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Malignant Catarrhal Fever Viruses in Tennessee Ruminants

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To the Graduate Council:

I am submitting herewith a dissertation written by Robin Lynn Cissell entitled "Malignant Catarrhal Fever Viruses in Tennessee Ruminants." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Robert L. Donnell, Major Professor

We have read this dissertation and recommend its acceptance:

Stephen Kania, Agricola Odoi, Graham Hickling

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Accepted for the Council:

Carolyn R. Hodges
Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Malignant Catarrhal Fever Viruses in Tennessee Ruminants

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Robin Lynn Cissell
August 2010

Dedication

I dedicate this dissertation to my late friend, Kathryn Gilpatrick. Her love of learning was exceeded only by her love of family and furry friends. Her friendship and guidance helped me throughout my years of graduate school.

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ABSTRACT

Malignant catarrhal fever (MCF) is a lymphoproliferative and inflammatory syndrome affecting primarily ruminant species. The disease, which is often fatal, is most often described as affecting bovids and cervids. No vaccines are available for prevention of MCFV infection. The primary method to control spread of disease is to prevent contact between carriers and clinically susceptible species. There is no known method to control infection of malignant catarrhal fever virus-white-tailed deer variant (MCFV-WTD), as the carrier animal of this virus is unknown.

To determine the prevalence of malignant catarrhal fever viruses in Tennessee ruminant populations, blood and/or lymph node samples were collected from farms, animal processing and disposal facilities, and hunter check-in stations from 2006-2008 from several species of animals including cervids, cattle, and goats. Strain-specific real time PCR was developed to detect ovine herpesvirus-2 (OvHV-2), caprine herpesvirus-2 (CpHV-2), and MCFV-WTD DNA. MCFV DNA was detected in all species of ruminants sampled. Although disease related to infection with MCFV-WTD and CpHV-2 has not been reported in Tennessee cattle or cervid populations, MCFV-WTD DNA was detected in 3 percent of cervid samples, and MCFV-WTD and CpHV-2 DNA was detected in 27 and 3 percent respectively of cattle samples from animal disposal facilities that process dead or debilitated animals. One hunter harvested deer (n=781) and 25 cattle (n=165) tested from animal disposal facilities were positive for OvHV-2 DNA.

This study demonstrated that healthy cattle and cervids can be infected with OvHV-2 and MCFV-WTD without apparent disease, and dead or debilitated cattle were infected with OvHV-2, MCFV-WTD and CpHV-2 at a higher percentage than healthy herd animals. Prevalence of CpHV-2 in Tennessee goat populations (7%) was significantly lower than reported in other goat populations (73%). Low prevalence of CpHV-2 in Tennessee goat populations likely explains why no evidence of infection was found in cervids tested, and the low prevalence of CpHV-2 infection in dead or debilitated cattle compared to prevalence of infection with OvHV-2 and MCFV-WTD. The discovery of infection in cattle with CpHV-2 and MCFV-WTD opens a new avenue of investigation into the pathology and virulence of MCFV's in domestic cattle.

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Chapter 1. Literature Review

Malignant catarrhal fever – Introduction

Malignant catarrhal fever (MCF) is a lymphoproliferative and inflammatory syndrome that primarily affects ruminant species. The disease, which is often fatal, has been most often described as affecting bovids, cervids, and certain other susceptible ruminant species, but is also recognized in domestic pigs (Bedelian, 2007; OIE, 2004), and has been shown experimentally in rabbits (Rossiter et al., 1977, 1978). Outbreaks of malignant catarrhal fever occur sporadically in all continents, and are attributed to large economic losses in domestic cattle, deer and bison herds (Berezowski et al., 2005; Brown and Bloss, 1992; Dabak and Bulut, 2003; Hamilton, 1990; Li et al., 2008; Li et al., 2006; O'Toole et al., 2002). Carrier animals have been identified and are believed to be the source of the disease in affected ruminants (Baxter et al., 1997; Li et al., 1995; Plowright et al., 1960). There are many strains of virus that cause this disease in susceptible species, all of which belong to the *Herpesviridae* subfamily *Gammaherpesviridae*.

Gammaherpesviruses

The host range of the *Gammaherpesvirinae* subfamily is primarily limited to the family or orders to which the natural host belongs. As with all viruses in the *Herpesviridae* family, their genomes are composed of linear double stranded

DNA. The genomic DNA is packed into an icosohedral capsid which is embedded in a complex amorphous layer composed of several proteins called the tegument. The entire structure is then enclosed by a glycoprotein containing lipid envelope (McGeoch et al., 2006). Viruses in the *Gammaherpesvirinae* subfamily are usually specific for T or B lymphocytes, and latency is frequently established in lymphoid tissue (Fields et al., 2001). Unlike alpha or beta herpes viruses, which seem to prefer lytic replication, gammaherpesviruses seem to favor the initial establishment of latency, while only a subset support lytic replication. The outcome of infections with the gammaherpesviruses depends not only on the virus but also the targeted animal. In vivo, viruses in this subfamily have evolved with their reservoir hosts to actively protect their latently infected cells from being destroyed by the hosts' immune response. Hosts have evolved to being infected and can transmit the viruses without showing symptoms of overt disease. In animals not adapted to infection, such as in animals that did not co-evolve with the virus, the development of lethal diseases such as malignant catarrhal fever or Kaposi's sarcoma occurs (Ackermann, 2006). In recent years many new gammaherpesviruses have been discovered and classified (McGeoch et al., 2005).

The *Gammaherpesvirinae* subfamily has been traditionally divided into two genera: *Lymphocryptovirus* and *Rhadinovirus* (Fields et al., 2001). The *Lymphocryptovirus* genus contains Epstein-Barr virus, and several other lymphocryptoviruses of primates. The *Rhadinovirus* genus contains herpes-

viruses with hosts of many mammalian taxa. Many of these viruses are of interest for medicine, veterinary medicine and biomedical research (Ackermann, 2006). There are many similarities between the two genomes. Given these similarities and the restriction of lymphocrytoviruses to primates, it is proposed that lymphocryptoviruses may have evolved from an early primate rhadinovirus (Knipe et al., 2001).

Gammaherpesviruses share many genes with limited or less obvious representation in the genomes of other herpes viruses. These include genes that encode several immediate-early or early regulators of viral gene expression, an anti-apoptotic Bcl-2 homolog, and two integral membranes (Knipe et al., 2001). Lymphocryptoviruses and rhadinoviruses have analogous, nonhomologous cis-acting DNA sequences and transacting nuclear proteins necessary for persistence of the genomes as episomes in dividing cells. The genomes of the lymphocryptoviruses and rhadinoviruses are much more related to each other than to other alpha or beta herpesviruses (Knipe et al., 2001).

Rhadinoviruses

Most rhadinoviruses have cellular genes including dihydrofolate reductase, interferon regulator factors, G-protein coupled receptors, chemokine analogs, and a cyclin homolog in common. These genes have not been detected in lymphocryptoviruses. Unlike lymphocrypto-viruses, rhadinoviruses are unable to immortalize B lymphocytes of

their natural host (Knipe et al., 2001). Malignant Catarrhal fever viruses have traditionally been characterized as rhadinoviruses, although in recent years it has been proposed that a new genus, the *Macaviruses*, be established in the gammaherpesvirus subfamily, and that these viruses be placed in this family (McGeoch et al., 2006). Several viruses have been identified within the MCF virus group, four of which: alcelaphine herpesvirus-1, ovine herpesvirus-2, caprine herpesvirus-2, and malignant catarrhal fever virus white-tailed deer variant, are known to be pathogenic (Li et al., 2003a).

Malignant Catarrhal Fever viruses as Interface diseases

The interaction of domestic animals and wildlife is increasingly becoming an issue of concern with regard to the spread and emergence of infectious animal diseases. Many important animal diseases cross-infect domestic animals and wildlife. These animals may interface in places such as fence lines, shared habitat and ranges, common water sources, and live animal markets (Bengis et al., 2002) (Fevre et al., 2006).

Many diseases are believed to be maintained in a region through infection of wildlife and domestic livestock. There has been a long-standing conflict between livestock owners and animal health authorities, as well as wildlife conservationists, regarding controlling diseases of livestock associated with wildlife. It is important to realize that where animals interface, transmission of

pathogens can be bidirectional: from wild to domestic animals, as well as domestic to wild animals (Bengis et al., 2002). This bidirectional transmission of pathogens allows a disease agent to maintain a cycle within a region, making it difficult to eradicate or control. The following malignant catarrhal fever viruses are most commonly associated with disease (Li et al., 2003a). The cycle of disease transfer which occurs in the spread of these viruses relies upon the interface between domestic and wildlife species.

Wildebeest-Associated Malignant Catarrhal Fever

Etiology

The virus known to cause malignant catarrhal fever in African bovids, alcelaphine herpesvirus-1, was first isolated from the leukocytes, spleen and lymph node suspensions of a blue wildebeest and described as being a herpesvirus in 1960 by Plowright and associates. Initially designated Bovid Herpesvirus 3 and believed to be similar to the betaherpesviruses (Roizman, 1973) (Plowright et al., 1960), it was later discovered to most closely resemble viruses associated with the gammaherpesvirus family (Rossiter et al., 1983b), (Mushi and Rurangirwa, 1981). Malignant catarrhal fever had been shown to occur in cattle after close association with apparently healthy blue or black wildebeests (Daubney, 1936; Mettam, 1923), but prior to the work by Plowright

and associates, the etiologic agent had not been identified or isolated. Having a method to obtain cell-free virus for use in experiments and development of diagnostics allowed for greater characterization of AlHV-1. In additional to this, the entire genome was sequenced in 1997 (Ensser et al., 1997).

Transmission of disease

First believed only to exist as a cell-associated virus (Rweyemamu et al., 1974) (Plowright et al., 1960), it was later discovered that it could survive as cell free virus and be passed in this form to cattle by nasal secretions of wildebeest (Mushi et al., 1981). It was also shown to be vertically transmitted to offspring transplacentally, when virus was isolated from the spleen of a fetus (Plowright et al., 1960). The main source of infection appears to be the wildebeest calf (Plowright, 1965), as virus has primarily been isolated from the ocular and nasal secretions of young wildebeest less than 3 months, and virtually all animals are infected by age 4 months (Barnard et al., 1989a; Mushi et al., 1980). Viral shedding in adults appears to be quite low, and occurs primarily during periods of stress or parturition (Barnard et al., 1989a; Rweyemamu et al., 1974). Originally, fetal membranes and fluids were also believed to be a major source of infection to cattle, but virus was not isolated

from either material (Rossiter et al., 1983a). It has been proposed that the membranes and fluids act as markers for pastureland which is heavily contaminated with malignant catarrhal fever virus from oculonasal secretions of wildebeest calves, therefore cattle should not be grazed at these locations (Rossiter et al., 1983a).

In Kenya and Tanzania, wildebeest associated MCF occurs primarily during calving season ((Mushi and Rurangirwa, 1981), but in South Africa disease occurs most often during the late winter and early spring, when calves are 8-10 months old (Barnard et al., 1989a). In zoological parks sporadic cases of wildebeest associated MCF can appear throughout the year (Castro et al., 1984; Hanichen et al., 1998).

AlHV-1 is not transmitted from one clinically susceptible host to another via natural methods, as the virus secreted from non-host animals is cell-associated and therefore extremely labile (Mushi and Rurangirwa, 1981; Plowright, 1968). As such, sick animals may be housed with healthy animals without fear of horizontal disease transmission (Plowright, 1965). In some instances it is possible for cattle to transmit the virus to their offspring transplacentally (Plowright et al., 1972).

Pathology

The length of incubation required to cause disease differs among species. Reports from studies of experimental exposure estimate incubation to last from 9 to 60 days or longer (Hatkin, 1980; Plowright, 1968; Plowright et al., 1960). In some instances, animals may recover from subclinical infection and the virus may recrudesce several months later (Heuschele et al., 1985). The disease caused by AlHV-1 is characterized by corneal opacity, erosions on the oral epithelium, salivation, anorexia, a mucopurulent nasal discharge, and increased body temperature and is typically referred to as the “head and eye” form of MCF (Pierson et al., 1979). Upon histologic examination, lympho-proliferation and generalized necrotizing vasculitis are the most commonly recognized signs (Metzler, 1991; Plowright, 1986).

Sheep-Associated Malignant Catarrhal Fever

Etiology

For almost a century, sheep were believed to be the source of non-wildebeest associated malignant catarrhal fever (Götze, 1930; Götze, 1929), but all attempts to isolate the causative agent were unsuccessful (Plowright, 1968; Selman et al., 1974). Attempts to understand the etiology and epidemiology of the agent causing this

disease have therefore been less direct than methods used with wildebeest strains. Lymphoblastoid cells have been propagated from cattle, deer and rabbits with sheep-associated malignant catarrhal fever (Reid et al., 1989; Reid et al., 1983; Schuller et al., 1990). One of these cell lines was used to construct a genomic library, and a clone from this library hybridized to cloned alcelaphine herpesvirus-1 DNA (Bridgen and Reid, 1991). From this it was suggested that the viral agent of sheep-associated malignant catarrhal fever and alcelaphine herpesvirus-1 are closely related gammaherpesviruses and the virus was named ovine herpesvirus-2 (OvHV-2) (Roizmann et al., 1992). Development of molecular techniques such as polymerase chain reaction has led to a greater understanding of OvHV-2. In 2007 the entire genome was published (Hart et al., 2007). Prior to that, inferences were made into how the virus reacted in a susceptible host based upon genes detected using molecular techniques (Coulter and Reid, 2002; Thonur et al., 2006).

Transmission of disease

Virtually all domestic sheep are believed to be infected with ovine herpesvirus-2 (Baxter et al., 1997; Li et al., 1995). Transmission of this virus occurs primarily from sheep after 3 months of age (Li et

al., 2001a). The majority of viral shedding comes from adolescent lambs, age 6 to 9 months. In general, the pattern of the appearance of viral DNA in nasal secretions occurred as a dramatic rise and subsequent fall within 24 to 36 hours. In adolescent sheep, this can occur multiple times between the ages of 6 and 9 months (Li et al., 2001a; Li et al., 2004). The frequency of viral shedding declines past the age of 9 months (Li et al., 2001a). Shedding episodes in adult sheep occur much less frequently than shedding in adolescent lambs (Li et al., 2001a; Li et al., 2004). Unlike wildebeest associated malignant catarrhal fever, shedding of the virus does not seem to be associated with lambing, and no seasonal trend in viral shedding of adult sheep has been identified (Barnard et al., 1994; Li et al., 2001a). This suggests that the likelihood of transmission from an adult sheep to a susceptible host occurs at a relatively stable, albeit infrequent, rate. Horizontal transfer between clinically ill cattle has not been shown in field observations and experimental data (Farquarson, 1946; Mare, 1977; Plowright, 1990).

Bali cattle, Asian swamp buffalo, the American bison, and deer species are reported to be more susceptible to disease caused by ovine herpesvirus-2 than *Bos taurus* and *Bos indicus* cattle species (Clark et al., 1970; Daniels et al., 1988; Hamilton, 1990; O'Toole et

al., 2002; Reid et al., 1987; Schultheiss et al., 2000). In general, cattle are regarded to be less susceptible to disease caused by OvHV-2 than AlHV-1 (Loken et al., 2009).

Pathology

In most cases, disease in cattle caused by OvHV-2 is virtually indistinguishable from the syndrome produced from infection with AlHV-1. Skin lesions occur more often in cattle and deer infected with the OvHV-2 strain than with AlHV-1 strain (Plowright, 1990). In deer, the syndrome appears to be peracute or acute, and animals succumb within 12 hours of onset of elevated temperature, mild diarrhea and inappetence without developing the characteristic signs of the disease (Reid, 1991), although some deer have developed acute haemorrhagic enteritis followed rapidly by death (Wilson et al., 1983). Bison appear to be one of the most highly susceptible animals to disease when infected with OvHV-2. Clinical onset of disease appears to be acute, with death coming within 1-3 days of onset (O'Toole et al., 2002), although chronic cases do occur (Schultheiss et al., 1998). Mortality in exposed bison herds is usually quite high (Schultheiss et al., 2000), although it has been shown definitively that bison do not spread virus to herdmates, as animals in an outbreak exposed prior to being added to an existing

herd of bison were the only animals exhibiting morbidity, even when 51% of animals succumbed to disease (n=825/1610) (Li et al., 2006). Goats can be infected with OvHV-2 (Li et al., 2001b), but disease associated with infection has not been reported.

Goat-Associated Malignant Catarrhal Fever

Etiology

In recent years, goats have been believed to be a source of malignant catarrhal fever virus where sheep and wildebeest were not present, but the agent of infection was believed to be OvHV-2 (Li et al., 1996; Wiyono et al., 1994). In 2001, a study was published by Li and coworkers in which a novel gammaherpesvirus in domestic goats was identified, and the two viruses reported appear to be the same (Li et al., 2001b). This virus was identified when OvHV-2-specific PCR failed to detect viral DNA in MCFV seropositive goats (antibody detected utilizing the CI-ELISA). Amplification product generated by degenerative primer PCR (Li et al., 2000) was analyzed and determined to be 71% identical to OvHV-2, 67% identical to AlHV-1, and 73% identical to MCFV-WTD. Based upon this information, the virus was characterized as a new member of the MCF group of viruses and designated caprine herpesvirus-2. At the same time as the Li study, Chmielewicz and

associates detected a herpesvirus in an apparently healthy pig, and the source of this virus was determined to be a goat housed with the animal. Upon analysis, it was observed that this virus also had high identity with OvHV-2 and AlHV-2, and was also characterized as a gammaherpesvirus and designated caprine herpesvirus-2 (Chmielewicz et al., 2001). The two viruses, which were characterized at the same time, appear to be the same.

Transmission of disease

The transmission pattern in goats of the virus appear to be similar to that of OvHV-2 in sheep, as kids separated from the herd at one week of age did not become infected with the virus, and adult goats were susceptible to CpHV-2 (Li et al., 2005).

Pathology

To date disease associated with infection of CpHV-2 has only been documented in cervid species, including white-tailed deer, sika deer, roe deer and moose (Chen et al., 2007; Crawford et al., 2002; Keel et al., 2003; Li et al., 2003b; Vikoren et al., 2006). Symptoms in susceptible animals are most commonly reported to be chronic weight loss, as well as mural folliculitis (mural pattern of inflammation of the hair follicle), dermatitis, and alopecia (Crawford et al., 2002). Upon histological examination the typical lesions

associated with the malignant catarrhal fever viruses are seen (lymphoproliferation and generalized necrotizing vasculitis) (Chen et al., 2007; Crawford et al., 2002).

Malignant Catarrhal Fever Affecting the White-Tailed Deer

Etiology

First described by Li and associates in 2000, malignant catarrhal fever virus white-tailed deer variant (MCFV-WTD) was the third virus attributed to the pathogenic group of gammaherpesviruses. It was detected when deer exhibiting clinical signs of the MCF syndrome as well as antibodies to a conserved epitope among MCF viruses did not test positive for either OvHV-2 or AlHV-1 by strain specific PCR. Degenerative primers specific to a conserved region of the DNA polymerase gene were then utilized, and amplification product occurred (Li et al., 2000). When this product was analyzed, it was found genetically to exhibit 82% identity to OvHV-2 and 71% identity to AlHV-1 (Kleiboeker et al., 2002; Li et al., 2000). All animals reported to have been infected with this virus were maintained in captive herds. The original reservoir host of this virus is unknown, although it is estimated to be a close relative of the sheep or goat (O'Toole and Li, 2008).

Transmission of disease

Based upon reports, cases of MCF associated with white-tailed deer occur in late fall or early winter, typically a time of high stress (Kleiboeker et al., 2002; Li et al., 2000). The mode of transmission of this virus is unknown, as the reservoir animal has not been identified (Kleiboeker et al., 2002; Li et al., 2000; O'Toole and Li, 2008).

Pathology

In affected deer, MCFV-WTD causes the classic symptoms of MCF syndrome described previously in cattle, with the exception of corneal opacity (Li et al., 2000).

Treatment, Control and Prevention of Disease

Currently there is no reliable method to treat MCF syndrome in affected animals. Occasionally supportive care with fluids and treatment with steroids and antibiotics has been effective in helping animals recover, but this does not occur consistently (Heuschele et al., 1985; Milne and Reid, 1990; Penny, 1998). Whether treatment is actually effective in helping animals' recovery is still not proven, as treatment has not been shown to increase recovery in animals. There are also many reports of animals recovering without treatment (Hamilton, 1990; Kalunda et al., 1981; O'Toole et al., 1997).

The best method for controlling the spread of disease in susceptible hosts such as bison, deer, Bali cattle, water buffalo, and to a lesser extent, European breeds of cattle, is to prevent exposure to known carrier animals: wildebeest, sheep and goats. It has been suggested that waiting until later in the day to graze cattle in wildebeest inhabited areas would greatly limit the exposure of cattle to AlHV-1, as virus is inactivated rapidly in sunlight (Rossiter et al., 1983a). This would allow wildebeest and cattle to share the best grazing land, and still limit the amount of disease in cattle. There is no reliable way to produce virus-free wildebeest calves, as some are infected transplacentally (Plowright et al., 1960), and virtually all animals in a herd are infected by 4 months of age (Barnard et al., 1989a; Mushi et al., 1980). A method to obtain virus-free sheep and goats has been shown (Cooley et al., 2008; Li et al., 2005; Li et al., 1998; Muller-Doblies et al., 2001). This consists of removing lambs from a positive flock by the age of 2.5 months. Kids were removed at 1 month and remained virus free. Derivation of virus free animals from MCFV positive females has important implications for disease control programs, especially in mixed species wildlife parks and zoological gardens.

Attempts to develop a vaccine to prevent infections with AlHV-1, have been made in the past without success (Plowright et al., 1975) . Currently the only method of prevention available is proper management of susceptible species.

Impact of Malignant Catarrhal Fever Viruses

There is a large variation in the impact that MCFV's have, depending on strain of virus and type of animal infected. Economic impact in Africa has been estimated to cause losses of up to 5-10% in domestic cattle herds (Barnard et al., 1989b; Bedelian, 2007; Plowright et al., 1975). In areas adjacent to wildebeest calving zones, Massai pastoralists believe MCF to be the most important disease with the largest impact on domestic cattle production, and in other areas where wildebeest were less prominent, it remained the fourth most common disease. Percent drop in sale price per animal infected with MCF in Africa was estimated at 50% in 2003-2004 (Bedelian, 2007). Several exotic species are also susceptible to AlHV-1, and losses in zoological gardens as well as in free-ranging African wildlife have been reported (Castro et al., 1984; Hamblin and Hedger, 1984; Hatkin, 1980).

Although economic losses related to sheep associated MCF have not been estimated, extremely high mortality rates have been reported in many herds of animals, especially deer and bison (Blood et al., 1961; Brown and Bloss, 1992; Clark et al., 1970; Hamilton, 1990; Li et al., 1999; Murray and Blood, 1961; O'Toole et al., 2002; Otter et al., 2002; Schultheiss et al., 2000; Tomkins et al., 1997). There have also been many reports of disease in free-ranging animals (Neimanis et al., 2009; Schultheiss et al., 2007; Vikoren et al., 2006). It is difficult to estimate the true losses of wildlife animals to sheep-associated MCF, as all cases are not recovered.

Losses in farmed and free-ranging cervids due to CpHV-2 have been reported and have been substantial in some cases (Chen et al., 2007; Vikoren et al., 2006). The symptoms of infection with CpHV-2 are not immediately detected (generally chronic weight loss and alopecia), thus the true impact of this disease may not be apparent, as it is most likely underreported.

To date, MCF associated with white-tailed deer has only been reported in captive white-tailed deer, and losses were varied in the two reports (Kleiboeker et al., 2002; Li et al., 2000). After discovery of this virus in captive white-tailed deer in Missouri, a further survey of several deer samples from both captive and wild white-tailed deer did not reveal MCFV-WTD infection and presence of the virus is not considered to be widespread in Missouri (Kleiboeker et al., 2002). A 2005 survey of samples from hunter harvested free-ranging white-tailed deer in east Tennessee revealed a rate of infection greater than 30%, with assumed subclinical infection (unpublished data). The impact of infection with MCFV-WTD in wild white-tailed deer is not yet understood.

Diagnostic Measures for MCF

Virus isolation

AlHV-1

Plowright and associates first isolated AlHV-1 in cell culture ((Plowright et al., 1960). He was later able to obtain cell-free virus of an isolate (WC11) after 49 calf kidney transfers and a further 5 or

10 passages as cell-free fluids in calf-thyroid cells (Plowright, 1968). After passage, it was still able to cause fatal MCF in cattle with a dose of 10^4 $TCID_{50}$. This isolate is still utilized today for diagnostics, and was essential in determining virus characteristics. Virus isolation is not very specific, as other viruses may grow in cell culture if the animal has a co-infection. Sensitivity can also be low, as sample type and proper management are crucial for maintaining live virus until it can be cultured (Mushi et al., 1980).

OvHV-2

Lymphoblastoid cells infected with OvHV-2 have been propagated from cattle, deer and rabbits with sheep-associated malignant catarrhal fever (Reid et al., 1989; Reid et al., 1983; Schuller et al., 1990), but cell-free virus has not yet been cultivated.

Virus Neutralization Assay

This assay was developed by Plowright in 1967, utilizing the WC11 virus isolate mentioned previously (Plowright, 1967). The virus neutralization assay in use today still employs the AlHV-1 virus as the target of neutralization, as cell free virus has never been isolated from other strains. This assay works best for antibody against the alcelaphine group of herpesviruses, and is used primarily in studying the range and extent of natural gammaherpesvirus infections in wildlife, zoological gardens and,

occasionally sheep populations. The virus neutralization assay is not used as a diagnostic test in clinically affected animals, as these animals are not able to produce virus neutralizing antibody (OIE, 2008). Animals with sheep-associated MCF do not produce virus neutralizing antibodies to AlHV-1 (Rossiter, 1983).

Serology

Indirect Immunoflorescence assay (IFA)

Although the IFA is not as specific as the virus neutralization assay, it is useful in detecting antibodies to several varieties of 'early' and 'late' antigens in AIHV-1-infected cell monolayers. These are antibodies that develop during the incubation period as well as during the clinical course of the disease. This test is not very specific, as other herpesviruses such as bovine herpesvirus-4 and infectious bovine rhinotracheitis virus cross-react (Rossiter et al., 1977). This assay can be utilized to detect sheep-associated MCF when this disease is suspected, but should be used in concert with another diagnostic method (OIE, 2008).

Enzyme-Linked Immunosorbent Assay (ELISA)

Many ELISA assays have been developed to detect antibody to MCFV's (Fraser et al., 2006; Frolich et al., 1998; Wan et al., 1988). The most commonly accepted method of detecting antibody to

MCFV infection is the competitive inhibition ELISA (OIE, 2008). Developed in 1994, this assay was designed to detect antibody to OvHV-2 using a MAb (15-A) targeting an epitope on a complex of glycoproteins that appears to be conserved among all MCF viruses. The antibody was raised against the wildebeest strain of MCF from a Minnesota isolate very similar to the WC11 strain (Hamdy, 1978; Li et al., 1994). Antibody to four MCFV's has been detected: AlHV-1,OvHV-2, CpHV-2 and MCFV-WTD, as well as one other very similar gammaherpesvirus: AlHV-2 (Li et al., 1994). Originally an indirect CI-ELIZA which utilized enzyme labeled anti-mouse immunoglobuins for antibody detection, this test was reformatted as a direct CI-ELISA in 2001 to increase sensitivity (Li et al., 2001c). The MAb 15A was conjugated directly with an enzyme label and a method was developed to precoat and store antigen-containing plates at 4degrees C for long periods without degradation. After modification, the sensitivity of this assay in cattle with clinical sheep-associated MCF approximates 95%, (80% in bison). The specificity was also increased, and is estimated to be 94% when utilized with cattle, deer, and bison. According to the OIE, the CI-ELISA has the advantage of being faster and more efficient than the IFA (OIE, 2008). In general the CI-ELISA method is frequently shown to be more sensitive than the IFA in detection of herpesviral

DNA (Nielsen and Vestergaard, 1996). Although this assay appears to be highly sensitive in most clinically affected animals, it has not been validated for use in detecting latent infection in non-diseased animals.

PCR

Nested Degenerative Herpesvirus PCR

First developed in 1996, this assay allowed for detection of many new gammaherpesviruses. Primary and secondary PCRs are performed with degenerate PCR primers targeted to a highly conserved region within the herpesviral DNA-directed DNA polymerase gene, using a nested format. This assay allows for the determination of partial herpesviral sequences for which no data have previously been reported. This is a sensitive (as little as 10 copies of DNA polymerase template per 100ng of DNA is detectable) and broadly applicable approach to the detection and identification of previously characterized herpesviruses present in human and animal tissues (VanDevanter et al., 1996). Adaptations of this PCR have been used to characterize new gammaherpesviruses in the MCF family (Kleiboeker et al., 2002; Li et al., 2000). The disadvantage of utilizing this assay for diagnostics is that it is quite time-consumptive, as well as

expensive to run. As the assay is not strain specific, product needs to be sequenced in order to correctly identify strains, which requires cloning and/or further processing. Also, several concentrations of DNA, as well as variations in the amount of enzymes and chemicals used must be evaluated in order to detect all virus positive samples, as the addition of reagents such as dimethyl sulfoxide (DMSO) has been shown to increase relative sensitivity in detection of some viruses, but decrease relative sensitivity of detection of others.

Strain specific PCR

Once a virus has been identified and genes have been characterized, strain specific assays have been developed for use in virus identification. These include traditional PCR assays (Baxter et al., 1993; Crawford et al., 1999; Li et al., 2001a; Li et al., 1995; Murphy et al., 1994; Wiyono et al., 1994), as well as real-time PCR assays (Cunha et al., 2009; Traul et al., 2007). The development of real-time strain specific assays for the detection of viral antigen in diseased animals has increased efficiency by increasing specificity, and has shortened the amount of time it takes to diagnose an animal with infection. Most recently a real-time PCR assay was developed to detect and differentiate malignant catarrhal fever

viruses in clinical samples (Cunha et al., 2009). In this assay, one pair of primers is utilized with fluorescently labeled probes specific for OvHV-2, CpHV-2, MCFV-WTD, MCFV-ibex, and AlHV-1 to identify these pathogenic MCFVs in clinical samples. All probes in this assay were able to detect as few as 50 copies of the specific viral DNA per reaction. Considering all five MCFV together, the multiplex real-time PCR assay has 97.2% sensitivity. When samples positive for other herpesviruses were analyzed, amplification did not occur, therefore this assay appears to be very specific for the viruses it was developed to detect. This assay should improve the length of time it takes to identify the strain of MCFV generating disease in an animal, but has not been validated for use in detecting latently infected animals.

A full description of the currently accepted diagnostic techniques for malignant catarrhal fever viruses can be found in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2008).

Malignant Catarrhal Fever viruses in Tennessee

Background information

The occurrence of malignant catarrhal fever in Tennessee ruminant populations has not been investigated and quantified, but has been

assumed to occur sporadically and to be most commonly associated with exposure to infected sheep. In 2005, a bison was presented to the University of Tennessee College of Veterinary Medicine necropsy service. Based upon gross and histological examination, it was believed the animal had succumbed to an infection of malignant catarrhal fever virus. Diagnostics were performed by National Veterinary Services Laboratory (NVSL) in Fort Collins, CO. Sheep-associated MCF was diagnosed by presence of OvHV-2 DNA. The animal had not been exposed directly to any sheep, and there were no sheep located within a 5 mile radius of the farm. Two additional cases of malignant catarrhal fever virus disease in local bison from intermingled livestock sources and 2 sheep-associated MCF affected cattle with no known exposure or proximity with sheep were then identified over the next six months.

The source of the infection in the bison and cattle was unknown, therefore it was hypothesized that white-tailed deer in the area may have transmitted the disease to the animals. Historically, deer have been suspected of being able to transmit OvHV-2 to cattle and bison (Imai et al., 2001), although this has yet to be proven. In the fall of 2005, an initial limited- survey of deer harvested in the region was undertaken. The cELISA was used to identify potentially infected deer, and DNA was extracted from the sodium heparinized blood of antibody positive samples. Based on the cELISA results there appeared to be widespread, moderate

rate (32 %: 30/92) of wild white-tailed deer in at least 12 counties in the eastern and middle areas of Tennessee that possessed antibody to a conserved epitope of the MCF family, but none of the animals were positive for OvHV-2 DNA (unpublished data). When additional tests were performed, it was determined that the antibody positive animals had been infected with the malignant catarrhal fever virus white-tailed deer variant (MCFV-WTD) (unpublished data).

Tennessee is second in the United States only to Texas in numbers of meat goats produced, with over 100,000 animals (National Agriculture Statistics Service, 2002). Due to the belief that CpHV-2 is endemic in domestic goats with infection rates similar to that of OvHV-2 in sheep (Li et al., 2005), as well as reports of serious disease in cervid species as a result of infection with this virus, (Crawford et al., 2002; Keel et al., 2003; Li et al., 2003b) we were prompted to investigate prevalence of CpHV-2 in goats and deer in the state of Tennessee.

Project Objectives

Based upon the prior experimental data, clinical information obtained by the necropsy and LACS services, and a review of the literature, we believe MCF viruses are present in an interface of ruminant species across the state of Tennessee. We have developed several objectives to investigate the prevalence of MCFV's in Tennessee ruminant populations.

Objective 1: Determine the prevalence of MCFV's in Tennessee deer populations

Objective 2: Determine the presence and prevalence of CpHV-2 in Tennessee goat herds

Objective 3: Determine if cattle have been infected with OvHV-2 and/or other MCFV's and if the prevalence of virus is higher in dead/debilitated animals than in healthy herd animals

Chapter 2. Malignant Catarrhal Fever virus-White Tailed Deer variant in Tennessee Wild and Domestic Cervid Populations

Abstract

For decades, malignant catarrhal fever viruses (MCFV's) have been reported to cause disease in cervids. Prevalence of MCFV's in Tennessee deer populations has not been investigated previously, so blood and/or lymph node samples were obtained from wild white-tailed deer harvested in the 2006, 2007, and 2008 Tennessee hunting seasons, as well from captive deer at local mixed species animal parks. Strain specific real-time polymerase chain reaction was performed to determine prevalence of infection with ovine herpesvirus-2, caprine herpesvirus-2, and malignant catarrhal fever virus white-tailed deer variant in individuals without apparent disease. Overall, prevalence of MCFV's in Tennessee cervids was less than 3%. MCFV's do not appear to be an issue of major concern for the health of Tennessee cervid populations, although these viruses are present in the deer population, and should be considered a health risk.

Introduction

Malignant catarrhal fever viruses have been reported to cause disease in several cervid species, and in farmed deer have been known to cause severe economic losses due to mortality (Brown and Bloss, 1992; Crawford et al., 2002; Heuschele et al., 1985; Imai et al., 2001; Keel et al., 2003; Kleiboeker et al., 2002; Klieforth

et al., 2002; Li et al., 2000; Li et al., 2003b; Reid, 1991; Reid et al., 1987; Reid et al., 1989; Schultheiss et al., 2007; Tomkins et al., 1997; Wilson et al., 1983). There have also been many reports of disease associated with OvHV-2 and CpHV-2 in free-ranging animals (Neimanis et al., 2009; Schultheiss et al., 2007; Vikoren et al., 2006). The population of free-ranging white-tailed deer in the state of Tennessee is estimated to exceed 900,000 animals. This species is considered to be the most important big game mammal of the eastern U.S., and hunting related expenditures associated with white-tailed deer hunting have an economic impact exceeding $650,000,000 annually in Tennessee (TWRA, 2010). Clinical signs of MCF vary depending on the strain of virus the animal is infected with. In deer, sheep-associated MCF appears to be peracute or acute, and animals are reported to succumb within 12 hours of onset of elevated temperature, mild diarrhea and inappetance, without developing the characteristic signs of the disease (Reid, 1991), although some deer have developed acute hemorrhagic enteritis followed rapidly by death (Wilson et al., 1983). It is difficult to estimate the true losses of wildlife to sheep-associated MCF, as few cases are recovered.

Losses in farmed and free-ranging cervids due to CpHV-2 have been reported and in some cases were substantial (Chen et al., 2007; Vikoren et al., 2006). The symptoms of infection with CpHV-2 are not easily detected (generally chronic weight loss and alopecia), thus the true impact of this disease may not be apparent, as it is most likely underreported. Tennessee is second in the United

States only to Texas in numbers of meat goats produced, with over 100,000 animals (National Agriculture Statistics Service, 2002). Due to the belief that CpHV-2 is endemic in domestic goats with infection rates similar to that of rates of OvHV-2 in sheep (Li et al., 2005), as well as reports of serious disease in cervid species as a result of infection with this virus (Crawford et al., 2002; Keel et al., 2003; Li et al., 2003b), we were prompted to investigate prevalence of infection with CpHV-2 in deer in the state of Tennessee.

First described by Li and associates in 2000, MCFV-WTD was the third virus attributed to the pathogenic group of gammaherpesviruses. The original reservoir host of this virus is unknown, although it is estimated to be a close relative of the sheep or goat (O'Toole and Li, 2008). Cases of MCF associated with white-tailed deer occur in late fall or early winter, typically a time of high stress (Kleiboeker et al., 2002; Li et al., 2000). MCFV-WTD causes most of the classic symptoms of MCF syndrome described previously in cattle, (i. e., serous ocular discharge, anorexia, depression, conjunctivitis, and periocular and nasal epithelial erosions, although not corneal opacity) in white-tailed deer (Li et al., 2000; O'Toole and Li, 2008).

To date, MCF associated with white-tailed deer has only been reported in white-tailed deer, and losses varied in the two reports (5 of 6 deer in one study, 1 deer each from 3 separate farms in another, where no other animals at the farms were affected)(Kleiboeker et al., 2002; Li et al., 2000). A 2005 preliminary survey of samples from wild white-tailed deer in east Tennessee revealed a rate of

infection with MCFV-WTD greater than 30%, with assumed subclinical infection (Robert Donnell, personal communication). The impact of infection with MCFV-WTD in wild white-tailed deer is not yet understood, and in Tennessee has not been extensively investigated. The objective of this study was to determine the prevalence of MCFV's in Tennessee cervid populations, primarily white-tailed deer. Although the most common method utilized to estimate prevalence of MCFV exposure in free-ranging wildlife has been the competitive inhibition enzyme linked immunosorbance assay (Frolich et al., 1998; Li et al., 1996; Zarnke et al., 2002), strain-specific real-time PCR was utilized in this study for detection of MCFV DNA. Real-time PCR was utilized because it can be developed to detect specific strains of MCFV, and is less time and resource consumptive than traditional PCR and degenerative herpesviral consensus primer PCR methods. Blood and lymph node samples were obtained from white-tailed deer harvested in the 2006-2008 hunting seasons, as well as from cervids in local mixed species animal parks.

Methods

Sample Collection

Samples were collected primarily at Tennessee Wildlife Resource Agency approved check-in stations during the 2006, 2007, and 2008 white-tailed deer hunting seasons (Table 2.1). The permitted harvest of antlerless deer (deer with no antlers, or those with antlers less than 3 inches in

length) varied per county (in some counties as little as 5 per season, in others as many as 3 per day). For bucks (any deer with at least one antler longer than 3 inches) the statewide limit allowed is 3 per season. All animals harvested must be >6 months of age. In addition to check-in stations, wild-game processors were utilized to collect samples from hunter-harvested deer, and sampling packets were distributed to hunters. Blood and/or lymph nodes were obtained from every animal where possible. The primary lymph node extracted was the inguinal lymph node, due to ease of access and decreased chance of meat contamination at this location. Pooled blood was removed from the body cavity of field dressed animals when available. Animals were considered to be in good health if they exhibited good body condition at the time of sampling.

A small number of blood samples were obtained in 2008 from cervid species at two local mixed species parks. Both parks contained several species of deer, goat and sheep, as well bison and exotic species (aoudad, zebra, ostrich, emu, etc). Where live animals were utilized to obtain samples, animals were handled in a manner approved by the University of Tennessee Institute of Animal Use and Care committee.

Locations of the sampled deer were categorized by Tennessee Wildlife Resource Agency Region (1-4) (Figure 2.1). All blood samples were stored in vacutainer tubes containing heparin at 4 degrees C until further processing. Lymph nodes were collected, placed in sterile plastic conical

tubes, and stored at -20 degrees C until further processing. When necessary, some lymph node samples were stored in 10% buffered neutral formalin until further processing, as these samples were obtained from either hunter sampling packets or areas a considerable distance from the university (primarily Region 1 samples), and length of transit would have led to degradation of the sample.

Table 2.1 Tennessee Wildlife Resource Agency Permanent Opening Dates for Deer Hunting

Samples were collected at TWRA checking stations in November and December, but samples were collected at wild-game processing facilities throughout the hunting seasons (2006-2008). Sample collection ended each year by December 24. Information obtained from the Tennessee Hunting and Trapping Guide (TWRA, 2009).

Hunting Type	Opening Date
Deer/Archery	Fourth Saturday in September
Deer/Muzzleloader	First Saturday in November
Deer/Gun	Saturday before Thanksgiving
Deer/Young Sportsman	Last Saturday in October

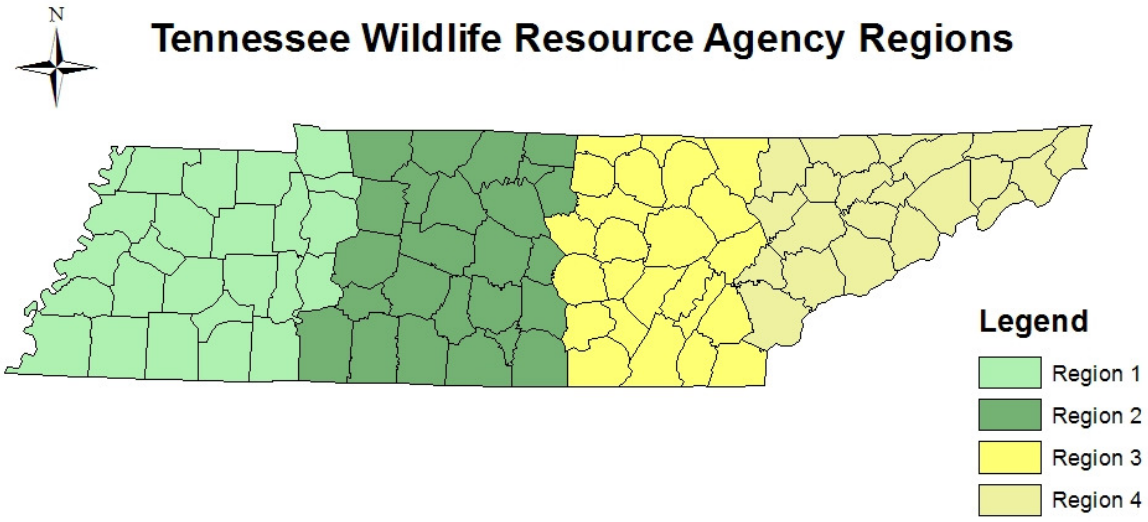

Figure 2.1 Tennessee Wildlife Resource Agency Region Map

Molecular analysis

Sample preparation

DNA was extracted from blood and/or tissue using the DNeasy blood and tissue kit according to the provided manufacture's protocol (Qiagen, Valencia, CA).

OvHV-2 and CpHV-2

Real-time PCR was performed according to the following protocol: per each reaction: 2 microliters extracted DNA, 10 microliters Taqman Universal PCR master Mix (Applied Biosystems, Foster City, CA) 0.4 microliters Rox Reference Dye II (Madison, WI), 0.9 microliters each of forward and reverse primer, 0.25 microliter

Probe, and 7.55 microliters DNase RNase free water. The reaction protocol is as follows: 50 degrees C for 30 seconds, 90 degrees C for 10 minutes, then 42 cycles of: 95 degrees C for 15 seconds, OvHV-2:61 degrees C for 1 minute /CpHV-2: 50 degrees C for 1 minute, and 72 degrees C for 30 seconds. Samples were considered positive if amplification occurred above the baseline prior to cycle 40.

OvHV-2 Primer and Probe set: F primer sequence: 5'- TGG TAG GAG CAG GCT ACC GT-3' R primer sequence: 5'-ATC ATG CTG ACC CCT TGC AG-3' Probe (P: 5'- 56-FAM/TCC ACG CCG TCC GCA TAA GA/3BHQ_1-3') (IDT, Coralville, PA),

CpHV-2 Primer and Probe set: F primer sequence: 5'- CAC TAC AAC ATC CTG TCC TT-3' R primer sequence: 5'- AGG GTA AAG AAT GCA TAC AG -3' Probe: 5'- 56-FAM/ AGA CGA AGA CAT AAT TAT CCA GAT ATC /3BHQ_1-3' (IDT, Coralville, PA).

OvHV-2 and CpHV-2 primer and probe sets were developed previously in the lab for use with another real-time PCR apparatus (personal communication). Temperatures were adjusted to accommodate the StepOne® unit from Applied Biosystems.

MCFV-WTD

To develop an assay mix to identify MCFV-WTD in cervids, the program File Builder v 3.1 was utilized (Applied Biosystems). The MCFV-WTD DNA polymerase gene (partial cds) sequence obtained in GenBank, accession number: AF387516 was imported, and a section not found in OvHV-2, AlHV-1 or CpHV-2 was designated for use within the probe. Areas within the sequence (> 12 base pairs) similar to human, mouse, bovine, and ovine DNA were eliminated from the segment. This segment was then submitted to Applied Biosystems for final primer and probe design. The StepOne® unit from Applied Biosystems was utilized for real-time PCR.

Real-time PCR was performed according to the following protocol: per each reaction: 2 microliters extracted DNA, 10 microliters Taqman Universal PCR master Mix (Applied Biosystems, Foster City, CA) 1 microliter of custom Taqman gene expression assay mix (Forward primer sequence: 5'- AGC AAA TAT GCC CAA CCC AGA TTA T-3'; Reverse primer sequence: 5'- GAG GCT AGC TTG TCG CTG AA-3'; Probe: 5'- 56-FAM/ AAT CGC CCC ACA CTA AC/3BHQ_1-3') (Applied Biosystems, Foster, CA), and 7 microliters DNase RNase free water. The reaction protocol is as follows: 50 degrees C for 2 minutes, 90 degrees C for 10 minutes, then 40

cycles of: 95 degrees C for 15 seconds, 60 degrees C for 1 minute. As this was a new assay, samples from a sheep infected with OvHV-2 and a deer infected with CpHV-2 (provided by Hong Li of the USDA-ARS) were utilized to confirm specificity. In addition, plasmid DNA containing a portion of the MCFV-WTD DNA polymerase gene obtained from Stephen Kleiboecher (formerly of the University of Missouri), as well as DNA from a deer that had died from MCFV-WTD (provided by Hong Li of the USDA-ARS) were utilized as positive controls. Finally, samples exhibiting amplification above the baseline prior to cycle 40 were rerun in duplicate and these products were sequenced at the University of Tennessee Molecular Biology Resource Facility to confirm presence of MCFV-WTD DNA.

Results

Sample Collection

Blood and lymph node samples were collected from check-in stations, farms, and processing facilities throughout the 4 TWRA regions. A list of assayed samples per region is given in Table 2.2.

MCFV-WTD Assay Development and Prevalence

The assay mix and protocol provided by Applied Biosystems worked well to detect MCFV-WTD DNA polymerase gene DNA. Both samples utilized as positive controls were detected by the assay. The relative sensitivity of the assay (based upon serial dilutions of a known copy number of the MCFV-WTD plasmid DNA positive control) showed that the probe was able to detect as few as 4 copies of viral DNA per reaction (data not shown). Also, the probe was highly specific, with no cross-reactivity detected with OvHV-2 or CpHV-2 positive samples.

Overall, prevalence of MCFV-WTD DNA was greater than any other MCFV in Tennessee cervids, at 2.9% (23/784), although this was much lower than preliminary data from the 2005 season (30 %). The highest prevalence (3.9%) was found in Region 4 (which was the region utilized for sample collection in the preliminary 2005 study. The lowest prevalence of MCFV-WTD (0.6%) was found in Region 1 (Table 2.3). All but one of the 23 positive samples were obtained from deer in the 2006 hunting season.

OvHV-2 Prevalence

Prevalence of OvHV-2 DNA in samples was quite low (0.1%), with only one deer testing positive of 781 sampled and tested. The only region with a positive sample was Region 3 (Table 2.3).

CpHV-2 Prevalence

There was no CpHV-2 DNA detected in any of 724 animals sampled and tested (Table 2.3).

Table 2.2 Samples Taken per TWRA Region and Assay Performed

Blood and/or lymph node samples were obtained from Tennessee cervids from 2006-2008, primarily during the Tennessee deer hunting seasons. Samples are from white-tailed unless otherwise indicated.

Region	Assay	Blood and Lymph Node	Blood Only	Lymph Node Only	Total Animals Tested
1	MCFV-WTD	69	30	56	155
	OvHV-2	87	18	68	173
	CpHV-2	67	38	62	167
2	MCFV-WTD	17	19	80	116
	OvHV-2	16	19	73	108
	CpHV-2	9	3	59	71
3	MCFV-WTD	38	48	93	179
	OvHV-2	40	42	96	178
	CpHV-2	30	48	90	168
4	MCFV-WTD	169	20*	145	334
	OvHV-2	162	12#	148	322
	CpHV-2	101	14*	203	318

* includes 4 sika deer, 2 Elk, and 1 Axis deer

includes 1 Axis deer

Table 2.3 Prevalence of MCFV per TWRA region

Strain Specific real-time polymerase chain reaction was performed on samples obtained primarily in the 2006, 2007, and 2008 Deer hunting seasons.

		MCFV DNA Present	
Region	**MCFV-WTD**	**OvHV-2**	**CpHv-2**
	Pos/Tested (%)	Pos/Tested (%)	Pos/Tested (%)
1	1/155 (0.6)	0/173	0/167
2	4/116 (3.5)	0/108	0/71
3	5/179 (2.8)	1/178 (0.6)	0/168
4	13/334 (3.9)	0/322	0/318
Total	23/784 (2.9)	1/781 (0.1)	0/724

Discussion

Data from this study strongly suggests that MCFV's are being maintained as subclinical infection in Tennessee cervid populations. This is believed to be the first report of MCFV-WTD and OvHV-2 infection in white-tailed deer without clinical disease, as all previous reports were of deer that had died of apparent MCF disease (Kleiboeker et al., 2002; Li et al., 2000; Vikoren et al., 2006). MCFV-WTD and OvHV-2 are believe to be highly virulent in deer, therefore reports of infection in deer not related to disease are valuable in understanding the nature of MCF viruses in deer. Although MCFV's have typically been characterized as highly virulent in cattle, these hosts have also been shown to be susceptible to OvHV-2 infection without succumbing to disease (Taus et al., 2006). Cattle are believed to be less susceptible to sheep-associated MCF than to wildebeest associated MCF (Loken et al., 2009). Similar to cattle, deer may

be less susceptible to disease caused by MCFV-WTD, as more deer were detected with infection with this MCFV than any other strain in this study.

The difference of prevalence reported in 2005 preliminary data (~30%) compared with this study could be related to several factors. Data from 2005 came primarily from animals harvested in Eastern Tennessee. During the hunting season of 2005, East Tennessee as well as the rest of the state experienced significant drought (Figure 2.2). Drought could have led to higher stress in deer populations, making them more susceptible to infection. Additionally, animals will have more contact with each other during drought as they congregate at common water and food sources, leading to increased transmission of pathogens.

Another factor which may have influenced the prevalence (or lack thereof) of MCFV-WTD infection in 2007 and 2008 hunting seasons may have been an outbreak of epizootic hemorrhagic disease (EHD) prior to and during the 2007 hunting season (Figure 2.3) (Hodge, 2007). Infection with an MCFV could have made deer less resistant to infection with EHD, leading to increased mortality of MCFV infected deer and therefore a lower number of infected deer at the times of the survey.

The presence of only one deer with OvHV-2 infection may be explained by reports that in deer this virus causes disease which is peracute or acute, and animals typically succumb to disease within 12 hours of infection.

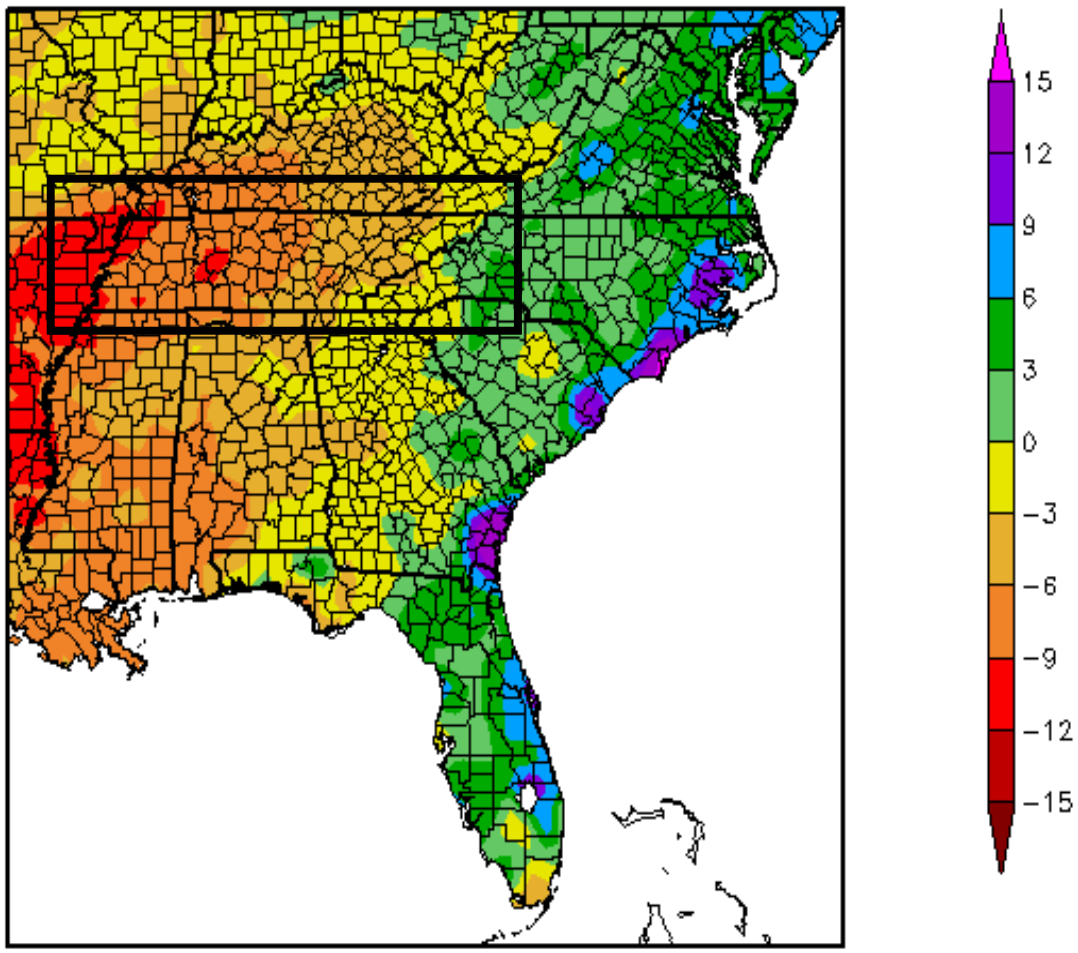

Figure 2.2 Departure From Normal Precipitation (inches) in East Tennessee During the 2005 Tennessee Deer Hunting Season (October 1, 2005 - December 31, 2005)

Tennessee is highlighted by a black box. This figure was adapted from the NOAA Southern Regional Climate Center website http://www.srcc.lsu.edu/maps/current/index.php?action=update_userdate&daterange=OND&year=05)

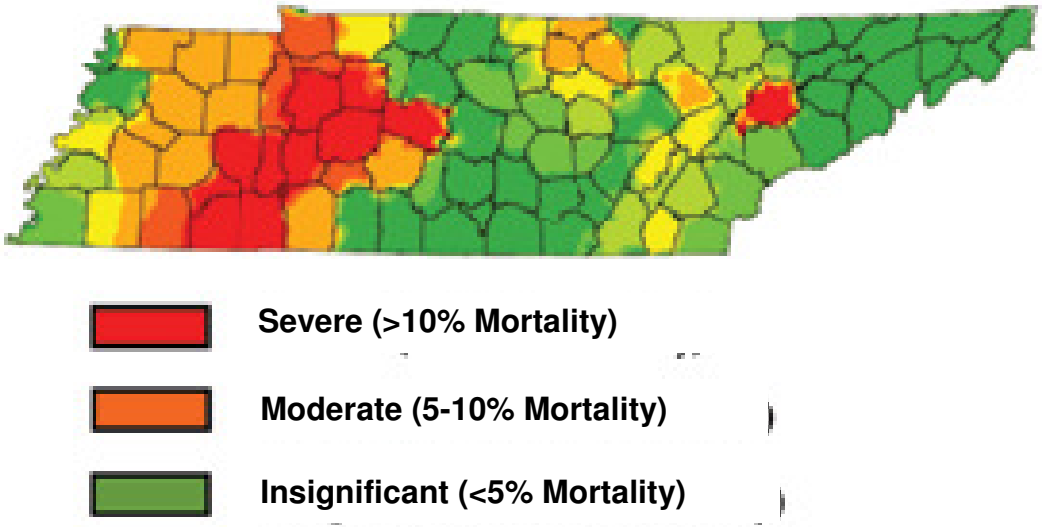

Figure 2.3 Severity of the 2007 EHD Outbreak in Tennessee Deer Populations

Figure taken from the 2009 Tennessee Hunting and Trapping Guide (TWRA, 2009)

The lack of CpHV-2 infection in Tennessee cervid populations, particularly in, the largest area of goat production in the state (region 2) was surprising, as CpHV-2 has been reported to cause chronic disease in cervids (Li et al., 2003b; Vikoren et al., 2006), and Tennessee is the second largest meat goat producing state in the U.S. In a recent study (unpublished), prevalence of CpHV-2 in Tennessee goat herds was much lower than that reported in other studies (7% compared to 73% of animals tested), which may explain the lack of CpHV-2 infection in Tennessee cervids.

Studies to determine susceptibility of deer to MCFV-WTD are impeded due to the lack of evidence supporting a specific carrier animal for MCFV-WTD. Without knowledge of a carrier animal, virus cannot be obtained to utilize in experimental infection studies, and naïve deer cannot be housed with a carrier animal to

investigate natural transmission. Although goats are the known reservoir of CpHV-2, studies have not been performed to determine susceptibility of deer to this virus, and a method of isolating virus from goats for utilization in experimental transmission has not been established.

Malignant catarrhal fever viruses are recognized to cause epizootics of high mortality in farmed deer (Brown and Bloss, 1992; Reid, 1991; Tomkins et al., 1997), but more information needs to be obtained to determine if infection in free-ranging cervids has as large an impact on the cervid population. With an estimated population of 900,000 free-ranging deer in the state of Tennessee, an MCFV-WTD prevalence of 3% indicates that an estimated 27,000 deer may be latently infected with this virus.

Chapter 3. Caprine-Herpesvirus-2 Prevalence and Shedding Patterns in Tennessee Goat Herds

Abstract

Virtually all domestic sheep are believed to become infected with OvHV-2. The carrier status of CpHV-2 in goats has been estimated to be similar to that of OvHV-2 in sheep. Research was needed to confirm that CpHV-2 infection in goats is similar. In the summer of 2008, 3-5 mls of whole blood were taken from goats at nine Middle and East Tennessee goat farms selected based upon convenience of location. Samples were analyzed for presence of caprine herpesvirus-2 DNA to estimate prevalence of this virus in Tennessee goat populations. To investigate infection patterns, goats from a local petting zoo were routinely sampled every 2-3 weeks over a period of 3 months. Of the nine farms sampled, 3 had animals which tested positive to CpHV-2 DNA, with prevalence ranging from 7 to 17 percent. Three of 15 petting zoo goats were shown to intermittently exhibit presence of viral DNA in blood samples obtained over the period of the study. It appears that in general, prevalence of CpHV-2 in goats is lower than OvHV-2 in sheep, but recrudescence of viral infection of CpHV-2 in goats and OvHV-2 in sheep is similar.

Introduction

Goats have been suggested to be a source of malignant catarrhal fever virus where sheep and wildebeest were not present, but the agent of infection was

believed to be OvHV-2 (Li et al., 1996; Wiyono et al., 1994). In 2001, a novel gammaherpesvirus in goats causing disease in cervid species was discovered and designated caprine herpesvirus-2 (CpHV-2) (Chmielewicz et al., 2001; Li et al., 2001b). Similar to ovine herpesvirus-2 (OvHV-2) in sheep and alcelaphine herpesvirus-1 (AlHV-1) in wildebeest, the goat appears to be the carrier animal and infection with CpHV-2 does not cause recognized disease in goats.

The infection and transmission pattern of this virus in goats has been estimated to be similar to that of OvHV-2 in sheep; virtually all animals are believed to be infected, and the young are not infected transplacentally, but are believed to be infected after 2 months of age. In a previous study, prevalence of CpHV-2 infection in goats sampled from multiple herds in several states was shown to be 73 percent by PCR (Li et al., 2001b). (In this report, prevalence of CpHV-2 DNA was reported in 84% of seropositive animals, the value of 73% was obtained by dividing the total number of positive animals (n=104) by the total number of animals sampled, regardless of antibody status (n=142).

Similar to OvHV-2 infection in sheep, when kids are separated from the herd at one week of age, they do not become infected with CpHV-2 virus (Li et al., 2005). Also, adult goats are susceptible to CpHV-2 when co-mingled with infected animals (Li et al., 2005). Goats have been shown to be co-infected with CpHV-2 as well as OvHV-2 (Li et al., 2001b; Li et al., 2005), but to date there have not been studies to show co-infection with these viruses in sheep.

Tennessee is the second highest producing state of meat goats in the United States (National Agriculture Statistics Service, 2002). Although these animals are raised for meat purposes, does can be kept for many years for breeding purposes, therefore if infection with CpHV-2 is occurring in goat herds, it is believed the virus will persist on the farm. To date there have been no surveys performed to determine CpHV-2 prevalence that have included goat herds in any of the southeastern states. Therefore this study was performed with the objective of investigating prevalence of CpHV-2 infection in Tennessee goat herds to determine if it is equivalent to that reported in goats in other states. In addition to this, a second objective was to investigate the pattern of latent infection and circulation of viral DNA in the goats' bloodstream, as little is known regarding infection patterns in goats. To determine the pattern of infection and latency of CpHV-2 in goats, animals in a local petting zoo were sampled over a period of 3 months to determine how often adult animals (>1 year of age) exhibited circulating viral DNA in the bloodstream when infected with CpHV-2. Presence of circulating viral DNA in the blood was used as an indication of the animal's ability to shed virus to other animals.

Methods

Animals and Sample Collection: Prevalence

Nine goat producing farms were selected based upon convenience of location (primarily East Tennessee, and one Middle TN farm) and owner

willingness to participate in research studies at the University of Tennessee. These farms included various breeds of domestic goats ranging in age, number and breed. In the summer of 2008, 3-5mls of whole blood was collected from each goat at the farms (with the exception of farm D, where a portion of animals (n=150) selected by the farm manager were sampled, as there were over 400 goats on the farm) under a protocol approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC). This blood was stored in vacutainer tubes containing sodium heparin (BD Medical, Franklin Lakes, NJ) at 4 degrees C until further processed.

Animals and Sample Collection: Infection Patterns

A group of animals from a local petting zoo including Nigerian Dwarf (n=15: 13 adults and 2 kids) and Oberhasli goats (n=2), as well as Tunis sheep (n=2) were sampled every 2-3 weeks for just over 3 months during the summer of 2008 (April 30-August 15). The kids were added to the sampling protocol as they were born. Whole blood, 3-5 mls., was collected from each animal under a protocol approved by the University of Tennessee IACUC. This blood was stored in vacutainer tubes containing sodium heparin (BD Medical, Franklin Lakes, NJ) at 4 degrees C until further processed.

Sample Processing and Molecular Analysis

DNA was extracted using the DNeasy blood and tissue kit according to the provided manufacture's protocol (Qiagen, Valencia, CA). Real-time PCR was performed according to the following protocol: per each reaction: 2 microliters extracted DNA, 10 microliters Taqman Universal PCR master Mix (Applied Biosystems, Foster City, CA) 0.4 microliters Rox Reference Dye II (Madison, WI), 0.9 microliters each of Forward and Reverse primer (F primer sequence: 5'- CAC TAC AAC ATC CTG TCC TT-3' R primer sequence: 5'- AGG GTA AAG AAT GCA TAC AG -3') 0.25 microliters Probe (P: 5'- 56-FAM/ AGA CGA AGA CAT AAT TAT CCA GAT ATC /3BHQ_1-3') (IDT, Coralville, IA), and 7.55 microliters DNase RNase free water. The reaction protocol is as follows: 50 degrees C for 30 seconds, 90 degrees C for 10 minutes, then 42 cycles of: 95 degrees C for 15 seconds, 50 degrees C for 1 minute, and 72 degrees C for 30 seconds. Samples were considered positive if amplification occurred above the baseline prior to cycle 40. This assay targeted a conserved region of the CpHV-2 DNA polymerase gene.

Statistical Analysis

A two-sample test of proportion was performed in STATA to compare previously reported prevalence to prevalence obtained in this study to

determine if there was a significant difference ($p<0.05$) between the prevalence reported previously, and that reported in this study.

Results

Prevalence

A total of 373 goats of various breed and age from nine Tennessee farms were sampled and tested by real-time PCR for presence of CpHV-2 DNA (Table 3.1). All ages were included, although the majority of animals sampled were believed to be >1 year (actual age of every animal sampled was not known). Of the animals with known age, a few were only a few weeks old, and others were as many as 12 years. Overall prevalence of infection was 7% (26/373) (CI: 4-10%). Of the nine farms, only farms C, F, and H had animals which tested positive for CpHV-2 DNA at the time of sampling. Prevalence at these farms was 6.7%, 16.7% and 15.1% respectively. Size of farm and breed of goat varied amongst the three farms. Prevalence of CpHV-2 infection in goats was significantly lower than the previously reported 73 percent ($P<0.0001$).

Infection and Recrudescence Patterns

Samples were taken from 13 adult goats and 2 adult sheep at the petting zoo up to 8 times over the period of the study. Three of the 13 adult goats were positive at different times over the period of the study, but were not

consistently positive every time sampled (Table 3.2). One of the animals that had tested positive gave birth to a kid within the study period. Both kids born during the study period were negative for CpHV-2 DNA within one week of birth, and remained negative through the end of the study (at the end of the study both kids were less than 2 months old). Neither sheep tested positive for CpHV-2 at any time.

Table 3.1 Prevalence of CpHV-2 in Domestic Goats from Middle and East Tennessee

Blood samples were taken in the summer of 2008 from goats at Tennessee farms and molecular analysis was performed to determine prevalence of CpHV-2 in Tennessee goat herds.

Farm Code	County	Breed(s)	Pos/Tested (%)	Date collected
A	Knox	Saanen and Oberhasli	0/16	6/10/2008
B	Union	Nigerian Dwarf	0/14	6/19/2008
C	Franklin	Boer X and Nubian X	10/150(6.7)	7/22/2008
D	Knox	Oberhasli and Lamancha	0/12	6/19/2008
E*	Knox	Fainting and Pygmy	0/28	6/11/2008 and 7/23/2008
F	Cocke	Saanen, Guernsey, and Nubian	8/48(16.7)	6/20/2008
G	Loudon	Pygmy	0/6	6/10/2008
H	Knox	Nubian X	8/53(15.1)	5/12/2008
I	Grainger	Nigerian Dwarf	0/46	7/25/2008
Total Collected			**26/373(7)**	

*This farm also contained sheep, several cervid species, aoudads, and other exotic ruminants

Table 3.2 Infection Patterns of CpHV-2 in a Petting Zoo

CpHV-2 specific real-time PCR amplification was performed on whole blood samples from animals in a petting zoo containing goats infected with CpHV-2.

Animal ID	4/30/08	5/14/08	5/28/08	6/11/08	6/25/08	7/9/08	7/23/08	8/15/08
1	-	-	-	+	-	-	+	ND
2	-	-	-	-	-	-	-	ND
3	-	-	-	-	-	-	-	ND
4	+	-	+	-	-	-	+	ND
5	-	-	-	-	-	-	-	ND
6	-	-	-	-	-	-	-	ND
7*1	N/A	N/A	N/A	N/A	N/A	-	-	-
8	-	-	-	-	-	-	-	ND
9	+	-	-	-	-	-	+	ND
10	-	-	-	-	-	-	-	ND
11*2	N/A	N/A	N/A	N/A	N/A	N/A	-	-
12	-	-	-	-	-	-	-	ND
13	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	ND
15	-	-	-	-	-	-	-	ND
16*3	-	-	-	-	-	-	-	ND
17*3	-	-	-	-	-	-	-	ND

*1 Number 4's kid
*2 Number 13's kid
*3 Tunis sheep

N/A: animal was not present at this time of the study
ND: Sample was not taken

Discussion

Goats have been considered a source of MCF for many years, as they have been known to be subclinically infected with OvHV-2, and hypothesized to be able to spread that virus to other animals (Wiyono et al., 1994). In the past decade, a novel gammaherpesvirus was discovered in goats and shown to cause disease in several cervid species (Chen et al., 2007; Crawford et al., 2002; Keel et al., 2003; Li et al., 2003b; Vikoren et al., 2006). Many similarities have been

drawn between CpHV-2 in goats and OvHV-2 in sheep. Sheep are believed to be ubiquitously infected with OvHV-2, and in a previous study, it appeared the same was true for CPHV-2 in goats sampled from several different geographical locations (Arizona, California, Florida, Minnesota, New York, Ohio, Oregon, Washington and Alberta, Canada) and several breeds (Li et al., 2001b). However, prevalence of CpHV-2 infection in goats included in the current study (7 %) was significantly lower than the previously reported 73 percent. It is unclear why there is such a dramatic divergence in prevalence between this study and previous reports. Sensitivity of the assay previously reported was reported to be 84%, but this number was obtained by dividing the number of PCR positive animals by the number of seropositive animals, therefore the actual sensitivity of this assay may be higher, as not all seropositive animals will have circulating viral DNA. Seroprevalence of animals tested in this study was not determined, therefore it was not possible to perform a sensitivity calculation similar to that in the 2001 study. Relative sensitivity of the assay utilized in this report (based upon serial dilutions of a known copy number of a reference plasmid DNA) showed that the probe was able to detect as few as 13 copies of CpHV-2 viral DNA per reaction (data not shown). Relative sensitivity of the assay utilized in the 2001 study was not reported. Perhaps the detection of CpHV-2 DNA would have been increased by utilizing peripheral blood lymphocytes instead of whole blood for DNA extraction, as MCFV's are believed to circulate in the lymphocytes of latently infected animals. Samples were frozen immediately

after they were obtained which prevented collection of these cells for extraction. It was not possible to perform DNA extraction immediately upon collection.

In this study, only herds with greater than 20 animals exhibited CpHV-2 infection. Stress has been shown to be instrumental in virus recrudescence. It is possible that herds with higher numbers of animals may be more stressed due to herd dynamics and space limitations. Animals tested at the petting zoo may have shown infection and recrudescence due to the stress of being located at the petting zoo, especially as traffic increases during the summer months, which was the study period. Pregnancy did not appear to strongly induce viral recrudescence, as only one of the two pregnant goats exhibited viral infection over the course of the study, but further studies need to be performed to confirm this. Neither kid born during the study had identifiable infection with CpHV-2, consistent with earlier reports that kids do not obtain virus transplacentally (Li et al., 2005). This is important information for virus control, as it is possible to produce CpHV-2 free animals with proper management of the animals after birth. Many mixed species parks co-mingle goats and susceptible cervid species (roe, sika, moose, white-tailed deer, etc), and it is very important to be able to obtain virus free goats to maintain good health in a mixed species environment. Further sampling of additional Tennessee goat herds, as well as additional sampling of animals in this study, may show higher prevalence of CpHV-2 overall, as adult goats were shown to intermittently have recrudescence of virus. However, it

seems unlikely that prevalence would approach that previously reported, as it was ten times lower in this study.

As goats have been shown to be co-infected with CpHV-2 and OvHV-2, another aspect of this study was to see if sheep were also able to be infected with both CpHV-2 and OvHV-2. The sheep as well as the goats at this petting zoo have been sampled and tested routinely over the past several years for presence of OvHV-2. Both sheep, as well as several of the adult goats, have shown previous OvHV-2 infection (data not shown, OvHV-2 DNA presence was not assessed at the time of this study). In this study, sheep did not exhibit infection with CpHV-2 at any time. It may be possible that due to infection with OvHV-2, sheep are refractory or immune to infection with CpHV-2. Further studies need to be performed with sheep not infected with OvHV-2 to see if they may be susceptible to CpHV-2.

Chapter 4. Malignant Catarrhal Fever Viruses in Cattle Populations: A Comparison of Healthy and Non-MCF Diseased Animals

Abstract

Malignant catarrhal fever is a lymphoproliferative disease that affects many ruminant species. Disease in North American cattle is most commonly associated with infection of OvHV-2, acquired by exposure to sheep. To date no disease in cattle has been associated with MCF-WTD or CpHV-2. We hypothesize that cattle may be infected with MCF viruses without succumbing to disease, and this infection may recrudesce when the animal becomes ill or debilitated due to complications other than MCF, contributing to the animal's morbidity. Blood samples from healthy or normal animals (n=156) were obtained from five healthy cattle herds and one slaughter facility, as well as animals (n=168) from 2 facilities which dispose of or screen dead and debilitated cattle to investigate the prevalence of MCFV infection. Real-time PCR amplification revealed MCF viral DNA was present in 31 percent of samples from dead or debilitated cattle, in contrast to 1 percent of samples from healthy animals.

Introduction

Malignant catarrhal fever is a disease syndrome associated with a high fatality rate in many ruminant species, most commonly cattle, deer and especially bison. This disease is caused by a group of several gammaherpesviruses within the

rhadinovirus subgroup. The strain of virus most commonly associated with disease in ruminants outside of the African continent is ovine herpesvirus-2, although both malignant catarrhal fever virus- white-tailed deer variant (MCFV-WTD) and caprine herpesvirus-2 have been reported to cause disease in cervids (Chen et al., 2007; Crawford et al., 2002; Keel et al., 2003; Kleiboeker et al., 2002; Li et al., 2000; Li et al., 2001b; Li et al., 2003b; Vikoren et al., 2006). Clinical signs vary depending on the species of animal infected, as well as the strain of virus causing disease (O'Toole and Li, 2008).

In cattle, clinical signs of disease associated with OvHV-2 infection most commonly include corneal opacity, persistent fever, enlarged lymph nodes, mucosal ulceration, mucopurulent nasal and ocular discharge, diarrhea and hematuria (Pierson et al., 1979). Sheep-associated MCF in cattle is most often fatal, although several cases have been reported where cattle have survived MCF following natural or experimental infection (Baxter et al., 1993; Hamilton, 1990; Milne and Reid, 1990; O'Toole et al., 1995; Otter et al., 2002). In other instances, OvHV-2 infection can occur in cattle without concurrent development of clinical MCF (Powers et al., 2005; Taus et al., 2006).

Two other rhadinoviruses within the MCF subgroup known to cause disease in white-tailed deer and other cervid species, MCFV-WTD and CpHV-2, have not yet been documented to cause disease in cattle.

Unlike alpha or beta herpes viruses, which seem to prefer lytic replication, gammaherpesviruses seem to favor the initial establishment of latency (Fields et

al., 2001). This could explain why many animals are able to become infected with an MCFV without developing clinical disease. According to a 2005 report, eight cattle from a dairy submitted for necropsy for reasons other than MCF had various diseases, but upon PCR analysis of tissue, 2 of the animals were positive for OvHV-2. This dairy had a history of sheep-associated MCF outbreaks, and was located adjacent to a sheep feedlot. Several animals at the dairy were positive for OvHV-2 without exhibiting any signs of clinical disease. It may be possible that cattle develop a latent infection with a MCFV, and upon immuno-suppression related to disease or injury, recrudescence of the virus occurs. The purpose of this study is to investigate if diseased or immunocompromised cattle exhibit a higher prevalence of infection with one or more of the gamma-herpesviruses within the MCF group than do cattle in good health status. This was done by collecting blood samples from healthy cattle on several farms, as well as from two facilities that process dead or debilitated cattle for diagnosis and/or disposal. Strain specific real-time PCR was performed to determine the prevalence of MCFV infection, and a comparison of the prevalence of MCFV's in the two groups was performed to determine if there was a statistically significant difference. This information may provide insight into the true nature of infection with MCFV's in cattle, as the prevalence of subclinical infection in cattle with OvHV-2 has not been extensively investigated, and infection of cattle with CpHV-2 and MCFV-WTD has not been reported.

Methods

Animals and Sample collection

Healthy animals

Six facilities were selected for this portion of the study based on accessibility and owner or manager's willingness to participate in a research study. Five of the facilities selected were UT Research and Education Centers (LD: dairy cattle, TN, FHB, AF and HR: beef cattle) with herds maintained by the University of Tennessee. The sixth facility was a private East Tennessee slaughter facility. Samples were obtained from May 2006 to December 2007. At the time of sampling, no animals with known MCF disease were identified within any of the herds. Blood samples (3-5 mls.) were obtained from each animal under a protocol approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC). This blood was stored in vacutainer tubes containing sodium heparin (BD Medical, Franklin Lakes, NJ) at 4 degrees C until further processed.

Dead/debilitated animals

Blood samples (3-5 mls.) from cattle submitted to the University of Tennessee Necropsy service (UTN) or to a regional (East Tennessee) contractor utilized by the USDA for bovine spongiform

encephalopathy surveillance were obtained for use in this portion of the study. Although lymph node, blood and brain samples were obtained from all animals in this category, blood samples were chosen for analysis to maintain consistency between the two sample groups. Information regarding cause of death was not obtained for the majority of samples taken. County of origin was recorded and this information was utilized in sample selection.

Sample Processing

DNA was extracted using the DNeasy blood and tissue kit according to the provided manufacture's protocol (Qiagen, Valencia, CA). DNA was stored at -20 degrees C for future use.

Molecular Analysis

Sample Selection

Due to the large number of samples obtained during the course of this study, systematic sampling methods were employed to select samples for molecular analysis. Samples were chosen based upon county location, and every third sample recorded for each county was selected.

MCFV-WTD

Real-time PCR was performed according to the following protocol: per each reaction: 2 microliters extracted DNA, 10 microliters Taqman Universal PCR master Mix (Applied Biosystems, Foster City, CA) 1 microliter of custom Taqman gene expression assay mix (Forward primer sequence: 5'- AGC AAA TAT GCC CAA CCC AGA TTA T-3'; Reverse primer sequence: 5'- GAG GCT AGC TTG TCG CTG AA-3'; Probe: 5'- 56-FAM/ AAT CGC CCC ACA CTA AC/3BHQ_1-3') (Applied Biosystems, Foster, CA), and 7 microliters DNase RNase free water. The reaction protocol is as follows: 50 degrees C for 2 minutes, 90 degrees C for 10 minutes, then 40 cycles of: 95 degrees C for 15 seconds, 60 degrees C for 1 minute. Samples were considered positive if amplification occurred above the baseline prior to cycle 40. The target of this assay was a conserved region of the MCFV-WTD DNA polymerase gene.

CpHV-2

Real-time PCR was performed according to the following protocol: per each reaction: 2 microliters extracted DNA, 10 microliters Taqman Universal PCR master Mix (Applied Biosystems, Foster City, CA) 0.4 microliters Rox Reference Dye II (Madison, WI), 0.9 microliters each of Forward and Reverse primer (F primer

sequence: 5’- CAC TAC AAC ATC CTG TCC TT-3’ R primer sequence: 5’- AGG GTA AAG AAT GCA TAC AG -3’) 0.25 microliters Probe (P: 5’- 56-FAM/ AGA CGA AGA CAT AAT TAT CCA GAT ATC /3BHQ_1-3’) (IDT, Coralville, PA), and 7.55 microliters DNase RNase free water. The reaction protocol is as follows: 50 degrees C for 30 seconds, 90 degrees C for 10 minutes, then 42 cycles of: 95 degrees C for 15 seconds, 50 degrees C for 1 minute, and 72 degrees C for 30 seconds. Samples were considered positive if amplification occurred above the baseline prior to cycle 40. The target of this assay was a conserved region of the CpHV-2 DNA polymerase gene.

OvHV-2

Real-time PCR was performed according to the following protocol: per each reaction: 2 microliters extracted DNA, 10 microliters Taqman Universal PCR master Mix (Applied Biosystems, Foster City, CA) 0.4 microliters Rox Reference Dye II (Madison, WI), 0.9 microliters each of Forward and Reverse primer (F primer sequence: 5’- TGG TAG GAG CAG GCT ACC GT-3’ R primer sequence: 5’-ATC ATG CTG ACC CCT TGC AG-3’) 0.25 microliters Probe (P: 5’- 56-FAM/TCC ACG CCG TCC GCA TAA GA/3BHQ_1-3’) (IDT, Coralville, PA), and 7.55 microliters DNase

RNase free water. The reaction protocol is as follows: 50 degrees C for 30 seconds, 90 degrees C for 10 minutes, then 42 cycles of: 95 degrees C for 15 seconds, 61 degrees C for 1 minute, and 72 degrees C for 30 seconds. Samples were considered positive if amplification occurred above the baseline prior to cycle 40. The target of this assay was a non-functional tegument protein.

Statistical Analysis

A two-sample test of proportion was performed in STATA to compare MCFV prevalence in healthy cattle samples to those from dead or debilitated animals to determine if there was a significant difference ($p<0.05$) between the two groups.

Results

A total of 156 samples from 5 counties: Marshall (n=93), Cumberland (n=33), Greene (n=21), Union (n=7), and Robertson (n=2), were chosen for molecular analysis from healthy animals, and 168 from 26 counties (Table 4.1) were selected from dead/down cattle. The majority of dead/debilitated cattle samples were obtained from 6 counties: Greene (n=50), Knox (n=22), Cocke (n=16), Sevier (n=14), Washington (n=14) and Jefferson (n=10). MCFV DNA was detected in 1 percent (CI: 0-3%) (n=2/156) of healthy cattle. In contrast MCFV DNA was detected in 31 percent (CI: 30-44%) (n=52/168) of samples from dead/debilitated cattle. Prevalence of MCFV DNA was significantly lower in

healthy cattle (1%) than in dead/debilitated cattle (31%) ($p<0.0001$). Thirteen percent (n=21/168) of dead/debilitated animals were positive for multiple MCFV's, and 2 (1%) of these were positive for all three strains. Individual percentages for each group and virus, as well as overall are listed in Table 4.2. The virus most prevalent in dead or debilitated animals was MCFV-WTD (27%), followed by OvHV-2 (15%), then CpHV-2 (3%). The only viral DNA detected in healthy animal samples was MCFV-WTD. No CpHV-2 or OvHV-2 DNA was detected in animals in good health.

Table 4.1 Location by County of Samples Obtained From Dead/Debilitated Animals

Every third sample obtained in each county was processed and utilized for detection of MCFV prevalence. Samples came from University of Tennessee necropsy services, as well as a local facility utilized by the USDA-APHIS for bovine spongiform encephalitis surveillance

County	Number of Samples Analyzed	County	Number of Samples Analyzed	County	Number of Samples Analyzed
Anderson	1	Greene	50	Monroe	2
Bledsoe	1	Hamblen	3	Roane	1
Blount	2	Hamilton	1	Scott	1
Carter	1	Hancock	1	Sevier	14
Claiborne	4	Hawkins	8	Sullivan	3
Cocke	16	Jefferson	10	Union	2
Cumberland	1	Knox	22	Washington	14
Fentress	1	Loudon	3	Williamson	1
Grainger	4	Meigs	1		
Total number of samples:					**168**

Table 4.2 Prevalence of MCFV-WTD, CpHV-2, and/or OvHV-2 DNA in Healthy and Dead/Debilitated Animals

Real-time PCR amplification was performed on samples from various facilities and percent infected animals was reported based on health status.

Sample site	**Health Status**	**MCFV-WTD** Pos/tested (%)	**CpHV-2** Pos/tested (%)	**OvHV-2** Pos/tested (%)
AF	Healthy	0/21	0/21	0/21
ANA	Healthy	0/7	0/7	0/7
FHB	Healthy	0/5	0/5	0/1
HR	Healthy	0/2	0/2	0/1
LD	Healthy	2/93 (2)	0/93	0/93
TN	Healthy	0/28	0/28	0/28
Total Healthy		**2/156(1)**	**0/156**	**0/151**
BSE*1	Dead/Debilitated	38/113 (34)	3/113(3)	22/113 (20)
UTN*2	Dead/Debilitated	7/55 (13)	2/55 (4)	3/55 (5)
Total Dead/Debilitated		**45/166 (27)**	**5/168 (3)**	**25/165(15)**
Total		**47/322 (15)**	**5/324 (1)**	**25/316 (8)**

*1 Disposal and screening facility, samples from multiple farms in 14 counties

*2 University of Tennessee Necropsy service, samples from multiple farms in 22 counties

Due to the surprisingly high prevalence of MCFV-WTD DNA detected in this study (27%), amplification product of a subset of samples (n=5) were sequenced by the University of Tennessee Biology Resource Facility to confirm that cross-reactivity was not occurring with any other bovine herpesvirus. All 5 assay positive samples (4 from dead or debilitated cattle, as well as one from the healthy cattle herd) exhibited 96-98% identity with the MCFV-WTD DNA sequence reported previously (Kleiboeker et al., 2002; Li et al., 2000).

Discussion

Infection of cattle with OvHV-2 has been shown to occur without development of sheep-associated MCF syndrome (Powers et al., 2005; Taus et al., 2006). In addition to infection without disease, recovery from sheep-associated MCF has been reported, and cattle that have recovered from infection with OvHV-2 have been demonstrated to remain positive by PCR for the virus for up to 24 months (O'Toole et al., 1997). In a previous study, in healthy cattle herds that had experienced an outbreak of sheep-associated MCF within the previous 5 years, OvHV-2 DNA was present in blood samples from 4 percent (n=15 /360) of cattle surveyed (Loken et al., 2009). Based upon this information, the 15 percent prevalence of OvHV-2 DNA (n=25/165) in dead/debilitated cattle could be explained by previous undiagnosed outbreaks in the herds from which these animals were submitted. Unfortunately parameters under which the study was set up did not allow identification/contact with the owners/herd managers of positive cases.

This is the first report of infection of cattle with MCFV-WTD. In this study, 27% prevalence of MCFV-WTD was recorded in dead/debilitated cattle. In a 2005 study performed in East Tennessee cervids, a similar prevalence (>30%) was recorded in presumed healthy harvested deer, followed by a decreased prevalence (3%) of MCFV-WTD in deer in 2006. It may be possible an outbreak of this virus occurred in ruminants in the region in 2005, without development of MCF symptoms. If cattle were infected with the virus, and did not become

diseased, they may have maintained a latent infection for several months following infection, similar to that reported with OvHV-2 infection, explaining infection at the time of sampling. Another explanation for the high prevalence of MCFV-WTD infection in dead/debilitated cattle is that viruses can recrudesce when animals are undergoing periods of high stress (infection with another pathogen, injury, overcrowding, heat stress, etc). If animals were infected in previous years with an MCFV, recrudescence may have occurred prior to death. This is also the first report of CpHV-2 infection in cattle. Prevalence of CpHV-2 infection in goat herds is lower in Tennessee (7%) than that reported in previous studies of CpHV-2 in goats (73%)(Li et al., 2001b), which may explain why the prevalence (3%, n=5/168) of this MCFV was not as high in the dead/debilitated cattle as that reported for other strains.

Further studies need to be performed to determine the impact MCFV-WTD and CpHV-2 may have on cattle populations and herd health. The carrier animal is unknown for MCFV-WTD, therefore it is not possible to attempt natural transmission of virus to cattle in a controlled setting. Natural transmission of CpHV-2 to cattle from an infected goat has not been attempted. Also, there is no known method of growing MCFV-WTD or CpHV-2 in vitro for use in experiment infection studies, therefore studies of these viruses and their effect on cattle populations are difficult to perform.

Chapter 5. Discussion and Conclusions

Multiple Malignant catarrhal fever viruses are present in several species of ruminants throughout the state of Tennessee. Occurrence of disease related to MCFV infection in Tennessee is presumed to be similar to that in Europe and other American states. Its incidence is sporadic and it is most commonly associated with exposure to infected sheep. Disease related to infection with MCFV-WTD and CpHV-2 has not been reported in Tennessee cattle or cervid populations.

This study demonstrated that healthy cattle and cervids can be infected with OvHV-2 and MCFV-WTD without apparent disease, and that dead or debilitated cattle were infected with CpHV-2. The previously unreported discovery of infection in cattle with CpHV-2 MCFV-WTD opens a new avenue of investigation into the pathology and virulence of MCFV's in domestic cattle. Perhaps infection of cattle with CpHV-2 or MCFV-WTD causes a previously unrecognized pattern of disease in this species. Based on the discovery of MCFV infection without concurrent signs of disease in cattle and deer, fatality rates related to infection with an MCFV may be much lower than previous estimates, especially in white-tailed deer (Brown and Bloss, 1992; Kleiboeker et al., 2002; Li et al., 2000; Otter et al., 2002). The prevalence of MCFV infection in dead or debilitated cattle was significantly higher than that in healthy cattle, which may provide some additional insight into the pathology of infection in cattle. The findings of co-infection of dead and debilitated cattle with multiple MCF viruses raises the possibility of long-term sub-clinical infection as it seems unlikely that the cattle

contracted the viruses simultaneously or from another single point source. It appears likely that MCFV's, like other herpes viruses, may recrudesce when cattle are in poor health. While it is tempting to speculate, additional information is needed to determine if infection with one or more of the MCFV viruses increases morbidity in cattle when experiencing co-infection with another unrelated pathogen.

The prevalence of CpHV-2 in goats is much lower in Tennessee than has been reported previously in other areas of the US (Li et al., 2001b). Perhaps the detection of CpHV-2 DNA would have been increased by utilizing peripheral blood lymphocytes instead of whole blood for DNA extraction, as MCFV's are believed to circulate in the lymphocytes of latently infected animals. Blood samples were frozen immediately after they were obtained which prevented collection of these cells for extraction. Due to time constraints and limited personnel, it was not possible to perform DNA extraction immediately upon collection.

Low prevalence of CpHV-2 in Tennessee goat populations likely explains why no evidence of infection was found in any of the cervids tested, and the low prevalence of CpHV-2 infection in dead or debilitated cattle compared to the rates of infection for OvHV-2 and MCFV-WTD. Additionally, cattle may be less susceptible to infection with CpHV-2 than other MCFV's.

While roughly equivalent percentages of the goat and deer populations were infected with CpHV-2 and MCFV-WTD respectively, and CpHV-2 infection was identified in a bovine, it is interesting that a much higher percentage of dead and debilitated cattle had evidence of MCFV-WTD than CpHV-2. Again, while it is tempting to speculate, more

detailed studies regarding the interface of cattle and deer versus cattle and goats are necessary to elucidate the reason for this divergence.

One of the underlying reasons for this study was to determine if deer were a source of OvHV-2 infection in cattle. OvHV-2 was identified in Tennessee deer but at a low prevalence. At the recognized level of infection within “healthy” animals it is difficult to ascribe a meaningful role to deer as a source of infection in cattle unless infected deer rapidly succumb and were not available for testing. Acquiring samples from diseased wild deer with known naïve cattle contact presents incredible difficult challenge.

No vaccines are available for prevention of MCFV infection. The primary method to control spread of disease is to prevent contact between carriers and clinically susceptible species. Studies have shown that it is possible to obtain OvHV-2 free lambs and CpHV-2 free kids if they are removed from infected herds in a timely matter (prior to 3 months for lambs, prior to nursing for kids) (Li et al., 2005; Li et al., 1998). There is no known method to control infection of MCFV-WTD, as the carrier animal of this virus is unknown. Information is needed regarding the pathology of this virus in free-ranging white-tailed deer as well as other ruminants to determine if MCFV-WTD can impact the health of ruminants other than captive white-tailed deer.

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Vita

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