WEST NILE VIRUS: FROM SURVEILLANCE TO PREDICTION USING SASKATCHEWAN HORSES

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By

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Keywords: WNV, horses, serologic prevalence, vaccine, predictive risk mapping

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Abstract

This thesis describes the West Nile virus (WNV) epidemic in horses by exploring all aspects: sub-clinical infection, development of clinical disease and case fatality. All of the collected data were then compiled to create predictive risk maps of WNV infection for the province of Saskatchewan.

During the 2003 season, 133 clinical cases were documented with laboratory testing. Week of onset of clinical signs, gender, and coat color were significant predictors of whether the horse died or was euthanized due to severity of clinical signs.

Studies of the serological response to vaccination and natural infection were examined to interpret the lab results from over 1100 samples taken from approximately 875 horses in 2003. A serologic study involving 212 horses on 20 farms determined the prevalence of sub-clinical infection (55.7% (95%CI, 44.9% to 65.8%)) and identifed risk factors for infection. The study found risk of infection was highest in the Grasslands ecoregions compared to the Boreal Transition ecoregion.

A case control study looked at risk factors for development of clinical disease. The study followed 23 case farms and control farms with a total of 300 horses sampled. This was the first field study to show that vaccination was efficacious in preventing the development of clinical signs.

The inclusion of horse surveillance data in the Saskatchewan Health WNV Integrated Surveillance Initiative was useful; however, it was discontinued due to time constraints, logistics, and declining monetary resources.

Since West Nile Virus is a mosquito-borne disease it is highly influenced by environmental changes, spatially and temporally. Discriminant analyses were used to partition Saskatchewan rural municipalities (RM) into categories of risk of infection with WNV based on acquired horse data and different environmental and meteorological data derived from both satellites or climate stations. The result was the creation of yearly predictive risk maps defining low to high risk of infection with WNV for each RM.

The 2003 epidemic provided a novel opportunity to study an important zoonotic disease emerging in a new environment. The information gathered will further the knowledge base upon which decisions for prevention of infection and clinical disease are made.

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To my husband…

It is finished, finally.

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List of Abbreviations

AAEP	American Association of Equine Practitioners
BC	British Columbia
C.	*Culex*
CCS	Census Consolidated Subdivision
CDC	Center for Disease Control and Prevention
CFIA	Canadian Food Inspection Agency
CCWHC	Canadian Cooperative Wildlife Health Center
DEM	Digital Elevation Model
ELISA	Enzyme-linked Immunosorbent Assay
EOS	Earth Observing System
EROS	Earth Resources Observation and Science
EVI	Enhanced Vegetation Index
GDD	Growing Degree Days
GIS	Geographical information systems
GPS	Global Positioning System
HRPO	Horse Radish Peroxidase
IDW	Inverse Distance Weighting
IFA	Immunoflourescent Anitbody
Ig(G)	Immunoglobulin G
Ig(M)	Immunoglobulin M
ISC	Information Services Corporation of Saskatchewan
LPDAAC	Land Process Distributed Acive Archive Center

LLL	Legal Land Location
LST	Land surface temperature
MODIS	Moderate Resolution Imaging Spectrometer
MB	Manitoba
NASA	National Aeronautics and Space Administration
NDVI	Normalized Difference Vegetation Index
NJLT	New Jersey Light Traps
OD	Optical Density
PBS	Phosphate buffered saline
PCR	Polymerase Chain reaction
PDS	Prairie Diagnostic Services
PQL	Penalized Quasi-likelihood
PRNT	Plaque Reduction Virus-Neutralization Test
PVC	Polyvinyl chloride
RHA	Regional Health Authority
RM	Rural Municipality
SAFRR	Saskatchewan Agriculture, Food and Rural Revitalization
SK	Saskatchewan
SLE	St. Louis Encephalitis
S/P ratio	Sample to Positive ratio
SRTM	Shuttle Radar Topography Mission
USA	United States of America
USDA	United States Department of Agriculture

USGS	United States Geological Survey
WCVM	Western College of Veterinary Medicine
WNV	West Nile Virus

1. Introduction

1.1 Background

West Nile virus (WNV) emerged in North America in 1999. The disease was first identified in humans, birds, and horses in New York State (Drexler 2002). The virus quickly spread across the North American continent. The first Canadian report was a bird in Southern Ontario in 2001 (Weese et al. 2003). In 2002, WNV was identified in birds and horses in Saskatchewan (Abutarbush et al. 2004). By 2003, the virus was found in all three of the prairie provinces and veterinary practitioners were receiving questions about the disease from horse owners. The questions included: "Is my horse at risk? What can I do to prevent the disease? Is the vaccine necessary, effective and safe?" However, there was very little evidence available to veterinarians on how to determine the extent of this new disease threat, how to prevent infection and to best manage clinical disease.

The horse industry is very important in Western Canada. Canadian Equine Federation commisioned a study to develop a profile of the industry and the horse herd in Canada (Equine Canada 1998). The 1998 National Horse Industry Study estimated the number of horses in Saskatchewan at 123,441 by sampling respondents from registries of different equine organizations. The survey also reported that the average horse industry participant was well educated, middle aged and of moderate to

high income (Equine Canada 1998). Horse owners invest significant resources into their animal's health and well being and the appearance of a new and potentially fatal infectious disease caused great concern to the horse-owning public. Despite the length of time that this virus has been present in Europe and Africa, there were few answers to address their questions. What was assumed based on the behavoir of the virus in Europe and Africa did not seem to apply in this outbreak. When WNV emerged in North America it became clear that the ecology of the virus differed in its' affects on birds (Drexler 2002).

The vast geographic differences that exist in North America also presented new challenges to the research of WNV. What was learned about the behavoir of WNV in one region would not necessarily apply across the continent. For example, the primary mosquito species in the transimssion cycle differs in the Prairies from the Eastern states and provinces (Curry 2004, Andreadis et al. 2001). Vastly different environmental conditions exist in North America from the temperate regions of southern Canada to the tropics of Florida. With these differences existing in North America, specific regional investigations would be required to address local concerns.

Following the experience of other areas in North America and the incursion of the virus into Saskatchewan in 2002, the potential for a WNV outbreak was very real for the 2003 season. Attempts to predict areas of risk in the province were limited to historical mosquito data from the 1960's when the last major outbreak of a mosquito-borne disease in Saskatchewan horses occurred (McLintock et al. 1966, personal communication with Phil Curry). Researchers and public health officials recognized the need to monitor the extent of WNV infection in 2003, and horse owners and

veterinarians were eager to help investigate this disease and find answers to their questions about prevention and control.

1.2 Investigative approach

In the spring of 2003, a comprehensive study was designed to look at the risks associated with WNV infection in horses. The overall objectives of the 2003 study were to work with sentinel veterinary practices to document the occurrence of clinical cases, to use serologic tests to measure prevalence of asymptomatic infection, and to explore risk factors for infection and for clinical disease manifestation. The answers to study questions would describe geographic areas of risk, factors associated with increased risk, and potential methods for controling the disease.

Once it was established where in the province the virus could exist and cause disease, the next step would be to see if there were methods of quantifying risk on a regional basis using minimal surveillance input. Surveillance initiatives while targetted at providing relevant data can be quite cost prohibitive. The primary push for surveillance is from the Public Health standpoint, making prediction of areas at high risk for humans the main goal. However, knowledge of regions and conditions of high risk of infection and disease of horses with this virus could provide critical information of risk for human infection.

The investigation began in 2003, which was the second year of recognized WNV incursion into Saskatchewan as well as the most extensive epidemic year to date. The investigation encompassed the southern portion of the province of Saskatchewan and used information from private veterinary practices and commercial

laboratories to minimize costs. The study continued in 2004 and 2005 as a component of Saskatchewan Health's integrated surveillance initiative.

This study forms the foundation for this thesis. Speculation for the studies in this thesis to be carried out was that 2003 would be an epidemic year for West Nile virus equine clinical disease which would allow research on many aspects of the ecology and epidemiology of West Nile virus.

Chapter 2 will review the pertinent literature to date for WNV infection in horses and, therefore, includes information that was not available at the time the studies were designed in 2003.

Chapter 3 deals with the clinical cases that occurred in Saskatchewan in 2003. The goal was to document the distribution of the cases as well as assess risk factors associated with mortality from clinical disease.

Chapter 4 summarizes a kinetics study that looked at changes in the concentration of IgG antibodies over time in horses exposed to WNV with different clinical and vaccination histories.

Chapter 5 reports the results of a serologic study which documented the extent of asymptomatic infection in horses and examined risk factors associated with infection. This chapter will explain the cutoff value that was used to determine the infection status for vaccinated horses.

Chapter 6 describes the case-control study based on a subset of the documented clinical cases. It focuses on risk factors associated with the development of clinical disease.

Chapter 7 provides a summary of the surveillance of horses over a 4 year period, 2002-2005. It assesses the progression of WNV in the province's horses population over that time period as well as the assessing the usefulness of this data in an integrated surveillance initiative by Public Health officials.

Chaper 8 describes the ecology of WNV and the creation of a predicitve risk model and subsequent risk map of infection with WNV in the province of Saskatchewan.

Chapter 9 will provide a summary of the major conclusions from this thesis with possible future implications.

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2. Literature Review

2.1 Introduction

West Nile Virus (WNV) has a long history spanning many continents and countries. Until its introduction into North America, WNV was not widely researched and was considered a minor arbovirus with only sporadic outbreaks in either humans or horses (Zeller et al. 2004, Gerhardt 2006). The resulting rapid spread across North America has prompted interest in studying arboviruses; in the possibility that seemingly harmless pathogens can become a threat in a new environment or that because of climate change arboviruses may re-emerge as public health concerns (Jonsson et al. 2000, Zeller et al. 2004, Gerhardt 2006) .

The first objective of this literature review was to present an overview of WNV as it was known until 1999 and then after the virus was introduced into North America in 1999. The second objective was to review what is known about how birds, mosquitoes and the environment are involved in disease transmission, specifically those factors that might be important in Saskatchewan (SK). The third objective was to summarize what has been reported about WNV infection in horses including: clinical manifestations, testing options available within the Canadian

prairie provinces, and vaccination. Finally, this review examined the potential use of spatial epidemiology to predict the risk of infection from this vector-borne disease.

The databases that were searched included CAB Abstracts and Agricola, through the University of Saskatchewan Library website. The search parameters included the words "West Nile virus" with occasional specific reference to horses, mosquitoes, humans and birds. This search was initially performed in early 2003, but was repeated in late 2006 to ensure that all of the most current published information was included. In late 2006, there was initially over 1800 references but through identification of duplicates and articles of limited usefulness, the list of usable references was decreased dramatically. In addition, the Yahoo internet search engine, was used to search for provincial and federal government sites, technical reports, and other grey literature articles on WNV in North America. As well, pertinent articles published in 2007 were included in the thesis as they were identified by the researchers involved in this thesis.

2.2 Old World experience with WNV

West Nile virus is a vector-borne zoonotic disease that is transmited between birds and mosquitoes with occasional spill-over into susceptible hosts, usually human or equine (Hayes 1989). The virus was first identified in the blood of a woman in the West Nile Province of Uganda in 1937 (Smithburn et al. 1940). Ecological studies in Egypt established the natural amplification cycle between birds and mosquitoes (Work et al. 1955, Taylor et al. 1956).

Until 1999, the virus was only reported in Africa, Europe and Asia (Hayes 1989). The virus is believed to have been transported from Africa to Europe through migrating birds (Hubalek et al. 1999, Durand et al. 2002). One such example was an outbreak in the summer of 1962 during which 50 horses with neurologic symptoms were diagnosed in the Camargue region in Southern France (Bouches-du-Rhộne Province) (Murgue et al. 2001). Several concurrent human cases were also reported. The area includes a large wet area corresponding to the delta of the Rhộne river which has a high mosquito density. The region is home to many wild bird species and is an important resting area for migratory birds (Murgue et al. 2001). There were no reports of avian species die-off associated with this outbreak.

Following this outbreak, a serosurvey was completed during 1963 and 1964 on 37 asymptomatic horses from the Camargue region. Six of the 37 horses were positive for antibodies to WNV (Durand et al. 2002). In 1965, WNV was confirmed as the cause of neurologic symptoms in three more horses. In 1975-1979 another serosurvey in the Camargue region showed a low frequency of antibody response to WNV (2%, 99 horses tested) (Murgue et al. 2001). It was not until the summer of 2000, that the area had another confirmed symptomatic case of WNV in a horse. A serosurvey conducted during and following the 2000 outbreak (combined with WNV history since 1962), showed that the seroprevalence rate did not increase with age, indicating that this was an epidemic area with periodic outbreaks followed by long silent periods (Durand et al. 2002).

Another serosurvey was conducted in southern France, near the French Riviera in 2003 (Durand et al. 2005). No unusual bird mortality was seen in the area

but an entomological study was conducted within the serosurvey region resulting in low mosquito numbers with all batches negative for the virus. Of most interest was that equine seroprevalence declined with increasing distance away from two important bird areas, with all stables where the Immunoglobulin (Ig)G seroprevalence was over 30% within 5 km and all IgM positive horses located within 3.5 km of these areas. In addition, stables with IgM positive horses had substantially higher IgG seroprevalence than stables with no IgM positive horses, 71% and 12% respectively.

Many European countries have reported outbreaks with human cases. Examples include Romania in1996 (Tsai et al. 1998) and Russia in 1999 (Platonov et al. 2001). Others have reported outbreaks in the horse population such as Italy in 1998 (Autorino et al. 2002), France in 2000 (Murgue et al. 2001), and Israel also in 2000 (Steinman et al. 2002). Although WNV antibodies have been identified in wild birds in many countries of Europe, Africa, and Asia (Malkison et al. 2002, Lundstrom 1999, Hayes 1989), clinical disease in wild birds was not reported prior to 1998 (Zeller et al. 2002). In September-October 1998, there was an outbreak of WNV in a flock of wild migrating white storks and a flock of domestic geese in Eilat, Israel (Malkison et al. 2002). Table 1 gives an overall view of the outbreaks that have occurred in the Old World since WNV was first recognized in Uganda.

Table 1: Overview of human, horse and bird outbreaks of West Nile Virus in the Old World from 1960 to present. Adapted from Hubalek et al. 1999 and Zeller et al. 2004.

Year	Country	Species affected
unknown	Former Yugoslavia	H
1960's	Spain	H
	France	H, Ho, M, B (wild)
	Italy	H, B (migrating, chickens), Domestic mammals, Rodents, Goats
	Portugal	C, S, Ho, H, M, B (wild)
	Bulgaria	H, Domestic animals, B (wetland)
	Romania	M, H
	Austria	B (wetland), Reptiles, Domestic/wild animals, H
	Russia	H, Ticks, B (water), M
1970's	Spain	Rodents
	France	H, H
	Greece	H, Domestic animals, Rabbits, B
	Bulgaria	M
	Hungary	Rodents, C, H
	Slovakia	H, M, B (migrating, pigeons), Game animals, C, S, Dogs
	Austria	B (wetland), Reptiles, Domestic/wild animals, H
	Belarus	H, B (wild)
	Ukraine	H, M, B
	Moldavia	Ticks, M, H
	Czechland	Domestic animals, Hares
1980's	Italy	Rodents
	Romania	H
	Ukraine	H
	Bohemia	Game animals
	Moravia	B (wetland, cormorants), M, H
1990's	Italy	Ho
	Romania	H, Domestic/wild animals, Dogs, B (wild)
	Poland	B (sparrows)
	Morocco	H, Ho
	Israel	H, Ho, Birds (geese, storks)
	Algeria	H
	Tunisia	H
	Russia	H
2000	France	H, Ho
	Morocco	Ho
	Israel	H, Ho
	Russia	H
Abbreviations: Humans (H), Mosquitoes (M), Horses (Ho), Birds (B), Cattle (C), Sheep (S).		

2.3 New World experience with WNV

West Nile Virus was introduced to the North American continent in 1999 (Drexler 2002, Gerhardt 2006); however, the route by which the virus entered the United States is unknown (Lanciotti et al. 1999). The North American experience with WNV has been very different than that in Europe, namely in the rapid spread of disease across the continent and the presentation of clinical disease in many wild bird species. Following the introduction of WNV into the North American continent and before the first human and horse cases were recognized, there were significant die offs of wild birds, specifically members of the corvid family (blue jays, crows, ravens, etc) . Corvids continue to be the sentinel for monitoring the progression of disease (Drexler 2002, Castillo-Olivares et al. 2004).

The first reported cases of neurologic disease in humans occurred in early August of 1999 in the suburb of Queens, New York city (Drexler 2002) and in horses in late August on Long Island, New York (Ostlund et al 2001). Originally, due to lack of specific testing, a diagnosis of St. Louis Encephalitis (SLE) was given for the human cases. It was the combination of bird deaths, zoo specimen deaths, human and horse cases, along with the diligence of human doctors and one veterinarian at the Bronx Zoo that finally led to the correct diagnosis of WNV rather than SLE (Drexler 2002). There were approximately 10,000 crows that died during the outbreak and state residents reported a total of 17,000 dead birds (includes crow numbers) in New York state alone.

After the initial year, the virus made a swift and relentless progression outward from New York State. Mosquitoes collected from sewers and dwellings in

New York City during the winter of 1999-2000 were found to be infected with the virus (CDC 2000) and, therefore, WNV was expected to reemerge the following summer. Using information on migratory bird involvement and the outbreaks seen in the Old World (Asia, Africa and Europe), one paper printed in July-August of 2000, suggested that the next likely spot for an outbreak would be the coastal plain of Georgia, northern Florida or Alabama (Rappole et al. 2000). However, the spread of WNV in the following year included only other Northeastern states (USDA 2001, Ostlund et al. 2001, Trock et al 2001). Despite this inconsistency, migratory birds have been a necessary element in understanding the large geographic spread of WNV in subsequent years (Peterson et al. 2003).

Few random serologic studies were conducted or reported that looked at the infection rates of asymptomatic horses. In a 1999 geographically limited New York State serosurvey of 69 asymptomatic stable mates of clinical cases, 20 (29%) had titers to WNV (Trock et al. 2001). In 2000, a Staten Island serosurvey of 91 clinically normal horses (located within 3 miles of a clinical horse case of WNV) found 7 (7.7%) seropositive horses (Trock et al. 2001). Further categorization of the serological results revealed that the seropositivity rate was higher on farms with clinical disease than without; in 1999, 23% to 8% respectively and, in 2000, 5% to <1% respectively (Kramer et al. 2001).

A 2000 case-control study from the northeastern states used “case equid” to represent both those that were confirmed clinical cases and those that were asymptomatic seropositive horses (USDA 2001). The percentage of infected horses using only horses from farms with at least one ‘case’ horse was 9% (54/594).

Approximately 40% of the horses (both clinical cases and asymptomatic horses) in this study were housed within a barn (USDA 2001). This study made recommendations for the protection of horses from clinical disease which included vaccination, housing and mosquito control.

The spread of WNV continued and by 2002 most eastern and mid-western states had reported the virus (CDC 2002). Up until this point, research on the virus and its spread involved detecting the virus in eastern moquito species and evaluating their transmission competency experimentally (Andreadis et al. 2001, Sardelis et al. 2001). Different mosquito species carried the virus from the natural amplification cycle to horses in the Eastern areas (C. *pipens,* C. *salinarius,* C. *nigripalpus, etc*) compared to the Western or Great Plains areas (C. *tarsalis*) of the continent (Andrealis et al. 2001, Sardelis et al. 2001, Gerhardt 2006). As WNV moved across the North American continent, inter-year trends began to emerge which included an introductory year followed by an epidemic year and then subsequent years of substantially less clinical disease (Bell et al. 2006). In addition, environment conditions were shown to have a huge influence on local WNV activity (Bell et al. 2005, Rainham 2005, Shaman et al. 2005).

A preliminary descriptive study of the 2002 outbreak in Colorado and Nebraska was published in early 2003 (Salazar et al. 2003). The statistical analysis of this outbreak was presented in a paper published in 2004 which identified gender, age, recumbency and vaccination as factors associated with clinical outcome of the symptomatic horses. Similar findings were reported in two other studies, one from Indiana and the other from North Dakota (Ward et al. 2004, Schuler et al. 2004). In

addition, a study from Colorado looked at factors associated with testing positive for WNV infection in equids exhibiting clinical signs consistent with WNV (Tanner et al. 2006). Factors significantly associated with testing positive compared to testing negative included vaccination status, severity of clinical signs, location of horses, duration of illness and season of illness. Age, breed and laboratory performing the testing were not significantly associated with test status in horses exhibiting clinical signs.

By the summer of 2001, the virus had been diagnosed in southern Ontario. By 2002, the virus had spread as far west as the southeastern area of Saskatchewan (Weese et al. 2003, Abutarbush et al. 2004). WNV was detected in horses, mosquitoes, domestic geese, corvids, sentinel chicken flocks and wild ducks across the southern one third of Manitoba in 2002 (Austin et al. 2004, personal communication, Shelagh Copeland). The number of reported horse cases in 2003 was dramatically less than 2002 (personal communication, Shelagh Copeland). The experience of WNV in Saskatchewan is the basis of this thesis and will be presented in the body of the thesis. The province of Alberta had its first case of WNV in 2003. A total of 170 symptomatic horses were confirmed IgM positive during a 5 month period beginning in July (Ollis et al. 2005). The outbreak was concentrated in southern Alberta with the majority of owners (98%) reportedly not using mosquito control programs or housing their horses outdoors at dusk and dawn.

Serosurveys in the United States were likely not performed after 2003 as there was widespread use of a killed vaccine which would complicate interpretation of serologic test results (Long et al. 2006). Instead, the majority of serosurveys reported

were from Central America, with approximately 16% to 53% of sampled clinically normal horses showing evidence of WNV infection (Estrado-Franco et al. 2003, Blitvich et al. 2003, Quirin et al. 2004, Lefrancois et al. 2005). In Guadelupe, the overall prevalence of WNV in the first year of the virus (July 2002) was 2.8% and this increased to 50% in July 2003 (Quirin et al. 2004).

In states and provinces in which the clinical disease emerged in humans and horses, clinical cases were usually preceded by avian die-offs at least a month or two before the first reported cases (Guptill et al. 2003, Zeller et al. 2004, Johnson et al. 2006). In Canada, a WNV surveillance program was developed and was based on the submission of dead birds for viral testing. Many provinces also provided documentation of clinical human and horse cases (CCWHC 2005, Saskatchewan Health 2006). Many research studies from both Canada and the United States that analyzed bird, mosquito, horse and human cases began to emerge with much emphasis on early warning and surveillance systems (Johnson et al. 2006, Marfin et al. 2001, Brownstein et al. 2004, Eidson et al. 2005, Orme-Zavaleta et al. 2006). These studies also provided the pros and cons of using of some or all of the different species in the surveillance of WNV.

In four short years, the virus had crossed an entire continent causing thousands of clinical horse cases with a wide range of mortality rates (Castillo-Olivares et al. 2004). As of 2006, WNV should be considered endemic to most of North and Central America. A few review papers have been compiled that provide an overview of WNV in both the New and Old World (Zeller et al. 2004, Castillo-Olivares et al. 2004, Gerhardt 2006).

2.4 Transmission cycle

WNV is a member of the Japanese encephalitis virus serogroup in the genus *Flavivirus*, family Flaviviridae (Hayes 1989). Examples of viruses that make up this group are Japenese Encephalitis virus and St Louis Encephalitis virus. These viruses fit into the category of arthropod-borne viruses or arboviruses. This means that they are all transmitted among vertebrate hosts by arthropod vectors such as mosquitoes.

WNV is maintained by a natural amplification cycle involving mainly birds (various species) and mosquitoes (mainly of the genus *Culex* (*C.*) but potentially other genera as well) (Hayes 1989). The particular species of mosquitoes involved in the cycle is geographically dependent. Virus containing mosquitoes can transmit the virus to susceptible mammals when conditions are favorable. Mammals, such as horses and humans, are considered dead-end or incidental hosts for the virus in that they do not produce enough virus to re-infect a mosquito and maintain the transmission cycle, but viremia in some cases is sufficient to result in clinical signs (Bunning et al. 2002). (See figure 1: Transmission cycle).

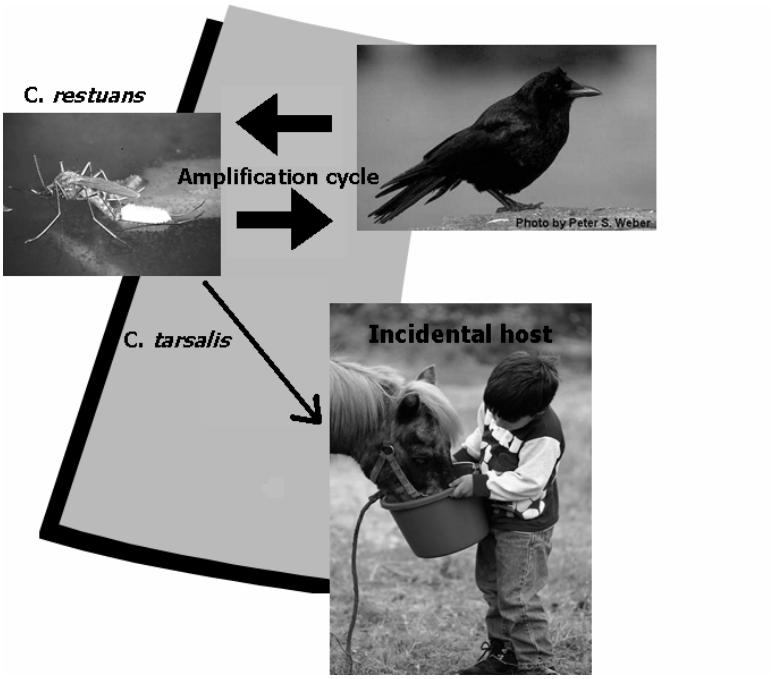

Figure 1: Transmission cycle (based on information known in 2003 with an emphasis on prairie mosquito species)

Culex restuans and *Culex tarsalis* are the main mosquito species involved in the transmission cycle of WNV in Saskatchewan (Curry 2004). In Saskatchewan, WNV has also been found in *Culiseta inornata,* but its role in transmission remains unknown (Curry 2004). *C. restuans* feeds primarily on birds and is thought to be involved in the bird to bird amplification of the virus (Curry 2004). *C. tarsalis* prefers to bite birds, but switches to a mammalian source of blood meals around mid-July after the birds fledge (Curry 2004). This species prefers sedge meadow areas where there are shallow pools of stagnant water, such as hoof prints. This species is known to bite several times, especially in hot weather and is considered the main vector of transmission to mammals. *Culiseta inornata* prefers permanent wetlands, usually bites once and prefers a mammalian source (Curry 2004).

Until the summer of 2003 when mosquito trapping became part of the Saskatchewan provincial surveillance program, the only reliable mosquito data was from the 1960's and 1970's (see figure 2: Historical *C. tarsalis* data). The distribution of *C. tarsalis* was limited to "at or below the Yellowhead (Number 16) highway" (personal communication with P. Curry, Saskatchewan Health). Since the 2003 summer season in which extensive mosquito trapping from all areas of the southern portion of Saskatchewan was carried out, the distribution map has changed dramatically to include areas as far north as Meadow Lake, SK.

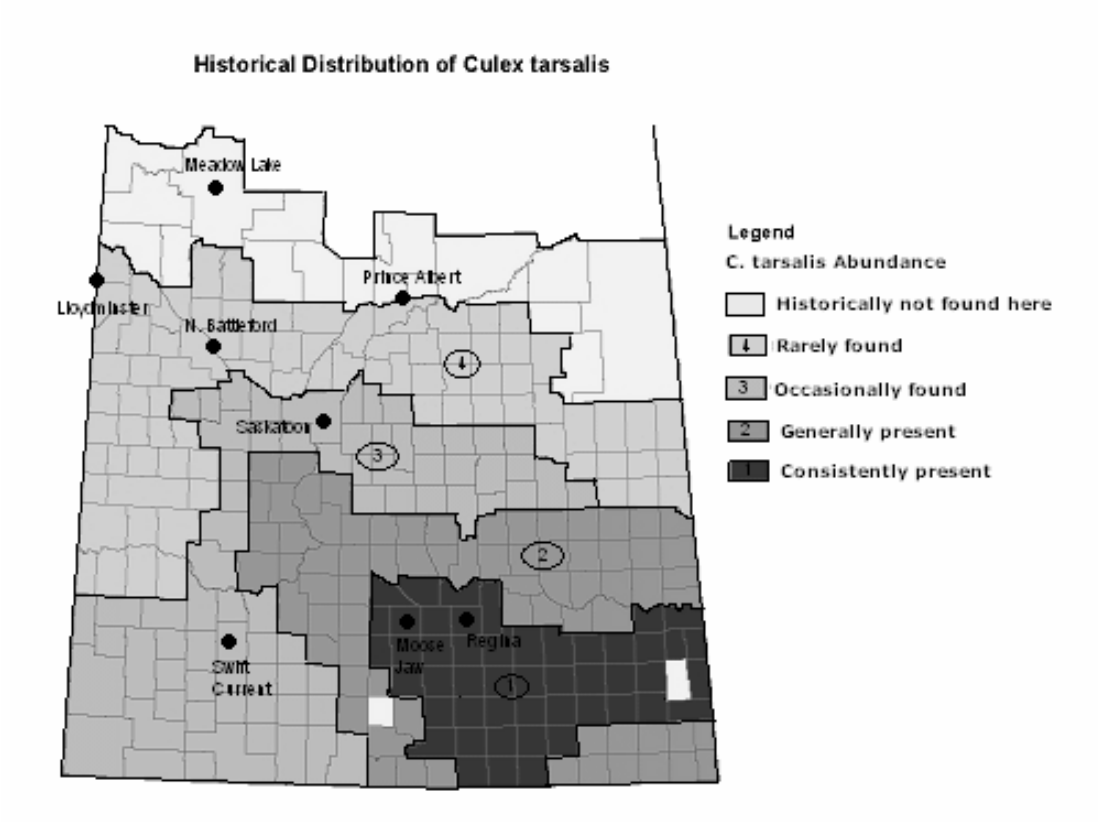

Figure 2: Historical *C. tarsalis* data (source: Phil Curry, Sask Health)

Birds are the other half of the natural amplification cycle. Crows, which are members of the corvid family of birds, are considered a hardy species that feed on everything from restaurant scraps to roadkill (Drexler 2002). However, when WNV first appeared in New York city in 1999, it was the massive die-off of crows and zoo specimens that finally assisted in identifying the correct diagnosis (Drexler 2002, Ludwig et al. 2002). During the winter of 1999-2000, study of dead birds continued with the first identification of WNV in the brain of a Red-tailed Hawk (Garmendia et al. 2000).

Researchers have since recognized more than 130 species of birds in addition to other members of the corvid family, such as Magpies, Gray Jays and Blue Jays, that can become infected and probably transmit WNV (CDC 2003). Experimental infection studies have estimated reservoir competency for a variety of species. The Blue Jay had the highest reservoir competency index. American Crows and House Sparrows were very similar in the mean duration of infectiousness and reservoir competency, but Crows had a higher number of fatal infections (Komar et al. 2003).

Experimental studies have also shown that bird to bird transmission can occur as well as transmission via mosquitoes (Komar et al. 2003). A report of an outbreak in domestic geese was recorded in Manitoba in 2002 (Austin et al. 2004). This report hypothesized that direct bird to bird transmission in addition to mosquito transmission was required for the seroprevalence patterns seen in this flock.

In Saskatchewan, the first positive bird was found in August 2002. In 2003, the first bird was found at the beginning of June in Regina, SK (personal communication, P Curry). The Canadian Cooperative Wildife Health Center

(CCWHC) coordinated the submission of dead birds across Canada starting in 2001. A recent CCWHC report indicated after 2003 there was a steady decline each year in the submission rate in all three prairie provinces (CCWHC 2005).

A potential concern of the wild bird surveillance is that it relies solely on the public for detecting and reporting of dead birds (Ward et al. 2006). Therefore, the numbers and locations of submission can be influenced by human-related factors such as population density, awareness and collection services. In 2005 and 2006, research involving identification of the virus in House Sparrows was initiated in Saskatchewan (personal communication, F. Leighton). The use of House Sparrows was thought to better reflect the rural areas of the province and possibly provide more localized evidence of WNV (CCWHC 2005).

2.5 Clinical manifestation in horses

Horses are incidental hosts or "dead-end hosts"; individuals which can exhibit clinical signs but are thought to rarely develop high enough viral levels in the blood to infect mosquitoes or be a component in sustaining the cycle of WNV (Castillo-Olivares et al. 2004). The incubation period is generally 6-10 days; however, it has been shown to be as long as 20 days (Smith 2002). In horses, the virus can produce varying degrees of clinical neurological symptoms ranging from mild ataxia, tremors or facial nerve paralysis to severe paralysis of one or more limb(s), recumbency and death (Oslund et al. 2000). A list of potential clinical signs is presented in Table 2; however, it is not an exhaustive list. Many infected horses show no clinical signs at all and without serologic testing remain unrecognized. In both field and experimental observations, it is estimated that up to 10% of horses infected with WNV will present

with neurologic disorders (Castillo-Olivaries et al. 2004, Bunning et al. 2002). Clinical attack rates calculated using popoulation at risk (not just infected horses) were estimated as 8.4% (Ward et al. 2006) and 12.9% (Salazar et al. 2004) for all premises with clinical cases, but <1% for the entire state of Texas (Ward et al. 2006).

The case fatality rate of horses generally ranges from 25 – 40% in horses (Ostlund et al. 2000, USDA 2001, Murgue et al. 2001, Ostlund et al. 2001, Trock et al. 2001). The prognosis is poor for horses that have severe paresis or paralysis of one or more limbs that results in recumbency (Cantile et al. 2000, Ostlund et al. 2000, Long et al. 2002, Salazar et al. 2004). This is especially true if the horse is recumbent and unable to get up, particularly for more then 3-5 days (Long et al. 2002, Salazar et al. 2004).

Duration of clinical signs for those that survive can range from days to weeks to months. In one study, 82% (276/338) of owners contacted reported "full recovery" 1-3 months after diagnosis (Salazar et al. 2003, Salazar et al. 2004). In another study, 16 of 19 (84%) owners contacted 6 months after discharge of their horse from the hospital reported their horses had recovered from WNV clinical signs (Porter et al. 2003). Some mild lingering effects reported in horses consisted of decreased stamina, weight loss and or loss of condition, reversible weakness or ataxia and behavioral changes (Salazar et al. 2003, Porter et al. 2003). Treatment of the clinical disease usually involves anti-inflammatory drugs and supportive care aimed at minimizing injury or adverse effects of prolonged recumbency (Ostlund et al 2000).

Table 2: Summary of clinical signs of WNV in horses. The percentages were compiled from multiple sources (Murgue et al. 2001, USDA 2001, Trock et al. 2001, Ostlund et al. 2001, Zeller et al. 2004, Ollis et al. 2005).

Sign	**Percentage**
Ataxia (rear or all 4 limbs)	75-95%
Weakness (mainly hind limbs)	48-69%
Muscle Fasciculation (mostly facial)	40-60%
Dull, Lethargic	61%
Recumbency	38-45%
Body stiffness or reluctance to move	43%
Decreased appetite	36%
Fever	23-35%
Altered mentation	31%
Hyperesthesia	30%
Cranial nerve deficits (droopy lips, muzzle ears, etc)	18-27%
Death	25-40%

2.6 Diagnostics for horses

There are several tests reported for use in diagnosing WNV infection in horses. The types of tests available are characterized by the type of sample collected (tissue versus serum), time-frame (detection versus diagnosis) and cost (research versus consumer). Virus isolation was available in some laboratories in 1999. However, because Level-3 laboratories are needed to isolate the virus, WNV diagnostics is usually based on the identification of antibodies to the virus (Ostlund et al. 2001). A plaque reduction virus-neutralization test (PRNT) that looks for virus-

neutralizing antibodies was used extensively in 1999 but it must be performed in a Level-3 facility (Ostlund et al. 2001).

In anticipation of the increase in samples for detection of WNV infections post-1999, an IgM-capture enzyme-linked immunosorbent assay (ELISA) was developed (Ostlund et al. 2001). Prior to 2001, both a positive IgM and PRNT titer were considered confirmatory of WNV infection in a horse (Long et al. 2006). ELISAs are the most widely used method of diagnosis, likely due to factors such as the cost effectiveness, the ability to perform the test in any lab and fast turn-around time for a diagnosis using a single blood sample (Ostlund et al. 2000, Ostlund et al. 2001, Long et al. 2006). However, while most diagnostic laboratories use this assay, it is not marketted as a commercial kit and as such the methodology and reagents do differ (personal comunication, Brian Chelack). A study conducted using the IgM ELISA from the National Veterinary Services Laboratory (USA), determined the sensitivity and specificity of the test to be 92% and 99% respectively (Long et al. 2006). Prairie Diagnostic Services (Saskatoon, SK) obtained reagents from BioReliance (Rockville, Maryland) for an IgM ELISA test. This was not an established, validated assay kit and as such did not have any sensitivity or specificity estimates. This laboratory also had reagents available to perform an IgG ELISA.

IgM antibody to WNV develops 8-10 days post infection and persists for less than 2-3 months (Ostlund et al. 2001). The presence of this antibody can be used to identify recently infected horses, specifically those which are ‘symptomatic’ or clinical cases of WNV displaying neurologic symptoms. It is less useful for

surveillance since the antibody response can be missed in some animals with sporadic sampling due to its short duration in the horse.

The IgG antibody develops within 3 weeks post infection and has been known to persist for greater than 2 years (Durand et al. 2002). This antibody is good for surveillance, but the potential persistence of the antibody for over 2 years and potential response to vaccination can complicate the interpretation. Little data are available on the kinetics of the IgG antibody over time in horses either naturally exposed to WNV or in horses that were vaccinated (Ng et al. 2003).

2.7 Equine vaccination and other control measures

In late 2001, a vaccine for prevention of clinical disease in horses was approved for use in horses in the United States and was fully licensed in 2003. In late 2002, this vaccine began entering the Canadian market (through emergency release) and was widely used after it was fully licensed in 2003 (personal communication, Larry Frischke, Wyeth Animal Health). The vaccine was a killed product produced by Wyeth Animal Health; West Nile-Innovator™ (Wyeth 2005). Label recommendations stated that naïve horses should be vaccinated intramuscularly twice three to six weeks apart with adequate time for the vaccine to stimulate immune response prior to the mosquito season.

In a study to assess the efficacy of the vaccine, development of viremia (surrogate for development of clinical disease) was assessed after a viral inoculation challenge conducted one year post vaccination (Ng et al. 2003). There was a significant difference between vaccinates and controls, demonstrating 94% efficacy. Preliminary field data from out of the USA was starting to emerge in 2003 which

appeared to show the vaccine was capable of increasing the survival rate of horses that did develop clinical signs (Salazar et al. 2003, Salazar et al. 2004, Schuler et al. 2004).

In late 2003, a modified-live recombinant virus vaccine was introduced and manufactured by Merial (Minke et al. 2004). The company claimed the new recombinent vector vaccine technology stimulated both humoral and cell-mediated immune responses (Merial 2004). The company recommended that for horses previously vaccinated with the killed product, only a single booster was required to maintain protection (Grosenbauhg et al. 2004, Siger et al. 2004). The vaccine is licensed to protect against clinical disease and has been demonstrated to protect horses against disease induced by a virulent strain of WNV administered intrathecally (Siger et al. 2006).

To date, only one study has tested the efficacy of the killed and the recombinant vaccines under natural field conditions (Gardner et al. 2007). Despite a small sample size, it seemed to show that both vaccines were effective in preventing clinical disease due to West Nile virus. A more indepth look at vaccination recommendations, specifically for pregnant mares, foals and weanlings can be found in the Proceedings of the 51st Annual Convention of the American Association of Equine Practitioners (AAEP) (Wilson 2005).

Other vaccine technologies are being developed but their use will likely be limited due to the efficacy of the previously mentioned vaccines. Fort Dodge Animal Health created a DNA vaccine which it claims can induce protective immunity in the presence of maternally derived antibodies (Chiang et al. 2005). In addition, a live

attenuated WNV Chimera vaccine for horses was created by Intervet (Long et al. 2005). Through clinical trials, this vaccine provided 95% protection against WNV disease.

In 2000, many vector abatement recommendations were listed that people could use to protect themselves and their horses from the virus. These recommendations were aimed at decreasing exposure to the virus. These include housing in an enclosed barn, the use of fans or altered lighting in barns, mosquito repellent application and elimination of common mosquito habitat and breeding areas (Ostlund et al 2000). A common form of mosquito control for horses on prairie pastures is the use of smudges; smoky fires designed to provide temporary areas of decreased adult mosquito populations.

2.8 Role of environment in the ecology of WNV

Warm winters with hot and dry conditions in the following summer are condusive to WNV outbreaks (Epstein 2001). Climate variations, including temperature and hydrologic conditions have effects on mosquito populations and the pathogens they can carry (Shaman et al. 2005, Mellor et al. 2000, Hay et al. 1996, Hay et al. 1998). Recently, vegetation cover, land cover and even elevation have been assessed as variables that affect or are indicative of vector habitat suitability (Ward et al. 2005, Curran et al. 2000). The ecology of vector-borne disease involves a complex interaction between the environmental determinants of vector abundance and distribution, the pathogen and the hosts (Kitron 1998). Incorporating this knowledge

into the epidemiological study of these diseases can greatly enhance the analysis (Rogers et al. 2003, Kitron 1998, Brooker et al. 2002).

Several new spatial tools have become readily available for use in the study of disease. These include geographic information systems (GIS), global positioning systems (GPS), remote sensing data (particularly satellite imagery) and the specialized field of spatial epidemiology (Kitron 1998). GIS is a database that allows storage of the geographical information (often obtained by GPS) for each observation that when combined with spatial analysis tools can offer ways to map and explain relationships in both time and space (Rogers et al. 2003, Kitron 1998). Remote sensing is the measurement of properties of objects by sensors that are not in direct physical contact with the object (Jensen 2005). Satellite-derived data is one form of remote sensing that has gained wide use in the study of vector-borne diseases because it provides continuous spatial data across vast regions on defined temporal scales (Hay et al.1996, Kitron 1998, Rogers et al. 2003).

By using remotely-sensed and other geographically linked data, epidemiologists can examine patterns, explain relationships and make predictions of disease potential, even in unsurveyed areas (Hay et al. 1998, Kitron 2000, Rogers et al. 2003). Examples of this have spanned a variety of different diseases such as malaria, schistosomiasis, Lyme disease, Hantavirus and Leishmaniasis (Dister et al. 1997, Glass et al. 2000, Hendrickx et al. 2000, Kuhn et al. 2002, King et al. 2004). Applications to other diseases are limited only by the present technology and knowledge of the ecology of specific diseases (Beck et al. 2000). Assessment of the accuracy of these predictive models is essential but often forgotten; especially

whether existing models can be applied to different locations (Brooker et al. 2002). Accurate predictive models can have application in both control of the disease, designing of intervention strategies and refining research questions and surveillance systems (Kitron 2000, Rainham 2005).

2.9 Conclusions and research needs

This literature review has summarized both the peer-reviewed literature and other pertinent local sources of information relating to WNV. Many questions remained in 2003, four years after the virus first appeared in North America. What was the distribution of clinical disease in the temperate climate of Saskatchewan? What was the distribution and proportion of asymptomatically infected horses? Was vaccination efficacious in the field for the prevention of clinical disease or mortality from clinical disease? What risk factors were important for clinical disease and asymptomatic infection? How did WNV activity change over several years? Can environmental and climatic factors predict the risk of infection? These questions formed the basis of the studies designed and conducted for this thesis. This review represents the knowledge to date and therefore, surpasses what was known in 2003. While the primary goal of this research was to further expand the information available on WNV in horses, the result could perhaps highlight effective methods to approach other emerging diseases of public health interest in the future.

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3. Factors associated with West Nile virus fatalities in horses.

3.1 Introduction

West Nile virus (WNV) is an arbovirus (arthropod-borne virus) that affects the nervous system of humans, horses, and birds, causing mild to severe illness and sometimes death (Hayes 1989). It was first reported in Africa in 1937 (Smithburn et al. 1940) and has been identified sporadically in European countries from the early 1960s to the present (Hubalek et al. 1999, Murgue et al. 2001, Autorino et al. 2002). Since it was diagnosed in New York state in 1999, WNV has spread across the North American continent (Ostlund et al. 2001, USDA 2001, Porter et al. 2003, Salazar et al. 2003).

In Canada, WNV infection was first seen in birds in southern Ontario in 2001 (Weese et al. 2003). Since 2001, throughout Canada, the date and location of birds found dead that tested positive for WNV were systematically recorded by the Canadian Cooperative Wildlife Health Centre. In 2002, the first equine cases (those showing clinical signs consistent with WNV infection) were identified in Ontario, Manitoba (MB), and Saskatchewan (SK) (Weese et al. 2003, Abutarbush et al. 2004). In SK, there was only a passive system to monitor the occurrence of clinical disease in horses, which, unfortunately, resulted in the numbers reported varying, depending on the source of information.

The virus is amplified in a natural cycle between birds and mosquitoes and incidentally infects humans and horses. In western Canada, *Culex* (*C.*) *restuans* and *Culex tarsalis* are the major mosquito species responsible for transmission in the amplification cycle. *Culex tarsalis* is considered the most important vector for transmission of WNV to humans and horses (Curry 2004). Horses can become infected when bitten by a mosquito carrying the virus, but they do not contribute to the spread or amplifcation of the virus in the natural cycle (Bunning et al. 2002). Once bitten, a horse may eliminate the virus uneventfully or show clinical signs including fever, depression, muscle tremors, weakness, lack of coordination, inability to rise, and paralysis (Ostlund et al. 2000). The incubation period of infection to manifestation of clinical signs is 5-15 d (CFIA 2005). Most reports suggest that around 25%-45% of those horses that show clinical signs die or require euthanasia (Ostlund et al. 2000, Murgue et al. 2001, Ostlund et al. 2001, Autorino et al. 2002, Porter et al. 2003, Salazar et al. 2004).

Factors significantly associated with fatality from WNV clinical disease include non vaccinated status for WNV; age; clinical signs such as inability to rise; early season onset of clinical disease; sex; and breed (Salazar et al. 2004, Schuler et al. 2004). Horses given either 1 or 2 doses of vaccine (even if it was not given according to the manufacturer's recommendations) seemed less likely to die than unvaccinated horses (Salazar et al. 2004, Schuler et al. 2004). In one of these studies, death was more likely if clinical signs occurred first from March 1 to August 19, the animal was female, the animal was over 18 years of age or was unable to rise at any time during clinical illness (Salazar et al. 2004).

Given the pattern of WNV spread across the continent, the number of horses in Saskatchewan showing clinical disease was expected to be higher in 2003 than in 2002. The first objective of this study was to document the date of onset, geographic location, and outcome of clinical cases of WNV infection in horses in Saskatchewan in 2003. The primary objective was to identify risk factors that could be associated with fatality in infected horses, including management, mosquito control, vaccination, individual horse characteristics, and environmental conditions.

3.2 Material and methods

3.2.1 Study population and protocol

Veterinarians in private practice, horse owners, and the staff of the regional diagnostic laboratory (Prairie Diagnostic Services (PDS), Saskatoon, SK) provided information on clinically affected horses in Saskatchewan during the summer and early fall of 2003. In June 2003, all large and mixed animal practices in the southern portion of Saskatchewan were contacted by facsimile and asked 1) to send any serum samples collected from horses where WNV infection was suspected to PDS in Saskatoon for Immunoglobulin (Ig)M enzyme-linked immunosorbant assay (ELISA) and 2) to allow the laboratory to notify the research team of any positive result from the samples submitted. Of the 79 veterinary clinics contacted, 51 responded to the facsimiles (signed) in June 2003. Twelve more practices were contacted by PDS at the time a submitted sample was declared positive, giving a total cooperation rate of 80% (63/79).

For the purposes of this study, the definition of a ‘clinical case’ was the presence of one or more of the classic signs of WNV clinical disease (ataxia, recumbency, paresis

or paralysis) or death; and a positive IgM ELISA. Once a 'clinical case' was identified, the veterinarian was contacted to obtain consent to contact the owner. If consent was given, owners were telephoned 2 to 4 weeks after the onset of clinical signs and asked for additional information. The telephone survey included questions about location, characteristics of the affected horse, management of that horse, vaccination status, other resident equids (horses, ponies, mules, donkeys), and mosquito control measures applied at both the farm and the individual animal level.

In October, after the mosquito season, large or mixed animal veterinary clinics in each Regional Health Authority (RHA) were contacted to estimate the number of untested probable cases of WNV per region. A random numbers procedure was used to rank all of the the veterinary clinics in each RHA. Clinics from each RHA were then contacted by facsimile in random order until at least one third had responded. The participating veterinarians were asked to estimate the number of horses that were known or reported to have shown clinical signs consistent with WNV but were not tested for IgM antibodies during the 2003 season.

3.2.2 Horse data

The legal land location of the clinical case at the estimated time of exposure (up to 2 weeks before the first clinical signs) was requested for all cases where follow-up was obtained. Legal land location(LLL) included quarter, section, township, range, and meridian. In one instance, a global positioning system (GPS) unit was used to obtain the latitude and longtitude. For those cases in which the owner was unsure or unwilling to give the location, directions from the nearest town or township/range data were collected.

This information was cross-referenced with an electronic map of Saskatchewan. Using commercial geographical information systems (GIS) software (ArcView GIS version 3.2 and ArcGIS version 8.0, Environmental Systems Research Institute Inc, Redlands, California, USA) spatial location was displayed as a point by using the centroid-of-the-polygon for the land location. Confidentiality was maintained in all publications by summarizing location data at the RHA level.

Statistics Canada's 2001 Census of Agriculture estimated the number of horses and ponies (horse data) in Saskatchewan to be approximately 71300 and provided horse data by Census Consolidated Subdivisions (CCS). The CCS has similar boundaries to rural municipalities (RM), allowing estimation of the horse population by RHA (aggregations of RMs) and the mapping of cases per 1000 horse population by RHA. Data provided by the post season survey were aggregated and mapped by RHA to compare and assess the accuracy of surveillance using only confirmed cases of WNV for surveillance in 2003.

The animals were classified by sex, color, age, and breed type, but not by specific breed. Breed type was divided into light horses, draft horses and crosses, ponies, and "other". The category of "other" was composed of 3 miniature horses and one mule. Sex was reported in 3 categories: mare, gelding, and stallion. Color was divided into three categories: dark (chestnut, sorrel, brown, black, or bay), light (gray, white, palomino, buckskin, or dun), and multicolored (roan, paint, pinto, and appaloosa). Age was classified into three categories: young (≤5 years), adults (6-18 years) and geriatrics (>18 years). Age was categorized because the odds of outcome was not thought to increase on a linear scale and so that the results were comparable with those of other studies (8).

Day of onset of clinical signs was aggregated into week of onset with Sunday as the start of the week. To allow for easier interpretation, week of onset was further categorized into weeks of the year, 31-33 (beginning of August), weeks 34-35 (epidemic peak, end of August), and weeks 36-38 (September).

Housing was classified as housed in an enclosed barn, access to a simple three-sided shelter with corral or pasture, or no access to a shelter in open pasture with or without trees. The method of mosquito control on farm was recorded as: smudges (smoky fire used to ward off mosquitoes), water removal, sealed barn, use of fans or special lighting in a barn, use of general insecticides, or no mosquito control attempted. The use of insecticide sprays on individual horses was recorded as yes (any amount or frequency) or no.

3.2.3 Mosquito data

Mosquito data were obtained from the 2003 Saskatchewan Health mosquito trapping program. Center for Disease Control (CDC) traps were used for the collection of live mosquitoes for virus testing and New Jersey Light traps (NJLT) were used for the collection of dead mosquitoes for the determination of mosquito species. *C. tarsalis* and *C. restuans* data were provided on a weekly basis from June to September as the average number of mosquitoes trapped per night. Variables of interest were (a) the highest weekly average for *C. tarsalis* from the whole season (Number of *C. tarsalis*), (b) the highest weekly average for *C. restuans* from the whole season (Number of *C. restuans*), (c) whether WNV was found in pooled mosquitoes of each species (Positive pool), and (d) the weekly average for *C. tarsalis* from the likely week of exposure for each case. For

each variable of interest, the results of the nearest mosquito CDC and NJLT trap were linked to each horse case.

3.2.4 Environment data

Daily average temperature and precipitation data for all climate stations in Saskatchewan were obtained from Environment Canada. These values were used to create a 'season average' for temperature and 'season total' for precipitation. These variables were calculated for each climate station, using daily values for June, July, and August 2003. The season average temperature was determined by dividing the sum of the daily temperature by the number of days; the season total for precipitation was the sum of the daily amounts of precipitation. The variables from the nearest climate station were linked to each horse case.

Cumulative growing degree days (GDD) are the sum of the positive daily differences between 16ºC (the threshold temperature for activity of *C. tarsalis* and virus transmission) and the mean temperature for that day (Woody Ornamentals 2003). 'Season GDD' was calculated for each climate station by using temperature data from May 1st to September 30th. Cases were assigned data from the nearest climate station with complete daily temperature and precipitation information.

3.2.5 Statistical analysis

The association between each risk factor and the final health outcome (fatality) was examined by using generalized linear mixed models with a binomial distribution and logit link function (Dohoo et al. 2003). The calculations were performed by using

penalized quasi-likelihood estimates and the second order of the Taylor series expansion (MLwiN version 2.0, Centre for Multilevel Modeling, Institute of Education, London, UK). The strength of the association between outcome and exposure was reported as odds ratio (OR) with 95% confidence intervals and *P* values. A two level hierarchical (RHA, Horse) logistic regression model was used to account for geographic clustering of observations and because the method of surveillance used clinics within RHAs (Dohoo et al. 2003).

The linearity assumption was assessed for all continuous risk factors considered in the modeling process. Exposures were reanalyzed after being classified into quartiles and the linearity assumption was examined by looking for an increasing (or decreasing) series of coefficients. The "effect" of exposure in each higher category, as determined by the odds ratio, was compared with the level of the outcome in the baseline or referrent exposure category to identify patterns consistent with a linear, monotonic, or threshold effect.

All exposure variables where the association with the odds of fatality was significant at $P < 0.25$ were considered in developing the final multivariable model. Nonexposure risk factors were defined as confounders, if removing or adding the factor changed the effect estimate for the exposure by more than 10%, in which case the factor was retained in the final model. Manual backwards elimination of variables was used to achieve a final model containing only the statistically significant exposure variables and any nonexposure risk factors that were either significant or acted as important confounders. Biologically reasonable interactions were assessed between significant risk factors ($P < 0.05$) in the final model.

An approximation method based on latent variables was used to estimate the intraclass (intraregion) correlation coefficient ($\rho=\sigma^2_r/(\sigma^2_r+\pi^2/3)$) to examine the clustering of clinical cases of WNV within RHA (Dohoo et al .2003).

3.3 Results

3.3.1 Onset date and location

The date of onset of clinical signs for the first WNV IgM positive clinical case was July 30th, 2003. The last recorded date of onset of clinical signs in a horse was September 19th, 2003. Of the 130 horses for which a questionnaire was completed out of the total 133 clinical cases, 57 died or were euthanized because of complications associated with clinical disease (43.8% case fatality rate, 95%CI 35.2, 52.4) and the remainder recovered completely. Case fatality rates differed by RHA (Table 1). The epidemic curve appeared to peak one week earlier for horses that died from WNV clinical disease than for horses that survived, suggesting that there could be a difference in risk of fatality associated with time of onset (Figure 1). This observation was explored in subsequent analyses.

For all 133 confirmed clinical cases, location was reported at the RHA level, based on the location of the owner (N=130) or of the submitting clinic (N=3). For the 130 clinical cases with further follow-up, exact legal land location was obtained for 117 cases (90%), exact GPS coordinates for 1 (1%), and partial legal land location (township and range) for 12 (9%).

Table 1: Post season survey results and case fatality rates by Regional Health Authority (RHA).

RHA	Surveyed Clinics (Total number of clinics in RHA)	Total probables [a]	Estimated probables [b]	Ratio (estimated probables: confirmed)	Confirmed cases ( ) [c]	Case fatality rates (%) [d]
Sun Country	4 (11)	20	55	5.5:1	10	50
Five Hills	3 (10)	58	193	16:1	12	75
Cypress	3 (5)	36	60	3:1	17 (1)	47
Regina Qu'Appelle	4 (10)	40	100	5.6:1	18	39
Sunrise	2 (6)	21	63	4.8:1	13	23
Saskatoon	5 (8)	7	11	0.5:1	22	41
Heartland	3 (6)	8	16	1.6:1	10	30
Kelsey Trail	1 (5)	2	10	2.5:1	4 (1)	75
Prince Albert Parkland	1 (4)	12	48	6:1	8	63
Prairie North	2 (7)	21	74	4.6:1	16 (1)	31
Totals	28 (72)	225	630	4.8:1	130 (3)	44

[a] from surveyed clinics
[b] based on proportion of surveyed clinics
[c] numbers in parentheses are cases with no further follow-up obtained
[d] case fatality rates are calculated on confirmed cases only

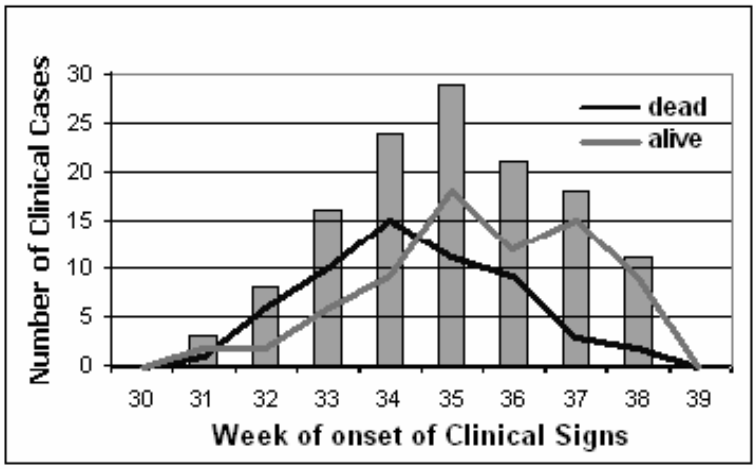

Figure 1: Number of clinical cases of West Nile virus infection in horses by week of onset of symptoms in 2003. The bars represent the total number of cases by week. The lines represent the number of cases by health outcome per week. Weeks begin on a Sunday and end on a Saturday, (Week 31 runs from July 27 to August 2, Weeks 31-35 correspond to the end of the month of July and the whole month of August 2003, and weeks 36-39 correspond to the month of September 2003).

Clinical cases of WNV infection were reported in all 10 RHA's in the southern portion of the province (Figure 2). When the raw data were adjusted for potential regional differences in reporting, based on the results of the post-season survey, the RHA with the highest number of clinical cases per 1000 horses was in the south central portion of the study area (Figure 3). Post-season results differed by RHA (Table 1), with the overall number of estimated untested probable clinical cases exceeding the tested clinical cases by approximately 4.8 to 1.

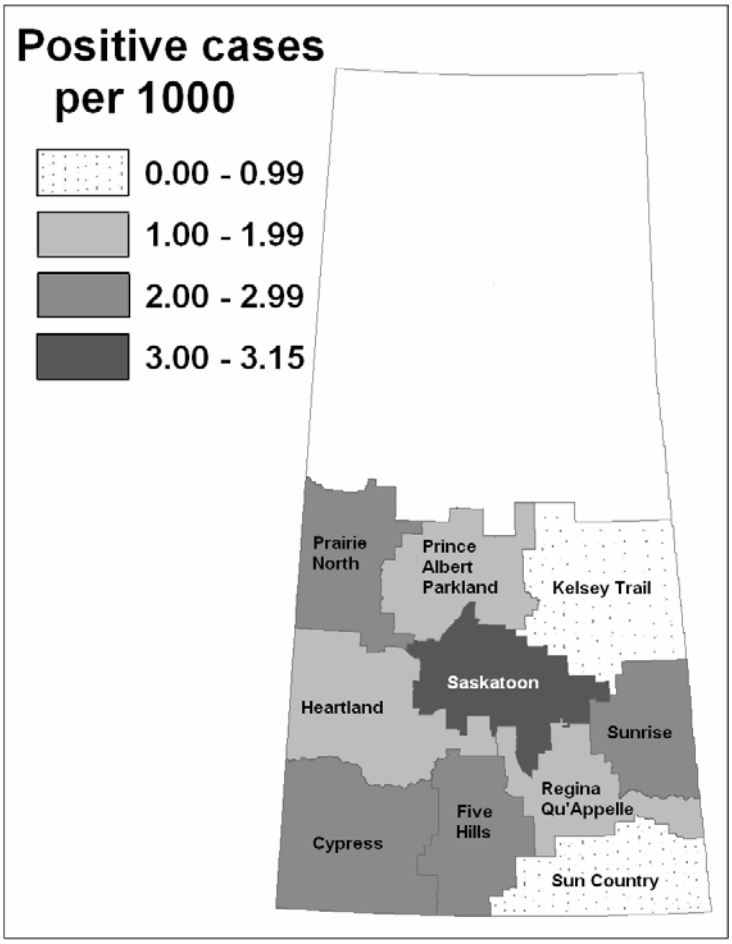

Figure 2: Confirmed positive IgM clinical cases of West Nile virus infection per 1000 horses at risk reported by Statistics Canada in each Regional Health Authority (RHA).

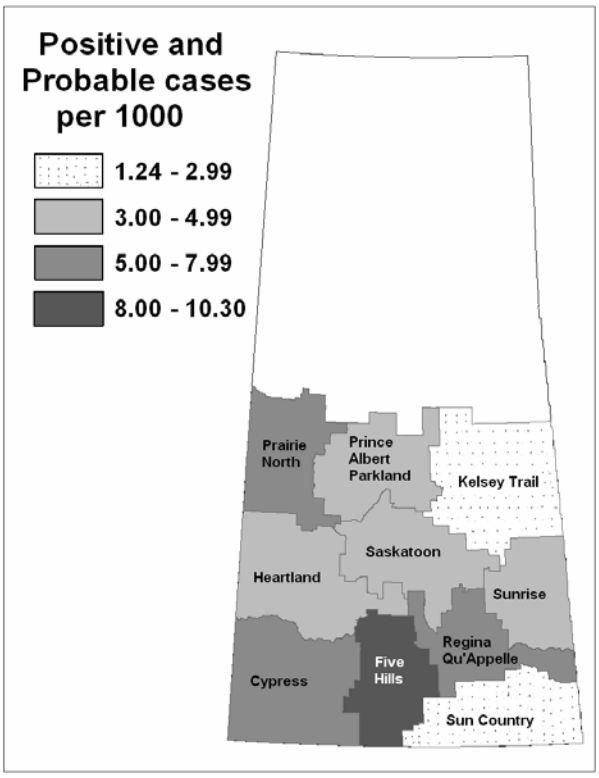

Figure 3: Combined number of diagnosed positive IgM clinical cases and undiagnosed probable clinical cases per 1000 horses at risk reported by Statistics Canada in each Regional Health Authority.

3.3.2 Description of affected horses

Most clinical cases occurred in horses ≤10 years; the fatality rate for this age group was 46% (38/83). The oldest horse that survived was 25 years old while the oldest horse that died was 32 years old. Most horses that died (as opposed to being euthanized) did so during the month of August (11/12 or 92%). The fatality rate was 36% (21/59) for mares, 45% (27/60) for geldings, and 82% (9/11) for stallions. Dark colored horses were most commonly affected (89/124), but they had a lower fatality rate (36%) than did those in other color classifications. Most of the clinical cases were light horse breeds and most were used for pleasure riding (Table 2).

Table 2: Summary of individual risk factors by survival outcome and case fatality rates (N=130).

Variable		Cases N	Health outcome		Case fatality Rate	Odds Ratio[a]	95% CI
			Dead	Alive			
Week of onset	31-33	27	17	10	63%	4.6	1.7, 12.5
	34-35	53	26	27	49%	2.5	1.1, 5.6
	36-38	50	14	36	28%	ref[b]	
Age	<5 years	44	22	22	50%	ref[b]	
	6-18 years	69	28	41	41%	0.7	0.3, 1.5
	>18 years	17	7	10	41%	0.7	0.2, 2.2
Gender	Gelding	60	27	33	40%	1.4	0.7, 3.0
	Mare	59	21	38	36%	ref[b]	
	Stallion	11	9	2	82%	8.5	1.6, 43.5
Breed	Pony	6	2	4	33%	0.6	0.1, 3.6
	Light	106	48	58	45%	ref[b]	
	Draft	11	4	7	36%	0.9	0.2, 3.3
	Cross	3	2	1	67%	2.6	0.2, 29.8
	Other	4	1	3	25%	0.4	0.04, 4.0
Color	Dark	89	32	57	36%	ref[b]	
	Light	19	14	5	74%	4.4	1.5, 13.1
	Multi-color	16	8	8	50%	1.9	0.6, 5.4
Individual shelter	No shelter	86	42	44	49%	2.7	1.2, 6.1
	Simple shelter	41	29	12	71%	ref[b]	
Individual insecticide use	no	108	51	57	47%	2.3	0.7, 7.4
	yes	19	5	14	26%	ref[b]	
Vaccine	No vaccine	121	53	68	44%	1.0	0.3, 3.9
	Vaccinated[c]	9	4	5	44%	ref[b]	
Primary use	Pleasure	108	49	59	45%	ref[b]	
	Breeding	13	6	7	46%	1.6	0.5, 4.9
	Farm work	5	2	3	40%	1.3	0.3, 5.4
	Competition	4	0	4	0%	[d]	
Herd size	10 or less	92	53	39	58%	ref[b]	
	11-25	21	10	11	48%	1.1	0.4, 2.5
	25 or more	13	8	5	62%	1.1	0.5, 2.7
Farm mosquito control	None	97	42	55	43%	0.9	0.4, 2.1
	Used at least one	29	13	16	45%	ref[b]	

[a] Univariable analysis adjusted for spatial location (RHA) by inclusion of a single random effect
[b] ref = reference category
[c] Vaccinated refers to fully vaccinated horses with the standard two vaccinations. Those horses with only one vaccination are considered non-vaccinates.
[d] Not enough data entries to converge

Of the 130 clinical cases with follow-up, only 9 were reported to have been fully vaccinated according to the manufacturer's recommendations (2 doses given 3-6 weeks apart, at least 2-3 weeks before the peak mosquito season), while 3 were vaccinated only once (Table 2). Initial vaccination dates for the 9 fully vaccinated horses were February (N=2) and April (N=7). The vaccination dates for the 3 horses given only one dose were May, August, and September. Of the 9 fully vaccinated horses, 5 recovered while 4 died or were euthanized (44% fatality rate). None of the three horses that were vaccinated once died or were euthanized.

Five of the 130 (3.8%) horses had been taken more than 15 km (10 miles) from their home location in the three weeks before onset of clinical sigs. Of these, two stayed within the same RHA, two travelled to a different RHA in SK and one travelled to British Columbia (BC). In 2003, BC had no evidence of WNV infection. Five horses had a change of residence 2-3 days before onset of clinical signs. The location at which they were most likely exposed (given an incubation period of 5-15 days) was, therefore, recorded as the location of the previous residence. Most of the clinical cases were in the same RHA as the veterinary clinic that submitted the blood sample (94%).

No horses were housed in a barn for any period of time and most horses (68%) did not have access to shelter. Nineteen of the horses had been sprayed periodically with insecticide during the mosquito season. None of the horses were blanketed for any period of time. The majority of clinical cases were kept on farms that did not use any on-farm mosquito control methods (97/126). For horses on farms that did use at least one method, the primary choice was smudges (18/29, 62%). Seven of the 8 horses on farms that used

water removal survived and 9 of the 18 horses on farms that used smudges survived (Table 2).

Herd size ranged from a single horse to 300 horses. The majority of horses were in small herds of 10 or fewer horses (73%). On most of the farms, all horses were kept under the same conditions; however, at one farm, the horses were housed in a barn, with the exception of the case horse. There were five farms that had 2 clinical cases and 5 farms that had at least one undiagnosed neurologic case in addition to the tested case.

3.3.3 Description of environmental and mosquito data for the reported cases

The mean 'season average temperature' was 18.7^{o}C and the mean 'season total precipitation' was 123 mm (Table 3); both of these values differed by climate station and by region. The mean 'season GDD' was 357. There was no obvious or statistically significant difference between these values for those horses that died and those that survived. Average temperature and total precipitation for the week prior to the estimated exposure period were also calculated, but, again, there was no apparent or statistically significant difference between values for those horses that died and those that survived (Table 3). The median distance between horse cases and the nearest climate station was 21 km (min, max = 2, 49).

Table 3: Summary of individual environmental data (continuous variables) by health outcome. These variables were calculated using only those climate stations or mosquito traps for which the data was matched up with the nearest case.

Variable	Health Outcome	Cases N	Temperature (°C) Min	Max	Mean	Overall Mean (°C)	Odds ratio [b] (95% CI)
Season Average Temperature [a] (°C/day)	Dead	57	16.7	20.3	18.7	18.7	1.0 (0.7, 1.5)
	Alive	73	16.4	20.3	18.6		
Season total Precipitation [a] (mm)	Dead	57	39.2	206	118	123	1.0 (1.0, 1.0)
	Alive	73	39.2	215	128		
Season GDD [a] (degree days)	Dead	57	199	490	350	357	1.0 (1.0, 1.0)
	Alive	73	178	507	355		
Exposure week Average Temperature (°C/day)	Dead	57	14.8	26.5	21.3	20.9	1.1 (1.0, 1.2)
	Alive	73	14	26.5	20.6		
Exposure week Total precipitation (mm)	Dead	57	0	44.6	7.65	7.30	1.0 (0.9, 1.0)
	Alive	73	0	39.4	6.85		
Exposure Week *Culex (C.) tarsalis* (number per night)	Dead	57	0	4.80	0.38	0.5	0.8 (0.5, 1.3)
	Alive	73	0	4.40	0.51		
Number of *C. tarsalis* (number per night)	Dead	57	0	10.40	1.67	1.9	0.9 (0.8, 1.1)
	Alive	73	0	15.00	1.98		
Number of *C. restuans* (number per night)	Dead	57	0	6.00	0.41	0.3	1.4 (0.8, 2.4)
	Alive	73	0	2.60	0.25		

[a] In this table growing degree days (GDD) is the cummulative growing degree days for the whole season (May to September), season average temperature is calculated with daily values from June to August divided by the total number of days and season total precipitation is the cumulative daily data from June to August.
[b] Univariable analysis adjusted for spatial location (Regional Health Authority) by inclusion of a single random effect

Variables summarizing mosquito data were similar for those horses that died or survived. The highest numbers of *C. tarsalis* and *C. restuans* over the whole season were 1.9 and 0.3, respectively. The number of C. *tarsalis* mosquitoes during the estimated exposure week of each case ranged from 0 to 4.8 (mean, 0.5) (Table 3). *Culex tarsalis* numbers were higher for those horses that survived, while *C. restuans* numbers were higher for those horses that died; however, the comparisons were not statistically significant.

WNV-positive *C. tarsalis* and *C. restuans* mosquito pools were found in the nearest CDC trap for 54% (70/130) and 39% (51/130) of clinical cases, respectively (Table 4). The median distance between horse cases and the nearest mosquito trap was 29 km (min, max = 4, 92).

Table 4: : Summary of individual environmental data (categorical variables) by survival outcome.

Variable		Cases	Health outcome		Odds ratio [a]
		N	Dead	Alive	(95% CI)
Positive pool *Culex*	no	60	28	32	ref [c]
tarsalis [b]	yes	70	29	41	0.8 (0.4, 1.7)
Positive pool *Culex*	no	79	35	44	ref [c]
restuans [b]	yes	51	22	29	1.0 (0.5, 2.0)

[a] Univariable analysis adjusted for spatial location (Regional Health Authority) by inclusion of a single random effect
[b] A positive pool of mosquitoes (collection of species of mosquito designated) at the nearest to the trap location of the case horse.
[c] ref = reference category

3.3.4 Association between individual horse risk factors, environmental variables, and the odds of fatality in clinical cases of WNV

Variables where there was a statistically significant association with fatality in the initial univariable mixed models that were adjusted for clustering by RHA included week of onset, coat color, gender, and availability of individual shelter. Horses affected in August had 3.1 (OR 95% C.I. 1.4, 6.8) times greater odds of dying than those affected in September. The month of August was further broken down into two categories, weeks 31-33 and weeks 34-35. The odds of dying in both weeks 31-33 and weeks 34-35 (August) was higher than the odds of dying in weeks 36-38 (September) (Table 2).

Table 5: Risk factors associated with survival outcomes in horses with clinical infection with West Nile virus (WNV): final mixed multivariable model [a].

Variable	Category	Cases N	Odds ratio	95% C.I.	*P*
Week of Onset	Week 31-33	27	6.3	1.9, 20.4	0.003
	Week 34-35	53	2.4	0.9, 6.2	0.08
	Week 36-38	50	ref [b]		
Gender	Mare	59	ref [b]		
	Gelding	60	1.2	0.5, 2.9	0.70
	Stallion	11	14.7	2.5, 87.4	0.005
Coat Color	Dark	89	ref [b]		
	Light	19	6.4	1.9, 21.6	0.004
	Multi-color	16	1.9	0.6, 6.5	0.31

[a] Adjusted for Regional Health Authority (RHA) by the inclusion of a single random effect
[b] ref = reference category

The odds of dying was greater for light colored horses than for dark colored horses, while multicolored horses were at similar risk to dark colored horses (Table 2). The odds of dying for males (stallions and geldings combined) was 1.9 times (95% C.I. 0.9, 3.8) greater than the odds of dying for females. Specifically, the odds of dying for

stallions was 8.5 times (95% C.I. 1.6%, 43.5%) greater than the odds of dying for females (Table 2). The odds of dying was greater for those with no shelter than for those with a simple shelter.

The final mixed model included week of onset, gender, and coat color (Table 5). Shelter did not remain statistically significant in the presence of the other variables and was removed from the final model. The final model was a mixed model with a random intercept to account for geographic clustering with RHA. The variance accounted for at the region level (σ^2_r) was 0.35 (*standard error*, 0.36) or approximately 9.5% of the overall variance in the model.

3.4 Discussion

West Nile virus infection and the development of associated clinical disease are influenced by many factors, including environment, mosquito populations, and individual susceptibility (Hubalek et al. 1999, Murgue et al. 2001, USDA 2001, Autorino et al. 2002). The onset dates of WNV clinical disease reflect the time necessary for development of climatic conditions required for mosquito reproduction, natural amplification of the virus, and shift in mosquito feeding patterns to allow transmission of the virus to horses (Curry 2004). The location of the cases corresponded to areas where mosquito populations capable of transmitting WNV and susceptible horse populations coexist.

3.4.1 Distribution of clinical cases

Travel history was essential in determining where horses were potentially exposed to WNV. However, as few horses had travel histories, exposure could be assumed to have occurred near their place of residence, even without complete travel history. Clinical cases and the submitting clinic were within the same RHA. Thus, on the basis of this study, we could assume WNV activity in the locality of either the submitting clinic or of the individual horses, depending on the degree of available location accuracy.

Most clinical cases were recorded in the 4 most southerly RHA's while the fewest occurred in the most northerly RHA's of the study area. This distribution corresponds mainly to differences in climate and mosquito populations; however, veterinarians and horse owners located in the Saskatoon RHA might have been more inclined to pursue veterinary services and diagnosis, as this is the location of PDS. No clinical cases were diagnosed from the northern portion of the province (outside the study area). This was probably because habitat and climate do not favor the principal vector, *Culex tarsalis* (Curry 2004).

The post-season survey of veterinary clinics suggested that there were numerous cases of neurologic symptoms in horses for which owners did not seek diagnosis. Clinical diagnosis by clinical signs alone could be mistaken for other neurological conditions in horses such as rabies, wobbler syndrome, equine herpesvirus ecephalomyelitis (EHV1), Equine protozoal myeloencephalitis (EPM) and western equine encephalomyelitis (WEE) (Ostlund et al. 2000); however, all of these are considered relatively unlikely in this

region. Thus, typical neurologic symptoms in an area known to have active WNV are reasonably predictive of WNV clinical disease presentation.

A mixed model was used to account for regional distribution of clinical cases because WNV has been shown to cluster by geographic area in other studies, including one recent study that identified statistically significant clusters of both horse and human cases of WNV infection in 2003 (USDA 2001, Brownstein et al. 2002, Corrigan 2005). In the current study, RHA was chosen as the unit to represent geographic location in the mixed model, because it was a reasonable geographic proxy for the veterinary clinics that were the main source of our information. Individual veterinary clinics were not used in the analysis, as most of these only sampled 1 or 2 cases. The boundaries of the RHAs often reflect patterns of travel from rural areas to larger centers for health services, and based on the maps appear to reflect a reasonable pattern of access to and use of veterinary services. In addition, case fatality rates differed by RHA, with the highest case fatality rate and highest post season survey ratio in the Five Hills RHA. Decisions on the form and timing of public education campaigns related to WNV and mosquito control programs are often made at the level of the RHA or by rural municipalities located within the RHA.

3.4.2 Risk of fatality for clinical cases

The only significant predictors of risk of fatality in the final model were week of onset of clinical signs, sex, and color, while controlling for region. A small amount of variation (9.5%) was accounted for by geographic region in the final mixed model (Dohoo et al. 2003). This could reflect variability of the RHA mosquito programs and

other inherent differences between the regions. Differences in clinic policy could have influenced the variation, but, overall, most clinics followed similar guidelines for diagnosis or treatment. Therefore, programs to control the outcome of clinical disease should be directed toward individual horses. Unfortunately, the variables found to be significant in this study are difficult to manage for disease control purposes.

The case fatality rate was similar to rates from other studies conducted in North America (Ostlund et al. 2001,Porter et al. 2003, Salazar et al. 2004). The increased survival rate of horses with clinical signs as the season progressed was similar to findings in the western United States (US). Salazar et al. (2004) found that horses were 1.7 times more likely to die if they showed clinical signs early (before August 19th, 2003) in the season. The increased risk of death in August could be a result of earlier recognition of clinical cases as the season progressed. Owners may have checked their horses for clinical signs more often when they heard of clinical cases in nearby areas, sought veterinary assistance sooner (subtler clinical signs) and begun treament earlier. This assumes that horses have a steady progression of clinical signs and that owners of horses that died merely missed the onset of mild signs. However, in this study, many owners reported seeing nothing unusual with their horses the day before the onset of moderate to severe clinical disease (recumbency, paresis, or paralysis of limbs). An alternate theory is that the incubation period for different doses of virus corresponding to different time periods results in the difference in immune response and the varying presentations and outcomes of clinical disease. Further clarification of this finding is warranted.

Stallions were more likely to die as a result of WNV clinical disease than either mares or geldings. In some mammals, it has been noted that while testosterone boosts sexual

characteristics it also simultaneously impairs immune system functioning (Folstad et al. 1992). Therefore, stallions may have a poorer immune response than either females or geldings. To compare our results to those of other studies from the United States, we recategorized the data as males (both stallions and geldings) and females (mares) for reanalysis. In both the other studies, the univariable analysis suggested that females were more likely to die, but this result was not statistically significant (Salazar et al. 2004, Schuler et al. 2004). There was no indication of pregnancy status of the mares in any of the studies mentioned which might affect this result. In our analysis, no statistically significant difference existed between mares and geldings, but rather between stallions and mares or geldings. Therefore the percentage of males that were intact stallions could influence this association. This was reported as 8% for one study (Salazar et al. 2004), compared to 15% for our study.

Prevention education programs for humans list wearing light colored clothing as a method to decrease the possibility of WNV infection (SK Health 2003). When seeking out potential explanations, no clear evidence for the reasoning behind the use of light colors as prevention of infection could be found. In this study, we looked at risk of outcome once infection and clinical disease had occurred. We found the light colored horses had greater odds of dying from WNV clinical disease than did dark colored horses. A possible explanation could be that the immune response is linked to color genetically. However, no references to justify this were found. Further information is needed to determine the role of coat color in the outcome of WNV clinical disease.

Other factors assessed in this study, particularly age and vaccination, were not significantly associated with the risk of fatality in clinical cases. In other studies, older

affected horses have been more likely to die (Salazar et al. 2004, Schuler et al. 2004). In this study, the case fatality rate was highest in horses 5 years or younger; however, this was not statistically significant. In this study only 7% of horses reported to have clinical signs in 2003 were vaccinated, so there was little power to assess vaccine effectiveness in prevention of fatality. Other studies have shown that the vaccine has a protective effect against fatality in clinically affected horses (Salazar et al. 2004, Schuler et al. 2004).

The environmental variables (temperature, precipitation, GDD, and mosquitoes) examined also were not associated with the individual horse risk of fatality. Calculation of these variables involved averaging 3 to 5 months of data which may not represent the exposure period of each clinically affected horse or the developmental period of the mosquitoes prior to exposure. However, when temperature and precipitation were calculated by using only data from the week before the estimated exposure date of each clinically affected horse, the results were still not statistically significant. With so much variation in environmental variables by local macro- and micro-climates, the use of the nearest climate station may not have represented the conditions at the actual location of the clinical horse. More likely, however, is the conclusion that environmental conditions would not play a role in an individual horse's health outcome once it had become infected with WNV, rather it would be associated with the risk of infection only.

The role of various other prevention strategies were also explored. Mosquito reduction and avoidance strategies were not widely used by the owners' of clinically affected horses. None of the mosquito reduction methods were significant predictors of fatality. Housing horses in a barn at night has been suggested to prevent infection and disease. In this study, no clinical WNV horses were housed in a barn at any time. When

variables sex, coat color, and onset of clinical signs were included in the analysis, the protective effect of simple shelter was not significant. Even with these findings, avoidance of mosquitoes is still the best method of prevention of clinical disease and death as a possible outcome. In addition, owners should be aware of the timing and risk of WNV infection in their geographic area, so that they can use all methods available to them in the prevention of infection and in having a favorable outcome of clinical cases.

3.5 Literature cited

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4. Serologic Responses of Horses Naturally Exposed to West Nile virus

4.1 Introduction

West Nile virus (WNV) was first isolated in horses in North America in the late summer of 1999 (Trock et al. 2001). The area of the epidemic was limited to New York State, specifically on Long Island. The 25 cases of WNV encephalitis were confirmed using the plaque reduction virus-neutralization test (PRNT). In 2000, 12 northeastern and mid-Atlantic states reported neurologic cases in horses due to WNV (Ostlund et al. 2001, Trock et al. 2001). Detection of these cases was by either PRNT or Immunoglobulin (Ig) M enzyme-linked immunosorbent assay (ELISA). As the virus continued to spread across the continent in the next two years, more horses developed neurologic disease and testing became routine with the IgM ELISA (Castillo-Olivares et al. 2004).

Serologic responses of horses exhibiting clinical signs have been documented by using IgM. IgM antibodies develop early becoming detectable approximately 8-10 days post infection and around the time of onset of clinical signs (Ostlund et al. 2000). Duration of the IgM antibody response is from 1 to 3 months in horses with the majority of antibody disappearing by 1-2 months after infection (Durand et al. 2002). This makes

IgM suitable for detection of clinical disease caused by WNV even in areas where WNV was present the previous year.

Little published data were found to show the change over time in IgG antibody in response to natural infection in horses. WNV neutralizing antibodies (via PRNT) are sometimes detectable within one week post infection and can persist for 18 months to greater than 2 years (Ostlund et al. 2000, Ostlund et al. 2001, Bunning et al. 2002, Durand et al. 2002). In one study, 18/20 (90%) of horses that were clinically affected still had PRNT titers greater than or equal to 1:100 5 to 7 months later (Davidson et al. 2005). However, the same has not been confirmed for IgG antibodies by the IgG ELISA test. A serosurvey of horses in Southern France in 2000 used indirect IgG ELISA to assess serology at one point in time and thus infection status of asymptomatic horses in an epidemic area (Murgue et al. 2001, Durand et al. 2002). The study then made the assumption that anti-WNV IgG usually persists for several years post infection to allow assessment of the epidemic versus endemic status of the study area.

Due to the overwhelming spread of the virus in North America and the increase in the numbers of neurologic WNV cases being reported each year, a killed vaccine was developed with a claim for protection from severe clinical disease (Ostlund et al. 2000). In a vaccination study to assess the efficacy of the vaccine and kinetics of the specific IgG response, sera from 8 vaccinated horses were tested using pan-flavivirus IgG Capture ELISA at a 1:100 dilution (Ng et al. 2003). The results showed significant IgG response detectable as early as 14 days post first vaccination with another rise after the second (booster) vaccination. The duration and the evolution of the IgG response was followed further but using PRNT instead of the IgG ELISA (Ng et al. 2003).

The introduction of the vaccine complicated surveillance efforts since both vaccinated and naturally infected horses could potentially create IgG antibodies. A vaccination study undertaken by this research team documented that no serologic IgG response to a two dose vaccination protocol was seen with a WNV-specific IgG Capture ELISA test (Epp et al. 2007). With this test, horses could be defined as naturally infected on the basis of IgG antibodies whether they had been vaccinated or not. The objective of this study was to document the changes in IgG concentrations (kinetics) resulting from natural infection over an 8 month period in both vaccinated and non-vaccinated horses.

4.2 Materials and methods

4.2.1 Study design

Data on the changes in antibody concentrations following natural exposure were required in order to use the ELISA test to differentiate between past and present season infections. To better understand the kinetics of the immune response as measured by the ELISA, the time-related variation in the concentrations of antibody to WNV were studied in several groups of horses (Table 1). These groups were chosen on the basis of their vaccination status and a positive IgG and/or IgM status during the 2003 mosquito season. These groups included 1) non-vaccinated WNV clinical case horses, 2) asymptomatic vaccinated horses with an IgG ELISA positive test result during August to October 2003, and 3) asymptomatic non-vaccinated horses with an IgG ELISA positive test result during August to October 2003. A horse was considered a confirmed positive "WNV clinical case" if it had one or more of the classic signs of WNV, such as ataxia,

recumbency, paralysis or paresis of limb(s), or death and a positive IgM ELISA test by the provincial laboratory.

Table 1: List of possible groups based on vaccination status, clinical history and the relationship with serum antibody levels that need to be distinguished in WNV surveillance.

Vaccination status	IgG status*	IgM status*	Groups to follow	Reason for not following group
No	Pos	Pos	Follow; Both asymptomatic and symptomatic groups	
No	Pos	Neg	Follow; Likely just the asymptomatic group	
No	Neg	Pos	Group not important to follow	Because IgM denotes recent infection
No	Neg	Neg	Group not important to follow	Because results denote animal is not infected
Yes	Pos	Pos	Follow; Both asymptomatic and symptomatic** groups	
Yes	Pos	Neg	Follow; Likely just the asymptomatic group	
Yes	Neg	Pos	Group not important to follow	Because IgM denotes recent infection
Yes	Neg	Neg	Group not important to follow	Because results denote animal is not infected

* Status was reported as a positive (pos) or negative (neg) test result that was reported during August to October 2003.
**Symptomatic vaccinated animals were not followed because of too few numbers (n=5)

For each group, a minimum of 10 horses that met the criteria were selected from studies on-going by the research team at the Western College of Veterinary Medicine (WCVM) at the University of Saskatchewan. The fourth group, 'vaccinated clinical horses' were not included as a group because there were only 9 clinical case vaccinated horses of which only 5 recovered. The horses in each of the three groups were bled during an eight month period at least three times: August or early September, October, December and March. These samples were tested with both the IgG and IgM ELISA and

results were reported as a sample-to-positive (S/P) ratio. Results were graphed (Microsoft Office Excel 2003, Microsoft Corporation, USA) and differences in S/P ratios among groups at different time points were analyzed using Generalized Estimating Equations analysis (proc GENMOD). Due to multiple comparisons, a Bonferroni correction was used with significance at P=0.006 for between groups and P=0.003 for within groups. The model contained a repeated term for horse, assumed a normal distribution and used an identity link function and an exchangeable covariance structure (PROC GENMOD, SAS for Windows ver. 8.2, SAS Institute Inc., Cary, NC).

4.2.2 Testing and reporting of results

Testing of the samples was done using an in house IgM and IgG WNV antibody-capture ELISA. All samples were classified as positive, suspicious or negative for both IgG and IgM based on the OD results of the appropriate ELISA. A sample-to-positive (S/P) ratio was then calculated for the IgG test results to allow comparison of results from multiple ELISA plates. The following formula was used (Adaska et al. 2002):

$$\text{S/P ratio} = \frac{\text{sample OD} - \text{negative control OD}}{\text{positive control OD} - \text{negative control OD}}$$

The optical density (OD) was determined as the difference in values between the duplicate wells on the plate for each test serum and control. Sera were tested on tissue culture derived WNV antigens in one of the wells. In the other well, sham infected tissue culture antigen preparations (control antigen) were used to ensure the specificity of the response was to WNV. The strong positive control from the top left corner was used in

the calculation of the above formula for the first four rows of the plate and the strong positive control at the bottom right corner of the plate for the bottom four rows.

Both the IgM and IgG assays used in this study were antibody capture ELISAs using reagents supplied by BioReliance Corporation (Rockville, Maryland). No sensitivity or specificity estimates were available for the reagents used in this test.

For the IgM ELISA, 100 microlitres of a 1:250 dilution of monoclonal antibody specific for Equine IgM in Phosphate buffered saline (PBS) was coated onto wells of a polyvinyl chloride (PVC) ELISA plate and incubated overnight at 4°C. The plates were washed 5 times between each step of the process. Control and test sera were then diluted in PBS-T, added to duplicate wells of the ELISA plate and incubated for 2 hours at 37°C. Then 100 microlitres of WNV antigen or control antigen preparations were diluted 1:5 in PBS-T, added to one of the duplicate wells and incubated for 2 hours at 37 °C. One hundred microlitres of a 1:100 dilution of WNV specific monoclonal antibody were added to the test wells. The monoclonal antibody was diluted in PBS-T + 5% skim milk. The plates were incubated for 2 hours at 37 °C. Then 100 microlitres of a 1:100 dilution of anti-mouse IgG –Horse Radish Peroxidase (HRPO) conjugate in PBS-T + 5% skim milk were added to each well and incubated for one hour at 37 °C. Finally 100 microlitres of ABTS (2,2'-azino-di(3-ethyl-benzthiazoline-6-sulfonate, KPL cat# 50-66-00) were added to each well and incubated for 30 minutes at 37 °C. The test was read using a spectrophotometer at 410 nm. A positive test was one that had a net O.D. of 0.2 or greater after subtraction of the control (duplicate) well O.D. and had at least 2 times the control well value. A suspicious test met one of these criteria and a negative test met none of the criteria.

For the IgG ELISA assay used in this study, 100 microlitres of a 1:50 dilution of a WNV specific monoclonal antibody is added onto wells of a polyvinyl chloride (PVC) ELISA plate and incubated overnight at 4 °C. The plates were washed 5 times between each step in the process. The WNV antigen and control antigen were diluted 1:50 in IgG lysis buffer (PBS+0.3% Igepal CA-630 Sigma #I3021) and incubated at room temperature for 15 minutes. Then 100 microlitres of the diluted antigen and control antigen were added to one of the duplicate wells on the ELISA plate and incubated at 37 °C for 2 hours. The dilutions of the control and test sera were prepared in PBS-T + 4% normal goat serum (all test sera are diluted 1:200). A total of 100 microlitres of the diluted samples and controls were added to the duplicate wells and incubated at 37 °C for 2 hours. Then 100 microlitres of anti-Equine IgG HRPO conjugate diluted in PBS-T + 5% skim milk + 4% normal goat serum was added. One hundred microlitres of ABTS (KPL cat# 50-66-00) were added to each well and incubated for 30 minutes at 37 °C. The test was read using a spectrophotometer at 410 nm. Positive, suspicious and negative test results are as defined above.

4.3 Results

Group 1 consisted of 14 clinical case horses (all were non-vaccinates) (Table 2). Of these, 64% (9/14) had 3 samples drawn between August 2003 and March 2004 while 36% (5/14) had only 2 samples drawn. Only the August time point differed from all other time points (P <0.0001).

Group 2 consisted of 11 vaccinated horses with positive IgG or IgM test results at the August or October 2003 sampling with the potential for natural exposure (Table 2).

All of the horses were vaccinated with the two shot standard protocol; nine horses were vaccinated in April, while two horses were vaccinated in September after clinical disease had occurred in a herd-mate. Using the cutoff values (0.305 for horses vaccinated two times; 0.76 for horses vaccinated three times) determined by a vaccination study performed by this research team, all horses were deemed to be naturally exposed (Epp et al. 2007). Of the horses in this group, 73% (8/11) had four samples drawn between August 2003 and March 2004 while 27% (3/11) had only three samples drawn. All time points differed (P <0.0001) except August and March, and October and December.

Group 3 consisted of 12 unvaccinated horses with positive IgG or IgM test results at the August or October 2003 sampling with the potential for natural exposure (Table 2). Of these, 1 horse had four samples drawn between August 2003 and March 2004 while 50% (6/12) had three samples drawn and 42% (5/12) had two samples drawn. Only the August time point differed (P <0.0001) from both the October and December time points.

Mean S/P ratios from non-vaccinated clinical case horses were higher than the mean S/P ratios from either the vaccinated or non-vaccinated asymptomatic naturally exposed groups (Figure 1). Average S/P ratios from clinical case horses were higher than from vaccinated asymptomatic naturally exposed horses at both the October (P=0.0007) and December (P=0.002) sampling dates and were also higher than S/P ratios from non-vaccinated asymptomatic naturally exposed horses in October (P= 0.005). The average S/P ratios from clinical case horses remained higher than for vaccinated asymptomatic naturally exposed horses through March (P<0.0001). Clinical case horses did not have significantly different S/P ratios than non-vaccinated asymptomatic naturally exposed horses in December (P=0.04) and March (P=0.09). Average S/P ratios of vaccinated and

non-vaccinated asymptomatic naturally exposed horses did not differ over any of the sample time periods.

Four positive IgM samples that were taken in late August or early September were negative when these horses were re-sampled anywhere between 41 to 60 days later. Two of the non-vaccinated asymptomatic horses and the two September vaccinated asymptomatic horses had positive IgM results in late August, but were negative when sampled approximately 100 days later. None of the horses vaccinated prior to exposure season had positive IgM samples despite showing increases in IgG S/P ratios over the sample period.

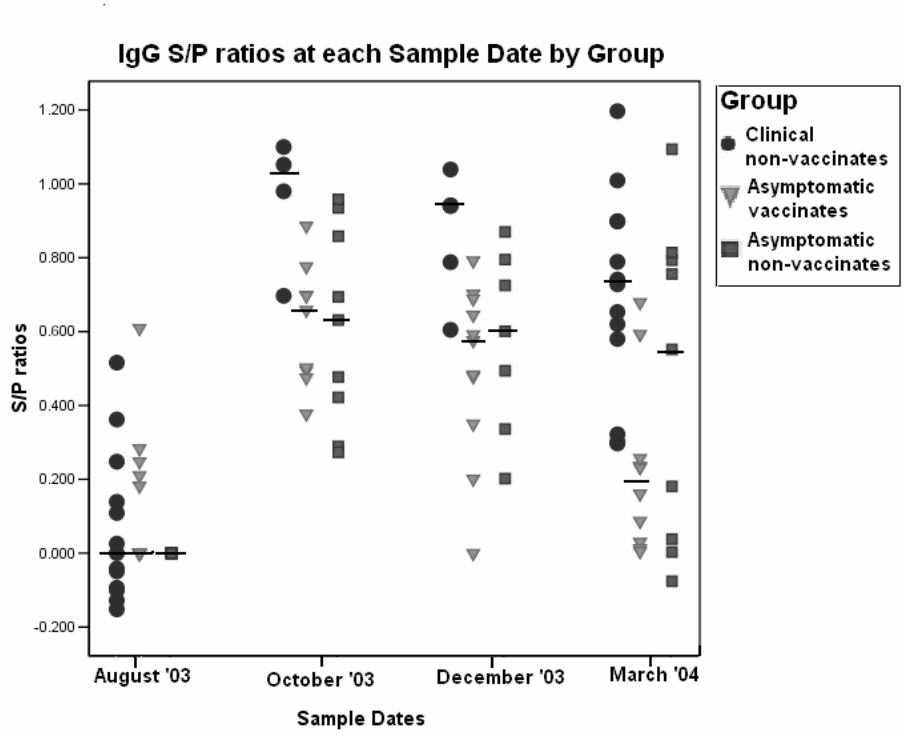

Figure 1: IgG S/P ratios for each sample date by group (Total N=37). (—) denote medians for each group by sample date as listed in Table II.

Table 2: The change in IgG antibody concentration over time as measured by S/P ratios for groups of horses with different clinical histories and vaccination status (N=37).

	N	n by month	Month and Year	Mean (*s* *)	median	IQR 25th	IQR 75th
Group 1: Clinical case horses	14	14	August 2003	0.06 (0.20)	0.00	-0.10	0.19
		4	October 2003	0.96 (0.18)	1.02	0.77	1.09
		6	December 2003	0.88 (0.16)	0.94	0.74	0.97
		13	March 2004	0.67 (0.24)	0.73	0.45	0.90
Group 2: Vaccinated naturally exposed asyptomatic horses	11	11	August 2003	0.14 (0.19)	0.00	0.00	0.25
		9	October 2003	0.62 (0.17)	0.66	0.49	0.74
		11	December 2003	0.50 (0.24)	0.58	0.35	0.69
		10	March 2004	0.23 (0.24)	0.20	0.03	0.34
Group 3: Non-vaccinated naturally exposed asymptomatic horses	12	7	August 2003	0.00 (0.00)	0.00	0.00	0.00
		9	October 2003	0.62 (0.27)	0.63	0.36	0.90
		7	December 2003	0.58 (0.25)	0.60	0.34	0.80
		9	March 2004	0.46 (0.43)	0.55	0.02	0.80

*s = standard deviation

4.4 Discussion

Sero-conversion was not documented by samples taken 14 days apart. Rather on the basis of a positive IgM ELISA or an increase in the S/P ratio of IgG ELISA of two consecutive samples all horses in the study were considered naturally exposed to WNV in 2003. The serologic responses of horses were followed over an 8 month period following the WNV season with 2-4 sampling times per horse. All groups of horses showed an

initial rise and subsequent fall of IgG antibodies. Clinical case horses showed significantly higher average S/P ratios at October, December and March time points compared to asymptomatic vaccinated horses while only at October for non-vaccinated horses. This indicates that clinical horses initially develop a greater IgG antibody response than non-clinical horses and may indicate that clinical disease is the result of an increased viral load which initially overwhelms the immune system of the horse or that clinical disease is the result of an overzealous immune system.

Other studies have noted that WNV neutralizing antibodies have been detected up to 18 months post infection (Durand et al. 2002). In this study, IgG antibodies to natural infection detected by the ELISA test initially rose and then declined over the 8 month sampling period. If the decline in average IgG S/P ratios is extrapolated, WNV antibodies should have become negative (less than 0.30 S/P ratio) using the ELISA within another 4-5 months (around July to August 2004) in the absence of additional exposure. This further supports the assumption that horses with positive IgG ELISA tests in the summer of 2003 were naturally exposed and infected with WNV during the summer of 2003.

Humoral immunity is considered essential in the immune response to WNV because neutralizing antibodies are thought to limit dissemination of infection (Diamond et al. 2003). The majority of studies published to date look at the mouse as a model. In this study, the IgG response of naturally exposed clinical horses was on average higher than naturally exposed asymptomatic horses whether vaccinated or not. This apparent relationship between increased IgG response and development of clinical signs in the horse warrants further investigation.

IgM is used as a means of identifying recent clinical cases of WNV. In humans, IgM antibodies are present in more than 50 % of patients 2 months after onset of clinical signs and are thought to be present in blood for a maximum of 7 months (Tardei et al. 2000, Prince et al. 2003). In this study, all of the 4 horses with two samples taken less than 2 months apart had sero-reverted and all of the 13 horses with two samples taken less than 4 months apart had sero-reverted. Thus, in horses IgM is useful for documentation of clinical disease due to WNV. However, unless frequent serial blood sampling is employed for surveillance, IgM would not be useful in monitoring infection status in a population as IgM antibodies usually do not persist in the serum longer than 1-2 months after exposure.

Serology using IgG ELISA in the presence of vaccinated horses may become more difficult to interpret as new vaccines become available and varied dosing regimens are employed. Further characterization of IgG responses of horses using the ELISA will be needed to assess the effect of more than two years of vaccination, vaccination of clinically infected individuals, different vaccination protocols (ie. multiple boosters per year) and the use of different vaccines.

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5. Serologic Prevalence and Risk Factors for Infection with West Nile virus in Saskatchewan Horses, 2003

5.1 Introduction

West Nile virus (WNV) was introduced to the North American continent in 1999 (Ostlund et al. 2001) and was first diagnosed in horses in Canada, including the province of Saskatchewan, in 2002 (Weese et al. 2003, Abutarbush et al. 2004). Horses that are infected with the virus may show clinical signs or go on to eliminate the virus uneventfully (Ostlund et al. 2001). Several studies have tried to assess the prevalence of asymptomatic WNV infections by either a random sampling of horses in an epidemic area or by sampling horses with known contact with clinical WNV cases (Trock et al. 2001, Autorino et al. 2002, Durand et al. 2002, Estrada-Franco et al. 2003). Reported prevalence varied from 1.2% in Yucatan, Mexico (Lorono-Pino et al. 2003) to 38% in Italy (Trock et al. 2001) in random sample surveys. A survey in France in 2000 showed geographical differences in prevalence from less than 5% to 58% (Durand et al. 2002). Serologic prevalence in horses tested in association with clinical case locations has ranged from 15% in Eastern United States (USA) in 2000 (6) to 43% on one ranch in Coahuila State, Mexico in 2002 (Blitvich et al. 2003).

No published studies to date solely look at risk factors for asymptomatic infection with WNV. A study from the Eastern USA looked at differences in individual horse characteristics between infected horses (with or without signs) and non-infected horses as defined by serology or virus isolation (USDA 2001). Horses used for pleasure riding were more likely to be infected while housing horses in a barn at night was protective against infection.

In July 2003, a seroprevalence study was initiated to measure the spread of the virus across Saskatchewan. However, the use of serology to identify infection status was complicated by the release of a new vaccine, an inactivated WNV vaccine that had been licensed for use in Canada during the spring of 2003 (West Nile®Innovator, Wyeth Animal Health, New Jersey, USA). The vaccine was widely used by horse owners across the province. Vaccinated horses were expected to produce immunoglobulin (Ig)G but not IgM antibodies in response to the vaccine (Ng et al. 2003) and to produce both IgG and IgM antibodies to natural infection. However, information on the IgM status of the horses in this study alone would not have been adequate to measure seroprevalence reflecting natural infection, because IgM antibodies persist for less than 2 months (Ostlund et al. 2001).

Reliance solely on IgM could lead to an underestimate of exposure to natural infection unless horses were sampled very frequently throughout the study period. In addition to IgM status, information on the concentration of IgG antibodies was necessary to identify all animals in this study with evidence of natural infection because of the timing of sample collection. To get the best potential estimate of seroprevalence, researchers had to first identify a cutoff value for the enzyme-linked imunosorbant assay

(ELISA) which would differentiate IgG antibody production in response to vaccination from that in response to natural infection.

This paper reports the results of an observational field study during the second year of WNV incursion into Saskatchewan and following the widespread use of a new inactivated vaccine. The first objective of this observational study was to assess WNV seroprevalence in Saskatchewan horses to determine geographically the proportion of horses exposed to the virus. The second objective of this study was to assess the effect of geographic region, climate, and other potential risk factors (herd or individual level) on prevalence of WNV infection.

5.2 Materials and methods

5.2.1 Serological response to vaccination – study animals and sampling protocol

Before the results from the seroprevalence study could be evaluated, it was necessary to generate a cutoff value to differentiate antibody production in response to the vaccine from antibody production in response to natural exposure or infection. Through a cooperative agreement, pre- and post-vaccination serum samples that had been obtained from a group of mares enrolled in a WNV vaccine study were made available for WNV serologic testing in our laboratory. The vaccinated and control mares were located on a ranch in Southern Manitoba and the samples had been collected over the winter and spring of 2002-2003 (outside the mosquito season). Testing of these samples using an IgG and IgM antibody capture ELISA provided data on the serologic response to WNV vaccination which were required for subsequent surveillance studies. Ethics approval was acquired by the researchers that performed the sampling.

On November 20, 2002, blood samples were collected from 40 mares (Table1). Twenty of the mares ("December vaccinates") were then vaccinated twice with three weeks between subsequent doses (West Nile-Innovator™, Wyeth Animal Health, Madison, NJ, USA). Blood samples were collected seven days after the first vaccination ("December vaccinates"). The remaining 20 mares (Controls) were given saline injections and blood samples were collected at the same time points as for the vaccinated group. All mares were sampled again three weeks later, on December 10, when a second dose of vaccine or saline was administered. A final blood sample was collected 10 days after the second vaccination.

Table 1: Schedule of dates and procedures followed for horses in the study to evaluate serologic responses to vaccination.

		Groups	
Dates	**Procedure**	**December vaccinates (20 mares)**	**Controls (20 mares)**
Nov 20, 2002	Pre-bleed [a]	20 (16 unexposed, 4 exposed)	20 (17 [b] unexposed, 3 exposed)
	Vaccination	First vaccination	Saline
Nov 27, 2002	Bleed	20	20
Dec 10, 2002	Bleed	20	20
	Vaccination	Second vaccination	Saline
Dec 20, 2002	Bleed	20	20
		March vaccinates (12 mares)	
March 6. 2003	Vaccination	First vaccination	
March 27, 2003	Vaccination	Second vaccination	
April-May 2003	Bleed	12 (10 unexposed, 2 exposed)	

[a] Horses with positive IgG test results in the pre-bleed are considered previously naturally exposed to WNV during the 2002 season. IgG results were initially reported as positive, negative and suspicious by the lab using a formula supplied by the supplier, Bioreliance (Rockville, Maryland).
[b] One horse was classified as suspicious on the first two samples and negative afterward but was labeled as negative/unexposed for the analysis.

In March 2003, 12 of the mares that had been used as controls in November 2002, were also vaccinated according to label recommendations (March vaccinates). Blood samples were not taken on these occasions but were collected 6 weeks after the first vaccination. Also in March 2003, 10 of the previously (December) vaccinated mares received a third vaccination and were bled in late April or early May. Following blood collection, the serum was separated by centrifugation and refrigerated until tested with both IgG and IgM capture ELISAs.

5.2.2 Serum IgG and IgM ELISA

All samples were classified as positive, suspicious or negative for both IgG and IgM based on the optical density (OD) results of the appropriate ELISA. A sample-to-positive (S/P) ratio was then calculated for the IgG test results as follows: S/P ratio = (sample OD-negative control OD)/(positive control OD – negative control OD).

The OD was determined as the difference in values between the duplicate wells on the plate for each test sera and controls. Sera were tested on tissue culture derived WNV antigens in one of the wells. In the other well, sham infected tissue culture antigen preparations (control antigen) were used to ensure the specificity of the response was to WNV. The strong positive control from the top left corner was used in the calculation of the above formula for the first 4 rows of the plate and the strong positive control at the bottom right corner of the plate for the bottom 4 rows.

Both the IgM and IgG assays used in this study were antibody capture ELISAs using reagents supplied by BioReliance Corporation (Rockville, Maryland). No sensitivity or specificity estimates were available for the reagents used in this test.

For the IgM ELISA, 100 microlitres of a 1:250 dilution of monoclonal antibody specific for Equine IgM in Phosphate buffered saline (PBS) was coated onto wells of a polyvinyl chloride (PVC) ELISA plate and incubated overnight at 4°C. The plates were washed 5 times between each step of the process. Control and test sera were then diluted 1:400 in PBS-T, added to duplicate wells of the ELISA plate and incubated for 2 hours at 37°C. Then 100 microlitres of WNV antigen or control antigen preparations were diluted 1:5 in PBS-T, added to one of the duplicate wells and incubated for 2 hours at 37 °C. One hundred microlitres of a 1:100 dilution of WNV specific monoclonal antibody were added to the test wells. The monoclonal antibody was diluted in PBS-T + 5% skim milk. The plates were incubated for 2 hours at 37 °C. Then 100 microlitres of a 1:100 dilution of anti-mouse IgG –Horse Radish Peroxidase (HRPO) conjugate in PBS-T + 5% skim milk were added to each well and incubated for one hour at 37 °C. Finally 100 microlitres of ABTS (2,2'-azino-di(3-ethyl-benzthiazoline-6-sulfonate, KPL cat# 50-66-00) were added to each well and incubated for 30 minutes at 37 °C. The test was read using a spectrophotometer at 410 nm. A positive test was one that had a net O.D. of 0.2 or greater after subtraction of the control (duplicate) well OD and had at least 2 times the control well value. A suspicious test met one of these criteria and a negative test met none of the criteria.

For the IgG ELISA assay used in this study, 100 microlitres of a 1:50 dilution of a WNV specific monoclonal antibody is added onto wells of a polyvinyl chloride (PVC) ELISA plate and incubated overnight at 4 °C. The plates were washed 5 times between each step in the process. The WNV antigen and control antigen were diluted 1:50 in IgG lysis buffer (PBS+0.3% Igepal CA-630 Sigma #I3021) and incubated at room

temperature for 15 minutes. Then 100 microlitres of the diluted antigen and control antigen were added to one of the duplicate wells on the ELISA plate and incubated at 37 °C for 2 hours. The dilutions of the control and test sera were prepared in PBS-T + 4% normal goat serum (all test sera are diluted 1:200). A total of 100 microlitres of the diluted samples and controls were added to the duplicate wells and incubated at 37 °C for 2 hours. Then 100 microlitres of anti-Equine IgG HRPO conjugate diluted in PBS-T + 5% skim milk + 4% normal goat serum was added. One hundred microlitres of ABTS (KPL cat# 50-66-00) were added to each well and incubated for 30 minutes at 37 °C. The test was read using a spectrophotometer at 410 nm. Positive, suspicious and negative test results are as defined above.

5.2.3 Serological response to vaccination – data analysis and establishment of cutoff value

The distribution of S/P ratios were examined graphically for all time points. The distribution of the serological data prior to vaccination in the late fall of 2002 was examined to identify horses with IgG antibody concentrations before vaccination that suggested they had been previously exposed to WNV. The difference in S/P ratios at all sampling points between previously exposed and non-exposed horses were examined using generalized estimating equations assuming a normal distribution, and using an identity link function and an exchangeable covariance structure (PROC GENMOD, SAS for Windows ver.8.2, SAS Institute Inc., Cary, NC). A histogram of the residuals was examined to assess the normality assumption.

Subsequently, all data from previously exposed horses were analyzed separately from the non-exposed horse data. IgG ELISA S/P ratio results were graphed and the differences between December vaccinates and concurrent controls, and December and March vaccinates were analyzed using Mann-Whitney-U test (SPSS version 13.0, SPSS Inc., Chicago, IL, USA).

The analysis of the change in antibody S/P ratios due to vaccination and subsequent cutoff value determination was performed using data from previously non-exposed horses that had been vaccinated two or three times. A cutoff value to differentiate horses that were likely infected from those that were non-infected was calculated as two times the standard deviation added to the highest recorded S/P ratio for all previously unexposed horses (Richardson et al. 1983) first for the horses that had been vaccinated twice and then for the horses that were vaccinated three times. A relatively conservative cutoff value was chosen to optimize specificity.

5.2.4 Seroprevalence study sample selection

Twenty herds of horses were selected as a geographically stratified random sample from across the southern portion of the province of Saskatchewan. Before sample selection, five geographic zones were defined by amalgamating the 10 regional health authority (RHA) boundaries. The 5 geographic zones were defined to ensure that the entire southern portion of the province was adequately represented by the sampling method.

From each of the 5 zones, 4 mixed or large animal veterinary practices were randomly selected from the listings of the provincial veterinary medical association using

a random number generator. Each selected clinic was asked to provide the names of 2 or 3 owners with 10 or more horses on their property. One of the farms from each practice list was randomly selected and contacted. If the owner declined the invitation to participate in the study or did not meet the criteria for enrollment, the next name on the list was contacted until one owner per clinic was enrolled in the study.

A minimum of ten horses from each herd was selected by the owner for sample collection. Approximately 5-10 ml of blood was collected from each horse in August and again in October, corresponding to the beginning of the risk period for clinical disease and the end of the period of risk of infection. If a horse was unavailable for sampling in October, a different horse was sampled. Samples were stored as serum in the refrigerator until testing was completed. Ethics approval for this study was obtained through the University of Saskatchewan Committee on Animal Care and Supply.

5.2.5 Classification of infected versus non-infected horses for seroprevalence study

In this study, serologic presence of antibodies to WNV was used as a proxy for natural exposure to or infection with WNV. A horse was considered naturally exposed or infected if the IgM result was positive or if the IgG S/P ratio of one or both samples (August and October) was above the appropriate cutoff value.

5.2.6 Geographic, mosquito and environmental data

At the time of sample collection, the legal land location (quarter, section, township, range, meridian) where the horses were kept, individual characteristics of all sampled horses, and information on the management of the herds and individual horses,

with emphasis on mosquito control measures, were recorded. Herd locations were mapped using ArcView GIS (version 3.2) and ArcGIS (version 8.0, Environmental Systems Research Institute Inc, Redlands, California). The location was displayed as a point using the centroid-of-the-polygon for the legal land location.

Mosquito data were obtained from the 2003 Saskatchewan Health mosquito trapping program which operated 42 traps across the southern portion of the province. CDC (Center for Disease Control and Prevention) traps were used for collection of live mosquitoes for virus testing and New Jersey Light traps (NJLT) were used for collection of dead mosquitoes for the determination of mosquito species. *Culex* (*C.*) *tarsalis* and *C. restuans* data were provided on a weekly basis from June to September as an average number of mosquitoes trapped per night. Variables of interest were: (a) the maximum weekly average for *C. tarsalis* from the whole season (Number *C. tarsalis*) (b) the maximum weekly average for *C. restuans* from the whole season (Number *C. restuans*) and (c) whether WNV was found in a pooled collection of mosquitoes of each species (positive pool). For each variable of interest, the results of the nearest CDC and NJLT trap were linked to each herd.

Geographic differences across the study area were described in the analysis by considering the ecoregion, which is a subdivision of ecological zones characterized by regional ecological factors such as vegetation, soil, climate, water and fauna (Marshall et al. 1999). Within the study area, the Prairie (Cypress Upland, Mixed Grassland, Moist mixed Grassland and Aspen Parkland ecoregions) and the Boreal Plain (Boreal Transition ecoregion) ecological zones are represented (Figure 1). The Mixed Grassland ecoregion is the driest area of the province with an absence of trees and scarce wetlands or

premanent bodies of water. About half of this area is cultivated while the rest is pasture for grazing. The Moist Mixed Grassland has semi-arid moisture conditions with small aspen groves and numerous undrained depressions or sloughs. Most of the land in this region is used for agricultural purposes. The Aspen Parkland is composed of a mosaic of aspen groves and farmed fescue grasslands, most of which are cultivated. The Boreal Transition is the sourthenmost limit to the boreal forest and the northernmost limit of arable agricultural land in the prairies.

Average daily temperature and total daily precipitation data for all climate stations in Saskatchewan were obtained from Environment Canada. Season average temperature and season total precipitation were calculated for each climate station in the southern half of the province using daily values for June, July and August 2003.

The suitability of environmental conditions for mosquito development was also summarized using cumulative growing degree days (GDD). GDD is the sum of the number of degrees above the base temperature of 16ºC (the threshold temperature of development and activity for *C. tarsalis*) for each day during a given season (McLintock 1948). GDD was calculated using the average daily temperature data minus 16ºC, with all positive values accumulated across the period May to September 2003. Season GDD was then calculated for each climate station in the southern half of the province by adding up monthly totals from May to September 2003. Herds were assigned data from the nearest climate station with complete daily temperature and precipitation information.

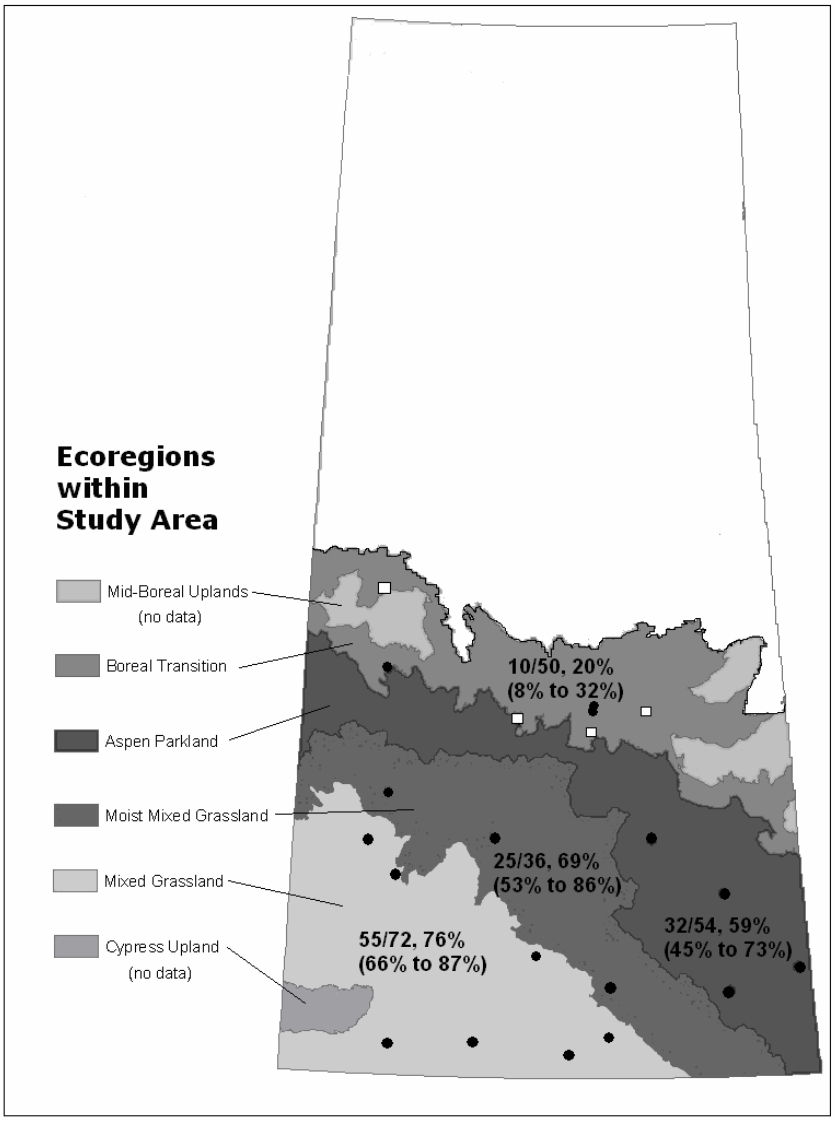

Figure 1: The locations of herds in the study by ecoregion. The vaccinated herds are represented by (●) (n=16 herds) and the non-vaccinated herds are represented by (□) (n= 4 herds). Included on the map is the proportion of infected horses over the total number of horses sampled in each ecoregion expressed as a fraction and a percentage with the 95% CI.

5.2.7 Herd and individual horse characteristics and management

Breed information was captured as class or category of equid (light, draft and draft cross, pony and other equids). Gender was recorded as mare, gelding or stallion. The various colors were divided into three categories, dark (chestnut, sorrel, brown, black or bay); light (gray, white, palamino, buckskin or dun); and multi-colored (roan, paint, pinto and appaloosa). Travel off the farm was defined as any excursion where the distance was greater than 10 miles. Housing was classified as having access to an enclosed barn, a simple three sided shelter with corral or pasture, or having no access to a shelter in open pasture with or without trees. On-farm mosquito control was classified as whether or not any method of mosquito control was used. When mosquito control was reported, the method was recorded as use of fans in a barn and/or sealed at night, smudges (smoky fire used to ward off mosquitoes), general insecticides, or no mosquito control attempted.

5.2.8 Epidemiological analysis of seroprevalence data

The associations between the outcome variable – infection status – and each putative risk factor were examined using mixed models with a binomial distribution and logit link function. The calculations were performed using penalized quasi-likelihood estimates (2nd order PQL) (MLwiN version 2.0, Centre for Multilevel Modelling, Institute of Education, London, UK). The strength of the association between outcome and exposure was reported as an odds ratio (OR) with 95% confidence intervals. Within-herd clustering was accounted for as a random intercept in all models (Dohoo et al. 2003).

A null model (ie. a model which contains only the intercept) was used to calculate the subject-specific and then population-averaged prevalence of WNV exposure and 95% confidence intervals using a formula which incorporates the herd variance and the intercept of the null two-level model (Dohoo et al. 2003). All risk factors were evaluated for association with infection-status in a series of unconditional models. Independent variables for which $P < 0.25$ were considered in developing a final multivariable model.

Manual backwards elimination of variables was used to achieve a final model containing only the statistically significant exposure variables and any nonexposure risk factors that were either significant or acted as important confounders. Nonexposure risk factors were defined as confounders if removing or adding the factor changed the effect estimate for the exposure by more than 10% and the factor was retained in the final model. Biologically reasonable interactions were assessed between significant risk factors (P <0.05) in the final model.

5.3 Results

5.3.1 Serological response to vaccination

None of the horses (exposed or unexposed) had a positive IgM test result just prior to or following the vaccination phase of the study. There was, however, a distinct pattern of IgG concentrations in the samples collected prior to the start of the study (Figures 2A and 2B). Of the 40 horses tested on November 20, 2002, 7 (18%) had a positive IgG response which suggested previous natural exposure to WNV during the summer season (Table 1: 4 vaccinates and 3 controls). The median serum IgG S/P ratio for the animals considered to be previously exposed was 0.86 (min. 0.54, max. 2.08).

(A) (B)

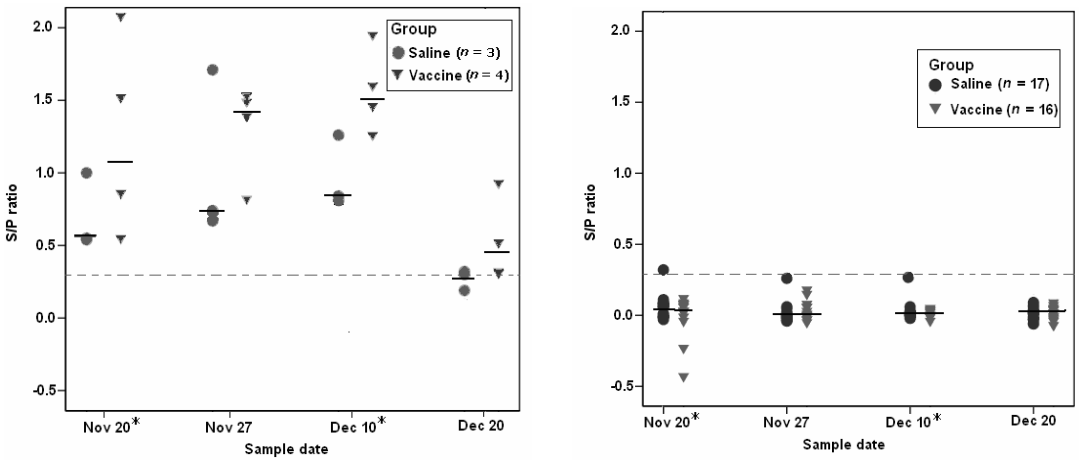

Figure 2: IgG ELISA S/P ratios by vaccination status for previously exposed (A) and unexposed horses (B). The dates of vaccination (*) were November 20 and December 10, 2002. The solid line (—) denotes median for each group. In the saline group, one horse was classified as 'suspicious' on the first two samples (S/P ratios of 0.32 and 0.26) but considered non-exposed for the analysis. Dotted line (- - -) shows the calculated cut-off point.

In a model that included date of sampling, vaccination status and previous exposure status, the mean IgG S/P ratios were significantly different between previously exposed and non-exposed horses (difference=0.96 S/P ratio, 95% CI 0.74, 1.17, $P<0.0001$). However, there were no significant differences in mean IgG S/P ratios between sampling time points ($P=0.08$) or vaccination groups ($P=0.16$).

Of the 16 unexposed December vaccinates, none had a positive IgG response prior to vaccination and none developed an IgG S/P ratio >0.3 after vaccination (maximum S/P ratio, 0.09) on Dec 20, 2002 (Figure 2B). Of the 10 unexposed March vaccinates, none had an IgG S/P ratio >0.3 prior to vaccination and none developed an IgG S/P ratio >0.3 after vaccination (maximum S/P ratio, 0.19) on April-May sampling dates. Of the 10 mares vaccinated a third time in March 2003, 2 were previously exposed and 2 of the 8 unexposed horses had IgG S/P ratio >0.3 at sampling (maximum S/P ratio, 0.44).

In the first step of the analysis, we found no difference in the S/P ratios between December vaccinates and concurrent controls (P= 0.26; Table 2) measured on the last sample date (Dec 20), 10 days after the second dose of either vaccine or placebo. In the second step of the analysis, there was no difference in the S/P ratios measured 10 days after the second December vaccination and from samples collected approximately 1 ½ months after the second March vaccination (P=0.19; Table 2) despite the different sampling times.

There was also no evidence of serologic response to vaccination in previously exposed horses; however, the small number of horses in this category precluded testing this hypothesis (Figure 2A).

Table 2: The difference in antibody response (IgG S/P ratio) between groups of non-exposed horses vaccinated on different dates with both two and three dose protocols and horses that were not vaccinated.

Analysis grouping	Group	N	Sample date used in analysis	Median S/P ratio	*P*-value
1	Group B: Controls	17	Dec 20, 2002	0.03	*P*=0.26
	Group A: December vaccinates	16	Dec 20, 2002	0.02	
2	Group A: December vaccinates	16	Dec 20, 2002	0.02	*P*=0.19
	Group C: March vaccinates	12	April-May 2003	0.04	

A cutoff value used to distinguish infected versus non-infected horses was determined using data on 26 previously unexposed horses that had been vaccinated twice (16 from Nov-Dec 2002 vaccination and 10 from March 2003 vaccination). The minimum and maximum S/P ratio for the 26 horses was -0.07 and 0.19 respectively and the median S/P ratio was 0.02 (interquartile range (IQR) -0.01, 0.05). The standard deviation of the 26 WNV2 samples was 0.058. Thus the cutoff value (for horses vaccinated twice) for the IgG ELISA test at 1:200 dilution was estimated to be 0.305.

All previously exposed horses were above the cutoff value at the first sampling time point which corresponds to 2 - 3 months after the 2002 exposure period (Figure 2A). In addition, the horse that had a suspicious result on the first two samples was also above the cutoff value on the first sample, making it possible that this horse was previously exposed earlier in the 2002 season. Removal of this horse from derivation of the cutoff value for horses vaccinated two times did not change the value itself.

A cutoff value for horses vaccinated three times was calculated with the same formula using the 8 previously unexposed horses sampled in April-May. The minimum and maximum S/P ratios were 0.02 and 0.44 respectively and the median S/P ratio was 0.09 (IQR 0.06, 0.36). The standard deviation of the 8 samples was 0.16. The calculated cutoff value for horses vaccinated three times was estimated as an S/P ratio of 0.76.

5.3.2 Seroprevalence study population

The recruited herds were distributed across the southern half of the province (Figure 1) with 76% of the horses in the Grasslands and Parkland ecoregions. The number of horses sampled per herd ranged from 10 to 15 and the herd sizes ranged from 10 to 250 horses. A total of 212 different horses were sampled in either August or October; 186 horses in the study were sampled in both time periods.

The majority of herd owners (16/20) did not use any type of mosquito control on the farm. Only two of the four herds where mosquito control was employed used more than one type of mosquito control measure. The primary methods of mosquito control in these 4 herds included housing some horses in a barn with fans (1/4), applying insecticide on the farm (1/4), and use of smudges (2/4).

The horses ranged in age from 1 month to 27 years (median, 6; IQR, 3, 9). The only foal in the study was born to a vaccinated mare but had no detectable IgG antibodies. Forty-seven percent of horses were mares (99/212), 40% were geldings (84/212) and 13% were stallions (29/212). Most of those sampled were dark colored (139/212, 66%), light breed (176/212, 83%), pleasure horses ((104/212, 49%).

Eighty-eight percent of the horses had no history of travel during the 2003 summer and fall season. Insecticide was applied to only a few horses (36/212, 17%) and only a few horses were blanketed (8/212, 4%) during the summer. Most horses (155/212, 73%) were kept in a pasture, 21% (45/212) had access to a three-sided shelter, and 6% (12/212) were housed in a barn at night. Most horses (79%, 167/212) were fully vaccinated according to the manufacturer's recommendations. Four horses were vaccinated three times (one vaccine given in fall of 2002) and one horse was vaccinated once in April 2003.

5.3.3 Serologic prevalence

Six horses were IgM positive on the first sample but none of the 212 horses were IgM positive on the second sample. Of the 6 IgM positive horses, 4 were <0.3 IgG S/P ratio on the first sampling and all were > 0.3 on the second sample. None of these horses showed signs consistent with WNV. The IgG S/P ratio was > 0.3 for either one or both samples from 58.4% of horses (124/212); 29%(60/204) on first sampling, 56% (108/193) on the second sampling, 24% (44/185) on both sampling, 5% (9/185) on first but not second sampling and 31% (57/185) on second but not first sampling.

The proportion of sampled horses in each herd classified as infected based on serum antibody concentrations ranged from 0% to 100%. The estimated population-averaged prevalence, adjusted for herd-level clustering, was 55.7% (95%CI, 44.9% to 65.8%).

The risk of a horse being classified as infected was highest in the Mixed Grasslands ecoregion, followed by the Moist Mixed Grasslands ecoregion, the Aspen

Parkland ecoregion and lowest in the Boreal Transition ecoregion (Figure 1). Ten of the 44 horses (23%) that were not vaccinated, the horse vaccinated once and 110 of the 163 horses (67%) that were vaccinated twice had at least one S/P ratios >0.305 and were classified as being infected when information from both sampling periods was considered. Three of the four horses that were vaccinated three times were classified as not infected since the S/P ratios for both were less than the elevated cutoff value of 0.760.

5.3.4 Risk factor analysis

In the initial herd-adjusted unconditional analysis, vaccination, ecoregion, season average temperature, season total precipitation and season cumulative GDD were associated with risk of infection (Table 3). The odds of infection were 1.7 times higher for horses in the Mixed Grasslands than those from the Moist Mixed Grasslands and 2.1 times greater for the Moist Mixed Grasslands than for the Aspen Parkland, which in turn had a 9.6 higher chance of infection than the reference ecozone Boreal Transition.

The final model included only two explanatory variables: herd mosquito control and ecoregion; all other variables were not significant after accounting for ecoregion as a fixed effect and herd as a random effect (Table 4).

Table 3: Unconditional analysis of risk factors for WNV infection[a] in a mixed model accounting for clustering by herd (N=212 horses, n=20 herds).

Variable	Categories	Odds Ratio	95% CI	*P*
Vaccination	No	reference category		
	Yes	4.3	1.2, 16.0	0.03
Herd Mosquito Control	Yes	reference category		
	No	7.3	0.8, 69.5	0.08
Individual Shelter	None	reference category		
	Simple	1.1	0.3, 4.2	0.8
	Barn	0.2	0.03, 1.3	0.09
Ecoregion	Boreal Transition	reference category		
	Mixed Grassland	34.3	4.2, 277	<0.001
	Moist Mixed Grassland	20.1	1.7, 243	0.02
	Aspen Parkland	9.6	1.2, 79.2	0.04
Temperature (°C)		3.0	1.3, 6.9	0.01
Precipitation (mm)		0.98	0.96, 0.996	0.01
GDD (degree days)		1.01	1.002, 1.02	0.006

[a] Infection status determined by IgG antibody in the serum with an S/P ratio > 0.3 suggesting natural exposure to the virus at some time within the current season.

Table 4: Multivariable model (adjusting for clustering by herd) showing the association between location and risk of infection[a] considering the presence or absence of herd mosquito control (N=212 horses, n=20 herds).

Variable	Categories	Odds Ratio	95% CI	*P*
Herd mosquito control	Yes	Ref		
	No	8.2	1.2, 57.8	0.03
Ecoregion	Mixed Grassland	34.6	4.9, 245.9	0.004
	Moist Mixed Grassland	14.0	1.5, 135.5	0.02
	Aspen Parkland	16.1	2.1, 124.4	0.008
	Boreal Transition	Ref		
Herd Variance (SE)		1.648 (0.788)		

[a] Infection status determined by IgG antibody in the serum with an S/P ratio > 0.3 suggesting natural exposure to the virus at some time within the current season.

The odds of being infected were 8.2 times higher for horses from herds where no method of mosquito control was used compared to those where any type of mosquito control method was used. Using the Boreal Transition as the reference region, the odds of being infected were greatest for horses residing in the Mixed Grassland and odds were similar for horses in both the Moist Mixed Grassland and the Aspen Parkland. The unexplained variance in infection status of horses within herds was 1.65 (S.E., 0.79). The intra-herd correlation coefficient, or proportion of total variance in the final model explained by between herd variation, was 0.33 (33%).

Only the primary type of herd mosquito control per farm was assessed in a mixed model with ecoregion. The odds of being infected were 57 times higher for herds where no mosquito control was used than for herds where horses were housed in barns with fans, and 8.5 higher than for herds where owners used smudges for mosquito control. The odds of being infected did not differ between herds that used insecticides compared to herds where no mosquito control was used (P=0.45).

5.4 Discussion

The prevalence of horses with antibody concentrations suggesting infection from this study was greater than any reported to date (Trock et al. 2001, Autorino et al. 2002, Durand et al. 2002, Blitvich et al. 2003, Estrada-Franco et al. 2003, Lorono-Pino et al 2003). However, the study area of southern Saskatchewan covered approximately 390,000 km^2 and there were substantial differences in seroprevalence identified across the study area. To explore the variation in risk of infection for horses with WNV, geographic differences that could be associated with changes in climate and vegetation

types were investigated by comparing seroprevalence among ecoregions. The proportion of infected horses differed greatly by area, from 20% in the northern Boreal Transition to 76% in the southern Mixed Grassland.

In the Boreal Transition ecoregion, both climate and mosquito habitat were less likely to facilitate WNV transmission than in the other ecoregions examined (curry 2004). Until the mosquito trapping was conducted in the 2003 season, historical trapping data suggested that *C. tarsalis* was not commonly found in the northern portion of the Aspen Parkland and the Boreal Transition. Therefore, the risk of exposure to WNV prior to 2003 was considered extremely low in this area. North of the study area (white area in Figure 1), there were no clinical cases reported and the risk of infection was negligible; therefore neither mosquito trapping nor blood sampling of horses was conducted in this area.

Ecoregion was a significant predictor of infection status. The odds of having been infected were more than 30 times greater in the Mixed Grassland than in the Boreal Transition. This finding is consistent with the fact that the Mixed Grassland ecoregion also had the most generations of the *C. tarsalis* mosquito in 2003 and the best climate and habitat for *C. tarsalis* mosquito growth (Curry 2004). There appeared to be a gradient of infection which progressed from high risk in the south-western most portion of the province to lower risk in the central region of the province, the northern most extent of documented WNV infection in any species (bird or human) in 2003 (Corrigan et al. 2006).

Vaccination was a significant predictor of infection status in an unconditional model adjusted only for clustering by herd. The odds of antibody concentrations

sufficiently high to suggest natural infection were 4 times greater for vaccinated horses than for non-vaccinated horses. The reason for this vaccine-related difference in antibody status was that the herds that were not vaccinated were most likely to be located in the northern area of the Aspen Parkland and the Boreal Transition ecoregions. In the spring of 2003, these areas were considered at low risk for infection due to climate and historical mosquito data. With this knowledge, horse owners in this region were less likely to vaccinate. When ecoregion was controlled for in the model, vaccine status was no longer significantly associated with IgG antibody concentrations and there was no evidence that vaccination prevented infection. This study did not examine whether the vaccine protected against clinical disease or whether the absence of detectable antibodies was related to efficacy of the vaccine or inference of immunity to WNV.

Mosquito trapping measures were not significantly associated with risk of infection but this could be a result of biases such as trap locations and trap malfunctions. Temperature, precipitation and GDD were significant predictors of infection status in the unconditional models, but were strongly correlated with ecoregion in the final model. As season average temperature increased, so did the odds of horses becoming infected. As season total precipitation increased, the odds of infection decreased. Temperature and precipitation differed for each ecoregion and tended to follow a south to north gradient. Lower rainfall and higher temperatures were generally in the southern areas of the province that coincided with the area where the greatest mosquito development occurred.

GDD is a measure of the suitability of meteorological conditions for mosquito growth. Increased GDD is associated with an increase in the number of generations of mosquitoes within a given time period and a resultant increase in the potential risk of

WNV infection. In the present study, as GDD increased the odds of infection increased. GDD was highly correlated with ecoregion and also decreased from south to north following a similar pattern as the ecoregions map. Because accounting for ecoregion explained much of the variability in meteorological conditions, the variables representing the meteorological data were eliminated from the final model.

Only use of herd-mosquito control and ecoregion were significantly associated with infection status when combined in the final model. Ecoregion was modeled as a fixed effect to estimate differences in infection status across geographic regions. The odds ratios show decreasing risk of infection from southwest to northeast. Housing in a barn with fans or a barn sealed at night to prevent mosquitoes from entering provided some protection against infection with WNV. Due to the small number of farms that used mosquito control methods, the absence of a significant protective effect associated with the use of smudges (2 herds, 20 horses total) does not necessarily exclude this as a useful prevention measure. There was also no evidence that insecticide use provided protection against infection. The effectiveness of smudges and insecticides are likely influenced by the timing and consistency of application.

This study used ELISAs for both IgM and IgG to evaluate serologic status and classify horses as infected or not and then to assess potential prevention tools. Few horses would have been classified as infected on the basis of IgM alone. As well, all IgM positive samples occurred during August. This antibody persists for a short time period and thus would not be helpful in serologic surveillance after the infection season (Ostlund et al. 2001). The use of a single IgM test at any point in time during the mosquito season will, therefore, underestimate the number of infected horses. The IgG ELISA was useful

in this study as this was the first season of vaccination for the majority of horses. Unknown vaccination status, multiple years of vaccination with the same or different vaccines, and natural infection with WNV from previous years will potentially complicate the use of IgG antibody tests in future serologic studies. Thus, future serologic surveillance will likely need to focus on the use of serial IgM testing during the period of risk of infection.

Because of Saskatchewan's large size and varying climate, regional differences were expected in the distribution of infection. The known distribution of *C. tarsalis*, the mosquito species capable of transmitting the virus to horses, is limited to the southern half of the province. Within this area, a gradient of infection risk was present and followed ecoregions, or temperature, precipitation and GDD gradients from the south to north end of the risk zone. The use of mosquito control measures at the farm level reduced the odds of infection within each ecoregion. Effective use of all the various mosquito control measures and knowledge of areas of risk should aid in the protection of horses against WNV infection.

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6. A case-control study of factors associated with development of clinical disease due to West Nile virus, Saskatchewan, 2003.

6.1 Introduction

West Nile Virus (WNV) was first diagnosed in horses in North America in the late summer of 1999 (Ostlund et al. 2001). Clinical signs in horses ranged from mild to severe with case fatality rates of 25-40% (Ostlund et al. 2001, Trock et al. 2001). Not all infected horses develop clinical signs. One review article suggested that approximately 10% of horses infected with WNV exhibit neurological symptoms, which have been corroborated by experimental equine infections (Castillo-Olivares et al. 2004, Bunning et al. 2002).

Researchers in the United States have examined the extent of sub-clinical WNV infection in horses. In a 1999 study conducted in New York state, serum samples were collected from 69 clinically normal horses kept on farms where at least one clinically diseased horse had been confirmed (Trock et al. 2001). Twenty of the 69 horses (29%) were antibody positive for the virus. In a 2000 study on Staten Island, 8% (7/91) of clinically normal horses from seven stables were seropositive; in one of the stables, 5/6 horses were seropositive (Trock et al. 2001). New York serosurveys conducted in 1999 and 2000 revealed higher seroconversion of clinically normal stable mates (23% and 5%

respectively) compared with seroconversion on farms without clinical disease (8% and 1% respectively) (Kramer et al. 2001).

Controlled challenge trials have demonstrated protection against viremia in horses vaccinated with West Nile Innovator (Wyeth Animal Health, Guelph, Ontario, Canada) (Ng et al. 2003). In other studies, all of the horses, which were vaccinated with sufficient time to develop immunity prior to showing clinical signs, survived (Salazar et al. 2003) and vaccination reduced the odds of death from clinical signs (Schuler et al. 2003). Another study reported that even if the vaccine was not administered in sufficient time prior to the infection season, it could reduce the risk of death by up to 44% (Ward et al. 2006). None of these studies was designed to assess whether vaccination would prevent development of clinical signs. In a cohort study that followed vaccinated and unvaccinated horses, vaccination seemed effective in preventing clinical disease (Gardner et al. 2007).

Previous studies have not yet identified other individual risk factors that were significantly associated with development of clinical disease. For example, the ages of case horses and clinically normal seropositive herd-mates were not significantly different in a study from New York in 1999 (Ostlund et al. 2001).

Based on the spread of WNV across the continent during the previous year, horses in Saskatchewan were expected to be at high risk of exposure to the virus in the summer of 2003. Most horses in the province of Saskatchewan are housed outside with little protection against exposure to mosquitoes. As a result, vaccination, in addition to other mosquito abatement methods was recommended for preventing clinical disease. A case-control study was undertaken in the summer of 2003 in anticipation of the outbreak to

identify risk factors for development of clinical disease in horses, which could potentially be used to make recommendations for control.

6.2 Material and methods

6.2.1 Study protocol

All laboratory-confirmed clinical cases of WNV in Saskatchewan (SK) were recorded in 2003. A confirmed 'clinical case' was any equid with clinical signs consistent with WNV and a positive Immunoglobulin (Ig)M enzyme-linked immunosorbent assay (ELISA) test from Prairie Diagnostic Services (PDS) in Saskatoon, SK. From the affected properties identified, a subset of case farms was randomly selected along with an equal number of control farms. Case farms were those having at least one clinical case of WNV on the premises in 2003, a minimum of five horses on the property and an owner willing to participate in our investigation. A control farm was the nearest property to a case farm with at least five horses and an owner willing to participate in the study, and where there were no confirmed or suspected clinical cases of WNV disease.

Study participants completed a form in the presence of the investigator that provided the legal land location, individual characteristics of all sampled horses, herd and individual management methods, and information about mosquito control measures used on farm. Approximately 10 ml of blood was collected from five to ten horses from each farm. All samples from both case and control farms were collected within a three-month period of the onset of disease on the case farm. The disease status of each farm was monitored until the end of the mosquito season to ensure control farms did not become case farms.

6.2.2 Sample to positive ratio and IgM and IgG ELISA

All samples were classified as positive, suspicious or negative for both IgG and IgM based on the optical density (OD) results of the appropriate ELISA. A sample-to-positive (S/P) ratio was then calculated for the IgG test results as follows: S/P ratio=(sample OD-negative control OD)/(positive control OD – negative control OD).

The OD was determined as the difference in values between the duplicate wells on the plate for each test and control sera. Sera were tested on tissue culture derived WNV antigens in one of the wells. In the other well, sham infected tissue culture antigen preparations (control antigen) were used to ensure the specificity of the response was to WNV. The positive control from the top left corner was used in the calculation of the above formula for the first four rows of the plate and the positive control at the bottom right corner of the plate for the bottom four rows.

Both the IgM and IgG assays used in this study were antibody capture ELISAs using reagents supplied by BioReliance Corporation (Rockville, Maryland). No sensitivity or specificity estimates were available for the reagents used in this test.

For the IgM ELISA, 100 microlitres of a 1:250 dilution of monoclonal antibody specific for Equine IgM in Phosphate buffered saline (PBS) was coated onto wells of a polyvinyl chloride (PVC) ELISA plate and incubated overnight at 4°C. The plates were washed five times between each step of the process. Control and test sera were then diluted in PBS-T, added to duplicate wells of the ELISA plate and incubated for 2 hours at 37 °C. Then 100 microlitres of WNV antigen or control antigen preparations were diluted 1:5 in PBS-T, added to one of the duplicate wells and incubated for 2 hours at 37 °C. One hundred microlitres of a 1:100 dilution of WNV specific monoclonal antibody

were added to the test wells. The monoclonal antibody was diluted in PBS-T + 5% skim milk. The plates were incubated for 2 hours at 37 °C. Then 100 microlitres of a 1:100 dilution of anti-mouse IgG –Horse Radish Peroxidase (HRPO) conjugate in PBS-T + 5% skim milk were added to each well and incubated for one hour at 37 °C. Finally 100 microlitres of ABTS (2,2'-azino-di(3-ethyl-benzthiazoline-6-sulfonate, KPL cat# 50-66-00) were added to each well and incubated for 30 minutes at 37 °C. The test was read using a spectrophotometer at 410 nm. A positive test was one that had a net O.D. of 0.2 or greater after subtraction of the control (duplicate) well O.D. and had at least 2 times the control well value. A suspicious test met one of these criteria and a negative test met none of the criteria.

For the IgG ELISA assay used in this study, 100 microlitres of a 1:50 dilution of a WNV specific monoclonal antibody was added onto wells of a polyvinyl chloride (PVC) ELISA plate and incubated overnight at 4°C. The plates were washed five times between each step in the process. The WNV antigen and control antigen were diluted 1:50 in IgG lysis buffer (PBS+0.3% Igepal CA-630 Sigma #I3021) and incubated at room temperature for 15 minutes. Then 100 microlitres of the diluted antigen and control antigen were added to one of the duplicate wells on the ELISA plate and incubated at 37 °C for 2 hours. The dilutions of the control and test sera were prepared in PBS-T + 4% normal goat serum (all test sera are diluted 1:200). A total of 100 microlitres of the diluted samples and controls were added to the duplicate wells and incubated at 37 °C for 2 hours. Then 100 microlitres of anti-Equine IgG HRPO conjugate diluted in PBS-T + 5% skim milk + 4% normal goat serum were added. One hundred microlitres of ABTS (KPL cat# 50-66-00) were added to each well and incubated for 30 minutes at 37 °C. The

test was read using a spectrophotometer at 410 nm. Positive, suspicious and negative test results are as defined above.

6.2.3 Determination of infection status

A positive IgM sample was indicative of recent WNV infection. Infection status for vaccinated horses was based on a positive IgM result or an IgG result above the appropriate cutoff value. An IgG cutoff value for infection status (yes or no) was determined to be equal to or greater than 0.305 S/P if vaccinated twice and equal to or greater than 0.760 S/P if vaccinated three times (Epp et al. 2007). Infection status for non-vaccinated horses was based on any positive (or suspicious) IgM or IgG ELISA result.

6.2.4 Horse data

Horses were classified by sex, color, age and breed type but not by specific breed. Breed type was divided into three groups: light horses, draft horses, and crosses and ponies which included miniatures and mules. Sex was reported in three categories: mare, gelding and stallion. Color was divided into three categories: darks (chestnut, sorrel, brown, black or bay), light (gray, white, palamino, buckskin or dun), and multi-colored (roan, paint, pinto and appaloosa). Size was recorded as the circumference of the girth and was divided into two categories: small girth ($\leq$ 69 inches) and larger girth ($>$ 69 inches). Age was divided into three categories: young (5 years or younger), middle-aged adults (6-18 years) and older adults (older than 18 years). Housing was also classified

into three groups: housed in an enclosed barn, access to a simple three sided shelter with corral or pasture, or no access to a shelter in open pasture with or without trees.

6.2.5 Farm level data

Herd locations were mapped using ArcView GIS (version 3.2) and ArcGIS (version 8.0, Environmental Systems Research Institute Inc, Redlands, California). The location was displayed as a point using the centroid-of-the-polygon for the legal land location.

Primary shelter of the majority of horses on the premise was recorded using the above categories. On-farm mosquito control was classified as whether or not any method of mosquito control was used. When mosquito control was reported, the method was recorded as use of fans in a barn and/or sealed at night, smudges (smoky fire used to ward off mosquitoes), general insecticides, or no mosquito control attempted. The proportion of sampled horses vaccinated on each farm was also recorded as: none (0%), part (1-99%) and all (100%).

6.2.6 Statistical analysis

Each potential risk factor was assessed to determine whether it was associated with clinical disease status of individual horses using data from all horses in case and control farms in a logistic regression model (SPSS ver 14.0, Chicago, IL, USA). In the second step, this process was repeated using only data from the horses classified as infected on both case and control farms.

To assess whether the infection pressure was similar on both case and control farms the proportion of serologically positive horses was analyzed with a logistic regression model (MlwiN ver 2.02, Centre for Multilevel Modelling, Institute of Education, London, UK). The average proportion across all farms was calculated from the estimate and standard error in a null model and the comparison of the case and control farm proportions was assessed in a model with farm status (case versus control) for a) all horses on each farm including clinical horses and b) only asymptomatic horses on each farm.

Vaccine efficacy or the attributable fraction exposed was calculated using EpiTable (EpiInfo version 6, CDC, Division of Surveillance and Epidemiology, Atlanta, Georgia, USA).

In the final step, each potential risk factor was assessed to determine whether it was associated with the farm disease status in a logistic regression model (SPSS ver 14.0, Chicago, IL, USA).

All independent variables with $P > 0.25$ in the univariate assessments were considered in developing the final multivariate models. Manual backwards elimination of variables was used to achieve a final model containing only the statistically significant exposure variables and any nonexposure risk factors that were either significant or acted as important confounders. Nonexposure risk factors were defined as confounders if removing or adding the factor changed the effect estimate for the exposure by more than 10% and the factor was retained in the final model. Biologically reasonable interactions were assessed between significant risk factors ($P < 0.05$) in the final model.

6.3 Results

Of 123 farms with a clinical case(s) in 2003, 63 (51%) farms had 5 or more horses on the property. However, only 44 case farms were willing to participate in the study. Of these, 23 (53%) were randomly selected and matched by location to 23 control farms. A total of 300 horses, including 25 clinical cases were sampled from these 46 farms. The median number of samples collected on each farm was 6 (range, 5 to 10). Two farms had two clinical cases each at the time of sample collection; one additional farm had a second clinical case after sampling was completed which was not included in analysis. Sampling was initiated on August 23 and concluded on November 1, 2003. The median proportion of each herd that was tested was 50% (range, 2% to 100%).

All samples from both case and control herds were collected within three months of the onset of clinical signs on the case farm. There were 7 positive and 3 suspicious non-clinical horses by IgM ELISA (n=275). All 25 clinical case horses were IgM positve. All positive IgM samples were collected before September 30th, 2003. Samples from non-clinical horses were also analyzed with IgG ELISA resulting in 172/275 (63%) positive, 12/275 (4%) suspicious and 91/275 (33%) negative results as assessed by the test protocol. For non-clinical horses, 7 were positive on IgM only, 171 were positive on IgG only, and no horses were positive on both IgG and IgM. Based on the appropriate cutoff value for the IgG ELISA, 78/133 (59%) vaccinated non-clinical horses were above the cutoff value and were considered exposed to WNV infection during the 2003 season.

Of the 300 horses sampled, 193 (64%) were classified as WNV infected; 70% (108/154) on case farms and 58% (85/146) on control farms (Table 1). Sixty-one percent

(168/275) of horses without clinical signs were infected with WNV; 64% (83/129) on case farms and 58% (85/146) on control farms. There was no significant difference in the proportion of asymptomatic infection between case and control farms ($P = 0.22$).

The first clinical disease case was reported in August 2003 in 65% (15/23) of case farms. Twenty-five of the 193 infected horses (13%) exhibited neurologic symptoms. Most individual horse characteristics were similar on case and control farms. The age distribution of both types of farms were skewed toward the younger age categories (Table 1). There were three foals (2 -3 ½ months old) with clinical disease in this study. Two of the foals were from mares that were vaccinated for WNV, of which one survived. The vaccination status of the horses differed between case and control farms as well as between individual clinical diseased and non-diseased horses (Table 1 and 2).

Table 1: Description of animal and management characteristics in both case and control farm horses; individual data.

WNV Infection status		**Case farm horses**				**Control farm horses**		
Variables		**Clinical infected**	**Asymp.[a] infected**	**Not infected**	**Total**	**Asymp.[a] infected**	**Not infected**	**Total**
		25	83	46	**154**	85	61	**146**
Class of equid	Light	18	68	34	**120**	69	44	**113**
	Pony /other	3	8	8	**19**	4	8	**12**
	Draft/draft cross	4	7	4	**15**	12	9	**21**
Coat color	Lights	6	14	5	**25**	10	10	**20**
	Darks	15	52	36	**103**	63	40	**103**
	Multis	4	17	5	**26**	13	10	**23**
Sex	Mare	9	39	19	**67**	43	33	**76**
	Gelding	13	41	24	**78**	41	26	**67**
	Stallion	3	3	3	**9**	1	2	**3**
Primary individual housing[b]	Barn	0	0	0	**0**	0	8	**8**
	Simple shelter	4	23	13	**40**	31	23	**54**
	No shelter	21	59	33	**113**	53	30	**83**
Age category	0-5 years	9	39	22	**70**	31	28	**59**
	6-18	13	41	22	**76**	46	27	**73**
	19+ years	3	3	2	**8**	8	6	**14**
Girth size category[b]	Small girth	0	1	9	**10**	0	6	**6**
	Medium girth	1	13	6	**20**	17	12	**29**
	Large girth	1	3	1	**5**	3	0	**3**
Vaccination	No	24	63	31	**118**	27	21	**48**
	Yes[c]	1	20	15	**36**	58	40	**98**
Travel	No	25	75	46	**146**	77	55	**132**
	Yes	0	8	0	**8**	8	6	**14**
Use	Pleasure	19	57	35	**111**	57	34	**91**
	Breeding	3	17	8	**28**	21	16	**37**
	Competition	1	3	1	**5**	7	11	**18**
	Farm/ranch work	2	6	2	**10**	11	0	**0**
Mosquito control (Individual)[b]	No	22	75	43	**140**	76	42	**118**
	Yes	3	7	3	**13**	8	19	**27**
Herd Size[b]	5-10 horses	12	43	20	**75**	24	20	**44**
	11-25 horses	9	30	18	**57**	39	22	**61**
	26-50 horses	2	3	6	**11**	6	6	**12**
	50+ horses	2	6	2	**10**	15	13	**28**

[a]Asymp. = asymptomatic,
[b]category has missing values
[c]vaccination status means fully vaccinated with two or more doses of vaccine by company protocol.

Table 2: Description of disease onset and herd management factors in both case and control farms; farm level data.

Variables		Case farms (N=23)	Control farms (N=23)
Number of clinical WNV equids	At least one	21	0
	Two or more	2	0
Date of onset of first equid with clinical signs	July	1	0
	August	15	0
	September	7	0
Farm mosquito control measures	At least one method	4	5
	Nothing	19	18
Primary herd shelter	None	18	14
	Simple	5	8
	Barn	0	1
Herd size	5-10 horses	12	7
	11-25 horses	8	10
	26-50 horses	2	2
	50+ horses	1	4
Farm vaccination category	No vacc	15	4
	Partly vacc	7	6
	All vacc	1	13

Based on the results of the initial unconditional analysis of individual horse disease status, vaccination and primary individual shelter were considered for inclusion in the final model (Table 3). In the final multivariable analysis, vaccination status was the only factor associated with disease status (diseased versus not diseased) (Table 4). The estimate of vaccine efficacy for the prevention of the development of clinical disease was 96% (95% CI 67% to 99%, $P<0.0001$).

Table 3: Unconditional analysis: for a) individual disease status (all horses showing clinical disease or not showing clinical disease), b) individual (infected horses only) disease status and c) farm status (case farm or control farm).

Model	Variable	Category	N	Disease Status		
				OR	95% CI	*P*
Individual	Vaccination	No	300	22.5	3.0, 168.5	0.002
		Yes		ref		
	Individual shelter[a]	None	298	2.9	1.0, 8.8	0.05
		Some shelter		ref		
Individual (infected Horses only)	Vaccination	No	193	20.8	2.8, 157.3	0.003
		Yes		ref		
	Individual shelter[a]	None	193	2.5	0.8, 7.7	0.10
		Some shelter		ref		
Model	**Variable**	**Category**	**N**	**Farm Status**		
				OR	**95% CI**	***P***
Farm	Herd Shelter[a]	None	46	2.3	0.6, 8.5	0.2
		Some shelter		ref		
	Farm Vaccination	No vacc	46	48.8	4.8, 493.0	0.004
		Part vacc		15.2	1.5, 152.5	
		All vacc		ref		

[a] Categories barn and simple shelter were combined to continue analysis (too few entries for barn, would not converge).

For the subset analysis based on the initial unconditional analysis of individual disease status for only the infected horses, vaccination and primary individual shelter were again considered for inclusion in the final model (Table 3). In the final multivariable analysis considering only the infected horses, vaccination was the only factor associated with disease status (diseased versus not diseased) (Table 4).

Table 4: Multivariable analysis: for a) individual disease status (all horses showing clinical disease or not showing clinical disease), b) individual (infected horses only) disease status and c) farm status (case farm or control farm).

Model	N	Variable	Category	Disease status		
				OR	**95% CI**	***P***
Individual	300	Vaccination	No vaccine	22.5	3.0, 168.5	0.002
			Yes vaccine	Ref		
Individual (infected horses only)	193	Vaccination	No vaccine	20.8	2.8, 157.3	0.003
			Yes vaccine	Ref		
Model	**N**	**Variable**	**Category**	**Farm status**		
				OR	**95% CI**	***P***
Farm	46	Farm Vaccination	No vacc	48.8	4.8, 493.0	0.004
			Part vacc	15.2	1.5, 152.5	
			All vacc	Ref		

At the farm level, there were some potential differences in the use of shelter, vaccination, and herd size between case and control herds (Table 2). Based on the initial unconditional analysis of farm status, farm vaccination status and primary herd shelter were considered for inclusion in the final model (Table 3). In the final multivariable analysis, farm vaccination status was associated with the farm status (case versus control farm) (Table 4). All three models were also assessed with random effects models (accounting for herd and location by ecological region), however, the variance of the random effects were zero in the null models, suggesting herd location was not an important confounder in this analysis, and thus was not pursued further.

6.4 Discussion

Although vaccination has been reported to have a protective effect with respect to disease outcome (recovery or death) when horses show neurological symptoms (Salazar et al. 2004, Schuler et al. 2004, Ward et al. 2006), this study provided some of the first field evidence that vaccination can protect against the development of clinical disease. A 96% vaccine efficacy from a field study in the face of a natural outbreak is remarkable. Using data from other concurrent studies during this outbreak, the vaccine efficacy remained at 97% (95% CI 93.5%, 98.4%) (Epp et al. 2005).

Vaccination had a substantial effect upon disease at the level of both the farm and the individual horse. Non-vaccinated horses were 23 times more likely to exhibit clinical signs of disease than vaccinated horses. This relationship was consistent even when only those horses exposed to and infected with WNV were used in the analysis. Farms were more likely to have had a clinical case when none of or only a portion of the horses in the herd were vaccinated. This study demonstrates that vaccination is highly efficacious and an important preventive measure against the development of clinical illness.

The proportion of asymptomatic infection in non-clinical stable mates on case farms was higher than studies in the eastern USA, but similar to the Californian cohort study (Trock et al. 2001, Kramer et al. 2001, Gardner et al. 2007). A key difference was that horses in the cohort study were housed on dry lots or irrigated pastures while the horses in the eastern states were housed in stables. In this study, the proportion of asymptomatic infection was similar in both the case and control farms, while the proportion of vaccinates was considerably higher on control farms. With similar

asymptomatic infection proportions, the difference in vaccination is a key risk factor that determines the occurence of clinical disease.

Other vaccines have entered the market since this study was conducted; Recombitek (Merial Canada Inc., Baie d'Urfe, Quebec, Canada), a DNA vaccine (Fort Dodge Animal Health, Fort Dodge, Iowa, USA) and a chimera vaccine, (PreveNile, Intervet, Inc. Millsboro, Deleware, USA). Of these, only the recombinant vaccine has been shown efficacious in a field study (Gardner et al. 2007). All vaccines claim to prevent viremia, a precursor for clinical disease development (Long et al. 2005, Chiang et al. 2005, Siger et al.2006). Due to the efficacy and availability of the first vaccine on the market, the subsequent introduction of the other vaccines into the market will increase consumer options and cost competitiveness.

Cost effectiveness of vaccination in the prevention of clinical disease could not be assessed in either the cohort study or our study (Gardner et al. 2007). However, it was inferred in the cohort study to be financially prudent based on a crude comparison of the average cost of each clinical case and the cost of vaccination. In this study, only one of the clinical horses was vaccinated for WNV, supporting the idea that even when administered correctly, some horses may respond poorly to vaccination. Further research into the vaccines may elicit reasons for these different responses.

WNV prevention strategies are presently based on vaccination, housing recommendations, and mosquito control measures. Methods such as on-farm mosquito control or housing in barns have been shown to be effective at reducing the risk of infection (USDA 2001). Failure of our study to show an effect of mosquito control upon the odds of development of clinical disease may have been due to limited adoption of

these strategies in the study population. However, there were no clinical horses housed in barns within this study. The mechanisms involved in which horses develop disease once infection occurs were also not evaluated in this study. This study has shown that vaccination provides remarkable protection against the development of disease, even in the absence of concerted efforts to reduce the risk of exposure to the virus.

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7. Equine West Nile Virus surveillance as a component of an integrated surveillance program over a four year period in Saskatchewan: 2002 - 2005

7.1 Introduction

When West Nile Virus (WNV) first emerged in North America in 1999, its clinical presentation in humans was mistaken for a different arbovirus in the same family, St. Louis Encephalitis virus (Drexler 2002). Public health officials later realized that earlier integration of information from birds and mammals could have unlocked the mystery sooner (Drexler 2002). With the subsequent spread of the virus, WNV surveillance became a central focus of both local and national governing bodies.

This paper documents equine surveillance in the province of Saskatchewan in four years (2002-2005) beginning with the introduction of the virus. It also explores the usefulness of equine surveillance as a component of the overall program for monitoring West Nile Virus in the province of Saskatchewan (SK).

7.2 Surveillance in Saskatchewan: 2002-2005

In 2002, serologically WNV positive horses were reported to Saskatchewan Agriculture Food and Rural Revitalization (SAFRR) by three different sources: Prairie

Diagnostic Services (PDS) in Saskatoon, Manitoba Provincial Laboratory in Winnipeg, and Canadian Food Inspection Agency (CFIA). No definition of a "clinical case" was provided by SAFRR and no information on the clinical signs of the tested horses was recorded. Reporting of cases was voluntary on the part of the horse owners, veterinarians, and laboratories.

In 2003, information was collected systematically by Western College of Veterinary Medicine (WCVM) researchers on all horses with neurological symptoms for which samples were submitted to the regional laboratory. All large and mixed animal practices in Saskatchewan were asked: 1) to send all samples for WNV detection into PDS in Saskatoon, SK for Immunoglobulin (Ig)M enzyme-linked immunosorbant assay (ELISA) and 2) to allow PDS to notify the WCVM research team of any positive result from samples submitted by the clinic. A "confirmed clinical case" was defined as a horse with one or more of the classic signs of WNV; such as ataxia, recumbency, paralysis or paresis of limb(s), or death and a positive IgM ELISA test. A "probable clinical case" did not have any confirmatory testing but had clinical signs consistent with WNV. Post-season surveillance of a random sample of clinics in the fall of 2003 was used to estimate the number of probable WNV cases per region. Information was shared with Saskatchewan Health's integrated surveillance initiative.

In 2004, twenty-two private veterinary clinics located within each of the ten Regional Health Authorities (RHA) in the southern portion of the province reported weekly on the number of laboratory-confirmed clinical WNV cases and probable clinical WNV cases each had seen or heard of in their area. Information collected included date of onset of clinical signs and location.

In 2005, nineteen veterinary clinics from nine RHAs reported weekly on the number of confirmed clinical cases and probable clinical cases each had seen or heard of in the respective area. Information collected included date of onset of clinical signs and location. To increase the number of confirmed cases, Saskatchewan Health sponsored the testing of probable clinical cases. In addition, all clinics within the province of Saskatchewan were asked for permission to allow the researchers to be notified of all positive IgM test results obtained by PDS (similar to 2003).

The full integrated surveillance initiative was coordinated by Saskatchewan Health and, in addition to horse statistics, included the following: dead bird surveillance information from Canadian Cooperative Wildlife Health Center (CCWHC website), mosquito speciation information, virus isolation from mosquito pools and human case statistics from Saskatchewan Health (Saskatchewan Health website), and daily temperature and precipitation information from Environment Canada (Environment Canada website). All components of the surveillance program were combined to confirm WNV activity in various geographic areas which drove the decision making process.

7.3 Surveillance results

In 2002, there were 29 documented positive diagnostic samples (Table 1). Only the location of the submitting clinic was recorded. No information was available on the presenting clinical signs for the tested horses nor was the health outcome of each horse reported, however six of the positives were from tests done on brain tissue. Although the date of onset of clinical signs was not recorded, no samples were tested prior to August 1. The primary focus of the reported cases was the southeast corner of the province.

In 2003, 82% (65/79) of clinics gave permission to access positive test results. A total of 133 positive IgM samples were documented by PDS (Table 1). Exact location was known for 130 cases, while the submitting clinic's location was known for the other 3 cases. The first onset date for clinical signs was July 30th, 2003 and the last date of onset was on September 19th, 2003. Clinical signs reported by owners were consistent with WNV. Of the 130 horses with known health outcome, 57 died or were euthanized (44%). Post season surveillance suggested that the number of confirmed clinical cases was approximately 1/5 of the potential number of cases in the province (Epp et al. 2007). The number of days from clinical onset to when a veterinarian visited the farm ranged from 0 to 49 with an average of three days. The number of days from clinical onset to a laboratory diagnosis ranged from 1 to 56 with an average of seven days.

In 2004, ten probable clinical horse cases were reported with confirmatory testing initiated in three cases, all three with negative results (Table 1). The first of these three cases was reported on July 24th. Clinical outcome was not reported. Locations were reported by RHA only.

In 2005, 49% of clinics (38/77) gave permission to access positive results. There were ten confirmed clinical cases and four probable clinical cases reported (Table 1). Outcome and vaccination status of the horses was not reported. The first date of onset of clinical signs was reported as July 26th and the last date was reported as September 24th.

Table 2 lists data for 2002 to 2005 for the following: bird data derived from the CCWHC website, human and mosquito derived from the Saskatchewan Health website, and temperature and precipitation data derived from the Environment Canada website.

Table 1: Results of surveillance: 2002 to 2005.

Year	RHA [a]	Clinics Reporting [b]	Potential Cases [c]	Laboratory confirmed
2002	**Sun Country**	--	--	**19**
	Five Hills	--	--	**1**
	Cypress	--	--	**1**
	Regina Qu'Appelle	--	--	**5**
	Sunrise	--	--	**1**
	Saskatoon	--	--	**1**
	Heartland	--	--	**1**
	Kelsey Trail	--	--	0
	P.A. Parkland	--	--	0
	Prairie North	--	--	0
2003	**Sun Country**	7	20	**10**
	Five Hills	4	28	**12**
	Cypress	5	48	**18**
	Regina Qu'Appelle	9	58	**18**
	Sunrise	7	21	**13**
	Saskatoon	9	7	**22**
	Heartland	6	8	**10**
	Kelsey Trail	5	2	**5**
	P.A. Parkland	3	12	**8**
	Prairie North	8	21	**17**
2004	**Sun Country**	3	1	0
	Five Hills	2	3	0
	Cypress	2	1	0
	Regina Qu'Appelle	3	0	1 negative
	Sunrise	2	0	0
	Saskatoon	3	0	0
	Heartland	3	0	1 negative
	Kelsey Trail	2	1	0
	P.A. Parkland	1	0	0
	Prairie North	2	1	1 negative
2005	**Sun Country**	3	0	**2**
	Five Hills	2	0	**2**
	Cypress	2	0	**1**
	Regina Qu'Appelle	3	0	**1**
	Sunrise	2	2	0
	Saskatoon	2	2	**2**
	Heartland	2	0	0
	Kelsey Trail	1	0	**2**
	P.A. Parkland	0	0	0
	Prairie North	2	0	0

[a] For RHA locations see map available online at http://www.health.gov.sk.ca/ph_rha_map.html
[b]"Clinics reporting was all participating clinics in each of the surveillance programs in each of the years.
[c]'Probable cases' in 2003 was recorded from a random 1/3 of all clinics within the RHA, but from only the reporting clinics in 2004 and 2005.

Table 2: Results of other components of the integrated surveillance; 2002 to 2005.

Year	RHA [a]	Humans[a]	Birds	Positive mosq. Pools[b]	Environment conditions[c]
2002	Sun Country		4		Temp: 18°C Prec: 196.9 mm
	Five Hills		8		
	Cypress		2		
	Regina Qu'Appelle		7		
	Sunrise		11		
	Saskatoon		5		
	Heartland		7		
	Kelsey Trail		0		
	P.A. Parkland		0		
	Prairie North		0		
2003	Sun Country	100	3	2	Temp: 18.6°C Prec: 97.5 mm
	Five Hills	225	9	4	
	Cypress	152	25	2	
	Regina Qu'Appelle	256	32	0	
	Sunrise	38	5	2	
	Saskatoon	62	37	6	
	Heartland	93	24	3	
	Kelsey Trail	2	5	0	
	P.A. Parkland	8	10	3	
	Prairie North	11	7	0	
2004	Sun Country	1	0	18	Temp: 15°C Prec: 228.2 mm
	Five Hills	2	2	1	
	Cypress	1	0	4	
	Regina Qu'Appelle	1	9	0	
	Sunrise	0	1	2	
	Saskatoon	0	9	0	
	Heartland	0	0	2	
	Kelsey Trail	0	1	1	
	P.A. Parkland	0	7	1	
	Prairie North	0	0	1	
2005	Sun Country	17	1	63	Temp: 16.2°C Prec: 267.5 mm
	Five Hills	6	0	3	
	Cypress	3	2	8	
	Regina Qu'Appelle	20	8	10	
	Sunrise	6	0	8	
	Saskatoon	5	2	12	
	Heartland	3	0	2	
	Kelsey Trail	0	0	3	
	P.A. Parkland	0	1	0	
	Prairie North	0	0	1	

[a] Human cases include WNV fever, asymptomatic and neurological cases. No cases in 2002.
[b] A positive mosquito pool is a collection of a species of mosquitoes that is positive for WN virus. No sampling in 2002.
[c] Environmental conditions are for Saskatoon Health region; overall average temperature (Temp.) and total rainfall (Prec.) for the months of June, July and August combined.

7.4 Discussion

7.4.1 Horse surveillance

One purpose of surveillance is to provide evidence of whether or not a disease exists in a geographic area (Salman 2003). Saskatchewan Health's integrated surveillance program included information on WNV activity from multiple different sources; wild and domestic animals, human cases, and vectors. All of these components have potential limitations. For example, wild bird submissions are highly influenced by human-related factors and can be limited geographically or temporally (Ward et al. 2006). Vector data, such as presence of WNV positive pools of mosquitoes, require an adequate number of monitoring locations to obtain a complete geographic picture, which can be difficult with a large study area. Access to human data is limited by confidentiality concerns. All of these surveillance components would be representative of urban locations but could be under-represented in more rural locations.

Equine surveillance has both advantages and limitations relative to other sources of data on the geographic spread of this virus. Advantages include: a) predominantly rural locations, b) few travel any distance away from the farm, c) most are housed outdoors 24 hours a day with little or no protection against mosquitoes, d) most are observed on a regular basis by their owners, and e) testing has a quick turnaround time with limited confidentiality concerns. Limitations include: a) reporting of WNV probable horses to veterinarians, especially mild cases decreases as public awareness and interest decreases and b) the cost of testing is the responsibility of the owner and may not be considered cost effective. Finally because vaccination of horses has been shown to decrease the chances of a horse developing clinical signs (Epp et al. 2005), widespread adoption of

vaccination could also potentially affect the interest and cooperation of veterinarians and owners in the surveillance program. In addition, surveillance solely based on identification of clinical horses would be under-representative of actual WNV activity in an area.

The use of an existing network of rural practitioners was a key component of the equine surveillance program in the summers of 2003 through 2005. While this was an effective way to gather information on an emerging disease, the case tracing system was time consuming. Permission to access information on samples submitted to the laboratory was required for confidentiality reasons. Laboratory submission forms did not provide complete information for tracking the disease and thus telephone interviews often were necessary. Although veterinarians were asked to report necessary information, such as location, date of clinical signs and vaccination status, compliance was highly variable. In 2004 and 2005, weekly faxed reminders and telephone follow-up were required for up to 50% of participating clinics. Even with additional monetary incentives for participating veterinary clinics, compliance in weekly reporting was highly variable. All of these factors could potentially be barriers to future use of horses in surveillance for WNV activity.

7.4.2 Interpretation of horse data

The Saskatchewan equine surveillance systems changed from 2002 through 2005. Data from 2002 was collected retrospectively from laboratories that received submissions and as such could have been an underestimate. Equine surveillance for 2003 through 2005 used a consistent case definition, accurate dates on onset and locations, involvement

of a network of veterinarians and one local laboratory. However, the number of clinics involved in surveillance was limited in 2004 and 2005 to include only those that were the most compliant in 2003 and were likely to see horses in their practice. Despite these differences, general inter-year trends in WNV activity from 2002 to 2005 can be explained by the ecology of the virus as reported in other areas of North America such as North Dakota (Bell et al. 2005).

In 2002, WNV activity in horses was low with the geographic extent primarily in the southeastern corner adjacent to the province of Manitoba. We presume this represents a late introduction of the virus into the province with insufficient time to promote extensive amplification of the virus. WNV was epidemic in 2003, evidenced by the high number of horse cases in addition to high dead bird numbers, positive mosquito pools, human cases, and suitable climate conditions. In 2004, unusually cool and wet conditions produced very little evidence of WNV activity in all surveillance components. We presume this represented prolonged mosquito development, prolonged extrinsic incubation of the virus, alteration of human behavior, and decreased congregation of birds and mosquitoes due to increased wetland habitat. In 2005, WNV activity in horses was decreased substantially from 2003 despite having favorable environmental conditions and evidence of high WNV activity in mosquitoes. A key difference between these years was improved wetland and surface water conditions across many areas of the province (personal communication, Phil Curry, Saskatchewan Health).

7.4.3 Use of horse data in the integrated system

Previous analysis associated within this equine surveillance program has showed that horses could serve as surrogates for humans but were not effective early predictors of human risk (Corrigan et al. 2006). Inclusion of information from all components of the integrated surveillance initiative gave the most complete picture of risk to humans. The combined information was used to determine levels of human risk geographically and facilitate decision making regarding interventions. For example, as the evidence of WNV activity in a locale increased, more intensive larviciding (or even adulticiding) might be suggested as well as initiation of targeted public awareness campaigns (personal communication, Phil Curry, Saskatchewan Health, WNV Coordinator).

In 2002, horse and bird surveillance provided the first indication that the virus was circulating in the province. In 2003, all health regions in the province reported WNV cases in humans and horses at roughly the same time. The horse cases did provide additional information on the geographic distribution of the virus, particularly in the rural portions of the RHAs where little information was available from mosquito, bird, or even human data. A cluster analysis of the 2003 data confirmed that horse case clusters continued to occur as the season progressed, indicating continued activity of WNV (Corrigan et al. 2006). A post-season evaluation of the 2003 Saskatchewan Health Integrated Initiative recommended the continuation of surveillance including horses for the coming year.

In 2004, horse surveillance did not detect any WNV activity. However, the number of probable horse cases by region was similar to WNV activity reported by at least one of the other surveillance groups: human cases, dead birds or positive mosquito

pools. Evidence of WNV activity was not identified by all components in all regions of surveillance in 2005. Only one region, Kelsey Trail, reported 2 confirmed horse cases but no confirmed human cases. In conclusion, since the integrated program based its response decisions (from communication to adulticiding) on a gradient scale of risk, equine surveillance was a useful component in providing evidence of WNV activity by region.

In 2006, the integrated surveillance program was reduced by excluding both dead bird and targeted equine surveillance. Instead, Saskatchewan Health relied on mandatory passive reporting of laboratory confirmed horse cases via federal and provincial government agencies. Reasons for the decision to exclude targeted horse surveillance included budget, the issue of vaccination and its effects on the identification of clinical cases, and the targeting of all surveillance towards urban versus rural areas. Mosquito surveillance is thought to provide the most measurable assessment of risk to humans and is to remain the cornerstone of the WNV surveillance in Saskatchewan (personal communication, Phil Curry).

Ultimately, the amount of public interest and the resulting resources available will determine the intensity of future WNV surveillance. This initiative has proven that lines of communication can be established between members of wildlife, veterinary and public health organizations. Many surveillance initiatives could be instituted for other important zoonotic diseases such as Hantavirus, Lyme disease, Q-fever, rabies and tularemia. This integrated initiative has proven that a collaborative approach to the surveillance of emerging and endemic zoonotic diseases is possible and will provide a template for future events.

7.5 Literature cited

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8.1 Introduction

The introduction of West Nile virus (WNV) into North America sparked an interest in predicting where and when the virus would appear (USDA 2000, Rogers et al. 2002a). Geographic tracking of individual cases showed they appeared to occur randomly, making prediction of when and where individual cases would occur impossible (USDA 2000). However, using geographical information systems (GIS), remotely sensed data (satellite imagery), ecological variables, and other spatial analysis techniques, production of maps identifying areas of higher risk of infection were possible (Kitron 2000, Rogers et al. 2002a). This approach has been used for other vector-borne diseases, such as Lyme disease and malaria (Dister et al. 1997, Rogers et al. 2002a).

Vector-borne diseases are particularly amenable to spatial and temporal analysis as they are highly influenced by regular, seasonal climate, and environmental changes (Kitron 2000). The classic epidemiologic triad adapted for West Nile Virus simplistically depicts the relationship between the pathogen (virus), vector, the hosts (birds and mammals), and environmental conditions (Gordis 2000) (Figure 1). Mosquitoes become infected with the virus and transmit it to multiple bird species, a cycle which amplifies

the virus. Governed by environmental conditions and host behaviors, mosquitoes can spread WNV to other incidental hosts, such as humans and horses. In the Canadian province of Saskatchewan (SK), the primary mosquito species involved is *Culex* (*C.*) *tarsalis* which begins to transmit the virus to incidental hosts around mid-July (Curry 2004).

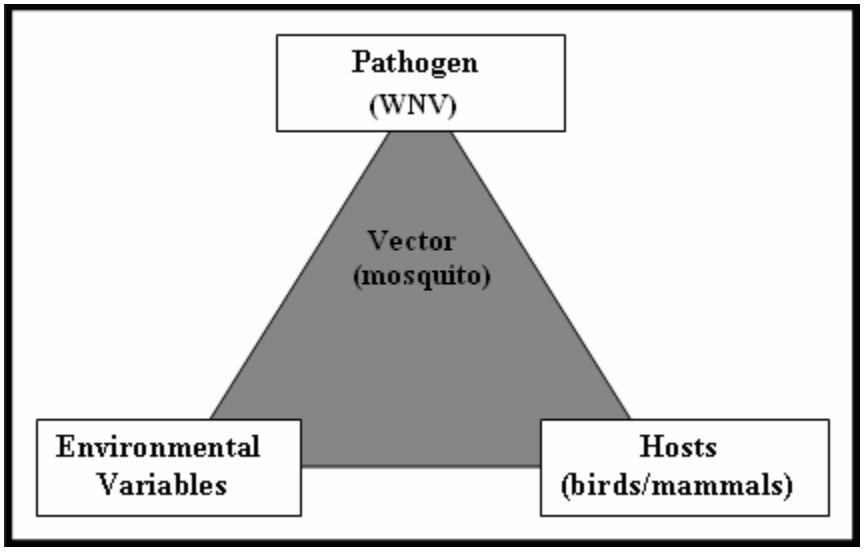

Figure 1: Classic Epidemiological Triad for West Nile Virus.

Environmental conditions determine the timing and intensity of the WNV cycle. Mosquitoes are cold-blooded and are therefore especially sensitive to regular seasonal changes in climate and environment, such as vegetation cover, rainfall, humidity, and temperature (Hay et al. 1996, Mellor et al. 2000). In addition, as the climate or habitat changes in an area due to human influence, mosquito species can establish in areas where they would otherwise not have flourished (Jonsson et al. 2000, Orme-Zavaleta et al. 2006). This is most notably demonstrated by the introduction of WNV to North America. The hot and humid conditions in New York City in August 1999 were the original

explanation for the numerous reported sightings of dead birds, mainly crows (Zellicoff at al. 2005). While the source of the introduction of WNV is not known, it is widely accepted that the favorable conditions which existed in 1999 allowed the establishment of WNV in the local mosquito and bird population (Epstein 2001, Rogers et al. 2002a).

Many components of the complex ecosystem-based transmission cycle are governed by fluctuations in climate (Rainham 2005). The extrinsic incubation period (time required from an infectious bloodmeal until transmission of the virus) is governed by temperature (Reisen et al. 2006). Congregation of mosquitoes and birds is essential to the amplification cycle and influenced by the availability of water sources (Kramer et al. 2001). Environmental conditions affect the behaviors of humans, specifically when time spent outdoors occurs at peak periods of infectivity, dusk or dawn (Loeb et al. 2005).

Defining the risk of acquiring infection with WNV is a key component to public health intervention strategies (Rainham 2005). Public Health officials use methods of mosquito control and public education to decrease the risk for human exposure. The programs are undertaken and prioritized largely based on environmental conditions conducive to mosquito development and surveillance for clinical disease in host species. Prioritization of vector-borne disease programs in the overall public health budget is a juggling act, affected by limited funding availability but a perceived risk by the public in the face of limited mortality or morbidity. Knowledge of the risk of infection would assist health officials by determining particular areas in which to concentrate control and surveillance programs thereby producing the maximum predictive advantage with minimum expense. If knowledge of risk was available earlier on a yearly basis, programs

could be adapted to match the predictions and possibly mitigate the potential for human infection in high risk areas.

Predictive risk mapping is a process by which components of the disease cycle (epidemiological, environmental and/or entomological) are used to create models and subsequent probability of risk category/group membership maps (Kitron 2000, Brooker et al. 2002). The methods have become more practical for a broader range of diseases and study locations because remote sensing can now provide environmental information at required spatial and temporal resolution (Beck et al. 2000, Rogers et al. 2002b) However, caution must be exercised to ensure that the appropriate resolution, variables of interest and methodologies are used to accurately address the questions being asked (Beck et al. 2000, Kitron 2000).

WNV was identified in both birds and horses Saskatchewan in 2002. A series of prospective cohort studies were undertaken in 2003 to monitor the progress of this emerging public health issue. This chapter details the creation of a model to define areas of low, medium, and high risk of WNV infection in the province of Saskatchewan in 2003 and assess its predictive ability using 2005 data.

8.2 Materials and methods

8.2.1 Proportion of WNV infected horses

Information on serological status of horses in the province was compiled and summarized at the rural municipality (RM) level. Blood samples had been collected from 923 horses during the summer of 2003 as part of a series of studies to determine factors affecting the risk of infection, clinical disease, and case fatality. For the first study, data

were summarized from 133 clinical cases with laboratory confirmation of WNV infection that were reported to the research team by the local laboratory (Epp et al. 2007a). A second study randomly sampled horses from 20 herds across SK at two separate time periods to determine serological status (Epp et al. 2007b). Results were also obtained from over 300 horses in a case-control study where the 23 case farms selected included a subset of the clinical case horses identified by the first study (Chapter 6). Finally, serological results were summarized from a rapid assessment study which sampled horses around seven cities based on positive mosquito trap locations (Corrigan 2005).

The samples were tested by both an in house Immunoglobulin (Ig)G and IgM enzyme-linked immunosorbant assay (ELISA) at Prairie Diagnostic Services (Epp et al. 2007b). The horses were defined as infected or not infected based on the ELISA results (Epp et al. 2007b). If a horse was IgM positive, it was considered recently infected whether showing clinical signs of WNV or not. If a horse was IgG positive but not vaccinated, it was considered infected, whether showing clinical signs of WNV or not. If a horse was vaccinated and IgG positive, the result was further characterized by calculating a sample-to-positive (S/P) ratio. If the horse was vaccinated twice and the S/P ratio was >0.30, the horse was considered infected. If the horse was vaccinated three times and the S/P ratio was > 0.78, the horse was considered infected with WNV. If the S/P ratio was less than either cutoff value, the horse was considered not infected.

The proportion of tested horses that were infected was calculated for each RM where 5 or more horses were sampled. Each RM with sufficient data to determine a proportion infected (known RM) was classified by category of risk of infection (group)

with WNV based on the 25th and 75th percentiles: low (0 – 29.9%), medium (30 – 77.7%), and high risk (77.8 – 100%).

In 2005, 196 horses were sampled and tested for WNV antibodies with an IgM ELISA only. Ten horses showed clinical signs of disease while the rest were sampled through cooperation with veterinarians already visiting the farms for other reasons, primarily Coggins testing (test for Equine Infectious Anemia). Infection status in horses was based on the blood sample results as previously described. The proportion infected was calculated by RM where 5 or more horses had been sampled. The same categories of risk of infection (groups) were used to label these sampled RMs (known RM-2005).

8.2.2 Environmental variables

8.2.2.1 Temperature

Land Surface Temperature (LST) was obtained through National Aeronautics and Space Administration (NASA) Earth Observing System (EOS) Gateway for the Moderate Resolution Imaging Spectrometer (MODIS) satellite. These data are distributed by the Land Process Distributed Active Archive Center (LPDAAC) located at the United States Geological Survey (USGS) center for Earth Resources Observation and Science (EROS): http://lpdaac.usgs.gov. The images were joined together (mosaicked) and clipped to show only the province of Saskatchewan using PCI Geomatica 9 (PCI Geomatics, Richmond, Ontario, Canada). Seventeen images were obtained as 8 day composites beginning May 1 and ending September 13 for 2003 and 2005. The images included daytime (maximum) and nighttime (minimum) temperatures and were manipulated to give a mean LST

(average of the day and nighttime images). For each time period, the average value per RM was calculated for all three variables for both 2003 and 2005 for each time period.

Minimum (min), maximum (max), and mean temperature (ºC) were also obtained from climate stations on a daily basis for both 2003 and 2005 through Environment Canada. Eight day composites (average min, mean, or max temperature per time period) were created to match the time periods of the remotely sensed data. For each time period, stations which were missing data from 50% or more of the days were assigned as missing. Interpolation between stations was accomplished with Inverse Distance Weighting (IDW) using ArcGIS 9.0 (ESRI Inc., Redlands, CA, USA). IDW is a quick deterministic interpolation of the data with minimal input of parameters. For each time period, the average value per RM was calculated for all three variables for 2003 and 2005.

Cumulative growing degree days (GDD) are the sum of the positive daily differences between 14.3ºC (the threshold for activity of *C. tarsalis* and virus transmission) and the mean temperature for that day (Woody Ornamentals 2003). The nine time periods used in analysis were defined as the cumulative GDD from May 1 until the beginning or middle of each month, ie. time period 1 was from May 1 until May 15 while time period 9 was from May 1 until September 15. GDD values were recorded as unknown at and after a time period in which there was missing data from that station. GDD were calculated for each climate station for 2003 and 2005 and interpolation between stations with complete data each time point was accomplished using the IDW method in ArcGIS 9.0. For each time period, the average value per RM was calculated for both 2003 and 2005.

8.2.2.2 Precipitation

Precipitation values (mm) were obtained for 2003 and 2005 on a daily basis from Environment Canada. Eight day composites (total precipitation per time period) were created to match the remotely sensed time periods. Interpolation between stations was accomplished using IDW method in ArcGIS 9.0. For each time period, the total value per RM was calculated for 2003 and 2005.

8.2.2.3 Vegetation

Normalized Digital Vegetation Index (NDVI) and Enhanced Vegetation Index (EVI) were obtained from EOS Gateway for the MODIS satellite (distributed by LPDAAC: USGS: EROS). NDVI is a simple index of vegetation cover based on near infra-red and red reflectance bands of satellite imagery which allows monitoring of seasonal and inter-annual changes in vegetation growth (Jensen 2005). EVI is a modified NDVI which incorporates a soil adjustment factor and correction for atmospheric scattering. The EVI has improved sensitivity for dense vegetation areas (Jensen 2005). Nine images were obtained as 16 day composites starting April 23 and ending September 13th for both 2003 and 2005. For each time period, the average value per RM was calculated using PCI Geomatica 9.0 for both variables.

8.2.2.4 Land cover

South Digital Land Cover dataset (North Digital and South Digital Land Cover) based on satellite imagery from 2000 for the province of Saskatchewan was obtained from Information Services Corporation of Saskatchewan (ISC). Classifications were

aggregated to make the number of categories used in the analysis more manageable. The aggregated categories included: water, wetland (bog, marsh, fen, etc), treed (pine, spruce, hardwood, softwood, etc), ground (rock, recent burn, cutovers, barren land), agriculture (cropland, pasture) and urban (settlements, roads). The percentage of RM covered by each of the categories was calculated using PCI Geomatica 9.0.

8.2.2.5 Elevation

Digital Elevation Model (DEM) was obtained from NASA: Jet Propulsion Laboratory: Shuttle Radar Topography Mission (SRTM) based on satellite images from 2000. The mean, majority, minimum, maximum and range of elevation were calculated for each RM.

8.2.2.6 Wetlands

The percentage of permanent and temporary wetlands by RM was obtained from Ducks Unlimited Canada's Habitat Inventory of wetlands and water bodies based on Landsat imagery from 1984 to 1993.

8.2.2.7 Census data

The number of horses per RM was obtained from Statistics Canada using 2001 Consolidated Census Subdivisions (CCS).

8.2.2.8 Latitude and Longitude

The latitude and longitude of the centroid of each RM was extracted from RM shape files using ArcGIS 9.0.

8.2.2.9 Ecoregion

Geographical differences exist across the province and can also be indicated by ecoregions. Ecoregions are a subdivision of ecological zones characterized by regional ecological factors such as vegetation, soil, climate, water, and fauna (Marshall et al. 1999). Within the geographic area covered by the RMs (does not include the northern half of the province), there are six ecoregions: Cypress Upland, Mixed Grasslands, Moist Mixed Grasslands, Aspen Parkland, Boreal Transition and Mid-Boreal Uplands. Using ArcGIS 9.0, each RM was assigned the ecoregion which comprised the largest proportion.

8.2.3 Statistical analysis

The whole dataset from 2003 was divided into a (a) training dataset (consisting of a random selection of 53 out of 67 known RMs) and (b) testing dataset (consisting of the remaining 14 known RMs and 231 unknown RMs). Two groupings of models were created: early season (May 1 to July 11) and whole season (May 1 to September 13).

Discriminant analysis was used to predict membership in the three mutually exclusive groups (low, medium and high risk) (SPSS 14.0, SPSS Inc. Chicago, IL, USA) (Klecka 1980). All variables and all time periods were introduced individually into the model for univariable analysis. Variables and time periods for which $P > 0.10$ were

excluded from any further analysis. An important assumption of discriminant analysis is that variables are not highly correlated with each other. Therefore, each variable with multiple statistically significant time periods by the univariate analysis was reduced using principle component analysis (SPSS 14.0, SPSS Inc. Chicago, IL, USA). This condenses the multiple statistically significant time points into one or two principal components which were then entered into multivariable models (Dohoo et al. 2003). The temperature related variables (GDD, temperature and LST) were also highly correlated with each other and were, therefore, used individually as the basis for separate models for both early and whole season.

Models used unequal weightings to adjust the posterior probabilities to account for prior knowledge of probable group membership. For example, since more than 50% of the known RM observations were in the medium risk category, this was given more weighting in the analysis than the other two categories. Separate matrices (to account for unequal group covariance matrices) were used when Box's M test was significant and the prediction accuracy percentage changed substantially from a model that used a common matrix for all groups.

RMs were classified into one of the risk categories by predicting the group (low, medium or high risk) to which the individual RM most likely belonged. The selection of the best model was determined using the overall classification or prediction accuracy percentage for the known RMs in both the training and testing datasets. This was defined as the proportion of known RMs correctly classified based on the pre-model classification (based on the proportion data) compared to post-model re-classification (based on the model classification) (Klecka 1980).

Individual RMs were assigned to a group (low, medium or high risk) based on which group the RM had the highest probability of belonging to (Klecka 1980). For example, an RM could have a probability of group membership in low risk of 90% , medium risk of 9% and high risk of 1%. Therefore, that RM would be classified in the low risk group with a probability of group membership of 90%. Caution must be exercised when RMs have probabilities that are less than 75%, as membership may be only slightly more probable in one risk group over another. Therefore, attempts were made to maximize the probability of group membership for all RMs in each group (low, medium, or high risk). Production of prediction maps from the best final models for both early and whole season was completed using ArcGIS 9.0.

The training dataset for 2003 (53 known RMs) was used to train the 2005 dataset (comprised of 10 known RMs and 288 unknown RMs). Then, the same variables as for the 2003 dataset were used for both the 2005 early and whole season models. Production of prediction maps from the early and whole season models was completed using ArcGIS 9.0.

8.3 Results

8.3.1 Distribution of infection in horses

The locations of clinically affected horses and serologically positive horses were compared between 2003 and 2005 (Figure 2, 3 and 4). There were 7 RMs with WNV infected horses in both years, 5 RMs with infected horses in 2005 that were adjacent to RMs with infected horses in 2003 and 1 RM with an infected horse in 2005 that was not sampled in 2003.

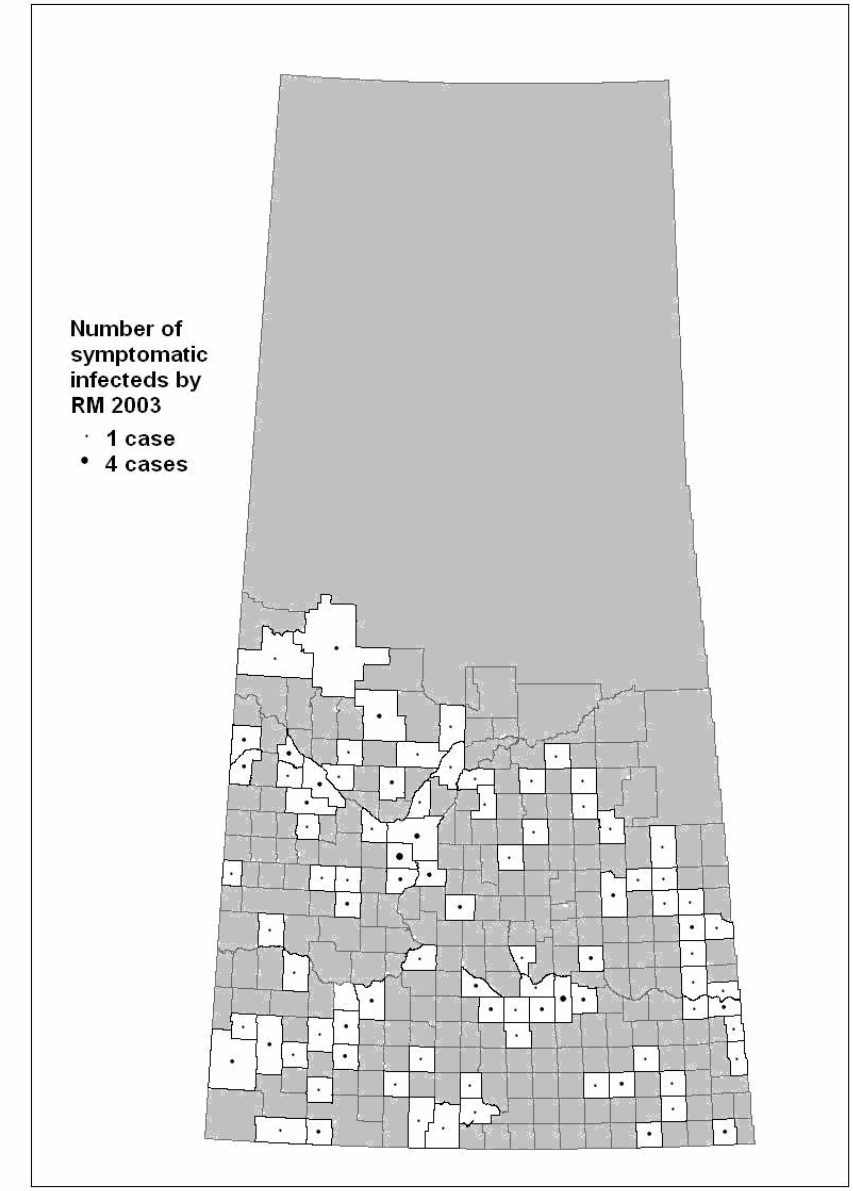

Figure 2: Distribution of the number of clinical cases (WNV clinically affected horses) for 2003 by RM.

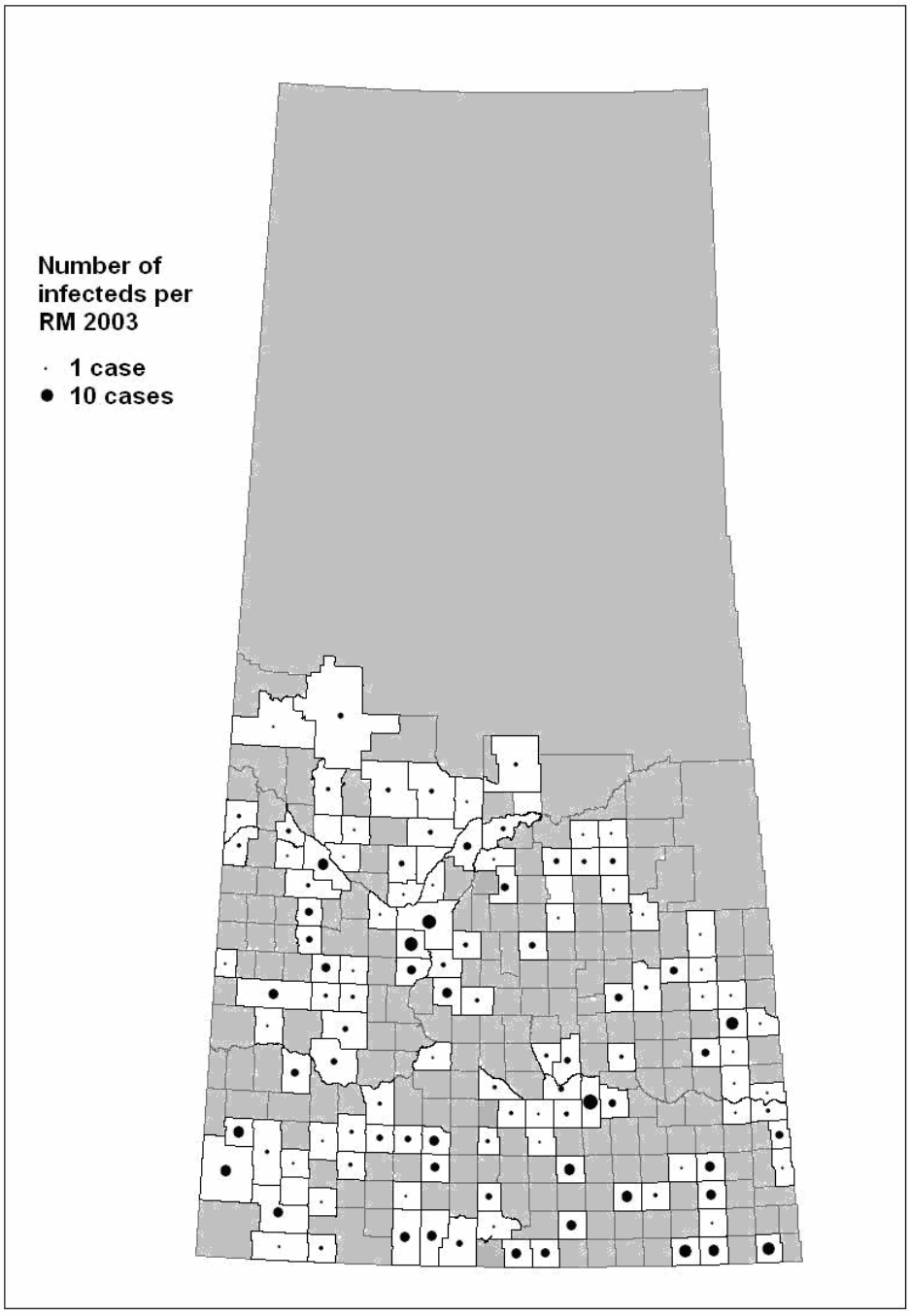

Figure 3: Distribution of the total number of WNV serologically positive and clinically affected horses for 2003 by RM.

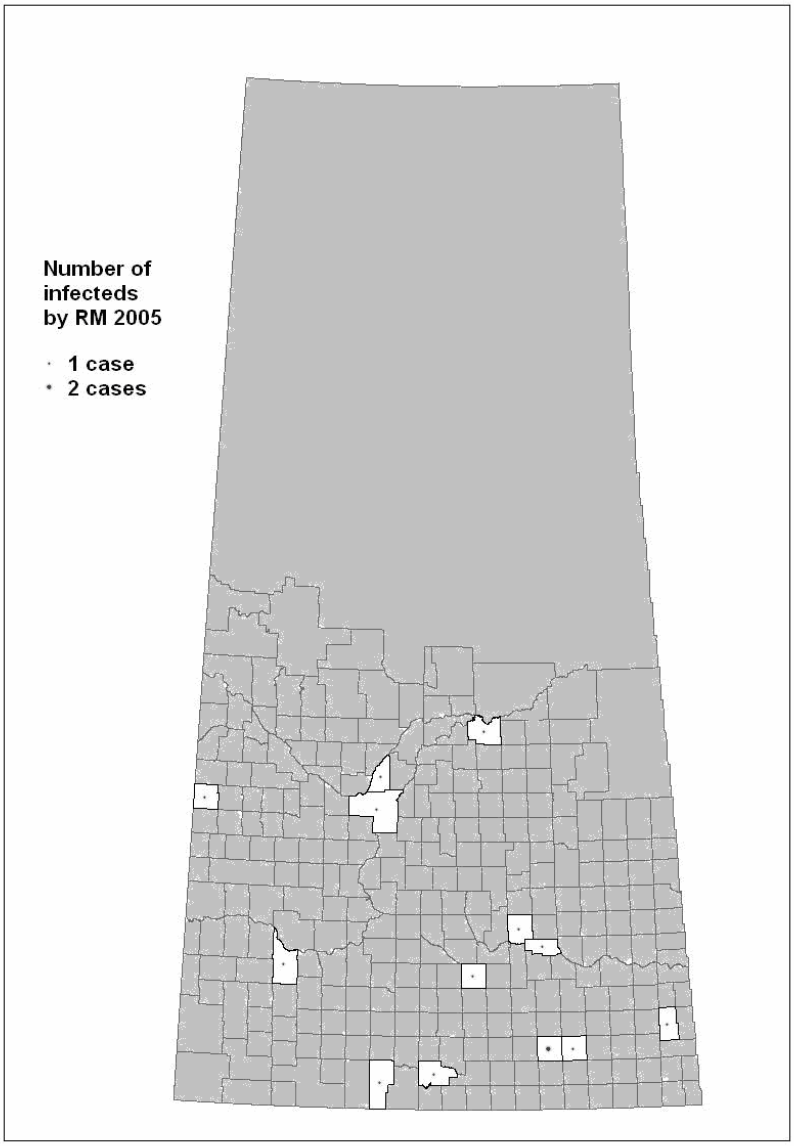

Figure 4: Distribution of the total number of WNV IgM serologically positive and clinically affected horses for 2005 by RM.

8.3.2 Creation of the Models - 2003

Of the 923 horses, 133 were clinical cases. The other 790 were asymptomatic, of which 395 (50%) were exposed to WNV. There were 67 RMs out of 298 included in the study area for which 5 or more horses were sampled. Based on calculating the proportion of infected horses by RM and the defined proportion per category, each RM was classified into a risk category: 16 low risk, 33 medium risk and 18 high risk.

The variables significant ($P<0.10$) in univariable models and used in further multivariable analysis included: GDD, mean temperature, mean LST, land cover (treed, water and wetland only), NDVI, mean DEM and precipitation (Table 1).

Table 1: Variables (with statistically significant time periods) tested in multivariable temperature based models for both early and whole season predictions. Bolded entries comprised the final prediction models for both early and whole season

Time-line	Model	Mean LST [a]	Mean Temp-erature [a]	GDD [c]	Precipitation [a]	NDVI [d]	Other time constant variables
Early Season	**1**	**June 26-July 11**	NA	NA	**June 2-25**	**April 23-May 8**	**DEM (mean)**, Latitude, Ecoregion, **Land Cover** [e]
	2	NA [b]	July 4- 11	NA	June 2-25	April 23-May 8	DEM (mean), Latitude, Ecoregion, Land Cover
	3	NA	NA	May 1-June 15	June 2-25	April 23-May 8	DEM (mean), Latitude, Ecoregion, Land Cover
Whole Season	**1**	**June 26-Sept.13**	NA	NA	**June 2-25, July 4-19**	**Aug 13-Sept 13**	DEM (mean), Latitude, Ecoregion, **Land Cover**
	2	NA	July 4-Sept. 5	NA	June 2-25, July 4-19	Aug 13-Sept 13	DEM (mean), Latitude, Ecoregion, Land Cover
	3	NA	NA	May 1-Sept 15	June 2-25, July 4-19	Aug 13-Sept 13	DEM (mean), Latitude, Ecoregion, Land Cover

[a] Remotely sensed time period (8 day composite), these underwent principle component analysis to be included in the model when more than one time period was significant.
[b] NA=not applicable, refers to variable not used in that models analysis.
[c] Bimonthly time period, cumulative GDD from May 1 until the end of the time period listed.
[d] Remotely sensed time period (16 day composites), these underwent principle component analysis to be included in the model when more than one time period was significant.
[e] Land cover included treed, water and wetland percentages.

The best early season model included variables: mean LST, precipitation, land cover, mean DEM and NDVI. The overall classification accuracy was 74% for training dataset and 64% for testing dataset. The group predictions and the probability of grouping by RM were mapped (Figure 5 and 6).

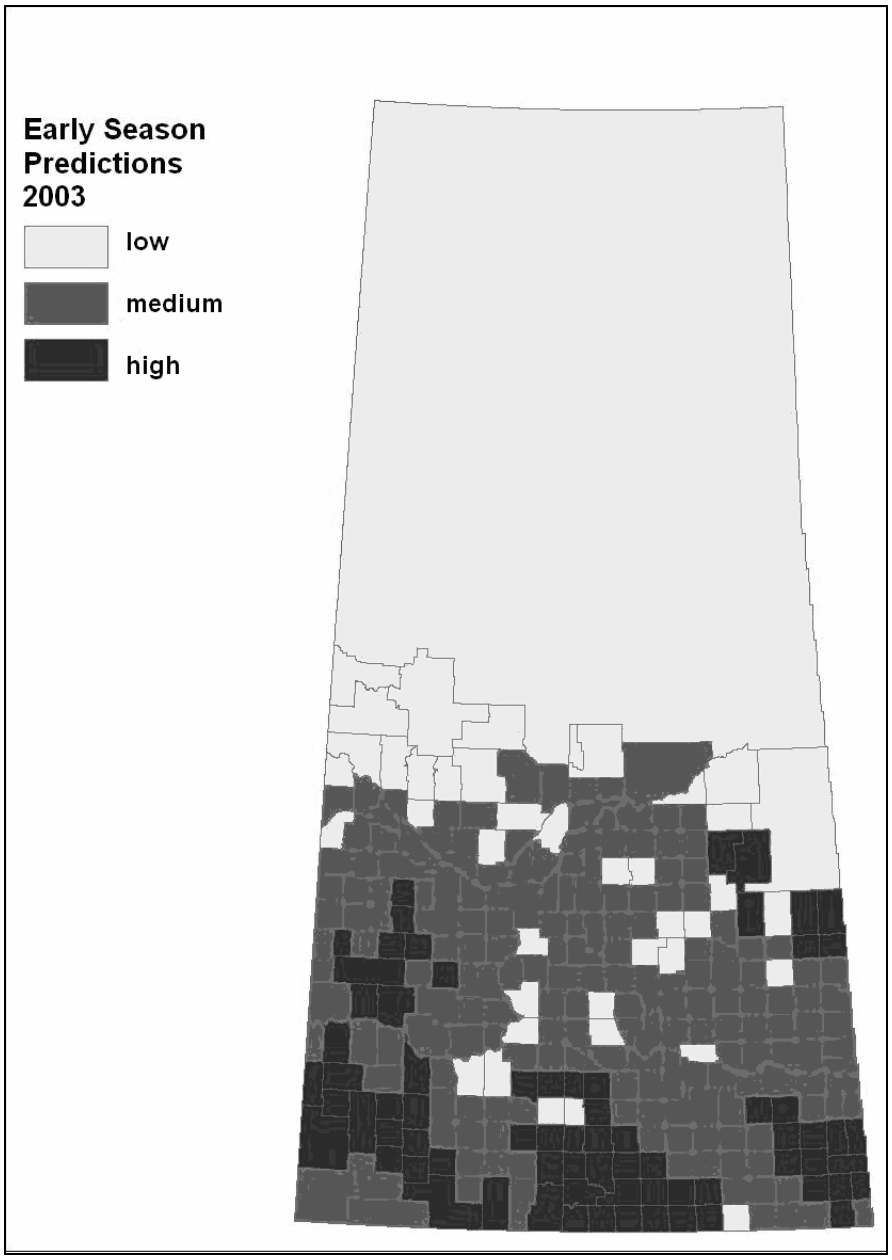

Figure 5: Early season (time periods from April 23 to July 11) model (LST, precipitation, NDVI, land cover, mean DEM). Predicted group membership (low, medium or high risk of infection) for 2003 by RM.

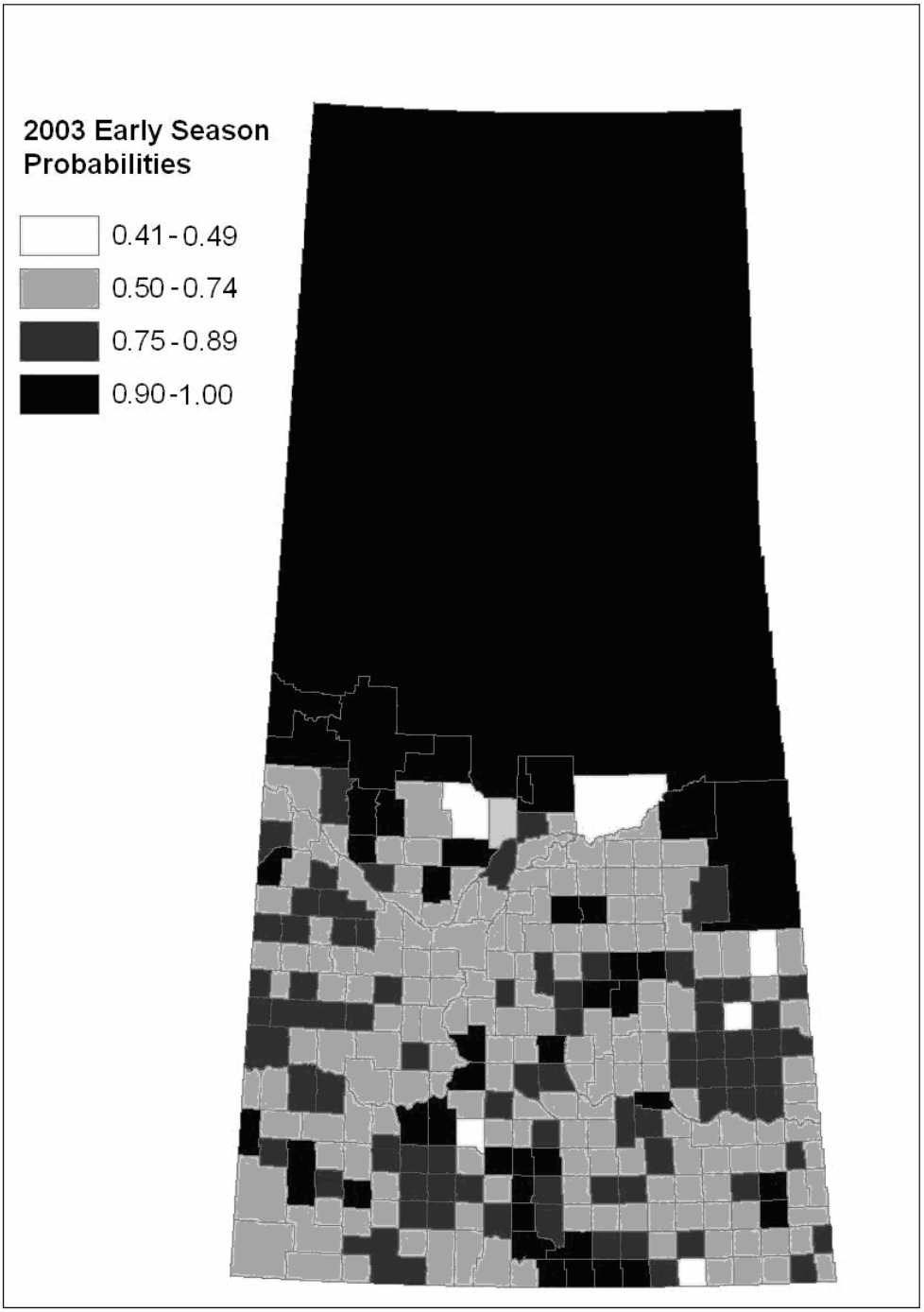

Figure 6: Early season (time periods from April 23 to July 11) model 1 (LST, precipitation, NDVI, land cover, mean DEM). Probability of group membership (low, medium or high risk of infection) for 2003 by RM.

Comparison of variables by group indicate that decreasing rainfall throughout June was the most important indicator for high risk group membership, followed by higher mean LST in July, and higher mean DEM. Lower percentage of treed, water and wetland area in the RM and slightly lower NDVI were secondary indicators of high risk group membership. The number of RMs in the three risk categories based on the results of the early season model was: 42 in the low risk group, 184 in the medium group, and 72 in the high risk group. The average probability of group membership for each category was 88% in the low risk group, 69% in the medium group, and 74% in the high risk group.

The best whole season model included variables: mean LST, precipitation, land cover and NDVI. The overall classification accuracy was 70% for training dataset and 71% for testing dataset. The group predictions and the probability of grouping by RM were mapped (Figure 7 and 8).

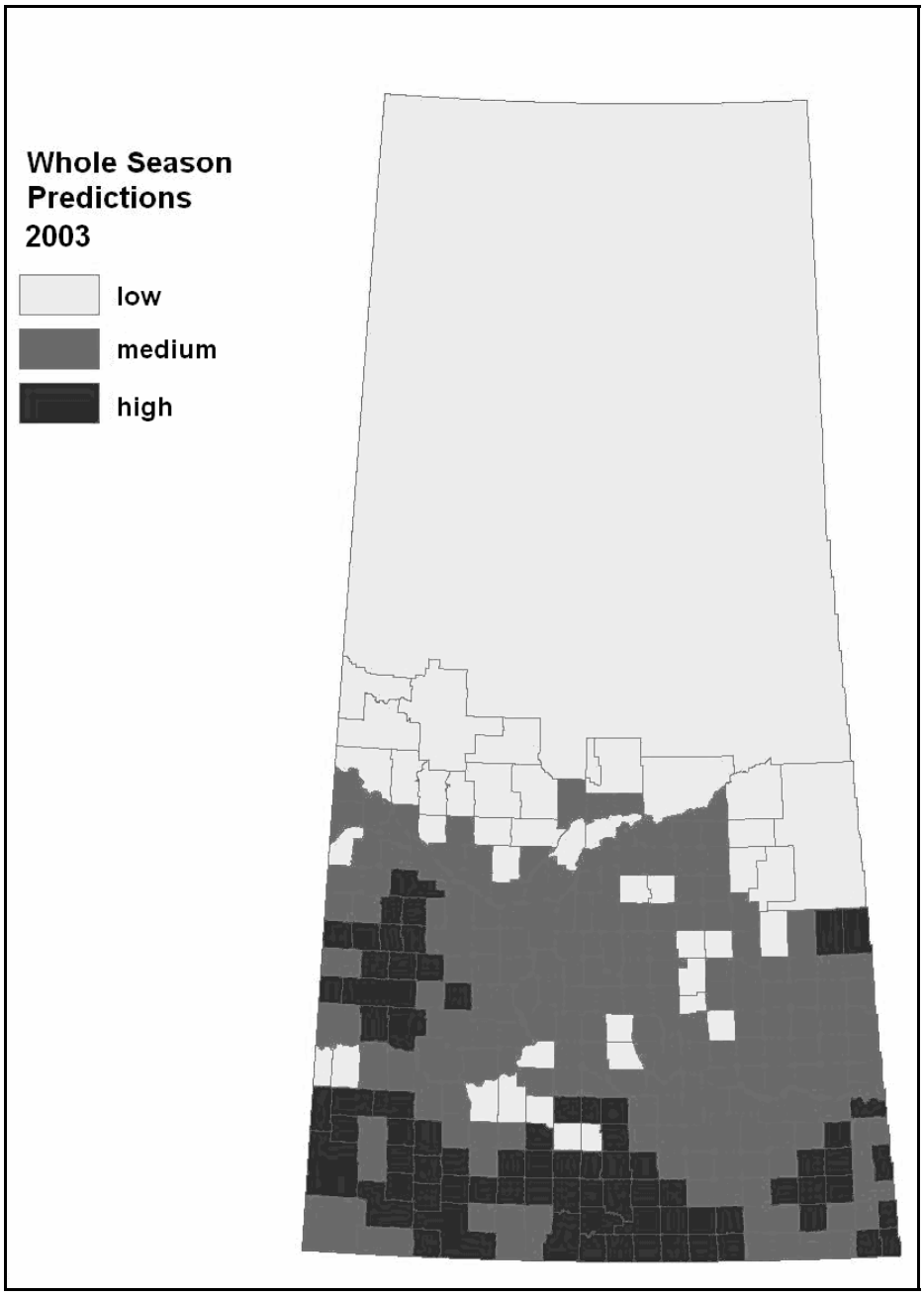

Figure 7: Whole season (time periods from May 1 to September 13) model (LST, precipitation, NDVI, land cover). Predicted group membership (low, medium, or high risk of infection) for 2005 by RM.

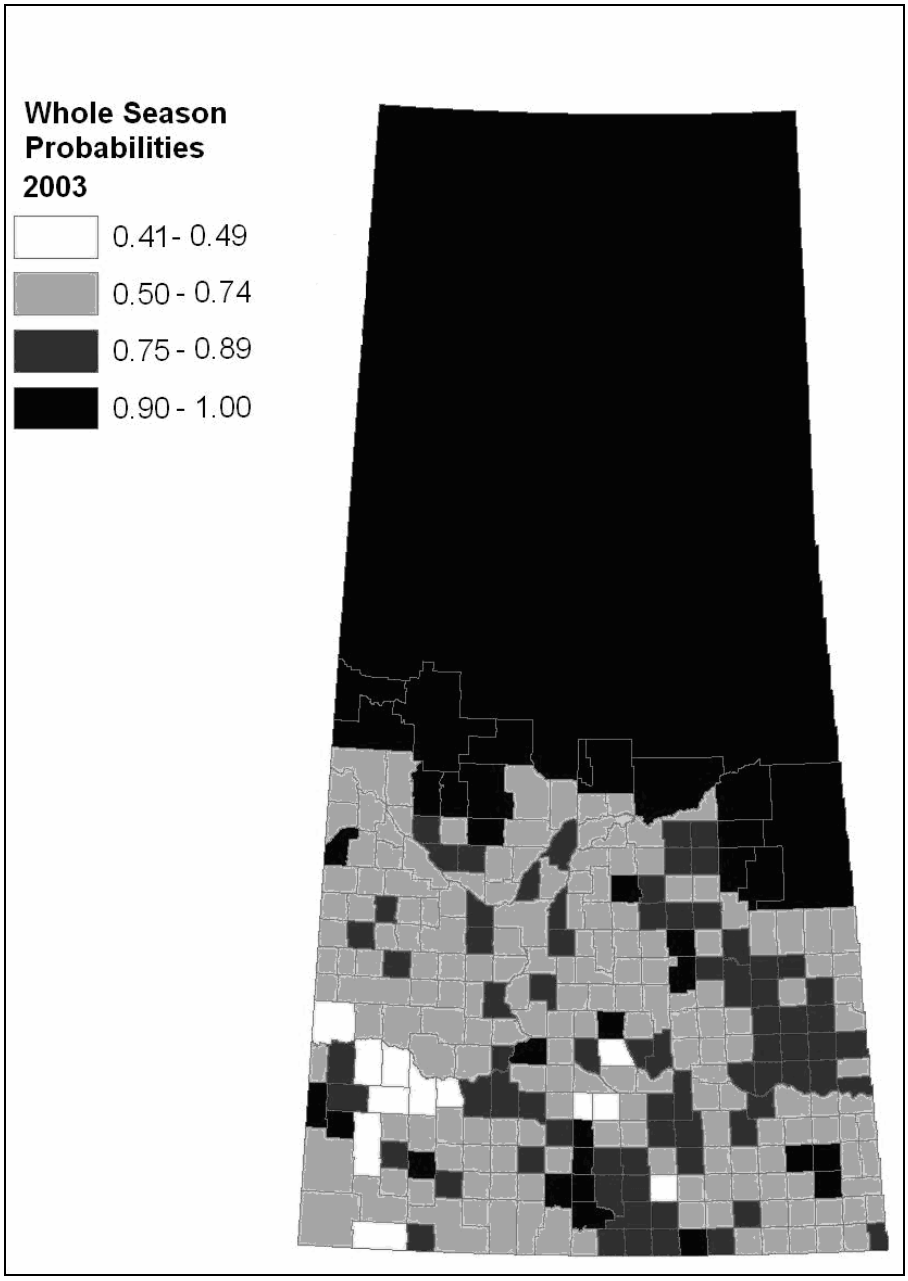

Figure 8: Whole season (time periods from May 1 to September 13) model (LST, precipitation, NDVI, land cover). Probability of group membership (low, medium, or high risk of infection) in 2003 by RM.

Comparison of variables by group showed that higher rainfall in June with lower rainfall in July was the most important indicator of high risk group membership followed by higher mean LST in July-September, lower NDVI at the end of August/beginning of September and lower percentage of water area per RM. Lower percentage of treed and wetland area per RM were secondary indicators of high risk group membership. The number of RMs in the three risk categories using the whole season model was: 44 in the low risk group, 178 in the medium group, and 76 in the high risk group. The average probability of group membership for each category was 85% in the low risk group, 67% in the medium group and 71% in the high risk group.

8.3.2 Predictive ability - 2005

The training dataset used for both the early season and whole season models was 53 known RMs from 2003. The testing dataset for 2005 only included 10 known RMs, all with the estimated category of membership as low risk. As such, the training and testing dataset overall classification accuracy percentages were not considered indicative of overall model accuracy.

For the early season model, the testing classification accuracy was 10% and the average probability of group membership was 89% in the low risk group, 69% in the medium group and 79% in the high risk group. The number of RMs in the three risk categories by the early season model was: 24 in the low risk group, 172 in the medium group, and 102 in the high risk group. Comparison of variables by group showed a similar pattern to 2003. The group predictions and the probability of correct grouping by RM were mapped (Figure 9 and 10).

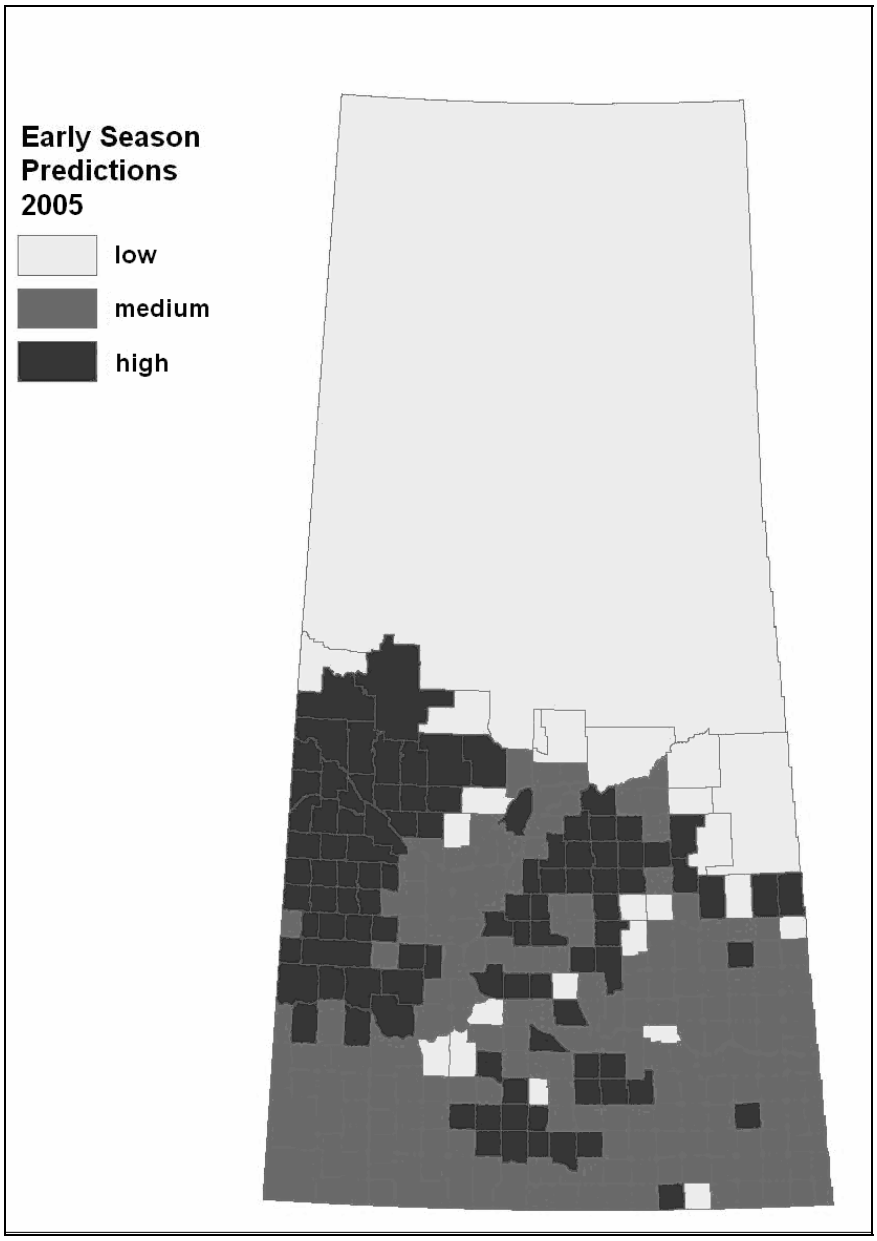

Figure 9: Early season (time periods from April 23 to July 11) model (LST, precipitation, NDVI, land cover, mean DEM). Predicted group membership (low, medium or high risk of infection) for 2005 by RM.

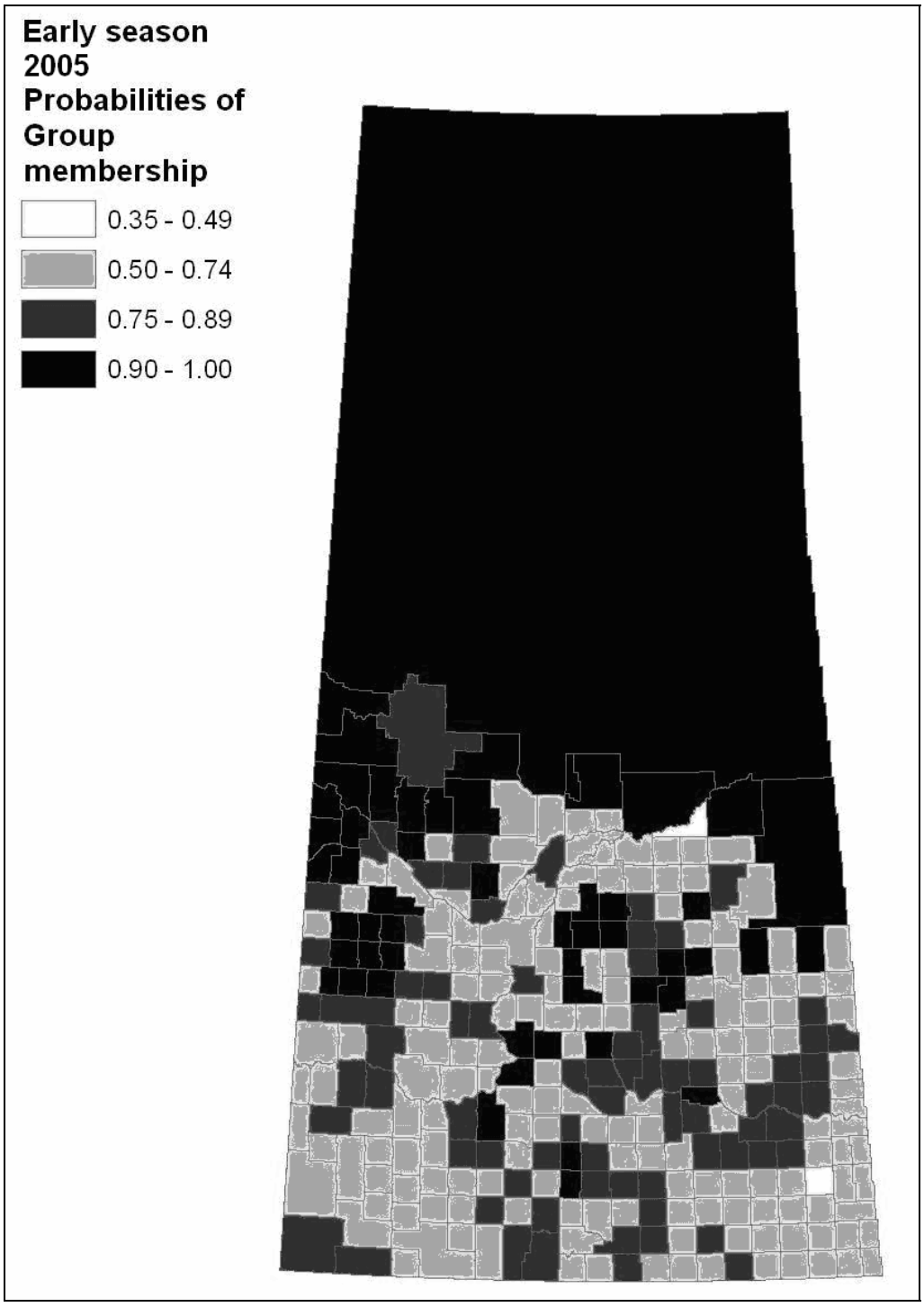

Figure 10: Early season (time periods from April 23 to July 11) model (LST, precipitation, NDVI, land cover, mean DEM). Probability of group membership (low, medium or high risk of infection) 2005 by RM.

For the whole season model, the testing classification accuracy was 30% and the average probability of group membership in 2005 was 78% in the low risk group, 68% in the medium group and 74% in the high risk group. The number of RMs in the three risk categories for the whole season model was: 57 in the low risk group, 148 in the medium group, and 93 in the high risk group. Comparison of variables by group showed a similar pattern to 2003. The group predictions and the probability of grouping by RM were mapped (Figure 11 and 12).

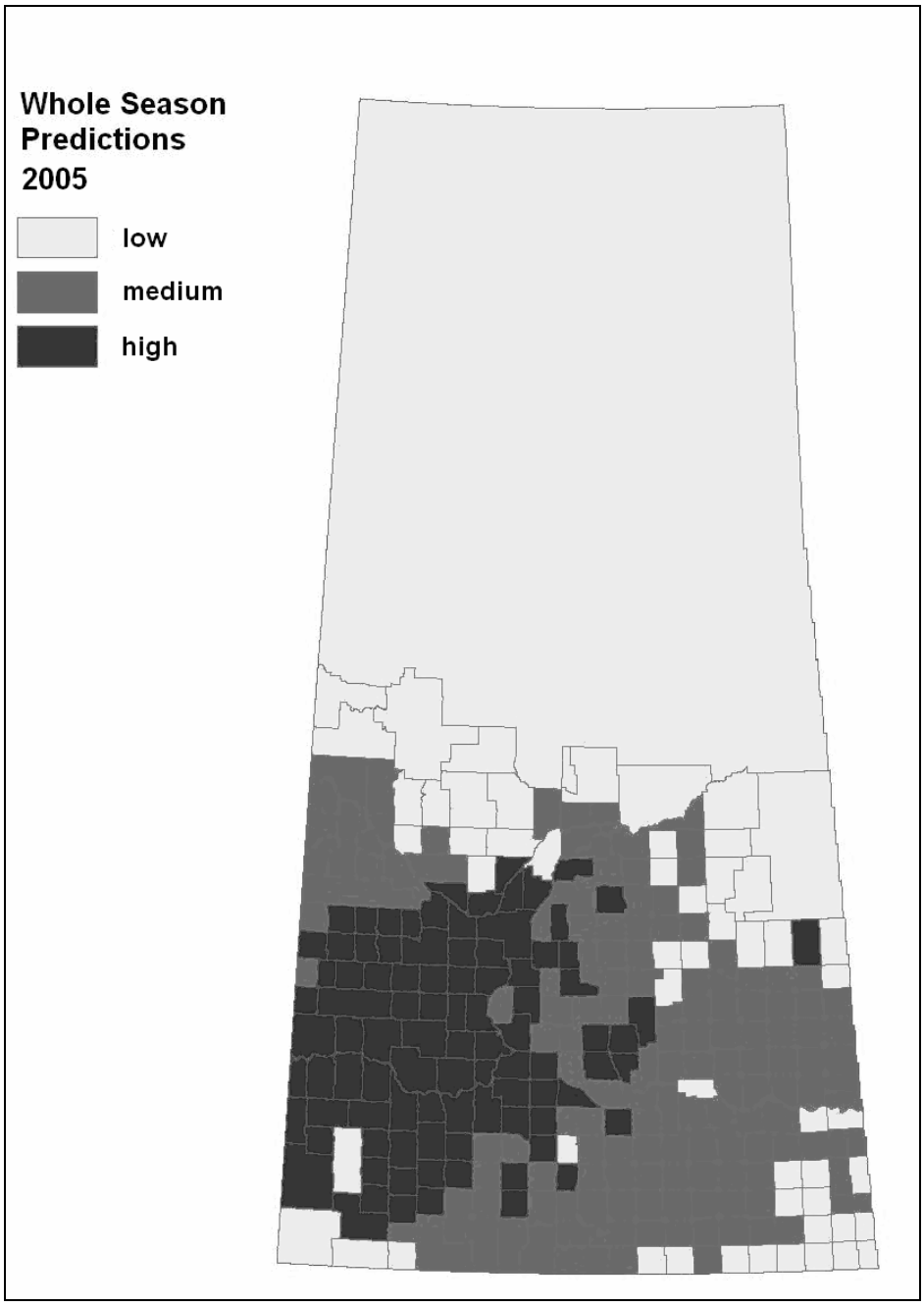

Figure 11: Whole season (time periods from May 1 to September 13) model (LST, Precipitation, NDVI, Land cover). Predicted group membership (low, medium or high risk of infection) for 2005 by RM.

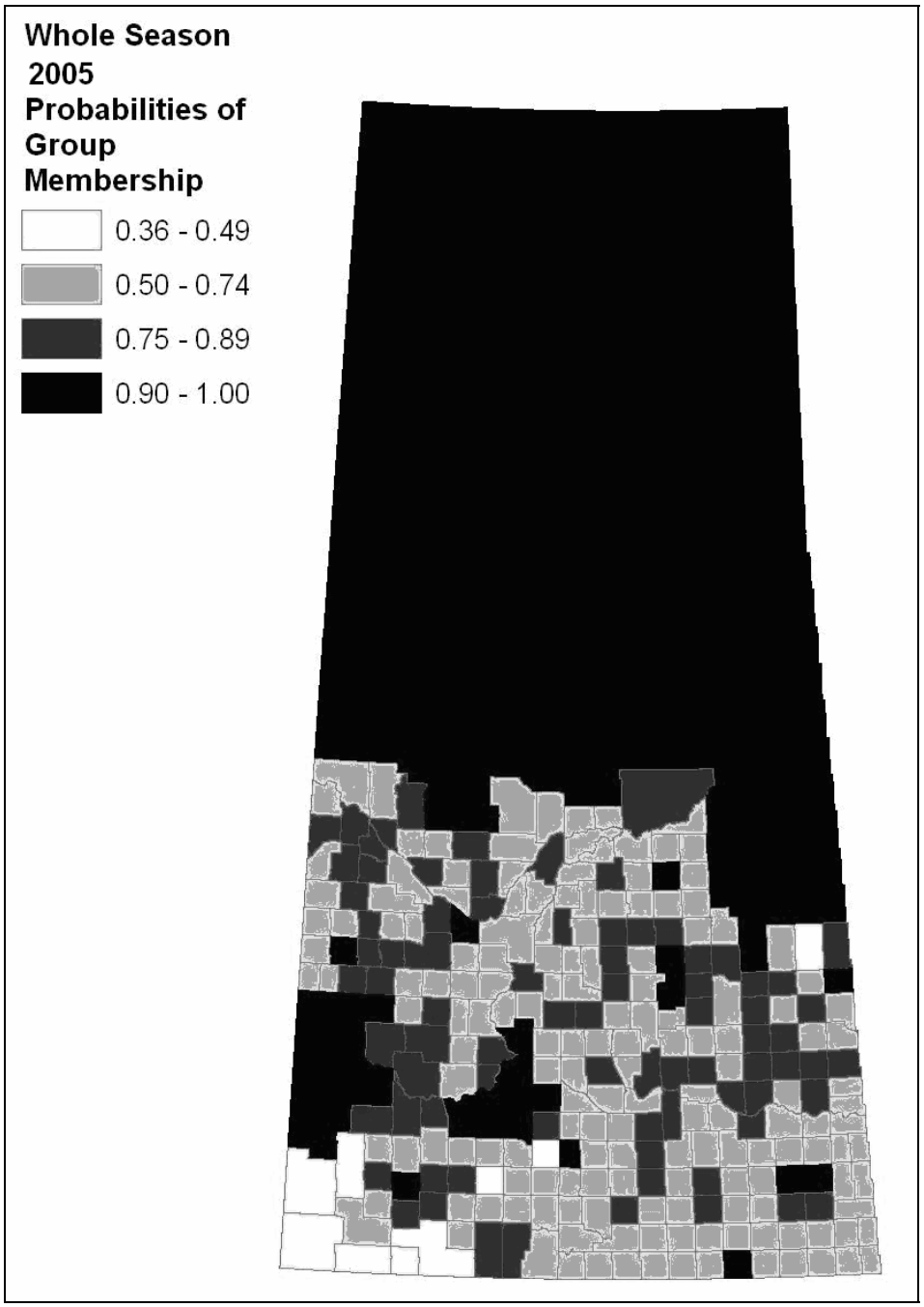

Figure 12: Whole season (time periods from May 1 to September 13) model (LST, Precipitation, NDVI, Land cover). Probability of group membership (low, medium or high risk of infection) for 2005 by RM.

8.4 Discussion

The models and predictive risk maps of WNV infection developed here are based on environmental conditions and not an estimate of risk based on clinical disease case load. The models in this study try to predict geographically which areas are at risk of infection (high, medium or low risk) using the proportion of horses infected, whether showing clinical signs or not, by region. Therefore, they were not affected by the rate of vaccination in these areas, as this would affect the number of clinical horses reported (Chapter 6).

To truly understand, model and ultimately control aspects of WNV infection, a picture of the complexity of the transmission cycle must be established. Expansion of the classic epidemiological triad provides a picture of how the environmental conditions affect each other as well as potential vector and host components (Figure 13). The important environmental variables included in this analysis were precipitation, temperature, elevation, vegetation and land cover, specifically wetlands, water and treed areas.

Using these environmental variables, a predictive model provided approximately 70-74% overall model accuracy in 2003 depending on whether the data were limited to early season or whole season time periods. Prediction of high and low risk RMs was better than for medium risk RMs. Obviously, the complexity of the cycle is not completely explained by these variables and their interactions. In addition, factors such as biodiversity, predators, parasites, food availability and spatial resources will affect interactions between the vector and hosts, while immune status of the hosts will become more important the longer the virus remains endemic in an area (Rainham 2005).

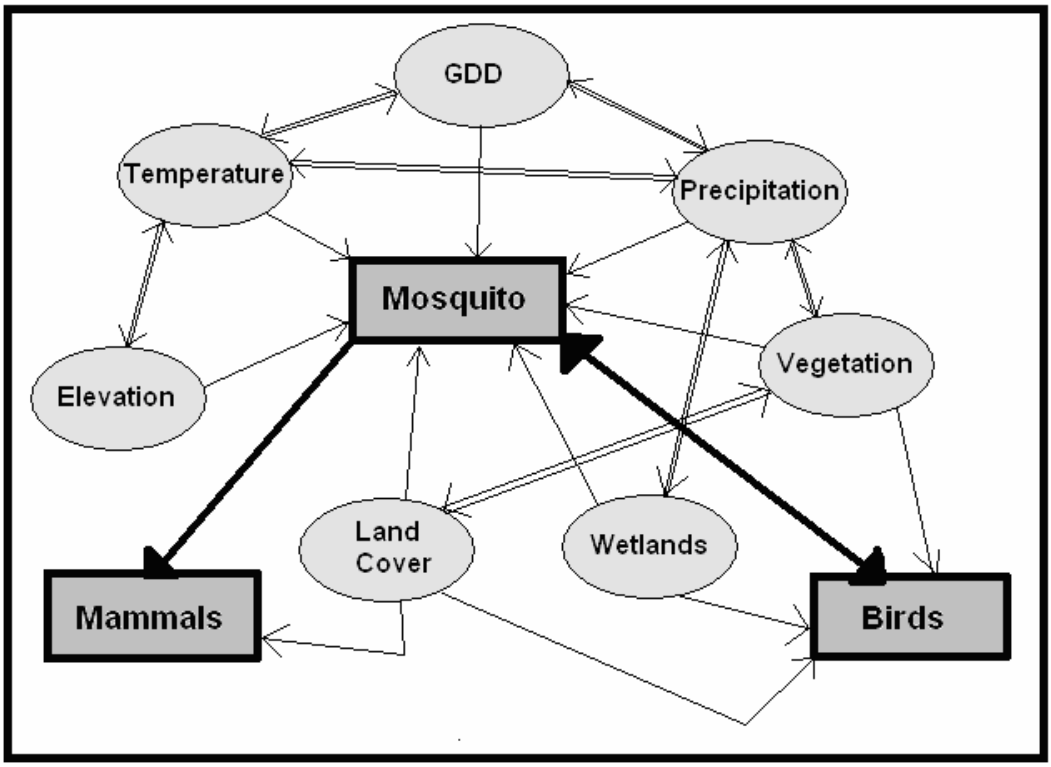

Figure 13: Detailed pathway of environmental influence on the cycle of West Nile Virus. Circles indicate environmental variables that influence all aspects of the amplification and spillover cycles (boxes), in addition to interactions between environmental variables.

The variable of primary importance in the prediction of risk of infection was precipitation, with precipitation from June important in the early season model and precipitation from June and July important in the whole season model. Mosquito life cycles (abundance and composition) and breeding site habitat (presence, size and persistence) are influenced by rainfall (Mellor et al. 2000, Shaman et al. 2005a). In Florida, drought-like conditions in spring followed by increased rainfall patterns in summer and fall facilitate WNV transmission (Shaman et al. 2005b). Different mosquito species will react differently to patterns of rainfall (Shaman et al. 2005a). *C. tarsalis* prefers rainfall followed by hot and dry conditions because it uses standing water with

increased organic content for oviposition which would be washed away by rainfall (Curry 2004, Shaman et al. 2005a). Both prediction models emphasize that higher rainfall at the beginning of June, decreasing towards the end of June, with minimal rainfall in July is associated with high risk areas whereas lower rainfall in June compared to July is indicative of low risk areas.

Another variable of importance was mean LST from July in the early season model and July and August in the whole season model. Increasing temperatures are linked to both increased survival of mosquitoes, increased biting habits and decreased extrinsic incubation period (Mellor et al. 2000, Reisen et al. 2006). Evidence of the effect of temperature on the transmission of WNV can be seen in 2004. In Central Red River Valley of North Dakota and Minnesota, temperatures and, therefore, the amount of thermal accumulations or degree-days was almost half of previous years with substantially lower number of human cases of WNV (Bell et al. 2005, Bell et al. 2006). A similar pattern of no confirmed horse cases and colder, wetter climate was observed in Saskatchewan during 2004 (Chapter 7). In the prediction models, higher rainfall in spring (June) followed by higher temperatures in July and August were indicative of high risk areas in both the early and whole season models.

Different temperature related variables were used in the models. LST was determined to be a slightly better predictor than either mean climate station temperature or GDD which was derived from the climate station data. This is likely because the climate station data required interpolation to provide a continuous map from which average data for each RM could be calculated, whereas the satellite data was provided on a continuous 500 meter scale. Studies have shown that LST (obtained on an 8 x 8 km or

even 1 x 1 km basis) and ambient temperatures (derived from climate stations) are quite similar, especially if other variables such as NDVI or latitude were included in the analysis (Hay et al. 1999, Green et al. 2002). Therefore, for large areas, LST has several advantages over climate station derived temperature data and will become a cornerstone of epidemiological studies in the future (Hay et al. 1999).

The amount and type of vegetation in an area is influenced by both temperature and precipitation (Hay et al. 1996). It has been proposed that WNV foci could be identified based on the presence of conducive mosquito habitat, specifically determined by water and vegetation (White 2001). A northern Indiana study looked at whether NDVI was associated with the occurrence of equine WNV cases (Ward et al. 2005). It determined that NDVI could be useful for predicting hotspots for implementation of mosquito control measures, but that a limiting factor for the use of NDVI would be the amount of agriculture in the area. In the present study, NDVI from the first week in May (early season) or the end of August and beginning of September (whole season) were important predictors. The NDVI from the beginning of the season could be an indication of the amount of previous fall and winter precipitation, especially in a year when warmer spring conditions facilitated earlier growth. The NDVI from the end of August and beginning of September would be indicative of vegetation amounts without a lot of agricultural influences, as in an average year, harvest would be well underway by these dates. Ward et al. hypothesized that once harvest is underway NDVI has a better discriminatory power to identify small areas where conditions are still suitable for vector habitat.

Land cover was another important variable in the model. The percentage of the RMs covered in trees, water or wetland areas differed between high risk (low percentages) and low risk (high percentages). *C. tarsalis* prefers shallow, often stagnant water of high organic content with little tree cover surrounding the sites, such as water-filled hoof prints near livestock watering sites (Curry 2004). A generalization would be that the RMs with high wetland and treed areas would not be considered ideal locations for WNV infection to occur. The predictive models identified these RMs as low risk areas. Elevation was a useful variable in the early season model. RMs with higher average elevation were more likely to be classified as high risk RMs. In the south central area of Saskatchewan in 2003, the human cases did follow the higher elevation of the Missouri Couteau, where the wetlands are concentrated in the low lying valleys and low depression areas (personal communication, Phil Curry, Saskatchewan Health).

The predictive ability of the model for 2005 data was compromised as all of the surveyed RMs were initially classified as low risk RMs. This was both due to a low number of sampled horses as well assessment of infection status based solely on IgM ELISA. This led to an inability to use the known 2005 RMs as a means to test the overall model's accuracy (training or testing datasets) or assessing predictor variable importance. Visually however, the risk predicted by environmental variables seems to indicate a shift in the high risk areas from 2003. The predicted area of high risk in the western portion of the study area (both early and whole season models) matches the area with the highest number of C. tarsalis mosquitoes trapped in 2006 (personal communication, P. Curry, Saskatchewan Health). On the other hand, the actual location of the RMs with positive cases in 2005 mirrors that of 2003. This indicates that although environmental risk of

WNV infection changes in a yearly basis, WNV activity and the spatial distribution of cases may not. This has been seen with other spatial analysis of bird and human cases in Ontario (Beroll et al. 2007). In addition, the area with the most positive pools of mosquitoes was in the southeastern corner and did not correspond to the model predictions. The complex relationship that exists between environmental risk, mosquito abundance and infectivity and actual disease transmission to various host species cannot be adequately summarized by a single predictive model from two years of limited horse data. The model is missing key information on the amplification cycle which would likely make it more predictive of risk of infection.

Finally, the models were created with a training set of data, comprised of a randomly selected portion of the RMs where sampling of horses occurred in 2003. A different randomly selected training dataset could have produced slightly different results. In addition, these models relied on summarized and interpolated environmental data for each RM, which can produce a loss of inherent heterogeneity in the data and introduce errors. As such, caution should be exercised when referring to any of the individual RM predictions made on the maps. Instead, the maps will be useful to indicate general but larger areas of high risk of infection.

8.5 Conclusions

The usefulness of the model as a predictor of high risk areas must be coupled with the knowledge of vector abundance and host population dynamics. If the environmental model predicts an area of high risk where there is not a large population of incidental hosts, the high risk pattern will not be mirrored in the distribution of cases in these populations. In addition, access to services for diagnosis of clinical cases in both equine

and humans is more limited in sparsely populated areas. Historically, maps of mosquito vectors indicate high risk areas to consistently be in the southeastern portion of the province. In 2005, these factors likely influenced the distribution of cases, although this theory could not be confirmed as only one location at the edge of the high risk RMs was sampled. As well, early season predictions can be negated by altered environmental conditions later in the season not highly conducive for mosquito development. More information on the connection between high environmental risk and WNV infection could be obtained from using the models in conjunction with other surveillance methods, particularly mosquito data.

The usefulness of the models as components of a surveillance initiative is the most promising. Indication of environmental conditions conducive to WNV would be useful in determining sites for intensified mosquito surveillance or control. By incorporating multiple years of data into the model, the accuracy and stability of the predictions will increase (personal communication, Martin Hugh-Jones). These prediction models were based solely on the epidemic year as the training set of data. Subsequent years did not produce the number of cases seen in 2003 in either the clinical cases or the asymptomatic horses, particularly because of the interference of vaccination despite environmental conditions of risk. Multiple years of data would reduce the dependency of the model on 2003 data. The distribution of cases in subsequent years indicates that WNV infection follows a more spatially conservative and consistent distribution rather than one that solely follows environmental risk (Chapter 7).

These models have identified time periods and variables to use in consideration of environmental contribution to WNV activity. They have also identified caution in

interpreting emerging disease models based on single epidemic year data. With further research, greater accuracy in predicting WNV risk of infection will occur. In addition, future incursions of mosquito-borne diseases will benefit from the knowledge gained from targeted surveillance accomplished now in the inter-epidemic period.

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9. Summary and Conclusions

9.1 Concluding remarks

In the spring of 2003, WNV was an emerging problem for both horses and humans in Saskatchewan and much of North America. The primary objective of this thesis was to describe the epidemic in horses by exploring individual, herd, and geographic risk factors for infection, for development of clinical disease, and for clinical disease outcomes. The second objective was to use the information collected from horses and predict risk of infection.

9.1.1 Clinical cases of WNV in horses in 2003 and factors associated with case fatality

The epidemic of WNV clinical disease in affected horses was documented by collecting data about management, vaccination status, horse characteristics, and location (Chapter 3). Horses in this study had mild to severe clinical signs of disease. The final outcomes of the disease documented in this study were death, euthanasia, or recovery. The data were analyzed to identify risk factors for fatality from clinical disease.

The highest number of clinically affected horses occurred in the grasslands ecoregion. This ecoregion consists of habitat and climatic conditions primarily favored by

the mosquito, *C. tarsalis*, which makes it a "hotspot" of infection and clinical disease. In 2003, West Nile virus made an appearance in horses beyond where infection was geographically predicted to occur based on historical mosquito distribution maps (personnal communication, Phil Curry, Saskatchewan Health). One explanation for the appearance of WNV as far north as Meadow Lake, SK, was that the climate and environmental condition were sufficient for establishment of the mosquito *C. tarsalis* in areas this species had not previously been reported.

The clinical epidemic began at the end of July and continued to the middle of September, while asymptomatically infected horses were found as early as June. The onset of clinical signs for the majority of cases was from middle of August until the begining of September. This pattern was consistent across all years of targetted surveillance, 2003-2005. This pattern was also consistent with studies from other regions of North America, with the season being progressively longer in the southern latititudes. For example, Texas had its' first reported case near the end of June with the majority of cases reported from September to October (Ward 2006).

In the analysis of the data from horses with clinical signs, the date of onset was an important predictor of fatality. This could be related to ecology of the virus or promptness of recognition of clinical signs by the owner and timing of supportive treatment. In addition, stallions and horses with a light coat color were also more likely to die from clinical disease. The reasons for these findings require further study.

Because the study was limited to inclusion of horses with serological testing results, there was potential for reporting bias. A post-season survey of veterinarians identified geographical differences in the reporting and testing of clinically affected

horses for WNV. Reasons for the lack of testing ranged from monetary to personnal recommendations to the owners.

9.1.2 Using serology to describe the infection status of horses

Serology was used to describe the infection status of horses across the province in 2003. Due to the introduction of a killed vaccine in the fall/winter of 2002, information was needed on how to interpret the immunoglobulin (Ig)G and IgM enzyme-linked immunosrobant assay (ELISA) in the face of vaccination. Two studies were completed, one describing the kinetics of naturally induced antibody titres over time and the other describing antibody titres in response to vaccination (Chapter 4). Using these data, cutoff values for identification of horses which were both naturally infected and vaccinated were established for use in the serologic prevalence study (Chapter 5) and the case-control study (Chapter 6).

Because of the introduction of other vaccines onto the commercial market after 2003, the reported cutoff values cannot be applied to future studies. Multiple years of different vaccination protocols and varying degrees of natural exposures would also interfere with interpretation of serological data after 2003. Therefore, the use of non-vaccinated horses would be recommended in any new serologic studies.

9.1.3 Serological study to investigate the risk of non-clinical infection

In addition to describing reports of clinical WNV infection (Chapter 3), a second study was completed to describe the distribution of non-clinical infections (Chapter 5). In this study, horses from across the province were sampled at two different times with the

intent to monitor the occurrence of seroconversion to WNV. For each horse sampled, investigators collected management data, vaccination status, horse characteristics, and location. The data were analyzed to identify risk factors for infection with WNV.

Serologically positive horses were found across the entire study area, but a south to north gradient of decreasing infection corresponding to ecological regions was observed. The grasslands ecoregions (Mixed and Moist Mixed) had the highest prevalence of asymptomatically infected horses while the northern most area of the study region (the start of the Boreal Forest) had the lowest prevalence. As well, the implementation of some form of mosquito control on the farm was associated with a lower risk of infection.

A serological study of this type would be hard to conduct in the future, due to the multiple vaccines and vaccine protocols being used. While there is no evidence that vaccination reduces the risk of infection, vaccination will limit the utility of the ELISA in determining infection status. The use of non-vaccinated horses would be the only feasible option. Because of the limited numbers of horses sampled in each ecoregion, it was not possible to determine if there was also a difference in prevalence from east to west with the available data.

9.1.4 Case-control study of factors associated with clinical disease

The objective of the next study was to identify risk factors for development of clinical disease (Chapter 6). A case-control study was designed to compare horses sampled on farms with clinical disease and horses from farms that did not have clinical disease. Data were collected for each sampled horse about management, vaccination

status, and horse characteristics. Because control farms were the nearest location to a case farm fitting the study criteria, location and geography variables were not considered as potential risk factors.

Both case and control farms had similar prevalences of infection in horses without any signs of clinical disease. Risk factors for the development of clinical disease in horses were compared across horses and herds enrolled in this study. One of the most important findings from this thesis was that vaccination with the killed vaccine was highly efficacious at preventing development of clinical disease.

As a side note, this study also emphasized that surveillance based solely on identification of clinically affected horses could have underestimated WNV activity. Specifically this could have occured in areas with higher vaccination rates, typically areas deemed to be at higher risk of infection.

9.1.5 Multi-year surveillance for WNV in Saskatchewan

The objectives of the next chapter of this thesis (Chapter 7) were to describe all data available on WNV activity in horses from 2002 to 2005 and to determine the role of equine studies in an integrated surveillance intiative by Saskatchewan Health over this four year period. Although the methods evolved over the four year period, the results were comparable to other components of the surveillance system as well as other similar geographic locations.

The inclusion of horse data (clinical cases and infection) were considered useful by Saskatchewan Health (internal evaluation of the surveillance initiative, Phil Curry, Saskatchewan Health). The inclusion of the horse data confirmed activity patterns seen

in other components of surveillance in 2003/2005 and identified regions of high activity where no human cases were found in 2005. However, the collection of horse data were time consuming, particularly because veterinarians did not completely fill out or complete laboratory submission or weekly reporting forms without repeated reminders.

Reliance on reporting of horses with clinical signs in the absence of active screening was also recognized to dramatically underestimate the epidemic as indicated by the previously mentioned post-season survey and the extensive use of vaccination. Therefore, with limited financial resources, the surveillance of horses as well as dead birds were discontinued in 2006. Likely, surveillance activities specifically for WNV will continue to decline; particularly if cases of WNV in humans remain low. This exercise did, however, suggest that the integrated component of the surveillance initative could be modified and employed again in the event of another emerging infectious zoonotic disease.

9.1.6 Predictive risk mapping of WNV infection

The culminating objective of this thesis was to see if a model could be created to predict areas of high risk without the need for costly surveillance (Chapter 8). Horse data collected in the previously mentioned studies were combined with other sources of environmental data to create predictive risk maps.

The modeling process demonstrated that using only one or two years of data was not ideal for model building and that predictive models should be based on mulitple years of data for better accuracy. More surveillance data from 2005 or data from more than one follow up year would have increased the utility of the resulting prediction model. The

number of horses sampled was too few to adequately train the model with 2005 data. As well, the RMs with adequate data to determine infection prevalence in 2005 all initially fell in the low risk group. This made assessment of the overall prediction accuracy of the 2005 models potentially unreliable.

However, the model did identify variables of interest in predicting risk. The variables included in the final model were land surface temperature, precipitation, vegetation cover, land cover (specifically trees, water and wetland), and possibly elevation. All of these variables affect the vector population which is the primary mode of transmission of the virus to susceptible hosts. In addition, the models did suggest specific time periods for meteorological and environmental data that were important predictors, such as precipitation in June and temperature during July and August. Use of these variables in future surveillance projects may aid in the detection of virus hotspots geographically and temporally.

9.2 Conclusion

The 2003 epidemic provided a novel opportunity to study an important zoonotic disease emerging in a new environment. The epidemic was described in detail, focusing on risk factors for infection with WNV, development of clinical disease, and finally, factors affecting the risk of fatality from clinical disease. The thesis demonstrated that horse data can be useful in an intergrated surveillance system for the benefit of human health. Finally, this thesis featured a modelling and mapping technique which could identify areas of high risk of infection for horses using readily accessible environmental data for minimal cost.

9.3 Literature cited

Ward MP. Spread of Equine West Nile virus encephalomyelitis during the 2002 Texas epidemic. Am J Trop Med Hyg 2006; 74:1090-1095.

Appendix A

Dear Colleagues

June 23, 2003

We are writing to seek permission to be informed of West Nile Virus positive horses in your practice and permission to contact the owners of affected animals. This initial contact is to inform you of the study and to prepare for the season ahead.

The research team is associated with the Western College of Veterinary Medicine, Saskatoon, Saskatchewan. This summer, we intend to conduct a case-control study in order to learn more about risk factors (including vaccination) related to the occurrence of clinical West Nile Virus infection (WNV) of horses in Saskatchewan. A critical aspect of this study is the identification of affected (clinical cases) and non-affected herds (no clinical cases) of horses in Saskatchewan.

We hope to locate clinically affected animals by having the Prairie Diagnostics Services (PDS) in Saskatoon inform us when they confirm the diagnosis of WNV (blood or postmortem) in Sask. horses. However, this will only be possible if veterinary practices in Saskatchewan sign a release allowing the laboratory to inform us regarding the identity of practices submitting one or more positive samples. The submission of any suspected WNV samples to PDS would help to facilitate this study.

In the event that a positive result does originate in your practice, we will first contact your clinic. We will not contact the horse owner without first contacting you and obtaining specific permission for follow-up. We will request your help in obtaining permission of the owner for us to conduct a detailed herd investigation and the collection of blood samples from other horses resident in the herd. Further, we will ask your assistance in identifying and obtaining the co-operation of the owner of a control herd (a herd with no clinical disease nearest to the affected herd) where a similar investigation will be conducted.

All data collected during our study, including the identity of all practices, practitioners, owners and horses will be held in confidence. Reports will identify the location of case and control premises by provincial region or health district only.

Thank you for your assistance,

Hugh G.G. Townsend DVM, MS, Cheryl Waldner DVM, PhD, Tasha Epp DVM

I/We hereby grant permission to Prairie Diagnostic Services Limited to inform Drs. Hugh Townsend, Cheryl Waldner or Tasha Epp whenever my/our practice submits a diagnostic specimen for the purpose of making/confirming the diagnosis of WNV infection in horses. We understand the data collected will be treated with confidentiality and only a summary of the data will be published. Complete & fax this form to (306) 966-7159 by June 27th.

Clinic Name_____

_____ _____ _____

Name Signature Date

Equine West Nile Virus Case Investigation Reporting Form

Owner's Name _____

Address _____City/Town_____

Province_____ Postal code_____ _____

Legal Land Description (where horse(s) is located most of the summer)
_____

Tel. H (_____) _____-_____ W (_____) _____-_____

Email _____

Veterinarian _____

Address _____City/Town_____

Province_____ Postal code_____ _____

Tel. W (_____) _____-_____ Fax (_____) _____-_____

Email _____

Clinical Case Definition:
An equine with clinical signs plus one or more of the following: detection of IgM and or IgG antibody to the virus by ELISA or by PCR on tissues. Clinical signs must include one or more of the following: ataxia (wobbly), inability to stand (recumbent), multiple or single limb paralysis or death.
Date of symptom onset of case horse: ____/____/_____

For office use:

Date of case sample sent to lab _____/_____ Type of sample sent_____ Date of on farm samples sent to lab _____/____ Number of samples sent _____ Results ? _____	Outcome of illness: __ Recovered __still ill ___unknown __Died (dd/mm) ___/___/___ ___ Euthanized (dd/mm) ___/___/___

Herd level factors

How many total equids (horses, ponies, donkeys, mules) are on the premises? _____

In three weeks prior to Case horse's onset of symptoms, overall, what have the horses primarily had access to?
____ simple shelter * ____no shelter** ____enclosed barn
* simple shelter is one of three sides, lean-to, etc **pasture/corrals with or without bush cover

What preventative measures have been taken to avoid/control mosquitoes (overall in the herd)? More than one answer can be marked but identify main control with a #1.

____ ____nothing
____ ____fans in barn
____ ____altered lighting around barn
____ ____insecticide on premises (brand name_____)
____ ____sealed barn (kept indoors at night)
____ ____smudges
____ ____habitat/water removal

Horse Information

*Non case horses are horses that did not show any symptoms of WNV during the mosquito season

	Case horse	Non-case* 1	Non-case 2	Non-case 3	Non-case 4
Name of horse					
Lab number					
Brand/identifier					
Breed	Pony Light Draft Cross (L*D) Other	Pony Light Draft Cross (L*D) Other	Pony Light Draft Cross (L*D) Other	Pony Light Draft Cross (L*D) Other	Pony Light Draft Cross (L*D) Other
Age (Year of birth)					
Sex	St F G	St F G	St F G	St F G	St F G
Body size (by girth tape)					
Primary use:	Pl Br C F/R R	Pl Br C F/R R	Pl Br C F/R R	Pl Br C F/R R	Pl Br C F/R R
Pl – Pleasure Br- Breeding C- Competition F/R – Farm/Ranch R - Racing					
Mosquito control?					
Insecticide (spray, etc)	Y N	Y N	Y N	Y N	Y N

Shelter	No Simple Barn	No Simple Barn	No Simple Barn	No Simple Barn	No Simple Barn
Blanketing	None Dusk to Dawn Continuous	None Dusk to Dawn Continuous	None Dusk to Dawn Continuous	None Dusk to Dawn Continuous	None Dusk to Dawn Continuous
Vaccination?					
Date of first Vaccination (dd/mm/yy)					
# of vaccinations	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
Who vaccinated?	Vet Owner	Vet Owner	Vet Owner	Vet Owner	Vet Owner

Did any of the horses travel **off farm** in the three weeks before onset of symptoms (a distance of greater than 10 miles)?

	Case Horse	Non-case 1	Non-case 2	Non-case 3	Non-case 4
Location					
Date (dd/mm to dd/mm)					
Location					
Date (dd/mm – dd/mm)					

Re: West Nile Virus Study 2003
Dear Veterinarian:

Thank you for participating in our studies this past mosquito season. We are in the process now of having the lab data organized and will then proceed to put the picture of WNV in Saskatchewan together.

Total cases reported this year by PDS for the province of SK was 133. What we would like to be able to estimate is the number of undiagnosed cases across the province as well as the potential vaccination rate across the province. We are hoping again for your cooperation in this matter.

Please take the time to read and answer the question below. This form can be faxed back to (306) 966-7159 Attention: Tasha Epp. If we have not heard back from you in 3 business days, we will be contacting you by phone.

1. Estimate the number of **possible** clinical cases of West Nile virus your clinic saw or conversed about over the phone about but **never** sent blood work for testing. A clinical case would be a horse that had mild to severe neurological symptoms.

_____

2. How much vaccine (doses) was sold by your clinic during these two time periods?

(A) prior to July 1st, 2003 _____

(B) after July 1st, 2003 _____

CLINIC _____

Thanks you for taking the time to answer these questions.

WCVM WNV team, Dr. Townsend, Dr. Waldner, Dr. Tasha Epp, Dr. Rebecca Corrigan

Appendix B

Dear Horse Owner

Since 1999, West Nile Virus (WNV) has spread across the continent with great speed, affecting birds, horses and humans. Last summer was the first time the province of Saskatchewan experienced WNV. This virus can manifest itself in severe clinical disease that debilitates horses or even results in death.

A team of investigators from the Western College of Veterinary Medicine in Saskatoon is working to get answer about WNV this summer. With the assistance of horse owners and veterinarians in the province, our team is attempting to determine what factors may influence the chances of horses becoming infected or show disease. Factors such as location, individual horse factors, management practices or vaccination.

What we are asking of you is to allow a visit to your premises by a research team member. During this visit we would ask to take blood samples from resident horses on the premises to test for antibodies to WNV. As well, we ask you to assist in filling out a simple questionnaire that addresses the areas of interest mentioned previously. The results of the blood testing will be reported back to you through your veterinarian to facilitate any questions that might arise.

This data (including the identity of all owners and horses) will be kept strictly confidential and only a summary of the data (location by provincial region or health district) will be published.
If at any time you wish to withdraw from this study, you are free to do so.

Thank you very much for your assistance,

Hugh G.G. Townsend DVM, MS, Cheryl Waldner DVM, PhD, Tasha Epp DVM

I hereby grant permission to the research team of Drs. Hugh Townsend, Cheryl Waldner or Tasha Epp for a herd visit, blood sampling for testing of WNV antibodies and the completion of an investigation form. I understand that the information will be kept confidential and I can withdraw from the study at any time. When the results of the testing are completed, the info will be reported to me through my veterinarian.

_____ _____ _____

Name Signature Date

Address_____

Phone number (where can be reached during the daytime)_____

Please indicate your veterinarian _____

Clinic_____

Equine West Nile Virus Seroprevalence Investigation Reporting Form

Sampling Date 1: ____/____/____ (dd/mm/yy)

Sampling Date 2: _____/_____/_____

Owner's Name _____

Address _____City/Town_____

Province_____ Postal code_____ _____

Legal Land Description (where horses are located most of the summer)
_____

Tel. H (_____) _____-_____ W (_____) _____-_____

Email _____

Veterinarian _____

Address _____City/Town_____

Province_____ Postal code_____ _____

Tel. W (_____) _____-_____ Fax (_____) _____-_____

Email _____

Additional Comments:
_____
_____
_____

FOR OFFICE USE:

Lab Information: Date of samples sent to lab_____
Number of samples sent_____
Results received? _____

Herd level questions:
How many equids (horses, ponies, donkeys, mules) are on the premises?_____

In the last three weeks, overall, what have the horses primarily had access to?
____simple shelter* _____ no shelter** _____enclosed barn
*simple shelter is three sided, lean-to, etc ** pasture/corrals with or without bush cover

Have you used any of these overall herd preventative measures to avoid mosquitoes in the last three weeks? More than one answer can be marked but identify main control with a #1.

_____ _____nothing
_____ _____altered lighting around barn
_____ _____fans in barn
_____ _____insecticide on premise (brand name_____)
_____ _____smudges
_____ _____habitat/water removal
_____ _____sealed barn (kept indoors at night)

HORSE INFORMATION

	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5
Horse's name					
Lab number					
Brand/ Identifier					
Breed	Pony Light Draft Cross(L*D) Other	Pony Light Draft Cross(L*D) Other	Pony Light Draft Cross(L*D) Other	Pony Light Draft Cross(L*D) Other	Pony Light Draft Cross(L*D) Other
Age (year of birth)					
Sex	St F G	St F G	St F G	St F G	St F G
Body size (by girth tape)					
Primary use	Pl Br C F/ R R	Pl Br C F/R R	Pl Br C F/R R	Pl Br C F/R R	Pl Br C F/R R
Pl – pleasure Br – Breeding C – Competition F/R – Farm/Ranch R - Racing					

Mosquito control?					
Insecticide use (sprays, etc)	Y N	Y N	Y N	Y N	Y N
Shelter	No Simple barn	No Simple barn	No Simple barn	No Simple barn	No Simple barn
Blanketing	None Dusk to Dawn Continuous	None Dusk to Dawn Continuous	None Dusk to Dawn Continuous	None Dusk to Dawn Continuous	None Dusk to Dawn Continuous
Vaccination?					
Date of 1st Vaccination (dd/mm/yy)					
# of vaccinations	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
Who Vaccinated?	Vet Owner	Vet Owner	Vet Owner	Vet Owner	Vet Owner

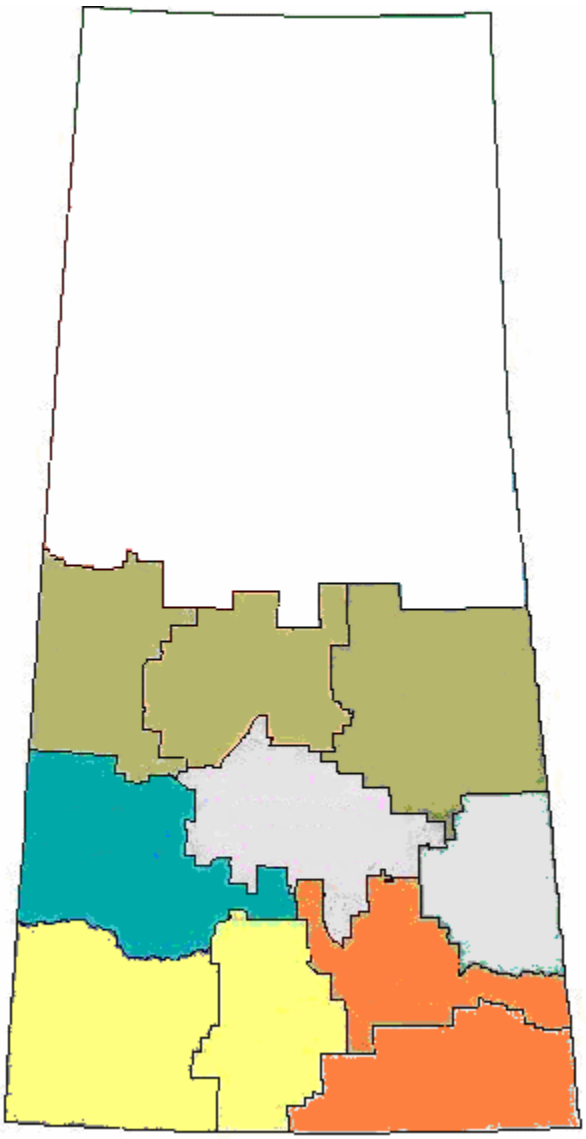
	Horse 6	Horse 7	Horse 8	Horse 9	Horse 10
Horse's name					
Lab number					
Brand/ Identifier					
Breed	Pony Light Draft Cross(L*D) Other	Pony Light Draft Cross(L*D) Other	Pony Light Draft Cross(L*D) Other	Pony Light Draft Cross(L*D) Other	Pony Light Draft Cross(L*D) Other
Age (year of birth)					
Sex	St F G	St F G	St F G	St F G	St F G
Body size (by girth tape)					
Primary use:	Pl Br C F/ R R	Pl Br C F/ R R	Pl Br C F/ R R	Pl Br C F/ R R	Pl Br C F/ R R
Pl – pleasure Br – Breeding C – Competition F/R – Farm/Ranch R – Racing					

Mosquito control?					
Insecticide use (sprays, etc)	Y N	Y N	Y N	Y N	Y N
Shelter No Simple	No Simple barn	No Simple barn	No Simple barn	No Simple barn	No Simple barn
Blanketing	None Dusk to Dawn Continuous	None Dusk to Dawn Continuous	None Dusk to Dawn Continuous	None Dusk to Dawn Continuous	None Dusk to Dawn Continuous
Vaccination?					
Date of First Vaccination (dd/mm/yy)					
# of vaccinations	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
Vaccinated by whom?	Vet Owner	Vet Owner	Vet Owner	Vet Owner	Vet Owner

Have any of the horses traveled **off farm** in the last three weeks (a distance greater than 10 miles)?

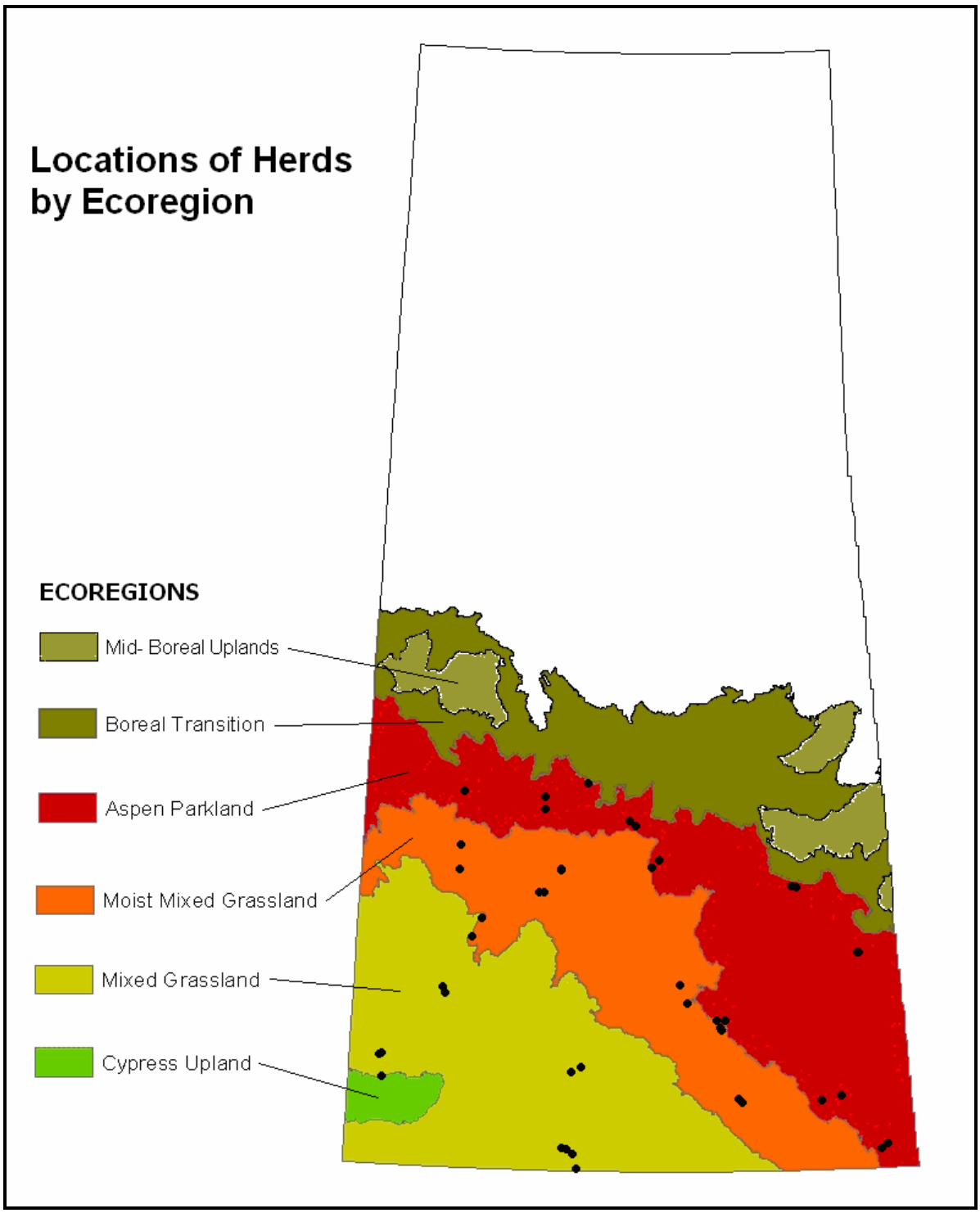
	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5
Location					
Date (dd/mm - dd/mm)					
Location					
Date (dd/mm –dd/mm)					

	Horse 6	Horse 7	Horse 8	Horse 9	Horse 10
Location					
Date (dd/mm – dd/mm)					
Location					
Date (dd/mm – dd/mm)					



Zones for seroprevalence study

Appendix C


Locations of Herds
by Ecoregion
ECOREGIONS
Mid- Boreal Uplands
Boreal Transition
Aspen Parkland
Moist Mixed Grassland
Mixed Grassland
Cypress Upland

Dear _____

As part of a West Nile Virus study at the Western College of Veterinary Medicine, University of Saskatchewan, we are asking your assistance in identifying control herds for our case control study. The following is a herd from your clinic that had a clinical case of West Nile Virus this season:

_____

The criteria for a control herd is that it has had no clinical (neurological, confirmed by lab work) case of West Nile Virus this season, it is as close as possible to the case herd and has as least 5 horses on the premises. If you can help us in this matter, please indicate the name and phone number of a potential herd.

_____ ph # _____

Also, due to time constraints we are looking to contract your clinic to take the blood work from these farms once they have agreed to the study (our research team will call and talk to them about what is involved). If you are willing to do this, please indicate whom to contact was the owners have been reached.

There is no time frame specific to do the blood taking within, but we hope to have this completed by the end of October. We would request that the blood work from the two farms be done within a few days of each other to ensure the best comparable results.

Thank you for your time,

Tasha Epp, DVM, grad student WCVM

To: _____

Here are the instructions in regards to the blood sampling for the West Nile Virus study.

1. Please label the tubes with the horse's name and the owners last name.

2. On the PDS form, put **"for the West Nile Virus study, RS-Townsend 7-01457"** so that it gets charged to our study and not the owners. Also, if you could put the date the samples were taken that would help a lot. Please indicate it is for **"WNV IgM Elisa"**.

3. Please have the bleeding done by the end of October, and **both case and control** farm within a few days of each other at the most.

4. Please take up to 8 blood samples (**minimum of 5**) from each place (both case and control farm). It does not matter if they are vaccinated or not. Whatever is available to bleed works for us (any age, breed, etc).

5. I will contact the owners for all the information about the horses and management once the blood work is complete. I will also contact the owners with the results of the testing at that time.

6. For the charges your clinic has for taking the blood samples (mileage fee and blood taking fee), please direct those to the study as well. The invoice can be sent to Tasha Epp, LACS, WCVM, U of S, 52 Campus Drive, Saskatoon, SK, S7N 5B4.

Thanks you for all of your help.

Tasha Epp

Equine West Nile Virus Control Investigation Reporting Form

Owner's Name _____

Address _____City/Town_____

Province_____ Postal code_____ _____

Legal Land Description (where horse(s) is located most of the summer)_____

Tel. H (_____) _____-_____ W (_____) _____-_____

Email _____

Veterinarian _____

Address _____City/Town_____

Province_____ Postal code_____ _____

Tel. W (_____) _____-_____ Fax (_____) _____-_____

Email _____

Control herd and Clinical Case Definition:

To qualify as a control herd, there must be and have been no case animals present on the farm during study period.

An case is an equine with clinical signs plus one or more of the following: detection of IgM and or IgG antibody to the virus by ELISA or by PCR on tissues. Clinical signs must include one or more of the following: ataxia (wobbly), inability to stand (recumbent), multiple or single limb paralysis or death.

For Office Use:

Lab information: Date samples sent to lab _____/_____/_____ (dd/mm/yy)
Number of samples sent _____
Results? _____

Herd level Factors

How many total equids (horses, ponies, donkeys, mules) are on the premises? _____

In the last three weeks, overall, what have the horses primarily had access to?
_____ simple shelter* _____ no shelter** _____enclosed barn
*simple shelter is three sided, lean-to, etc ** pasture/corrals with or without bush cover

Have you used any of these overall herd preventative measures to avoid mosquitoes in the last three weeks? More than one answer can be marked but identify main control with a #1.

____ _____nothing
____ _____fans in barn
____ _____altered lighting around barn
____ _____insecticide on premises (brand name_____)
____ _____ sealed barn (kept indoors at night)
____ _____smudges
____ _____habitat/water removal

Individual Factors

	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5
Name of horse					
Lab Number					
Brand/identifier					
Breed	Pony Light Draft Cross (L*D) Other	Pony Light Draft Cross (L*D) Other	Pony Light Draft Cross (L*D) Other	Pony Light Draft Cross (L*D) Other	Pony Light Draft Cross (L*D) Other
Age (year of birth)					
Sex	St F G	St F G	St F G	St F G	St F G
Body size (by girth tape)					
Primary use:	Pl Br C F/R R	Pl Br C F/R R	Pl Br C F/R R	Pl Br C F/R R	Pl Br C F/R R
Mosquito Control?					
Insecticide (spray, etc)	Y N	Y N	Y N	Y N	Y N
Shelter	No Simple Barn	No Simple Barn	No Simple Barn	No Simple Barn	No Simple Barn
Blanketing	None Dusk to Dawn	None Dusk to Dawn	None Dusk to Dawn	None Dusk to Dawn	None Dusk to Dawn

	Continuous	Continuous	Continuous	Continuous	Continuous
Vaccination?					
Date of first vaccination (dd/mm/yy)					
# of vaccinations	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
Who vaccinated?	Vet Owner	Vet Owner	Vet Owner	Vet Owner	Vet Owner

Have any of the horses travel **off farm** in the last three weeks (a distance of greater than 10 miles)?

	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5
Location					
Date (dd/mm to dd/mm)					
Location					
Date (dd/mm to dd/mm)					

Appendix D

West Nile Reporting Form (2004):

Clinic name: _____

Week #: _____

To be completed weekly and sent in for surveillance compilation. ** starred entries are required, others only if known.

**Probable (P) or confirmed positive (L)	Date of onset (if known)	Outcome of illness (if known)	**RM /nearest town	WNV Vaccine history (if known)

Probable (P) : A horse showing classic symptoms of WNV but not tested by the lab

Confirmed Positive (L): A horse with classic symptoms that was tested positive for IgM antibodies by the lab.

Please fax to___(306) 966-7159___ Attention: Tasha Epp or Rebecca Corrigan.

Thank you

2004 Summary Report of Clinical WNV Horse Case Surveillance Evaluation

Purpose: The purpose of the clinical WNV horse case surveillance was to provide an estimation of which RHAs had WNV cases in the horse population in order to evaluate potential risk of WNV in the human population, in conjunction with other surveillance being done by Saskatchewan Health

Key Accomplishments:

- Surveillance was initiated in the Sun Country, Five Hills, Cypress, Regina/Qu'Appelle, Sunrise, Saskatoon, Heartland, Kelsey Trail, Prince Albert Parkland and Prairie North health regions
- 22 private veterinary clinics and the Western College of Veterinary Medicine participated in surveillance
- A total of 10 suspicious clinical horse cases were recorded
- Confirmatory testing was initiated in 4 cases; no confirmed clinical horse cases occurred during 2004

RHA	Sun Country	Five Hills	Cypress	Regina Qu'Appelle	Sunrise	Saskatoon	Heartland	Kelsey Trail	Prince Albert Parkland	Prairie North
Number of Clinics Reporting	3	2	2	3	2	3	3	2	1	2
Number of Suspicious Horse Cases	1	3	1*	1**	0	0	1**	1	0	2 (1**)
Number of Confirmed Horse Cases	0	0	0	0	0	0	0	0	0	0
Week(s) of Occurrence	34	29, 31, 33	34	--	--	--	Unknown	Unknown	--	32, 33

*Was to undergo IgM ELISA testing for confirmation but was lost
**Underwent confirmatory testing with IgM ELISA and came back negative

Highlights of 2004 Surveillance:

- A good response came from private clinics throughout Saskatchewan. They were very interested in participating in the surveillance and were extremely helpful
- Weekly follow-up via fax or telephone for all clinics was easy to do
- A large number of clinics participated from each RHA which resulted in an extensive coverage of the province

Recommendations for 2005:

- Confirmatory testing with IgM ELISA in suspicious cases was low—setting aside money to confirm "x" number of tests of the first reported suspicious cases in each RHA may help to increase the number of confirmed cases of WNV
    - Veterinary clinics would need to be made aware of this potential funding early in the season in order to advise clients with horses displaying potential clinical signs of WNV
    - A timeline of testing, based on the rainfall, mosquito activity and other predictors of WNV ecology, would need to be established to avoid testing horses before potential WNV cases could likely occur

Dear Colleagues June 2005

We are writing to seek permission to be informed of West Nile Virus positive horses in your practice. This initial contact is to inform you of the study and to prepare for the season ahead.

The research team is associated with the Western College of Veterinary Medicine, Saskatoon, Saskatchewan. This summer, we intend to conduct surveillance of the occurrence of clinical West Nile Virus infection (WNV) of horses in Saskatchewan and test blood samples submitted for Coggins tests from July 10th to September 15th for antibodies to WNV.

We hope to locate clinically affected animals by having the Prairie Diagnostics Services (PDS) in Saskatoon inform us when they confirm the diagnosis of WNV (blood or postmortem) in Sask. horses. However, this will only be possible if veterinary practices in Saskatchewan sign a release allowing the laboratory to inform us regarding the identity of practices submitting one or more positive samples. The submission of any suspected WNV samples to PDS would help to facilitate this study.

We hope to obtain permission to test blood samples sent in for Coggins tests during the specified time period by having you the veterinarian submit a release form, signed by the owner at the time of sample collection. The release form must be included with the Coggins submission and the sample sent to PDS. The sample will be tested for IgM antibodies and the result will be sent back to you, the submitting veterinarian to be forwarded to the owner.

We will not require any contact with the horse owners for either portion of surveillance. However, we will request your help in obtaining the location of where the horse was residing in the three weeks prior to symptoms developing. This can be an exact legal land description or merely the Rural Municipality name/number. Please include this information on the submission form to the lab for clinical cases and on the release form for the Coggins test submissions.

All data collected during our study, including the identity of all practices, practitioners, owners and horses will be held in confidence. Reports will identify the locations by rural municipality or health district only.

Thank you for your assistance, Hugh G.G. Townsend DVM, MS, Cheryl Waldner DVM, PhD, Tasha Epp DVM

I/We hereby grant permission to Prairie Diagnostic Services Limited to inform Drs. Hugh Townsend, Cheryl Waldner or Tasha Epp whenever my/our practice submits a diagnostic specimen for the purpose of making/confirming the diagnosis of WNV infection in horses. We understand the data collected (specifically horse location) will be treated with confidentiality and only a summary of the data will be published. Complete & fax this form to (306) 966-7159 by June 27th. For any questions, phone Tasha Epp at (306) 966-7166.

Clinic Name_____

_____ _____ _____

Name Signature Date

WNV Release Form:
This form is to accompany the submission of a blood sample for Coggins testing. Please photocopy as needed.

Time period allow for submission of samples: samples taken between July 10th and September 15th, 2005

The samples will be tested for IgM antibodies to West Nile Virus. This test indicates whether the horse has been exposed to WNV recently. If the horse is showing no symptoms of WNV and the test is positive, the horse will likely not show any symptoms but instead will clear or may have already cleared the infection with the virus uneventfully. Only the location and test result information will be used for research purposes.

This form must be completed for testing to ensue, so please ensure all areas are completed as indicated.

I/We (owner of the horse) hereby give permission to the West Nile Virus (WNV) research team at the Western College of Veterinary Medicine (WCVM) to use the submitted sample and the following information for research purposes. The sample is to be tested at Prairie Diagnostic Services Limited (PDS) in Saskatoon, SK for IgM antibodies to West Nile Virus (WNV). The test result is to be sent to my veterinary listed below. We understand the data collected (specifically horse location) will be treated with confidentiality and only a summary of the data will be published.

_____ _____ _____
Name of owner Signature Date

_____
Legal land location (of the horse in the two weeks prior to sampling)

Veterinarian: _____

Vet clinic: _____

Clinic fax number: _____

Clinic phone number: _____

West Nile Reporting Form (2005):

Clinic name: _____

Week #: _____

To be completed weekly and sent in for surveillance compilation.

Probable (P) or confirmed positive (C)	Test result (indicate if awaiting result)	Week (or date) of onset of symptoms	RM/nearest town

Probable (P): A horse showing classic symptoms of WNV but not tested by the lab

Confirmed Positive (C): A horse with classic symptoms that tested positive for IgM antibodies by the lab. (Please indicate horses that are being tested but the result is not confirmed yet in this category as well.)

Please fax to___(306) 966-7159___ Attention: Tasha Epp

Thank you

2005 Summary Report of Clinical WNV Horse Case Surveillance Evaluation

Purpose: The purpose of the clinical WNV horse case surveillance was to provide an estimation of which RHAs had WNV cases in the horse population in order to evaluate potential risk of WNV in the human population, in conjunction with other surveillance being done by Saskatchewan Health

Key Accomplishments:

- Surveillance was initiated in the Sun Country, Five Hills, Cypress, Regina/Qu'Appelle, Sunrise, Saskatoon, Heartland, Kelsey Trail and Prairie North health regions (only Prince Albert Parkland did not have representation)
- 19 private veterinary clinics and the Western College of Veterinary Medicine participated in surveillance
- A total of 10 confirmed and 4 probable clinical horse cases were recorded
- Confirmatory testing was charged to Saskatchewan Health in 7 cases

RHA	Sun Country	Five Hills	Cypress	Regina Qu'Appelle	Sunrise	Saskatoon	Heart-land	Kelsey Trail	Prince Albert Parkland	Prairie North
Number of Clinics Reporting	3	2	2	3	2	2	2	1	0	2
Number of Probable Horse Cases	0	0	0	0	2	2	0	0	0	0
Number of Confirmed Horse Cases	2	2	1	1	0	2	0	2	0	0
Total number of cases	2	2	1	1	2	4	0	2	0	0
Week(s) of Occurrence	30, 35	35, 36	35	33	32	33, 38	--	30, 37	--	--

Week 30 corresponds to July 24 – 30th and week 37 corresponds to September 11 -17th, 2005.

Highlights of 2005 Surveillance:

- A good response came from private clinics throughout Saskatchewan. They were very interested in participating in the surveillance and were extremely helpful
- Weekly follow-up via fax or telephone for all clinics was easy to do but did become time consuming as the weekly response rate did drop off significantly in September
- Clinics response rate to the weekly faxing ranged from 20 – 100%
- A large number of clinics participated from each RHA (except from Prince Albert Parkland) which resulted in an extensive coverage of the province; reporting clinics also informed researcher of other clinics to contact in regards to other potential clinical cases
- Coverage of a small number of confirmatory tests with IgM ELISA was a success
    - Veterinary clinics were aware of this potential funding early in the season and were able to use this to test horses displaying potential clinical signs of WNV where the owner didn't want to test

- The timeline of testing confined to between mid-July and mid-September was effective in confirming a few negative horses in mid-July despite these horses showing symptoms seemingly consistent with WNV, defining the first confirmed positive case in late July and the last recorded positive case in mid-September.

Recommendations for future surveillance:

- Continue the free testing portion of the program to ensure confirmation of at least the first positive per RHA
- Continue the use of sentinel veterinary clinics only if there will be a person solely in charge of reminders (phone calls as vets do not read faxes) for clinics to report weekly
- Weed out the clinics with poor response rates and potentially replace with other clinics in those regions; keep at least 2 clinics per RHA for the most comprehensive coverage of the area (RHAs 8 and 9 could get away with only one clinic per region)
- Continue the recording of both probable and confirmed cases of WNV clinical disease in horses as a part of an integrated surveillance initiative