THE EPIDEMIOLOGY OF

MALIGNANT CATARRHAL FEVER VIRUSES IN BISON

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ABSTRACT

A competitive inhibition enzyme linked immunosorbent assay was validated and used to estimate the prevalence of MCF-group virus infections in a sample of Alberta farmed bison. Prevalence among 995 slaughterhouse specimens was 21.8%. Among 953 samples from a serum bank the prevalence was 23.9%, and among 646 samples from five bison herds the prevalence was 23.4%. Test results from samples from one isolated bison herd collected over a period of six years provided evidence that an MCF-group virus was being transmitted across generations in the absence of exposure to any other ruminant sources of MCF-group viruses. Study of an outbreak of MCF in bison following a brief exposure to sheep provided very strong evidence that transmission of MCF among bison does not occur. Culturing lymphocytes from 12 healthy adult bison in the presence of the phorbol ester, 12-*O*-tetradecanoyl phorbol-13-acetate and testing of DNA extracted from these cultures with consensus herpesvirus PCR allowed the identification of viruses very similar to ovine herpesvirus two in five of the bison samples.

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LIST OF ABBREVIATIONS

A1HV-1	Alcelaphine herpesvirus-1
A1HV-2	Alcelaphine herpesvirus-2
ACD	Acid citrate dextrose
BHV-1	Bovine herpesvirus-1
BHV-4	Bovine herpesvirus-4
BIV	Bovine immunodeficiency virus
BLHV	Bovine lymphotrophic herpesvirus
BSV	Bovine syncitial virus
BVDV	Bovine virus diarrhea virus
cDNA	Complimentary DNA
CFIA	Canadian Food Inspection Agency
CI-ELISA	Competitive inhibition enzyme linked immunosorbent assay
CpHV-2	Caprine herpesvirus-2
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanidine triphosphate
dTTP	Deoxythymidine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EHV-2	Equine herpesvirus-2
ELISA	Enzyme linked immunosorbent assay

HHV-8	Human herpesvirus-8
HI/EJ	Hanging Ice/Edjericon
HiHV-1	Hippotragine herpesvrius-1
HLCH	Hook Lake Captive Herd
HVS	Herpesvirus samiri
IIF	Indirect immunoflourescent assay
Mab-15-A	Monoclonal antibody against antigenic epitope 15A
Max	Maximum
MBS	Mackenzie Bison Sanctuary
MCF	Malignant catarrhal fever
MCFV-WTD	Malignant catarrhal fever virus of white-tailed deer
MHV-68	Murine herpesvirus-68
Min	Minimum
N	Number
OD	Optical density
OR	Odds ratio
OvHV-2	Ovine herpesvirus-2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Percent inhibition
Prg	Rogan Gladden prevalence
r	Pearson's product moment correlation coefficient

ROC	Receiver operating characteristic
RRV	Rhesus monkey rhadinovirus
SA-MCF	Sheep associated malignant catarrhal fever
Se	Sensitivity
Sp	Specificity
Taq	Thermus aquaticus
TG-ROC	Two graph receiver operating characterisitc
TPA	12-*O*-tetradecanoyl phorbol-13-acetate
WBNP	Wood Buffalo National Park
WD-MCF	Wildebeest derived malignant catarrhal fever

1. INTRODUCTION

Malignant catarrhal fever (MCF) is a highly fatal disease syndrome of many ruminant species throughout the world. Five gamma herpesviruses of the rhadinovirus genus have been causally associated with MCF. These viruses, which include alcelaphine herpesvirus-1 (AlHV-1) (Plowright, Ferris *et al.* 1960), alcelaphine herpesvirus-2 (AlHV-2) (Klieforth, Maalouf *et al.* 2002), ovine herpesviruse-2 (OvHV-2) (Collins, Bruns *et al.* 2000) , caprine herpesvirus-2 (CpHV-2) (Li, Keller *et al.* 2001) and an as yet unnamed virus that causes MCF in white-tailed deer (MCFV-WTD) (Li, Dyer *et al.* 2000) have recently been placed into the tentatively named MCF group of gamma herpesviruses (Li, Keller *et al.* 2001)

Although MCF is a sporadic disease in most ruminant species, it is an important cause of mortality in farmed American bison (*Bison bison*). Reported mortalities in bison have ranged from sporadic to as high as 100% in small herds (Schultheiss, Collins *et al.* 2000). In one large herd of 900 bison 300 MCF mortalities were reported (Schultheiss, Collins *et al.* 2000). Mortalities have not been restricted to cow calf operations. One bison feedlot reported mortality rates as high as 9.4% per year (O'Toole, Li *et al.* 2002). In a survey of bison farmers in Canada, MCF was the most common cause of infectious mortality (Berezowski, Haigh *et al.* 2000). In a survey of submissions to veterinary diagnostic pathology laboratories in Western Canada and the Western United States during the period from 1990 to 2000, MCF was the most common infectious cause of submissions (Berezowski, Haigh *et al.* 2000).

In Canada the population of farmed bison has been estimated to be expanding at the rate of 26% per year since 1996 (Armstrong, Ayars *et al.* 1998). Although the Canadian bison industry is small compared to other livestock industries, as it expands, it's economic importance will also expand. Diseases such as MCF, which can cause significant mortalities in farmed bison populations have the potential to dramatically impact the bison industry in the future. It is therefore in the bison industry's best interest to institute research with the goal of understanding the epidemiology of MCF-group viruses in Canadian farmed bison populations. The purpose of this thesis is to begin that process.

In the broadest sense this thesis is concerned with answering the questions: how many healthy bison are infected with MCF-group viruses and are MCF-group viruses being maintained within healthy bison populations? These questions initially seems counter intuitive, since mortalities due to MCF in North American bison are generally considered to be the result of transmission of an MCF-group virus, ovine herpesvirus-2 (OvHV-2), from sheep to bison (Collins, Bruns *et al.* 2000). It seems unlikely that a virus which causes a highly fatal disease, often occurring in large outbreaks in bison would also be maintained within bison populations. A virus with these characteristics would be expected to leave few survivors. This however may not be the case for the relationship between bison and MCF-group viruses. Personal communications with other researchers has suggested that in a few very small preliminary studies, there were a significant proportion of healthy bison that had antibodies to MCF-group viruses (Crawford 2000; Li 2000; O'Toole 2000). An initial explanation for these findings might

be that bison which are exposed to OvHV-2 don't all die from the infection. Some infected bison may clear the virus from their body, and it is these bison that are being identified by these preliminary studies. OvHV-2, however is a herpesvirus. Herpesviruses, and especially those herpesviruses closely related to OvHV-2, tend to produce life long infections (Murphy, Gibbs *et al.* 1999; Timoney, Gillespie *et al.* 1999), making it unlikely that bison with MCF-group virus antibodies would have cleared their MCF-group virus infections. If the prevalence of MCF-group virus infections in farmed bison populations is high, are these infections all a direct result of the exposure of bison to sheep, or is it possible that MCF-group virus infections are being maintained in farmed bison populations by some form of bison to bison transmission?

Clearly the first step in studying MCF-group viruses in bison is to establish the proportion of farmed bison that are infected with MCF-group viruses. Before this can be done a test must be selected that correctly identifies those bison which are infected. In this thesis, this selection process was initiated by conducting an "armchair" evaluation of candidate tests through a critical review of the literature. The second chapter of this thesis contains a review of the literature relating to diagnostic tests. Chapter two also presents a review of the literature relating to the epidemiology of other MCF-group viruses in their host species populations, as well as a short review of the literature relating to MCF in bison. The test selected for this thesis project was a direct competitive inhibition immunosorbent enzyme linked antibody test (CI-ELISA). This test detects a monoclonal antibody specific for an antigenic epitope, 15-A, which is conserved among all known viruses in the MCF-group of viruses (Li, Mcguire *et al.*

2001). Since this test has been poorly validated in bison, the third chapter of this thesis presents the results of studies that evaluated the performance of this CI-ELISA in bison. The fourth chapter estimates the prevalence of MCF virus infections in bison from sero-surveys using the CI-ELISA in populations of farmed bison in Alberta. The fifth chapter lookes at the prevalence of CI-ELISA positive bison in isolated bison populations in Northern Canada, where there is no exposure to sheep. The sixth chapter presents results from a study demonstrating that MCF was not transmitted among bison in a naturally occurring outbreak of MCF. The seventh chapter presents the results of surveys of small populations of bison for the presence of MCF-group viruses using PCR assays.

2. LITERATURE REVIEW

2.1 Introduction.

The purpose of this review is to provide the reader with a broad understanding of malignant catarrhal fever and the epidemiology of MCF-group viruses in animal populations. Since this thesis is concerned with the epidemiology of MCF-group viruses, there will only be a minor discussion of the clinical signs and pathology associated with MCF, except with respect to bison. The bulk of this review will be concerned with the transmission and epidemiology of MCF-group viruses as well as the diagnostic tests used to diagnose infection with MCF-group viruses and clinical MCF.

2.2 General overview of malignant catarrhal fever.

Malignant catarrhal fever is a sporadic, highly fatal disease syndrome of many ungulate species. The syndrome has a world wide distribution and has been reported in 33 species of ungulates including cattle, bison, buffalo, swine and many deer species (Loken, Aleksandersen *et al.* 1998; Metzler 1991; Reid 1992). In susceptible species, MCF is characterized clinically by high fever, profuse nasal discharge, leukopenia, opthalmitis, corneal opacity, generalized lymphadenopathy, erosions of the upper respiratory tract and alimentary tract, and occasionally diarrhea and neurologic signs (Heuschele 1988; Metzler 1991; Mushi Rurangira 1981; Plowright 1990). Histologically the disease is characterized by hyperplasia of lymphoid organs, accumulation of

lymphocytes in many tissues, and generalized lymphocytic vasculitis (Liggitt, DeMartini 1980; Plowright 1990; Schultheiss, Collins *et al.* 2000).

There are two very similar, well known forms of MCF. They are differentiated not by clinical signs or pathology, but rather by epidemiological and etiological differences. The first is wildebeest derived malignant catarrhal fever (WD-MCF), so named because of the association between exposure to wildebeest (*Connochaetes* spp.) and the occurrence of MCF in cattle in Africa (Daubney, Hudson 1936). The second was named sheep-associated malignant catarrhal fever (SA-MCF) because of the association between MCF in cattle and their exposure to sheep (Goetze, Liess 1929; Goetze, Liess 1930). SA-MCF is considered to be the form of MCF that occurs most frequently throughout the world, excluding Africa (Plowright 1990).

A third, less well known form of MCF has recently been reported in white-tailed deer (*Odocoileus virginianus*) (Li, Dyer *et al.* 2000). This form of MCF is similar clinically and pathologically to the SA-MCF and WD-MCF (Li, Dyer *et al.* 2000). Other forms of MCF with varying clinical presentations and pathology have been reported in white-tailed deer (Li, Wunschmann *et al.* 2003), sika deer (*Cervus nippon)* (Crawford, Li *et al.* 2002; Keel, Patterson *et al.* 2003) and barbary red deer (*Cervus elaphus barbarus*) (Klieforth, Maalouf *et al.* 2002). At the present time little is known about these syndromes.

The association between blue wildebeest (*Connochaetes taurinus*) and WD-MCF in cattle was known to Maasai pastoralists of East Africa since early times (Mushi, Rurangira 1981; Plowright, Ferris *et al.* 1960). They named the disease "Ngati" or

"Ugonjwa wa nyumbu" which translates as "disease of wildebeest" in Kiswahili (Mushi, Rurangira 1981). The Maasai believed cattle contracted WD-MCF by grazing over ground contaminated by the after-birth of calving wildebeest or by grazing over ground on which wildebeest calves had lain and shed their neonatal hair coats (Daubney, Hudson 1936). These beliefs may have arisen from their observation that most WD-MCF mortalities occurred in cattle during, and for a few months following the wildebeest calving season (Mushi, Rurangira 1981). The Maasai also recognized that WD-MCF is a fatal disease and routinely slaughtered any cattle that developed clinical signs that were suggestive of WD-MCF early in the course of the disease (Mirangi, Kang'ee 1999). Timely slaughter allowed them to salvage the meat from affected cattle. Early European travelers in Africa encountered WD-MCF when they grazed trek oxen over ground that was frequented by wildebeest (Mushi, Rurangira 1981). They called the disease "snot sickness" or "snottsiekte" in Africaans because of the copious nasal discharge produced by oxen with WD-MCF (Mushi, Rurangira 1981).

Sheep associated malignant catarrhal fever has been recognized in Europe since the late 1800's (Goetze, Liess 1929; Goetze, Liess 1930). The association between sheep and SA-MCF in cattle was suggested by early epidemiological studies in Germany, where it was found that a high proportion of cattle that died of MCF were pastured or stabled closely to sheep (Goetze, Liess 1929; Goetze, Liess 1930).

WD-MCF and SA-MCF were initially considered to be two separate, distinct diseases (Daubney, Hudson 1936; Goetze, Liess 1929; Goetze, Liess 1930). In 1930, Goetze suggested that since the African (WD-MCF) and the European (SA-MCF) forms

of the disease were very similar, they should be classified as one disease caused by an infectious agent that was present in both wildebeest and sheep (Goetze, Liess 1929; Goetze, Liess 1930). Although it has since been accepted that the two diseases are one syndrome, considerable debate has remained about the etiology of MCF. In 1936, Daubney suggested that the infectious cause of MCF was a virus (Daubney, Hudson 1936). It was not until the 1960's when Plowright cultured the virus that causes WD-MCF (Plowright, Ferris *et al.* 1960) and the advent of DNA base sequencing in the 1990's, that it was determined that the causative agents of SA-MCF and WD-MCF are closely related but separate viruses (Bridgen, Herring *et al.* 1989; Bridgen, Reid 1991; Coulter, Wright *et al.* 2001; Ensser, Pfanz *et al.* 1997).

2.3 Etiologies of MCF

There are five gamma herpesvirus belonging to the genus rhadinovirus that have been causally associated with MCF. These viruses are alcelaphine herpesvirus-1 (AlHV-1) (Plowright, Ferris *et al.* 1960), alcelaphine herpesvirus-2 (AlHV-2) (Klieforth, Maalouf *et al.* 2002), ovine herpesviruse-2 (OvHV-2) (Collins, Bruns *et al.* 2000; Li, Shen *et al.* 1996b; Li, Shen *et al.* 1995) (Muller Doblies, Li *et al.* 1998), caprine herpesvirus-2 (CpHV-2) (Crawford, Li *et al.* 2002; Keel, Patterson *et al.* 2003; Li, Keller *et al.* 2001; Li, Wunschmann *et al.* 2003) and an as yet unnamed virus which causes MCF in white-tailed deer (MCFV-WTD) (Li, Dyer *et al.* 2000). Some other members of the rhadinovirus genus include herpesvirus samiri (HVS), human herpesvirus-8 (HHV-8), murine herpesvirus-68 (MHV-68), rhesus monkey rhadinovirus (RRV), equine

herpesvirus-2 (EHV-2), bovine herpesvirus-4 (BHV-4) and hippotragine herpesvirus-1 (HiHV-1) (Coulter, Wright *et al.* 2001; Li, Keller *et al.* 2001; Mushi, Rossiter *et al.* 1981; Reid Bridgen 1991). Pathogenic members of this group are characterized by their ability to cause lymphoproliferative diseases, establish latent infections in lymphoid tissue and to replicate in vitro in lymphoblastoid cells (Coulter, Wright *et al.* 2001). Many of these viruses are well adapted to their natural host species populations, and as is the case with A1HV-1, A1HV-2, CpHV-2 and OvHV-2, only produce disease when they infect hosts which belong to less well adapted species (Imai, Nishimori *et al.* 2001; Mushi Rurangira 1981; Plowright, Ferris *et al.* 1960, Klieforth, Maalouf *et al.* 2002, Crawford, Li *et al.* 2002).

Viruses in the genus are classified as rhadinoviruses by their shared properties as well as similarities in their genomic structure (Coulter, Wright *et al.* 2001). OvHV-2, CpHV-2 and MCFV-WTD, however, have been classified as rhadinoviruses based only on their DNA base sequence homology to other viruses in this group, since none of these three viruses have been isolated or cultured in tissue culture (Bridgen Reid 1991; Li, Dyer *et al.* 2000; Li, Keller *et al.* 2001). Li has proposed, based on their genomic similarity, that A1HV-1, A1HV-2, OvHV-2, CpHV-2, HiHV-1 and MCFV-WTD be classified as a separate subgroup of gamma herpesviruses called the MCF subgroup (Li, Keller *et al.* 2001).

Alcelaphine herpesvirus-1, which sub-clinically infects wildebeest, causes WD-MCF in cattle in Africa (Plowright, Ferris *et al.* 1960) and susceptible ungulate species in zoos and wildlife parks worldwide (Heuschele, Swansen *et al.* 1983; Whitenack,

Castro 1981). The causal link between A1HV-1 and WD-MCF has been firmly established through epidemiological studies and transmission experiments. A1HV-1 has been isolated and characterized from bovine cell cultures (Castro, Heuschele *et al.* 1985; Plowright, Macadam *et al.* 1963), and recently the complete genomic sequence of A1HV-1 has been reported (Ensser, Pfanz *et al.* 1997). Plowright and others were able to transmit WD-MCF to healthy cattle by parenteral injection of cellular suspensions from A1HV-1 infected bovine cell cultures and injection of cell free A1HV-1 from cell culture supernatants (Kalunda, Dardiri *et al.* 1981; Plowright 1963; Plowright, Ferris *et al.* 1960). These studies firmly established that, at least in the laboratory, A1HV-1 alone is sufficient to cause WD-MCF. Studies of naturally occurring WD-MCF mortalities in which A1HV-1 has been found by isolation of A1HV-1 in tissue culture and identification of A1HV-1 DNA by PCR, suggest that A1HV-1 is also a necessary cause of naturally occurring WD-MCF (Barnard, Van De Pypekamp *et al.* 1989; Mirangi, Kang'ee 1999; Murphy, Klieforth *et al.* 1994; Plowright 1963; Plowright, Ferris *et al.* 1960).

Several different viruses have been isolated from SA-MCF mortalities in cattle. These include a bovine syncytial virus, a morbillivirus, a parvovirus, a toga virus, an enterovirus, and several herpesviruses (Reid, Buxton *et al.* 1984). Even though these viruses have been isolated from SA-MCF mortalities, some of these viruses have failed to experimentally reproduce MCF, and all are considered to have no causal relationship to the disease (Reid, Buxton *et al.* 1984). There have been several viruses, including a reovirus, an adenovirus and some unclassified viruses, which have been isolated from

sheep incriminated in outbreaks of SA-MCF (Hoffman, Young 1989). Researchers have not experimentally produced MCF with any of them, and they are not considered to be causally associated with SA-MCF (Hoffman, Young 1989). OvHV-2 has remained the only candidate for the causal agent of SA-MCF (Bridgen, Reid 1991; Collins, Bruns *et al.* 2000; Crawford, Li *et al.* 1999; Ensser, Pfanz *et al.* 1997; Li, Shen *et al.* 1995; Muller Doblies, Li *et al.* 1998; O'Toole, Li *et al.* 1997; Plowright 1990; Schuller, Cerny *et al.* 1990; Schultheiss, Collins *et al.* 2000). Since OvHV-2 has resisted culture in tissue culture, cell free OvHV-2 has been unavailable, making transmission experiments using infectious virus particles impossible. The association between OvHV-2 and SA-MCF has therefore been established by less direct methods.

Support for a causal association between sheep and SA-MCF initially came from epidemiological reports of mortalities in cattle that were exposed to sheep (Aleska. 1935; Bindel 1937; Goetze, Liess 1929; Goetze, Liess 1930; Magnusson 1940; Piercy 1954). In many of these studies, sheep were penned closely with healthy cattle and deer over various periods of time and in some cases sheep exposed cattle and deer developed MCF (Goetze, Liess 1930; Magnusson 1940; McAllum, Mavor *et al.* 1982). Although these studies provided the first indication that sheep may be a risk factor for MCF in cattle and deer, they must be considered with some caution since transmission was not consistent and the researchers failed to include unexposed control cattle or deer in their studies. In a recent study, Japanese deer (*Cervus nippon)* that died of MCF after exposure to sheep were found to have OvHV-2 DNA fragments that were identical to those found in the sheep they were exposed to (Imai, Nishimori *et al.* 2001). Although this study does

provide more compelling evidence that sheep and OvHV-2 are causally linked to MCF, these authors failed to use unexposed controls in their study. By failing to use unexposed controls in the study, the authors failed to demonstrate that Japanese deer which don't have SA-MCF did not have OvHV-2 DNA in their tissues.

The strongest support for a causal link between sheep, OvHV-2 and MCF comes from PCR studies in which OvHV-2 DNA is found in a large proportion of healthy sheep worldwide (Baxter, Pow *et al.* 1993; Li, Shen *et al.* 1994; Li, Shen *et al.* 1996; Li, Shen *et al.* 1995; Li, Snowder *et al.* 1998), as well as in cattle and bison that died of MCF, but not, or rarely in cattle or bison that died of other causes (Baxter, Pow *et al.* 1993; Collins, Bruns *et al.* 2000; Crawford, Li *et al.* 1999; Dunowska, Letchworth *et al.* 2001; Hussy, Stauber *et al.* 2001; Li, Shen *et al.* 1995; Muller Doblies, Li *et al.* 1998; Tham 1997; Wiyono, Baxter *et al.* 1994). Although the authors did not do so, the data presented in 4 of these studies can be used to calculate odds ratios for the strength of the association between OvHV-2 and mortalities due to MCF. The odds ratios calculated from these 4 studies (table 2.1) are very large, ranging from 110 to 3984, which indicates that the association between OvHV-2 and MCF in these studies, is very strong. The number of subjects in each study however is quite small, ranging from 19 to 140. This has resulted in very broad 95% confidence intervals around each odds ratio estimate.

Table 2.1 References, odds ratios, 95% confidence interval of odds ratios, number of study subjects and *p* values for studies associating OvHV-2 and SA-MCF in cattle and bison.

Reference[1]	**Odds Ratio**	**Confidence Interval**	**Number In Study**	***P* Value**
1	110	10.2-1187.0	19	<.0005
2	357	82.3-1548.0	77	<.0005
3	718	213.3-2427.0	140	<.0005
4	3984	923.9-17180.0	133	<.0005

[1] 1.Li, Shen *et al* 1995, 2.Crawford, Li *et al* 1999, 3.Muller Doblies, Li *et al* 1998, 4.Colins, Bruns *et al* 2000

None of the 95% confidence intervals encompasses one, nor do any of the *p* values approach or exceed 0.05. This suggests that although the sample sizes were small, the association between OvHV-2 and MCF was statistically significant in these studies.

Calculating odds ratios from the data presented in these studies assumes that the authors of the original studies used case-control study designs for determining the association between OvHV-2 and MCF. Although all of the authors were interested in this association, none of them discussed the use of case-control study designs, nor did they address the potential sources of bias which case-control studies are prone. Since these authors did not design their studies to reduce potential sources of bias, using their data for inferences for which their data was not originally intended may result in biased inferences. The odds ratios calculated from these studies are, however, quite large and would require equally large flaws in study design to have produced spurious odds ratios of the magnitude seen here. It is unlikely that such flaws would have existed in all four of these studies.

Defining the relationship between OvHV-2 and SA-MCF has been very difficult. Since there have been no transmission experiments performed with cell free OvHV-2 virus particles, it's relationship to SA-MCF must only be considered to be an association. Although the association is a strong one, it cannot be defined as a causal association. At best it can be stated that OvHV-2 is necessary for the production of SA-MCF, but lacking transmission experiments with cell free OvHV-2, it must also be stated that OvHV-2 alone may not be sufficient for the production of SA-MCF.

In 2000, Li (Li, Dyer *et al.* 2000) described an outbreak of MCF that caused 5

white-tailed deer (*Odocoileus virginianus*) mortalities in a zoo in the United States. The deer exhibited clinical signs that were similar to those observed in deer with SA-MCF (Li, Dyer *et al.* 2000; Li, Westover *et al.* 1999; Reid 1992), as well as gross pathology and histopathology that was similar to that seen in cattle and deer with SA-MCF or WD-MCF (Li, Dyer *et al.* 2000). Li was unable to detect the presence of A1HV-1 or OvHV-2 DNA fragments in tissues from any of the dead deer. Using consensus primers directed at a region of the herpesviral DNA polymerase gene, Li was able to amplify a 230 base pair DNA fragment from peripheral blood lymphocytes of the affected deer. This fragment was sequenced and found to be 82% identical to OvHV-2 and 72% identical to A1HV-1. Based on these observations Li concluded that this virus was a newly discovered pathogenic MCF virus that was similar to, but distinct from A1HV-1 and OvHV-2. Since a primary host for this new virus was not identified, the virus has been given the temporary name of MCF virus of white-tailed deer (MCFV-WTD). The causal association between MCFV-WTD and MCF is tenuous at this time. Li suggested that MCFV-WTD had a causal association with MCF in white-tailed deer, based on detecting this new virus in white-tailed deer that died of a disease similar to MCF, in the absence of other known pathogenic MCF viruses. In addition, the new virus had genetic similarities to other pathogenic MCF viruses. In order to strengthen this association Li looked for, but was unable to find MCFV-WTD, in the peripheral blood leukocytes of three healthy white-tailed deer. Since a sample of three could not possibly be representative of the general population of white-tailed deer, firm acceptance of a causal link between MCFV-WTD and MCF in white-tailed deer will await the results of further

studies that look for the presence of this virus in healthy white-tailed deer populations and MCF mortalities.

Alcelaphine herpesvirus-2 which sub-clinically infects Jackson's hartebeest (*Alcelaphus buselaphus jacksoni*) (Klieforth, Maalouf *et al.* 2002) and CpHV-2 which sub-clinically infects domestic goats (Li, Keller *et al.* 2001) have recently been associated with MCF like syndromes in sika deer (Crawford, Li *et al.* 2002; Keel, Patterson *et al.* 2003), white-tailed deer (Li, Wunschmann *et al.* 2003) and barbary red deer (Klieforth, Maalouf *et al.* 2002). Causal associations between these two viruses and MCF should be considered tentative at this time since there are few studies reported and none of them have critically examined causal associations.

2.4 Transmission of malignant catarrhal fever viruses

2.4.1 Transmission of A1HV-1 among cattle

Early MCF transmission studies demonstrated that WD-MCF could be transmitted horizontally through transfusion of whole blood, or by parenteral injection of tissue homogenates from cattle with WD-MCF to other susceptible cattle (Daubney, Hudson 1936; Piercy 1952; Piercy 1952; Plowright 1963; Plowright, Ferris *et al.* 1960; Rinjard 1935). Although these early studies clearly demonstrated that WD-MCF was experimentally transmissible between cattle, these studies failed to demonstrate the transmission of WD-MCF horizontally among cattle by any but iatrogenic means. In these studies many cattle with experimentally induced WD-MCF were stabled closely to susceptible cattle. In all instances, WD-MCF failed to be transmitted from cattle with

WD-MCF to susceptible cattle. In addition, cattle which were stabled next to cattle with WD-MCF, failed to produce antibodies to A1HV-1 (Kalunda, Dardiri *et al.* 1981). From these studies it has been concluded that cattle with WD-MCF do not transmit this disease, or A1HV-1 to other cattle (Plowright 1990).

A1HV-1 has consistently been found in leukocyte fractions from cattle with WD-MCF (Plowright 1963; Plowright, Ferris *et al.* 1960). Transmission studies, in which leukocytes from peripheral blood buffy coats were successful at transmitting WD-MCF, failed to demonstrate transmission through parenteral injections of serum or cell free supernatants of tissue suspensions (Piercy 1953; Plowright 1982). This suggests that cattle with WD-MCF produce A1HV-1 only in a leukocyte bound form. Kalunda found A1HV-1 in nasal and salivary secretions of cattle with WD-MCF by virus isolation, but not in fecal, urinary, vulvar or ocular secretions (Kalunda, Dardiri *et al.* 1981). However, Kalunda did not determine whether the A1HV-1 found in the nasal and ocular secretions of the cattle in his study was in a cell free or leukocyte bound form.

Culturing A1HV-1 in tissue culture allowed researchers to study the stability of A1HV-1 in both a cell free and cell bound form. Although A1HV-1 rapidly lost infectivity in cellular suspensions, it was found to be very stable in a cell free form (Mushi, Rossiter *et al.* 1980). Cell free A1HV-1 retained infectivity for at least 30 days on filter paper at 22 degrees C in relative humidity of 100% (Mushi, Rossiter *et al.* 1980). These studies suggest that in a natural setting, effective transmission of WD-MCF is most likely to occur when A1HV-1 is in a cell free form (Plowright 1990). Since cattle infected with A1HV-1 were found to contain A1HV-1 only in a cell bound form (Piercy

1953; Plowright 1963; Plowright 1982; Plowright, Ferris *et al.* 1960), and cellular suspensions were found to be unstable (Mushi, Rossiter *et al.* 1980), it has been postulated that cattle fail to transmit MCF horizontally because cattle infected with A1HV-1 produce and excrete little or no cell free A1HV-1(Plowright 1982; Plowright 1990).

Although horizontal transmission of A1HV-1 has not been demonstrated in cattle there is some evidence to suggest that adult cattle may become persistently infected with A1HV-1 and possibly transmit the virus vertically to their calves. Plowright studied a dairy cow that had been experimentally infected with A1HV-1 (Plowright, Kalunda *et al.* 1972). The cow was viraemic for 15 weeks following infection, but did not develop WD-MCF. When it was killed 84 months after infection, virulent A1HV-1 was cultured from its tissues. During its lifetime the cow produced 6 calves, 4 of which were shown to be infected with A1HV-1 at birth or shortly after birth. In a study of an outbreak of WD-MCF in cattle, Barnard was able to show an association between cows that died of WD-MCF and calves that died of WD-MCF (Barnard 1990). In this outbreak calves that died of WD-MCF were 79.5 times more likely to have mothers that died of WD-MCF than calves that didn't die of WD-MCF (95% CI = 29.5 - 214.5, $p<.0001$). This association may not, however, have been due to vertical transmission of A1HV-1, but rather to the close spatial relationship, and thus shared potential exposure of cows and their calves to infectious wildebeest that were in the area.

2.4.2 Transmission of A1HV-1 from wildebeest to cattle

In 1936, Daubney and Hudson reported the experimental induction of WD-MCF in cattle by stabling them in close proximity to a wildebeest calf (Daubney Hudson 1936). In 1965, Plowright repeated the same study and found the incubation period following exposure to wildebeest calves, ranged from 30 to 81 days (Plowright 1965). Plowright's work indicated that by the time wildebeest calves were 3 months of age very few of them were viraemic, and he suggested that after that age wildebeest calves rarely transmitted A1HV-1 to cattle (Plowright 1965). Mushi was able to isolate cell free A1HV-1 in nasal and ocular secretions of wildebeest calves by filtering nasal mucus through a 450nm millipore membrane filter and from explant cell cultures of nasal mucosa and cornea (Mushi, Rossiter *et al.* 1980). Barnard demonstrated that the peak age of shedding of A1HV-1 in nasal and ocular secretions occurred in wildebeest calves aged 1 to 2 months (Barnard, Bengis *et al.* 1989). By the time wildebeest calves reached the age of 6 months there was very little virus in their nasal and ocular secretions (Barnard, Bengis *et al.* 1989). A1HV-1 infected free ranging adult wildebeest were rarely found to shed A1HV-1 in their nasal or ocular secretions unless they were stressed by captivity or treated with corticosteroids (Barnard, Bengis *et al.* 1989; Rweyemamu, Karstad *et al.* 1974). The inability to detect A1HV-1 in nasal and ocular secretions of unstressed adult wildebeest, as well as the failure to detect A1HV-1 in wildebeest fetal fluids or placentas (Rossiter, Jessett *et al.* 1983) combined with the observation that WD-MCF mortalities occur in cattle during the time when wildebeest calves are secreting the most virus in their nasal and ocular secretions has led to the acceptance that wildebeest calves are the

main source of A1HV-1 that infects cattle in Africa (Mushi, Rurangira 1981; Plowright 1982).

The exact mechanism of transmission of A1HV-1 from wildebeest calves to cattle is not known. Blood sucking external parasites were rejected as a vector of transmission by the observation that the quantity of A1HV-1 in infected wildebeest blood was much lower than that of other viruses which are known to be transmitted by blood sucking parasites (Plowright 1965). Attempts to experimentally transmit A1HV-1 with African face flies (*Musca xanthomelas*) have failed, as have attempts to isolate A1HV-1 from African face flies exposed to wildebeest calves (Barnard, Bengis *et al.* 1990). Attempts to transmit MCF to cattle by lice found on wildebeest have been unsuccessful (Mushi, Rurangira 1981). Since cell free A1HV-1 is known to retain it's infectivity for extended periods of time, in conditions of high relative humidity (Mushi, Rossiter *et al.* 1980), and cell free A1HV-1 is found in wildebeest calf nasal secretions (Mushi, Rossiter *et al.* 1980), it has been suggested that A1HV-1 is most likely spread to cattle by wildebeest calves that aerosolize droplets containing infectious virus particles (Mushi, Rurangira 1981; Plowright 1982).

A1HV-1 DNA has been detected in the urine of 3 month old wildebeest calves by DNA hybridization (Michel 1993), raising the possibility that urine may be a source of A1HV-1 for infection of cattle. Even though A1HV-1 is stable in environments of high humidity, there have been no reports that wildebeest urine is infectious to cattle.

2.4.3 Transmission of OvHV-2 among cattle, deer and bison

Horizontal transmission of SA-MCF has been sporadically successful by intravenous, subcutaneous, intraperitoneal and intradermal injection, as well as oral administration of large quantities of whole blood in cattle (Blood, Rowsell *et al.* 1961; Goetze 1930; Goetze, Liess 1929; Goetze, Liess 1930; Horner, Oliver *et al.* 1975; Magnusson 1940; Selman, Wiseman *et al.* 1974; Selman, Wiseman *et al.* 1978), red deer (*Cervus elaphus*) (Huck, Shand *et al.* 1961; Oliver, Beatson *et al.* 1983), water buffalo (*Bubalus bubalus*) (Wiyono, Baxter *et al.* 1994) and bison (*Bison bison*) (Liggitt, McChesney *et al.* 1980). However, there have also been numerous unsuccessful attempts at transmission (Blood, Rowsell *et al.* 1961; Duncan 1956; Horner, Oliver *et al.* 1975; Pierson, Storz *et al.* 1974; Reid, Buxton *et al.* 1979). Lymphocyte cell lines have been successfully cultured from cattle, red deer and Pere David's deer (*Elaphurus davidianus*) with SA-MCF (Reid, Buxton *et al.* 1989). In one instance lymphocytes from cell culture have been infectious to rabbits, but they have not been infectious to cattle or deer (Reid, Buxton *et al.* 1989). Attempts to transmit the disease with nasal or ocular secretions have failed (Imai, Nishimori *et al.* 2001; Schofield Bainf 1941). Natural transmission of SA-MCF to healthy cattle stabled closely to cattle with SA-MCF has invariably been unsuccessful (Goetze 1930; Goetze, Liess 1929; Goetze, Liess 1930; Piercy 1954; Pierson, Thake *et al.* 1973). This has not always been the case with red deer. In 1986, Reid reported horizontal transmission of SA-MCF in one healthy red deer penned with a red deer that had SA-MCF (Reid, Buxton *et al.* 1986). Reid suggested that horizontal transmission among red deer was not the main method by which SA-MCF was

transmitted in red deer, since this happened to only one of seven red deer,. The observation of horizontal transmission among red deer has not subsequently been reported.

Failure of natural transmission of OvHV-2 among cattle is thought to be due to the cell bound nature of OvHV-2 in cattle with SA-MCF, and the inability of cattle with SA-MCF to produce and shed cell free OvHV-2 in a large enough quantity to be infectious to other cattle (Michel, Aspeling 1994; Plowright 1990). It has been postulated that OvHV-2 is cell bound because the viral DNA of OvHV-2 becomes incorporated in the cellular genome of infected cells or exists as an episome in infected cells (Metzler 1991; Reid, Buxton *et al.* 1986; Westbury, Denholm 1982). This may explain why attempts at transmission of SA-MCF among cattle were successful only when methods of transmission ensured the integrity of the donor cells used for transmission (Westbury, Denholm 1982). Understanding the mechanism of transmission of OvHV-2 has been difficult because of the inability to isolate the virus or culture it in cell culture. Tests to detect OvHV-2 in tissues and secretions have all been PCR based tests (Baxter, Pow *et al.* 1993; Bridgen, Reid 1991; Li, Shen *et al.* 1995). These tests can detect the presence of defined segments of OvHV-2 viral DNA, but can make no inferences about whether these DNA segments are associated with viable infectious virus particles.

2.4.4 Transmission of OvHV-2 from sheep to cattle, deer and bison.

The mechanism by which OvHV-2 is transmitted from sheep to animals of other species has not been determined. The inability of researchers to isolate or culture OvHV-

2 has prevented them from performing transmission experiments with cell free OvHV-2. MCF has not been successfully transmitted to animals of any species by the inoculation of adult sheep blood, tissues, secretions (Blood, Rowsell *et al.* 1961; Goetze 1930; Goetze, Liess 1929; Goetze, Liess 1930; Hoffman, Young 1989; Metzler 1991; Pierson, Storz *et al.* 1974; Plowright 1990), or external parasites (Goetze 1930; Goetze, Liess 1929; Goetze, Liess 1930). MCF has, however, been transmitted from healthy adult sheep to cattle and deer by stabling them in close proximity to sheep (Aleska. 1935; Bindel 1937; Goetze, Liess 1929; Goetze, Liess 1930; Imai, Nishimori *et al.* 2001; Magnusson 1940; Piercy 1954).

Through the use of PCR technology it has been shown that adult sheep and lambs older than 5 months have OvHV-2 DNA in their nasal secretions (Baxter, Pow *et al.* 1993; Baxter, Wiyono *et al.* 1997; Imai, Nishimori *et al.* 2001; Li, Hua *et al.* 2001; Li, Snowder *et al.* 1998; Li, Snowder *et al.* 2000). Li found the number of copies of OvHV-2 DNA in lamb nasal secretions to be highest in lambs aged 6 to 8 months of age (Li, Hua *et al.* 2001). The copy numbers in nasal secretions of this age group exceeded the copy numbers in their peripheral blood leukocytes by as much as 100,000 fold (Li, Hua *et al.* 2001). Li found that over the course of one year the number of copies of OvHV-2 viral DNA in nasal secretions of most adult sheep remained constant and low (Li, Hua *et al.* 2001). Some individual sheep within the flock, however, sporadically produced high DNA copy numbers in their nasal secretions (Li, Hua *et al.* 2001). These sporadic increases in copy numbers did not follow a seasonal pattern (Li, Hua *et al.* 2001). Based on these findings, Li suggested that it was unlikely that the high number of copies of

OvHV-2 DNA found in nasal secretions of 6 to 8 month old lambs and occasional adult sheep was due to the presence of latently infected lymphocytes, but rather was due to viral replication of OvHV-2 in the epithelium of the sheep's naso-pharynx (Li, Hua *et al.* 2001). Li proposed that nasal secretions of lambs and adult sheep could be a source of virus for transmission of OvHV-2 to other species. Adult sheep could be sporadically infective at any time of the year, but lambs were only infective once they reached the age of 5 to 6 months (Li, Hua *et al.* 2001).

There is no direct evidence that there is cell free, or infectious OvHV-2 in sheep nasal secretions, nor is there evidence that sheep nasal secretions are infectious to other animals (Imai, Nishimori *et al.* 2001; Li, Hua *et al.* 2001). In one study, nasal swabs from sheep known to be infected with OvHV-2 failed to produce SA-MCF in Japanese deer when the swabs were repeatedly rubbed in the nasal passages of the Japanese deer (Imai, Nishimori *et al.* 2001). The only conclusion that can be made about the transmission of OvHV-2 by sheep is that sheep are infected with OvHV-2, OvHV-2 DNA can be detected in their nasal secretions, and sheep can transmit OvHV-2 to animals of other species, when they are stabled or penned closely to them (Imai, Nishimori *et al.* 2001; Li, Snowder *et al.* 2000).

2.5 Epidemiology of MCF viruses in their natural host populations.

2.5.1 Alcelaphine herpesvirus-1 and wildebeest.

Although few adult wildebeest are viremic with AlHV-1 (Barnard, Bengis *et al.* 1989; Plowright 1965), almost all of them have been shown to be latently infected with

AlHV-1 through detection of AlHV-1 virus neutralizing antibodies in their serum (Plowright 1967; Rossiter, Jessett *et al.* 1983). A proportion of wildebeest calves are born infected with AlHV-1. This was demonstrated by Plowright who found AlHV-1 in the spleen of a wildebeest fetus and in the blood of 3 of 7 recently born wildebeest calves (Plowright 1965; Plowright 1965) as well as by Mushi *et al* who found AlHV-1 in the nasal secretions of a 4 day old wildebeest calf (Mushi Rurangira 1981). Barnard was unable to demonstrate AlHV-1 viremia, or virus in the nasal secretions of near term pregnant free ranging wildebeest cows or in near term pregnant wildebeest cows that were stressed by recent capture (Barnard, Bengis *et al.* 1989). Barnard suggested that recrudescence of AlHV-1 in wildebeest cows was unlikely, except under extreme conditions, and wildebeest cows were not a common source of infection for wildebeest calves under field conditions (Barnard, Bengis *et al.* 1989). Based on Barnard's, Mushi's and Plowright's findings it is likely that, under natural conditions, wildebeest cows do not, or only rarely, infect their calves post calving. Rather, they initiate infection in the population of wildebeest calves by producing a small number of *in utero* infections.

In free ranging wildebeest populations, Plowright found the proportion of wildebeest calves that were viremic with AlHV-1 was highest at 31% in 3 month old calves, and then dropped to less than 2% in calves 6 months old (Plowright 1965). In Plowright's study only one wildebeest calf among 44 calves aged 13 to 18 months, and none of 106 wildebeest over 18 months of age, were viremic (Plowright 1965). Barnard repeated Plowright's study in 1989. His observations were similar to those of Plowright except the highest prevalence of viremia was found to occur in slightly younger calves;

aged 1 to 2 months (Barnard, Bengis *et al.* 1989). Plowright suggested the mechanism of transmission of A1HV-1 in wildebeest populations was by spread of the virus *in utero* to a proportion of wildebeest calves and then by horizontal spread among wildebeest calves within the herd. The culmination of this process resulted in the infection of most wildebeest calves by the time they reached the age of 4 to 5 months (Plowright 1965). In order to verify this theory Plowright studied a group of neonatal wildebeest calves. In this group of calves he observed that one congenitally infected calf spread A1HV-1 horizontally to 8 pen mates over a period of 15 weeks (Plowright 1965). Plowright's theory gained further support by the finding that wildebeest calves had cell free A1HV-1 in their nasal and ocular secretions until they reached the age of 3 or 4 months (Barnard, Bengis *et al.* 1989; Mushi, Rossiter *et al.* 1980). The cessation of cell free virus shedding in wildebeest calf nasal secretions was found to coincide with the appearance of virus neutralizing antibodies in their nasal secretions (Barnard, Bengis *et al.* 1989; Mushi Rurangira 1981). It was therefore suggested that transmission of A1HV-1 among wildebeest calves occurs through production of aerosolized droplets containing cell free A1HV-1 and that wildebeest calves produce infectious aerosols only during the short period from infection until they produce their own virus neutralizing antibodies (Plowright 1990). An alternative, or additional source of virus may be wildebeest calf urine. Michel was able to detect A1HV-1 viral DNA in 3 month old wildebeest calf urine by DNA hybridization (Michel 1993).

The infection of wildebeest calves does not seem to be hindered by the presence of circulating maternal antibodies. Mushi *et al* demonstrated the presence of virus

neutralizing antibody within the colostrum of wildebeest cows (Mushi Rurangira 1981). Maternally derived virus neutralizing antibody titers were found to be high in neonatal wildebeest calves after colostrum consumption and then to decline over the first 4 months of their life, the time during which they were becoming infected with A1HV-1 from their herd mates (Plowright 1967; Plowright 1990).

Serum neutralizing antibody titers follow a biphasic pattern in wildebeest calves. Plowright found titers to be low at birth and then, as expected, they rose and peaked shortly after colostrum consumption (Plowright 1967; Plowright 1990). This peak was followed by a steady decline in titers until calves reached 4 months of age (Plowright 1967; Plowright 1990). After 4 months of age mean virus neutralizing antibody titers were found to increase steadily, presumably in response to of infection with A1HV-1, until calves reached 18 months of age (Plowright 1967; Plowright 1990; Rossiter, Jessett *et al.* 1983). Barnard observed a similar pattern of antibody titers in wildebeest calves, but found the second peak of antibody titers to occur when wildebeest calves were 6 to 7 months of age (Barnard, Bengis *et al.* 1989). In both studies the second peak of antibody titers was followed by a slight decline to amounts that were similar to those found in adult wildebeest (Barnard, Bengis *et al.* 1989; Plowright 1967; Plowright 1990; Rossiter, Jessett *et al.* 1983).

Barnard observed a seasonal variation in mean virus neutralizing antibody titers in adult wildebeest. A1HV-1 virus neutralizing antibody titers were found to peak in adult wildebeest at the same time of the year that they peaked in wildebeest calves. Barnard suggested this rise in antibody titers was due to exposure of adult wildebeest to

the large quantities of A1HV-1 that was being produced by wildebeest calves, rather than recrudescence of latent infection in adult wildebeest (Barnard, Bengis *et al.* 1989).

2.5.2 Ovine herpesvirus-2 and sheep

The pattern of transmission of OvHV-2 among sheep is markedly different from that of A1HV-1 in wildebeest. Nearly all of the adult populations of both species are infected with their respective MCF viruses (Baxter, Wiyono *et al.* 1997; Li, Mcguire *et al.* 2001; Li, Shen *et al.* 1995; Li, Shen *et al.* 1995; Metzler 1991; Mirangi, Kang'ee 1997; Muluneh, Liebermann 1992; Plowright 1968; Plowright 1990; Plowright, Ferris *et al.* 1960; Rossiter 1981). The prevalence of viremia varies between the two species. Only a few adult wildebeest are viremic with A1HV-1, as determined by cell culture, but almost all adult sheep have OvHV-2 DNA in their peripheral blood leukocytes, as determined by PCR assay (Baxter, Pow *et al.* 1993; Li, Mcguire *et al.* 2001; Li, Shen *et al.* 1995; Mirangi, Kang'ee 1997; Wiyono, Baxter *et al.* 1994). PCR assay may be a more sensitive test, but it can only detect the presence of specific segments of viral DNA and cannot differentiate between infectious virus particles and noninfectious fragments of viral DNA. It may not be appropriate to compare the PCR studies in sheep to virus isolation studies in wildebeest. However, there have been no PCR studies of large populations of wildebeest reported.

In contrast to wildebeest, pregnant ewes rarely transmit OvHV-2 to their lambs *in utero* , nor do lambs become infected with OvHV-2 within the first few months of life (Li, Snowder *et al.* 1998). Li examined 4 sheep fetuses and demonstrated that they did

not have OvHV-2 antibodies in their serum, nor did they have OvHV-2 viral DNA in their lymph nodes or peripheral blood leukocytes (Li, Snowder *et al.* 1998). Even though OvHV-2 viral DNA is present in the colostrum and milk of ewes, Li was able to determine that OvHV-2 infections were not established in lambs until they reached the age of 3 to 3.5 months (Li, Snowder *et al.* 1998). Li suspected the OvHV-2 DNA which was present in ewe colostrum and milk was present in lymphocytes, not in a cell free infectious form (Li, Snowder *et al.* 1998). Alternatively the OvHV-2 in colostrum may have been in an infectious form, but inactivated by the presence of antibodies within the colostrum. Some OvHV-2 infected lymphocytes may have been transferred passively to lambs when they suckled, resulting in Li's and Baxter's separate observations of a few weak OvHV-2 PCR positive test results from peripheral blood leukocytes of lambs that had consumed colostrum (Baxter, Wiyono *et al.* 1997; Li, Snowder *et al.* 1998). These lymphocytes although they contained OvHV-2 DNA were probably not infective, since they did not establish OvHV-2 infection in lambs. Li determined in a subsequent study that transfusion of peripheral blood leukocytes from latently infected sheep that were PCR positive for OvHV-2 DNA was relatively inefficient for transmitting OvHV-2 in sheep (Li, Snowder *et al.* 2000). Li firmly established that OvHV-2 was not transmitted *in utero* or by the consumption of colostrum and milk in lambs, by demonstrating that lambs weaned at 2 to 2.5 months of age and reared in isolation from other sheep, remained uninfected with OvHV-2, even though their dams were determined to be infected by demonstrating the presence of OvHV-2 DNA in their peripheral blood leukocytes (Li, Snowder *et al.* 1998).

It remains unclear why lambs under the age of 2 months are impervious to OvHV-2 infection. It is likely that the environment, at least within large OvHV-2 infected sheep flocks is constantly infective over time. In Li's study, lambs that were weaned at 2 to 2.5 months of age and returned to an OvHV-2 infected sheep flock after 10 to 14 days rapidly became infected with OvHV-2 (Li, Snowder *et al.* 1998). Li also observed that all 20 uninfected 1 and 2 year old sheep that were introduced into an OvHV-2 infected flock became infected with OvHV-2 (Li, Snowder *et al.* 2000). Some of the uninfected sheep became infected as early as one month after their introduction into the infected flock (Li, Snowder *et al.* 2000). This latter study suggests that once lambs which have consumed colostrum gain the age of 3 months they become susceptible to OvHV-2 infection and remain susceptible until 2 years of age and possibly for life. The inability of very young lambs to become infected with OvHV-2 may be related to passively acquired immune components, age specific susceptibility to infection or other unknown factors.

In Li's study, lambs in a sheep flock rapidly became infected with OvHV-2 after the age of 3 months. Eighty seven percent of lambs in the flock had OvHV-2 DNA in their peripheral blood leukocytes at 3.5 months and 100% of lambs by 5.5 months of age (Li, Hua *et al.* 2001; Li, Snowder *et al.* 1998). Li observed a lag between the age that OvHV-2 was detected in peripheral blood leukocytes and the age that OvHV-2 was detected in nasal secretions of the lambs. There were only 5% of lambs with OvHV-2 DNA in their nasal secretions at 3.5 months of age, when 87% of the lambs had detectable OvHV-2 DNA in their peripheral blood leukocytes (Li, Snowder *et al.* 1998).

The prevalence of lambs with OvHV-2 DNA in their nasal secretions increased to 33% at 5.5 months, and peaked at 88% at 7.5 months, which was well after the age when 100% of the lambs in the flock had detectable OvHV-2 DNA in their peripheral blood leukocytes (Li, Snowder *et al.* 1998). There is no good explanation for this time lag. It does, however, suggest that most lambs become infected with OvHV-2, or at least have OvHV-2 DNA in their peripheral blood leukocytes, before the age when the virus is replicating in their nasal mucosa and therefore before the age when they would potentially be shedding the virus in large quantities in their nasal secretions.

The mechanism by which lambs become infected with OvHV-2 is not known. It is probable that, similar to wildebeest and AlHV-1, the mechanism of spread of OvHV-2 is through aerosolized cell free virus particles, but the source of the virus is not known. It may originate from sporadically shedding adult sheep within the flock; from a few lambs in which virus replication occurs in the nasal passages early in the course of their infection or it may originate from cell free virus that is made available to lambs by some other, as yet unknown, means.

OvHV-2 antibodies follow a biphasic pattern in lambs that is similar to that seen in wildebeest calves. Lambs are born without OvHV-2 antibodies, but become seropositive as soon as they suckle colostrum (Li, Snowder *et al.* 1998). Maternal antibodies remain detectable until lambs reach about 2.5 months (Li, Snowder *et al.* 1998). After 2.5 months of age lambs gradually become seropositive, with 50% becoming seropositive at 7 to 8 months of age and 80 to 90 % becoming seropositive at 1 year of age (Li, Snowder *et al.* 1998).

2.5.3 Caprine herpesvirus 2 and goats

Little is known about the transmission of CpHV-2 in goats. A large proportion of adult goat populations are considered to be infected with an MCF virus, since antibodies to MCF viruses can be found in their sera (Li, Shen *et al.* 1996a). With the development of PCR primers specific to CpHV-2 it has been determined that most goats are infected with CpHV-2 rather than other MCF viruses (Li, Keller *et al.* 2001). Li found that 87% of 142 goats, had CpHV-2 DNA in their peripheral blood leukocytes (Li, Keller *et al.* 2001). Goats can be infected with OvHV-2 as well as CpHV-2. Wiyono found that 17% of Indonesian goats had OvHV-2 DNA (Wiyono, Baxter *et al.* 1994), and Li found that 9% of 124 goats had OvHV-2 DNA, in their peripheral blood leukocytes (Li, Keller *et al.* 2001). In addition, a small proportion of the goats in Li's study were found to be co-infected with both CpHV-2 and OvHV-2 (Li, Keller *et al.* 2001). Although it is highly likely that CpHV-2 infections are maintained in goat populations by some form of transmission between goats, it remains a matter of speculation whether OvHV-2 infections in goats are maintained by transmission of OvHV-2 from other goats or from sheep.

Prevalence of antibodies to CpHV-2 follows a biphasic pattern in goat kids, which is similar to that observed in wildebeest calves and lambs. Kids are born without CpHV-2 antibodies, but become seropositive after colostrum consumption (Li, Keller *et al.* 2001). Maternal antibodies degrade until they are undetectable in kids 3 month of age, after which the percentage of seropositive kids increases to 40% at 9 months and 100% at 12 months (Li, Keller *et al.* 2001). This biphasic pattern suggests that, similar to lambs

and wildebeest calves, few or no kids are born infected with CpHV-2, and most of them gain infection at some time after birth. The exact mechanism of transmission has not been determined.

2.5.4 Other MCF viruses.

The mechanism of transmission of Hippotragine herpesvirus-2 and A1HV-2 is not known. Since the natural host of MCFV-WTD remains to be elucidated, studying it's transmission is impossible. A1HV-2 has been isolated from the hartebeest (*Alcelaphus buselaphus cokei*) and topi (*Damaliscus korrigum*) (Mushi, Rossiter *et al.* 1981; Reid, Rowe 1973; Seal, Heuschele *et al.* 1989). Forty to sixty percent of hartebeest and topi populations have been found to have antibodies to MCF viruses in their sera (Reid, Plowright *et al.* 1975; Seal, Heuschele *et al.* 1989). HiHV-1 has been isolated from the roan antelope (*Hippotragus equinus*) (Reid Bridgen 1991), of which 45 percent have antibodies to MCF viruses (Mushi, Rossiter *et al.* 1981; Seal, Heuschele *et al.* 1989). The high prevalence of antibodies to MCF viruses in all three of these host species suggests that a large proportion of each population is infected with their respective MCF virus. The mechanism by which MCF virus infections are maintained in each of these populations has not been determined.

The prevalence and titer of virus neutralizing antibodies to MCF viruses has been determined in the sera of captive fringe-eared oryx (*Oryx beisa callotis*) from one game farm in Africa (Mushi Karstad 1981). All of the 50 adults tested were found to have antibodies to MCF viruses. Calves had high antibody titers following colostrum

consumption. After this time titers began to decline until calves reached 6 to 8 months of age, when titers began to rise again. This biphasic pattern of antibody levels is similar to that seen in lambs, goats and wildebeest calves. It suggests that this fringe-eared oryx population was maintaining an ongoing infection with an MCF virus by transmission of the virus to neonates, or among neonates, at some time before they reached adult age. However, there has never been an MCF virus identified from this, or any other fringe-eared oryx population. This failure may have been due to difficulties associated with virus isolation and culture, or it may have been a result of the fringe-eared oryx in this study having been infected with an MCF virus which, similar to OvHV-2, is refractory to isolation and culture.

2.6 MCF in bison.

MCF is a disease of economic importance to the bison industry. MCF mortalities have been reported in bison herds in Colorado, Wyoming, Kansas, New Mexico, Minnesota, Nevada, Utah, North Dakota, South Dakota, Alberta, Saskatchewan and in a bison feedlot in the American Midwest (Collins, Bruns *et al.* 2000; O'Toole, Li *et al.* 2002; Berezowski, Haigh *et al.* 2000 ; Berezowski, Middleton *et al.* 2000; Liggitt, McChesney *et al.* 1980; Ruth, Reed *et al.* 1977; Schultheiss, Collins *et al.* 2000). Mortality rates have ranged from sporadic single mortalities to rates as high as 100 percent. In one feedlot the mortality rates ranged from 0.87% to 9.4% during the period from 1996 to 1999 (O'Toole, Li *et al.* 2002). Mortalities have been evenly distributed through all age groups, with the exception that only rare mortalities have occurred in pre-

weaned bison calves (Collins, Bruns *et al.* 2000; O'Toole, Li *et al.* 2002; Schultheiss, Collins *et al.* 1998; Schultheiss, Collins *et al.* 2000). Mortalities have not been limited to one gender (Collins, Bruns *et al.* 2000). Although occasional MCF mortalities have occurred in bison during any month of the year, the vast majority have occurred during the months of November through May (Collins, Bruns *et al.* 2000). The reason for this seasonal distribution of mortalities is unclear. Mortalities have been temporally associated with handling of bison, occurring 3 to 14 days following handling of bison for any reason (O'Toole, Li *et al.* 2002). It has been suggested that the stress associated with handling of bison may precipitate disease, but this theory has not been explored, and should be considered speculative at this time.

The clinical course of MCF in bison is very short. Most bison that die of MCF are either found dead, or die within 8 to 48 hours of the onset of illness (Schultheiss, Collins *et al.* 1998). In rare cases, bison with MCF have survived for longer periods. In one report a bison cow survived for 80 days following an acute episode of MCF (Schultheiss, Collins *et al.* 1998).

The earliest clinical signs of MCF are separation from the herd, epiphora, listlessness and refusal of food. This rapidly progresses to severe depression, recumbency and death. Other clinical signs, which include diarrhea, melena, hematuria, stranguria, corneal opacity, severe ocular and nasal discharge, blindness, convulsions and coughing may be observed, but not consistently in all cases. The clinical presentation of bison with MCF is of little or no diagnostic value to veterinarians, since the clinical signs are both highly variable, and common to many other bison diseases (Collins, Bruns *et al.* 2000;

Liggitt, McChesney *et al.* 1980; O'Toole, Li *et al.* 2002; Ruth, Reed *et al.* 1977; Schultheiss, Collins *et al.* 1998; Schultheiss, Collins *et al.* 2000).

The most common gross pathological changes associated with MCF in bison are corneal opacity, erosions throughout the gastrointestinal tract, and hemorrhagic cystitis. Other gross changes include congestion and edema of the lungs, erosions of the tracheal and bronchial mucosa, conjunctivitis, focal white lesions in the kidney, erythema of the turbinate mucosa, and lymphnode enlargement. The gross pathology is often subtle and inconsistent among cases, making confirmation of a diagnosis of MCF in bison difficult based on gross pathology alone (Liggitt, McChesney *et al.* 1980; O'Toole, Li *et al.* 2002; Ruth, Reed *et al.* 1977; Schultheiss, Collins *et al.* 1998; Schultheiss, Collins *et al.* 2000).

In bison and other species, MCF is defined microscopically, by the presence of lymphocytic infiltrations and lymphocytic vasculitis throughout many tissues and organs in the body as well as erosions or ulcerations of the mucosa of the gastrointestinal and urinary tracts. Lymphocytes infiltrate the adventitia and media of both arterioles and venules. Mucosal erosions are often associated with lymphocytic infiltration of the adjacent mucosa and submucosa, but may or may not be associated with underlying or adjacent vasculitis. Diffuse accumulations of lymphocytes may be observed within many tissues; commonly hepatic portal triads and renal cortices. Although mortality rates are higher, and outbreaks more common in bison than in cattle, the lesions which are found in bison are often more subtle than those found in cattle with MCF. In bison there is less widespread lymphocytic vasculitis and less severe lymphocytic infiltration. Mucosal erosions are often not associated with underlying vasculitis, lymphocytic infiltration or

inflammation. Although the defining lesions are always present, in many cases a wide range of tissues must be examined before characteristic lesions are observed (Liggitt, McChesney *et al.* 1980; O'Toole, Li *et al.* 2002; Ruth, Reed *et al.* 1977; Schultheiss, Collins *et al.* 1998; Schultheiss, Collins *et al.* 2000).

MCF mortalities in farmed North American bison are considered to be associated with exposure to sheep, and infection with OvHV-2 (Collins, Bruns *et al.* 2000; O'Toole, Li *et al.* 2002; Schultheiss, Collins *et al.* 1998; Schultheiss, Collins *et al.* 2000). Although the reports are minimal, the association between OvHV-2 and MCF in bison is reasonably well supported. The association between exposure to sheep and outbreaks of MCF in bison is not as well documented.

OvHV-2 DNA has been detected, by PCR analysis of tissues, in 27 of 29 (Schultheiss, Collins *et al.* 2000), 12 of 14 (O'Toole, Li *et al.* 2002), 15 of 15 (O'Toole, Li *et al.* 2002) and 52 of 53 (Collins, Bruns *et al.* 2000) bison that have died of MCF. OvHV-2 DNA was not detected in 10 of 10 (Collins, Bruns *et al.* 2000), 4 of 4 (Schultheiss, Collins *et al.* 1998) and 4 of 4 (O'Toole, Li *et al.* 2002) bison that died of other causes. There has been no association demonstrated between MCF mortalities in bison and bovine herpesvirus 4 (BHV-4), bovine lymphotrophic herpesvirus (BLHV), bovine syncitial virus (BSV), bovine immunodeficiency virus (BIV), and bovine virus diarrhea virus (BVDV) in 26 bison that died of MCF (Collins, Bruns *et al.* 2000). The weakness in these reports lies with the small number of non-MCF mortalities that were tested and the failure of the authors to look for OvHV-2 DNA in a representative sample of normal healthy bison. These studies demonstrate fairly convincingly that OvHV-2

DNA is present in bison that die of MCF, but they do not adequately demonstrate that OvHV-2 DNA is not present in bison that are healthy or bison that die of other causes.

Demonstrating a clear and consistent link between exposure to sheep and MCF mortalities in bison is difficult. There have been 4 outbreaks reported in which there is well documented exposure to sheep (Ruth, Reed *et al.* 1977; Schultheiss, Collins *et al.* 2000), 2 with an equivocal exposure (O'Toole, Li *et al.* 2002; Ruth, Reed *et al.* 1977), and 8 which failed to report whether there was or was not exposure to sheep (Collins, Bruns *et al.* 2000; Ruth, Reed *et al.* 1977; Schultheiss, Collins *et al.* 1998). In many of these studies, the main focus was to describe the lesions associated with MCF in bison or the association between OvHV-2 and MCF in bison. The failure to firmly establish an association with sheep may simply have been the result of researchers failing to investigate the existence of an association. Alternatively, the association may not have been there. None of the authors of these studies, however, unequivocally stated that they investigated and found no association between sheep and their reported outbreaks of MCF in bison.

Since the association between sheep, OvHV-2 and MCF in cattle is well established (Baxter, Pow *et al.* 1993; Collins, Bruns *et al.* 2000; Crawford, Li *et al.* 1999; Li, Shen *et al.* 1994; Li, Shen *et al.* 1996b; Li, Shen *et al.* 1995; Li, Snowder *et al.* 1998; Muller Doblies, Li *et al.* 1998; Wiyono, Baxter *et al.* 1994), and OvHV-2 is consistently found in bison with MCF (Collins, Bruns *et al.* 2000; O'Toole, Li *et al.* 2002; Schultheiss, Collins *et al.* 2000), it is seems likely that, similar to cattle, sheep are associated with MCF outbreaks in bison. Firm establishment of this association must,

however, await more detailed bison MCF outbreak investigations or controlled sheep exposure trials.

2.7 Diagnostic tests for MCF and MCF viruses

MCF is a disease syndrome, which is defined by the observation of characteristic histopathological changes in carcasses on postmortem examination. It is only when researchers attempt to establish causal associations between MCF and MCF-group viruses, or try to understand the epidemiology of MCF-group viruses in natural and susceptible host populations that difficulties arise. These difficulties are mainly due to the latent nature of herpesvirus infections. Even though infections with herpesviruses, such as those belonging to the MCF-group of viruses, are considered to be lifelong, these viruses are often difficult to find in animals that are infected with them (Michel, Buchholz *et al.* 1995). The sensitivity of tests that are designed to isolate or identify herpesviruses in latently infected animals are often low. Tests that detect antibodies to herpesviruses may perform poorly because antibody levels in animals infected with herpesviruses are often not specific to one herpesvirus and antibody levels in latently infected ruminants are often variable over time.

2.7.1 WD-MCF and Infection with A1HV-1

The first method used to confirm WD-MCF in cattle was through the use of transmission experiments. Blood or tissue from animals suspected of having WD-MCF were injected into healthy cattle and rabbits. Induction of disease, having the

characteristic clinical signs and pathological changes of MCF in the recipients was considered to be diagnostic for WD-MCF. Successful transmission of the disease, however, was not always consistent and transmission studies were mostly performed during experiments to study the disease and not for routine diagnostic testing (Daubney, Hudson 1936; Piercy 1952; Piercy 1952).

2.7.1.1 Virus isolation

Once methods for culturing A1HV-1 in tissue culture were established and A1HV-1 was accepted as the causative agent of WD-MCF, a diagnosis of WD-MCF could be confirmed by culturing the virus in bovine tissue culture (Plowright, Ferris *et al.* 1960; Seal, Klieforth *et al.* 1988). Tissue cultures infected with A1HV-1 demonstrated a cytopathic effect, consisting of multinucleate cellular focii, which was characteristic of A1HV-1. Intranuclear, cytoplasmic and extracellular herpes-like particles could be demonstrated by electron microscopy in A1HV-1 infected tissue cultures (Plowright, Macadam *et al.* 1963). A1HV-1 could be cultured from the blood of cattle with WD-MCF as well as from lymphnodes of cattle that died of WD-MCF (Plowright 1968). Culturing A1HV-1 however, was a very cumbersome and often unsuccessful diagnostic procedure. Tissue cultures were difficult to maintain and samples were required to be taken from suspect animals shortly after death in order for the virus to be cultured successfully (Plowright 1968).

2.7.1.2 Virus neutralizing antibody test

The first test to detect the presence of antibodies against A1HV-1 was a virus neutralization or virus neutralizing antibody test. Virus neutralizing antibody tests were conducted by inoculating tissue cultures with a mixture of serum from a test animal and live A1HV-1. Serum was considered to contain antibodies against A1HV-1 and was classified as positive if it inhibited growth of A1HV-1 in tissue culture (Plowright, Macadam *et al.* 1963). The test was not very sensitive for diagnosing WD-MCF since only a portion of the cattle that died of WD-MCF produced serum neutralizing antibodies to A1HV-1 prior to death (Plowright 1968; Rossiter, Jessett *et al.* 1980).Virus neutralizing antibody tests have therefore been used mainly for the detection of latent infections of ruminants of various species with MCF viruses (Hamblin, Hedger 1984; Rossiter, Jessett *et al.* 1980; Seal, Heuschele *et al.* 1989).

The virus neutralizing antibody test is considered to be specific to A1HV-1. It does not detect antibodies produced against other bovine herpes viruses or OvHV-2 (Muluneh, Liebermann 1992; Plowright, Macadam *et al.* 1963; Rossiter 1983; Seal, Heuschele *et al.* 1989). An A1HV-1 cDNA clone has been identified and shown to express a protein which has been recognized by A1HV-1 neutralizing sera (Lahijani, Sutton *et al.* 1995). Even though the virus neutralizing antibody test has gained acceptance as a good test for detecting latent A1HV-1 infections, and has been used as a gold standard for validating other tests, there have been no reports of studies aimed directly at validating it (Seal, Heuschele *et al.* 1989; Wan, Castro *et al.* 1988). There is one small study of wildebeest, that although not directly aimed at validating the virus

neutralizing antibody test, can be used to make inferences about the validity of the test (Lahijani, Sutton *et al.* 1994). In this study, the virus neutralizing antibody test was applied to serum samples taken from a population of 33 wildebeest. The sensitivity and specificity of the test were 68.4 (95% CI = 47.5-89.3, N = 19) and 100.0 (95% CI = 73.2-100, N = 14) respectively. A PCR assay which amplified a piece of A1HV-1 DNA in peripheral blood leukocytes was used as the gold standard. A kappa statistic for comparing the agreement between the two tests was 0.648 (95% CI = 0.308-0.967, N = 33) indicating a good degree of statistical agreement, beyond chance, between the two tests.

Although laboratory studies suggest that the virus neutralizing antibody test is specific for detecting A1HV-1 antibodies, without adequate studies to evaluate the test's ability to correctly classify the latent infection status of ruminants, the validity of the virus neutralizing antibody test for the detection of latent A1HV-1 infections must remain unknown.

Virus neutralizing antibody tests are very expensive, and labor intensive procedures. In order to conduct virus neutralizing antibody tests, tissue cultures and cell free A1HV-1 must be maintained in the laboratory (Plowright 1990). The difficulty, expense and time required to perform these tests in the laboratory has compelled researchers to search for alternative tests, which are more easily adapted to diagnostic laboratory environments (Wan, Castro *et al.* 1988).

2.7.1.3 ELISA test

An whole virus enzyme linked immunosorbent assay (ELISA) has been developed that detects multivalent antibody responses to A1HV-1 (Wan, Castro *et al.* 1988). This test did not detect antibodies produced against bovine herpesviruses 1, 2 or 4. The sensitivity and specificity for detecting A1HV-1 antibodies were 85.3 (95% CI = 79.1-91.4, N = 129), and 83.9 (95% CI= 76.2-91.6, N = 87), in a diverse population of ruminants. An A1HV-1 virus neutralizing antibody test was used as the gold standard. Agreement between the two tests was good (Kappa = 0.69, 95% CI = 0.55-0.82). This suggests that the two tests were probably detecting the same antibodies in serum, but since the virus neutralizing antibody test has never been validated for detecting latent A1HV-1 infections, it's suitability for use as a gold standard is questionable, and therefore the validity of this ELISA for detecting latent A1HV-1 infections also remains in question.

2.7.1.4 Other serology tests

There have been several other tests developed for detecting antibodies to A1HV-1, including immunoflourescence (Rossiter 1981; Rossiter, Mushi *et al.* 1977), immunoperoxidase(Rossiter 1981; Rossiter 1982), immunodiffusion (Rossiter 1980), counter-immunoelectrophoresis (Rossiter 1980), compliment fixation (Rossiter Jessett 1980), and flourescent antibody tests (Ferris, Hamdy *et al.* 1976). All of these test have been either very poorly validated or not validated at all. Those for which validation studies have been reported have performed very poorly. For these reasons, this review

will not provide any further discussion of these tests.

2.7.1.5 PCR tests

DNA base sequencing of portions of the A1HV-1 viral genome has allowed several researchers to develop diagnostic tests which use PCR technology to amplify, and gel electrophoresis to identify, segments of A1HV-1 DNA found in tissue or peripheral blood leukocytes of animals infected with A1HV-1 (Bridgen 1991; Bridgen, Herring *et al.* 1989; Hsu, Shih *et al.* 1990; Seal, Klieforth *et al.* 1990).

The first report of a PCR test for amplifying A1HV-1 DNA was a test that used two, 20 base primers to amplify a 1028 base pair segment of a tissue culture strain (WC 11) of A1HV-1 (Hsu, Shih *et al.* 1990). The sequence of the primers and the amplified segment of A1HV-1 DNA were determined from a previous report of the base sequence of a 3389 base pair segment of the genome of the WC11 strain of A1HV-1 (Hsu, Shih *et al.* 1990). The amplified DNA fragment was cleaved, with restriction endonuclease enzyme *xba*I, into two fragments which were determined by gel electrophoresis to contain the number of base pairs predicted from the previously known sequence of the amplified DNA fragment. The authors reported that the test performed well, but only reported using the test on DNA derived from small numbers of infected cell cultures. In a subsequent report the same primers from this test were used to identify A1HV-1 DNA in peripheral blood leukocytes of 6 bovine calves that were experimentally infected with A1HV-1 (Michel, Buchholz *et al.* 1995). In this study, the results of the PCR test were compared to those of a hybridization test in which a 2.0 Kb DNA probe (SW15) (Michel

1993), specific to A1HV-1 DNA, was used to detect A1HV-1 DNA by hybridization in samples from peripheral blood leukocytes. Both tests positively identified A1HV-1 DNA in the peripheral blood leukocytes of three infected calves that developed WD-MCF and three infected calves that did not. The authors reported both PCR and hybridization test results from multiple testings of all of the calves throughout the course of this study. Although both tests yielded positive results for all 6 calves, the results were not consistently positive over the 142 day period of the study. The authors assumed that all of the calves in the study were truly infected with A1HV-1, even though 3 of them didn't develop WD-MCF. Based on this assumption they made the recommendation that, in order to firmly establish a diagnosis of latent infection with A1HV-1, multiple testings of suspect animals would be required. If the assumption that all 6 calves were truly infected with A1HV-1 can be accepted, then a crude estimate of the sensitivity of both of these tests can be calculated form the data presented in the report. The sensitivity for the PCR and DNA hybridization test are 65.5 (95% CI = 56.0-74.2) and 40.2 (95% CI = 33.8-46.8) respectively. From these calculations it would appear that both of these tests performed poorly when used to diagnose latent infections with A1HV-1. The sample size in this study was, however, very small. Establishing the true validity of these two tests would require studies with much larger numbers of known A1HV-1 infected and uninfected animals. There have been no subsequent reports of studies aimed at validating either of these two tests.

A two stage nested PCR amplification test was developed (Katz, Seal *et al.* 1991) using two sets of primers, which were selected from the base sequence of a DNA clone

(clone B25) representing the *Hin*dIII fragment D of the WC11 strain of A1HV-1 (Seal, Klieforth *et al.* 1990). The 2 outer primers amplified a 487 base pair DNA fragment and the two inner pairs a 172 base pair fragment. From the known base sequence of the amplified fragments, restriction endonucleases *PVU* II and *STU* were used to cleave the amplified fragments into segments which were determined to be of the predicted number of base pairs by gel electrophosesis. The test did not amplify DNA from bovine herpesviruses 1, 2, and 4, but did amplify DNA from 5 different strains of A1HV-1. Although studies to validate this PCR test were not reported, the authors did report that the test amplified DNA from both A1HV-2 and A1HV-1, suggesting that the test may not be specific for A1HV-1.

In another study, a pair of PCR primers were developed from the base sequence of the B25 clone of genomic DNA of the WC11 strain of A1HV-1 (Murphy, Klieforth *et al.* 1994).These two 30 base primers were used to design a diagnostic PCR test which successfully amplified a 201 base pair segment of A1HV-1 DNA. In addition to PCR amplification of a specific segment of A1HV-1 DNA, the authors designed a 27 base DNA probe that successfully hybridized to the PCR amplified segment of DNA. The PCR successfully amplified DNA from 4 strains of A1HV-1, but not DNA from other herpesviruses including; human cytomegalovirus, Epstein-Barr virus, bovine herpesvirus-1, and bovine herpesvirus-2. The test positively amplified and identified A1HV-1 DNA in 6 of 8 tissues taken at postmortem from an Indian gaur (*Bos gaurus gaurus*) that died of WD-MCF. In a second study, this test as well as an A1HV-1 virus neutralizing antibody test, were used to identify latent A1HV-1 infections in blood

samples from 86 ruminants belonging to 31 species of Bovidae and Cervidae (Lahijani, Sutton *et al.* 1995). As expected, among wildebeest, which are known to be infected with A1HV-1, the agreement between the two tests was good (Kappa = 0.648, 95% CI = 0.328 - 0.967). Of the 33 wildebeest in the study, 13 were classified as positive by both tests,14 were negative by both tests, and 6 were positive by PCR, but negative for virus neutralizing antibody. Among other species of ruminants, for which the MCF virus infection status is not known, the agreement between the two tests was very poor (Kappa -0.1124, 95% CI = -0.650-0.425). The agreement between samples with negative PCR test results and their virus neutralizing test results was fairly good. The virus neutralizing test yielded 25 negative test results from the group of 28 samples which had negative PCR results. The virus neutralizing test however, yielded no positive test results from the group of 25 ruminants that had positive PCR results. Since the A1HV-1 virus neutralizing antibody test is considered to be specific for A1HV-1 antibodies (Muluneh, Liebermann 1992; Plowright, Macadam *et al.* 1963; Rossiter 1983; Seal, Heuschele *et al.* 1989), this latter finding suggests that this PCR test may be capable of detecting DNA from other viruses that do not stimulate their hosts to produce A1HV-1 virus neutralizing antibodies. Obvious candidates would be other MCF group viruses such as A1HV-2, HiHV-1, or OvHV-2, but may also include other as yet unidentified viruses. An alternative explanation would be that those ruminants that are positive by PCR for A1HV-1 DNA, but negative for A1HV-1 neutralizing antibodies, may be capable of being infected with A1HV-1 without producing antibodies. Either alternative suggests that one or both of these tests may not be a good test for correctly classifying ruminants

as being latently infected or non-infected with A1HV-1. Further studies are required to validate both of these tests.

Tham developed a PCR assay using a pair of 20 base primers from the published base sequence of gene A of the WC 11 strain of A1HV-1 (Hsu, Shih *et al.* 1990), which amplified a 413 base pair fragment of A1HV-1 DNA (Tham, Ng *et al.* 1994). The amplified DNA fragment was cleaved with restriction endonuclease *Xba*I into two segments each with the number of base pairs predicted from the known base sequence of the amplified fragment. In addition, a 25 base DNA probe was developed which hybridized to the amplified DNA fragment. The test did not amplify DNA from bovine herpesvirus-1, caprine herpesvirus-1, suid herpesvirus-1, gallid herpesvirus-1 or canine herpesvirus-1. The test was used to identify A1HV-1 DNA in rabbits and red deer that were infected with blood from a red deer which had clinical SA-MCF. The PCR test successfully amplified the expected 413 base pair fragments of DNA from the infected rabbits. This result would suggest that the genome of OvHV-2 contains a segment of DNA that is homologous to the segment of DNA of gene A, of the A1HV-1 genome. It would not be unreasonable to assume that these two viruses have homologous sections of their respective genomic DNAs, since these two viruses are known to be antigenically related (Li, Shen *et al.* 1994). The PCR test did not, however, amplify the expected 413 base pair fragment of DNA from the OVHV-2 infected red deer. Rather, it consistently amplified a 600 base pair fragment. The authors of this study failed to put forward any theories as to why a fragment of 600 base pairs was amplified in these deer with SA-MCF, nor did they discuss any attempts made to sequence the fragment, or identify it's

origin. The identity of this fragment might possibly be determined if the nucleotide sequence of OvHV-2 was known. At the very least this report suggests that the set of primers used in this PCR amplification may amplify DNA segments from OvHV-2 and possibly other unknown viruses, as well as those of A1HV-1. In a subsequent study which compared this PCR assay to a PCR assay which used OvHV-2 specific primers (Baxter, Pow *et al.* 1993), it was shown that there was almost complete agreement between the two tests (Kappa = 89.43, 95% CI = 83.00-95.80), when they were used on blood and tissue samples from cattle, red deer and buffalo that were either normal, had died of SA-MCF or had died of other diseases (Tham 1997). Since the base sequence of the PCR primers used in Tham's PCR amplification were developed from the known base sequence of gene A of the WC 11 strain of A1HV-1, this study provides further support for hypothesis that both OvHV-2 and A1HV-1 share this same fragment of DNA in their genomes. It also suggests that Tham's PCR assay was unable to differentiate between OvHV-2 and A1HV-1 viral DNA.

There have been two reports of A1HV-1 DNA probes being used to hybridize to A1HV-1 viral DNA found in tissue samples, blood, urine, nasal mucus, and ocular secretions of animals infected, or suspected of being infected with A1HV-1. In the first report a 950 base pair clone of the *hin*dII sequence of genomic DNA of the WC 11 strain of A1HV-1 was used in an attempt to identify A1HV-1 DNA in the tissue of rabbits experimentally infected with A1HV-1 (Bridgen, Munro *et al.* 1992). The DNA clone hybridized with only a small number of tissues from the infected rabbits. The authors suggested that the poor hybridization observed in this study was a result of their being

little or no A1HV-1 viral DNA in tissues of rabbits dying of WD-MCF. This suggestion was based on their observation that the clone was successful at hybridizing with A1HV-1 DNA from tissue cultures that were infected with A1HV-1. There have been no further reports of this hybridization test being used in any experimental or field studies. The validity of this hybridization test for detecting A1HV-1 in the tissues of ruminants must await the reporting of further studies.

An A1HV-1 specific DNA probe was developed that used two DNA fragments; a 2.0 Kb (SW 15) fragment and a 3.0 Kb fragment (SW 2) (Michel 1993). The probes were developed from the WC 11 strain of A1HV-1 , and hybridized successfully with it. Neither of these two fragments hybridized with infectious bovine rhinotracheitis virus, bovine herpesvirus 2 or bovine herpesvirus 4. The SW 2, SW15 probe successfully detected A1HV-1 DNA in the peripheral blood leukocytes of 7 of 10, three month old, free ranging wildebeest calves. Although it is likely that these calves were infected with A1HV-1, the cell cultures which were used to demonstrate the cytopathic effect characteristic of A1HV-1 failed to do so. The failure was due to death of the cultures and the results were considered to be inconclusive. The SW 15 probe failed to detect A1HV-1 DNA in the peripheral blood leukocytes of 40 cattle from a herd which had no contact with wildebeest or sheep and no previous MCF mortalities. Even though it is likely that these cattle were not infected with A1HV-1, their true infection status can only be suggested, since the cell cultures used to confirm the presence of A1HV-1 in this study provided inconclusive results. If, however, the wildebeest in this study were truly infected with A1HV-1 and the cattle were not, then the performance of this test for

detecting latent A1HV-1 infection would be reasonable (sensitivity = 70.0%, 95% CI 35.4-91.9, specificity = 100.0%, 95% CI 89.1-100.0). Establishing the true validity of this test will require further studies with known infected and uninfected animals.

There have been many tests developed for the detection of latent infections of ruminants with A1HV-1. All of these tests either performed poorly or their performances have not been adequately evaluated. This is surprising, since A1HV-1 has been successfully cultured and identified from infected animals for over 40 years (Plowright, Ferris *et al.* 1960). The major difficulty encountered when assessing the validity of newly developed tests is finding or producing study subjects of known infection status. The infection status of A1HV-1 uninfected ruminants could be established by virus isolation, multiple testing with different tests, or the production of specific pathogen free ruminants. The negative status of these ruminants could be strengthened by selection of study subjects from populations that are not exposed to other known MCF virus carrying ruminants. Since cell free strains of A1HV-1 exist, it would be possible to infect some of these ruminants with A1HV-1, thereby positively establishing the infection in these ruminants. Gold standard positive and negative blood, sera, and tissue samples could be collected from these known infected and uninfected ruminants and used to validate newly developed serology, PCR and other tests.

2.7.2 SA-MCF, and infection with OvHV-2

SA-MCF is a syndrome that is defined by the same histopathological observations that define WD-MCF (Plowright 1990; Schultheiss, Collins *et al.* 2000).

Classifying mortalities as being due to SA-MCF is therefore no more difficult than classifying mortalities as being due to WD-MCF. However, since the two diseases are pathologically the same, differentiating between SA-MCF and WD-MCF can be difficult. In the past, the two syndromes have often been differentiated by documenting the exposure of ruminants that die of MCF to latently infected hosts. MCF mortalities that have occurred in East Africa or in zoos where there has been exposure to wildebeest have been considered to be the result of WD-MCF (Plowright 1990). MCF mortalities that have occurred in other parts of the world, or where there has been exposure to sheep have been considered to be due to SA-MCF (Goetze 1930; Goetze, Liess 1929; Goetze, Liess 1930). Geographical location and exposure to sheep or wildebeest have not always been good predictors of the causative agent of MCF mortalities. An outbreak of MCF mortalities in Minnesota, in which there was an association with sheep, turned out to be caused by A1HV-1(Li, Shen *et al.* 1994; Li, Shen *et al.* 1996b). Both OvHV-2 and A1HV-1 have been independently identified from African MCF mortalities (Michel, Aspeling 1994; Plowright, Ferris *et al.* 1960). The identification of a novel MCF virus which was associated with MCF mortalities in white-tailed deer raises the possibility that in addition to SA-MCF and WD-MCF, there may be other forms of MCF (Li, Dyer *et al.* 2000). Since the syndromes of MCF cannot reliably be differentiated by pathology or epidemiological associations with MCF virus infected host species, their classification can only be achieved by demonstrating the presence of A1HV-1, OvHV-2, or some other MCF virus in the carcasses of MCF mortalities.

Developing diagnostic tests to detect infection with OvHV-2 has been severely

hindered by the resistance of OvHV-2 to propagation in tissue culture. Since OvHV-2 has not been propagated, cell free OvHV-2 has been unavailable for infecting study subjects. Failing to culture OvHV-2 has also made it difficult to positively establish the infection status of study subjects. Culturing OvHV-2 from a study subject would provide strong evidence of infection. Since OvHV-2 resists culture, failure to culture the virus from a study subject cannot be considered evidence that a subject is free from infection. Therefore gold standards for developing new diagnostic tests and establishing their validity have been difficult to obtain, and infection status of study subjects has been determined by other means.

The inability to propagate OvHV-2 in tissue culture, has made it impossible to conduct virus neutralizing antibody tests directly against OvHV-2. Although OvHV-2 shares some antigenic properties with A1HV-1 (Li, Shen *et al.* 1995), antibodies from sheep infected with OvHV-2, and cattle with SA-MCF have not neutralized A1HV-1 growth in tissue culture (Muluneh, Liebermann 1992; Rossiter 1981; Rossiter 1983). As a result, virus neutralizing antibody tests using A1HV-1 tissue cultures have been unsuccessful at detecting OvHV-2 antibodies.

2.7.2.1 Indirect immunofluorescent antibody test

The antigenic similarity between OvHV-2 and A1HV-1 has prompted the use of other A1HV-1 antibody detecting tests to detect antibodies to OvHV-2. The test most commonly used has been an indirect immunofluorescent antibody test (IIF). This test has been used to detect OvHV-2 antibodies in sheep (Li, Shen *et al.* 1994; Muluneh,

Liebermann 1992; Rossiter 1981), healthy cattle (Rossiter 1983), and cattle with SA-MCF (Rossiter 1983). The specificity of this test is considered to be poor since the test detects antibodies to other herpesviruses such as bovine herpesvirus 1, 2 and 4, as well as OvHV-2 and A1HV-1 (Li, Shen *et al.* 1994; Muluneh Liebermann 1992). Interpretation of this test's results must therefore be done with caution.

2.7.2.2 ELISA tests

Li (Li, Shen *et al.* 1995; Li, Shen *et al.* 1994) developed an indirect competitive inhibition enzyme linked immunosorbent assay (CI-ELISA) that detected an immunoglobulin G2b (monoclonal antibody 15-A) which binds to a carbohydrate dependent epitope (epitope 15-A) of MCF viruses. The antigenic epitope 15-A, was demonstrated to be conserved among 3 isolates of A1HV-1, as well as OvHV-2 and MCFV-WTD (Li, Shen *et al.* 1994). The authors concluded that antigenic epitope 15-A was an immunodominant epitope conserved among all known isolates of MCF viruses, and most ruminants would produce immunoglobulin G2b in response infection with an MCF virus. It was their opinion that the indirect CI-ELISA test would detect antibodies in most ruminants to infection with any MCF virus (Li, Shen *et al.* 1995; Li, Shen *et al.* 1994). The test did not detect antibodies to 13 other common sheep and cattle viruses including: ovine herpesvirus 1, caprine herpesvirus 1, OPPV, caprine arthritis encephalitis vuris, ovine adenoviruses 5 and 6, ovine respiratory syncytial virus, ovine parainfluenza virus 3, bovine herpesviruses 1, 2,and 4, bovine virus diarrhea virus, and bovine respiratory syncytial virus (Li, Shen *et al.* 1994). A cut-off value for the test was

established using sera from 5 to 6 month old lambs that were defined as being antibody negative by their age and by having negative indirect immunoflourecence (IIF) and immunoprecipitation (IPP) tests. Test sera were considered to be positive when the percent inhibition of the test sera was greater than three standard deviations above the mean percent inhibition of the negative sheep sera. The performance of the CI-ELISA test was compared to the IIF test in a sample of 149 sheep. The agreement between the two tests was good (Kappa = 73.83, 95% CI = 58.30 - 89.30). All of the CI-ELISA positive samples (N = 88) were positive when tested with the IIF test. Of the 61 sera that tested negative with the CI-ELISA test, 43 were negative, but 18 were positive when tested with the IIF test. The authors suggested a possible reason for the IIF test classifying 18 of the CI-ELISA negative sera as positive might be the IIF test's ability to detect antibodies to other herpesviruses which may have been infecting the study sheep (Li, Shen *et al.* 1994). In a subsequent study 136 of 143 sheep, which had OvHV-2 DNA in their peripheral blood leukocytes as determined by PCR assay, were CI-ELISA positive for OvHV-2 antibodies (Li, Shen *et al.* 1995). If PCR detection of OvHV-2 DNA in peripheral blood leukocytes can be considered an indication of true infection with OvHV-2, then the sensitivity of the CI-ELISA was 95.11 (95% CI = 89.79 - 97.84) in sheep. In the same study all serum samples from 59 pre-suckling lambs were CI-ELISA negative for OvHV-2 antibody. If pre-colostral lambs can be considered to have no OvHV-2 antibodies in their serum, then in this study, the specificity of the CI-ELISA test to detect latently infected sheep was 100.00 (95% CI = 92.13 - 100.00). These studies, although small, indicate that the indirect CI-ELISA performs well as a test for

classifying sheep with respect to their latent OvHV-2 infection status.

The indirect CI-ELISA test has never been validated as a test for classifying the latent OvHV-2 infection status of ruminants other than sheep. It has, however, been validated as a test for diagnosing SA-MCF mortalities in cattle (Muller Doblies, Li *et al.* 1998). The test was found to have a sensitivity of 59.46% (95% CI = 43.64 - 75.28) and a specificity of 100.00% (95% CI = 94.18 - 100.00) in a sample that included 75 healthy cattle, 37 cattle that died of MCF and 3 cattle that died of other diseases. The low sensitivity of the test was attributed to the rapid progression of MCF in cattle. It was suggested that some of the cattle with MCF died before they had time to mount an immune response (Muller Doblies, Li *et al.* 1998).

The indirect CI-ELISA was modified to a direct CI-ELISA by conjugating the monoclonal antibody15-A directly to horseradish peroxidase and by producing precoated dried antigen plates (Li, Mcguire *et al.* 2001). The new direct test had increased sensitivity when compared to the indirect CI-ELISA. The cut off value for the test was established using sera from a flock of sheep that had been weaned at 2 to 2.5 months of age and then maintained in isolation. The negative status of the sheep was confirmed by monthly PCR assays for OvHV-2 DNA. Serum samples were classified as positive when their percent inhibition was greater than 25%, which was three standard deviations above the mean percent inhibition of the uninfected sheep sera. The specificity of the test was 99.33% (95% CI = 97.35 - 99.89) in a sample of 300 sera, drawn by multiple samplings, from a flock of 19 OvHV-2 uninfected sheep. In a sample of 20 healthy bison, cattle and deer that were determined to be OvHV-2 antibody negative by IIF test; the direct CI-

ELISA had a specificity of 95.00% (95% CI = 85.18 - 98.70). In a sample of 48 adult sheep determined to be infected with OvHV-2 by PCR assay for OvHV-2 DNA; the sensitivity of the test was 100.00% (95% CI = 90.77 - 100.00). In a sample of 17 mouflon sheep determined to be OvHV-2 infected by PCR assay for OvHV-2 DNA; the sensitivity was 94.12% (95% CI = 69.24 - 99.69). The sensitivity of the test as a diagnostic test for SA-MCF was 98.25% (95% CI- 89.37 -99.91) in a sample of 57 cattle mortalities that were confirmed to be SA-MCF mortalities by histopathology and PCR assay for OvHV-2. From these limited data, it can be concluded that the direct CI-ELISA performs well as an instrument to correctly classify sheep with respect to their latent OvHV-2 virus infection status. The test also performs well as a test to detect OvHV-2 virus antibodies in cattle that died of SA-MCF. The ability of this test to correctly classify the latent MCF virus infection status of other ruminants cannot be established from this study, since the number of ruminants other than sheep in the study was very small.

2.7.2.3 PCR tests

Although OvHV-2 has not been cultured in tissue culture, infected lymphocytes from ruminants that have died from SA-MCF have been propagated in vitro (Reid, Buxton *et al.* 1983). Segments of OvHV-2 genomic DNA have been cloned from infected lymphocytes, and their base sequences determined (Bridgen Reid 1991). Although there was a great deal of similarity between the genomic DNA of A1HV-1 and OvHV-2, the authors were able to find DNA fragments in which there were base

sequence differences between the two viruses. From the λ8a subclone of a 5.2 Kb *Hind* III insert from lymphocyte line MF629, Baxter (Baxter, Pow *et al.* 1993) developed two sets of primers for a nested OvHV-2 PCR assay. The primers were considered to be unique to OvHV-2. The outer set of primers (556 and 755) amplified a 422 base pair fragment of OvHV-2 DNA. The nested primers (556 and 555) amplified a 238 base pair fragment. Both of the fragments could be cleaved with restriction endonuclease *RsaI* into fragments that were consistent with their predicted size from the known base sequence of the λ8a subclone. The primers did not amplify DNA from BHV-1, BHV-2, BHV-4, or A1HV-1.

Baxter's PCR assay has been used in many studies to detect latent OvHV-2 infections in sheep. The assay has detected OvHV-2 DNA in the peripheral blood leukocytes of 83% of 7 (Wiyono, Baxter *et al.* 1994), 99% of 144 (Li, Shen *et al.* 1995), 60% of 10 (Baxter, Wiyono *et al.* 1997), 90% of 178 (Mirangi, Kang'ee 1997), and 100% of 20 (Frolich, Li *et al.* 1998) healthy adult sheep. It consistently amplified OvHV-2 DNA in peripheral blood leukocytes drawn monthly from 10 adult ewes over a 12 month period (Li, Hua *et al.* 2001). These studies would suggest that the sensitivity of this PCR assay for detecting latent infections of sheep with OvHV-2 may be high. If it can be assumed that all of the sheep in these studies were infected with OvHV-2, then the sensitivity of the PCR ranged from 60% to 100% in these studies. However none of these studies attempted to establish the true prevalence of OvHV-2 infections in their study subjects. The true sensitivity of this PCR assay for detecting latent OvHV-2 infections in sheep cannot be established from these studies.

Baxter's PCR assay amplified OvHV-2 DNA in the peripheral blood leukocytes of 6.3% of 16 (Li, Shen *et al.* 1995), and 5.2% of 77 (Li, Snowder *et al.* 1998) pre-suckling lambs, as well as 0% of 4 lamb fetuses (Li, Snowder *et al.* 1998). If these lambs were truly uninfected with OvHV-2, then the specificity of Baxter's PCR was high in this study. It was 100% for lamb fetuses and ranged from 93.7% to 94.8% for lambs. The assumption that these lambs were not infected with OvHV-2 is a reasonable assumption. Lambs weaned younger than 2.5 months of age and raised in isolation from other sheep have been shown to remain uninfected with OvHV-2 as long as they are kept separate from other sheep (Li, Snowder *et al.* 1999; Li, Snowder *et al.* 2000). In one flock of 19 OvHV-2 uninfected sheep, raised under these conditions and sampled monthly for 18 months, all of the samples were negative for OvHV-2 DNA, when tested with Baxter's PCR (Li, Mcguire *et al.* 2001). Although the number of subjects were small, these studies suggest that the specificity of this PCR assay is quite high in sheep.

There have been no studies reported which validate Baxter's PCR assay for detecting latent OvHV-2 infections in other species of ruminants.

Baxter's PCR assay has proven to be a useful test for detecting OvHV-2 DNA in ruminants that died of SA-MCF. There have been several studies reported, which validated the PCR for this purpose. In all of these studies ruminant mortalities were classified as being SA-MCF mortalities by histopathology. Ruminants were classified as not being SA-MCF mortalities by routine diagnostic tests, or not having SA-MCF if they were alive and healthy. The results from these studies are presented in table 2.2.

The majority of the studies reported were conducted in cattle. There was only one

study of bison and one of red deer. The sensitivities ranged from 91.7% to 100.0%, with an over all sensitivity of 97.6% (95% CI = 95.2 - 99.1) and a median of 100%. The specificities ranged from 40.0% to 100.0%, with an over all mean of 90.7%(95% CI = 86.9 - 93.6) and a median of 100.0%.

Baxter's PCR assay performed very well as a test to detect OvHV-2 DNA in cattle and bison SA-MCF mortalities. The overall sensitivity of 97.6% suggests that the test produced on average only 2.4% false negative results. A comment should not be made on the test's ability to detect OvHV-2 DNA in red deer, since there was only one red deer SA-MCF mortality present in these studies. In most of the studies, the PCR produced very few false positive results. This was especially evident for cattle. In 5 of 7 studies that reported using the PCR on non-SA-MCF mortalities or healthy cattle, the test did not detect OvHV-2 DNA in any of the study subjects. This was not the case with red deer, where the test detected OvHV-2 DNA in 60% of the healthy red deer. There are several reasons why the PCR may have had poor sensitivity in red deer. The most probable is that there was some form of laboratory error that occurred during the performance of the assay. Alternatively the test may have been detecting some other MCF virus, or the red deer in the study may have actually been latently infected with OvHV-2. There is some support for this last hypothesis. It has been shown that 45% of weaned red deer fawns, (N = 40) and 45% of yearling red deer fawns (N = 40) had OvHV-2 DNA in their peripheral blood leukocytes, as detected by Baxter's PCR (Mackintosh, Tham 1998). In another study, PCR primers, developed from the base sequence of the WC11 strain of A1HV-1, amplified a 600 base pair fragment of DNA

Table.2.2 Reference number (Ref), species of study subjects, sensitivity (Se), specificity (Sp), 95% confidence intervals (95% CI), and number of study subjects (N) from studies that validated Baxter's PCR assay for OvHV-2 DNA in pathology specimens.

Ref[1]	Species	Se	95% CI	N	Spe	95% CI	N
1	Bovine	100.0	56.1 - 100.0	7	100.0	62.9 - 100.0	9
2	Bovine	91.7	59.8 - 99.6	12	100.0	71.7 - 100.0	13
3	Bovine	100.0	62.9 - 100.0	9	N/A		
4	Bovine	100.0	84.5 - 100.0	27	62.8	46.7 - 76.6	43
8	Bovine	96.7	81.5 - 99.8	31	100.0	88.6 - 100.0	38
5	Bovine	97.4	84.6 - 99.7	38	96.9	90.7 - 99.2	98
6	Bovine	91.9	72.0 - 97.9	37	100.0	89.3 - 100.0	41
7	Bovine	100.0	31.0 - 100.0	3	N/A		
9	Bovine	100.0	92.8 - 100.0	63	100.0	89.9 - 100.0	43
4	Red deer	100.0	5.5 - 100.0	1	40.0	17.6 - 67.1	15
8	Bison	100.0	91.6 - 100.0	53	90.9	57.1 -99.5	11
Total	**All**	**97.6**	**95.2 - 99.1**	**281**	**90.7**	**86.8 - 93.6**	**311**

[1] 1. Baxter, Pow *et al.* 1993, 2. Wyono, Baxter *et al.* 1994, 3. Li, Shen *et al.*1995, 4. Tham 1997, 5. Muler Doblies, Li *et al.* 1998, 6. Crawford, Li *et al.* 1999, 7.Hua, Li *et al.*1999, 8. Collins, Bruns *et al.* 2000, 9.Hussy, Stauber *et al.* 2000.

from red deer peripheral blood leukocytes (Tham, Ng *et al.* 1994). The red deer in this study were infected with leukocytes originating from red deer that died of SA-MCF. The amplified 600 base pair fragment was of unknown origin, since the primers from this assay were shown to amplify a 413 base pair fragment from both A1HV-1 and OvHV-2 genomic DNA. These studies suggest that red deer may be latently infected with some other, as yet unknown, MCF virus.

A PCR assay has been reported that amplified a 274 base pair fragment of OvHV-2 genomic DNA (Schenz, Paernthaner *et al.* 2000). The assay successfully amplified OvHV-2 DNA fragments in 18 SA-MCF cattle mortalities. The mortalities were confirmed to be SA-MCF mortalities by histopathology. There have been no subsequent validation studies reported for this PCR assay.

Hussy *et al* (Hussy, Stauber *et al.* 2001) developed a quantitative flourogenic PCR assay that successfully amplified a 131 base pair segment of OvHV-2 genomic DNA. In peripheral blood leukocytes from a sample of 20 known OvHV-2 infected and 20 known OvHV-2 uninfected sheep, the sensitivity and specificity of this PCR assay for classifying sheep with respect to their latent OvHV-2 infection were both100% (95% CI = 80.0 - 100.0). The PCR assay amplified OvHV-2 DNA in the peripheral blood leukocytes of all 63 cattle that were confirmed by histopathology to have died of SA-MCF. From this sample the sensitivity of the assay for detecting OvHV-2 in SA-MCF mortalities was 100.0% (95% CI = 92.8 -100.0). The PCR assay failed to amplify OvHV-2 DNA in all of the peripheral blood leukocyte samples of 43 healthy cattle. The specificity of the PCR for detecting OvHV-2 infection in healthy cattle in this study was

100.0% (95% CI = 89.8 - 100.0).The performance of Hussey's PCR assay was compared to that of Baxter's PCR assay (Hussy, Stauber *et al.* 2001). In both the sheep and cattle populations cited above there was complete agreement between these two tests. Although the number of study subjects was small, this study suggests that Hussey's PCR assay is a good test for classifying cattle SA-MCF mortalities with respect to their OvHV-2 infection status, and sheep with respect to their latent OvHV-2 infection status. The assay has not been validated as a test for classifying the latent OvHV-2 infection status of other ruminants

2.8 Which test to use.

Selection of a diagnostic test to classify bison with respect to their latent OvHV-2 infection status, has been difficult. An ideal test would be one that is both sensitive and specific for OvHV-2 infection. If the validity of the selected test had not been established for bison, it should at least have been established for other ruminants. If such validation studies were available they could be used to make inferences about the potential performance of the test in bison. In addition to being valid, the selected test should be readily available, easy to perform, reliable and economical for large scale use.

All of the diagnostic tests that have been developed to detect both latent A1HV-1 infections of ruminants, and A1HV-1 infections of WD-MCF ruminant mortalities have been poorly validated. This alone would be enough to reject these tests for use in this study. Although OvHV-2 and A1HV-1 are closely related viruses (Bridgen, Reid 1991; Li, Shen *et al.* 1995), there are differences in both their genomic DNA base sequences

(Bridgen, Reid 1991) and their antigenic characteristics (Mulune, Liebermann 1992; Rossiter 1981; Rossiter 1983). These differences ensure that before any of these tests could be justifiably used to detect latent infections of bison with OvHV-2, their complete validation would be required.

There have been two types of diagnostic tests developed for detecting MCF virus infections. These are PCR assays and CI-ELISA tests. Baxter's PCR assay for OvHV-2 DNA is the most likely of the PCR candidates (Baxter, Pow *et al.* 1993). It is the most extensively validated PCR test and is the test that is used for most routine diagnostic procedures (O'Toole, Li *et al.* 2002). In addition this test has been validated, in one small study, as a test for detecting OvHV-2 DNA in bison that have died of SA-MCF (Collins, Bruns *et al.* 2000). The assay was very good for detecting OvHV-2 infections in sheep (Baxter, Wiyono *et al.* 1997; Li, Shen *et al.* 1994; Li, Shen *et al.* 1995), but has not been validated as a test for detecting latent OvHV-2 infections in bison, or any other ruminants.

It may be inappropriate to use the studies that validated Baxter's PCR assay in sheep to estimate the validity of the test in bison. Where as most adult sheep are both CI-ELISA positive for OvHV-2 antibodies in their serum and PCR positive for OvHV-2 DNA in their peripheral blood leukocytes (Li, Shen *et al.* 1994; Li, Snowder *et al.* 2000), this may not be the case with bison. In one bison feedlot study, only 8 of 71 bison that were CI-ELISA positive for MCF virus antibodies were PCR positive for OvHV-2 DNA in their peripheral blood leukocytes (O'Toole, Li *et al.* 2002). There may be several explanations for these findings. The PCR may have failed to detect OvHV-2 DNA that

was present in bison peripheral blood leukocytes, or bison that were latently infected with OvHV-2 did not have OvHV-2 DNA in their peripheral blood leukocytes. The PCR products from the 8 PCR positive bison were not sequenced, so it is possible that the PCR assay, although using primers specific for OvHV-2, may not have detected OvHV-2, but rather some other closely related virus that infected the PCR positive bison. Conversely the CI-ELISA may have detected antibodies produced by infection with some other closely related virus in the 71 CI-ELISA positive bison.

A biologically plausible hypothesis for the difference in the performance of these two tests is that those bison which were CI-ELISA positive were truly infected with an MCF virus, and that Baxter's PCR assay failed to detect OvHV-2 DNA in their peripheral blood leukocytes. Baxter's PCR may have failed to detect OvHV-2 DNA in these bison because they were either infected with another MCF virus, which was stimulating MCF virus antibody production, or they were infected with OvHV-2, but were not viremic at the time they were sampled. It would not necessarily be uncharacteristic of a host species population that was latently infected with an MCF virus to have few viraemic individuals in the population at any one time. Of the two host species for which most of the research has been reported, adult sheep are viremic all of the time, as determined by PCR (Li, Snowder *et al.* 2000), where as adult wildebeest are rarely viremic as determined by virus isolation (Plowright 1990). However, there have been no reports published that assayed the peripheral blood leukocytes of a large population of wildebeest for the presence of AlHV-1 DNA using a PCR test. Although this hypothesis cannot be directly supported by any experimental or observational

studies, if it were accepted, it would suggest that the CI-ELISA was a much more sensitive test for detecting latent MCF virus infections than Baxter's PCR assay. It was for this potential lack of sensitivity that Baxter's PCR assay was not selected for use in this study.

Since OvHV-2 is a herpesvirus, it should, like other herpesviruses, produce life long infections in bison that survive infection. Although antibody levels can change over time in animals infected with herpesviruses, their infections should provide reasonably wide, repeated windows of opportunity for antibody detection. Cross sectional studies provide the best estimate of infection prevalence if the infecting agent is present in the study subjects for long periods of time, or if antibodies are repeatedly or continually detectable. Since the direct CI-ELISA detects antibodies, it was thought that it would provide greater opportunity to detect bison that were latently infected with OvHV-2 than would a PCR assay.

The final reason for selecting the CI-ELISA for this study was the availability and low cost of the test. Both of which allowed for testing of a large number of bison. In addition to looking at the prevalence of OvHV-2 infection of bison, the low cost of the test enabled the performance of validity studies on this test in bison

The challenges associated with the use of the CI-ELISA in this study will be those relating to the interpretation of the test results. Since the CI-ELISA detects antibodies produced in response to infection with any MCF virus, it will be difficult to draw conclusions as to which virus is infecting those bison which have positive test results.

3. CUT-OFF ESTIMATION AND RELIABILITY OF THE CI-ELISA

3.1 Introduction

In order for livestock disease surveys to be meaningful to farmers, veterinarians, animal health officials and researchers, survey instruments must provide reliable estimates of the disease or infection status of the animals being tested. The performance of a diagnostic test is characterized by two independent properties; accuracy and precision. Accuracy is a measure of the agreement between a test result and the true value being measured by the test. Precision, also called repeatability or reliability, is a measure of the amount of agreement between test results when the test is repeatedly applied to the same animal, or the same sample (Greiner, Gardner 2000). Precision and accuracy can be further subdivided into analytical precision and accuracy, and field or diagnostic precision and accuracy.

The analytical precision and accuracy of a test are concerned with the test's ability to correctly measure the specific antibody or analyte it is designed to measure, over repeated measurements, within an acceptable margin of error. Analytical precision and accuracy are performed in the laboratory with samples of known concentrations of the target analyte or antibody. They are usually the first studies carried out to evaluate a test's performance. If a test performs well in the laboratory it is further evaluated to establish the test's diagnostic precision and accuracy (Jacobson 1998).

The analytical accuracy of a test is measured by estimating it's analytical sensitivity and specificity. Analytical sensitivity relates to the ability of the test to detect

the target analyte at varying concentrations in test solutions. Analytical specificity relates to the tests failure to detect other closely related analytes and provides an estimate of the test's propensity to generate false positive results (Saah, Hoover 1997).

The analytical sensitivity and specificity of the CI-ELISA for detecting antibodies to an antigenic epitope (epitope 15-A) conserved among MCF viruses have been reported (Li, Mcguire *et al.* 2001; Li, Shen *et al.* 1994; Li, Shen *et al.* 1996b). Briefly, the test was able to detect antibodies produced against the15A antigenic epitope in sera from rabbits that were infected with 4 strains of MCF viruses, a calf infected with A1HV-1 and sheep infected with A1HV-1, over dilutions up to 1:640. The test did not react to 8 other common sheep and 5 other common bovine viruses. Readers are directed to the original reports for a more complete description of the analytical accuracy of the CI-ELISA (Li, Mcguire *et al.* 2001; Li, Shen *et al.* 1994; Li, Shen *et al.* 1996b). No studies of the CI-ELISA's analytical precision have been reported.

Diagnostic accuracy and precision are measures of the ability of a test to correctly classify animals with respect to their true infection or disease status, over repeated measurements (Jacobson 1998). Studies have been reported which assess the CI-ELISA's accuracy as a test for classifying the OvHV-2 infection status of cattle and bison that have died of SA-MCF (Li, Mcguire *et al.* 2001; Li, Shen *et al.* 1994; Li, Shen *et al.* 1996b; Muller Doblies, Li *et al.* 1998) and sheep that are latently infected with OvHV-2 (Li, Mcguire *et al.* 2001; Li, Shen *et al.* 1994; Li, Shen *et al.* 1996b). There have been no reported studies evaluating the accuracy of the CI-ELISA as a test for classifying bison with respect to their latent MCF virus infection status.

The diagnostic accuracy, (also called validity) of a test is evaluated by estimating the diagnostic sensitivity and specificity of the test in populations of study subjects with known infection status. Diagnostic sensitivity is an estimate of the probability that the test will yield a positive result when applied to a known infected animal. Diagnostic specificity is an estimate of the probability that the test will yield a negative result when applied to a known uninfected animal (Greiner, Gardner 2000).

The diagnostic accuracy of a test will be directly dependant on the tests ability to correctly classify animals as being positive or negative with respect to their disease or infection status based on their test results. Test results for many tests including ELISA tests are not binary, positive/negative distributions, they are continuous distributions. A value within the distribution must be selected in order to change the continuous distribution to a binary distribution. This value, called the cut-off value, splits the distribution into two groups, positive and negative. The diagnostic accuracy of a test with continuously distributed results will be heavily dependant on the cut-off value (Greiner, Gardner 2000).

ELISA test results are expressed as absorbance on a single serum dilution, the optical density (OD) of which will be a reflection of the antibody activity in the serum. In order to allow comparisons of the optical density of serum samples between test plates, the optical density of each sample is compared to that of negative or positive control sera that are included in each plate of test sera. The CI-ELISA for MAb 15-A uses uninfected sheep sera as a negative control and the comparison is expressed as a ratio or percent inhibition (PI) of the negative control sera. Since the OD of each sample is compared to a

control which is constant across all plates, the PI is used to calculate a cut-off value, which can be used on all test plates (Wright, Nilsson *et al.* 1993). The cut-off value is used to classify serum samples as either containing or not containing the test antibody based on the sample PI and ultimately to classify animals as having or not having the test antibody in their serum. Since PI values are continuously distributed, the cut-off value that is selected will directly affect the diagnostic sensitivity and specificity of the test (Barajas-Rojas, Riemann *et al.* 1993).

A cut-off of 25 PI of uninfected sheep control sera has been established for the CI-ELISA as a test to classify latent OvHV-2 infections in sheep and a small population of cattle and bison (N = 20) (Li, Mcguire *et al.* 2001). In this study the CI-ELISA performed reasonably well. Using a 25 PI cut-off, the CI-ELISA had a diagnostic sensitivity of 100% (95% CI = 90.77 - 100.00) and a diagnostic specificity of 99.33% (95% CI = 97.35 - 99.89) in sheep. In 20 healthy bison the test had a diagnostic specificity of 95.00% (95% CI = 85.18 - 98.70), and in bison that died of SA-MCF the test had a diagnostic sensitivity of 98.25% (95% CI = 89.37 - 99.91).

When known infected subjects are unavailable, cut-off values for ELISA tests have been calculated from the distribution of test results from populations of uninfected animals. The cut-off value is estimated by adding 2 or 3 standard deviations to the mean of the distribution of test results. An alternative method using the percentiles of the distribution has been suggested for cut-off estimation if the test results are not normally distributed (Jacobson 1998). This method of validation is not the preferred method since it provides no information about the diagnostic sensitivity of the ELISA and it is

recommended that when possible known infected animals should be included in the validation study (Greiner, Sohr *et al.* 1995). Two graph receiver operating characteristics (TG-ROC) curve analysis has been reported as a method for estimating the most efficient cut-off value from samples of known infected and un-infected study subjects. TG-ROC curve analysis estimates the cut-off value which maximizes the sensitivity and specificity at a point on the graph at which the sensitivity and specificity are equal (Greiner, Sohr *et al.* 1995)

Reported here are two studies. The first is a validity study which established a cut-off value for the CI-ELISA test when the test was used to classify bison with respect to their latent MCF virus infection status. The cut-off value was estimated from sera collected from two bison populations. One was a population of bison determined to be uninfected with OvHV-2 by biological means and the other a very small population of healthy bison that were determined to be infected by detecting OvHV-2 DNA in their peripheral blood leukocytes by PCR assay. A cut-off value was estimated using the mean plus standard deviations method as well as with TG-ROC analysis. Since the populations were very small, a boot strapping method was used to estimate confidence intervals for the cut-off value. Bootstrapping is a computer simulation method that can be used to estimate confidence intervals for population parameters, such as a population mean or in this case a cut-off value. In this study a large number of hypothetical data sets (10,000) were created by randomly drawing values, with replacement, from the original study population data set. Cut-off values were calculated from each hypothetical data set, and bootstrapped confidence intervals were estimated from the distribution of these cut-off

values (Efron, Tibshirani 1993).

This chapter also reports a reliability study which estimated the precision of the CI-ELISA test in paired bison sera. The precision was estimated from both the distributions of PI in the paired samples and the classifications of the paired sera using a 25 PI cut-off.

3.2 Materials and Methods

3.2.1 Validation study

Bison sera used in this study came from two sources. Uninfected sera were obtained from captive bison held in the Hook Lake bison recovery project isolation facility at Fort Resolution, North West Territories. This bison herd was established by capturing bison calves with a net gun from a helicopter shortly after calves were born. Calves were captured from free ranging bison cows belonging to the Hook Lake free ranging bison herd, which is a sub-population of the Wood Buffalo National Park bison herd. The herd is located in Northern Alberta and the North West Territories of Canada. A total of 62 calves were captured during the period from 1996 to1998. Four calves died shortly after capture, leaving 58 bison as the foundation stock for the herd. Since the study began there have been 28 calves born in the herd. The herd has been maintained in confinement since it's inception in 1996, with no contact being allowed between captive bison and any other ruminants. There were no domestic or free ranging sheep within hundreds of kilometers of the facility, nor has there ever been reported cases of MCF in the Hook Lake captive herd or it's parent herd. A total of 132 serum samples were

obtained for CI-ELISA analysis from sampling's during the years, 1998, 2000, and 2001. In October of 2001, samples of peripheral blood leukocytes were obtained from the Hook Lake captive herd for PCR analysis for OvHV-2 DNA.

Sera from known infected bison were obtained from a bison feedlot in the American Midwest. At capacity the feedlot held 4,600 bison and 5,000 cattle. From 300 bison enrolled in a previously reported study of MCF in this feedlot (O'Toole, Li *et al.* 2002), 11 male bison aged 12 to 24 months were identified as being infected with OvHV-2 by detecting OvHV-2 DNA in their peripheral blood leukocytes. Sera from these 11 bison were obtained for CI-ELISA analysis.

3.2.2 Reliability study

Sera for the reliability study came from 47 adult male, and 230 adult female bison on 15 farms. The number of bison sampled on each farm ranged from 8 to 42. The mean number of bison sampled per farm was 18. Bison were blood sampled by a Canadian Food Inspection Agency (CFIA) veterinarian during routine brucellosis and tuberculosis surveillance. Each bison was sampled on two occasions, with approximately 72 hours between the first and second sampling. Farms that were selected for this study included all those farms from which farm owners requested to have their bison tested for brucellosis and tuberculosis in one veterinary district in North Western Alberta, during the winter of 1999 and 2000. The bison selected on each farm were a convenience sample, selected by individual farm owners. Each sample was labeled with a number that was coded in such a way as to blind the laboratory to the identity of the sample.

3.2.3 Analysis

CI-ELISA tests were performed, as previously reported (Li, Mcguire *et al.* 2001; Li, Shen *et al.* 1994) at the Department of Veterinary Microbiology and Pathology at the Washington State University in Pullman, Washington. PCR analysis was performed at Prairie Diagnostic Services, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, using previously reported methods (Baxter, Pow *et al.* 1993; Muller Doblies, Li *et al.* 1998).

Descriptive statistics and Pearson's correlation coefficients were calculated with SPSS®10.0 (SPSS® Inc, Chicago Illinois, USA) statistical software. Confidence intervals for the mean percent inhibition of the feedlot, Hooklake and reliability samples were 95% confidence intervals estimated from the 2.5th and 97.5th percentiles of the distribution of the means of 10,000 non-parametric bootstrapped samples drawn from each original sample, using the bootstrapping module of MlwiN® software (Institiue of Education, London England) (Effron, Tibshirani 1993). Differences between the percent inhibition of the Hooklake and feedlot bison were tested for statistical significance at the 5% significance level using Kruskal-Wallis one way analysis of variance with SPSS®10.0 software. An optimal cut-off value for the CI-ELISA was estimated with two graph receiver operating characteristic curve analysis using CMDT software (Jens Briesofsky, Institute for Parasitology and Tropical Medicine, Berlin, Germany) (Greiner, Sohr *et al.* 1995). The confidence intervals for the cut-off value was a 95% confidence intervals estimated from 2.5th and 97.5th percentiles of the distribution of the cut-off values calculated for each of 10,000 bootstrapped samples of the feedlot and Hooklake

samples using CMDT software. The area under the receiver operating characteristic curve was also estimated using CMDT software. Confidence intervals for sensitivities and specificities were exact binomial 95% intervals for proportions calculated with Epi Info® 6 version 6.04 software (Centers for Disease Control and Prevention (CDC), USA and World Health Organization (WHO), geneva, Switzerland). Lin's concordance correlation coefficient for agreement between the percent inhibition of the first and second samples of the reliability study was calculated as previously reported (Shourkri, Pause. 1999).

3.3 Results

3.3.1 Validation study

For the purposes of this study the Hook Lake bison were considered to be truly uninfected with MCF viruses. PCR assays of peripheral blood leukocytes of these bison failed to detect OvHV-2 viral DNA. The distribution of the PI values (figure 3.1) in the Hook Lake population were significantly lower than those of both the feedlot bison and the reliability study bison (Kurskal-Wallis $p < 0.001$). In addition the Hook Lake bison were removed from their dams at a very early age, and were subsequently raised in isolation from any other ruminant sources of MCF viruses.

In order to allow easy visualization of the PI data distributions, the PI's were converted from percentages to decimals and value of one was added to each PI value. As a result of this transformation, samples with a PI = 1 had antibody activity that was the same as that of the negative control serum. Samples with PI < 1 had antibody activity

that was less than that of the control sera and those with PI > 1 had antibody activity that was greater than that of the control sera. The distribution of the percent inhibitions of the Hook Lake bison sera are presented in figure 3.1, and the descriptive statistics are presented in table 3.1.

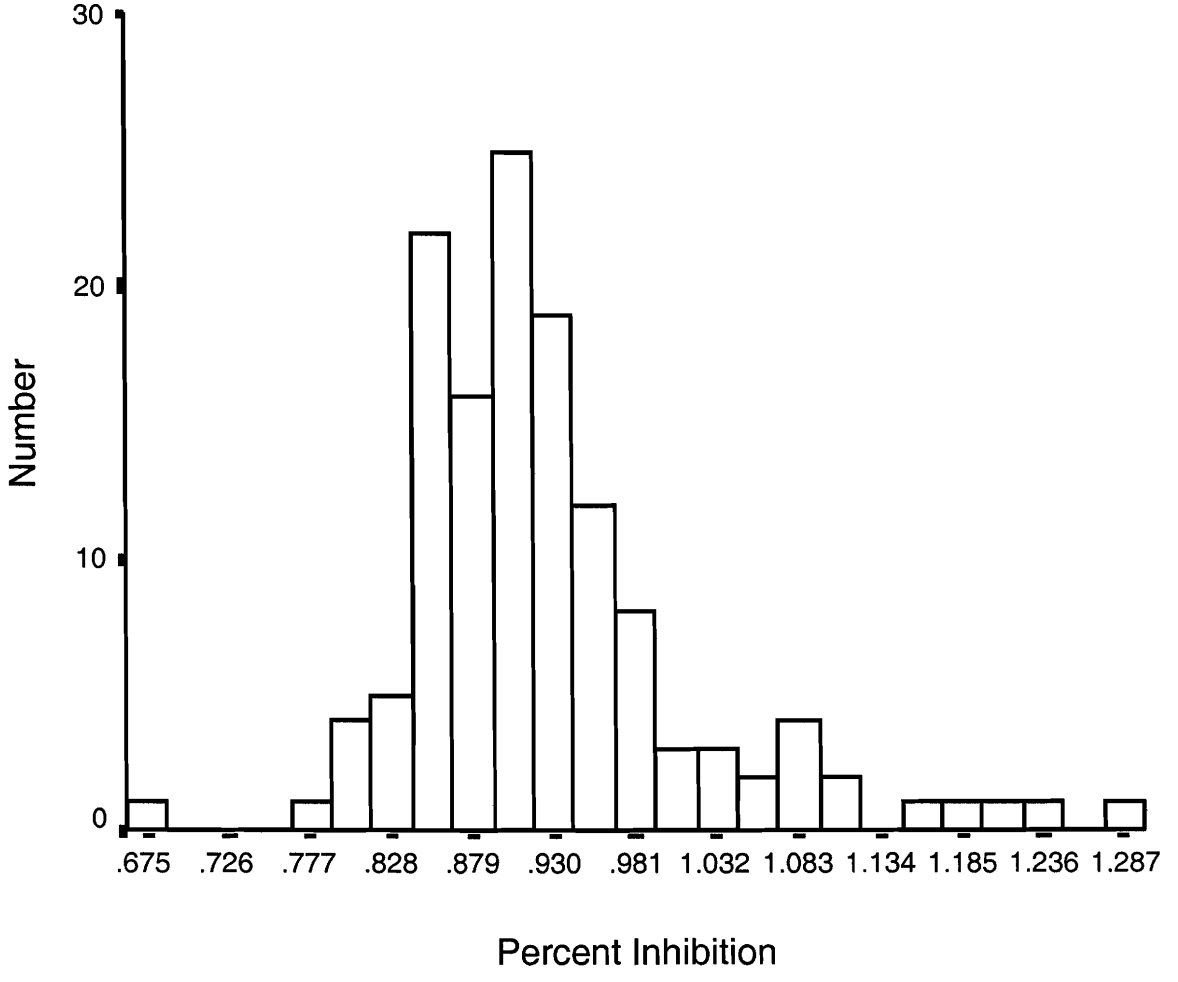


Figure 3.1 Histogram of the percent inhibition of sera from the Hook Lake bison.

Table 3.1 Number in group, mean, 95% confidence interval of the mean, standard

deviation(SD), minimum(Min) and maximum(Max) of the distributions of the percent inhibition of the feedlot (infected), Hook Lake(uninfected) bison and the first and second sampling's of bison for the reliability study (Reliability 1 and Reliability 2).

	Feedlot	Hook Lake	Reliability 1	Reliability 2
N	11	132	277	277
Mean	1.470	0.925	1.135	1.112
95% CI of mean[1]	1.392-1.554	0.910-0.941	1.108-1.163	1.082-1.141
Median	1.460	0.910	1.095	1.064
SD	0.143	0.091	0.237	0.253
Min	1.263	0.663	0.345	.13
Max	1.741	1.281	1.820	1.79

[1] 95% confidence intervals were calculated by bootstrapping with MLwiN, using 10,000 non-parametric iterations. Confidence intervals were chosen from the 2.5 and 97.5 percentiles

The distribution resembled a normal distribution, with a skew to the right. The mean of the distribution was 0.925 and the standard deviation was 0.091. The mean plus three standard deviations was 1.20, or 20 PI., which was on the 97.5 percentile of the distribution. If this value was used as a cut-off, the estimated specificity of the CI-ELISA in this population would be 97.727 (95% CI = 93.502 - 99.529). In normally distributed populations the mean plus three standard deviations should be close to the 99.9 percentile, which in this distribution was 1.269. If 1.25 were used as a cut-off, the estimated specificity of the CI-ELISA from the distribution of PI's in this population would be 99.242 (95% CI = 95.851 - 99.981).

For TG-ROC analysis, the Hook Lake population of bison were considered to be the uninfected or "gold standard" negative population and the 11 feedlot bison, by virtue of having OvHV-2 DNA in their peripheral blood leukocytes, were considered to be the infected or "gold standard positive" population. Figure 3.2 is a histogram of the combined distributions of the percent inhibition of the two populations. It can be seen from figure 3.2 that there is little overlap between the two distributions. There is wide separation between the means of the two populations and the 95% confidence intervals of the two means do not overlap. The two distributions were significantly different (Mann-Whitney U $p < .001$). The TG-ROC curve is presented in figure 3.3. The area under the ROC curve, although not evident from figure 3.3 was 0.999. The cut-off value which optimized both the sensitivity and specificity was estimated to be 1.256. Using this cut-off value the sensitivity and specificity estimates from these two populations are 100% (95% CI = 71.50 - 100.00) and 99.242 (95% CI = 95.851 - 99.981)

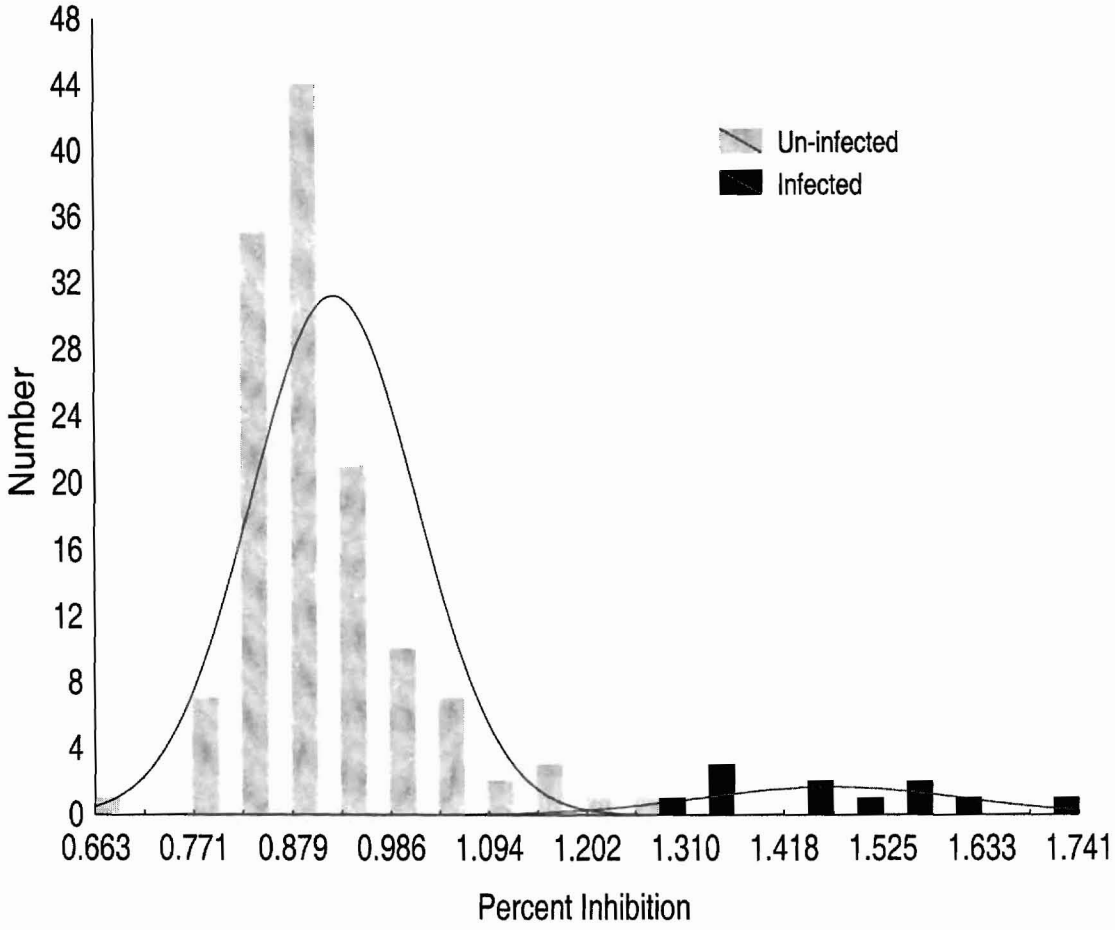


Figure 3.2 Combined histograms of the PI of infected (feedlot) and uninfected (Hook Lake) bison. Included are the estimated normal curves for the two distributions.

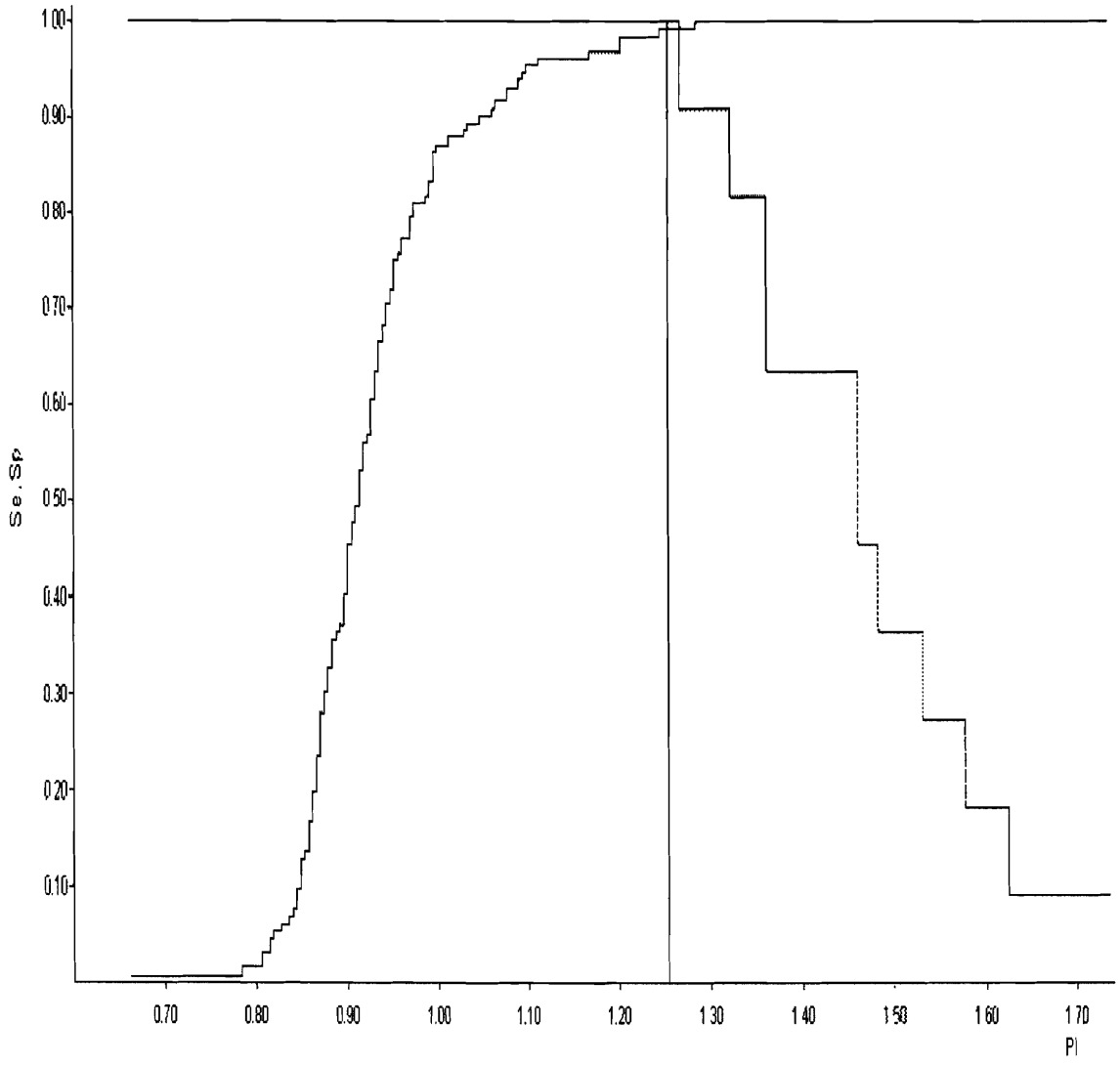


Figure 3.3 Two graph receiver operating characteristics curve of the sensitivity (Se) and specificity (Sp) of the CI-ELISA (Y axis) for all PI cut-off points (X axis). Included in the graph is the vertical line at the optimal cut-off PI value of 1.256 PI. The sensitivity curve begins on the lower left of the graph and rises to the right and the specificity curve begins on the lower right and rises to the left.

respectively. The cut-off estimated from 10,000 random samples drawn with bootstrapping was 1.257. The 95% confidence intervals from the distribution of 10,000 bootstrapped cut-off values was 1.186 - 1.301.

3.3.2 Reliability study

The descriptive statistics for the distributions of the two samples of the reliability study are presented in table 3.1 The mean PI of the two distributions are similar and there is overlap of the 95% confidence intervals of the means. To illustrate the agreement between the PI of paired samples, the PI of the first sample was plotted against the PI of the second sample for each pair of samples. Figure 3.4 presents this scatterplot with an estimated regression line. It can be seen from this graph that except for a few outliers, most of the points are well clustered around the regression line indicating a good deal of agreement between the paired PI's. Figure 3.5 presents a plot of the mean of the PI of the paired samples versus one half of the difference between paired sample PI, with the accompanying estimated regression line. From this plot it can be seen that most of the differences in PI between the paired samples cluster around the regression line regardless of the size of the mean PI, except for very low mean PI's. The smallest mean PI values had the largest difference between paired PI values. This gives the plot an arrow shaped appearance with the tip pointing in the direction of the largest PI values, and gives the estimated regression line a negative slope (slope = -0.038, intercept = 0.054, r = -0.118, p = 0.05).

Linn's concordance coefficient for agreement between the PI of the first and

second of the paired samples was 0.811 (95% CI = 0.772 - 0.851), which indicated a good deal of agreement between the paired PI.

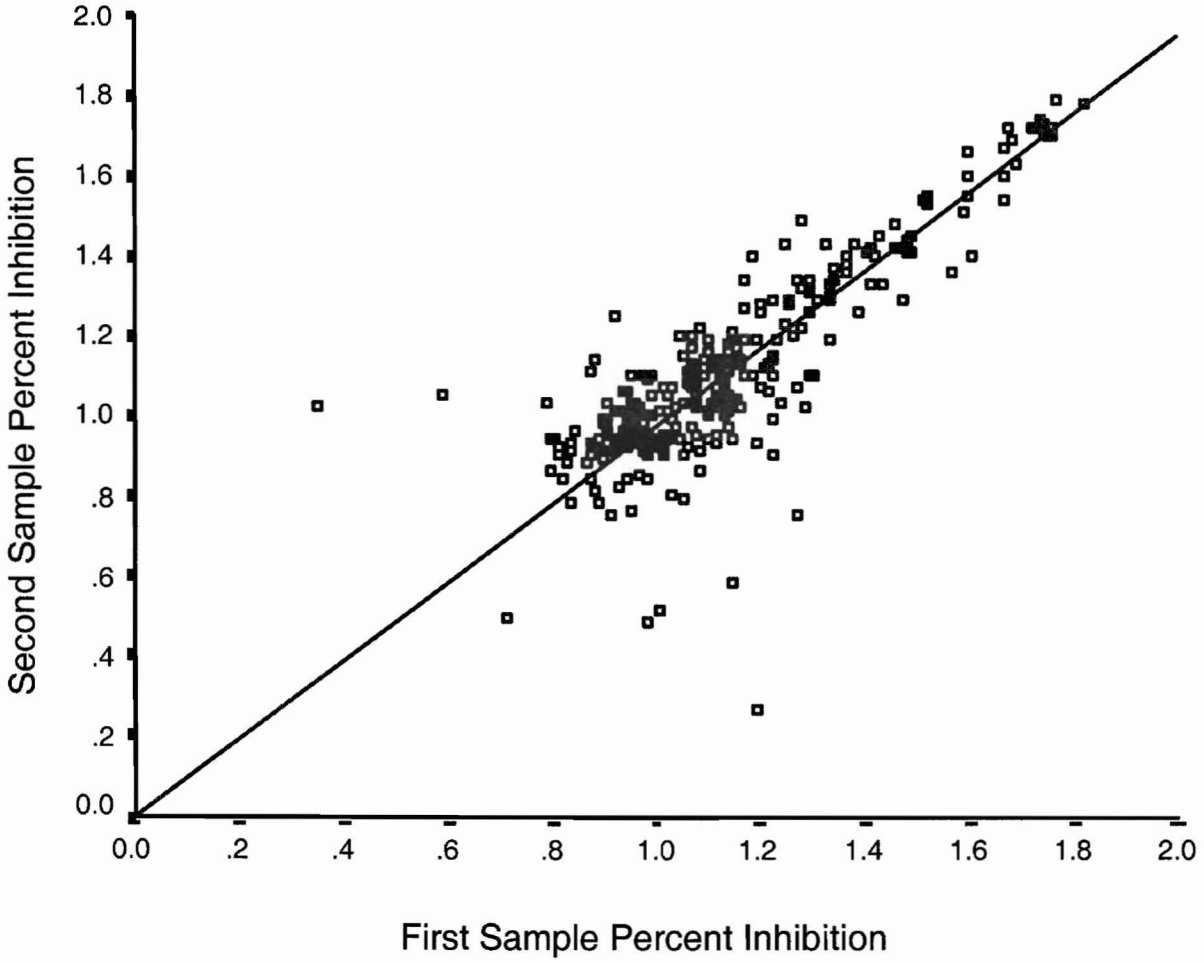


Figure 3.4 Scatterplot of the PI of the first sample against the PI of the second sample for paired serum samples from bison in the reliability study, with estimated regression line.

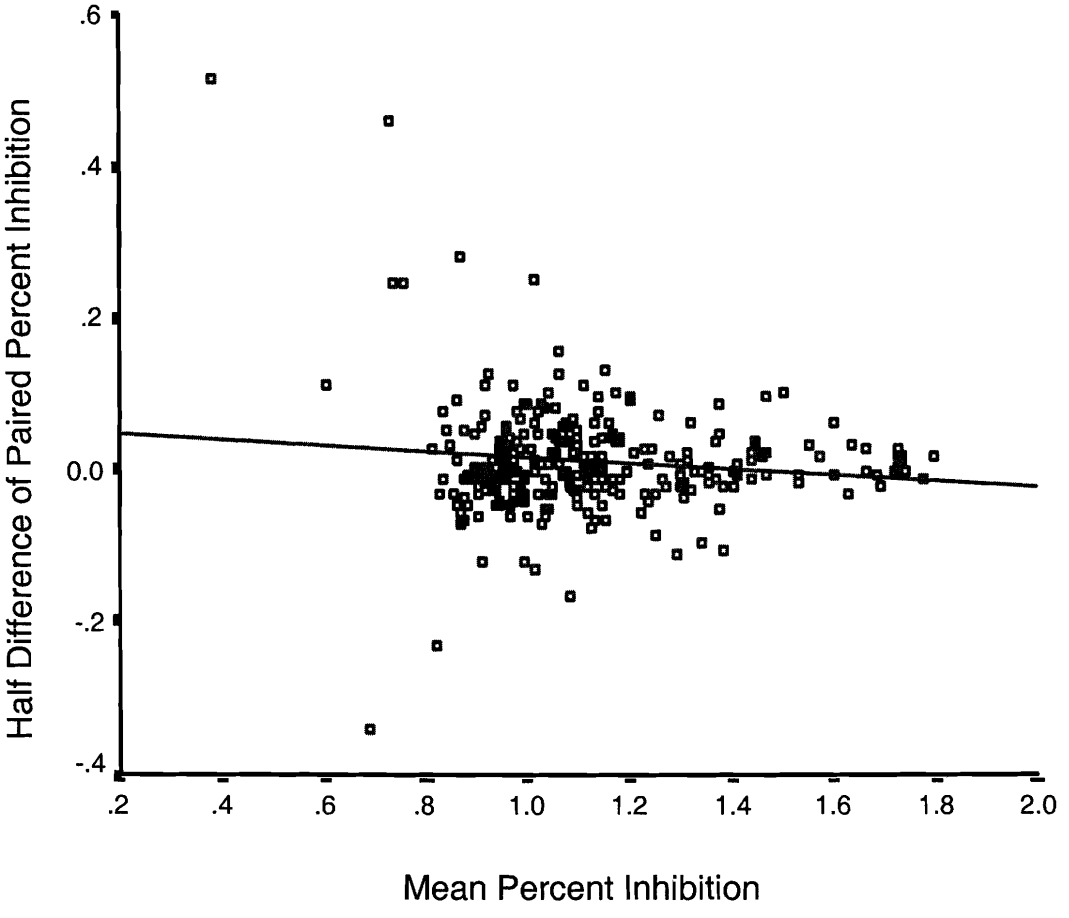


Figure.3.5 Scatterplot of the mean percent inhibition of paired bison serum samples (X axis) versus one half the difference of the percent inhibition of the same paired samples (Y axis), for the reliability study, with estimated regression line.

3.4 Discussion

3.4.1 Validity Study

Estimating the sensitivity and specificity of diagnostic tests for classifying

the infection status of animals requires the tests to be applied to populations of known infected and uninfected animals. Establishing the true infection status of animals is often difficult or impossible. In many cases the only methods available for verifying infection status require the use of other less than perfect tests (Jacobson 1998). It is possible in some situations however, to establish through biological or other means that animal populations are not infected with infectious agents. When this can be accomplished, the population of uninfected animals can be used to establish a test cut-off value. From the distribution of test results from this population a cut-off value is selected which is either 2 or 3 standard deviations away from the mean for normal distributions. If the distribution is skewed a cut-off value is selected that lies on a high percentile, usually the 99.9th percentile of the distribution (Jacobson 1998).

In order for this method to meaningfully classify animals with respect to their infection status, it is imperative that the test's ability to detect it's target antibody or analyte has been firmly established in previous studies (Jacobson 1998). Bison which die of SA-MCF are considered to be truly infected with OvHV-2 (Collins, Bruns *et al.* 2000; Dunowska, Letchworth *et al.* 2001; O'Toole, Li *et al.* 2002; Schultheiss, Collins *et al.* 1998; Schultheiss, Collins *et al.* 2000). These bison elicit CI-ELISA positive test results from their serum, indicating that the CI-ELISA does detect MAb 15-A in sera of bison that are known to be infected with OvHV-2 (Li, Mcguire *et al.* 2001; Muller Doblies, Li *et al.* 1998).

Populations can be classified as being uninfected by a number of methods. They can be selected from geographical regions where there is no infection, and from herds

with no history of infection. The population may be further defined as uninfected by testing with multiple tests, or by using biological criteria, such as lack of vectors for vector transmitted diseases (Jacobson 1998). The Hook Lake captive bison herd can be considered to be uninfected with OvHV-2 for a number of reasons. The bison which were initially captured to form the herd were removed from their dams very shortly after they were born. Removing lambs from their parent flock before they reach the age of 2.5 months, and raising them in isolation from other sheep successfully blocks the transmission of OvHV-2 from the sheep flock to the lambs (Li, Snowder *et al.* 1999). Even if the parent herd of the Hook Lake captive herd were infected with OvHV-2, it is probable that in a manner similar to lambs, removing the calves from the herd at a very early age effectively blocked transmission of OvHV-2 from the parent herd to the calves that formed the Hook Lake captive herd. Once the calves were captured, they were raised in complete isolation from any ruminants including sheep and other bison for the duration of the study. Maintaining the Hook Lake captive herd in isolation from other ruminants would have prevented infection of this herd with MCF viruses after their capture. The negative infection status of the herd was further confirmed by the failure of PCR assays to detect OvHV-2 DNA in the peripheral blood leukocytes of the bison in this herd.

Using the Hook Lake captive bison herd as a population of MCF virus uninfected bison allows the estimation of a cut-off value for the CI-ELISA test that should have a very high specificity. The distribution of PI in this population has a skew to the right. This is not unique to MCF viruses and this CI-ELISA test. ELISA's for other antibodies

commonly produce similar distributions when they are applied to sera from other uninfected populations (Jacobson 1998). Since the study subjects in these populations were uninfected, the mean PI for the population is always low. Using the mean plus three standard deviations as a method for calculating a cut-off value from populations such as these will result in the test producing a higher than predicted number of false positive results when the test is applied to other populations. The preferred alternative is to use percentiles to estimate the cut-off value (Jacobson 1998). The 99.9th percentile of the Hook Lake captive herd was 26.912 PI (1.26912), which is close to the cut-off of 25 PI established from studies of sheep (Li, Mcguire *et al.* 2001). With 25 PI as the cut-off, the estimated specificity of the CI-ELISA test in the Hook Lake captive herd was still quite high at 99.242% (95% CI = 95.851 - 99.981).

Every population of test positive animals is made up of two sub-populations. The first is a sub-population of animals that are truly infected, and the second is a sub-population of animals that are not infected, but produce false positive test results. Maximizing the specificity of a test minimizes the proportion of uninfected animals that are classified as being positive by the test. In other words, it maximizes the probability that an animal which tests positive is truly infected. The drawback to using only known uninfected animals to establish a test cut-off is that since there are no known infected animals available for test validation, the proportion of all infected animals that the test is able to detect remains unknown. Using a population of known uninfected animals to establish a cut-off maximizes the predictive value of a positive test result, but provides no information about the predictive value of a negative test result (Greiner, Sohr *et al.*

1995).

Identifying healthy live bison that are infected with MCF viruses is very difficult. Bison that die of MCF can be readily classified as being infected with OvHV-2 by demonstrating the presence of OvHV-2 DNA in their tissues (Collins, Bruns *et al.* 2000; Muller Doblies, Li *et al.* 1998). Since this study was aimed at classifying healthy bison with respect to their MCF virus infection status, as opposed to detecting the presence of OvHV-2 antibodies in bison that died of MCF, it was considered inappropriate to use bison that died of MCF as truly infected animals for the validity analysis. It is very difficult to identify bison that are truly infected with OvHV-2 in populations of healthy bison, since few healthy bison have been shown to have OvHV-2 DNA in their peripheral blood leukocytes (O'Toole, Li *et al.* 2002). This study was able to identify only 11 healthy bison that could be considered to be infected with OvHV-2, using PCR assays.

Although the sample of infected bison in this study was very small, the results of the TG-ROC analysis support the use of a 25 PI cut-off for classifying bison with respect to their MCF virus infection status.

Analysis of ROC curves and TG-ROC curves are methods which examine the covariance of sensitivity and specificity over various cut-off values (Greiner, Sohr *et al.* 1995). ROC curves are plots of the sensitivity versus one minus the specificity for all cut-off values. Measuring the area under the ROC curve provides an estimate of the tests efficiency or ability to discriminate between the infected and uninfected study subjects. The area under the curve for tests which have perfect sensitivity and specificity is one.

Tests with less than perfect sensitivity and specificity have steadily decreasing areas under the curve as the efficiency of the test decreases. ROC curves, while providing a good way of estimating the efficiency of a test, provide no easy method for estimating the most efficient cut-off value (Greiner, Sohr *et al.* 1995).

TG-ROC curve analysis provides an easier method for selecting cut-off values. With TG-ROC analysis the sensitivity and specificity are plotted as separate variables for each cut-off value. The point at which the two curves cross is the most efficient cut-off value, since it is the cut-off value at which the specificity and sensitivity are equal. In this study the most efficient cut-off value was estimated to be 1.256 (25.6 PI) (figure 3.3).

It is evident from figure 3.2 that the distributions of PIs from infected and uninfected bison populations in this study were separated. The normal curves of the two populations were distinct and there was little overlapping of the two curves. A cut-off value, selected in the area of the histogram where the two population distribution curves overlap should be very efficient, since the two curves cross at a point close to zero on the Y axis. The area under the ROC curve from this small validation study of the CI-ELISA test was 0.999. This value is very close to one, which indicates that in this study the CI-ELISA performed very well at discriminating between infected and uninfected bison. This is further reflected by the high sensitivity (100.0%) and specificity (99.242%) of the test when a cut-off value of 1.25 was used. Bootstrapping was used to assess the effect which random variation may have had on the selection of a cut-off value. In a population of 10,000 random samples drawn with replacement from this population, the mean cut-

off value (1.254) was very close to that which was calculated from the study population (1.256). The 95% confidence interval from the bootstrapping population (1.186 - 1.301) provides a estimate of where the mean cut-off value would lie, 95% of the time in studies of the same size. The large spread of the confidence interval is a direct reflection of the small sample sizes used in this study. Since the number of infected subjects was much smaller than the number of uninfected subjects in this study, mis-classifications are more likely to occur with negative test results than with positive. This is evident form the much larger confidence intervals estimated for the sensitivity of the test (71.50 - 100.00) as compared to those for the specificity (95.851 - 99.981).

The large confidence interval around the sensitivity estimate for the CI-ELISA suggests that negative test results may have poor predictive value when prevalence in the population under study is high. For example if the sensitivity of the CI-ELISA was 70% and the prevalence in the population was 75% the predictive value for a negative test result would be 52.43%, just slightly better than tossing a coin. Having low negative predictive value is unlikely to severely bias prevalence estimates from surveys unless the prevalence in the population was extremely high. However low negative predictive value would severely reduce the utility of this test for ruling out an MCF virus infection in a single bison in which a diagnostician had other evidence to suggest may be infected with an MCF virus. In this case the test would provide no additional information if the test result was negative.

Selecting 25 PI as the cut-off value for the CI-ELISA will ensure that test positive bison have a very high probability of being infected with an MCF virus. Since

there is less confidence in negative than positive test results, the CI-ELISA is more likely to under estimate the prevalence of MCF virus infections in bison populations than it is to over estimate it.

3.4.2 Reliability Study

Precision, or reliability is a very important property of a diagnostic test. The reliability of a test is a measure of the amount of random variation, or error that is inherent in a tests measurement. Tests which have poor reliability are tests which have a large amount of variation in test results when the test is applied repeatedly to the same study subject. A test with poor reliability will always perform poorly as a diagnostic test, since a test that demonstrates a large amount of variation when applied repeatedly to the same individual, will have a large amount of variation inherent within single measurements on one individual (Streiner, Normal 1995).

If there were complete agreement between paired test results from study subjects in a population, the scatter plot of the first result against the second result would produce a straight line with a 45 degree angle, which passes through the origin (Shourkri, Pause 1999). The scatter plot of the first versus the second of the paired PI's of the reliability study (figure 3.4) suggests that there is a good deal of agreement between the paired test results. The points cluster tightly around the estimated regression line and the regression line passes through the origin. The amount of agreement between the paired PI's could be assessed by calculating a Pearson product moment correlation coefficient. Pearson's coefficient measures a linear relationship, but fails to detect any departure of pairs from

the 45 degree line which passes through the origin. Lin's concordance coefficient provides a better measure of the agreement between pairs of test results, since it evaluates the degree to which paired results fall on the 45 degree line though the origin (Shourkri, Pause 1999). Lin's concordance coefficient for the paired PI test results from this study was 0.817, indicating a high degree of agreement between the pairs.

A scatter plot of one half of the difference between the paired PI's versus the mean of the paired PI's provides an indication of whether the precision of the test was uniform over the complete range of test values. In figure 3.5 it can be seen that in general the precision did not vary greatly over the complete range of mean test values except for the very lowest mean values. This indicates that the CI-ELISA had more variation or did not perform as well when the test results were at the low range of possible results. Since the extreme low values are far from the cut-off value, poor reliability in this range of test results should not significantly compromise the tests's ability to correctly classify bison with respect to their latent MCF virus infection status.

In addition to providing information about the performance of the CI-ELISA test, the reliability study also provides some information about the variability of the levels of antibody produced against antigenic epitope 15-A of MCF viruses in bison sera over short periods of time. Since the bison in this study were sampled at 72 hour intervals, the high degree of agreement between paired CI-ELISA test results, suggests that antibody levels did not vary significantly in bison over this time period. Establishing that there is little variation in test results over time provides some confidence in test results from bison sampled on only one occasion. The time interval, however is very short and

provides no information about long term fluctuations of antibody levels in bison sera.

There is some evidence from a previous study that levels of antibody in bison sera, produced against antigenic epitope 15-A of MCF viruses, may fluctuate over long periods of time (O'Toole, Li *et al.* 2002). In this feedlot study bison were classified with respect to their latent MCF virus infection status with the direct CI-ELISA using a cut-off of 25 PI. From three samplings of 256 bison over a 13 month period, 93 (36.4%) were classified as being positive on at least one of the three tests. Of these 93 bison, 34 (36.5%) were positive on only one test, 26 (28.0%) were positive on two tests and 33 (35.5%) were positive on all three tests. Viruses belonging to the MCF group of viruses are known to produce long term latent infections in their hosts (Coulter, Wright *et al.* 2001). If infection with MCF viruses in bison can be considered to be long term, at least of a longer duration than the time span of the feedlot study, and the specificity of the CI-ELISA can be accepted as being high, then the results of this study suggest that there may be considerable variation in antibody levels against antigenic epitope 15-A of MCF viruses within the sera of bison over long time periods. These results also suggest that the CI-ELISA will probably under estimate the prevalence of MCF virus infections in bison populations.

3.4.3 Summary

The CI-ELISA has been demonstrated to be a reliable test. A cut-off of 25 PI for the direct CI-ELISA, previously established for sheep, has been demonstrated to be an appropriate cut-off value for classifying bison with respect to their latent MCF virus

infection status. The long term variation of antibody levels against antigenic epitope 15-A in bison, and the wide confidence interval of the diagnostic sensitivity estimate suggest that this test may under estimate the prevalence of latent MCF virus infections in bison populations.

4. SERO-SURVEY OF FARMED BISON IN ALBERTA

4.1 Introduction

In North America it has been accepted that MCF is most commonly caused by a virus which infects large proportions of domestic sheep populations (Collins, Bruns *et al.* 2000; Muller Doblies, Li *et al.* 1998). This virus, although not as yet isolated or propagated in tissue culture has been named ovine herpes virus-2 (OvHV-2) (Coulter, Wright *et al.* 2001). In Europe and North America, MCF has been called sheep associated malignant catarrhal fever (SA-MCF) because of the observed association between sheep and MCF mortalities (Daubney, Hudson 1936; Goetze, Liess 1929; Goetze, Liess 1930). Although the association between OvHV-2 and MCF has been well documented in bison, there has been little support for an association between sheep and MCF in bison (Collins, Bruns *et al.* 200; O'Toole, Li *et al.* 2002; Schultheiss, Collins *et al.* 1998; Schultheiss, Collins *et al.* 2000).

OvHV-2 is a gamma herpesvirus which is genetically closely related to a group of viruses called the MCF-group (Li, Mcguire *et al.* 2001). Within this group are pathogenic viruses such as Alcelaphine herpesvirus-1(A1HV-1) and an un-named virus which causes MCF in white tailed deer (MCFV-WTD), as well as other viruses such as Hippotragine herpesvirus-1 (HiHV-1) which have not been shown to be pathogenic (Coulter, Wright *et al.* 2001; Li, Keller *et al.* 2001). Viruses within this group are well adapted to certain host species with which they are thought to have co-evolved (Coulter,

Wright *et al.* 2001; Dunowska, Letchworth *et al.* 2001). Large proportions of the populations of these species are sub-clinically infected, and it is only when the virus is transmitted to other less well adapted host species that MCF is produced (Dunowska, Letchworth *et al.* 2001). A1HV-1 sub-clinically infects large proportions of wildebeest populations, and produces MCF when the virus infects cattle (Plowright, Ferris *et al.* 1960). PCR studies have indicated that OvHV-2 sub-clinically infects large proportions of domestic sheep populations and produces MCF when it infects cattle, bison and deer (Collins, Bruns *et al.* 2000; Imai, Nishimori *et al.* 2001; Li, Snowder *et al.* 2000; Muller Doblies, Li *et al.* 1998). Large proportions of both adult sheep and adult wildebeest populations have been shown to have antibodies to their respective MCF viruses (Li, Shen *et al.* 1996a; Li, Shen *et al.* 1994; Plowright 1967). Sero-surveys of other ruminant species have demonstrated that only small proportions of the populations of susceptible species have antibodies to MCF viruses (Frolich, Li *et al.* 1998; Heuschele 1988; Li, Shen *et al.* 1996a). Bison, having mortalities ranging from sporadic to 100% in small herds (Collins, Bruns *et al.* 2000; Ruth, Reed *et al.* 1977; Schultheiss, Collins *et al.* 2000), can be considered to be a species that is highly susceptible to MCF.

A previous survey of bison using an indirect CI-ELISA (Li, Shen *et al.* 1994) indicated that only 2% of 103 bison had antibodies to MCF viruses (Li, Shen *et al.* 1996a). A more recent serological survey of a small group of bison in Alberta and the United states, using a more sensitive direct CI-ELISA (Li, Mcguire *et al.* 2001), demonstrated that 18% of 270 bison had antibodies to MCF viruses (Li, Mcguire *et al.* 2001). A prevalence of 18% in healthy bison suggests that a large proportion of the

bison in this study were exposed to MCF viruses without developing clinical MCF. This study, however was based on a small sample size and may not be representative of the entire bison population.

The purpose of chapter 4 of this thesis will be to establish the prevalence of latent MCF virus infections in a large sample of farmed bison in Alberta. Bison serum samples were collected from a bison serum bank located at Fairview Alberta, two Alberta slaughter plants and five bison herds. Bison were classified as being latently infected using a direct CI-ELISA test which detects an antibody response to antigenic epitope 15-A in the serum of bison. Antigenic epitope 15-A is conserved among all viruses belonging to the MCF group. A cut-off of 25 PI of negative control sheep serum was used to classify bison with respect to their infection status. Prevalence estimates with confidence intervals were calculated with a Rogan-Gladen prevalence estimator. Rogan-Gladen prevalence estimates and confidence intervals are sensitive not only to the size of the population under study, but also to the size of the populations used to establish the test's sensitivity and specificity (Greiner, Gardner 2000).

4.2 Materials and methods

4.2.1 Fairview serum bank

Beginning in November 1996, Canadian Food Inspection Agency (CFIA) veterinarians established a bank of bison sera located at the Alberta Agriculture Animal Health Laboratory in Fairview Alberta. Sera for the serum bank were the extra aliquots of serum from those bison tested during routine brucellosis surveillance in the Peace

River district of Northwestern Alberta. The farms sampled were self selected by those farm owners who requested their bison be tested in order to maintain their negative herd status, or to allow the movement of bison off the farm. Bison sampled were all 18 months of age or older. Samples collected from individual farms could have included the entire population of bison on the farm which were over 18 months of age, or only a part of that population. If a part of the on farm population was sampled, the sample was selected as a convenience sample by the farm owner.

Within the serum bank, sera were organized into pods. Pods were labeled with a pod number and the date of collection. Each pod could contain sera collected from one or more farms. Information as to the number of farms in each pod or the number of bison belonging to each farm within each pod was not available to this study. At the time of the study there were 115 pods containing 4384 serum samples available for sampling. The mean, median and mode of the distribution of the number of samples per pod were 38, 19, and 10 respectively. A histogram of the distribution of pods by their size is presented in figure 4.1

A sample size calculation for a population of 4384 with a prevalence of 25% and a precision of 2.5% estimated the sampling fraction for the serum bank to be 913, if random sampling procedures were used. This sampling fraction would provide an exact binomial 95% confidence interval of 22.57 - 27.43 if there was an apparent prevalence of 25% in the population and 0.44 - 1.56 if there were an apparent prevalence of 1%.

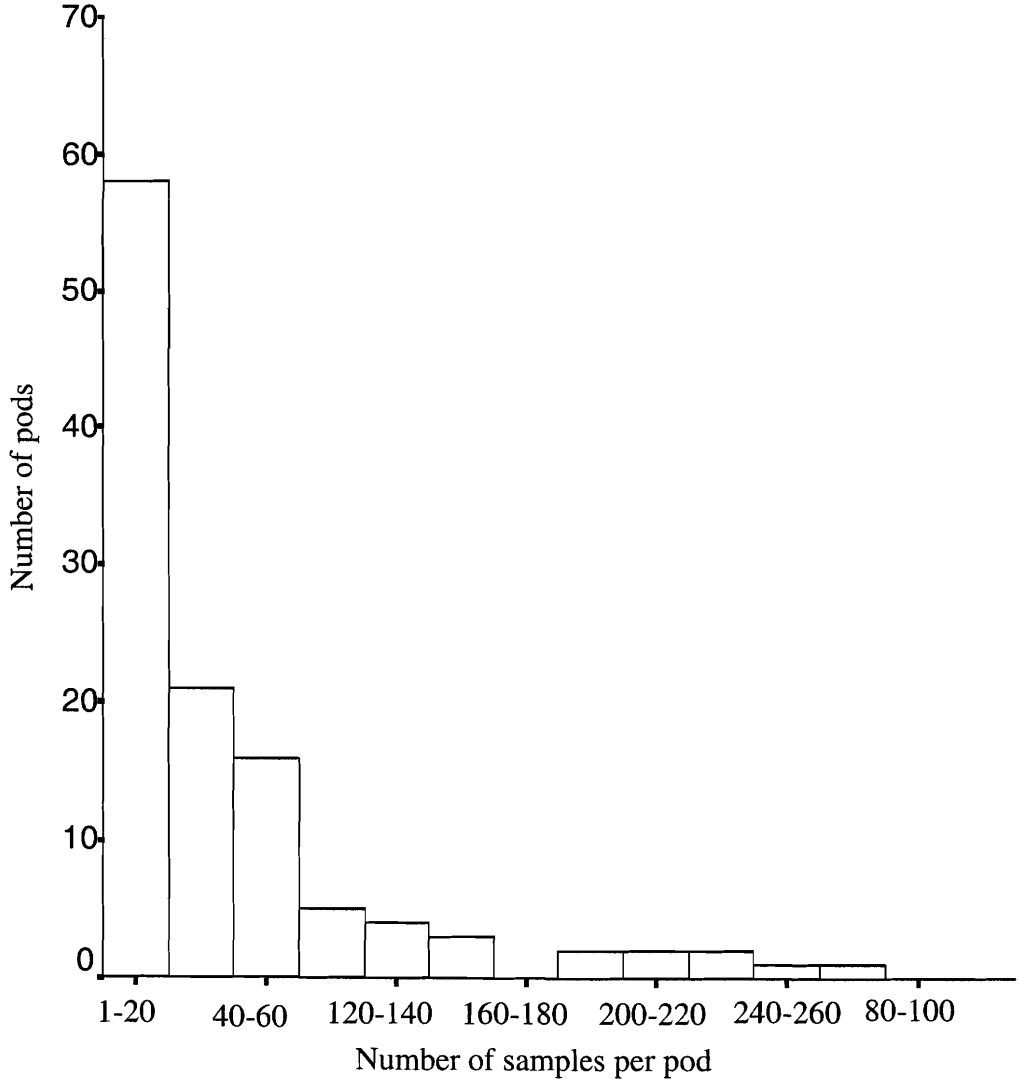


Figure 4.1 Histogram of the number of serum samples in each pod of the Fairview serum bank

Individual identification numbers were not available for each sample, so the pods were sampled proportional to their size. In general every 5^{th} sample was selected from each pod until the required number of samples were collected. There were 953 samples collected from 115 pods. The mean, median and mode of the distribution of the number of samples per pod were 8, 5 and 1. A histogram of the distribution of pods by the number of samples collected per pod is presented in figure 4.2.

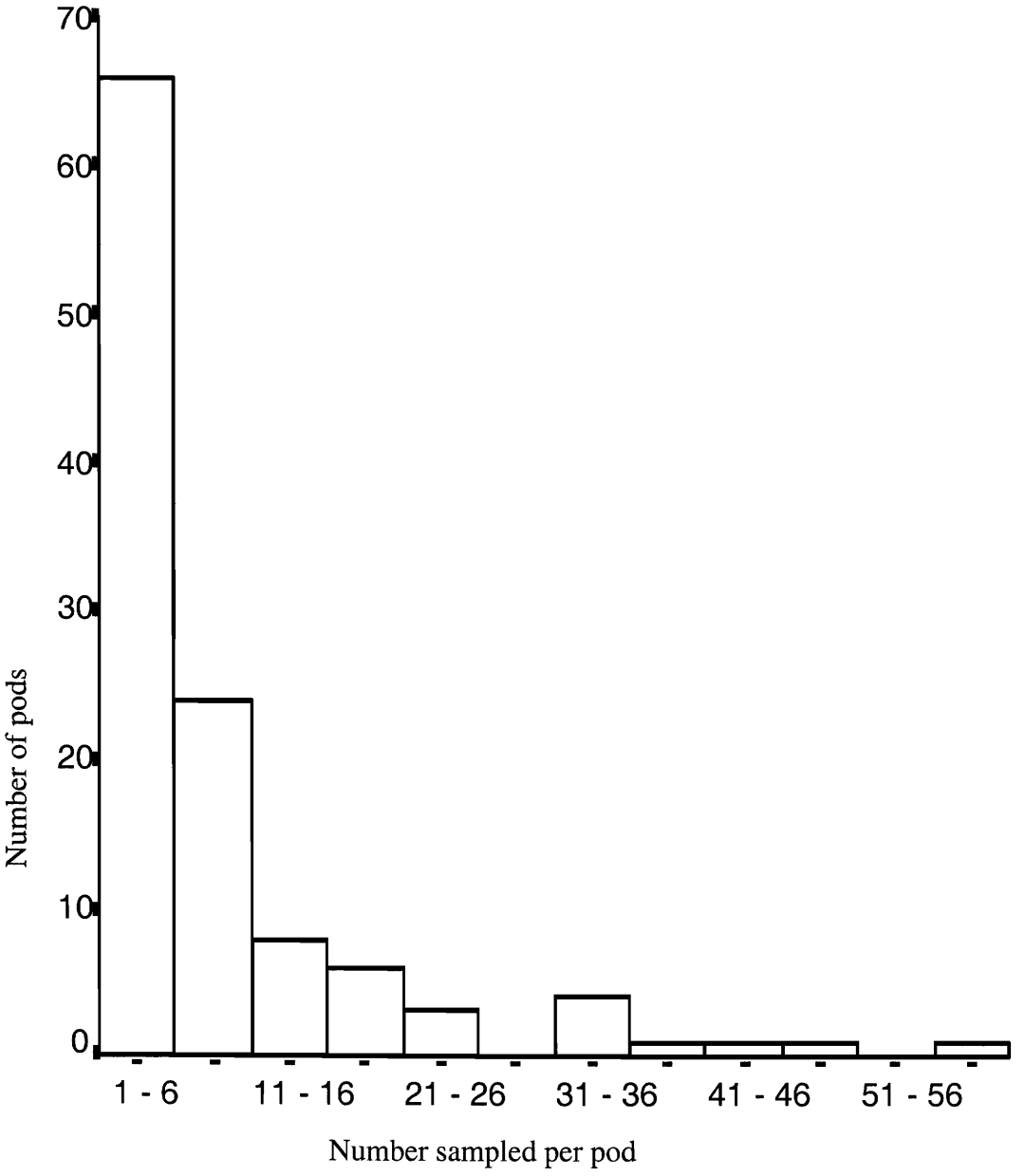


Figure 4.2 Histogram of the number sampled per pod from the Fairview serum bank.

4.2.2 Slaughter survey

During the period from April 7th, to September 8th of 2000, serum samples were collected from bison that were slaughtered at two slaughter plants in Alberta. The age and sex of the slaughtered bison was unknown. However, it has been reported that almost all bison commercially slaughtered in Canada are males between the ages of 18 and 30 months, with an average live weight of 950 pounds (Armstrong, Ayars *et al.* 1998; Nixdorf, Barber *et al.* 1998). Information about the origin of the bison prior to reaching the slaughter houses was not available to the study.

Collections were made on all days in which bison were being slaughtered during the period of the study. On each slaughter day, all bison submitted for slaughter were collected. Free flowing blood from severed jugular veins was collected into glass tubes at the time of slaughter. In total 995 blood samples were collected on 24 slaughter dates. The number of bison sampled per slaughtered date ranged from 8 to 71 samples. The mean median and mode of the number of samples collected on each slaughter date were 41.4, 44.0 and 31.1 respectively.

4.2.3 Cow-calf study

During the period from October 17, 2000 to February 20, 2001, serum samples were obtained from 5 bison herds. Four of the herds were located within a 100 km radius of the city of Edmonton, Alberta, and one herd was located in the Peace River district of North Eastern British Columbia. Herds selected for the study were restricted to those herds from which the herd owners allowed the principle investigator to collect samples.

Herds were also selected to provide a more or less even distribution of collection dates over the course of the winter. Herds 2 and 5 had never experienced mortalities due to MCF. Herds 1, 3 and 4 had reported mortalities due to MCF within the last 6 months prior to sampling. An attempt was made to collect serum from all of the cows and calves on each farm. Mechanical failure of equipment due to cold weather, escape of bison, and other logistical problems prevented the complete collection of all bison cows and calves in herds 2, and 3. Samples were collected into glass tubes by coccygeal or jugular venipuncture.

4.2.4 Analysis

CI-ELISA tests were performed, as previously reported (Li, Mcguire *et al.* 2001; Li, Shen *et al.* 1994) at the Department of Veterinary Microbiology and Pathology at the Washington State University in Pullman, Washington. In order to control for potential effects of pod and slaughter day clustering on odds ratios, the odds ratio and confidence interval for the comparison of the CI-ELISA positive prevalence between slaughter survey bison and Fairview serum bank bison were calculated in a mixed-effect generalized linear model with slaughter day/pod as a random effect using PROC.GLIMMIX, SAS® version 8.1 software (SAS Institute Inc, Cary, NC.). In order to control for the potential effect of clustering by herd, the odds ratio and confidence interval for the comparison of the CI-ELISA positive prevalence between cow and calf bison were also calculated in a mixed-effects generalized linear model with herd as a random effect using PROC.GLIMMIX, SAS® version 8.1 software (SAS Institute Inc,

Cary, NC.). All other odds ratios, *p* values (Chi-square approximations) and confidence intervals were calculated with Epi Info® 6 version 6.04 software (Centers for Disease Control and Prevention (CDC), USA and World Health Organization (WHO), Geneva, Switzerland). A 5% significance level was used for hypothesis testing. Descriptive statistics were calculated with SPSS® 10.0 (SPSS Inc, Chicago Illinois, USA) software. Sample size calculations were for estimating prevalence and were performed with Win Episcope® 2.0 software (Ignacio de Blas, Carmelo Ortega, University of Zaragoza Spain; Klaas Frankena, Wageningen University, The Netherlands; Jos Noordhuizen, Utrecht University, The Netherlands; Michael Thrusfield, University of Edinburgh, United Kingdom). Rogan-Gladen prevalence estimates and confidence intervals were calculated as previously reported (Greiner, Gardener 2000).

4.3 Results

A histogram of the distribution of the CI-ELISA positive apparent prevalence among the pods of the Fairview serum bank is presented in figure 4.3. It is evident from this distribution that zero prevalence was the predominant prevalence among the Fairview pods. Many of the pods were small and since pods were sampled proportional to size, the median and mode number of samples per pod was 5 and 1.

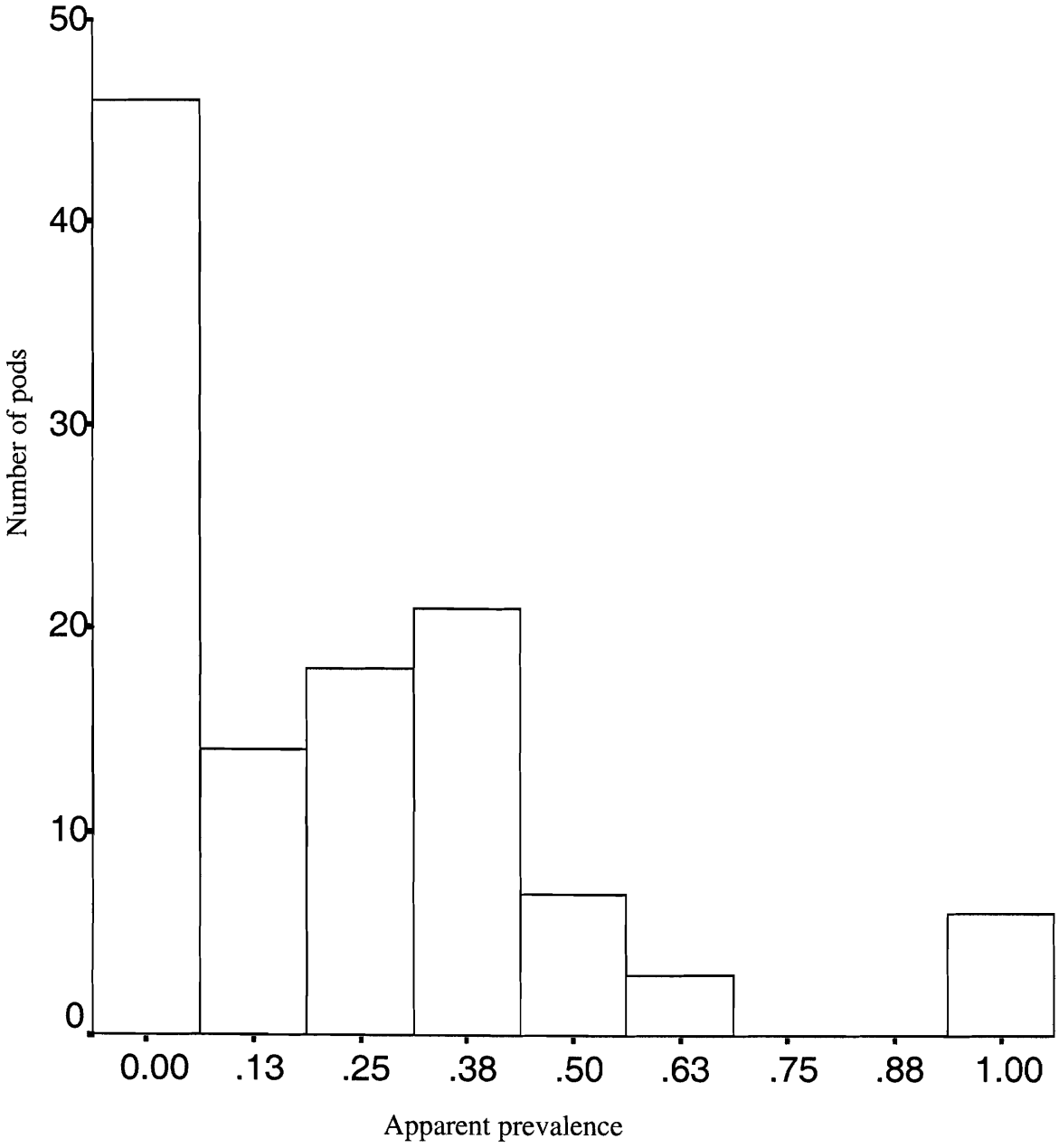
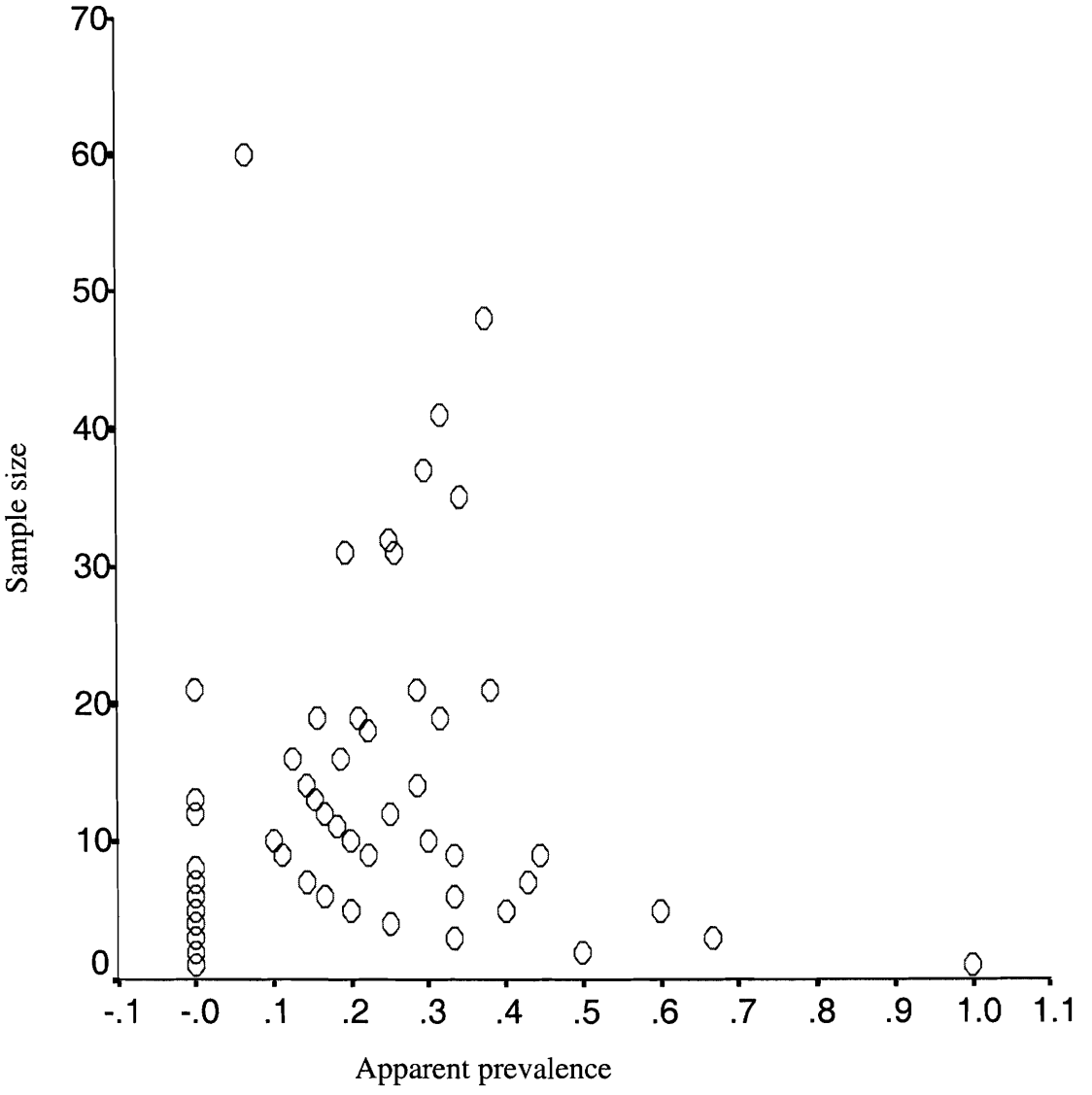


Figure 4.3 Histogram of the apparent prevalence of CI-ELISA positive bison sera among the pods in the Fairview serum bank

The relationship between sample size and prevalence is presented in figure 4.4, which is a scatterplot of the pod sample size versus the apparent prevalence of the pods in the Fairview serum bank. Figure 4.4 demonstrates that most of the variation in apparent prevalence occurred among those pods whose samples sizes were small, especially those pods with less than 10 samples per pod. Variation in prevalence was much less among pods with larger sample sizes. Pods with zero prevalence were predominantly found among those pods with small sample sizes. Since many pod sample sizes were small, and there was considerable variation in prevalence among small pods, the prevalence estimates for the Fairview serum bank were made using the entire sample population as the denominator, rather than combining the prevalence estimates from individual pods.

The CI-ELISA positive apparent prevalence among the slaughter dates of the slaughter survey ranged from 10.0 to 40.0. The mean, median and mode of apparent prevalence among slaughter dates were 23.8, 22.4 and 29.0 respectively. Figure 4.5 presents a scatterplot of the apparent prevalence among slaughter dates versus the number of samples collected (sample size) on each date. From this scatterplot it can be seen that there was a considerable amount of variation in the apparent prevalence among slaughter dates. The variation, however was relatively uniform over all slaughter dates, regardless of the slaughter date sample size. Since there was a considerable range in sample size and apparent prevalence for individual slaughter dates, prevalence estimates for the slaughter survey were made using the entire slaughter population as the



denominator, rather than combining prevalence estimates from each slaughter date.

Figure 4.4 Scatterplot of the CI-ELISA positive apparent prevalence of pods versus the sample size of pods in the Fairview serum bank

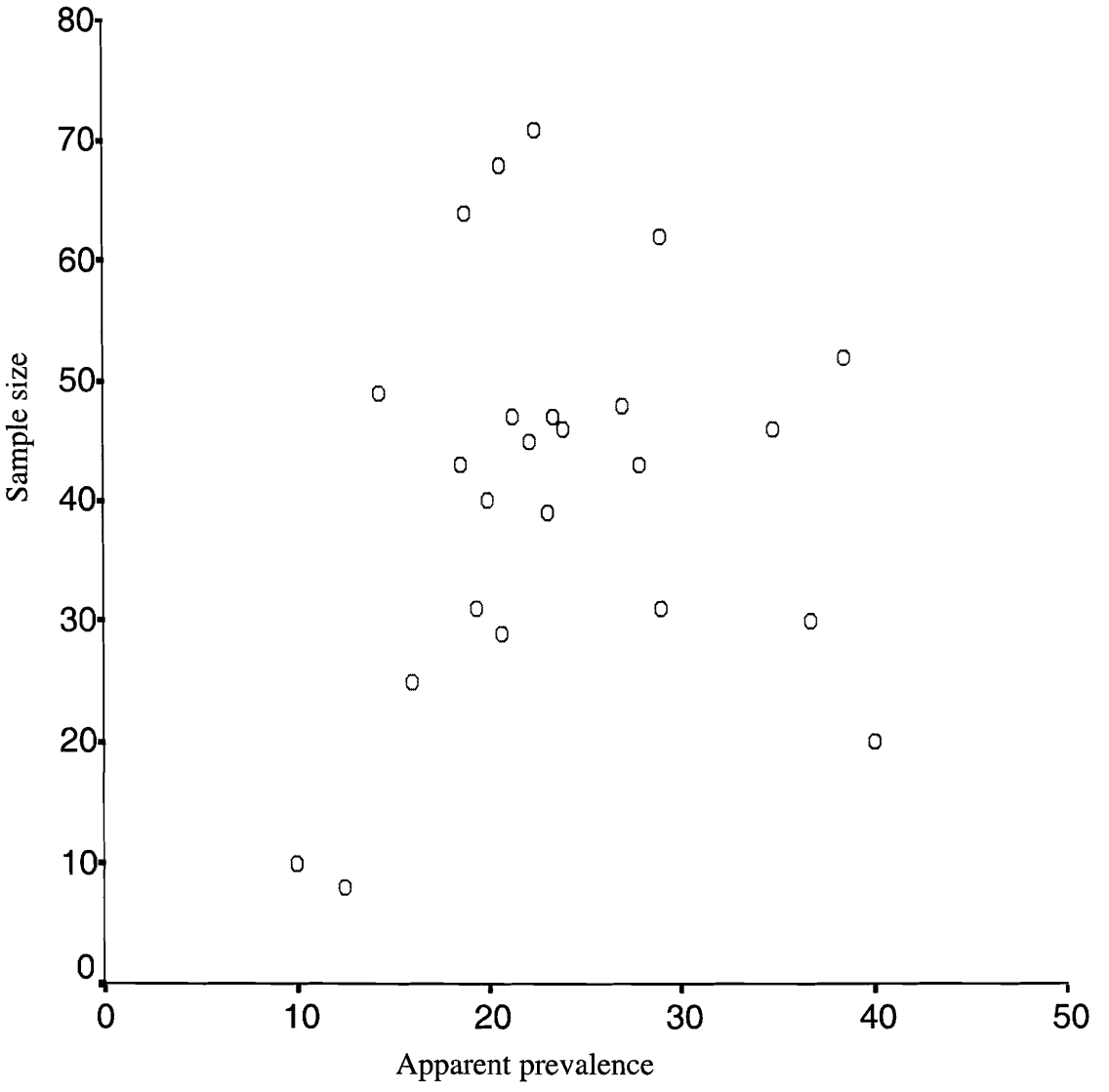


Figure 4.5 Scatterplot of the CI-ELISA positive apparent prevalence for each slaughter day (X axis) versus the number of samples collected (sample size) on each slaughter day (Y axis).

The apparent prevalence and Rogan-Gladen prevalence (Prg) estimates with accompanying Rogan-Gladen confidence intervals for the Fairview serum bank and the slaughter survey are presented in table 4.1. The overall Prg was 22.9% and there was no difference in apparent prevalence between the Fairview and samples (OR = 1.162, 95% CI = 0.908 - 1.483).

The apparent prevalence, Prg estimates and Rogan-Gladen confidence intervals for the 5 herds of bison in the cow-calf study are presented in table 4.2. The over all Prg of CI-ELISA positive bison in these 5 herds was 16.2, with a 95% confidence interval of 13.7 - 18.7. There was no difference in the apparent prevalence of CI-ELISA positive bison among the total populations of all 5 herds, nor was there a difference in apparent prevalence among the cows in these herds. There was a significant difference between the apparent prevalence in cows (22.8) of all herds combined, compared to the calves (8.7) of all herds combined (OR = 3.176, 95% CI = 2.226 - 4.532). This was not true for individual herds in the cow-calf study. In table 4.3 it can be seen that the cows in herds one to four were more likely to be CI-ELISA positive than were the calves in those herds (OR's 3.437 to 21.083, confidence intervals all >1). In herd five, however there was no difference between the apparent prevalence in cows versus calves (OR = 1.458, 95% CI = 0.905 - 2.348). This is not due to the cows in herd five having a higher prevalence than the cows in the other four herds, rather it is due to the higher prevalence of CI-ELISA positive calves in herd five.

Table 4.1 CI-ELISA positive prevalence in sera from the Fairview serum bank and the slaughter survey. Included in the table are the number of samples tested in each group, the number CI-ELISA positive, the apparent prevalence, the Rogan-Gladen prevalence estimate and the Rogan-Gladen 95% confidence interval for both groups and the combined groups (Total).

	Fairview	**Slaughter**	**Total**
Number tested	953	995	1948
Number positive	209	240	449
Apparent prevalence	22.0	24.1	23.1
Rogan-Gladen Prevalence	21.8	23.9	22.9
95% Confidence Interval	19.0-24.5	21.1-26.7	20.8-24.9

Table 4.2 CI-ELISA positive prevalence estimates for bison cows and their calves from 5 bison herds. The table includes the date sampled (Date), The herd identification number (Herd), the number of bison sampled from each herd (No), the apparent prevalence (AP), the Rogan-Gladen prevalence (Prg) and the Rogan-Gladen 95% confidence interval (CI). Totals are included for each class of bison, each farm and an over all total for the 5 herds.

			Date			
Date	**Oct 17**	**Nov 20**	**Dec 3**	**Feb 2**	**Feb 20**	**Total**
Herd	1	2	3	4	5	
Cows						
No	141	119	79	17	290	646
Ap	23.4	24.4	31.7	29.4	20.3	23.4
Prg	22.8	23.8	31.1	31.1	19.7	22.8
CI	15.7-30.0	15.9-31.7	20.7-41.5	7.0-50.7	14.9-24.6	19.3-26.3
Calves						
No	140	105	46	18	208	517
AP	1.4	8.6	6.5	0	14.9	8.7
Prg	0.7	7.8	4.5	0	14.3	8.7
CI	-0.01-3.1	0.03-13.4	-0.01-11.1	-0.01-0.01	9.2-19.3	8.4-9.0
Total						
No	281	224	125	35	498	1163
AP	12.5	17.0	22.4	14.3	18.1	16.9
Prg	11.8	16.3	21.8	13.6	17.5	16.2
CI	7.7-15.9	11.2-21.4	14.4-29.3	0-25.4	13.8-21.1	13.7-18.7

Table 4.3 Comparisons of the prevalence of CI-ELISA positive bison between cows and calves for all 5 herds combined; comparison of the prevalence of CI-ELISA positive bison between cows and calves for individual herds, and the comparison of the prevalence of CI-ELISA calves in herd 5 to the calves in the rest of the herds combined. Included are the odds ratios (OR), 95% confidence intervals (CI) and *p* values.

Comparison	OR	CI	*P* value
Cows vs calves all herds combined	3.176	2.226 - 4.532	< 0.001
Herd 1 cows vs calves	21.083	4.949 - 89.817	< 0.001
Herd 2 cows vs calves	3.437	1.542 - 7.658	0.003
Herd 3 cows vs calves	6.634	1.877 - 23.449	0.003
Herd 4 cows vs calves[1]	8.769	1.194 - 64.420	0.044
Herd 5 cows vs calves	1.458	0.905 - 2.348	0.121
Herd 5 calves versus calves of herds 1 to 4 combined	3.690	1.911 - 7.126	< 0.001

[1] One of the cells of the two by two table for the comparison of the cows and calves for herd 4 contained a zero. For this comparison a value of one was added to each cell for the calculation of the odds ratio, confidence interval and *p* value. The *p* value was calculated with a Fisher's exact test.

The calves in herd five had a significantly higher apparent prevalence than the calves in the other four herds combined (OR = 3.690, 95% CI = 1.911 - 7.126). In addition, the confidence intervals for the estimates of the Prg of CI-ELISA positive calves included zero, or were very close to zero for all herds except herd number five, indicating that the Prg for calves in herds one through four may have been zero.

There were no differences in the apparent prevalence of CI-ELISA positive cows or calves between the herds which reported MCF mortalities and those which did not.

There was very little variation in the combined apparent prevalence or Prg among the adult bison in the slaughter, Fairview serum bank and cow-calf studies. The Fairview bison had the lowest Prg (21.8%), and the Slaughter bison had the highest (23.9%). The apparent prevalence and Prg among all bison over the age of 1 year, in the slaughter, Fairview and cow-calf studies combined were 23.17% and 22.94 (95% CI = 21.14 - 24.73) respectively.

4.4 Discussion

Generalization of the results of this study to the bison population in Alberta must be done with caution. None of the sampling procedures employed in this study were random, nor were sources for sampling a random or even representative sample of the bison farmed in Alberta. Bison are not as amenable to handling as cattle are. Injuries are more often associated with handling bison than cattle. Bison producers are in general reluctant to handle their bison, or allow extra procedures to be performed on them, unless the procedures are deemed absolutely necessary. As a result, this study obtained

samples only from those sources that were readily available. The number of samples collected for this study however, was large and came from diverse groups of bison.

The Fairview serum bank contained samples that were collected over a period of five years. Although samples came from only one area of Alberta; the Peace River district, it is the area of Canada having the largest bison population. This geographical region is estimated to be home to more than half of all Canadian bison (Armstrong, Ayars *et al.* 1998). The average size of bison herds in Alberta was estimated to be 68 in 1996, with breeding females making up 50% of the herd (Nixdorf, Barber *et al.* 1998). In that same year it was estimated that there were 22,782 bison in Alberta. The Canadian bison population has been estimated to be growing at a rate of 26% per year since 1996 (Armstrong, Ayars *et al.* 1998). From this estimated rate of growth the population in Alberta may have been as high as 55,000 in the year 2000. The number of bison farms in Alberta was estimated to be 334 in 1996 (Armstrong, Ayars *et al.* 1998). If the number of farms increased at the same rate which the Canadian bison population increased, then there may have been as many as 800 bison farms in Alberta in 2001. Bison were sampled for the serum bank over a continuous period of time, during which the Alberta bison population was rapidly expanding. It is therefore not possible to estimate the fraction of the Alberta bison population which the Fairview serological survey represented. However, the serum bank contained sera from at least 115 farms, which ranges from 34% of farms in 1996 to an estimated 14% of farms in 2001.

It is not known how many farms the slaughter plant samples came from. It has been estimated that there were 2,854 bison slaughtered in Canada in 1998 (Hobbs,

Sanderson 2000). If the number of bison slaughtered in Canada has increased at the same rate as the Canadian bison herd, the number of bison slaughtered in Canada may have been as high as 4,500 in the year 2000. If this were the case, the slaughter survey sampled a potential 22% of all bison slaughtered in Canada during the 2000 slaughter year.

The accuracy of prevalence estimates from serological surveys are heavily dependant on the validity of the tests used to classify the infection status of study subjects. Validity studies are seldom conducted on the same study subjects that are the object of serological surveys. They are most often conducted on known infected and uninfected study subjects that may not be a sub-population of the population that is the focus of the serological study. Selection of infected and un-infected sub-populations may introduce bias into the prevalence estimates (Greiner, Gardener 2000). The Rogan-Gladen prevalence estimator provides an unbiased estimate of the prevalence in the survey populations if the sensitivity and specificity of the test have been previously established. Confidence intervals can be calculated for the Rogan-Gladen prevalence estimate which include the effect on the Rogan-Gladen variance of not only the size of the survey sample, but also the size of the samples which were used to establish both the sensitivity and specificity of the diagnostic test. Although this method results in a decrease in test precision, reflected as a widening of the confidence interval, it provides a more valid estimate of the prevalence and confidence intervals in the survey populations (Greiner, Gardener 2000).

The CI-ELISA test has been validated as a test to classify bison with respect to

their latent MCF virus infection status (see chapter 3). Although the validity studies were small, the effect of their size on prevalence estimates has been accounted for by using a Rogan-Gladen estimator to calculate prevalence estimates and their confidence intervals (Greiner, Gardner 2000). The prevalence estimates generated by this study can therefore be considered to be the result of valid classifications of the infection status of the bison in this study, at the time of the study, within their respective confidence intervals. However, since it has been shown that the level of antibody response to antigenic epitope 15-A may vary in bison sera over time (O'Toole, Li *et al.* 2002), it is possible that these prevalence estimates may be low compared to the true prevalence of latent MCF virus infections in these populations.

The Rogan-Gladen estimates of the prevalence of CI-ELISA positive bison in this study indicate that a large proportion (>20%) of the study bison were latently infected with MCF viruses. This is especially true of those study bison which were older than one year of age. The Prg of all the three groups was similar and ranged from 21.8 to 23.9 percent. Although it can be stated with reasonable confidence that a large proportion of these bison were infected with an MCF virus, the identity of the virus they were latently infected with remains unknown, since the CI-ELISA was based on an monoclonal antibody (MAb 15-A) which was produced against an epitope conserved among all MCF viruses.

The prevalence of antibodies to MCF viruses is reported to be low in those species that are susceptible to MCF (Frolich, Li *et al.* 1998; Heuschele 1988; Li, Shen *et al.* 1996a). Although the reason for this has never been clearly established, there are

some hypotheses that may be put forward. Since susceptible hosts are not well adapted to MCF viruses, it may be that most or all of those susceptible hosts in which an infection becomes established die of MCF, leaving only a few latently infected survivors. Alternatively, it may be that many susceptible hosts become infected, some of which die, but most clear the infection. The latter suggestion seems unlikely since viruses in the MCF group are known to produce long term, frequently life long, latent infections in their host species (Coulter, Wright *et al.* 2001; Murphy, Gibbs *et al.* 1999; Plowright 1990).

It has become accepted that MCF viruses are not transmitted between members of susceptible host species (Heuschele 1988; Plowright 1990). This would suggest that animals of susceptible host species attain their infections only through exposure to other host species in which MCF viruses have co-evolved. MCF viruses which are well adapted to a host species become highly infectious within that species (Dunowska, Letchworth *et al.* 2001). Both the MCF virus and the co-evolved host species develop methods for transmitting the MCF virus quickly and effectively among most or all of the members of host species populations (Barnard, Bengis *et al.* 1989; Li, Snowder *et al.* 2000). OvHV-2 should not be considered to be well adapted to bison since it produces significant MCF mortalities in bison populations. Bison, however do not fit the pattern of a susceptible host species, since the prevalence of antibodies in bison populations as demonstrated by this study, is relatively high. These infections must either come directly from exposure to adapted host species, the most likely candidate in Canada being domestic sheep, or through the maintenance of MCF virus infections within bison

populations via some form of bison to bison transmission. In a survey of bison farmers in the year 2000, only 5.6% of 479 bison farmers reported having any contact between sheep and their bison (Berezowski, Haigh *et al.* 2000). The demonstration by this study that a large proportion of farmed bison have antibodies to an MCF virus suggests that bison may be maintaining MCF virus infections within their populations by bison to bison transmission. Since the CI-ELISA test used in this study does not differentiate between MCF virus species, the identity of the MCF virus infecting bison remains uncertain.

Unlike the older bison in this study, the prevalence of CI-ELISA positive bison calves was not uniform among all the populations of calves in this study. Within the first four herds sampled the prevalence of CI-ELISA positive bison calves was low, or non-existent. In contrast, the last herd sampled, herd number five, had a much higher prevalence. This difference is hard to explain. It may have been due to the effect of chance, since the number of herds sampled for this study was small. Furthermore, the number of herds sampled for each age class of calves was only one. Both of these sample sizes is far to small to draw any firm conclusions.

4.4.1 Summary

The prevalence of CI-ELISA positive bison in three large samples of farmed Alberta bison older than one year of age, ranged from 21.8 to 23.9 percent. In five herds of farmed bison the prevalence of CI-ELISA positive bison calves ranged from zero to 14.3%. Since the CI-ELISA test used in this study detects an antibody produced against an antigenic epitope which is conserved among MCF viruses, it is likely that positive

bison were exposed to and possibly latently infected with an MCF virus of unknown identity.

5. SERO-SURVEY OF NORTHERN CANADIAN BISON

5.1 Introduction

The MCF group of viruses are a group of closely related gamma herpes viruses in the genus Rhadinovirinae. Viruses within this group share many properties including antigenic similarity and base sequence homology (Li, Keller *et al.* 2001). The most studied viruses within this group are OvHV-2 and A1HV-1. OvHV-2 has co-evolved with sheep and inapparently infects large proportions of adult domestic sheep populations (Li, Snowder *et al.* 2000). A1HV-1 has co-evolved with wildebeest and inapparently infects large proportions of adult wildebeest populations (Plowright, Ferris *et al.* 1960). Latent infections in the adult populations of either of these two species can be demonstrated by detecting antibodies to MCF viruses in their sera (Li, Shen *et al.* 1996a; Plowright 1967). In both of these species the prevalence of antibody positive animals is high (Li, Shen *et al.* 1996a; Plowright 1967). These viruses are maintained over generations in their respective host populations by transmission from the adults in the herd to neonates and then by horizontal transmission from infected to uninfected neonates (Li, Snowder *et al.* 1998; Plowright 1965). Viral transmission to neonates is very rapid in both species. By the time neonates reach the age of one year most or all of them have become infected (Li, Snowder *et al.* 1998; Plowright 1965). Transplacental transmission of A1HV-1 occurs in wildebeest (Plowright 1965), but not in sheep with OvHV-2. Lambs which are removed from their flock at less than 2.5 months of age and

raised in isolation from other sheep remain un-infected with OvHV-2, even though they are susceptible to OvHV-2 infection for the remainder of their life (Li, Snowder *et al.* 1999).

In the previous chapter it was demonstrated that 21.8 to 23.9 percent of bison in a large sample of Alberta bison had antibodies to MCF viruses. Since MCF-group viruses produce life long sub-clinical infections in other species (Li, Snowder *et al.* 2000; Plowright, Ferris *et al.* 1960) it is probable that many of the bison tested were sub-clinically infected with an MCF virus. These infections may have originated directly from exposure to sheep, or an alternative as yet unexplored hypothesis suggests that a significant proportion of bison may be infected with an MCF virus that is being maintained within bison populations by bison to bison transmission. The purpose of this chapter is to investigate whether MCF virus infections are being maintained in bison populations, independent of exposure to sheep. Four bison populations that were either partially or completely isolated from other ruminants including sheep and other bison were tested to determine their CI-ELISA positive prevalence. The CI-ELISA used in this study measures an antibody produced against antigenic epitope 15-A which is conserved among all MCF viruses (Li, Shen *et al.* 1994). Two of these herds were maintained in isolation for a long enough period to give birth to subsequent generations of bison. These two herds formed the basis of a natural experiment that tested whether there was cross generational transmission of MCF viruses in bison that were isolated from other known ruminant sources of MCF viruses.

5.2 Materials and methods

Figure 5.1 presents a map of the geographical location of the 4 herds of bison tested in this study.

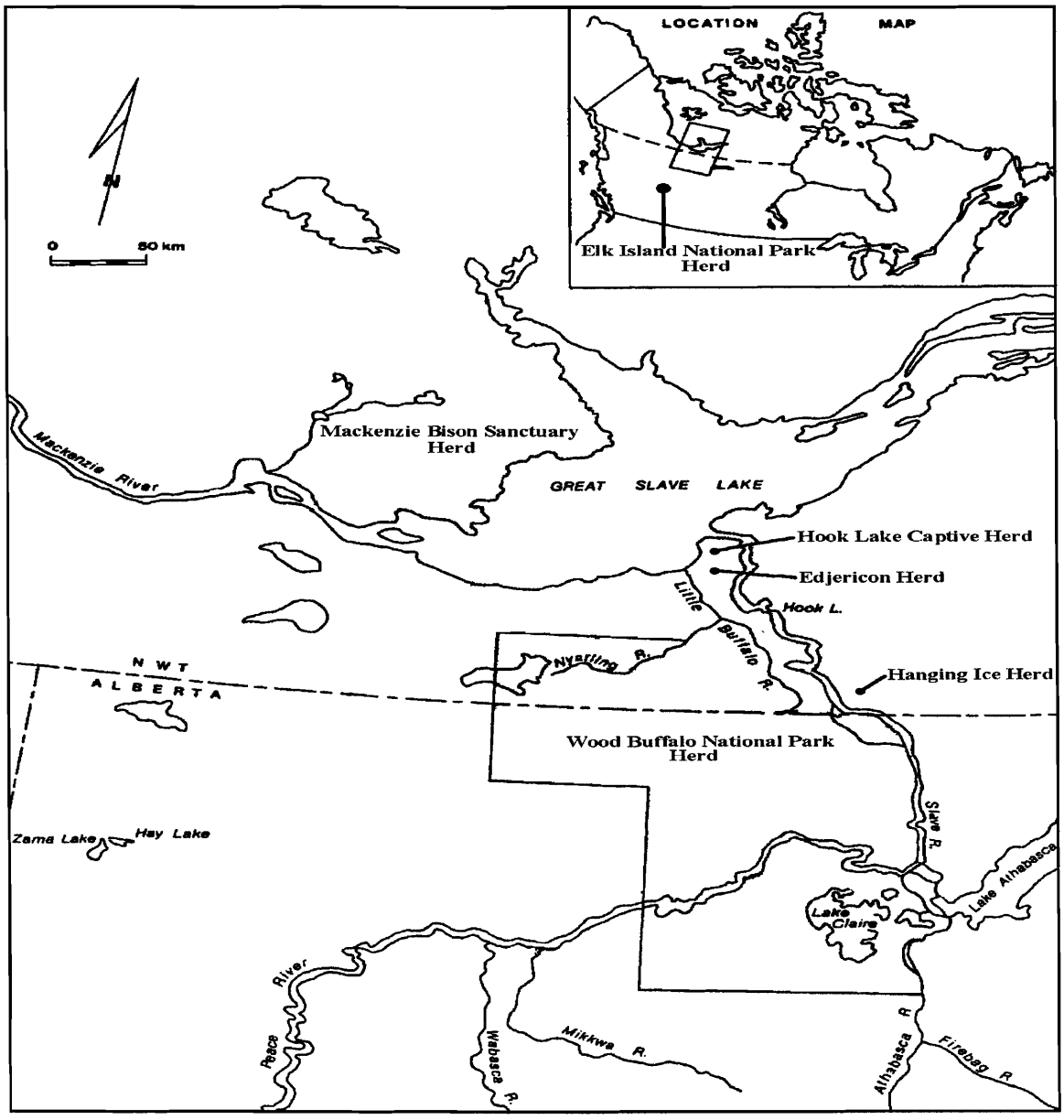


Figure 5.1 Map of the location of the bison herds tested for chapter 5. (Adapted from S. Tessaro 1990, Northern diseased bison. Report of the environmental assessment panel, Federal Environmental Assessment Review Office, Ottawa, Canada.)

5.2.1 Wood Buffalo National Park

Wood Buffalo National Park is a large tract of wilderness located in Northern Alberta and the Northwest territories. The park was created in 1922 to protect the few remaining wood bison (*Bison bison athabascae*) in Northern Canada from extinction. During the years from 1925 to 1928, however 6,673 plains bison (*Bison bison bison*) were translocated to the park from the National Buffalo Park in Wainwright Alberta. The translocated plains bison interbred with woods bison in the park and the population grew to reach 12,000 bison by 1934. For a number of reasons including disease, drowning and predation, the bison population within the park has since declined to 2,137 bison, estimated in the year 2000 (Nishi, Elkin *et al.* 2000). From a study which was commissioned to assess the affect of brucellosis and tuberculosis on the growth of the bison population in Wood Buffalo National Park (Joly 2001), 104 bison serum samples were obtained for the present study. These samples were collected from free ranging bison which were blood sampled after capture by net gun or darting with carfentanil and xylazine hydrochloride from a helicopter. Capture methods were designed to provide a sample representative of the geographical distribution of bison in the park (Joly 2001). Bison were captured from February 24, 1998 to March 15, 1998. The sample population consisted of female bison. The mean, median and mode of the distribution of ages of sampled bison were 5.2, 4.0 and 2.0 years of age respectively. Bison in the park were exposed to other free ranging ruminants, such as moose (*Alces alces*). The park is isolated from commercial agriculture, but it is possible that since the park was established, bison may have ranged into farming areas and been exposed to sheep and

cattle. Mortalities due to MCF have never been reported in the Park herd.

5.2.2 Hanging Ice/Edjericon

The Hanging Ice ranch was a private bison ranch, first established in 1990 and 1991 with surplus bison of various ages from Elk Island National Park. The ranch was located 30 km north of Fort Smith in the North West Territories of Canada, on the east side of Slave Lake. In 1992 most of the bison of reproductive age were moved to a new location, the Edjericon ranch which was located near Fort Resolution in the North West Territories. Bison were maintained in confinement on both ranches and there were no additions to this herds since it's inception. The bison at both ranches have had no contact with other domestic ruminants from the time they left Elk Island National Park. Since Elk Island National Park is located in the midst of a farming community east of Edmonton, Alberta, it is possible that bison in the park were exposed to sheep in the past. Removals from the Hanging Ice and Edjericon ranches were due to mortalities, sale of bison and slaughter for meat production. One hundred twenty eight serum samples were obtained from a blood collection in 1994 and another 65 samples were obtained from a collection in 2000. In both cases the collections were complete herd tests. Mortalities due to MCF have never been reported in this herd.

5.2.3 Mackenzie Bison Sanctuary

The Mackenzie Bison Sanctuary bison herd is a free ranging herd, located on 8000 square kilometers of wilderness north of Great Slave Lake and the Mackenzie

River in the Northwest Territories of Canada. The herd was established in 1963 by translocating 18 bison from Wood Buffalo National Park to an area northeast of Fort Providence. In 1998 the herd was estimated to contain 1908 +/- 200 bison (Gates, Stephenson *et al.* 2000). The range of the herd is confluent with the range of other northern free ranging ruminants, most commonly moose (*Alces alces*). Due to the northern geographical location of the sanctuary, the herd has never been in contact with any other northern bison herds nor has their been contact with any farmed ruminants. Fifty eight archived serum samples collected from bison in the sanctuary during the years 1984 to 1987 were obtained for CI-ELISA analysis. The bison were sampled for disease monitoring purposes. Information about sampling procedures and techniques were unavailable to this study. Mortalities due to MCF have never been reported in this herd.

5.2.4 Hook Lake Captive Herd

The Hook Lake captive bison herd is located at the Hook Lake bison recovery project isolation facility at Fort Resolution, North West Territories. This bison herd was established by capturing bison calves with a net gun from a helicopter shortly after they were born. Captured calves were from free ranging bison cows belonging to the Hook Lake free ranging bison herd, which is a sub-population of the greater Wood Buffalo National Park bison herd. A total of 62 calves were captured during the period from 1996 to1998. Four calves died shortly after capture, leaving 58 bison as the foundation stock for the herd. Since the study began there have been 28 calves born in the herd. The

herd has been maintained in confinement since its inception in 1996, with no contact having occurred between captive bison and any other ruminants. For the duration of the study there were no domestic or free ranging sheep within hundreds of kilometers of the facility, nor has there ever been reported cases of MCF in the parent herd. Serum samples were obtained from all 86 of the bison in this herd in the year 2000.

5.2.5 Analysis

CI-ELISA tests were performed, as previously reported (Li, Mcguire *et al.* 2001; Li, Shen *et al.* 1994) at the Department of Veterinary Microbiology and Pathology, Washington State University in Pullman, Washington. Rogan-Gladen prevalence estimates and confidence intervals were calculated as previously reported (Greiner, Gardener 2000). Odds ratios, *p* values (Chi-square approximations) and confidence intervals for odds ratios were calculated with Epi Info® 6 version 6.04 software (Centers for Disease Control and Prevention (CDC), USA and World Health Organization (WHO), Geneva, Switzerland). A 5% significance level was used for hypothesis testing.

5.3 Results

Table 5.1 presents the overall prevalence and confidence intervals for all four of the bison herds sampled for this study. The Mackenzie Bison Sanctuary (MBS) herd and the Hook Lake Captive herd (HLCH) samples both had very low Rogan-Gladen prevalence (Prg). The accompanying confidence intervals of the Prg for the MBS and HLCH both included zero. The HLCH sample was a complete census of the HLCH,

Table 5.1 CI-ELISA positive prevalence in four northern bison herds. Herds included are the Wood Buffalo National Park herd (WBNP), Hanging Ice/Edjericon herd (HI/EJ), Mackenzie Bison Sanctuary Herd (MBS), and the Hook Lake captive herd (HLCH). The table presents the size of each population, number of bison tested from each population (Sample size), apparent prevalence, Rogan-Gladen prevalence estimate and 95% confidence intervals of the Rogan-Gladen prevalence estimate.

	WBNP	HI/EJ	MBS	HLCH
Population size	2137[1]	160	1908[1]	86
Sample size	104	160	58	86
Apparent prevalence	6.7	38.8	5.2	1.2
Rogan-Gladen prevalence	6.4	38.6	4.9	0.9
95% Confidence interval	1.5 - 11.3	31.0 - 46.2	-0.9 - 10.7	-1.6 - 3.4

[1] The sizes of the Wood Buffalo National Park and Mackenzie Bison Sanctuary bison herds are both approximations.

making it possible to conclude with some degree of confidence from the Prg and its accompanying confidence interval that the HLCH had a CI-ELISA positive prevalence of zero and therefore was uninfected with an MCF virus. Since the MBS prevalence estimate was based on a relatively small sample of the parent herd, it may not be correct to assume the MBS herd was uninfected with an MCF virus even though the confidence interval of the Prg included zero. Information about sampling procedures was unavailable for this herd and therefore sampling methods may have affected the prevalence estimate.

The Prg estimate for the Wood Buffalo National Park (WBNP) herd was also low. Although the Prg confidence interval did not include zero, it approached zero, making it difficult to assume that WBNP herd was free from infection with an MCF virus.

The prevalence estimates for the Hanging Ice/Edjericon (HI/EJ) sample were high. The CI-ELISA positive Prg was 38.6 with a confidence interval well above zero (31.0 - 46.2).

Both the HLCH and the HI/EJ herds were formed from bison that originated from other herds. The HLCH came from neonatal bison calves that were captured form the WBNP herd and the HI/EJ herd from surplus bison of various ages from the Elk Island National Park herd (herd number 2 in chapter 4). Once these two herds were established, they were maintained in captivity, free from exposure to other ruminants for a period of time sufficient to allow the cows within these herds to give birth to subsequent generations of calves. There were 28 calves born into the HLCH and 92

calves born into the HI/EJ herd (table 5.2). The Prg estimates for these two herds are widely divergent. The translocated HI/EJ bison had a much higher Prg than did the HLCH bison (OR = 38.2, 95% CI = 9.5 - 153.5). Among bison born after translocation, the HI/EJ bison also had a much higher Prg than did the HLCH (OR = 18.0, 95% CI = 3.8 - 85.7). It is difficult to firmly establish whether the herd of origin of the HLCH was infected with an MCF virus, since the Prg and confidence interval of the herd of origin were low. Among translocated HLCH bison the Prg was close to zero (Prg = 1.7, 95% CI = -2.1 - 4.9) and among HLCH bison born after translocation the Prg was zero (95% CI = -1.3 - 0.7).

The Prg of the herd of origin of the HI/EJ herd was similar to that of other farmed bison (Prg = 23.8, 95% CI 15.9 - 30.0) and the Prg of the translocated bison was high (Prg = 39.5, 95% CI = 27.8 - 51.2). The Prg of the bison born after translocation was high (Prg = 37.8, 95% CI = 27.8 - 47.8).

Table 5.2 CI-ELISA positive prevalence for the Hanging Ice/Edjericon and Hook Lake captive bison herds. Included in the table are the number of bison tested (N), the Rogan-Gladden prevalence, and the 95% confidence intervals of the Rgoan-Gladen prevalence for the herd of origin, the bison that were translocated and the bison that were born after translocation.

	Hanging Ice/Edjericon	Hook Lake Captive Herd
Herd of origin	N = 119[1]	N = 104[2]
Rogan-Gladen prevalence	23.8	6.4
Confidence interval	15.9 - 30.0	1.5 - 11.3
Translocated bison	N = 68	N = 58
Rogan-Gladen prevalence	39.5	1.7
Confidence iterval	27.8 - 51.2	-2.1 - 4.9
Born after translocation	N = 92	N = 28
Rogan-Gladen prevalence	37.8	0
confidence interval	27.8 - 47.8	-1.3 - 0.7

[1] The herd of origin of the Hanging Ice/Edjericon herd was herd number 2 in chapter 4.

[2] The herd of origin of the Hook Lake captive herd was the Wood Buffalo National Park herd

5.4 Discussion

It is difficult to firmly establish the CI-ELISA positive prevalence within the WBNP and MBS bison populations from the results of this study. This is mainly due to inadequacies in the study design. The sampling fractions of both herds were low. In addition, the prevalence estimates from the samples of these two herds were low compared to those expected in MCF virus infected bison populations (Chapter 4). Small sample sizes and low numbers of test positive animals have the combined effect of generating low prevalence estimates with wide confidence intervals that can easily approach or encompass zero. Both the WBNP and MBS herds are populations of free ranging bison that exist on large tracts of wilderness in remote areas of Canada. It is very difficult to sample bison in either of these herds, and this study was forced to use only those samples which were available from previous studies.

The CI-ELISA positive prevalence estimates for the HLCH and the HI/EJ herds in this study can be considered to be more valid estimates of the CI-ELISA positive prevalence within these two populations, since both of the herd samples were a complete census of the populations alive at the time of sampling. The CI-ELISA positive prevalence estimates for these two populations suggest that two different processes were occurring within these populations.

The prevalence of CI-ELISA positive bison in the parent herd of the HI/EJ herd was similar to that found in other farmed bison herds (table 5.2). Bison of various ages were translocated to form the HI/EJ herd, and it is likely that the translocated bison carried MCF viruses into the newly formed HI/EJ herd. The bison in this herd were

isolated from other ruminant sources of MCF viruses for the duration of the study. The high CI-ELISA positive prevalence in second generation bison suggests that MCF viruses were transmitted from translocated bison to subsequent generations of bison in the HI/EJ herd.

The bison which formed the HLCH were uninfected when the herd was formed and have remained uninfected into the next generation of bison (table 5.2). The bison which formed the HLCH were captured as neonatal calves, but it is not known whether removing these calves from their parent herd at an early age actually blocked transmission of MCF viruses to these calves, since the prevalence estimates from their parent herd were equivocal. The parent herd may not have been infected with MCF viruses.

The HLCH and HI/EJ herds can be considered to be parts of a natural experiment in which the HLCH serves as a control herd or herd with which the HI/EJ herd can be compared. Both herds were maintained in Northern Canada, under similar conditions, during the same period of time. Both herds were kept in confinement, unexposed to other ruminants for a long enough period to produce subsequent generations of bison. The major difference between the two herds was the HI/EJ herd was infected with MCF viruses at its inception and the HLCH was not. The HI/EJ herd has maintained MCF virus infections in subsequent generations of bison, in the absence of other ruminant sources of MCF viruses. This suggests that MCF virus infections have been transmitted from the translocated bison of the HI/EJ herd to subsequent generations of bison within this herd. The HLCH, however has maintained it's uninfected status across generations

in the absence of other sources of MCF viruses. It is likely that both herds were effectively isolated from other ruminant sources of MCF viruses for the duration of the study.

It has been clearly established that herpesviruses belonging to the MCF group are transmitted from adults within their co-evolved species populations to the next generation of neonates. A1HV-1 is transmitted from adult wildebeest to neonatal wildebeest (Plowright 1965), and OvHV-2 is transmitted from adult sheep to lambs (Li, Snowder *et al.* 1998). The findings of this study suggest that a similar phenomenon may have occurred among the HI/EJ bison. The mechanism of cross generational transmission of MCF viruses in HI/EJ bison remains unknown. The CI-ELISA test used in this study detects an antibody produced against an epitope which is conserved among all known MCF group viruses (Li, Shen *et al.* 1994). It is therefore not possible from the results of this study to determine the identity of the virus which was transmitted among the bison in the HI/EJ herd.

5.4.1 Summary

Using a CI-ELISA test, this study demonstrated that MCF virus infections were being maintained and transmitted across generations in one isolated bison herd, in the absence of other ruminant sources of MCF viruses. Since the CI-ELISA detects antibodies produced against an antigenic epitope which is conserved among all MCF viruses, the identity of the virus infecting this bison herd could not be determined.

6. TRANSMISSION OF MCF IN BISON

6.1 Introduction

Malignant catarrhal fever(MCF) is a fatal disease of many ruminants including cattle, bison, buffalo, swine and deer (Castro, Daley *et al.* 1982; Heuschele, Fletcher *et al.* 1984; Liggitt, McChesney *et al.* 1980; Loken, Aleksandersen *et al.* 1998; Metzler 1991; Reid, Buxton *et al.* 1984). There are two commonly recognized forms of MCF, wildebeest associated MCF (WD-MCF) and sheep associated MCF (SA-MCF). Wildebeest associated MCF is so named because wildebeest, most of whom are latently infected with a herpesvirus named Alcelaphine herpesvirus 1 (A1HV-1), produce outbreaks of WD-MCF when they transmit A1HV-1 to cattle or other susceptible ruminant species (Plowright, Ferris *et al.* 1960). A1HV-1 has been isolated in tissue culture and the complete DNA base sequence of its genome has been reported (Ensser, Pfanz *et al.* 1997; Plowright, Ferris *et al.* 1960). Sheep associated malignant catarrhal fever was originally named because of the observed association between exposure to sheep and the occurrence of malignant catarrhal fever in susceptible ruminant species, especially cattle (Goetze 1930; Goetze, Liess 1929; Goetze, Liess 1930). Ovine herpesvirus-2 (OvHV-2), a gamma herpesvirus closely related to A1HV-1, has become accepted as the causal agent of SA-MCF even though it has never been isolated in tissue culture or observed by electron microscopy (Bridgen, Reid 1991; Dunowska, Letchworth *et al.* 2001; Heuschele, Reid 2001). OvHV-2 has been identified only from it's DNA base sequences (Baxter, Pow *et al.* 1993; Bridgen, Reid 1991; Coulter, Reid 2002;

Dunowska, Letchworth *et al.* 2001). PCR studies have demonstrated that a large proportion of sheep are latently infected with OvHV-2, or at least have OvHV-2 DNA base sequences in their peripheral blood leukocytes (Baxter, Pow *et al.* 1993; Li, Shen *et al.* 1995; Li, Snowder *et al.* 1998). PCR studies have also demonstrated a strong association between the presence of OvHV-2 DNA base sequences and mortalities attributed to SA-MCF in ruminants (Baxter, Pow *et al.* 1993; Collins, Bruns *et al.* 2000; Crawford, Li *et al.* 1999; Li, Shen *et al.* 1995; Muller Doblies, Li *et al.* 1998; Tham 1997; Wiyono, Baxter *et al.* 1994).

A third, less well known form of MCF, has been reported in one herd of white-tailed deer (*Odocoeleus) virginianus*) from a zoo in the United States (Li, Dyer *et al.* 2000). Deer suspected of dying from MCF had gross pathology and histopathology typical of malignant catarrhal fever, but evidence of neither A1HV-1 or OvHV-2 could be found in their carcasses. However, a 230 base pair DNA fragment with homology to other MCF virus DNA was identified in peripheral blood leukocytes of affected deer. It had 82% similarity to the corresponding region of OvHV-2 DNA polymerase and 72% similarity to A1HV-1 DNA polymerase, suggesting that there may be another herpesvirus similar to A1HV-1 and OvHV-2 which is capable of producing MCF in white-tailed deer. Further studies of the base sequence of this virus's DNA polymerase and DNA packaging genes have supported it's close relationship to OvHV-2 and A1HV-1 (Kleiboeker, Miller *et al.* 2002). The virus has been putatively named MCF virus of white-tailed deer (MCFV-WTD). The reservoir host for this virus has not been reported.

Although SA-MCF is the most common form of malignant catarrhal fever world

wide, little is known about the epidemiology of the disease (Heuschele, Reid 2001). The resistance of OvHV-2 to propagation in vitro has been a major impediment to researchers studying MCF. Prior to the advent of PCR technology there was no method available for positively identifying animals infected with OvHV-2 (Baxter, Pow *et al.* 1993). The inability to replicate OvHV-2 in vitro has made it difficult to study the virus's stability in the environment, it's incubation period, or the mechanisms by which it is transmitted from sheep to cattle or other susceptible species.

PCR and antibody prevalence studies have demonstrated that most sheep are infected with OvHV-2, and that OvHV-2 is highly infectious within sheep populations (Baxter, Wiyono *et al.* 1997; Li, Mcguire *et al.* 2001; Li, Shen *et al.* 1995; Li, Snowder *et al.* 1998; Li, Snowder *et al.* 2000; Metzler 1991; Mirangi, Kang'ee 1997; Muluneh, Liebermann 1992; Rossiter 1981; Wiyono, Baxter *et al.* 1994). OvHV-2 or at least SA-MCF is probably not transmitted directly from ruminants with SA-MCF to other healthy susceptible ruminants. Transmission of SA-MCF has been sporadically successful by injection of blood or tissue from affected cattle or bison to healthy cattle or bison (Blood, Rowsell *et al.* 1961; Goetze 1930; Goetze, Liess 1929; Goetze, Liess 1930; Horner, Oliver *et al.* 1975; Liggitt, McChesney *et al.* 1980; Magnusson 1940; Selman, Wiseman *et al.* 1974; Selman, Wiseman *et al.* 1978). In contrast, horizontal transmission of SA-MCF from affected cattle to healthy cattle stabled next to them has invariably failed (Goetze 1930; Goetze, Liess 1929; Goetze, Liess 1930; Piercy 1954; Pierson, Storz *et al.* 1974). Attempts to transmit SA-MCF with nasal and ocular secretions from affected cattle or deer with SA-MCF to healthy cattle or deer has also failed (Imai,

Nishimori *et al.* 2001; Schofield, Bainf 1941).

There are several published reports of MCF outbreaks in bison (Collins, Bruns *et al.* 2000; Liggitt, McChesney *et al.* 1980; O'Toole, Li *et al.* 2002; Ruth, Reed *et al.* 1977; Schultheiss, Collins *et al.* 1998; Schultheiss, Collins *et al.* 2000). In these reports sources of OvHV-2 and details relating to the exposure of bison to sheep have been either unreported or poorly defined, making it difficult to infer the mechanism of transmission of OvHV-2 from sheep to bison.

This study reports the results of a natural experiment in which bison were exposed to sheep for a period of less than one day at a public auction sale in Saskatchewan. Following the auction sale, bison attendant at the sale were transported to 11 different farms where they were mixed with 1010 other bison. There were no sheep on or near any of the destination farms. Over the next 220 days, 45 bison died of SA-MCF on the destination farms. Only those bison exposed to sheep at the auction sale died of SA-MCF.

The purpose of this chapter is to demonstrate that in this natural outbreak of SA-MCF, there was no horizontal transmission of SA-MCF among bison.

6.2 Outbreak History

On November 7th , 2000, 163 bison, originating from 8 different Saskatchewan farms were assembled for sale at an auction market in Saskatchewan. The number of bison on each submitting farm ranged from 4 to 170 bison, with a mean herd size of 72.3 bison. The proportion of bison sold from each farm ranged from 5.8 to 100.0 percent of

each herd. The crude percent sold over all farms was 28.2 percent of all bison on the submitting farms. Following the removal of sale bison to the auction market there were 415 bison remaining on the farms of origin. Table 6.1 presents the number of bison submitted from each farm to the November 7th auction sale and the number of bison remaining on each farm after the sale bison were removed. Buyer 10 is listed as a seller because he not only sold bison at the auction sale on November 7th, but also bought other bison at the sale and transported them to his farm. None of the farms of origin reported any previous cases of MCF, nor were any of the sale bison exposed to sheep on their farms of origin. The farms were widely separated geographically and there was no exchange of bison between submitting farms prior to the November 7th sale. Most of the bison were brought to the auction market on Sunday November 5th. The remainder were brought to the auction market on Monday, November 6th. Bison at the sale consisted of 87 calves, 57 yearlings and 19 adults.

During the morning of November 6th , 216 sheep from several different Saskatchewan sheep farms were assembled at the auction market for transport to Alberta for slaughter. The sheep remained in the auction market until late in the afternoon of November 6th. In addition, the method by which bison at the auction market were exposed to the sheep has not been clearly established. However, since the sale was held during the winter, both sale bison and sheep were housed within the same building during their stay at the auction market. Sheep were not housed in the same room as sale bison, but were housed in an adjoining room. The two rooms were connected by two large openings that did not restrict air movement.

Table 6.1 The number of bison sold from each farm that submitted bison to the November 7th auction sale, percent of the total herd that was sold, number of mortalities in each group of bison submitted to the sale that were suspected to be MCF mortalities, number of mortalities in each group of bison submitted to the sale that were confirmed to be MCF mortalities and the number of bison left on each submitting farm after the sale bison were transported to the sale. There were no mortalities reported among bison left at home during the study period.

	Bison Sold At Auction Sale				Bison Left On Farm
Seller ID	**Number**	**Percent Sold**	**Suspect MCF**	**Confirmed MCF**	**Number**
Buyer 10	30	29.7	3	1	71
Seller 1	20	34.5	4	6	38
Seller 2	2	5.6	1	0	34
Seller 3	38	32.8	5	5	78
Seller 4	24	41.3	4	5	34
Seller 5	5	14.3	1	0	30
Seller 6	40	23.6	4	4	130
Seller 7	4	100.0	2	0	0
Total	**163**	**28.2**	**24**	**21**	**415**

During the day of the sale there was movement of people and equipment through these two openings. It is unlikely that bison and sheep would have been in direct contact since bison were confined to pens within the auction market until the time they were sold. No other livestock were present at the auction market during the time the sale bison were there. This auction market has held many bison sales in the past. No sheep were present at any of the previous sales nor were any of the previous sales associated with MCF outbreaks.

After the sale, the bison were transported to 11 different bison farms. The number of bison on these farms ranged from 0 to 270 bison, with a mean herd size of 91.2 bison. The number of sale bison transported to each destination farm ranged from 2 to 37 bison. On destination farms sale bison were mixed with resident bison of the same age to form new groups of bison. Sale bison ranged from 3.3 to 100.0 (mean = 31.7) percent of the bison in each newly formed group. In total, sale bison were mixed with 1010 other bison on destination farms. Table 6.2 presents the number of sale bison purchased by each buyer. There were no sheep within 2 miles of any of the destination farms prior to the arrival of sale bison or during the term of the study. There were no cases of MCF reported on any of the destination farms prior to the November 7th sale. The destination farms were very widely dispersed geographically. Table 6.3 presents the number of other bison that sale bison were mixed with on their destination farms. Buyer 11 did not purchase any bison at the November 7th auction sale. The sale bison purchased by buyers number 4 and 9 spent one month at buyer 11's farm mixed with his bison before being moved to the farms of buyers 4 and 9.

Table 6.2 Number of bison purchased by each buyer, number of suspected MCF mortalities, number of MCF mortalities confirmed by postmortem examination, crude MCF mortality rate, including both suspect and confirmed MCF mortalities, and the number of mortalities by other causes. There were no mortalities for reasons other than MCF reported in these bison.

Buyer ID	Number of Bison Purchased	Suspect MCF Mortalities	Confirmed MCF Mortalities	MCF Mortality Rate
Buyer 1	33	3	6	27.3%
Buyer 2	5	0	1	20.0%
Buyer 3	16	5	2	43.8%
Buyer 4	7	0	0	0.0%
Buyer 5	2	0	0	0.0%
Buyer 6	23	3	3	26.1%
Buyer 7	37	6	4	27.0%
Buyer 8	31	6	5	35.5%
Buyer 9	5	0	0	0.0%
Buyer 10	4	1	0	25.0%
Total	**163**	**24**	**21**	**27.6%**

Table 6.3 Number of bison on destination farms that were exposed to bison purchased at the November 7th auction sale, number of mortalities due to all causes and the crude mortality rate.

Buyer ID	Exposed Bison	Mortalities	Mortality Rate
Buyer 1	22	0	0.0%
Buyer 2	30	0	0.0%
Buyer 3	105	0	0.0%
Buyer 4	198	0	0.0%
Buyer 5	20	0	0.0%
Buyer 6	107	1	0.9%
Buyer 7	270	3	1.1%
Buyer 8	7	0	0.0%
Buyer 9	0	0	0.0%
Buyer 10	74	0	0.0%
Buyer 11	177	0	0.0%
Total	**1010**	**4**	**0.4%**

On December 28, 2000 one of the bison that had been purchased at the auction market on November 7th died. Subsequently a total of 45 bison purchased at the November 7th sale died on 7 destination farms. Twenty one were confirmed as being MCF mortalities by postmortem examination, and 24 were suspected of being MCF mortalities based on descriptions of clinical signs provided by farmers.

The outbreak began quite suddenly on the 50th day following the sale. The peak of the outbreak occurred during the 60th to 70th days or January 6th to 16th (mean = 81 days, median = 65 days, mode = 60 days). The rate of occurrence of mortalities slowed by the 100th day or February 12th, and then were sporadic until the last mortality on the 220th day or June 14th . Figure 6.1 compares the number of mortalities that occurred in each 10 day period on destination farms following the auction sale.

There were no MCF mortalities in any of the 1010 bison that were mixed with sale bison on destination farms. However, two bison died as a result of handling trauma and 2 died from pneumonia as determined by postmortem examination. There were no mortalities among the 415 bison remaining on the farms of origin for the duration of the study.

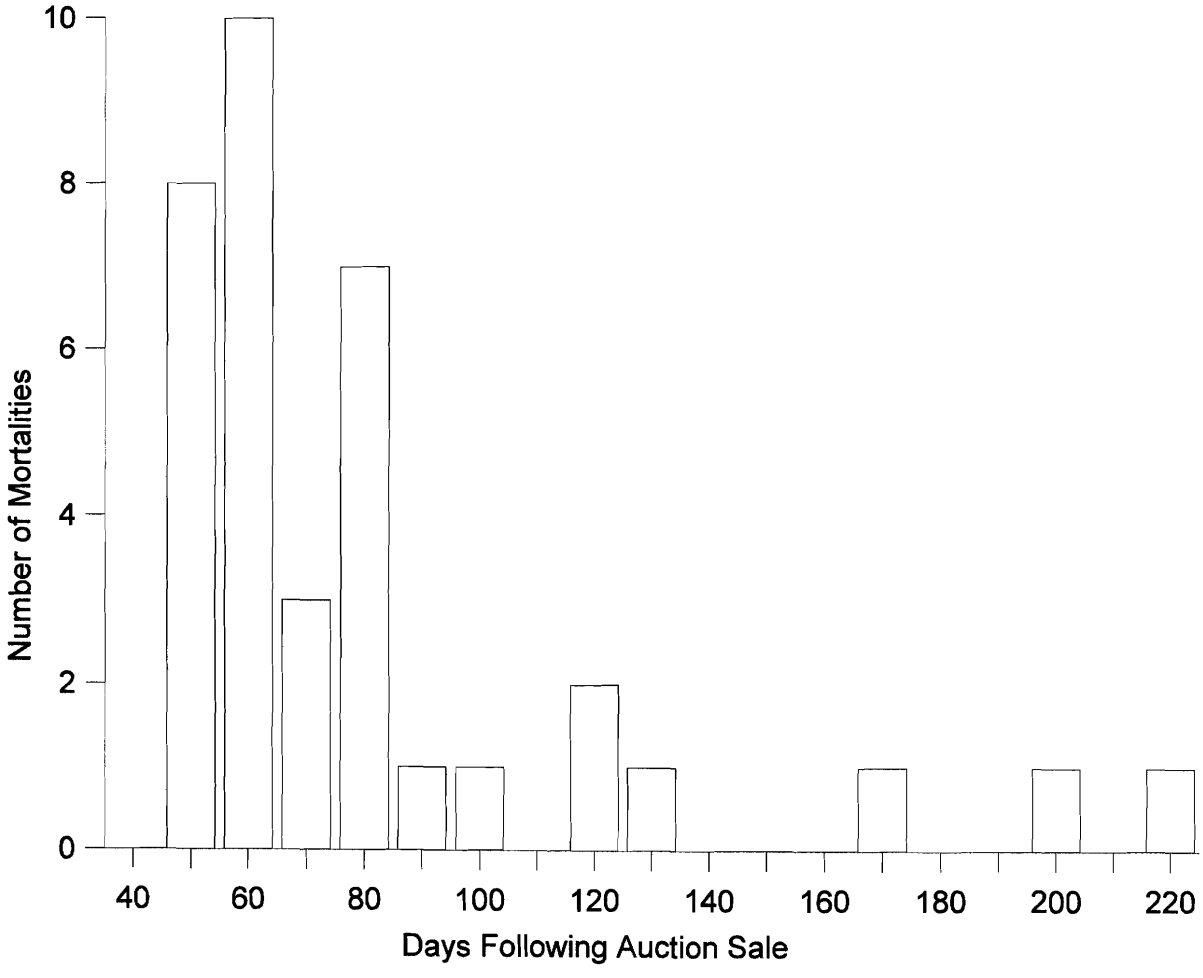


Figure 6.1 Bar chart of the number of mortalities that occurred in the MCF outbreak for each 10 day period following the auction sale. Day zero is the day of the auction sale, November 7th.The first mortality occurred on December 28th, the 50th day following the auction sale. The last mortality occurred on June 14th, the 220th day following the auction sale.

6.3 Materials and methods

Names of farmers submitting bison to the November 7th action sale as well as names of farmers buying bison at the sale were obtained from information supplied by the auction market. Information about bison herds from both submitting and purchasing farms was collected by directly interviewing farm owners. Once contact was established with farmers, all farms involved were monitored by telephone consultation for a period of 18 months following the November 7th auction sale. During this period farmers were encouraged to immediately report any mortalities that occurred in their bison herds. Dead bison were transported to the Prairie Diagnostic Services veterinary diagnostic pathology laboratory at Saskatoon, Saskatchewan for postmortem examinations.

Bison mortalities were classified as confirmed MCF mortalities by postmortem examination and positive PCR tests for the presence of OvHV-2 DNA. Gross pathology included erosions and ulcerations in any part of the gastro-intestinal tract or bladder, conjuntivitis, and keratitis. Histopathology included multisystemic mononuclear vasculitis, erosions and ulcerations in the gastro-intestinal tract or bladder, conjunctivitis, keratitis and uveitis. PCR tests were performed as previously reported ((Li, Shen *et al.* 1995)) at Prairie Diagnostic Services.

Bison mortalities were classified as suspect MCF mortalities from clinical signs provided by farm owners. Clinical signs included sudden death, diarrhea, ocular discharge, nasal discharge, anorexia and severe depression.

From February 9th to March 6th, 2001, 145 bison were blood tested on 4 farms that received bison from the November 7th auction sale. Bison sampled included all of the sale bison on each farm (N= 37) that were alive at the time of sampling and a random

sample of 108 of their penmates.

Peripheral blood leukocytes from all bison were tested for the presence of OvHV-2 DNA with a PCR assay as previously reported (Li, Shen *et al.* 1995) at Prairie Diagnostic Services. Serum was tested for the presence of antibodies to MCF viruses with a CI-ELISA test, as previously reported (Li, Mcguire *et al.* 2001), at the Department of Veterinary Microbiology and Pathology at the Washington State University in Pullman, Washington. Serum samples were classified as being positive for MCF virus antibodies when they had a percent inhibition greater than 25 percent. Rogan-Gladen prevalence estimates and confidence intervals were calculated as previously reported (Greiner, Gardener 2000).

In order to control for the potential effect of clustering by herd, the odds ratio and confidence interval for comparisons of the CI-ELISA positive prevalence between sale bison and bison they were mixed with on destination farms were calculated in a mixed-effects generalized linear model with herd as a random effect using PROC.GLIMMIX, SAS® version 8.1 software (SAS Institute Inc, Cary, NC.).

Two by Two tables for all other comparisons of groups had cells with values of zero or less than 5 and produced unstable odds ratios when estimated with PROC GLIMMIX. Odds ratios with accompanying confidence intervals for these comparison were calculated as crude odds ratios, unadjusted for clustering. For two by two tables with cells values of zero a value of one was added to each cell. Confidence intervals for these comparisons were exact binomial confidence intervals and p values were Fischer's exact p values. A 5% level of significance was used for hypothesis testing.

6.4. Results

The overall SA-MCF mortality rate in this outbreak was 27.6 percent of bison that were sold at the November 7th auction sale. SA-MCF mortalities occurred among all groups of bison submitted to the sale. Mortality rates ranged from 13.3 percent to 50.0 percent for bison from each farm submitting bison to the sale (table 6.1). There were SA-MCF mortalities on 7 of the 11 farms that purchased bison following the sale. Mortality rates on destination farms ranged from 0.0 percent to 43.8 of bison purchased at the November 7th sale (table 6.2).

Following the removal of sale bison to the November 7th sale there were 415 bison remaining on submitting farms. During the 18 month period of observation there were no mortalities reported among these 415 bison.

Bison sold at the November 7th auction sale were mixed with 1010 bison on 11 destination farms. There were 4 mortalities among these bison, none of which were attributed to SA-MCF. Sale bison were 95.5 (95% CI = 55.0 - 167.1, $p < 0.001$) times more likely to die from any cause than bison they were mixed with on their destination farms. A comparison of the rate of confirmed SA-MCF mortalities among sale bison to the rate of SA-MCF mortalities among bison they were mixed with, estimated that sale bison were 155.5 (95% CI = 65.2 - 370.9, $p <0.001$) times more likely to die of SA-MCF than bison they were mixed with after the sale. When both confirmed and suspect SA-MCF mortalities were combined, sale bison were 390.0 (95% CI = 195.5 - 781.1, $p <$ 0.001) times more likely to die of SA-MCF than bison they were mixed with on

destination farms.

Among the 37 sampled sale bison and 108 sampled bison they were mixed with on their destination farms, the overall prevalence of bison that were CI-ELISA positive for MCF virus antibodies was 20.0 percent (Rogan Gladen prevalence = 19.8%, 95% CI = 13.2 - 26.3). The difference in prevalence between sale bison and bison they were mixed with was not statistically significant. Sale bison had a CI-ELISA positive prevalence of 24.3 percent (Rogan Gladen prevalence = 24.1%, 95% CI = 10.2 - 38.0) and bison they were mixed with had a CI-ELISA positive prevalence of 18.5 percent (Rogan Gladen prevalence = 18.3, 95% CI = 10.9 - 25.6) The 95% confidence intervals for the two prevalences overlapped and the odds ratio for the comparison of the two prevalences was 1.9, with a 95% confidence interval that encompassed one (95% CI = 0.73 - 5.0, $p = 0.19$).

The prevalence of bison that were PCR positive for OvHV-2 DNA among the 37 bison sampled on destination farms and a random sample of 108 of their penmates was similar and low. The overall PCR positive prevalence for both groups combined was 2.10 percent (95% CI = 0.4 - 5.9). The prevalence among sale bison was 2.70 percent (1/37) (95% CI = 0.1 - 15.8) and among their penmates the prevalence was 1.85 percent (2/108) (95% CI = 0.2 - 6.5). The odds ratio estimate of 1.5 for the comparison of the two prevalences was not statistically significant (95% CI = 0.13 - 16.63, $p = 1.00$).

When the confirmed SA-MCF moralities among the sale bison were included with the 37 sale bison tested on destination farms, the OvHV-2 PCR positive prevalence was 37.93 percent (95% CI = 25.51- 51.62). The odds ratio for the comparison of the

PCR positive prevalence in this group of bison to the PCR prevalence in the group of 108 bison on destination farms was statistically significant at 32.3 (95% CI 1 = 0.95 - 95.80, $p < 0.001$).

6.5 Discussion

The events that led to this outbreak of SA-MCF, namely the transport of 163 bison originating from 8 geographically separated farms transported to one location where they were exposed to sheep and then transported to 11 other widely separated farms where they were mixed with 1010 bison, can be considered a natural experiment with three cohorts. Bison sold at the auction sale were a cohort exposed to sheep. Those bison remaining on originating farms following removal of sale bison formed a second cohort. This cohort served to demonstrate that bison sold at the November 7th auction sale did not become infected with the agent causing this outbreak of SA-MCF while on their farms of origin. The third cohort were bison that were mixed with sale bison on destination farms. This cohort served to demonstrate that bison sold at the November 7th auction sale did not become infected with the agent causing this outbreak of SA-MCF on their destination farms, and that exposed bison did not transmit SA-MCF to any of the bison they were mixed with following the sale. It is highly unlikely that this outbreak of SA-MCF was precipitated by any means other than the exposure of bison to sheep for a short period at the November 7th auction sale.

Even though the study of this outbreak was not begun until well into the outbreak it should still be considered a prospective observational study. The period of observation

commenced on the day bison farmers selected and transported bison from their farms to the auction sale and ended 18 months after the sale. From the time of the sale to the end of the observation period, all bison on both submitting and purchasing farms were accounted for.

The bison farms involved in this outbreak were not a random selection of Saskatchewan bison farms, nor were bison sold at the auction market a random selection of the bison on each farm. However, failing to randomize was unlikely to have affected this outbreak. There is no reason to believe that bison sold at the auction mart were not representative of other Saskatchewan bison. Nor is there any reason to believe that bison sold at the auction sale were more predisposed to developing MCF than their pen-mates on their farms of origin. It is unlikely that on the 8 farms submitting bison to the sale, farm owners would have purposely selected for sale only those bison that were in some way predisposed to developing MCF. It is more probable that the outbreak was caused by exposure of bison to sheep at the auction sale. The probability of this having occurred by chance alone was extremely low (odds ratio 160.8, 95% CI 62.2 - 396.6, p value < 0.001)

Although feeding and management of bison on the farms involved in this outbreak was not under the direct control of the author, it is unlikely that bison sold at the auction sale were managed differently from unexposed bison. In all but one destination farm, sale bison were mixed in pens with bison of the same age. All bison within each pen were provided the same feed and were maintained under the same environmental conditions. If differential treatment of sale bison and un-exposed bison

had occurred, it did not have an effect on the relative risks estimated from the outbreak, since there were no SA-MCF mortalities recorded in either the bison remaining on the farms of origin or the bison mixed with sale bison on destination farms..

Performing stressful procedures on bison such as processing them in handling facilities has been associated with the occurrence of SA-MCF mortalities in one bison feedlot (O'Toole, Li *et al.* 2002). Selling bison through an auction sale is a stressful experience for bison. However, it is unlikely that the sole act of selling bison through an auction sale caused this outbreak. Many bison are sold through auction sales every year in North America. There have been no outbreaks of MCF reported in association with other auction sales. In addition, the auction market that was central to this outbreak of SA-MCF has held previous bison sales, none of which were associated with SA-MCF outbreaks.

The criteria used for classifying bison mortalities as being due to SA-MCF was not rigorous for all bison mortalities that occurred in this outbreak. By the time the author was made aware of the outbreak, over half of the bison mortalities had already occurred. Any subsequent mortalities that occurred were collected and subjected to complete postmortem examinations. Mortalities were classified as being MCF mortalities by gross pathology, histopathology and positive PCR assays for OvHV-2 DNA. However, postmortem examinations were performed on only 21 of 45 MCF mortalities. The other 24 mortalities were classified as SA-MCF mortalities based only on clinical histories provided by farmers. Although the method of classification for these latter mortalities, based on history alone, may not have been as rigorous as the former, it

is likely that most, if not all of them were SA-MCF mortalities. The rate of non-SA-MCF mortality among bison remaining on the farms of origin following removal of the November 7th sale bison was zero. Since sale bison originated from these farms it is unlikely that they would have experienced mortalities due to causes other than SA-MCF at a much higher rate than those bison remaining on the farms of origin. Likewise, the rate of non-SA-MCF mortality among the 1010 bison that sale bison were mixed with following the sale was low and it is unlikely that the sale bison would have had a much higher non-SA-MCF mortality rate than their penmates on destination farms.

Failing to accurately classify 24 of the 45 mortalities that occurred within the group of sale bison would have had little effect on the associations inferred from this outbreak. Excluding the suspect SA-MCF mortalities from the relative risk calculations would have reduced the magnitude of the relative risk estimates for comparisons of SA-MCF mortality rates among sale bison and un-exposed bison, but would not have changed their direction or made them statistically insignificant. It is also unlikely that there was mis-classification of mortalities among the un-exposed bison. The number of mortalities among the un-exposed bison was very low (4 mortalities among 1425 bison). Two of the mortalities were due to trauma and the other two were classified as being due to pneumonia by post mortem examinations. Besides there were only a small number of mortalities within the un-exposed bison, their mis-classification would not have significantly changed the strength or direction of the relative risks estimated from the outbreak.

The association between sheep and MCF in cattle has been recognized since the

late 1700's (Heuschele, Reid 2001). Early epidemiological studies demonstrated that cattle stabled next to sheep were often at risk of developing MCF (Aleska. 1935; Bindel 1937; Goetze 1930; Goetze, Liess 1929; Goetze, Liess 1930; Magnusson 1940; Piercy 1954). There have been several viruses isolated from SA-MCF mortalities, however, none of them have reproduced MCF (Reid, Buxton *et al.* 1984). OvHV-2, although never isolated in tissue culture, has remained the only candidate as a causal agent for SA-MCF (Bridgen, Reid 1991; Collins, Bruns *et al.* 2000; Crawford, Li *et al.* 1999; Ensser, Pfanz *et al.* 1997; Li, Shen *et al.* 1995; Muller Doblies, Li *et al.* 1998; O'Toole, Li *et al.* 1997; Plowright 1990; Schuller, Cerny Reiterer *et al.* 1990; Schultheiss, Collins *et al.* 2000). Large proportions of sheep populations are infected with OvHV-2 (Baxter, Pow *et al.* 1993; Li, Shen *et al.* 1996a; Li, Shen *et al.* 1994; Li, Shen *et al.* 1996; Li, Shen *et al.* 1995; Mirangi, Kang'ee 1997). Support for OvHV-2 as the causal agent for SA-MCF comes from studies demonstrating that most cattle and bison that die of MCF have OvHV-2 DNA in their carcasses, but healthy cattle and bison or cattle and bison that die of other causes rarely have OvHV-2 in their blood or carcasses (Baxter, Pow *et al.* 1993; Collins, Bruns *et al.* 2000; Crawford, Li *et al.* 1999; Dunowska, Letchworth *et al.* 2001; Hussy, Stauber *et al.* 2001; Li, Shen *et al.* 1995; Li, Snowder *et al.* 1998; Muller Doblies, Li *et al.* 1998; Tham 1997; Wiyono, Baxter *et al.* 1994).

The outbreak reported here provides further support for sheep being a common source of OvHV-2 in outbreaks of SA-MCF. The presence of OvHV-2 DNA was demonstrated in all MCF mortalities that were examined. The measured associations between exposure of bison to sheep for a short period of time and the occurrence of SA-

MCF mortalities in bison were very strong in this outbreak. Among all of the 1588 bison included in this study it was only those bison exposed to sheep at the November 7th auction sale that experienced SA-MCF mortalities. It is highly unlikely that bison sold at the November 7th sale were infected with OvHV-2 on their farms of origin, since there were no SA-MCF mortalities among bison remaining on these farms before and during the study period. It is also highly unlikely that bison sold at the auction sale were infected with OvHV-2 at their destination farms since there were no SA-MCF mortalities among any of the bison they were mixed with on their destination farms for the duration of the study.

The mechanism by which SA-MCF is transmitted from sheep to cattle or other susceptible species has never been clearly demonstrated. Attempts to transmit MCF to cattle and deer by inoculating them with sheep's blood, tissues, nasal secretions and external parasites have failed (Blood, Rowsell *et al.* 1961; Goetze 1930; Goetze, Liess 1929; Goetze, Liess 1930; Hoffman, Young 1989; Metzler 1991; Pierson, Storz *et al.* 1974; Plowright 1990). However, there are many reports of MCF being transmitted from sheep to cattle and deer by housing sheep in their close proximity (Aleska. 1935; Bindel 1937; Goetze, Liess 1929; Goetze, Liess 1930; Imai, Nishimori *et al.* 2001; Magnusson 1940; Piercy 1954). Goetze reported the close stabling of sheep to cattle in 37 of 40 farms on which MCF occurred (Goetze, Liess 1930). In this outbreak the exact mechanism by which OvHV-2 was transmitted from sheep to bison is unknown. Because of the wild and dangerous nature of farmed Saskatchewan bison, the bison at the November 7th auction sale were confined to stout pens for the duration of their stay at the

auction market. Auction market workers were unlikely to be in direct contact with bison since entering pens containing unrestrained bison would have been very dangerous. Confining sale bison in such a manner would have precluded any direct physical contact between bison and sheep at the sale. Furthermore, sheep present at the auction market were penned in a room adjacent to the room where bison were penned. A possible mechanism for transmission of OvHV-2 from sheep to bison at the auction market would have been via air movement from sheep pens to bison pens through two large openings that connected the two rooms. OvHV-2 DNA has been shown to be present in the nasal secretions of many sheep (Li, Hua *et al.* 2001; Li, Snowder *et al.* 1998; Li, Snowder *et al.* 2000). Although the presence of OvHV-2 DNA in nasal secretions cannot be considered unequivocal evidence for the presence of infectious OvHV-2 virus particles, aerosolized water droplets containing OvHV-2 virus particles from air exhaled by sheep has been speculated as a mechanism by which sheep transmit OvHV-2 to susceptible ruminants (Li, Hua *et al.* 2001).

Susceptible hosts that develop SA-MCF are thought to be unable to transmit OvHV-2 to other susceptible hosts (Heuschele 1988; Heuschele, Reid 2001; Plowright 1990). The reason for this remains unclear. It has been speculated that OvHV-2 has only limited viral replication within susceptible hosts resulting in little or no cell free infectious virus being produced on epithelial surfaces (Heuschele, Reid 2001). Horizontal transmission of SA-MCF has been sporadically successful by intravenous, subcutaneous, intraperitoneal, and intradermal injection as well as oral administration of large quantities of blood in cattle (Blood, Rowsell *et al.* 1961; Goetze 1930; Goetze,

Liess 1929; Goetze, Liess 1930; Horner, Oliver *et al.* 1975; Magnusson 1940; Selman, Wiseman *et al.* 1974; Selman, Wiseman *et al.* 1978), red deer (*Cervus elaphus*) (Huck, Shand *et al.* 1961; Oliver, Beatson *et al.* 1983), water buffalo (*Bubalus bubalus*) (Wiyono, Baxter *et al.* 1994) and bison (*Bison bison*) (Liggitt, McChesney *et al.* 1980). However, horizontal transmission of SA-MCF from cattle with SA-MCF to healthy cattle stabled next to them has always failed (Goetze 1930; Goetze, Liess 1929; Goetze, Liess 1930; Piercy 1954; Pierson, Thake *et al.* 1973).

The outbreak reported here provides strong evidence that bison with SA-MCF do not transmit SA-MCF to other susceptible bison. In this outbreak 45 bison that died of SA-MCF failed to transmit SA-MCF to any of 1010 bison they were penned with on 11 different farms.

Although this outbreak provides strong evidence against horizontal transmission of SA-MCF, evidence against horizontal transmission of OvHV-2 is less persuasive. Prevalence of OvHV-2 PCR positive bison in a group of 37 surviving auction sale bison and a random sample of 108 of their penmates provided inconclusive evidence for horizontal transmission of OvHV-2 having occurred among the bison tested. The presence of OvHV-2 DNA in peripheral blood leukocytes was demonstrated by PCR in only one surviving sale bison and two of their penmates. The pre-sale PCR status of the bison tested could not be determined. Therefore those bison that were positive may have been positive before the sale. The low PCR positive prevalence among surviving sale bison may have resulted from most OvHV-2 infected sale bison having died of SA-MCF prior to being tested. If horizontal transmission of OvHV-2 had occurred it was expected

that some of those bison that were mixed with sale bison would have died of MCF, or that the PCR positive prevalence among these bison would have been higher, possibly in the range of the prevalence of SA-MCF mortalities among sale bison. This was not observed.

Although the OvHV-2 PCR prevalence results provide no evidence supporting the occurrence of horizontal transmission of OvHV-2 in bison, they cannot be considered definitive proof that horizontal transmission of OvHV-2 did not occur. There are no reports of studies establishing the sensitivity of PCR assays for detecting OvHV-2 infections in healthy bison. It may very well be that the analytical sensitivity of the PCR assay for detecting OvHV-2 infected bison is very low in populations of healthy OvHV-2 infected bison. For this reason the assay may have failed to detect healthy bison that were infected with OvHV-2 as a result of horizontal transmission.

The CI-ELISA prevalence of bison with MCF antibodies among 37 surviving sale bison and 108 of their penmates provided no direct evidence for or against the occurrence of horizontal transmission of OvHV-2 among the bison in this outbreak. The CI-ELISA positive prevalence was slightly higher among surviving sale bison, being 24.3 percent, as compared to 18.5 percent among their penmates . Surviving sale bison were expected to have a higher CI-ELISA positive prevalence than their penmates since they were known to have been exposed to OvHV-2 at the auction market. Their penmates were not. However the difference between the two groups was not statistically significant, possibly due to their small sample sizes. The pre-sale CI-ELISA status of the bison could not be determined. The CI-ELISA positive prevalence of both groups of

bison were within the range expected for bison herds that had no SA-MCF mortalities (see chapter 4). It cannot be concluded from these CI-ELISA test results that OvHV-2 was or was not horizontally transmitted among the bison tested following this outbreak

There has been some suggestion that bison may be latently infected with OvHV-2 for long periods before developing SA-MCF (O'Toole, Li *et al.* 2002). However, there is little support for this theory in the literature. Liggit (Liggitt, McChesney *et al.* 1980) reported an incubation period of 24 days for the occurrence of SA-MCF following transfusion of blood from an infected bovine calf into a single yearling bison. Schultheiss (Schultheiss, Collins *et al.* 1998) reported one bison that survived for 80 days following an acute episode of SA-MCF before going on to die of a recrudescence of SA-MCF. There have been no reports of the length of time bison may be latently infected with OvHV-2 before developing SA-MCF following exposure to sheep. In this outbreak the earliest time that SA-MCF occurred in bison was 50 days following a single short exposure to sheep. The median time was 65 days and the mean was 81 days. The last bison to die in the outbreak died 220 days following the auction sale, suggesting that bison can be latently infected with OvHV-2 for a period of at least 220 days before developing SA-MCF. Since the sensitivity of the PCR assay for detecting OvHV-2 infection in healthy bison is not known, it is possible that not all of the bison infected with OvHV-2 at the auction sale died of SA-MCF during the observation period. Some of the bison infected with OvHV-2 at the auction sale may have remained alive and healthy through the study period without developing SA-MCF.

It has been previously established that at least 20 percent of farmed bison have antibodies to MCF viruses (chapter 4), suggesting that a relatively large proportion of farmed bison carry MCF virus infections. Since the CI-ELISA test used to detect

antibodies to MCF viruses is sensitive to all known MCF viruses (Li, Shen *et al.* 1994), the identity of the MCF virus infecting these bison is unknown. But it is possible that this un-identified virus may be OvHV-2. However, OvHV-2 has been shown to be closely associated with MCF mortalities in bison (Collins, Bruns *et al.* 2000; O'Toole, Li *et al.* 2002; Schultheiss, Collins *et al.* 1998; Schultheiss, Collins *et al.* 2000). In this outbreak 27.6 percent of the bison exposed to sheep died from MCF. Those MCF mortalities tested were all positive for OvHV-2 DNA. It has also been shown that MCF virus antibodies can be maintained in bison across generations in the absence of exposure to sheep or any other ruminant sources of MCF virus infection (chapter 5). This latter finding provides some support for the theory that the MCF virus infections observed in healthy farmed bison may be the result of bison to bison transmission of an unidentified MCF virus. In the outbreak reported here, bison were infected with OvHV-2 for as long as 220 days before they died of MCF. During that time none of the 45 bison that died of MCF successfully transmitted MCF to any of the 1010 bison they were in close association with. If OvHV-2 was transmitted among the bison in this outbreak, it did not produce MCF in any bison. Since the MCF virus infecting farmed bison is unidentified, it can only be speculated that if OvHV-2 is in fact the un-identified MCF virus, it must lose it's pathogenicity when it passes through bison.

6.5.1 Summary

This outbreak provides strong evidence for a causal association between exposure to sheep and the subsequent occurrence of MCF in bison. It also provides strong evidence that SA-MCF is not transmitted among bison and that bison can be infected with OvHV-2 for up to 220 days before developing MCF. However it provides

no evidence for or against the horizontal transmission of OvHV-2 among the bison. At best, it can be speculated that either OvHV-2 was not horizontally transmitted among the bison in this outbreak or if it was, it was at a low frequency and the virus was no longer pathogenic once it was transmitted from one bison to other bison.

7. PCR ASSAYS FOR MCF VIRUSES IN BISON

7.1 Introduction

Malignant catarrhal fever is a fatal disease of many ungulates including cattle, bison, water buffalo and deer (Heuschele Reid 2001; Plowright 1990). It is most commonly caused by one of two distinct but closely related gamma herpesviruses belonging to the provisionally named MCF sub-group of ruminant rhadinoviruses (Heuschele Reid 2001; Li, Keller *et al.* 2001). Wildebeest (*Connochaetes species*), most of which are sub-clinically infected with Alcelaphine herpesvirus-1 (AlHV-1), produce wildebeest-associated MCF (WD-MCF) when they transmit AlHV-1 to susceptible ruminants in Africa and zoos or game farms where wildebeest are penned (Heuschele Reid 2001). Similarly sheep, most of which are sub-clinically infected with ovine herpesvirus-2 (OvHV-2), produce sheep-associated MCF (SA-MCF) when they transmit OvHV-2 to susceptible ruminants worldwide (Heuschele Reid 2001; Li, Westover *et al.* 1999). Recent reports suggest that other MCF group viruses may be causally associated with MCF. White-tailed deer (*Odocoeleus virginianus*) that died of MCF were shown to have viral DNA in their tissues belonging to a previously unreported MCF group virus (Kleiboeker, Miller *et al.* 2002; Li, Dyer *et al.* 2000). Caprine herpesvirus-2 (CpHV-2), a virus that sub-clinically infects domestic goats, caused chronic disease in a sika deer (*Cervus nippon*) and Alcelaphine herpesvirus-2 (AlHV-2), demonstrated to sub-clinically infect Jackson's hartebeest (*Alcelaphus buselaphus jacksoni*) has been reported to cause MCF in barbary red deer (*Cervus elaphus barbarus*) (Crawford, Li *et al.* 2002;

Klieforth, Maalouf *et al.* 2002).

Ovine herpesvirus-2, the most common cause of MCF among farmed ruminants throughout the world (Heuschele Reid 2001; Plowright 1990) has been reported to cause MCF in North American farmed bison (*Bison bison*) (Collins, Bruns *et al.* 2000; O'Toole, Li *et al.* 2002; Schultheiss, Collins *et al.* 1998; Schultheiss, Collins *et al.* 2000). Susceptible hosts, such as bison, that develop SA-MCF are generally considered to be unable to transmit OvHV-2 to other susceptible hosts (Heuschele 1988; Heuschele Reid 2001; Plowright 1990) (also see chapter 6). Since MCF virus infections are often fatal to susceptible hosts it is logical that relatively few members of susceptible host species populations would be infected with MCF viruses. Only 4% of 232 reindeer (*Rangifer tarandus*), which are susceptible to MCF, were seropositive for antibodies to MCF viruses (Zarnke, Li *et al.* 2002). Similarly, only 3% of 63 white-tailed deer were demonstrated seropositive for MCF virus antibodies (Li, Shen *et al.* 1996a). In contrast to white-tailed deer and reindeer, recent sero-surveys have reported that 17% of 197, 23.7% of 300 and 22.9% of 2594 healthy bison had antibodies to MCF viruses (O'Toole, Li *et al.* 2002; Zarnke, Li *et al.* 2002) (also see chapter 4). In addition, it has been reported that an MCF virus was being transmitted among bison in one confined bison population in the absence of any known ruminant sources of MCF viruses (chapter 5). These studies demonstrated MCF viral infections by detecting a serum antibody that is conserved among all known MCF viruses (Li, Mcguire *et al.* 2001), making it impossible to be certain of the identify of the MCF virus infecting healthy bison. Studies reporting the use of more specific tests are sparse. In one study, PCR assays detected OvHV-2 DNA in the peripheral blood leukocytes of only 8 out of 111 healthy bison from a feedlot during an outbreak of MCF (O'Toole, Li *et al.* 2002).

The purpose of this report is to present the results of three studies aimed at establishing the identity of MCF group viruses that infect healthy farmed bison. In the first study, PCR assays specific for OvHV-2 were used to assay the peripheral blood of 127 healthy bison involved in an outbreak of MCF for the presence of OvHV-2 DNA. In the second study PCR assays specific for OvHV-2 were used to assay the peripheral blood, kidneys and livers of 99 healthy adult bison for the presence of OvHV-2 DNA. In the third study peripheral blood lymphocytes from 12 healthy adult bison were cultured in vitro in the presence of the phorbol ester, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), which has been shown to induce lytic growth of gamma herpesviruses in cell cultures (Rolf, Zhong *et al.* 1996). DNA was extracted from the lymphocyte cell cultures and assayed for the presence of herpesvirus DNA with PCR assays using consensus herpesvirus DNA polymerase primers and primers specific for OvHV-2.

7.2 Materials and methods

7.2.1 Samples

7.2.1.1 Bison associated with MCF outbreaks

Whole blood and serum were collected from 52 female and 75 male bison aged 8 to 20 months. Bison were selected as a convenience sample from three herds that had confirmed cases of MCF in the last three months prior to testing. Herds contained 115, 200 and 307 bison. Whole blood was tested with OvHV-2 specific PCR assays and serum was tested with a CI-ELISA specific for MCF viruses.

7.2.1.2 Bison not associated with MCF outbreaks or sheep

Whole blood and serum as well as kidney and liver samples were collected from

99 adult female bison during a complete herd depopulation. MCF was never reported in this herd and there were no sheep within 2 miles of the herd. DNA extracted from whole blood, kidney and liver were tested with OvHV-2 specific PCR assays and serum was tested with a CI-ELISA specific for MCF virus antibodies.

7.2.1.3 Bison sampled for lymphocyte culture

Approximately 24 ml of whole blood was collected into ACD (22.0 g/L trisodium acetate, 8.0 g/L citric acid, 22.0 g/L dextrose) anticoagulant vacutainer tubes by venipuncture from12 adult female bison from two different bison herds. Blood samples were maintained at room temperature and processed within 4 hours for cell culture. DNA was extracted from lymphocytes prior to culturing and after culture for 72 to 144 hours and then tested by PCR with primers specific for OvHV-2 as well as consensus herpes DNA polymerase primers.

7.2.1.4 Controls for PCR assays

Both positive and negative controls were included with each batch of samples that were assayed. Positive controls came from whole blood from sheep that were demonstrated to have OvHV-2 DNA in their blood by PCR assays. Negative controls were PCR reaction mixtures with sterile water added instead of sample DNA.

7.2.2 Sample Analysis

7.2.2.1 Extraction of DNA

Two hundred microliters of whole blood or lymphocyte cell culture suspension was mixed with 400 µL of D solution (4M GITC, 25mM sodium citrate (pH 7.0), 0.1 M

2-mercapto-ethanol, 0.5% sacrosyl) and vortexed for 1 minute. Three hundred microliters of phenol-chloroform (1: v/v) was added, the mixture was vortexed for 30 seconds and then centrifuged at 10,000 G at 4° C for 5 minutes. Three hundred microliters of the supernatant was transferred to 200 µL of phenol-chloroform, vortexed for 30 seconds and centrifuged at 10,000 G at 4° C for 5 minutes. Two hundred microliters of the supernatant was added to 500 µL of 95% ethanol and centrifuged at 10,000 G at 4° C for 10 minutes. The supernatant was poured off, the DNA pellet dried and re-suspended in 25 to 50 µL of water prior to PCR amplification

Kidney and liver samples were macerated with a scalpel, suspended in 500 µL of Lysis solution (100mM sodium chloride, 500mM Tris (pH 8) and 10% SDS) with 5 µL of proteinase K (1/100 dilution) and incubated for 2 hours at 65° C. Following incubation 500 µL of phenol chloroform was added to the mixture. The mixture was vortexed for 30 seconds and centrifuged at 15,000 G for 5 minutes. The upper aqueous layer was removed and mixed with 500 µL of phenol chloroform and centrifuged at 15,000 G for 5 minutes. The upper aqueous layer was removed, mixed with 1000 µL of ice-cold salted 95% ethanol and incubated at minus 20° C for 10 minutes. Following incubation the mixture was centrifuged at 15,000 G at 4° C for 15 minutes. The supernatant was poured off, 200 µL of 80% ethanol was added and the mixture centrifuged at 15,000 G at 4° C for 5 minutes. The ethanol was drained off, the DNA pellet dried and then re-suspended in 25 to 100 µL of water prior to PCR amplification.

7.2.2.2 PCR assays

PCR protocols for amplifying OvHV-2 DNA were adapted from Baxter *et*

al.(Baxter, Pow *et al.* 1993). For the primary reaction 5 μL of extracted DNA suspensions were added to a mixture of 35.7 μL water, 5 μL 10X PCR buffer (Qiagen), 2.5 μL (50 mM) $MgCl_2$, 0.5 μL (25 mM) each of dATP, dGTP, dCTP, and dTTP, 1.0 μL (20 pmol) each of primer 556 (AGTCTGGGTATATGAATCCAGATGGCTCTC) and primer 755 (AAGATAAGCACCAGTTATGCATCTGATAAA), 0.3 μL (5 U/μL) Taq DNA polymerase (Qiagen) and 1 drop of mineral oil. For the secondary reaction 5 μL of the primary reaction mixture was added to 35.2 μL water, 5 μL 10X PCR buffer (Qiagen), 2 μL (50 mM) $MgCL_2$, 0.5 μL (25 mM) each of dATP, dGTP, dCTP and dTTP, 1.0 μL (20 pmol) each of primers 556 and 555 (TTCTGGGGTAGTGGCGAGCGAAGGCTTC), 0.3 μL (5 U/μL) Taq DNA polymerase (Qiagen) and 1 drop of mineral oil. Thermal cycling protocols for both the primary and secondary reactions were 95° C for 5 minutes followed by 35 cycles of 94° C for 60 seconds, 55° C for 60 seconds and 72° C for 60 seconds. After cycling the mixture was incubated at 72° C for 5 minutes and then held at 4° C. PCR assays for OvHV-2 DNA were classified as positive when the reaction produced a PCR product of approximately 238 base pairs.

Consensus herpes virus PCR protocols were adapted from VanDevanter *et al.*(VanDevanter, Warrener *et al.* 1996). For the primary reaction 5 μL of each sample with 0.1 to 2.0 μg total DNA was added to a mixture of 27.25 μL water, 5 μL 10X PCR buffer (Qiagen), 4 μL (25 mM) $MgCL_2$, 1.25 μL DMSO (for a final concentration of 2.5%), 0.5 μL (25 mM) each of dATP, dGTP, dCTP and dTTP, 1 μL (5 U/μL) Taq DNA polymerase (Qiagen) and 2 μL (20 pM) each of upstream primers ILK (TCCTGGACAAGCAGCARNYSGCNMTNAA), DFA

(GAYTTYGCNAGYYTNTAYCC) and downstream primer KG1 (GTCTTGCTCACCAGNTCNACNCCYTT). One drop of light mineral oil was added to the mixture. For the secondary reaction 5 µL of the primary reaction was added to a mixture of 29.25 µL water, 5 µL 10X PCR buffer (Qiagen), 4 µL (25 mM) $MgCL_2$, 1.25 µL DMSO (for a final concentration of 2.5%), 0.5 µL (25 mM) each of dATP, dGTP, dCTP and dTTP, 1 µL (5 U/µL) Taq DNA polymerase (Qiagen) and 2 µL (20 pM) each of primers TGV (TGTAACTCGGTGTAYGGNTTYACNGGNGT) and 1YG (CACAGAGTCCGTRTCNCCRTADAT). One drop of light mineral oil was added to the mixture. Thermal cycling settings for both the primary and secondary reactions were 94° C for 5 minutes followed by 45 cycles of 94° C for 30 seconds, 46° C for 60 seconds and 72° C for 60 seconds. After cycling the reaction mixture was incubated at 72° C for 7 minutes and held at 4° C. Consensus PCR DNA polymerase assays were classified as positive when the PCR reaction produced a PCR product of approximately 230 base pairs.

For PCR product visualization 10 µL of amplified PCR products were analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining.

7.2.2.3 Lymphocyte cultures

Vacutainer tubes containing whole blood were centrifuged at 2000 rpm for twenty minutes. Buffy coats were aspirated and mixed with equal volumes of PBS and Elseviers solution (2 parts PBS to 1 part Elseviers solution). Five mls of this mixture was layered over 5 mls of 70 % percol and centrifuged for 25 minutes at 2500 rpm.

Lymphocytes were aspirated from percol gradients and suspended in equal volumes of PBS and Elsevier's solution (2 parts PBS to 1 part Elseviers solution). Lymphocytes were cultured in concentrations ranging from 10^6 to 10^7 cells/ml in 2ml volumes in 24 well tissue culture plates (Falcon, Becton Dickenson) and 5ml volumes in Falcon T-25 25cm^2 tissue culture flasks (Becton Dickenson). Culture media consisted of RPMI media (Invitrogen) plus 10% (V/V) fetal calf serum, 100units/ml penicillin G, 100 μg/ml streptomycin 0.3ng/ml L-glutamine, 0.5 mM 2-mercaptoethanol, 20mM Hepes solution with 5ug/ml Concovalin A and 20 or 50 ng/ml 12-*O*-tetradecanoyl phorbol-13-acetate. Cultures were incubated at 37°C in 97% humidity with 5% CO_2. Lymphocytes were harvested for DNA extraction after 72 and 144 hours in culture.

7.2.2.4 MCF virus antibody detection

MCF viruses antibodies were detected using a recently reformatted direct competitive inhibition enzyme linked immunosorbent assay (CI-ELISA), as previously reported (Li, Mcguire *et al.* 2001) at the Animal Disease Research Unit, USDA-ARS, and the Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington. The assay detects an antibody produced against an antigenic epitope (15A) conserved among all known MCF group herpesviruses. Sera were classified positive when they inhibited binding of a monoclonal antibody against the 15A antigenic epitope by 25% more than the inhibition of negative control sera.

7.2.2.5 DNA sequencing

Consensus herpesvirus secondary PCR product bands (approximately 230 base

pairs) were sliced out of ethidium bromide stained gels and purified using an agarose gel DNA extraction kit (QIAquick gel extraction kit, Qiagen, Washington, USA). Purified PCR products were sequenced directly with both TGV and IYG primers with fluorescent dye terminators and Taq DNA polymerase on an automated DNA sequencer (ABI Prism 3700 capillary electrophoresis DNA analyzer, Applied Biosystems Foster City California) at the Plant Biotechnology Institute, National Research Council, Saskatoon, SK.

7.2.3 Data analysis

7.2.3.1 Sequence alignments

DNA sequences were aligned by the clustal-w method using Editseq and MegAlign® (DNA star, Madison, Wisc.) (Higgins Sharp 1989). Sense and anti-sense DNA base sequences for each DNA polymerase consensus positive sample were aligned in Editseq and those portions at either ends of each strand which contained errors were removed from the final sequence for the sample.

7.3 Results

7.3.1 Bison associated with MCF outbreaks.

Sera from 26 of the 127 bison tested were positive for MCF virus antibodies when tested with the CI-ELISA (prevalence = 20.5%, 95% CI = 13.8-28.5). OvHV-2 PCR assays of whole blood samples were positive for 3 bison (prevalence = 2.3%, 95% CI = 0.5-6.7), one of which was CI-ELISA positive.

7.3.2 Bison not associated with MCF outbreaks or sheep.

Sera from 22 of 99 bison tested were positive for MCF virus antibodies (prevalence = 22.2%, 95% CI = 14.8-31.7). PCR assays detected OvHV-2 DNA in one kidney sample from a bison that was CI-ELISA negative, all other samples were PCR negative (prevalence = 1.0%, 95% CI = 0.0-5.4).

7.3.3 Bison sampled for lymphocyte culture.

Prior to culture, DNA extracted from lymphocytes of all bison were negative when tested with PCR assays for OvHV-2 DNA and when tested with herpes consensus DNA polymerase PCR assays. Following culture for 72 to 144 hours, only one lymphocyte culture, bison number 8, was positive for OvHV-2DNA. Seven of the cultures including bison number 8 and the sheep whole blood sample, produced PCR products of approximately 230 base pairs when amplified with herpes DNA polymerase consensus primers (figure 7.1). Sequences from 2 of the bison lymphocyte cultures contained many errors and were excluded from further analysis. Following alignment and trimming of sequences, the remaining 5 bison lymphocyte cultures and one sheep whole blood sample yielded sequences ranging from 133 to 162 base pairs. These 5 bison lymphocyte culture DNA sequences were 99.2 to 100.0 percent similar (figure 7.2). They were 99.2 to 100.0 percent similar to the DNA sequence from sheep whole blood and 99.2 to 100.0 percent similar to a previously reported OvHV-2 DNA polymerase gene sequence (Chmielewicz, Goltz *et al.* 2001)(Genbank accession number AF327831).

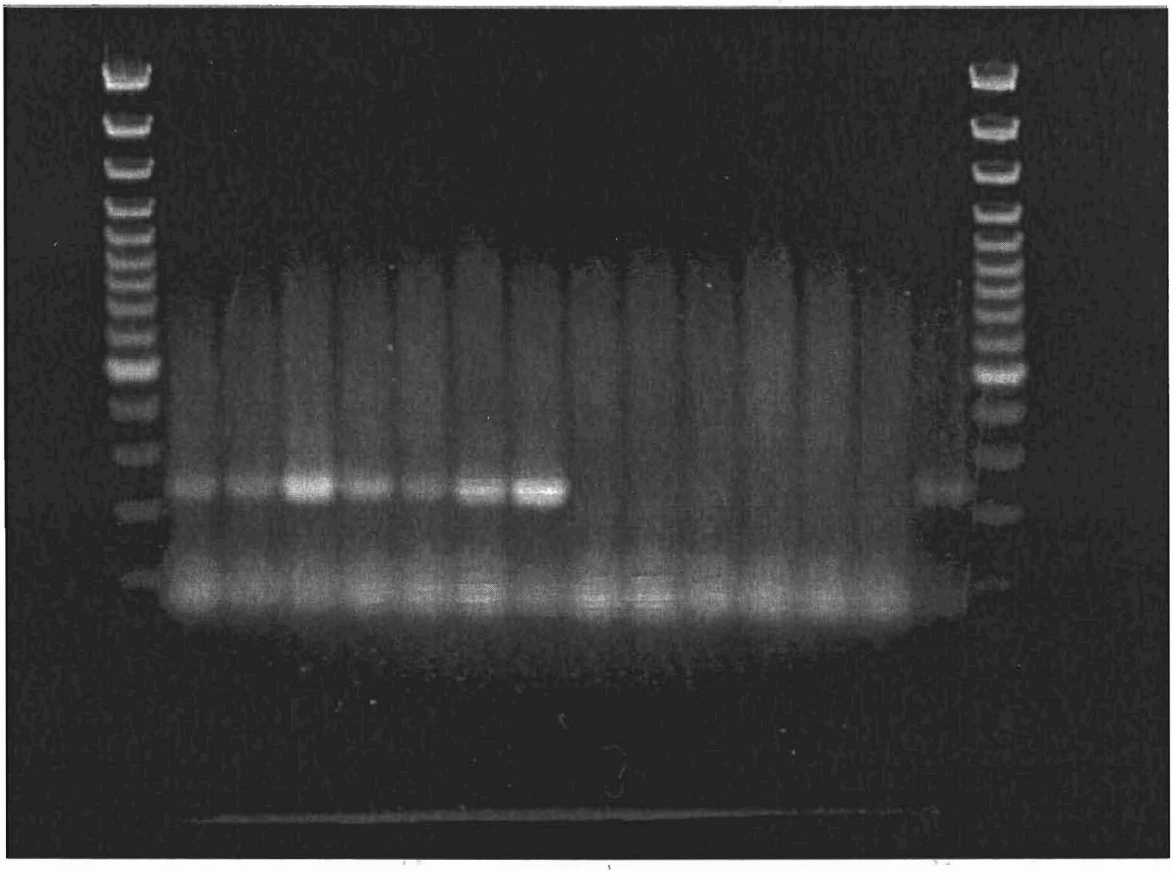


Figure 7.1 Gel electrophoresis of PCR products from consensus herpesvirus DNA polymerase PCR reactions for DNA extracted from12 bison lymphocyte cultures, one negative control and one positive control (DNA extracted from sheep whole blood). The first and last lanes are 100 Bp ladders. Lanes 2 to 13 are bison lymphocyte cultures. Lane 14 is the negative control and lane 15 is the positive control.

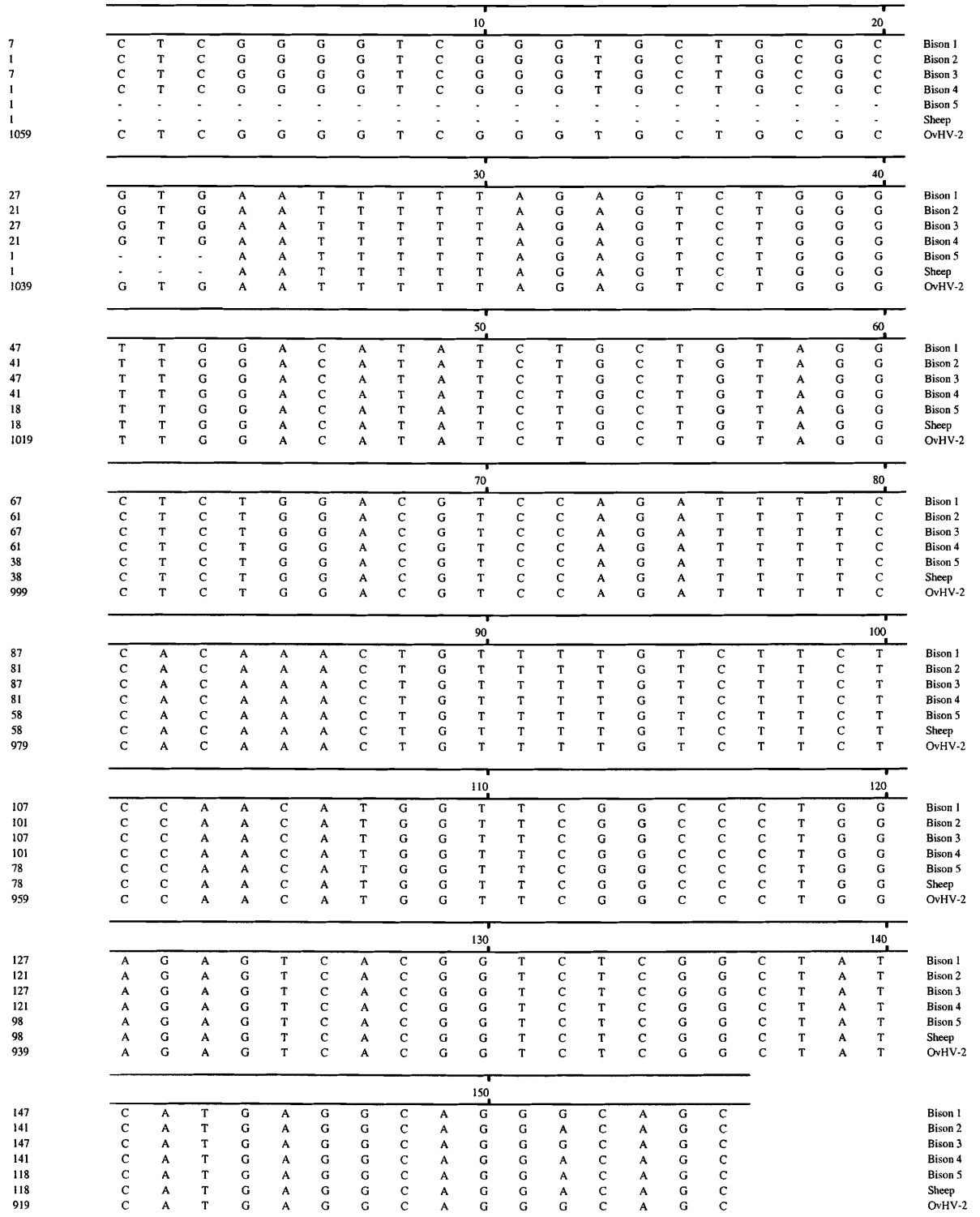


Figure 7.2 Sequences of DNA extracted from the lymphocytes of 5 bison following 50 to 60 hours of cell culture, sheep whole blood and OvHV-2 DNA polymerase gene (Genbank accession number AF327831)

7.4 Discussion

The prevalence of MCF virus antibody positive bison among the 226 bison tested in this study is similar to the prevalence reported for bison in previous studies. Although 48 of these bison had antibodies to MCF viruses, OvHV-2 specific PCR assays were able to detect OvHV-2 DNA in only 2 of them. The reason for this remains unclear. It might be speculated that in bison that are MCF virus antibody positive, the proportion of circulating lymphocytes that are infected with MCF viruses is low, possibly too low for PCR assays to detect.

The phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate has been demonstrated to induce gamma herpesvuses infecting lymphocytes in cell cultures to switch from latent to lytic growth cycles (Rolf, Zhong *et al.* 1996). Induction of lytic cycles would result in the production of large numbers of copies of herpes viral DNA that should be easily detected with appropriate PCR assays. Although there is no direct evidence, it can be surmised that a herpesvirus resembling OvHV-2 was induced into lytic cycles in 5 of the lymphocyte cultures in this study. Both OvHV-2 specific PCR assays and herpesvirus consensus DNA polymerase PCR assays were unable to detect herpesvirus DNA in pre-culture lymphocytes. However, following growth in cell culture with 12-*O*-tetradecanoyl phorbol-13-acetate, 5 lymphocyte cultures were demonstrated by PCR assay and sequence analysis to contain DNA sequences very similar to a sequence reported for the DNA polymerase gene of OvHV-2.

It is difficult to explain why OvHV-2 specific primers were able to amplify DNA from only one of the lymphocyte cultures in this study, when consensus herpesvirus DNA polymerase primers were able to amplify DNA from 7 of them. It may be possible that OvHV-2 specific primers were less sensitive than consensus herpesvirus DNA

polymerase primers. It may also be speculated that the DNA base sequences of the MCF virus infecting the bison in this study may be sufficiently different from those of OvHV-2 to prevent annealing of OvHV-2 specific primers.

This study was based on only a small number of bison and the results although preliminary, suggest that culturing bison lymphocytes in the presence of 12-*O*-tetradecanoyl phorbol-13-acetate has the potential of becoming a useful method for identifying MCF viruses that sub-clinically infect bison.

7.4.1 Summary

Tissues and blood from 226 healthy bison tested with OvHV-2 specific PCR primers were able to identify OvHV-2 in two bison. Culturing lymphocytes from 12 healthy bison in the presence of the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate allowed the identification of MCF viruses that were very similar to OvHV-2 in 5 bison using consensus herpesvirus PCR primers

8. DISCUSSION

The two major objectives of this thesis were to estimate the prevalence of latent MCF virus infections in farmed bison populations in Alberta and to establish wether MCF viruses are being maintained within bison populations, independent from exposure to other MCF virus carrying host species. Both of theses objectives could only be met by using a diagnostic test that could correctly classify healthy bison with respect to their latent MCF virus infection status. The direct CI-ELISA test which detects antibodies against an epitope conserved among known MCF viruses was shown to meet the needs of this study.

The validity of the direct CI-ELISA test was initially established by estimating the test's diagnostic specificity in a group of bison that were determined by biological means to be free from infection with MCF viruses. Test results from the uninfected bison population indicated that the diagnostic specificity of the test was high. Tests with high diagnostic specificity are likely to produce few false positive results, and therefore provide a considerable amount of confidence in the validity of positive test results.

Diagnostic specificity, however only provides half of the analysis required to adequately evaluate the performance of a diagnostic test. The other half is diagnostic sensitivity. Establishing the diagnostic sensitivity of a test requires application of the test to known infected study subjects. Known healthy MCF virus infected bison were very hard to obtain for this study, and only a very small number were tested. Although they were few, when included in the evaluation of the CI-ELISA, they supported a similar cut-off value to that which was established from the specificity estimation. This small

number of known MCF virus infected bison, therefore provided further support for the CI-ELISA having high diagnostic specificity. Although vigorous attempts were made to obtain a larger sample of MCF virus infected bison, the sample of infected bison remained small, and sensitivity estimates from this study will remain both subject to question and a weakness of this work.

The CI-ELISA test was demonstrated to be a reliable test when the test was applied to paired serum samples drawn from bison at 72 hour intervals. These results indicated that the CI-ELISA had both high analytical and diagnostic precision. They suggest that anti-MCF antibody levels as detected by the CI-ELISA varied little in bison over a 72 hour period. Results of a previous study however, suggest that anti-MCF virus antibody levels as measured by the CI-ELISA may vary in bison over periods of months (O'Toole, Li *et al.* 2002). It has been well established that herpesviruses belonging to the MCF group of viruses produce life-long infections in their hosts (Coulter, Wright *et al.* 2001). If this is the case, and anti-MCF virus antibody levels can vary above and below the CI-ELISA cut-off value in bison that are infected with MCF viruses, then it is likely that the CI-ELISA will have true diagnostic sensitivity that is lower than estimated by this study. This will have a direct effect on prevalence estimates from cross sectional sero-surveys that use the CI-ELISA to identify MCF virus infected bison. CI-ELISA positive prevalence studies such as those conducted for this thesis are likely to underestimate the true prevalence of MCF virus infections in bison populations.

CI-ELISA positive prevalence estimates from samples of farmed Alberta bison were higher than expected for a species which is very susceptible to MCF. Although information about the origin of the serum samples from the Fairview serum bank and the slaughter plants were unknown, the sample sizes were large. Within these 1,948 samples

and the 646 adult bison from the cow-calf herds, the prevalence of MCF virus infections ranged from 21.8 to 23.9 percent. Since the true diagnostic sensitivity of the CI-ELISA may be lower than estimated by this study, it is possible that these prevalence estimates are under estimates of the true prevalence.

It is not possible to state with certainty the mechanisms by which the bison within this study were infected with MCF viruses. It might be speculated that these MCF virus infections were the result of bison to bison transmission. Demonstrating the presence of MCF virus antibodies in bison born in isolation from all other ruminants except their MCF virus infected parents as was the case with the Edjericon herd provides some support for this speculation. It might also be speculated that these infections were OvHV-2 infections resulting from transmission of OvHV-2 during exposures to sheep. Although the PCR studies reported in chapter seven were based on a very small number of bison they demonstrated that at least some healthy bison are infected with either OvHV-2 or a virus very similar to it. However, the auction market outbreak demonstrated that many bison infected with OvHV-2 of sheep origin die of MCF and are unable to transmit MCF to other bison. Auction market bison that died of SA-MCF or that were sub-clinically infected with OvHV-2 were either unable to transmit OvHV-2 to other bison, or if transmission did occur, the virus became attenuated during it's initial passage through a bison.

This study has fulfilled it's objectives. The prevalence of MCF virus infections within farmed bison populations in Alberta was established, and estimated to range from 21.8 to 23.9 percent in adult bison. This study demonstrated that MCF virus infections can be maintained within bison populations independent from exposure to other ruminants by cross generational transmission and provided some preliminary evidence

that OvHV-2 or a very close relative may be infecting some of these bison

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