populations are suspected to have led to the emergence and persistence of BTV 8 within Northern Europe. Circulation of BTV 8 has been identified throughout India, Malyasia, Nigeria, Pakistan, Kenya, Sudan, Malawi, South Africa, Dominican Republic, Trinidad, Barbaros, and Puerto Rico. This is the first isolation of BTV-8 within the European Union. *C. imicola*, the primary vector of BTV-8, has not been found in sufficient numbers throughout northern Europe where BTV-8 was efficiently transmitted. This suggests climatic and environmental conditions may only be part of the explanation of BTV-8 emergence in Europe. [22,23] Instead, *C. obsoletus* and *C pulicaris* complexes have been more frequently identified and are probably part of the transmission cycle in this outbreak. [24,25] Although not completely understood, increased incidence among cattle might be due to the exchange of dsRNA segments (reassortment) that can occur when

two different BTV (serotypes) infect the same cells, resulting in new BTV variants that have increased virulence in bovine cells.[6] These new BTV variants are causing devastating economic losses globally.

1.3 National Distribution

1.3.A Disease status

Although the first isolation of BTV from California was reported in 1953, the first comprehensive serologic survey within the U.S. was not conducted until the winter months of 1977-1978 on blood serum samples from slaughter cattle. Statewide serum antibody prevalence ranged from 0-79% with a national prevalence of 18.2%.[26] A series of state-level serological studies of cattle have been conducted by USDA/APHIS on a regular basis from 1979 until 2004. These surveys in addition to the present study demonstrate similar findings and regional differences with the lowest seroprevalence within the northeastern states and higher seroprevalence among the southwest and southeastern states. [18]

The national surveys conducted by USDA/APHIS help determine BTV free states that are capable of exporting cattle to Canada without additional BTV testing requirements. A seroprevalence threshold of 2% among all ruminants is used to differentiate BTV free zones from BTV endemic areas. During the latest survey conducted from 1991-2004, it was concluded that BTV was endemic in all states excluding Alaska, Hawaii, Michigan, Minnesota, New York, Wisconsin, and New England. [18] Serologic surveys conducted less frequently within wildlife populations

have demonstrated that white-tailed deer, black-tailed deer, mule deer, elk, pronghorn, and bighorn sheep are also infected by BTV. [27]

Although sporadic outbreaks have occurred throughout the United States - primarily affecting sheep flocks in the southwestern states, the most significant outbreak reported within the last decade occurred throughout southern Montana and Wyoming during November of 2007. Over three-hundred domestic sheep died as the result of BTV-17 infection, which also affected wildlife populations of pronghorn antelope, white-tailed deer, and mule deer. [28] This further establishes the importance of BTV as a virulent, persistent, and threatening virus of the United States livestock industry.

1.3.B Serotype Status

Initial plaque reduction neutralization assays performed during the 1970's revealed four antigenically distinct serotypes (10,11,13,17) circulating within 13 U.S. states. [29,30] These continue to be the predominant serotypes, with serotype 17 occurring most commonly, followed by 11, 13, and 10, based on serological evaluation of submissions to the National Veterinary Services Laboratories. However, most BTV cases are not sent for serotyping, so the true frequency of BTV serotypes in the United States may be different.[18] Other serotypes that have been rarely isolated in the U.S. include BTV 2 that occurred during a 1982 outbreak in Ona, Florida and BTV 1 during a 2004 outbreak in deer from Louisiana. [31-33] More recently, six additional serotypes (3,5,6,14,19,22) have been isolated from domestic and wild ruminants during 2007 in the southeastern region. [34]

It has been suggested that these additional serotypes were introduced from the Carribbean ecosystem where they are considered to be endemic and transmitted by *Culicoides insignis*. [35] After recent outbreaks in Europe with suspected wind-borne transmission, these new serotypes within the United States have provoked concern about a possible expansion of the Carribbean's ecosystem which might regularly exchange *Culicoides spp*. with the southeastern United States irrespective of international boundaries. [36]

1.3.C Vector status

Culicoides spp. have been implicated as the primary vector of BTV in North America by experimental infection of sheep with BTV from titrated *Culicoides* captured during an outbreak in Texas .[11] Further investigations performed in 1963 revealed the first transmission of BTV from sheep to sheep by *Culicoides spp*. gnats. [37] After these experiments the first descriptions of the *Culicoides variipennis* complex were described including five subspecies *C.v variipennis*, *C.v. sonorensis*, *C.v. occidentalis*, *C.v. australis*, and *C.v. abertensis.* [37,38] This taxonomic classification was later disputed and studies demonstrated that *C.v variipennis*, *C.v. occidentalis*, and *C.v. sonorensis* were genetically distinct populations. [15,37,39] Currently, the only *Culicoides* species that has been extensively studied within the United States is *Culicoides sonorensis*. Although *Culicoides insignis* has been identified in Florida, research is lacking to demonstrate the true vector epidemiology and prevalence of other *Culicoides* populations that might serve as important vectorial species for BTV transmission.[15]

The confirmation of *C. sonorensis* as the primary vector of BTV was based on evidence of blood feeding on livestock, oral susceptibility for replication and dissemination of BTV, transmission of BTV to vertebrate animals under experimental conditions, and isolation of BTV from *C. sonorensis* in field populations. [37] Seventeen species of *Culicoides* have been identified in North America that feed on livestock; however, *C. sonorensis* continues to be recognized as the primary and proven vector of BTV transmission.[15] Other North American *Culicoides spp.*, including members of the *Avaritia* subgenus , which have also been found throughout Northern Europe, have been investigated as potential vectors but their role in BTV transmission has not been established within the United States. [40]

Understanding the distribution and vector biology of *Culicoides* in relation to USDA's classification of BTV free and cattle-exporting zones is important. Serological surveys have repeatedly demonstrated that the northeastern states have low (<2%) prevalence of antibodies to BTV yet *C. variipennis* is a common vector found on dairy farms throughout that area. In contrast, BTV endemic areas in the southeast are populated by *C. sonorensis.* [18] This emphasizes that a variety of factors contribute to variation in virus serotype infecting different vectors including altered susceptibility to BTV among *Culicoides spp*. and variation of environmental conditions. [23]

Environmental factors known to support larval populations of *Culicoides sonorensis* include standing or slow moving sunlight-exposed aquatic environments often contaminated with manure. [40] It has also been demonstrated that larval habitats of *C. sornorensis* commonly have increased salinity concentrations when compared to those of

C. variipennis. [41] The greatest abundance of adult *C. sonorensis* populations occurs with temperatures ranging from 28-30°C; therefore, the typical vector season extends from July-November within Northern Hemisphere areas classified as BTV seasonally free zones. [16] Recent studies are just beginning to explore novel environmental factors such as altitude, terrain slope, percentage of area covered by forests, normalized difference vegetation index, and aridity index that might influence populations of *Culicoides*. It is becoming more apparent that each *Culicoides* subspecies prefers a particular subset of biotic and abiotic factors for survival and transmission of BTV.[42]

A crucial knowledge gap in BTV epidemiology has been the "overwintering" mechanism of the virus. It has been demonstrated that *C. sonorensis* overwinters in temperate winter environments but the mechanism of viral overwintering continues to baffle the BTV research community. Neither transovarial nor transovum transmission has been confirmed, but recent studies demonstrated that viral RNA was present in larva and pupae of *C sonorensis* collected from the field that may be suggestive of vertical transmission.[43,44]

Other concerns have revolved around the emergence of new viral vector interactions that might lead to a more virulent viral strain. The isolation of BTV-2 in Florida stimulated further research in understanding the vector competence of *C. sonorensis* with exotic serotypes of the virus. It was demonstrated that BTV-2 does not replicate as well as other US serotypes in colonized *C. sonorensis* which may suggest that *C. sonorensis* may not be a capable vector for BTV-2 despite periodic isolations within the United States. [23] Therefore, *C. insignis* and other vector species might be necessary

in order to transmit what were classically designated exotic serotypes. This further demonstrates the importance of understanding the prevalence, species, ecology and BTV status among vectors within different ecosystems to fully understand current BTV status.[45]

1.4 Virus, vector, host relationships

1.4. A Bluetongue Virus

Bluetongue is a species of *Orbivirus* within the family *Reoviridae,* and is a non-enveloped, double stranded RNA virus composed of ten linear segments.[46] Many viruses belonging to the *Orbivirus* genus are pathogenic to animals but can also infect humans, plants, or insects and are transmitted by arthropod vectors. Bluetongue virus (BTV) is closely associated with epizootic hemorrhagic disease virus (EHDV) of deer, African horse sickness virus (AHSV) and equine encephalitis viruses (EEV) all of which are transmitted by *Culicoides* spp. gnats and can result in significant morbidity and mortality among their susceptible host species. BTV is the most common *Orbivirus* throughout the world and has resulted in significant economic loss secondary to infection and control efforts that have been traditionally established to limit transmission by ruminant hosts. [47]

Unlike most single stranded RNA viruses, the *Orbiviruses* are relatively stable throughout infection, and point mutations do not appear to arise as frequently as those seen in non-segmented ssRNA viruses such as West Nile Virus .[48] Some of the structural proteins that have proven to be most significant include VP2 which is the major determinant of BTV serotype and VP5 which is more conserved but shows some degree of serotype variation related to geographic origin .[6]

BTV interacts with the cell by the binding of VP2 with the cell surface. After the virus is internalized, VP2 dissociates and causes VP5 fusion with the endosomal membrane, delivering a transcriptionally active core into the cell cytoplasm. The VP1 molecules begin to transcribe positive sense ssRNA from each of the ten segments composing the BTV genome. The viral mRNA then serves as a template for translation of additional viral proteins which can begin two hours after infection. Exchanges of dsRNA segments can and do frequently occur when two different BTV serotypes infect the same cell, which allows for the evolution of BTV through the process of reassortment. It has been demonstrated that some segments are more often exchanged than others, but the reasons for this have not been clearly identified. [49]

The differences in the nucleotide sequence of each of the ten distinct dsRNA segments leads to the genetic heterogeneity of field strains that can occur due to genetic drift and genetic shift. Genetic shift is a result of reassortment of viral genes during mixed infections of vertebrate or invertebrate hosts. [50] It has been proposed that this process is responsible for selection of individual BTV genes that occurs over a period of time finally leading to the creation of genetically distinct, region-specific genotypes (topotypes) of each virus gene. [4] The differences in genetic and phenotypic properties among and within serotypes affect differences in virulence and can also create difficulties when developing PCR assays that are nucleic acid based detection methods. [51]

1.4.B Virus Vector Interactions

It has been postulated that *Orbiviruses*, particularly Bluetongue, have evolved in response to selective pressure from the vector, *Culicoides spp.*.[50] Sequence analysis has

established geographically distinct genetic virus groupings, known as topotypes that are related to vector distribution. [52] The NS3 protein is encoded by RNA segment 3 and is involved in virus budding and highly expressed in insect cells.[51] These RNA segments have been demonstrated to segregate virus serotypes and strains into distinct topotypes, but it is thought this could be attributed to founder effect versus selection pressure by the insect vector. [50] Founder effect is thought to occur in arboviral infections when virus populations become very small during the vertebrate-invertebrate transmission cycle. When the *Culicoides* vector consumes a blood meal from vertebrate host with a low titer viremia, very few virus particles are present within the vector which can act as a genetic bottleneck.[53] A single variant within the vertebrate host can be selected from the quasispecies within the invertebrate host and amplified allowing the genotype to be fixed.[54] Other evidence of selection pressure on virus topotypes by the insect vector came from sequence analysis performed using segment 7 which encodes VP7. [55] The VP7 viral protein is involved with virus binding in insect cells and virus topotypes are associated with insect vector distributions, but absolute evidence of selection pressure by the vector still remains to be completely proven.[6]

In addition to phylogenetic analysis, further understanding of the viral and vector genetics and the vector's immune system will aid in explaining the infection, replication, and transmission of viruses with respect to vector competence. [56] The composition of the midgut differs among competent and incompetent vector species, and it is still uncertain if salinity in the environment has a relationship with the composition or capacity of different vectorial species. [57] Although the entire relationship between the virus and

vector is not understood, environmental and genetic factors are important determinants of Bluetongue activity within the vector and its ecosystem.

1.4.C Bluetongue Virus Infection of Ruminants

Clinical severity and host susceptibility of BT among individual species can vary remarkably although the primary pathogenesis is similar among all ruminant species. [58] After the initial insect bite, the virus replicates in adjacent lymph nodes and then spreads to infect vascular endothelium, macrophages, and dendritic cells in a variety of tissues and organs. Injury to the endothelial cells in small blood vessels results in vascular thrombosis and ischemic necrosis of the tissues. [59] This leads to oral ulcerations, coronitis, muscle necrosis, and vascular leakage. Facial and pulmonary edema develops as a result of vascular leakage; other clinical signs associated with vascular leakage include pleural or pericardial effusion. [60]

Differences in clinical disease observed among different ruminant species are thought to be due to inherent differences in the response of their vascular endothelium.[59] A common theme among all ruminants is the duration of viremia, which is characterized as prolonged but not persistent. The long duration of viremia is important because it increases the likelihood of infecting feeding insects with BTV. Neutralizing antibodies are detected in the blood 14 days after infection and have been detected as early as 9 days by cELISA but the close association of the virus with blood cells protects the virus from complete immune clearance by neutralizing antibodies.[19]

The prolonged nature of viremia is thought to be due to the cell associated nature, particularly the red blood cell, of BTV infections. [19] Ruminants infected with a particular

BTV serotype are likely to have lifelong immunity to the homologous serotype with only partial or no protection against heterologous BTV serotypes .[61] The prolonged viremia in conjunction with immunity only to homologous serotypes further compromises boundaries between BTV-free and infected zones when novel serotypes or strains are identified.

Although viremia is known to be prolonged, studies of the duration of viremia reported varying results. Lack of agreement on duration of viremia further complicates trade issues and implementation of control measures and quarantine periods for ruminants. Recent reports suggest that the duration of viremia reflects the lifespan of circulating red blood cells that harbor viral RNA but are not permissive for viral replication. [51] Studies conducted in Europe using serotypes 2,4,9, and 16 established viremia periods of 14-45 days in experimentally infected sheep, and other studies have found viremia to persist 24-78 days. [62] In contrast, duration of BTV nucleic acid can be found up to 7 months after inoculation possibly making PCR an overly sensitive diagnostic tool when attempting to screen animals for productive BTV infection. [51] The OIE has adopted a period of 60 days in which livestock are considered to be infectious to *C. sonorensis.* [19]

In utero vertical transmission of BTV has been recognized in ruminants, but these forms of transmission are considered to be insignificant in the natural transmission of this virus. Bluetongue was first suspected of causing congenital brain malformations in 1955 after the administration of a live-attenuated BTV vaccine in pregnant ewes.[63] The adaptation of field viruses for the use in vaccines has been known to increase their ability

to cross the placenta and cause teratogenesis within the developing fetus. [60] Infections early in gestation can result in fetal death or congenital defects, whereas fetuses that survive infection through the first half of gestation develop congenital defects and can have virus-specific antibody without infectious virus present at the time of birth. Fetuses infected during the second half of gestation do not develop congenital malformations but can be born viremic usually clearing the virus within months. [64]

Recent outbreaks of BTV 8 within Northern Europe have revived the issue of vertical transmission and persistence among calves as a possible overwintering mechanism. [21] In contrast to European reports of congenitally-infected calves, only rare cases of congenital BTV have been attributed to natural infections within the United States and most cases have been the result of experimental infection with live attenuated vaccine strains which are known to cross the placental barrier resulting in vertical transmission when administered to pregnant dams. [65] Over the past 20 years, only a few animals have been infected in utero with natural BTV infection and all fetuses had severe brain lesions that were not compatible with post-partum life. Although vertical transmission or the possibility of persistently infected calves may play a role in the overwintering process of BTV, this role is not likely to be significant among ruminants in the United States. [65]

Other explanations of the overwintering process that have appeared more plausible include: vertical transmission of the virus in vector insects; a complicated overwintering cycle that involves some unidentified intermediate host such as reptiles or birds; prolonged survival of infected adult *Culicoides* insects; prolonged infection of

cattle; and an ongoing low level cycle of infection between cattle and *Culicoides* insects throughout the overwintering period. [66] Most of these overwintering hypotheses are consistent with findings by others that have substantiated BTV as a persistent virus of the vector and not the host.[16]

1.5 Regulatory Issues

1.5.A Control Strategies

A number of control strategies have been established to either protect naive animals by vaccination or limit naive animals' exposure to *Culicoides spp*. Only one nationally licensed vaccine can be administered to sheep within the United States, and it is a modified live vaccine that is only effective against serotype 10. [67,68] The other vaccine utilized most frequently within the United States is produced by the California Woolgrowers Association and is only approved for use in California. It is also a modified live vaccine that protects against serotypes 10, 11, and 17. Both of these vaccines have limitations inherent to modified live vaccinations, which may lead to abortions in pregnant animals or development of mild clinical signs. [63] A recombinant canarypox vaccine has been developed and has demonstrated a high level of protection among sheep with minimal side effects but it is not available commercially.[69]

The primary control recommendation is to prevent exposure of livestock to feeding *Culicoides* by using insecticides and housing animals indoors during periods in which *Culicoides* are most active, late evening and early morning. [9] However, recent studies have found that *Culicoides* are active throughout the day and feed within barn facilities

quite frequently, so, housing recommendations do not adequately protect against exposure. [7]

1.5.B Trade Restrictions

Bluetongue is considered a notifiable disease by the Office International des Epizooties (OIE) based on its ability to cause substantial morbidity and mortality with a potential for rapid international spread in regions with competent vector populations.[12] Inclusion on the notifiable disease list allows countries with BTV-free status to impose nontariff trade restrictions on cattle imported from BTV endemic countries. [1] Within the United States, Bluetongue virus is the only OIE-notifiable disease that affects a substantial portion of the United States based on data collected from United States Department of Agriculture/Animal and Plant Health Inspection Service (USDA/APHIS) national surveillance. [18] In 1998, 66 countries had imposed 159 BTV-based import measures on US ruminants and by-products leading to annual losses as high as $144 million. [5]

Bluetongue was initially included on the OIE's list secondary to outbreaks that were thought to be caused by the movement of ruminants in addition to historical evidence that cattle were capable of persistent infections.[19] However, now that it is established that movement and expansion of *Culicoides* into new geographic areas, rather than animal movement, was the primary cause of BTV 8 emergence among European cattle, many countries now recognize that bluetongue is a disease influenced by climatic and environmental conditions. This has lead to modification of their livestock trade policies.[70] The OIE's Terrestrial Manual designates three classifications of BTV status

affecting transportation and free trade of ruminants. These designations are based on surveillance surveys conducted by USDA/APHIS where a threshold of less than 2% seroprevalence is a considered BTV free zone. Seroprevalence levels greater than 2% are classified as BTV infected zones and limit the ability to trade freely with other countries that have BTV-free status. [18]

Throughout the past 30 years, science based approaches have been utilized to understand the duration of viremia and limitations of diagnostics in detection of active infection. [19] The OIE now considers the infective period for cattle to be 60 days, which reflects the maximal period when ruminants are thought to be infective to *Culicoides*. In contrast, many countries with BTV free status deny transport of animals based on pre-arrival antibody detection methods. [1] Antibodies can be detected as early as 14 days post infection, and it is believed that they can persist for long durations in the field providing lifelong immunity. Thus, serum antibodies to BTV are an inadequate measurement of active infection. [71] Other limitations with serological tests include the inconsistency of diagnostic techniques. Although the cELISA has become the preferred method of testing for BTV antibodies, some countries still utilize the AGID for export certification. The BTV AGID serologic test cross reacts with antibodies of other *Orbiviruses* including EHDV and can result in false-positive test results when compared with BTV-specific serum neutralization assays. [9,14]

Issues concerning trade restrictions within the United States have demonstrated a great deal of progress in understanding that BT is an endemic but non-contagious disease among ruminants. APHIS now recognizes the primary cause for virus introduction into

new geographic areas is a result of vectors instead of movement of viremic livestock. Vector ecosystem expansion is related to prevalent vector species, climatic events, ecology, and environment rather than livestock movement. [70]

The prevalence of Bluetongue within the United States has been historically based on antibody detection methods in cattle and has not focused on the significant morbidity experienced in sheep. This study was established in order to understand the impact of BTV among Colorado's sheep flocks and identify risk factors associated with BTV infection and bluetongue disease so that proper preventative measures could be suggested and implemented by producers throughout the state. The objectives of this study are to measure the herd-level prevalence of BTV antibodies, BTV RNA, and clinical signs of BTV infection in Colorado sheep, and to identify risk factors associated with BTV seroprevalence, presence of viral RNA, and clinical signs. Chapter 2 presents the results from the cross-sectional prevalence study and Chapter 3 delves into understanding the risk factors involved with infection rates among sheep. Chapter 4 offers conclusions from our study while outlining future research opportunities for the BTV community.

1.6 References

1. Singer RS, MacLachlan NJ, Carpenter TE. Maximal predicted duration of viremia in bluetongue virus-infected cattle. *J Vet Diagn Invest* 2001;13:43-49.
2. Hoar BR, Carpenter TE, Singer RS, et al. Regional risk of exporting cattle seropositive for bluetongue virus from the United States. *Am J Vet Res* 2003;64:520-529.
3. MacLachlan NJ. The pathogenesis and immunology of bluetongue virus infection of ruminants. *Comp Immunol Microbiol Infect Dis* 1994;17:197-206.
4. MacLachlan NJ, Zientara S, Stallknecht DE, et al. Phylogenetic comparison of the S10 genes of recent isolates of bluetongue virus from the United States and French Martinique Island. *Virus Res* 2007;129:236-240.
5. Tabachnick WJ, MacLachlan NJ, Thompson LH, et al. Susceptibility of Culicoides variipennis sonorensis to infection by polymerase chain reaction-detectable bluetongue virus in cattle blood. *Am J Trop Med Hyg* 1996;54:481-485.
6. Schwartz-Cornill I, Mertens PP, Contreras V, et al. Bluetongue virus: virology, pathogenesis and immunity. *Vet Res* 2008;39:46.
7. Meiswinkel R, Goffredo M, Leijs P, et al. The Culicoides 'snapshot': A novel approach used to assess vector densities widely and rapidly during the 2006 outbreak of bluetongue (BT) in The Netherlands. *Prev Vet Med* 2008;87:98-118.
8. Baylis M. When will bluetongue transmission start in 2008? *Vet Rec* 2008;162:526; author reply 527.
9. World Organisation for Animal Health. Article 8.3.19, Bluetongue. OIE Terrestrial Manual 2008.
10. Monath. *Orviviruses and Coltiviruses, In: Fields*. Boston: Lipinncott, 1996.
11. Price DA, Hardy WT. Isolation of the bluetongue virus from Texas sheep-Culicoides shown to be a vector. *J Am Vet Med Assoc* 1954;124:255-258.
12. McKercher DG, McGowan B, Howarth JA, et al. A preliminary report on the isolation and identification of the bluetongue virus from sheep in California. *J Am Vet Med Assoc* 1953;122:300-301.
13. Greiner EC, Alexander FC, Roach J, et al. Bluetongue epidemiology in the Caribbean region: serological and entomological evidence from a pilot study in Barbados. *Med Vet Entomol* 1990;4:289-295.
14. Pearson JE, Carbrey EA, Gustafson GA. Bluetongue virus in cattle: complement fixing antibody response and viremia in experimentally infected animals. *Proc Annu Meet U S Anim Health Assoc* 1973:524-531.
15. Gibbs EP, Greiner EC, Alexander FC, et al. Serological survey of ruminant livestock in some countries of the Caribbean region and South America for antibody to bluetongue virus. *Vet Rec* 1983;113:446-448.
16. Ostlund E, Moser K. Distribution of bluetongue in the United States of America, 1991-2002. *Vet Ital* 2004;40:83-88.
17. MacLachlan NJ, Osburn BI. Impact of bluetongue virus infection on the international movement and trade of ruminants. *J Am Vet Med Assoc* 2006;228:1346-1349.

18. Tabachnick WJ. Culicoides variipennis and bluetongue-virus epidemiology in the United States. *Annu Rev Entomol* 1996;41:23-43.
19. Mellor PS, Boorman J. The transmission and geographical spread of African horse sickness and bluetongue viruses. *Ann Trop Med Parasitol* 1995;89:1-15.
20. Purse BV, Mellor PS, Rogers DJ, et al. Climate change and the recent emergence of bluetongue in Europe. *Nat Rev Microbiol* 2005;3:171-181.
21. Clercq D. Transplacental infection and apparently immunotolerance induced by a wild type bluetongue virus serotype 8 natural infections. *Transbound Emerg Dis* 2008;55:352-259.
22. Gloster J, Burgin L, Witham C, et al. Bluetongue in the United Kingdom and northern Europe in 2007 and key issues for 2008. *Vet Rec* 2008;162:298-302.
23. Gerry AC, Mullens BA, Maclachlan NJ, et al. Seasonal transmission of bluetongue virus by Culicoides sonorensis (Diptera: Ceratopogonidae) at a southern California dairy and evaluation of vectorial capacity as a predictor of bluetongue virus transmission. *J Med Entomol* 2001;38:197-209.
24. Meiswinkel R, Baldet T, de Deken R, et al. The 2006 outbreak of bluetongue in northern Europe-The entomological perspective. *Prev Vet Med* 2008;87:55-63.
25. De Deken G, Madder M, Deblauwe I, et al. Vector monitoring at Belgian outbreak sites during the bluetongue epidemic of 2006. *Prev Vet Med* 2008;87:64-73.
26. Metcalf HE, Pearson JE, Klingsporn AL. Bluetongue in cattle: a serologic survey of slaughter cattle in the United States. *Am J Vet Res* 1981;42:1057-1061.
27. Stallknecht DE, Kellogg ML, Blue JL, et al. Antibodies to bluetongue and epizootic hemorrhagic disease viruses in a barrier island white-tailed deer population. *J Wildl Dis* 1991;27:668-674.
28. Barrett B. Bluetongue, ovine, USA: Wyoming. ProMED mail; 2007, archive number 20071109.3635.
29. Barber TL, Jochim MM. Serologic characterization of selected bluetongue virus strains from the United States. *Proc Annu Meet U S Anim Health Assoc* 1973:352-359.
30. Hourrigan JL, Klingsporn AL. Epizootiology of bluetongue: the situation in the United States of America. *Aust Vet J* 1975;51:203-208.
31. Bram RA, George JE, Reichar RE, et al. Threat of foreign arthropod-borne pathogens to livestock in the United States. *J Med Entomol* 2002;39:405-416.
32. Mecham JO, Johnson DJ. Persistence of bluetongue virus serotype 2 (BTV-2) in the southeast United States. *Virus Res* 2005;113:116-122.
33. Johnson DJ, Ostlund EN, Stallknecht DE, et al. First report of bluetongue virus serotype 1 isolated from a white-tailed deer in the United States. *J Vet Diagn Invest* 2006;18:398-401.
34. Johnson, D.J., Identification of new United States bluetongue types. In: *Proc Ann Mtg US Anim Health Assoc*. 2007; 111,:209-210.
35. Gibbs EP, Greiner EC. The epidemiology of bluetongue. *Comp Immunol Microbiol Infect Dis* 1994;17:207-220.

36. Gloster J, Mellor PS, Manning AJ, et al. Assessing the risk of windborne spread of bluetongue in the 2006 outbreak of disease in northern Europe. *Vet Rec* 2007;160:54-56.
37. Foster NM, Jones RH, McCrory BR. Preliminary Investigations on Insect Transmission of Bluetongue Virus in Sheep. *Am J Vet Res* 1963;24:1195-1200.
38. Wirth WW, Dyce AL. The current taxonomic status of the Culicoides vectors of bluetongue viruses. *Prog Clin Biol Res* 1985;178:151-164.
39. Holbrook FR. Research on the control of bluetongue in livestock by vector suppression. *Prog Clin Biol Res* 1985;178:617-620.
40. Mullens BA, Tabachnick WJ, Holbrook FR, et al. Effects of temperature on virogenesis of bluetongue virus serotype 11 in Culicoides variipennis sonorensis. *Med Vet Entomol* 1995;9:71-76.
41. Green AL, Dargatz DA, Schmidtmann ET, et al. Risk factors associated with herd-level exposure of cattle in Nebraska, North Dakota, and South Dakota to bluetongue virus. *Am J Vet Res* 2005;66:853-860.
42. Conte A. Novel environmental factors influencing the distribution and abundance of Culicoides imicola and the obsoletus complex in Italy. *Vet Ital*;43.
43. White DM, Wilson WC, Blair CD, et al. Studies on overwintering of bluetongue viruses in insects. *J Gen Virol* 2005;86:453-462.
44. White DM, Blair CD, Beaty BJ. Molecular epidemiology of Bluetongue virus in northern Colorado. *Virus Res* 2006;118:39-45.
45. Baldet T, Delecolle JC, Cetre-Sossah C, et al. Indoor activity of Culicoides associated with livestock in the bluetongue virus (BTV) affected region of northern France during autumn 2006. *Prev Vet Med* 2008;87:84-97.
46. Mertens PP, Diprose J. The bluetongue virus core: a nano-scale transcription machine. *Virus Res* 2004;101:29-43.
47. Roy P. Bluetongue virus: dissection of the polymerase complex. *J Gen Virol* 2008;89:1789-1804.
48. Osburn BI, de Mattos CA, de Mattos CC, et al. Bluetongue disease and the molecular epidemiology of viruses from the western United States. *Comp Immunol Microbiol Infect Dis* 1996;19:181-190.
49. Pritchard LI, Gould AR. Phylogenetic comparison of the serotype-specific VP2 protein of bluetongue and related orbiviruses. *Virus Res* 1995;39:207-220.
50. Bonneau KR, Mullens BA, MacLachlan NJ. Occurrence of genetic drift and founder effect during quasispecies evolution of the VP2 and NS3/NS3A genes of bluetongue virus upon passage between sheep, cattle, and Culicoides sonorensis. *J Virol* 2001;75:8298-8305.
51. Bonneau KR, Topol JB, Gerry AC, et al. Variation in the NS3/NS3A gene of bluetongue viruses contained in Culicoides sonorensis collected from a single site in southern California. *Virus Res* 2002;84:59-65.
52. Pritchard LI, Sendow I, Lunt R, et al. Genetic diversity of bluetongue viruses in south east Asia. *Virus Res* 2004;101:193-201.
53. Weaver SC, Rico-Hesse R, Scott TW. Genetic diversity and slow rates of evolution in New World alphaviruses. *Curr Top Microbiol Immunol* 1992;176:99-117.

54. Weaver SC, Bellew LA, Rico-Hesse R. Phylogenetic analysis of alphaviruses in the Venezuelan equine encephalitis complex and identification of the source of epizootic viruses. *Virology* 1992;191:282-290.
55. Xu G, Wilson W, Mecham J, et al. VP7: an attachment protein of bluetongue virus for cellular receptors in Culicoides variipennis. *J Gen Virol* 1997;78 (Pt 7):1617-1623.
56. Campbell CH, Breese SS, Jr., McKercher PD. Antigenic and morphologic comparisons of Ibaraki and bluetongue viruses. *Can J Microbiol* 1975;21:2098-2102.
57. Campbell CH. Immunogenicity of bluetongue virus inactivated by gamma irradiation. *Vaccine* 1985;3:401-406.
58. Barratt-Boyes SM, MacLachlan NJ. Pathogenesis of bluetongue virus infection of cattle. *J Am Vet Med Assoc* 1995;206:1322-1329.
59. DeMaula CD, Jutila MA, Wilson DW, et al. Infection kinetics, prostacyclin release and cytokine-mediated modulation of the mechanism of cell death during bluetongue virus infection of cultured ovine and bovine pulmonary artery and lung microvascular endothelial cells. *J Gen Virol* 2001;82:787-794.
60. MacLachlan NJ, Crafford JE, Vernau W, et al. Experimental reproduction of severe bluetongue in sheep. *Vet Pathol* 2008;45:310-315.
61. Verwoerd DW, Huismans H. Studies on the in vitro and the in vivo transcription of the bluetongue virus genome. *Onderstepoort J Vet Res* 1972;39:185-191.
62. Savini G, Goffredo M, Monaco F, et al. Transmission of bluetongue virus in Italy. *Vet Rec* 2003;152:119.
63. Schultz G, Delay PD. Losses in newborn lambs associated with bluetongue vaccination of pregnancy ewes. *J Am Vet Med Assoc* 1955;127:224-226.
64. MacLachlan NJ, Nunamaker RA, Katz JB, et al. Detection of bluetongue virus in the blood of inoculated calves: comparison of virus isolation, PCR assay, and in vitro feeding of Culicoides variipennis. *Arch Virol* 1994;136:1-8.
65. Maclachlan NJ, Osburn BI. Induced brain lesions in calves infected with bluetongue virus. *Vet Rec* 2008;162:490-491.
66. Nevill EM. Cattle and Culicoides biting midges as possible overwintering hosts of bluetongue virus. *Onderstepoort J Vet Res* 1971;38:65-71.
67. Kemeny L, Drehle LE. The use of tissue culture-propagated bluetongue virus for vaccine preparation. *Am J Vet Res* 1961;22:921-925.
68. Luedke AJ, Jochim MM. Clinical and serologic responses in vaccinated sheep given challenge inoculation with isolates of bluetongue virus. *Am J Vet Res* 1968;29:841-851.
69. Osburn BI, McGowan B, Heron B, et al. Epizootiologic study of bluetongue: virologic and serologic results. *Am J Vet Res* 1981;42:884-887.
70. Boone JD, Balasuriya UB, Karaca K, et al. Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. *Vaccine* 2007;25:672-678.
71. DeHaven. Bluetongue viruses and trade issues: a North American perspective. *Vet Ital* 2004;40:683-687.

72. Osburn BI. Role of the immune system in bluetongue host-viral interactions. *Prog Clin Biol Res* 1985;178:417-422.

CHAPTER 2

PREVALENCE OF BLUETONGUE IN COLORADO SHEEP FLOCKS

Abstract

Objective- To estimate flock-level prevalence of clinical signs, antibodies, and Bluetongue virus (BTV) infection among Colorado sheep, to estimate the proportion of infected sheep within affected flocks, and to identify clinical signs associated with the presence of viral RNA.

Design- Cross-Sectional study

Sample Population- 2544 serum and whole blood samples obtained from sheep in 108 flocks in Colorado.

Procedures- Participating flocks were visited between July and November 2007 to coincide with the BTV vector season. The animals were observed for clinical signs of BTV infection and blood was obtained from up to ten ewes, ten lambs, and five rams from each of one hundred and eight flocks for cELISA and RT-PCR testing. Flock-level prevalence (proportion of positive flocks) was estimated, as was proportion of infected sheep within infected flocks, and multivariable logistic regression analysis was utilized to determine the relationship between detection of BTV viral RNA and presence of specific clinical signs.

Results- Flock level seroprevalence was 28.70% (95% CI, 20.41% to 38.20%), viral RNA was detected in 22.22% (95% CI, 14.79% to 31.24%) and clinical disease was observed in 19.44% (95% CI, 12.46% to 28.17%) of flocks tested. Animal level seroprevalence ranged from 7.6% to 83 % within positive flocks with a mean of 27.09% (95%CI, 23.87% to 30.51%), viral RNA prevalence ranged from 4.8% to 48% within

positive flocks with a mean of 25.62% (95%CI, 21.93% to 29.59%), and clinical disease ranged from 16.7% to 41.7% with a mean of 24.24% (95% CI, 20.38% to 28.43%). Animal and flock level seroprevalence was higher among the adult population whereas prevalence of viral RNA and clinical disease was higher among the lambs. Positive flocks were distributed heterogeneously throughout the state and all but three flocks that demonstrated clinical disease were identical to those with detectable viral RNA. The two most significant clinical signs associated with detection of viral RNA were weight loss (OR, 12.366, 95% CI, 2.057-74.343) and oral ulcerations (OR, 11,756, 95% CI, 1.061-130.243).

Conclusions and Clinical Relevance- Bluetongue affects a substantial number of sheep flocks within Colorado and the prevalence is distributed heterogeneously throughout the state. Higher seroprevalence among adults suggests continued exposure with the development of an acquired immune system whereas increased detection of nucleic acid with concurrent observation of clinical signs may be due to the naïve immunologic status of the younger population in the face of the first exposure to Bluetongue virus by *Culicoides spp.* Clinical signs associated with the odds of having viral RNA represent both acute illness (oral ulcerations) and chronic disease (weight loss). These findings aid in understanding the epidemiological and clinical features of BTV infection.

Introduction

Bluetongue virus (BTV) is the causative agent of Bluetongue, an economically important emerging arboviral disease belonging to the family Reoviridae, genus *Orbivirus*. Twenty-four serotypes of BTV are recognized globally and five are considered endemic within the United States (2,10,11,13,17). The virus is transmitted by *Culicoides* spp. biting midges, which affect a large host range including both wild and domestic ruminant species.[1] Within the United States domestic sheep and wild deer populations are the most significantly affected populations leading to morbidity rates as high as 70% and mortality rates as high as 50% in naïve sheep populations.[30] Annual losses to the US livestock industry have been estimated as high as $144 million dollars due to decreased production in clinically affected animals as well as non-tariff trade restrictions on BTV positive animals and animal germplasm.[2]

Bluetongue virus has been identified on all continents excluding Antarctica, and changes in environmental parameters appear to support new habitats for *Culicoides spp*..[22,35] The unique regional distribution of distinct BTV strains (virus topotypes) with respect to their vector populations is beginning to change. [8] BTV activity, defined by the Office international des epizooties (OIE) Terrestrial Manual, has been predominantly found within the latitude boundaries of 53°N and 34°S, and the OIE defines three BTV status classifications, which affect transportation and free trade of ruminants. These include BTV free zones, BTV seasonally free zones, and BTV infected zones of which Colorado is considered a BTV seasonally free zone. Seasonally-free status restricts

interstate and international trade, which impacts Colorado's sheep industry during seasonal ram sales and shows. [9]

Clinical severity and host susceptibility of Bluetongue (BT) among individual species can vary remarkably; however, the primary pathogenesis is similar among all ruminant species. [58] After the initial insect bite, the virus replicates in adjacent lymph nodes and then spreads to infect vascular endothelium, macrophages, and dendritic cells in a variety of tissues and organs. Injury to the endothelial cells in small blood vessels results in vascular thrombosis and ischemic necrosis of the tissues. [59] As a result of pathogenesis, the most significant clinical signs seen in sheep include transient fevers as high as 106°F followed by nasal discharge, inflammation of the nasal mucosa ("soremuzzle"), ulcerations of the gingiva and tongue leading to depression, weight loss, wool loss, diarrhea, and lameness, all of which can result in substantial economic losses to the producer.

Even in the face of the detrimental effects BTV can have upon the small ruminant population, much of the epidemiology and prevalence of BTV small ruminant infections within the United States remains unknown. The first comprehensive serologic survey within the U.S. was not conducted until the winter months of 1977-1978 on blood serum samples from slaughter cattle. At the state level, serum antibody prevalence in cattle ranged from 0-79% with a national mean prevalence of 18.2%. Cattle prevalence levels within nine southwestern states including Arizona, California, Colorado, Kansas, Nebraska, Nevada, New Mexico, Oklahoma, and Texas were found to be between 18.0% and 53.2%. [26] Serological studies have been randomly conducted by USDA/APHIS from

1979 until 2004 and have confirmed similar findings and regional differences with the lowest cattle prevalences in the northeastern states and higher prevalence among the southwest and southeastern states. [18] During the latest cattle USDA survey conducted from 1991-2004, BTV was found to be endemic in all states excluding Alaska, Hawaii, Michigan, Minnesota, New York, Wisconsin, and New England. [18]

The surveys done by the USDA have primarily involved testing of slaughter cattle; little is known about BTV prevalence in US sheep. Within endemic states, the proportion of infected sheep flocks, proportion of infected animals within an infected flock, and the distribution of infection and clinical disease in different sheep production groups such as lambs, ewes, and rams is unknown. Sporadic outbreaks have occurred throughout the United States primarily affecting sheep flocks in the southwest. The most significant outbreak reported within the last decade occurred throughout southern Montana and Wyoming during November of 2007. Over three-hundred domestic sheep died as the result of BTV-17 infection that also affected wildlife populations of pronghorn antelope, white-tailed deer, and mule deer. [28]

To minimize disease spread, science-based approaches have been implemented to understand the duration of viremia and the limitations of current diagnostic tests for detection of active infection. [19] The OIE now considers the infective period for cattle to be 60 days which reflects the maximal period when ruminants are thought to be infective to *Culicoides*. In spite of this, many countries with BTV free status continue to deny transport of animals based on pre-arrival antibody detection methods as determined by competitive ELISA techniques. [1] Antibodies can be detected as early as 14 days post

infection but can persist for long durations thus creating an inadequate measurement of active infection. [71]

Control strategies focus on protecting the animal either by vaccination or by limiting exposure to *Culicoides spp*. Currently only one nationally licensed sheep vaccine is available in the United States: a modified live vaccine that is only effective against serotype 10. [67,68] An additional modified live vaccine that protects against serotypes 10, 11, and 17 is produced by the California Woolgrowers Association but is only approved for use in California. Both of these modified live vaccines may cause abortions in pregnant animals or development of mild clinical signs. [63] Vaccination with modified live vaccines have demonstrated variable results for protection against BTV and may worsen the situation if animals develop clinical disease.[72] The other primary control recommendation is to prevent exposure of susceptible animals to feeding *Culicoides*, and this may be accomplished by use of insecticides or housing animals indoors during periods in which *Culicoides* are supposedly most active including late evening and early morning. [9] However, recent studies have found that *Culicoides* are active throughout much of the day and feed within barn facilities quite frequently, so housing recommendations appear to inadequately protect against BTV exposure. [7] Due to frequent inconsistencies and inadequacies of available control measures, effective prevention measures for producers in BTV endemic states who need to transport animals across state lines do not exist.

Issues concerning trade restrictions within the United States have demonstrated a great deal of progress in understanding that BT is an emerging but non-contagious

disease of ruminants. Although much is known about the distribution of BTV in US cattle, little is known about the distribution and impact of disease in US sheep. The objectives of this study were to estimate the prevalence of BTV antibodies and nucleic acid among Colorado sheep, determine if there was a significant association between the detection of viral nucleic acid by reverse transcriptase PCR (RT-PCR) and clinical signs of disease, and describe the distribution of BTV flocks within the state in order to further understand environmental and ecological parameters that influence BTV within the state.

Materials and Methods

Study Population- Participating flocks were recruited through the distribution of questionnaires to five hundred and six members belonging to the following groups: Colorado sheep feedlots; Colorado State University (CSU) and CSU extension sheep clients; Colorado Woolgrowers Association members; sheep club associations including state fairs and shows; and clients of Colorado veterinarians belonging to the American Association of Small Ruminant Practitioners (AASRP). One-hundred and eight questionnaires (21.3%) were returned with adequate information to schedule a flock visit during the months of July until November 2007 to coincide with the *Culicoides* vector season. Thirteen questionnaires were returned with inadequate information or refusal of participation leaving a remainder of three-hundred and eighty-five flock owners that did not respond to the survey.

A total of 25 animals (10 ewes greater than or equal to 1 years of age, 10 lambs less than 6 months of age, 5 rams greater than or equal to 1 year of age) were selected from each flock for sampling of serum and whole blood. A sample size of 10 animals

was chosen within each age/sex group so that it would allow us to detect a prevalence at least >23% in an infected flock (assuming an average of 30 ewes/flock).[73] A smaller number of rams (5) was targeted because fewer numbers of rams are typically present within a flock.

Flock visits consisted of recording a brief history from the primary caretaker of the animals and recording the proportion of the flock with clinical signs supporting bluetongue infection: fever; coronitis; cyanotic tongue; edematous ears; edematous muzzle; lameness; wool loss; weight loss; reproductive infertility; and oral lesions/ulcerations. Clinical disease at the flock level was considered to be the presence of three or more clinical signs present in each of at least five animals.

Sample Collection and Testing- Serum and whole blood were collected from animals within each flock and were stored at 4°C until analyzed by cELISA and reverse transcriptase polymerase chain reaction (RT-PCR).[74] The cELISA was utilized in this study because it is less subjective than Agar Gel Immunodiffusion (AGID) techniques and is able to discriminate BTV from another closely associated *Orbivirus*, Epizootic Hemorrhagic Disease virus (EHDV). Serum collection tubes were centrifuged at 3,000 x g for five minutes, serum was removed using a sterile transfer pipette, and run according to manufacturer's instructions (cELISA; VMRD Inc., Pullman, WA). Results were reported as positive or negative based on optical density readings when compared with positive and negative controls.

Viral RNA was extracted from whole blood samples utilizing MagMax™ 96 total RNA isolation kit. Reverse transcriptase nested PCR was performed utilizing the Qiagen

one step RT-PCR kit and procedures previously described. [74] Results were reported as positive or negative based on detection of amplified DNA product by use of agarose gel electrophoresis and staining with ethidium bromide. Extracted samples were stored at -80°C and whole blood was stored with serum samples at -20°C.

Data Analysis- Prevalence of clinical disease, nucleic acid, and antibodies was defined as the proportion of flocks and animals with positive results within each age/sex category. Comparisons of prevalence of antibody detection, viral RNA and clinical disease among rams, ewes, and lambs were made by use of 95% confidence intervals, odds ratios obtained from logistic regression, and chi-square for homogeneity. Within each flock, relative frequencies were calculated for each clinical sign and a multivariable logistic regression analysis was utilized to assess clinical signs as predictors of viral RNA detection at the flock level.

Viral RNA results were reported as positive or negative based on RT-PCR. Independent variables included: fever; coronitis; cyanotic tongue; edematous ears; edematous muzzle; lameness; wool loss; weight loss; reproductive infertility; and oral lesions/ulcerations. The final model was presented utilizing odds ratios, standard error, confidence intervals, and p-values considered significant at a critical alpha of 0.05.

Results

A total of 2,544 samples were obtained from 1,058 ewes, 992 lambs, and 494 rams located in 108 sheep flocks throughout Colorado. Flock level seroprevalence was 28.70% (95% CI, 20.41% to 38.20%), viral RNA was detected in 22.22% (95% CI,

14.79% to 31.24%) and clinical disease was observed in 19.44% (95% CI, 12.46% to 28.17%) of flocks tested. Positive flocks were distributed heterogeneously throughout the state and all but three flocks that demonstrated clinical disease were identical to those with detectable viral RNA. A similar relationship was found among flocks that had detectable viral RNA and concurrent BTV antibodies where all but 10 locations were identical.

Prevalence was further characterized within each age and sex group. The flock level seroprevelance was higher among the adult population. The proportions of flocks with rams and ewes testing positive were 15.74% (95%CI, 9.45% to 24.00%) and 25.93% (95% CI, 17.97% to 35.25%), respectively, as compared to 3.70% (95% CI, 1.02% to 9.21%) flocks with lambs with detectable antibodies to BTV by cELISA. In contrast, detection of viral RNA and observation of clinical signs was higher in the lamb population. Viral RNA was identified in lambs in 17.59% (95%CI, 10.94% to 26.10%) of flocks and clinical disease was identified in lambs from 16.67% (95%CI, 10.19% to 25.06%) of flocks compared to viral RNA identified in the adult ram population in 5.56% (95%CI, 2.07% to 11.70%) of flocks, and in the adult ewe population in 7.41% (95%CI, 3.25% to 14.07%) of flocks. Clinical disease was observed in the adult ram population in 5.56% (95%CI, 2.07% to 11.70%) of flocks and in the adult ewe population in 4.63% (95%CI, 1.52% to 10.47%) of flocks (Table 2.1).

A total of 716 animals were evaluated from flocks that had BTV antibodies. Seroprevalence ranged from 7.6% to 83% within positive flocks with a mean of 27.09% (95%CI, 23.87% to 30.51%). Seroprevalence was highest among ewes with values of

44.09% (95%CI, 38.51% to 48.78%) and rams 27.78% (95%CI, 20.17% to 36.46%) and lowest among the lambs 20.22% (95%CI, 15.65% to 25.44 %). A total of 523 animals were evaluated from flocks that had detectable viral RNA. Viral RNA prevalence ranged from 4.8% to 48% within positive flocks with a mean of 25.62% (95%CI, 21.93% to 29.59%). As observed in flock level prevalence, viral RNA prevalence was greatest in the lamb population with a value of 47.68 (95%CI, 40.51% to 54.95%) in contrast to the adult population with values of 11.4 (95%CI, 5.65% to 20.12%) among rams and 12.86 (95%CI, 8.91% to 17.76%) among ewes. A total of 458 animals were evaluated from flocks that had clinical disease and the proportion of affected animals within a flock ranged from 16.7% to 41.7% with a mean of 24.24% (95% CI, 20.38% to 28.43%). The prevalence of clinical disease was also similar to the trends observed in flock level viral RNA prevalence with clinical signs present in 31.76% (95%CI, 24.85% to 39.33%) of lambs in infected flocks compared to 6.67% (95% CI, 2.20% to 14.88%) of rams and 8.23% (95% CI, 5.02% to 12.55%) of ewes (Table 2.2). Evaluation using the chi-square for homogeneity revealed that the proportion of rams, ewes, and lambs with detectable viral RNA was not significantly different ($p=0.271$) from the proportion of rams, ewes, and lambs with clinical disease whereas the proportion of lambs, ewes, and rams with BTV antibodies was significantly different ($p\leq0.001$) from the proportions with clinical disease.

Three logistic regression models were utilized to estimate the association between age/sex group (rams, ewes, lambs) and the odds of being seropositive, testing positive for viral RNA, and detection of clinical signs, respectively. These models demonstrated that ewes had significantly higher odds of detectable antibodies for BTV than the ram

population, but not significantly higher odds of detectable viral RNA or of clinical signs (Table 2.3). The lambs were not significantly less likely to have antibodies than rams, but were significantly more likely to have viral RNA and clinical signs respectively as compared with the ram population (Table 2.3).

The association between specific signs of clinical disease and detection of viral RNA was evaluated at the flock level with the use of multivariable logistic regression analysis. The regression model was constructed to determine the odds of a flock having viral RNA based on the following clinical signs: fever, abortion, cyanotic tongue, infertility, lameness, oral ulcerations, edematous muzzle or ears, wool-loss, and weight loss. Results of univariable analyses are shown in Table 2.4.

All variables were found to be significant at a level of 0.25 after univariable analysis. Collinearity was identified among the majority of covariates that were included in the multivariable analysis excluding lameness and wool loss. Clinical signs resulting from vasculitis such as coronitis, edematous muzzles and ears, and cyanosis of the tongue had similar relative frequencies (Table 2.5). On multivariable analysis, only weight loss and oral ulcerations were significantly associated with presence of viral RNA. After these two variables were in the model, incorporating additional clinical sign variables did not significantly improve model fit. Oral ulcerations represent a defining acute clinical feature of BT in sheep; and flocks with this lesion were about 12 times more likely (OR, 11.756, 95% CI, 1.061-130.243) to have viral RNA detectable by RT-PCR than flocks without clinical ulcerations. In this model, flocks with weight loss were also 12 times more likely to have viral RNA detectable by RT-PCR than animals without weight loss (OR, 12.366, 95% CI, 2.057-74.343) (Table 2.6).

Discussion

Sparse information has been published on BTV prevalence levels within Colorado sheep since the first national survey conducted during the late 1970's.[26] This cross-sectional study was performed following a field investigation during the Fall of 2006 which raised many questions concerning control measures, prevention, and travel restrictions on BTV test positive animals. Many of the trade restrictions within the United States are still based on antibody detection methods, which are an inaccurate measure of active disease and viremia. There is also little known about the serological status, virus presence, and prevalence of clinical signs under field conditions within the United States, yet this information is key in interpreting whether an animal is infectious to *Culicoides* vectors. In order to understand limitations in prevention and control, it was important to first determine the serological, viral, and clinical Bluetongue status of sheep and flocks within Colorado.

Our study demonstrates a substantial number of flocks and sheep were affected by BTV during the 2007 vectorial season. Higher seroprevalence among adult sheep was likely due to acquired immunity gained over multiple years of exposure to BTV throughout multiple BTV vector seasons whereas higher viremia and clinical disease among lambs was the result of their naïve immunologic status in the face of their first exposure to BTV. Viral RNA was detected in almost all animals that demonstrated clinical disease, the majority of which were lambs. However, seroprevalence among the lambs was low in the face of such high viral RNA prevalence, which has been seen in

previous studies where viremia is detected but antibody titers have remained low for long durations of time.[75]

The role of cytotoxic immunity in BTV infections continues to be poorly understood,[76] and this affects interpretation of the study's finding of low seroprevalence in the face of high viral RNA prevalence. Experimentally infected mice have demonstrated the ability to mount a cell mediated immune response following inoculation with live BTV. Although circulating BTV antibody may not be detected within the blood of clinically affected sheep inoculated with BTV, adequate antibodies are thought to be present due to resolution of clinical signs. [77] A recent study performed in cattle detected BTV antibodies that peaked at six weeks following experimental infection with BTV 2, but there was no statistical difference of animals that mounted an immune response among infected and control groups. [76]

Previous studies have reported BTV to inhibit lymphocyte proliferation during experimental infection, and this might explain why antibody detection was so low in this study among lambs with concurrent viral RNA .[71] A lag in BTV antibody production following parturition has been described as a "non-defined parturition associated immunosuppression" that results in prolonged viremias with the lack of antibody production. [71] Another explanation for the absence of serologic response in the face of viral detection could be the sensitivity of nested PCR diagnostics and potential for laboratory contamination leading to false positive results. [74]

Many clinical signs associated with viral RNA detection were correlated with each other. This correlation was not surprising because many of these clinical signs

appear acutely in the first stage of infection and result from the same pathophysiology: endothelial damage and vascular leakage. Coronitis, cyanotic tongue, edematous ears, edematous muzzle, and oral lesions/ulcerations are all caused by endothelial damage and vascular leakage, and these signs in addition to fever lead to secondary symptoms of weight loss, depression, wool loss, and infertility. [3] In this study, the two most significant clinical signs (oral ulcerations and weight loss) associated with detection of viral RNA represent the two most common clinical features reported in current outbreaks within the United States and northern Europe, in the acute and chronic stages of disease, respectively. Progression of disease begins with clinical signs secondary to vascular injury such as oral ulcerations that lead to depression and weight of animals who continue to decline without appropriate intervention. [3,6]

In the current study, a flock was considered to have clinical disease if three or more clinical signs were present in at least five animals at the time of the visit. Because this definition was rather strict, flocks with fewer clinically ill animals, or flocks with animals displaying fewer than three clinical signs, were classified as negative for clinical disease. The study might have underestimated the proportion of flocks with clinical disease.

Although the study identified some of the most predominant clinical features of BTV within Colorado sheep, it also had limitations. One of the most significant limitations was the potential for non-responder bias with 398 producers that did not respond to the questionnaire. Reasons for non-response may have included trepidation from potential government involvement, lack of monetary compensation, and time

commitments during the fall involving ram sales and shows. This bias may affect the validity of extrapolating results from the current study to the entire sheep population of Colorado due to the fact that most of the responders were typically knowledgeable about the disease and its effect on their sheep. In addition, most of the responders were those who chose to support academic studies and had learned of the study through referring veterinarians or extension services.

Although many producers did not respond, those who did were distributed evenly throughout the state, providing an opportunity to evaluate BTV activity in a range of environments in Colorado (Fig. 2.1,2.2, 2.3). Given the sample size selected from each flock and prevalence values obtained within this study, only five animals would have to be infected in order to detect BTV within the flock. Utilizing a questionnaire as the form of recruitment, all participants were voluntary responders, which could have resulted in over-estimating of the true prevalence of BTV within the state of Colorado at the flock level. However, it is expected the cross sectional study design limited sampling and follow up of positive results, under-estimating the prevalence at the animal level. A potential resolution would be following these animals over an entire season, which would have established a better understanding of the trends and development of BTV infection within these flocks.

The results of this study help in understanding the prevalence of BTV infection among Colorado sheep flocks during 2007 but do not provide information about BTV within flocks over a multitude of seasons, which could demonstrate variable prevalence values based on the change in climatic factors that support BTV transmission. Each year

brings a new variety of ecological and environmental parameters to which the virus, vector, and host must adapt, leading to fluctuations in susceptibility of the host and virulence of the virus. Future investigations should be focused on identification of risk factors that are associated with seropositivity in small ruminants and identification of a time course of infection and disease ecology through long term studies with cohorts of animals. Vector species and environmental parameters that support their existence should be studied to further understand relationships among vector, host, virus, and environment. This study offers a sturdy platform to start identifying some of these variables in order to adequately address prevention and control measures.

References

1. Singer RS, MacLachlan NJ, Carpenter TE. Maximal predicted duration of viremia in bluetongue virus-infected cattle. *J Vet Diagn Invest* 2001;13:43-49.
2. Hoar BR, Carpenter TE, Singer RS, et al. Regional risk of exporting cattle seropositive for bluetongue virus from the United States. *Am J Vet Res* 2003;64:520-529.
3. MacLachlan NJ. The pathogenesis and immunology of bluetongue virus infection of ruminants. *Comp Immunol Microbiol Infect Dis* 1994;17:197-206.
4. MacLachlan NJ, Zientara S, Stallknecht DE, et al. Phylogenetic comparison of the S10 genes of recent isolates of bluetongue virus from the United States and French Martinique Island. *Virus Res* 2007;129:236-240.
5. Tabachnick WJ, MacLachlan NJ, Thompson LH, et al. Susceptibility of Culicoides variipennis sonorensis to infection by polymerase chain reaction-detectable bluetongue virus in cattle blood. *Am J Trop Med Hyg* 1996;54:481-485.
6. Schwartz-Cornill I, Mertens PP, Contreras V, et al. Bluetongue virus: virology, pathogenesis and immunity. *Vet Res* 2008;39:46.
7. Meiswinkel R, Goffredo M, Leijs P, et al. The Culicoides 'snapshot': A novel approach used to assess vector densities widely and rapidly during the 2006 outbreak of bluetongue (BT) in The Netherlands. *Prev Vet Med* 2008;87:98-118.
8. Baylis M. When will bluetongue transmission start in 2008? *Vet Rec* 2008;162:526; author reply 527.
9. Health. WOfA. Terrestrial animal health code: bluetongue, Sept, 2008.
10. Monath. *Orviviruses and Coltiviruses, In: Fields*. Boston: Lipinncott, 1996.
11. Price DA, Hardy WT. Isolation of the bluetongue virus from Texas sheep-Culicoides shown to be a vector. *J Am Vet Med Assoc* 1954;124:255-258.
12. McKercher DG, McGowan B, Howarth JA, et al. A preliminary report on the isolation and identification of the bluetongue virus from sheep in California. *J Am Vet Med Assoc* 1953;122:300-301.
13. Greiner EC, Alexander FC, Roach J, et al. Bluetongue epidemiology in the Caribbean region: serological and entomological evidence from a pilot study in Barbados. *Med Vet Entomol* 1990;4:289-295.
14. Pearson JE, Carbrey EA, Gustafson GA. Bluetongue virus in cattle: complement fixing antibody response and viremia in experimentally infected animals. *Proc Annu Meet U S Anim Health Assoc* 1973:524-531.
15. Tabachnick WJ. Culicoides variipennis and bluetongue-virus epidemiology in the United States. *Annu Rev Entomol* 1996;41:23-43.
16. Mellor PS, Boorman J. The transmission and geographical spread of African horse sickness and bluetongue viruses. *Ann Trop Med Parasitol* 1995;89:1-15.
17. Gibbs EP, Greiner EC, Alexander FC, et al. Serological survey of ruminant livestock in some countries of the Caribbean region and South America for antibody to bluetongue virus. *Vet Rec* 1983;113:446-448.
18. Ostlund E, Moser K. Distribution of bluetongue in the United States of America, 1991-2002. *Vet Ital* 2004;40:83-88.

19. MacLachlan NJ, Osburn BI. Impact of bluetongue virus infection on the international movement and trade of ruminants. *J Am Vet Med Assoc* 2006;228:1346-1349.
20. Purse BV, Mellor PS, Rogers DJ, et al. Climate change and the recent emergence of bluetongue in Europe. *Nat Rev Microbiol* 2005;3:171-181.
21. Clercq D. Transplacental infection and apparently immunotolerance induced by a wild type bluetongue virus serotype 8 natural infections. *Transbound Emerg Dis* 2008;55:352-259.
22. Gloster J, Burgin L, Witham C, et al. Bluetongue in the United Kingdom and northern Europe in 2007 and key issues for 2008. *Vet Rec* 2008;162:298-302.
23. Gerry AC, Mullens BA, Maclachlan NJ, et al. Seasonal transmission of bluetongue virus by Culicoides sonorensis (Diptera: Ceratopogonidae) at a southern California dairy and evaluation of vectorial capacity as a predictor of bluetongue virus transmission. *J Med Entomol* 2001;38:197-209.
24. Meiswinkel R, Baldet T, de Deken R, et al. The 2006 outbreak of bluetongue in northern Europe-The entomological perspective. *Prev Vet Med* 2008;87:55-63.
25. De Deken G, Madder M, Deblauwe I, et al. Vector monitoring at Belgian outbreak sites during the bluetongue epidemic of 2006. *Prev Vet Med* 2008;87:64-73.
26. Metcalf HE, Pearson JE, Klingsporn AL. Bluetongue in cattle: a serologic survey of slaughter cattle in the United States. *Am J Vet Res* 1981;42:1057-1061.
27. Stallknecht DE, Kellogg ML, Blue JL, et al. Antibodies to bluetongue and epizootic hemorrhagic disease viruses in a barrier island white-tailed deer population. *J Wildl Dis* 1991;27:668-674.
28. Barrett. Bluetongue, ovine, usa (wyoming), 2007.
29. Barber TL, Jochim MM. Serologic characterization of selected bluetongue virus strains from the United States. *Proc Annu Meet U S Anim Health Assoc* 1973:352-359.
30. Hourrigan JL, Klingsporn AL. Epizootiology of bluetongue: the situation in the United States of America. *Aust Vet J* 1975;51:203-208.
31. Bram RA, George JE, Reichar RE, et al. Threat of foreign arthropod-borne pathogens to livestock in the United States. *J Med Entomol* 2002;39:405-416.
32. Mecham JO, Johnson DJ. Persistence of bluetongue virus serotype 2 (BTV-2) in the southeast United States. *Virus Res* 2005;113:116-122.
33. Johnson DJ, Ostlund EN, Stallknecht DE, et al. First report of bluetongue virus serotype 1 isolated from a white-tailed deer in the United States. *J Vet Diagn Invest* 2006;18:398-401.
34. Johnson DJ. Identification of new United States bluetongue types. In: Proceedings of the Annual Meeting of the United States Animal Health Association ip, ed, 2007.
35. Gibbs EP, Greiner EC. The epidemiology of bluetongue. *Comp Immunol Microbiol Infect Dis* 1994;17:207-220.
36. Gloster J, Mellor PS, Manning AJ, et al. Assessing the risk of windborne spread of bluetongue in the 2006 outbreak of disease in northern Europe. *Vet Rec* 2007;160:54-56.

37. Foster NM, Jones RH, McCrory BR. Preliminary Investigations on Insect Transmission of Bluetongue Virus in Sheep. *Am J Vet Res* 1963;24:1195-1200.
38. Wirth WW, Dyce AL. The current taxonomic status of the Culicoides vectors of bluetongue viruses. *Prog Clin Biol Res* 1985;178:151-164.
39. Holbrook FR. Research on the control of bluetongue in livestock by vector suppression. *Prog Clin Biol Res* 1985;178:617-620.
40. Mullens BA, Tabachnick WJ, Holbrook FR, et al. Effects of temperature on virogenesis of bluetongue virus serotype 11 in Culicoides variipennis sonorensis. *Med Vet Entomol* 1995;9:71-76.
41. Green AL, Dargatz DA, Schmidtmann ET, et al. Risk factors associated with herd-level exposure of cattle in Nebraska, North Dakota, and South Dakota to bluetongue virus. *Am J Vet Res* 2005;66:853-860.
42. Conte. Novel environmental factors influencing the distribution and abundance of Culicoides imicola and the obsoletus complex in Italy. *Vet Ital*;43.
43. White DM, Wilson WC, Blair CD, et al. Studies on overwintering of bluetongue viruses in insects. *J Gen Virol* 2005;86:453-462.
44. White DM, Blair CD, Beaty BJ. Molecular epidemiology of Bluetongue virus in northern Colorado. *Virus Res* 2006;118:39-45.
45. Baldet T, Delecolle JC, Cetre-Sossah C, et al. Indoor activity of Culicoides associated with livestock in the bluetongue virus (BTV) affected region of northern France during autumn 2006. *Prev Vet Med* 2008;87:84-97.
46. Mertens PP, Diprose J. The bluetongue virus core: a nano-scale transcription machine. *Virus Res* 2004;101:29-43.
47. Roy P. Bluetongue virus: dissection of the polymerase complex. *J Gen Virol* 2008;89:1789-1804.
48. Osburn BI, de Mattos CA, de Mattos CC, et al. Bluetongue disease and the molecular epidemiology of viruses from the western United States. *Comp Immunol Microbiol Infect Dis* 1996;19:181-190.
49. Pritchard LI, Gould AR. Phylogenetic comparison of the serotype-specific VP2 protein of bluetongue and related orbiviruses. *Virus Res* 1995;39:207-220.
50. Bonneau KR, Mullens BA, MacLachlan NJ. Occurrence of genetic drift and founder effect during quasispecies evolution of the VP2 and NS3/NS3A genes of bluetongue virus upon passage between sheep, cattle, and Culicoides sonorensis. *J Virol* 2001;75:8298-8305.
51. Bonneau KR, Topol JB, Gerry AC, et al. Variation in the NS3/NS3A gene of bluetongue viruses contained in Culicoides sonorensis collected from a single site in southern California. *Virus Res* 2002;84:59-65.
52. Pritchard LI, Sendow I, Lunt R, et al. Genetic diversity of bluetongue viruses in south east Asia. *Virus Res* 2004;101:193-201.
53. Weaver SC, Rico-Hesse R, Scott TW. Genetic diversity and slow rates of evolution in New World alphaviruses. *Curr Top Microbiol Immunol* 1992;176:99-117.
54. Weaver SC, Bellew LA, Rico-Hesse R. Phylogenetic analysis of alphaviruses in the Venezuelan equine encephalitis complex and identification of the source of epizootic viruses. *Virology* 1992;191:282-290.

55. Xu G, Wilson W, Mecham J, et al. VP7: an attachment protein of bluetongue virus for cellular receptors in Culicoides variipennis. *J Gen Virol* 1997;78 (Pt 7):1617-1623.
56. Campbell CH, Breese SS, Jr., McKercher PD. Antigenic and morphologic comparisons of Ibaraki and bluetongue viruses. *Can J Microbiol* 1975;21:2098-2102.
57. Campbell CH. Immunogenicity of bluetongue virus inactivated by gamma irradiation. *Vaccine* 1985;3:401-406.
58. Barratt-Boyes SM, MacLachlan NJ. Pathogenesis of bluetongue virus infection of cattle. *J Am Vet Med Assoc* 1995;206:1322-1329.
59. DeMaula CD, Jutila MA, Wilson DW, et al. Infection kinetics, prostacyclin release and cytokine-mediated modulation of the mechanism of cell death during bluetongue virus infection of cultured ovine and bovine pulmonary artery and lung microvascular endothelial cells. *J Gen Virol* 2001;82:787-794.
60. MacLachlan NJ, Crafford JE, Vernau W, et al. Experimental reproduction of severe bluetongue in sheep. *Vet Pathol* 2008;45:310-315.
61. Verwoerd DW, Huismans H. Studies on the in vitro and the in vivo transcription of the bluetongue virus genome. *Onderstepoort J Vet Res* 1972;39:185-191.
62. Savini G, Goffredo M, Monaco F, et al. Transmission of bluetongue virus in Italy. *Vet Rec* 2003;152:119.
63. Schultz G, Delay PD. Losses in newborn lambs associated with bluetongue vaccination of pregnancy ewes. *J Am Vet Med Assoc* 1955;127:224-226.
64. MacLachlan NJ, Nunamaker RA, Katz JB, et al. Detection of bluetongue virus in the blood of inoculated calves: comparison of virus isolation, PCR assay, and in vitro feeding of Culicoides variipennis. *Arch Virol* 1994;136:1-8.
65. Maclachlan NJ, Osburn BI. Induced brain lesions in calves infected with bluetongue virus. *Vet Rec* 2008;162:490-491.
66. Nevill EM. Cattle and Culicoides biting midges as possible overwintering hosts of bluetongue virus. *Onderstepoort J Vet Res* 1971;38:65-71.
67. Kemeny L, Drehle LE. The use of tissue culture-propagated bluetongue virus for vaccine preparation. *Am J Vet Res* 1961;22:921-925.
68. Luedke AJ, Jochim MM. Clinical and serologic responses in vaccinated sheep given challenge inoculation with isolates of bluetongue virus. *Am J Vet Res* 1968;29:841-851.
69. Boone JD, Balasuriya UB, Karaca K, et al. Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. *Vaccine* 2007;25:672-678.
70. DeHaven. Bluetongue viruses and trade issues: a North American perspective. *Vet Ital* 2004;40:683-687.
71. Osburn BI. Role of the immune system in bluetongue host-viral interactions. *Prog Clin Biol Res* 1985;178:417-422.
72. Osburn BI, McGowan B, Heron B, et al. Epizootiologic study of bluetongue: virologic and serologic results. *Am J Vet Res* 1981;42:884-887.

73. M Thrusfield CO, I de Blas, JP Noordhuizen, K Frankena. Winepiscope 2.0. 2001.
74. Shad G, Wilson WC, Mecham JO, et al. Bluetongue virus detection: a safer reverse-transcriptase polymerase chain reaction for prediction of viremia in sheep. *J Vet Diagn Invest* 1997;9:118-124.
75. Stott JL, Barber TL, Osburn BI. Immunologic response of sheep to inactivated and virulent bluetongue virus. *Am J Vet Res* 1985;46:1043-1049.
76. Francesco D. Cell-mediated response in cattle experimentally infected with bluetongue virus serotype 2. *Veterinaria Italiana* 2004;41:34-45.
77. Waldvogel AS, Stott JL, Squire KR, et al. Strain-dependent virulence characteristics of bluetongue virus serotype 11. *J Gen Virol* 1986;67 (Pt 4):765-769.

Table 2.1—Flock level BTV seroprevalence by use of cELISA, prevalence of viral RNA by use of RT-PCR, and observation of clinical disease signs supportive of BTV infection in 108 Colorado sheep flocks.

	Age/sex category	No. positive flocks	Prevalence (%)	95% CI
Seroprevalence	Rams	17	15.74	9.45 -24.00
	Ewes	28	25.93	17.97-35.25
	Lambs	4	3.70	1.02-9.21
Viral RNA	Rams	6	5.56	2.07-11.70
	Ewes	8	7.41	3.25-14.07
	Lambs	19	17.59	10.94-26.10
Clinical disease	Rams	6	5.56	2.07-11.70
	Ewes	5	4.63	1.52-10.47
	Lambs	18	16.67	10.19-25.06

Table 2.2— Bluetongue seroprevalence by use of cELISA, prevalence of viral RNA by use of RT-PCR, and observation of clinical disease signs supportive of BTV infection in sheep from subsets of 108 Colorado sheep flocks that tested positive for each factor.

	Age/sex category	No. of animals	No. positive animals	Prevalence (%)	95% CI
Seroprevalence n=31 flocks	Rams	126	35	27.78	20.17-36.46
	Ewes	313	138	44.09	38.51-48.78
	Lambs	277	56	20.22	15.65-25.44
Viral RNA n=24 flocks	Rams	87	10	11.4	5.65-20.12
	Ewes	241	31	12.86	8.91-17.76
	Lambs	195	93	47.69	40.51-54.95
Clinical Disease n=21 flocks	Rams	75	5	6.67	2.20-14.88
	Ewes	213	19	8.23	5.02-12.55
	Lambs	170	54	31.76	24.85-39.33

Table 2.3- Odds ratios and respective 95% confidence intervals of being seropositive, testing positive for viral RNA, and having observable clinical signs for a total of 2544 animals from 108 flocks represented by ewes and lambs as compared to rams at the animal level.

Category	**Seroprevalence OR**	**95% CI**	**Viral RNA OR**	**95% CI**	**Clinical Signs OR**	**95% CI**
Rams (reference group)	1.0		1.0		1.0	
Ewes	2.111	1.34-3.31	1.70	0.56-5.18	1.27	0.44-6.32
Lambs	0.678	0.41-1.10	8.14	2.82-23.46	7.72	3.66-16.26

Table 2.4- Results of univariable logistic regression analysis for odds of having detectable viral RNA based on the presence of clinical signs of disease in 2544 sheep from 108 flocks in Colorado.

Variable	OR	95% CI	p-value
Coronitis	34.17	3.94-296.11	0.001
Ulcerated Tongue	20.49	3.97-105.61	0.003
Lameness	24.17	4.94-112.99	0.001
Infertility	41.49	4.85-355.06	0.001
Fever	41.49	4.85-355.06	0.001
Esophagitis (determined by necropsy)	>999.999	<0.001->999.99	0.96
Oral ulcerations	41.49	4.85-355.06	0.001
Edematous ear/muzzle	34.17	3.94-296.11	0.001
Wool loss	>999.999	<0.001->999.999	0.96
Weight loss	29.28	5.79-148.04	<.0001

Table 2.5- Relative frequency of each clinical sign present in at least one animal that also tesetd positive by viral RNA at the flock level.

Variable	Relative Frequency (proportion of flocks with specifiedclinical sign divided by the total flocks positive for viral rna)
Abortion	0.93%
Coronitis	7.41%
Cyanotic Tongue	7.41%
Edematous ears/muzzles	7.41%
Fever	9.26%
Infertility	8.33%
Lameness	6.48%
Oral Ulcerations	8.33%
Weight loss	11.11%
Wool loss	5.56%

Table 2.6- Results of multivariable logistic regression analysis for likelihood of having detectable viral RNA based on the presence of clinical signs of disease, among all flocks that demonstrated at least three or more clinical signs in at least five aimals.

Factor	Estimate	SE	OR	95% CI	p-value
Intercept	-1.8561	0.2996	NA	NA	<0.0001
Weight Loss	2.51	0.9152	12.36	2.05- 74.34	0.001
Oral Ulcerations	2.46	1.2271	11.75	1.06-130.24	0.04

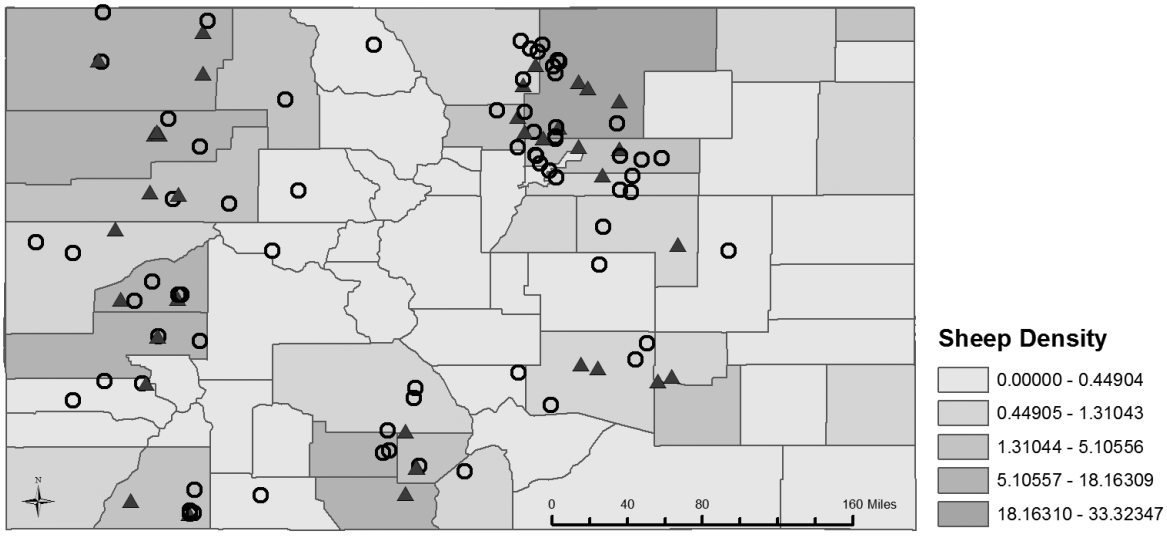

Figure 2.1—Map of Colorado sheep flocks that tested positive for BTV antibodies by cELISA, of 108 flocks tested. Triangles represent seropositive flocks, circles represent seronegative flocks, and the percentage density of sheep per county is represented by shading with darkest shading indicative of greater density (NASS, USDA).

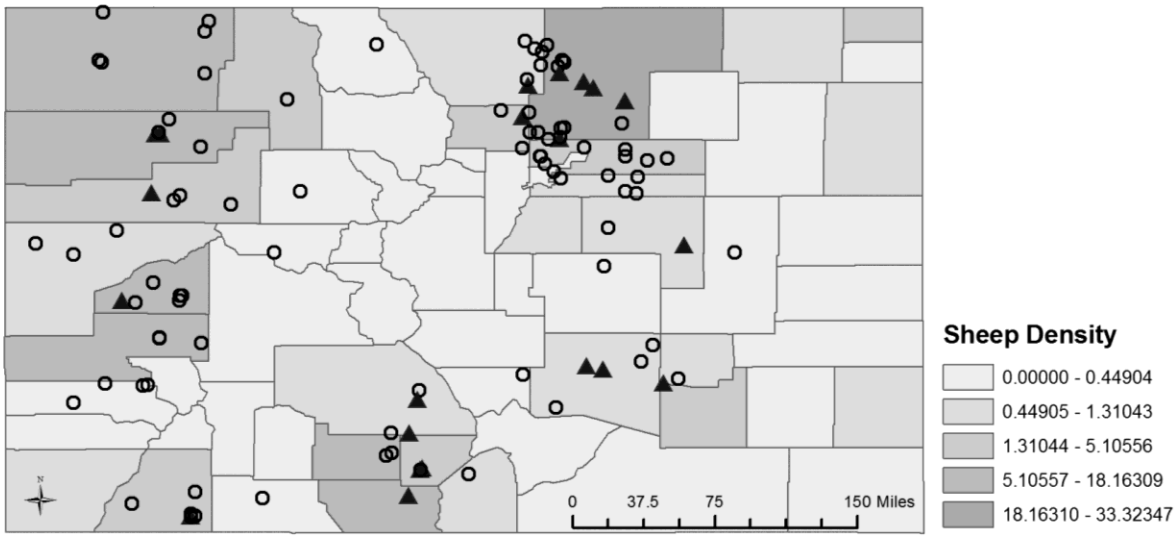

Figure 2.2—Map of Colorado sheep flocks that tested positive for BTV viral RNA by RT-PCR. Triangles represent positive flock, circles represent negative flocks, and percentage density of sheep per county is represented by shading with darkest shading indicative of greater density (NASS, USDA).

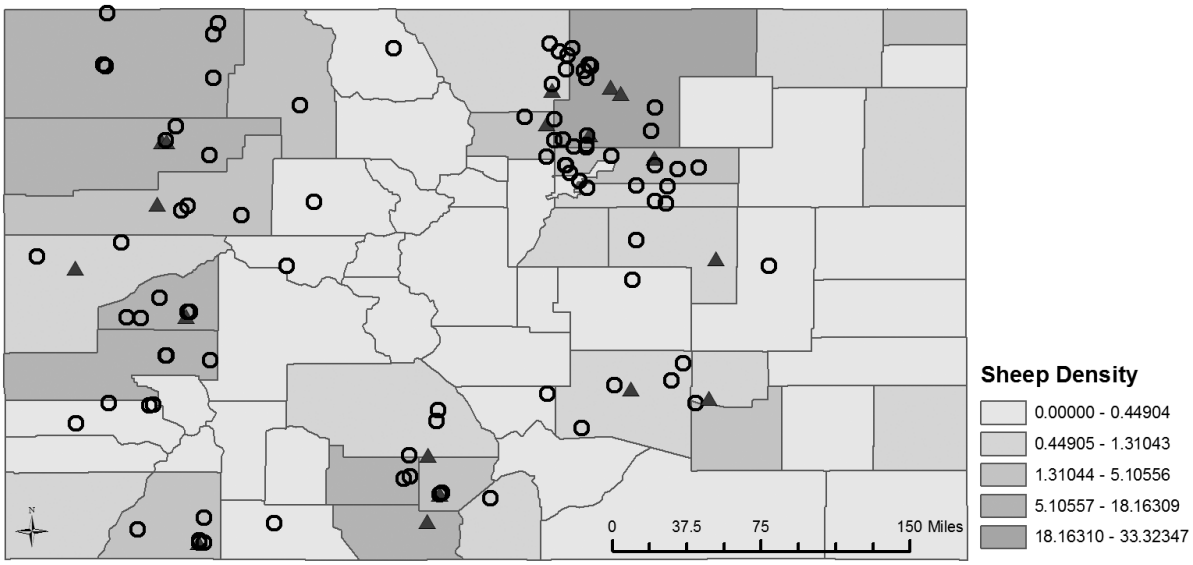

Figure 2.3—Map of Colorado sheep flocks with animals demonstrating clinical disease based on the criteria of three or more clinical signs present in at least five sheep. Triangles represent clinically affected flocks while circles represent flocks that were not clinically affected, and percentage density of sheep per county is represented by shading with darkest shading indicative of greater density (NASS, USDA).

CHAPTER 3

RISK FACTORS ASSOCIATED WITH BLUETONGUE IN COLORADO SHEEP

Abstract

Objective- To identify environmental and husbandry factors that contribute to prevalence of Bluetongue virus and clinical signs in Colorado sheep.

Design- Cross sectional Study

Sample Population- 2544 serum and whole blood samples obtained from sheep in 108 flocks from clientele of local practitioners belonging to the American Association of Small Ruminant Practitioners, Colorado State University Veterinary Teaching Hospital, Colorado State University Extension, Colorado Woolgrowers Association, Club Associations, and State Fairs and Shows.

Procedures- Participants were recruited by the submission of a questionnaire to the groups in the sample population. Upon completion of the questionnaire, a flock visit was made throughout the months of July until November to coincide with the vector season. Flocks were classified as either commercial flocks or club and show market flocks. The animals were observed for clinical signs of BTV infection and blood was obtained from ten ewes, ten lambs, and five rams from each of one hundred and eight flocks for cELISA and RT-PCR testing in conjunction with collection of geolocation coordinates utilizing a global positioning system (GPS). Temperature and rainfall data were collected from the weather station closest to each flock's GPS coordinates. Climate and questionnaire data were entered into a database and multivariable analysis was utilized to assess the relationship of husbandry and environmental factors with the likelihood of having viral RNA or antibodies to BTV.

Results- Two logistic regression models were utilized to assess environmental and husbandry factors that affect viral RNA and antibody detection. The two variables most

strongly associated with viral RNA detection were flock classification as commercial (OR 3.594;95% CI, 0.851- 15.177) and previous BTV vaccination (OR 15.949; 95% CI, 4.514- 56.350). The effects of flock purpose and vaccination were confounded by environmental variables of maximum temperature at the time of flock visit, proximity to water, and cumulative precipitation thirty-five days previous to the time of flock visit. The variable most strongly associated with antibody detection was vaccination (OR 9.360; 95%CI, 3.046-28.764). The effects of vaccination were confounded by proximity to water and elevation.

Conclusions- Findings from this study identified environmental and husbandry factors that contribute to the likelihood of detecting viral RNA and antibodies to BTV within Colorado sheep flocks. It is not surprising that the modified live BTV vaccination was the variable most strongly associated with detecting viral RNA and antibodies because their purpose is to initialize an immune response. However, more importantly, there was a significant association ($p \leq 0.001$) between vaccination and clinically affected flocks. It is likely that commercial flocks vaccinate more frequently and routinely than club or show flocks, but causality among the use of vaccines and clinical disease cannot be assessed within the current study. Temporal associations of vaccine delivery and previous flock history of Bluetongue were not accounted for within the model. Environmental factors that were related to both antibody and viral RNA detection were environmental parameters that typically support *Culicoides* populations. Two environmental parameters vary widely across Colorado (temperature and elevation). The risk factors evaluated within this study aid in understanding the drivers of Bluetongue infection and disease are more complicated than identifying environmental parameters

alone. There is a much more complex ecosystem reliant upon additional husbandry practices acting in concert to support the virus within the environment, invertebrate, and vertebrate hosts.

Introduction

Bluetongue virus (BTV) is the causative agent of an economically important emerging arboviral disease belonging to the family Reoviridae, genus *Orbivirus*. Twenty-four serotypes of BTV are recognized globally, five of which are considered endemic within the United States (2,10, 11,13,17). The virus is transmitted by *Culicoides spp*. biting midges that feed on a large host range including both wild and domestic ruminant species.[1] Clinical signs are primarily identified in sheep and are the result of a vasculitis. Typically during the acute phase, transient fevers are noted followed by oral ulcerations, coronitis, facial edema, and profuse nasal discharge leading to weight loss, infertility and wool loss classically known as "wool break".[2] The recent invasion of the southeastern United States by at least seven previously exotic serotypes (1, 3, 5, 6, 14, 19, 22, 24) as well as the rapid spread of 6 serotypes of BTV throughout both northern and southern Europe since 1998 has confirmed the importance of surveillance and understanding risk factors associated with disease. [3]

Bluetongue virus has been identified on all continents excluding Antarctica, and the unique regional distribution of distinct BTV strains (virus topotypes) with respect to their vector populations on all other continents is beginning to change. [4] BTV activity, defined by the OIE's Terrestrial Manual, is predominantly found within the latitude boundaries of 53°N and 34°S and three classifications of BTV status have been defined affecting transportation and free trade of ruminants.[5] These include BTV free zones, BTV infected zones, and BTV seasonally free zones such as Colorado. Due to its BTV

seasonally-free status, interstate and international trade restrictions related to BTV continue to impact Colorado's sheep industry during seasonal ram sales and shows.[5]

The distribution and vector biology of *Culicoides* are important and affect classification of BTV free zones and exportation of cattle. A variety of factors contribute to variation of transmission competence within the vector including altered susceptibility to BTV among *Culicoides spp*. and variation of environmental conditions that can support them. [7] One of the most important environmental components that contribute to the greatest abundance of adult *Culicoides* populations is temperature with peak vector abundance occurring when temperatures range from 28-30°C; therefore, the typical vector season extends from July-November within areas such as Colorado that are classified as BTV seasonally free zones. [8] Larval populations of *Culicoides sonorensis* are typically found around standing or slow moving sunlight-exposed aquatic environments often contaminated with manure, and larval habitats of *C. sonorensis* commonly have increased salinity concentrations when compared to those of *C. variipenni*s. [9] [10-11]

Recent studies are just beginning to explore novel environmental variables such as altitude, terrain slope, percentage of area covered by forests, normalized difference vegetation index, and aridity index that might influence populations of *Culicoides*, and these studies of environmental effects on *Culicoides* are finding that each subspecies prefers a particular subset of biotic and abiotic factors for survival and transmission of BTV. [16] Both altitude and latitude have been identified as risk factors for cattle herds testing positive to one or more BTV serotypes.[9] The mean altitude of positive operations

was 721 m above sea level whereas mean altitude of negative operations was 587 m above sea level. Sites with an altitude of 2134m or higher are not favorable conditions for survival of *Culicoides sonorensis* . [4,9,17]

Control strategies to protect animals from BTV infection include either vaccination or limiting exposure to *Culicoides spp*. Currently there is only one nationally licensed vaccine that can only be administered to sheep within the United States. This is a modified live vaccine that is only effective against serotype 10. [18-19] Limitations inherent to modified live vaccinations include abortions in pregnant animals or development of mild clinical signs. [20] A recombinant canarypox vaccine has been developed and has demonstrates a high level of protection among sheep with minimal side effects but it is not available commercially. [22]

The other primary BTV control recommendations aim to prevent exposure to feeding *Culicoides*. These practices have historically included the use of insecticides and housing animals indoors during late evening and early morning when *Culicoides* are most active.[5] However, recent studies have found that *Culicoides* will actively feed throughout the day and feed within barn facilities quite frequently, so these recommendations do not provide adequate protection for ruminant species. [23-26] The purpose of this study is to identify risk factors associated with BTV seroprevalence, viral DNA prevalence, and clinical infection in sheep so that rational control measures can be developed and recommended. By identifying risk factors associated with BTV infection, appropriate steps can be identified to manage and minimize environmental risks.

Methods and Materials

Sample population- Participants were recruited as described in Chapter 2 (Appendix 1). Of 506 questionnaires distributed, 108 were returned with adequate information to schedule a flock visit during the July-November 2007 vector season. Criteria for entry required that each location had sheep, completed the survey, and was located in Colorado.

Flock visits and identification of clinically infected flocks were conducted as described in Chapter 2. The location of each herd was established utilizing a handheld global positioning system (GPS) where latitude, longitude, and elevation were recorded at the site of blood collection. Additional environmental data was obtained from the National Oceanic and Atmospheric Administration for the weather station closest to each flock's GPS coordinates. These data included precipitation and temperature on the day of the flock visit, and average daily temperature and precipitation for the 35 days prior to the flock visit. Average values were calculated from daily reported temperature and precipitation. Blood samples were collected, described, and analyzed as described in Chapter 2.

Data Analysis- Data was entered into an electronic database and checked for errors. The outcome of interest was categorized as positive or negative animals on the basis of RT-PCR, cELISA, and clinical disease results as described in Chapter 2, and three multivariable logistic regression analyses were constructed to assess the most significant husbandry and environmental predictors of antibody detection, viral RNA, and clinical disease utilizing SAS 9.1.3. Fourteen flock variables were analyzed for potential inclusion in the model, including: latitude coordinates; longitude coordinates; elevation

(meters above sea level); precipitation the day of the flock visit (inches); cumulative precipitation thirty-five days before the flock visit (inches); proximity to surface water (meters) characterized as lake, river, stream, creek, or irrigation ditch; maximum and minimum temperature at the time of the flock visit (Fahrenheit); average maximum and minimum temperature thirty-five days before the flock visit; stocking density (sheep/acre); breed classification (Suffolk, Hampshire, Rambouillet, Columbia, Polypay); primary purpose of the flock (commercial, show/club); and BTV vaccination history (vaccinated, unvaccinated). Descriptive statistical analyses were performed to identify sparse data and collapse categorical variables if needed.

Variables that were significant at the 0.25 level following univariate analysis were entered into a multivariable logistic regression model utilizing a purposeful selection model building strategy that utilizes aspects of both forward and backward selection.[27] Variables that were significant at the level of 0.05 were retained as the main effects model and all other variables were forced back into the model to assess for confounding by a change in odds ratios. Any variables that resulted in a >10% change in the estimates of the main effects were considered to be confounders and retained within the model. All first order interactions were assessed at the 0.10 level of significance. Scale assessment was performed utilizing two methods in order to identify any necessary transformations of continuous predictor variables: graphical representation of predictor variable with the log odds of the outcome variable and fractional polynomial to assess all first and second power transformations and their impact within the model.[28-29]

Model fit was assessed by use of Hosmer-Lemeshow test and predictive ability was assessed utilizing sensitivity, specificity, positive, and negative predictive value.[28] A probability cut-point of 0.25 was used for predicting the outcome of viral RNA and a cut-point of 0.50 was utilized for predicting the outcome, antibody detection. Outliers were assessed evaluating four measures: leverage; delta chi-square; difference in deviance; and delta beta hat.[30] Each outlier was deleted from the model separately and then in conjunction with all other outliers to assess differences in parameter estimates and model fit compared to the full model. The final model was presented and interpreted utilizing odds ratios, standard error, confidence intervals, and p-values.[29]

Results

Antibody Detection

The most significant predictors of antibody detection were vaccination and elevation (Table 3.2). Linearity of the two continuous variables (proximity to water, elevation) was assessed utilizing graphical representation and fractional polynomials. The use of fractional polynomials did not reveal a significant one or two power transformation; however, graphical representation revealed that the combination of variables would be an appropriate resolution for stability of the model. Dichotomous variables were created for both continuous variables so that proximity to water was categorized as 2.10-1004.4 meters or 1004.5-42380.3 meters from the nearest natural body of water and elevation was categorized as 1322.832-1634.032 meters or 1634.337-2635.910 meters above sea level.

The Hosmer-Lemeshow test for model fit was most appropriate due to the similarities in covariate patterns (108) and sample size (108).[29] The test revealed a p-value of 0.86 which demonstrated evidence of model fit and based on a cut point of 0.50, the model had a sensitivity of 38.7% and specificity of 93.4%. The positive predictive value at 10% antibody prevalence was 39.5% and the negative predictive value was 93.2%. As the prevalence of BTV antibodies increased to 30%, the positive predictive value was 71.6% with a negative predictive value of 78.1% which is to be expected because probability of identifying BTV antibodies within a population with higher prevalence is greater than that of a lower prevalence. The area under the ROC curve was 0.778 which revealed acceptable discrimination of the model in determining seropositive and seronegative flocks. There were three outliers within the model that did not appear to alter the model fit after deletion compared to the full model. The variable that demonstrated the most significant change was elevation. The covariate patterns of the outliers revealed that all three had antibodies at elevations of 7545, 7566, and 7693 meters above sea level. All outliers were retained within the model.

Viral RNA

The two most significant predictors of viral RNA detection were flock purpose and vaccination Confounders included maximum temperature at the time of flock visit, proximity to water, and precipitation thirty-five days previous to the time of flock visit (Table 3.1). No first order interactions were found to be significant in the prediction of viral RNA.

The Hosmer-Lemeshow test for model fit was most appropriate due to the similarities in covariate patterns (108) and sample size (108).[29] The test revealed a p-value of 0.91 which demonstrated evidence of model fit and based on a cut point of 0.25, the model had a sensitivity of 58.3% and specificity of 84.5%. The positive predictive value at 10% prevalence of viral RNA was 29.5% and the negative predictive value was 94.8%. As the prevalence of viral RNA increased to 30%, the positive predictive value was 61.8% with a negative predictive value of 82.6% which is to be expected because the probability of identifying viral RNA within a population with higher prevalence is greater than that of a lower prevalence. The area under the ROC curve was 0.858 which revealed excellent discrimination of the model in determining viral RNA positive and negative flocks.

There were four outliers within the model that did not appear to alter the model fit after deletion compared to the full model. The two variables that demonstrated the most significant change were proximity to water and precipitation thirty-five days previously. The covariate patterns of the outliers revealed that two flocks did not have detectable viral RNA but were approximately 7000 meters from water and had cumulative precipitation levels of 0.05-1.05 inches thirty-five days previously. The other two outliers had covariate patterns where animals had detectable viral RNA but were 12-20 meters from the nearest water source and had no detectable precipitation levels thirty-five days previously. There were no coding errors in the database and an explanation for these values could be related to the unequal numbers of each age/sex (rams, ewes, lambs) class among the flocks based on prevalence data in which viral RNA prevalence was higher among lambs than ewes or rams. A decision was made to retain the outliers

within the model recognizing the covariate patterns. In future studies, it would be appropriate to collect additional data that would better represent the dataset with relation to environmental and specific animal variables.

Clinical Disease

The most significant predictor of clinical disease was vaccine administration with confounding environmental variables of maximum temperature at the time of the flock visit and proximity to groundwater. The Hosmer-Lemeshow test for model fit was most appropriate as described for the other two models. The test revealed a p-value of 0.89 which demonstrated evidence of adequate model fit. Based on a cut point of 0.25, the model had a sensitivity of 54.3% and specificity of 86.2%. The positive predictive value at 10% prevalence of viral RNA was 32.3% and the negative predictive value was 97.8%. As the prevalence of viral RNA increased to 30%, the positive predictive value was 60.8% with a negative predictive value of 79.4% which is to be expected because the probability of identifying viral RNA within a population with higher prevalence is greater than that of a lower prevalence. The area under the ROC curve was 0.928 which revealed excellent discrimination of the model in determining viral RNA positive and negative flocks. There were five outliers within the model that did not appear to alter the model fit after deletion compared to the full model. The variable that demonstrated the most significant change was proximity to water as seen within the viral RNA model.

Discussion

Findings in this study confirm that both environmental and management parameters are associated with BTV incidence of infection. In all multivariable logistic regression models, it was found that vaccination was the variable most strongly associated with viral RNA, antibodies, and clinical disease within Colorado sheep. The vaccines are primarily utilized by commercial producers to protect them from financial losses as a result of clinically diseased animals in addition to secondary production losses due to trade restrictions, decreased wool production, and infertility. The only licensed vaccine that is available for use in Colorado is a modified live vaccine preparation which is weakened (attenuated) in cell culture and can potentially result in development of mild clinical disease. [31] Given the way in which modified live vaccines work, it was not surprising to find that vaccination was associated with viral RNA or antibodies, which is the primary purpose for administration. However, it is surprising that vaccination was strongly associated with clinical disease. Without appropriate attenuation of the virus in modified live vaccines, administration could have resulted in infection of naive animals leading to clinical signs of BT.

Vaccination had a stronger effect on the odds of viral RNA detection than on the odds of antibody detection because the vaccines administered were modified live in nature, which potentially resulted in a rise of viral RNA before antibodies could be detected within the blood. In addition, sheep may have been demonstrating clinical signs of disease before administration of the vaccine. Vaccination may also have been associated with clinical signs of disease by needle re-use: producers using a single needle for multiple injections. This may have infected naïve sheep with needles previously inserted into naturally infected animals, especially in flocks where Bluetongue infection

is more common. In vaccinated flocks, the date of vaccine administration was not accounted for; therefore, no assessment of the temporal lag until development of clinical signs could be made. Serotype identification and sequence analysis of the vaccine and field strains are some diagnostic strategies that could further aid in understanding relationships among these samples, but diagnostic modalities such as virus neutralization were not available and the time and sample integrity was compromised for further sequence analysis.

Additional methods of controlling BTV without leading to BTV serpositivity include limiting *Culicoides* exposure or use of killed or new generation (canarypox vectored) vaccines. Limiting exposure of ruminants to *Culicoides spp*. requires a better understanding of the actual feeding habits, ecological variables and activity of *Culicoides*. Although environmental factors are known to affect development and survival of *Culicoides spp.*, they did not significantly affect the odds of viral RNA, antibody detection, or clinical disease once the effect of management factors (vaccination, flock type) was modeled. The effect of vaccination differed depending on these environmental variables, but the environmental variables themselves were not associated with increased odds of viral RNA or antibody detection.

Some of the environmental confounders, such as high temperatures, have been explained extensively in previous studies in which temperature increased infection rates, rates of virogenesis, and transmission of BTV within *Culicoides.*[8] Fundamental research performed within the 1970's identified the cyclical nature of BTV throughout the year and found that precipitation data thirty days previous to identification of BTV in

Culicoides was one of the most significant predictors in BTV transmission to ruminants. Previous work has also found that lower elevations were more likely to have BTV antibodies than higher elevations as also demonstrated in our study. [7,15,34]

A limitation of this study was the short time period in which the study was conducted. It would have been interesting to conduct the cross sectional study throughout the course of an entire year so that adequate comparisons could be made between the vector season in July-November and the winter months. Newly developed recombinant vaccines are highly effective and have provided promise for short term control but disadvantages always revolve around ensuring protection against multiple serotypes while providing safety for the animals.[22] Although true causation was not demonstrated in the current cross-sectional study, the strong association observed between vaccination and clinical signs indicative of Bluetongue should be followed by clinical trials to measure the effects of vaccine intervention after exposure, both directly from BTV inoculation and the feeding of BTV infected *Culicoides* vectors.

References

1. Singer RS, MacLachlan NJ, Carpenter TE. Maximal predicted duration of viremia in bluetongue virus-infected cattle. *J Vet Diagn Invest* 2001;13:43-49.
2. Osburn BI. Role of the immune system in bluetongue host-viral interactions. *Prog Clin Biol Res* 1985;178:417-422.
3. Schwartz-Cornill I, Mertens PP, Contreras V, et al. Bluetongue virus: virology, pathogenesis and immunity. *Vet Res* 2008;39:46.
4. Baylis M. When will bluetongue transmission start in 2008? *Vet Rec* 2008;162:526; author reply 527.
5. World Organisation for Animal Health. Article 8.3.19, Bluetongue. OIE Terrestrial Manual 2008
6. Ostlund E, Moser K. Distribution of bluetongue in the United States of America, 1991-2002. *Vet Ital* 2004;40:83-88.
7. Gerry AC, Mullens BA, Maclachlan NJ, et al. Seasonal transmission of bluetongue virus by Culicoides sonorensis (Diptera: Ceratopogonidae) at a southern California dairy and evaluation of vectorial capacity as a predictor of bluetongue virus transmission. *J Med Entomol* 2001;38:197-209.
8. Mellor PS, Boorman J. The transmission and geographical spread of African horse sickness and bluetongue viruses. *Ann Trop Med Parasitol* 1995;89:1-15.
9. Green AL, Dargatz DA, Schmidtmann ET, et al. Risk factors associated with herd-level exposure of cattle in Nebraska, North Dakota, and South Dakota to bluetongue virus. *Am J Vet Res* 2005;66:853-860.
10. Meiswinkel R, Goffredo M, Leijs P, et al. The Culicoides 'snapshot': A novel approach used to assess vector densities widely and rapidly during the 2006 outbreak of bluetongue (BT) in The Netherlands. *Prev Vet Med* 2008;87:98-118.
11. Tabachnick WJ. Culicoides variipennis and bluetongue-virus epidemiology in the United States. *Annu Rev Entomol* 1996;41:23-43.
12. Mullens BA, Rodriguez JL. Colonization and response of Culicoides variipennis (Diptera: Ceratopogonidae) to pollution levels in experimental dairy wastewater ponds. *J Med Entomol* 1988;25:441-451.
13. Bonneau KR, Topol JB, Gerry AC, et al. Variation in the NS3/NS3A gene of bluetongue viruses contained in Culicoides sonorensis collected from a single site in southern California. *Virus Res* 2002;84:59-65.
14. Bonneau KR, Mullens BA, MacLachlan NJ. Occurrence of genetic drift and founder effect during quasispecies evolution of the VP2 and NS3/NS3A genes of bluetongue virus upon passage between sheep, cattle, and Culicoides sonorensis. *J Virol* 2001;75:8298-8305.
15. Gloster J, Mellor PS, Manning AJ, et al. Assessing the risk of windborne spread of bluetongue in the 2006 outbreak of disease in northern Europe. *Vet Rec* 2007;160:54-56.
16. Conte A. Novel environmental factors influencing the distribution and abundance of Culicoides imicola and the obsoletus complex in Italy. *Vet Ital*;43.
17. Gibbs EP, Tabachnick WJ, Holt TJ, et al. U.S. concerns over bluetongue. *Science* 2008;320:872.

18. Kemeny L, Drehle LE. The use of tissue culture-propagated bluetongue virus for vaccine preparation. *Am J Vet Res* 1961;22:921-925.
19. Luedke AJ, Jochim MM. Clinical and serologic responses in vaccinated sheep given challenge inoculation with isolates of bluetongue virus. *Am J Vet Res* 1968;29:841-851.
20. Schultz G, Delay PD. Losses in newborn lambs associated with bluetongue vaccination of pregnancy ewes. *J Am Vet Med Assoc* 1955;127:224-226.
21. Osburn BI, McGowan B, Heron B, et al. Epizootiologic study of bluetongue: virologic and serologic results. *Am J Vet Res* 1981;42:884-887.
22. Boone JD, Balasuriya UB, Karaca K, et al. Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. *Vaccine* 2007;25:672-678.
23. Mellor PS, Carpenter S, Harrup L, et al. Bluetongue in Europe and the Mediterranean Basin: History of occurrence prior to 2006. *Prev Vet Med* 2008;87:4-20.
24. Purse BV, Baylis M, Tatem AJ, et al. Predicting the risk of bluetongue through time: climate models of temporal patterns of outbreaks in Israel. *Rev Sci Tech* 2004;23:761-775.
25. Baldet T, Delecolle JC, Cetre-Sossah C, et al. Indoor activity of Culicoides associated with livestock in the bluetongue virus (BTV) affected region of northern France during autumn 2006. *Prev Vet Med* 2008;87:84-97.
26. Meiswinkel R, Baldet T, de Deken R, et al. The 2006 outbreak of bluetongue in northern Europe-The entomological perspective. *Prev Vet Med* 2008;87:55-63.
27. Hosmer DW, Hjort NL. Goodness-of-fit processes for logistic regression: simulation results. *Stat Med* 2002;21:2723-2738.
28. Lemeshow S, Hosmer DW, Jr. A review of goodness of fit statistics for use in the development of logistic regression models. *Am J Epidemiol* 1982;115:92-106.
29. Hosmer DW, Lemeshow S. Confidence interval estimates of an index of quality performance based on logistic regression models. *Stat Med* 1995;14:2161-2172.
30. Hosmer DW, Hosmer T, Le Cessie S, et al. A comparison of goodness-of-fit tests for the logistic regression model. *Stat Med* 1997;16:965-980.
31. Osburn BI, de Mattos CA, de Mattos CC, et al. Bluetongue disease and the molecular epidemiology of viruses from the western United States. *Comp Immunol Microbiol Infect Dis* 1996;19:181-190.
32. Berry LJ, Osburn BI, Stott JL, et al. Inactivated bluetongue virus vaccine in lambs: differential serological responses related to breed. *Vet Res Commun* 1982;5:289-293.
33. Ghalib HW, Cherrington JM, Adkison MA, et al. Humoral and cellular immune response of sheep to bluetongue virus. *Prog Clin Biol Res* 1985;178:489-496.
34. Foster NM, Jones RH, McCrory BR. Preliminary Investigations on Insect Transmission of Bluetongue Virus in Sheep. *Am J Vet Res* 1963;24:1195-1200.

Table 3.1 ─Univariable odds of detecting antibodies to bluetongue virus for specified husbandry and environmental parameters in 108 Colorado sheep flocks.

Variable	Value	OR	95% CI	p-value	P<0.25
Breed	Black Face	3.85	0.92-16.01	0.06	X
	White Face				
Purpose	Commercial	0.72	0.30-1.76	0.48	
	Club/show/4-H				
Vaccination	Vaccinated	9.37	3.29-26.76	<.001	X
	Unvaccinated				
Latitude		1.00	1.00-1.00	0.37	
Longitude		1.00	1.00- 1.00	0.78	
Elevation		1.00	1.00 1.00	0.35	
Precip. at time of flock visit (inches)		1.86	0.53-6.51	0.32	
Proximity to water (meters)		1.00	0.99-1.00	0.07	X
Temperature minimum at time of visit (ºF)		1.00	0.97-1.03	0.99	
Temperature maximum at time of visit (ºF)		1.01	0.98-1.04	0.50	
Cumulative temperature minimum for 35 days (ºF)		1.01	0.97-1.05	0.59	

temperature maximum for 35 days (ºF)	0.98	0.94-1.01	0.32	
Density	1.00	0.99-1.00	0.21	X

Table 3.2─Multivariable analysis of herd-level risk factors for BTV seropositivity in 108 Colorado sheep flocks.

Factor	Category	Estimate	SE	OR	95% CI	p-value
Intercept	NA	-3.29	0.842	NA	NA	<.001
Vaccination	Vaccinated	2.23	0.572	9.36	3.04-28.76	<.001
***Proximity to water (meters)**	2.10-1004.4m	1.16	0.699	3.19	0.81-12.56	0.09
***Elevation (meters)**	1322.832-1634.032m	1.13	0.660	3.09	0.84-11.30	0.08

*Variables added into the model based on confounding , $\geq$ 10%. Reference category is an unvaccinated flock at elevation 1634.1 to 2636 m with water proximity of 1004.5 to 42380 m

Table 3.3─Univariable odds of detecting BTV viral RNA for specified husbandry and environmental parameters in 108 Colorado sheep flocks.

Variable	Comparison	OR	95% CI	p-value	P<0.25
Breed	**Black Face**	2.956	0.55-15.72	0.20	X
	White Face				
Purpose	**Commercial**	2.636	0.82-8.44	0.10	X
	Club/show/4-H				
Vaccination	**Vaccinated**	13.30	4.46-39.57	<.001	X
	Unvaccinated				
Latitude		1.00	1.00-1.00	0.96	
Longitude		1.00	1.00-1.00	0.40	
Elevation		1.00	0.99-1.00	0.19	X
Precipitation at time of flock visit		0.32	0.02-4.77	0.40	
Cumulative 35 day precipitation		3.55	0.15-80.14	0.42	
Proximity to water		1.00	0.99 -1.00	0.12	X
Temperature minimum at time of visit		1.01	0.97-1.04	0.44	
Temperature maximum at time of visit		1.03	0.99-1.08	0.05	X
Cumulative temperature minimum for 35 days		0.98	0.97-0.99	0.87	
Cumulative temperature maximum for 35 days		1.01	0.97-1.05	0.56	
Flock Density		1.00	0.99-1.00	0.21	X

Table 3.4– Multivariable analysis of herd-level risk factors for BTV viral RNA positivity in 108 Colorado sheep flocks.

Factor	Category	Estimate	SE	OR	95% CI	p-value
Intercept	NA	-6.19	2.329	NA	NA	0.01
Purpose of flock	Commercial Club	1.27	0.735	3.59	0.85- 15.17	0.08
Vaccination	Vaccinated Unvaccinated	2.76	0.644	15.94	4.51- 56.35	<.001
***Maximum temperature at flock visit**	NA	0.04	0.026	1.04	0.98- 1.09	0.12
***Proximity to water (meters)**	NA	-0.01	0.001	1.00	0.99- 1.00	0.14
***Precipitation 35 days previously**	NA	2.54	2.193	12.70	0.17- 935.84	0.25

*Variables added into the model based on confounding , $\geq$ 10%.

Table 3. 5 ─ Univariable odds of detecting BTV clinical signs for specified husbandry and environmental parameters in 108 Colorado sheep flocks.

Variable	Comparison	OR	CI	p-value	P<0.25
Breed	**Black Face**	2.11	1.22-10.44	0.1864	X
	White Face				
Purpose	**Commercial**	3.11	1.808-	0.1225	X
	Club/show/4-H		12.889		
Vaccination	**Vaccinated**	12.66	5.88-45.87	<.0001	X
	Unvaccinated				
Latitude		1.00	1.00-1.00	0.7889	
Longitude		1.00	1.00-1.00	0.5579	
Elevation		1.00	1.00-1.00	0.4556	X
Precipitation at time of flock visit		0.56	0.22-6.77	0.8895	
Cumulative 35 day precipitation		2.99	0.49-66.75	0.8876	
Proximity to water		1.00	0.99-1.00	0.1673	X
Temperature minimum at time of visit		1.00	0.97-1.00	0.6678	
Temperature maximum at time of visit		1.04	0.99-1.06	0.0668	X
Cumulative temperature minimum for 35 days		0.95	0.76-0.88	0.775	
Cumulative temperature maximum for 35 days		1.04	1.00-1.06	0.8805	
Density		1.00	1.00-1.88	0.2226	X

Table 3.6-- Multivariable analysis of herd-level risk factors for BTV clinical signs in 108 Colorado sheep flocks

Factor	Category	Estimate	SE	OR	95% CI	p-value
Intercept	NA	-4.84	2.185	NA	NA	0.02
Vaccination	Vaccinated Unvaccinated	2.12	0.617	8.33	2.48-27.95	0.001
***Maximum temperature at flock visit**	NA	-0.04	0.025	1.04	0.99- 1.09	0.11
***Proximity to water (meters)**	NA	-0.09	0.001	0.99	0.99-1.00	0.15

CHAPTER 4

SUMMARY AND CONCLUSIONS

Estimating the prevalence and identifying risk factors associated with Bluetongue virus infection and disease within Colorado sheep flocks is important for the Colorado sheep industry, the Colorado veterinary community, and the future of BTV research. Within this study, the odds of viral RNA detection were 15.9 times greater among vaccinated compared with non-vaccinated sheep. Vaccination in combination with previous exposure could have resulted in greater seroprevalence among the older populations thereby masking the relationship of measuring viral RNA or clinical disease. However, because modified live vaccines are used among naïve populations, vaccination might be contributing to increased viral RNA prevalence and clinical signs typically observed in conjunction with BTV infection. It is important to recognize clinical signs may be secondary to the use of modified live vaccines, and might be avoided with the use of inactivated or new generation vaccines.[2-3] Although true causation was not demonstrated in the current cross-sectional study, the strong association observed between vaccination and clinical signs indicative of Bluetongue should be followed by clinical trials to measure the effects of vaccine intervention after exposure, both directly from BTV inoculation and the feeding of BTV infected *Culicoides* vectors.

Culicoides spp. serve a critical role in the maintenance and transmission cycle of the virus. Environmental parameters play a critical role in sustaining these vector populations, but within this study environmental factors were not associated with an increase in risk of BTV detection once vaccination and flock type were included as risk factors in the model. Some of the primary environmental risk factors that have been attributed to increased abundance of *Culicoides sonorensis* include temperatures ranging from 81-86°F (27-30°C); lower elevations (0-400ft.); increased precipitation and

increased humidity.[1] Elevation, precipitation, and humidity changed drastically among the sites tested in 2007 within Colorado, depending on location and time of collection. This offered ranges of environmental data that might have been directly related to the BTV status (seroprevalence, viral RNA, clinical disease) in addition to the two primary risk factors in our model: vaccination and flock type. Commercial flocks observed more stringent vaccination protocols due to intermingling of animals and typically housed their animals on multiple locations depending on season, which exposed them to a multitude of different environments. Due to the cross-sectional nature of this study, animals were not followed over several seasons; therefore, seasonality of infection cannot be accounted for. In addition, the impact of vaccination on detection of BTV may mask the true environmental contribution that typically supports *Culicoides* vectors and transmission of the virus.

These findings are a much needed contribution since the last intensive BTV surveillance program conducted in Colorado occurred in the 1970s when only antibody detection was utilized to identify BTV prevalence. At that time, the prevalence was estimated to be approximately 30% among slaughter cattle.[4] This prevalence estimate among cattle is similar to that identified within surrounding states including Wyoming, Nevada, Texas, and California, but stands in marked contrast to lower prevalence estimates identified among the Northeastern states.[4] With the introduction of nested PCR, this study has allowed us to introduce a more sensitive tool in identifying early infection through detection of viral RNA. The increased sensitivity of this and future diagnostic modalities will increase our ability to establish stronger associations among husbandry and environmental risk factors and the relationship with BTV infection status.

In part, many ecological and environmental changes that are occurring globally and nationally are contributing to the northward expansion of *Culicoides spp*. and BTV within previously uninhabitable environments.[5] In addition, the research community is just beginning to identify some of the fundamental concepts concerning *Culicoides* ecology and differences in abiotic and biotic environmental parameters that exist among vector species.[6] The conclusions from this study have allowed us to identify husbandry practices that contributed to BTV viral RNA prevalence among Colorado sheep and may also be useful in understanding appropriate prevention measures and predicting future activity.

Future studies should evaluate whether novel environmental risk factors (i.e. wind, vegetative index, soil indices, salinity), which potentially contribute to the seasonality of BTV infection among Colorado sheep and ruminants nationally, are associated with use of vaccines. Within our study, vaccination was identified as the primary contributor for BTV infection and disease among sheep. Potential confounding variables (purpose of flock, management, environmental predictors) could have affected the relationship between vaccination and BTV infection. The strong association of vaccination with identification of BTV viral RNA and clinical signs warrants further investigation through a clinical trial where causation can be assessed. This type of study could firmly establish BTV vaccination recommendations for producers and veterinarians, and offer a better understanding of the consequences of BTV vaccination. In further expanding the horizons of vaccination within the arboviral community, control strategies among the vector and the host should be explored.

The ecology of arboviral diseases and their key players are changing, in part as a result of climate change. Temperatures and precipitation parameters are known to affect the vectors that harbor Bluetongue virus; however, it is not known what specific ecosystem parameters have the greatest impact on BTV infection rates or incursion of exotic serotypes. As climate change progresses, weather may become more variable and alter ecosystem health by disassociating long standing relationships and feedback systems among the host, vector, and virus. One goal of this and future studies is to better identify and define risk factors associated with endemic and exotic strains of BTV within Colorado and the United States.

References

1. Mellor PS, Boorman J. The transmission and geographical spread of African horse sickness and bluetongue viruses. *Ann Trop Med Parasitol* 1995;89:1-15.
2. Boone JD, Balasuriya UB, Karaca K, et al. Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. *Vaccine* 2007;25:672-678.
3. Roy P, Bishop DH, LeBlois H, et al. Long-lasting protection of sheep against bluetongue challenge after vaccination with virus-like particles: evidence for homologous and partial heterologous protection. *Vaccine* 1994;12:805-811.
4. Metcalf HE, Pearson JE, Klingsporn AL. Bluetongue in cattle: a serologic survey of slaughter cattle in the United States. *Am J Vet Res* 1981;42:1057-1061.
5. Gloster J, Mellor PS, Manning AJ, et al. Assessing the risk of windborne spread of bluetongue in the 2006 outbreak of disease in northern Europe. *Vet Rec* 2007;160:54-56.
6. Conte A. Novel environmental factors influencing the distribution and abundance of Culicoides imicola and the obsoletus complex in Italy. *Vet Ital*;43.