Transmission of West Nile Virus in the Niagara Region Among a Population At Risk for Exposure

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ABSTRACT

Background. West Nile Virus (WNV), a mosquito-borne flavivirus, is one of an increasing number of infectious diseases that have been emerging or re-emerging in the last two decades. Since the arrival of WNV to Canada to present date, the Niagara Region has only reported 30 clinical cases, a small number compared to the hundreds reported in other regions of similar conditions. Moreover, the last reported human case in Niagara was in 2006. As it has been demonstrated that the majority of WNV infections are asymptomatic, the question remains whether the lack of clinical cases in Niagara truly reflects the lack of transmission to humans or if infections are still occurring but are mostly asymptomatic.

Objectives. The general objective of this study was to establish whether or not active WNV transmission could be detected in a human population residing in Niagara for the 2007 transmission season. To fullfil this objective, a cross-sectional seroprevalence study was designed to investigate for the presence of anti-WNV antibodies in a sample of Mexican migrant agricultural workers employed in farms registered with the Seasonal Agricultural Workers Program (SAWP). Due to the Mexican origin of the study participants, three specific research objectives were proposed: a) determine the seroprevalence of anti-WNV antibodies as well as anti-Dengue virus antibodies (a closely related virus prevalent in Mexico and likely to confound WNV serology); b) analyze risk factors associated with WNV and Dengue virus seropositivity; and c) assess the awareness of study participants about WNV infection as well as their understanding of the mode of transmission and clinical importance of the infection.

Methodology: After obtaining ethics clearance from Brock University, farms were visited and workers invited to participate. Due to time constraints, only a small number of farms were enrolled with a resulting convenience and non-randomized study sample. Workers' demographic and epidemiological data were collected using a standardized questionnaire and blood samples were drawn to determine serum anti-WNV and anti-Dengue antibodies with a commercial ELISA. All positive samples were sent to the National Microbiology Laboratory in Winnipeg, Manitoba for confirmation with the Plaque Reduction Neutralization Test (PRNT). Data was analyzed with Stata 10.0. Antibody determinations were reported as seroprevalence proportions for both WNV and Dengue. Logistic regression was used to analyze risk factors that may be associated with seropositivity and awareness was reported as a proportion of the number of individuals possessing awareness over the total number of participants.

Results and Discussion. In total 92 participants working in 5 farms completed the study. Using the commercial ELISA, seropositivity was as follows: 2.2% for WNV IgM, 20.7% for WNV IgG, and 17.1% for Dengue IgG. Possible cross-reactivity was demonstrated in 15/20 (75.0%) samples that were positive for both WNV IgG and Dengue IgG. Confirmatory testing with the PRNT demonstrated that none of the WNV ELISA positive samples had antibodies to WNV but 13 samples tested positive for anti-Dengue antibodies (14.1% Dengue sereoprevalence). The findings showed that the ELISA performance was very poor for assessing anti-WNV antibodies in individuals previously exposed to Dengue virus. However, the ELISA had better sensitivity and specificity for assessing anti-Dengue antibodies.

Whereas statistical analysis could not be done for WNV seropositivity, as all samples were PRNT negative, logistic regression demonstrated several risk factors for Dengue exposure. The first year coming to Canada appeared to be significantly associated with increased exposure to Dengue while lower socio-economic housing and the presence of a water basin in the yard in Mexico appeared to be significantly associated with a decreased exposure to Dengue. These seemingly contradictory results illustrate that in mobile populations such as migrant workers, risk factors for exposure to Dengue are not easily identified and more research is needed. Assessing the awareness of WNV and its clinical importance showed that only 23% of participants had some knowledge of WNV, of which 76% knew that the infection was mosquito-borne and 47% recognized fever as a symptom. The identified lack of understanding and awareness was not surprising since WNV is not a visible disease in Mexico. Since WNV persists in an enzootic cycle in Niagara and the occurrence of future outbreaks is unpredictable, the agricultural workers remain at risk for transmission. Therefore it important they receive sufficient health education regarding WNV before leaving Mexico and during their stay in Canada.

Conclusions. Human transmission of WNV could not be proven among the study participants even when due to their occupation they are at high risk for mosquito bites. The limitations of the study sample do not permit generalizable conclusions, however, the study findings are consistent with the absence of clinical cases in the Niagara Region, so it is likely that human transmission is indeed neglible or absent. As evidenced by our WNV serology results, PRNT must be utilized as a confirmatory test since false positivity occurs frequently. This is especially true when previous exposure to Dengue virus is likely.

ACKNOWLEDGEMENTS

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I would like to acknowledge Dr. Mark Loeb for fulfilling the role as External Examiner and for providing the confirmatory serum tests, for without these the laboratory work would not have been completed.

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My love and thanks to my wife June and my sons for their support during these past two years. My role as husband and father were compromised during this work and I am profoundly appreciative of the patience and love they provided.

This work is dedicated to the Mexican migrant agricultural workers.

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AFP: Acute Flaccid Paralysis

C: Capsid

CDC: Centers for Disease Control and Prevention

CCWHC: Canadian Cooperative Wildlife Health Centre

CNS: Central Nervous System

CSF: Cerebrospinal Fluid

DEET: Diethyl-methyl-toluamide

DNA: Deoxyribonucleic Acid

E: Envelope

ELISA: Enzyme Linked Immunosorbent Assay

GBS: Guillain Barré Syndrome

HI: Hemagglutination Inhibition

HRP: Horseradish Peroxidase

IFN: Interferon

Ig: Immunoglobulin

IP: Inducible Protein

M: Membrane

MCP: Monocyte Chemoattractant Protein

MHC: Major Histocompatibility Complex

MIF: Macrophage Inhibiting Factor

MIG: Monokine Induced Gamma Interferon

mRNA: Messenger Ribonucleic Acid

NCR: Non-coding Region

NIAID: National Institute of Allergy and Infectious Disease

NIH: National Institutes of Health

NK: Natural Killer Cells

NS: Non-structural

NVT: Non-viremic Transmission

ORF: Open Reading Frame

PHAC: Public Health Agency of Canada

PrM: Premembrane

PRNT: Plaque Reduction Neutralization Test

RdRp: RNA-dependent RNA polymerase

RNA: Ribonucleic Acid

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

SAWP: Seasonal Agricultural Workers Program

siRNA: Small Interfering Ribonucleic Acid

TMB: Tetramethylbenzidine

TNF: Tumour Necrosis Factor

US: United States

USGS: United States Geological Survey

WNND: West Nile Neurological Disease

CHAPTER 1: INTRODUCTION

Globalization of infectious diseases have become more evident with the movement of West Nile Virus (WNV) to the American continent [1]. An RNA flavivirus, WNV is considered the most geographically widespread arbovirus in the world and exists in an enzootic cycle between birds as the natural and amplifying reservoir and mosquitoes as the arthropod vector [2]. Humans and horses are the most susceptible hosts, although the attained level of viremia is not sufficient to allow further vectorial transmission [3]. Worldwide, human WNV infection is asymptomatic in approximately 80% of all those infected, with the remaining 20% manifesting flu-like symptoms and sometimes a roseolar skin rash. Clinical and epidemiological studies have demonstrated that less than 1% of those with symptoms will develop neurological disease [4, 5].

West Nile Virus was considered a minor human pathogen until 1996 when a number of epidemics began to occur resulting in an unprecedented increase of neurological disease and death [6, 7]. First isolated in 1937, from a woman with febrile disease living in the West Nile province of Uganda [8], the virus is now endemic in Africa, Asia, Europe, Australia, and, as of 1999, North America [4]. Within North America, the unpredictability of WNV transmission during the last ten years has been puzzling. The virus entered the continent apparently through New York City in 1999, when animals and humans were unequivocally identified with a WNV strain 99.8% homologous to a strain causing an epizootic in geese in Israel [9, 10]. At present, although the year-to-year number of cases varies considerably, WNV is considered endemic in the United States

(US), as human, bird, or mosquito infections have been reported from all states except Hawaii and Alaska [11].

In Mexico, thanks to active surveillance systems, serological evidence of WNV transmission was found in birds and horses in 2002. Isolation of the virus was first demonstrated in a raven in 2003 and the first human case was reported in 2004 [12-16]. To date, despite favorable ecological conditions, human cases are almost non-existent in the country. Moreover, as opposed to the case of US and Canada, the infection in horses and other mammals in Mexico have shown exceptional low rates in both morbidity and mortality [12].

In Canada, on the other hand, WNV activity started in August 2001, when the virus was found in dead birds and mosquito pools in southern Ontario [17]. The first human case was detected in Ontario in 2002 and in the following years the number of cases have varied tremendously: an upward trend in Canada was identified from 2002 to 2003 (with 414 and 1,481 cases for each year, respectively), followed by reduced numbers in 2004 to 2006 (25, 225 and 151 cases, respectively), which rose again in 2007 (2,215 cases). In 2008, at the end of the WNV transmission season, a total of only 36 cases had been reported in Canada [17]. Human cases of WNV infections in the Niagara Peninsula have shown similar unpredictable trends: the largest-albeit small-numbers of cases were reported from 2002 and 2003 (18 and 5 cases, respectively), with very few cases reported from 2004 to 2006 (1, 3, 3 cases, respectively) and no cases have been reported in 2007 and 2008 [18].

When comparing the widespread WNV transmission in the United States with the isolated epidemics in Canada and the almost non-existent human infection in Mexico, it is difficult to explain the differences of WNV transmission in North America. The epidemiological triangle with the ever changing interactions between agent, host, and the environment, is naturally at the heart of the explanation as to why the disease patterns of WNV are occurring with no apparent consistent predictors. Could it be that WNV is a virus that requires very specific elements of the epidemiological triad to be fulfilled in order for the virus to be successful in causing epidemics and establishing itself as an endemic virus to any particular geographical area? Historically, through Europe and the Middle East, a pattern of recurrent outbreaks have occurred in previously established endemic areas [2]. Silent periods as long as two decades have been observed between epidemics/epizootics in Europe and the Mediterranean basin. Although Niagara currently appears to have low transmission, WNV is still present in the avian population as of 2008 and has been found in one mosquito pool to date of this year [18]. Thus it is prudent to continue surveillance and preventive measures as a new epidemic could occur.

To advance the understanding of WNV transmission in Niagara Region, the present study aimed to establish whether or not active WNV transmission (as determined by the presence of specific circulating antibodies) could be detected in a population, that due to their occupation as agricultural workers, were more likely than the general population to have been exposed to vectorial transmission should this be taking place at sufficient levels in Niagara.

CHAPTER 2: REVIEW OF LITERATURE FOR WEST NILE VIRUS AND WEST NILE VIRUS INFECTION AND DISEASE

2.1 History of West Nile Virus infection and disease

Alexander the Great was thought to have succumbed in Babylon in 323 BC to the neurological manifestations of WNV [19, 20]. Babylon, located in the area of present day Iraq, was along the Tigris and Euphrates rivers as well as being surrounded by swamp. The geography at the time is thought to have been able to support a large mosquito as well as large bird populations. Plutarch, the Greek historian wrote, "when he arrived before the walls of the city he saw a large number of ravens flying about and pecking one another, and some of them fell dead in front of him"[20]. Alexander is thought to have developed a febrile disease accompanied by signs of encephalitis and acute flaccid paralysis. Polioencephalomyelitis, influenza, poisoning, and other parasitic diseases are considered; however, the behaviour and the deaths of ravens preceding Alexander's death are similar to the situation that occurred in New York City in 1999. In a letter to the editor, Cunha (2004) attempted to discount this proposal and indicated that typhoid is the most likely explanation for the demise of Alexander the Great [19]. It is possible that WNV has been around for many centuries and was not recognized until 1937 when a Ugandan woman suffering from febrile disease was diagnosed [8].

Since 1937 the virus has established itself as an endemic infectious disease of humans on every continent except the Antarctica. Initially endemic in Africa, Asia, Europe, and the Middle East the virus crossed the Atlantic in 1999 to infect North America. The virus exists in an enzootic cycle between the mosquito vector and birds with humans and

horses being the most common incidental hosts. Prior to 1996 the disease appeared as sporadic epidemics with rare neurological cases and deaths. Since 1996 the virulence of this virus has increased through a series of accumulated mutations, particularly in North America, where it is the most common arbovirus to have affected the continent [4]. The virus has not yet been diagnosed beyond very rare cases in Mexico, Central America, and South America [12].

2.2 Etiological viral agent

2.2.1 Taxonomy

WNV is a single stranded enveloped RNA virus with a positive sense polarity. RNA viruses are categorized into 3 superfamilies based on their RNA-dependent RNA polymerases (RdRP) with WNV belonging to superfamily 2. WNV is part of the family Flaviviridae and genus Flavivirus. It is further categorized into the Japanese encephalitis serocomplex based on serological cross-reactivity. Other viruses within the Japanese encephalitis serocomplex include Alfuy, Cacipacore, Japanese encephalitis, Koutango (a distant African variant of WNV), Kunjin, Murray Valley encephalitis, St. Louis encephalitis, Rocio, Stratford, Usutu, and Yaounde. Other viruses in the genus Flavivirus but not in the Japanese serocomplex include Dengue and Yellow Fever [21].

The virus is further categorized into 2 distinct phylogenetic lineages based on nucleotide sequences. Lineage I viruses have been isolated from all continents except Antarctica and are responsible for most of the neurological disease and death. Lineage I is further categorized into 4 clades: Kunjin, Indian, A, and B. Clade B has been responsible for the epidemic in North America. The virus strain introduced into New York City in 1999

(NY99) demonstrated an increase in virulence over the other strains circulating in the Mediterranean area at the time. NY99 was found to be 99.7% homologous with the concurrent epidemic occurring in Israel. Lineage II is confined to South Africa and Madagascar and has not been associated with epidemic transmission [4].

2.2.2 Morphology

The viral particle measures 50nm with a nucleocapsid core of 30nm in diameter. It is an enveloped virus with a lipid bilayer containing two enveloped glycoproteins known as the envelope (E) and premembrane (prM) proteins. The icosahedral nucleocapsid core contains a single strand of positive sensed RNA complexed with multiple copies of capsid protein known as C. The genome is 11 kb in length with a single open reading frame (ORF) flanked by a 5' (100 nucleotides) and a 3' (400-700 nucleotides) noncoding region (NCR) with a 5' cap but lacking a 3' tail. The DNA encodes for a single polypeptide which is cleaved by viral and host enzymes into 3 structural proteins (C, prM, and E) responsible for virus particle formation and 7 nonstructural proteins responsible for virus replication and virion assembly (fig.1) [4, 22].

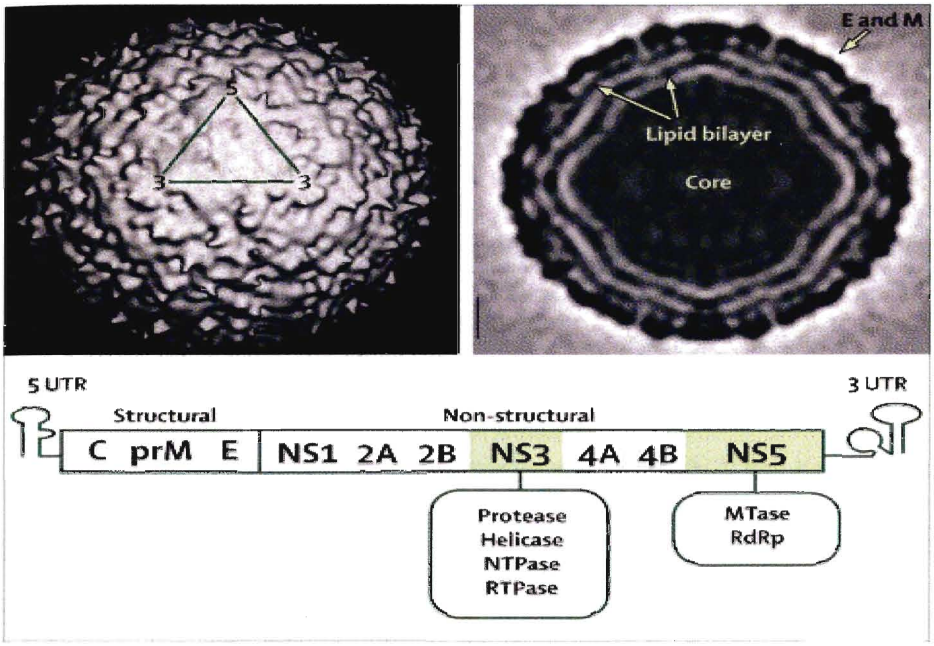


Fig. 1. West Nile virion and genome. WNV structure as reconstructed by cryo-electron microscopy. A surface-shaded view with one asymmetric unit of the icosahedron indicated by the triangle. Central section of the reconstruction showing the concentric layers of mass density. WNV genome, a single-stranded positive-sense RNA, approximately 11 kb in length consisting of a 5' untranslated region (UTR), a single long open-reading frame, and a 3' UTR. The open-reading frame encodes three structural and seven nonstructural proteins. Kramer, Steyer, and Ebel (2008) [2].

2.2.3 Life cycle

Following a mosquito bite, WNV initially begins its replication in the intradermal dendritic cells. Domain III of the E glycoprotein, a hemagglutinin, is responsible for viral receptor binding to the host cell (fig.2-1st step) [5, 23]. The virus then enters the cell by endocytosis creating a vesicle around the viral particles (fig.2-2nd step). Once inside the cell there is a drop in the pH causing a conformational change in the E protein exposing a hydrophobic domain, allowing fusion between the host and viral membranes (fig.2-3rd step). The virus then fuses with the prelysosomal endocytic compartment where the fusion between the virus and the membrane releases the nucleocapsid core (fig.2-4th step). The viral RNA is released into the cytoplasm making it available for translation

and replication (fig.2-5th step). The viral RNA acts as mRNA and is translated on the host ribosomes into a polyprotein. Through enzymatic cleavage with both host and viral enzymes the polyprotein is cleaved in 3 structural proteins and 7 nonstructural proteins (fig.2-6th step). These proteins copy and assemble the viral RNA into new virions. Synthesis of a genome length minus strand of viral RNA is replicated on the endoplasmic reticulum which then serves as a template for production of further strands of positive sense RNA. The production of the minus strand of RNA has been detected within 3 hours of initial WNV infection. There are a least 10 positive strands of RNA produced for every minus strand template. Viral RNA accumulates in vesicles along the borders of the smooth endoplasmic reticulum (fig.2-7th step). The E and PrM proteins form a heterodimer. PrM is cleaved by furin through the Golgi network and the virion is rendered mature, moves to the cell surface (fig.2-8th step), is enclosed in a lipid bilayer and exits the cell by exocytosis (fig.2-9th step) [22, 24].

After replication in the dendritic cells, the virus moves through the lymphatic system to the bloodstream and then to other organs and tissues including the central nervous system. The pathogenesis of WNV will be discussed later.

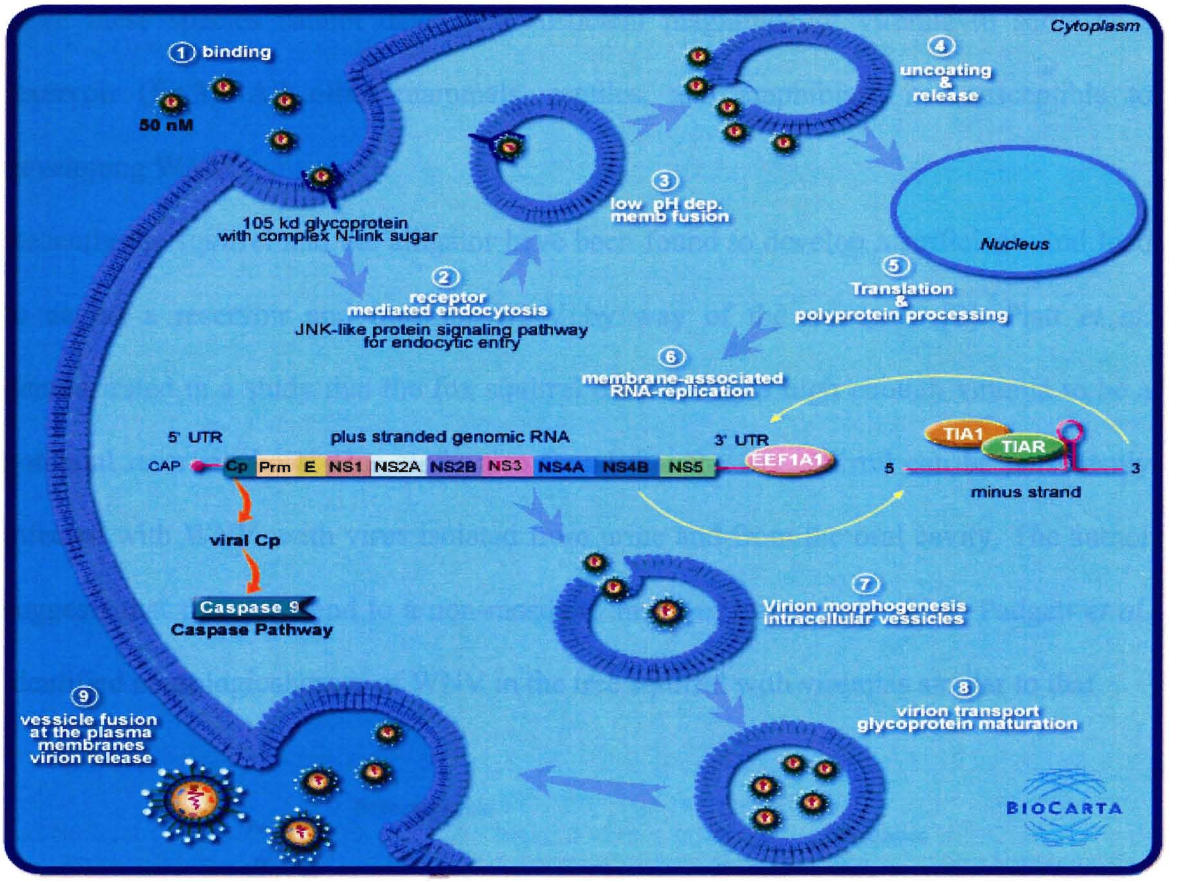


Fig. 2. Viral intracellular replication. Biocarta (2007) [25].

2.3 Clinical aspects of West Nile Virus infection

2.3.1 Transmission

2.3.1.1 Vector transmission

WNV exists in an enzootic transmission cycle with over 65 species of mosquitoes, most commonly *Culex* species, as the vectors and over 300 species of wild birds as the amplifying hosts. Migratory birds introduce the virus into new geographic areas and local mosquitoes then serve as the vectors to spread the virus to native wild and domestic birds to complete the enzootic cycle. Horses and humans are incidental or dead-end hosts,

since these species cannot develop a sufficient magnitude of viremia to serve as a reservoir (fig.3). All other mammals, reptiles, and amphibians are susceptible to developing WNV.

Recently the squirrel and the alligator have been found to develop a sufficient viral load to act as a reservoir and transmit WNV by way of the mosquito [2]. Platt *et al.* demonstrated in a study that the fox squirrel can develop a high enough viremia to be a potential reservoir host. This study also found that the fox squirrel can be persistently infected with WNV, with virus isolated from urine and from the oral cavity. The author suggests that this may lend to a non-mosquito mode of transmission [26]. Padgett *et al.* identified neurological cases of WNV in the tree squirrel with viremias similar to that

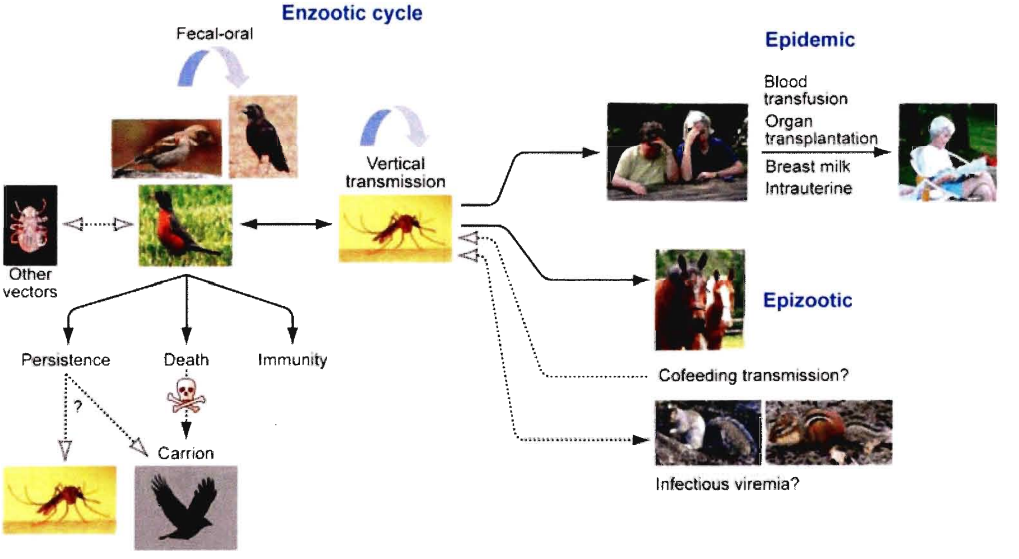


Figure 3

WNV transmission cycle. Primary enzootic amplification by birds and mosquitoes may be supplemented by bird-to-bird transmission, amplification in nonavian hosts, and transmission between cofeeding mosquitoes. Alternative vectors most likely have a less important role. Persistent infection in vertebrates may allow subsequent infection in susceptible scavengers, predators, or mosquitoes. Vertical transmission by mosquitoes provides one mechanism of virus overwintering. Equines and humans are incidental hosts; however, human-to-human transmission may occur through blood transfusion, organ transplantation, breast milk, or in utero. Solid arrows represent confirmed transmission pathways; dotted arrows represent proposed pathways that have not been confirmed in nature.

Fig.3. West Nile Virus transmission cycle. Kramer et al (2008) [2].

found in symptomatic birds suggesting that the tree squirrel may play a role in the ecology of WNV [27].

Feral swine have become established in 20 states of the United States. Since these pigs are already being surveilled for brucellosis and pseudorabies, Gibbs et al (2006) evaluated the blood samples for WNV antibodies and found a prevalence of 22.5% for 222 samples collected over the years 2001-2004. The result of this study suggested that other mammals including the feral pig can serve as sentinels of WNV transmission [28].

Passerine birds, including the corvids (crows, ravens, and blue jays), have been thought to be of major importance in North America for the transmission of WNV. However this may not be the case. The corvids develop high viremias quickly but also die before serving as a long term reservoir of the virus. They have served as sentinels for monitoring movement and introduction of WNV into a new geographic area, however, other birds such as sparrows and finches may be better long term reservoirs [29]. Sparrows and finches develop sufficient viral loads to act as reservoirs and also survive for longer periods of time, even over the winter, to pass the virus to the vector. Migrating birds are important for spreading the virus long distances whereas mosquitoes and non-migrating birds are more important for sustaining the virus in any given geographic location [4].

Transmission is a dynamic mechanism involving mosquitoes, birds, and the prevailing environmental conditions. Hatchlings play a role in transmission as they have no established immunity and are more prone to mosquito bites and thus rapidly amplify WNV transmission. Warm dry summers are associated with an increase in WNV transmission. Drought conditions increase contact between mosquitoes and birds at the reduced water sources [30].

2.3.1.2 Non-viremic transmission in mosquitoes

Non-viremic transmission (NVT) between mosquitoes has been proposed by Higgs *et al.* (2005) as a potentially important means of transmission and long term survival for WNV. NVT refers to the transfer of WNV by co-feeding mosquitoes before a viremia is established in the host. If the quantity of WNV in the saliva secreted by the mosquito is of sufficient concentration then co-feeding adjacent mosquitoes can become infected from dermal areas without assistance from the host. The mosquito population density would be the limiting factor. NVT enhances transmission times and changes the role of the incidental host, giving them a key role with the regards to the long term survival of WNV [31, 32].

2.3.1.3 Other modes of transmission

Horizontal transmission among birds has also been proposed by direct contact from bird to bird through preening or fighting, through the fecal-oral route, and from ingestion of infected carrion [2]. Banet-Noach *et al.* (2003) demonstrated horizontal transmission of WNV in flocks of geese through feather picking and cannibalism [33]. Organ transplantation has also been documented as means of transmission. In August of 2002, four U.S. patients received various organs from a common donor and became ill with a fever. Three of the four patients developed encephalitis. All 4 tested positive for WNV. The source of WNV in the donor was traced back to a blood transfusion the donor received after sustaining life threatening injuries [34].

In the United States in 2002, 23 patients developed WNV through the blood supply. Thirteen of these donors developed meningoencephalitis [34]. Since 2003 both Canada and the United States screen all blood for the presence of WNV using nuclear

amplification antigen tests. In 2002, WNV also appeared in the Mexican blood supply. An evaluation of the Mexican blood supply was performed in a study by Sanchez-Guerrero et al (2006) which discovered one positive sample among 3856 specimens [35]. This study suggested that 0.03% of the blood supply was viremic for WNV. The donor was a 41 year old farmer from the state of Chihuahua. His home was close to a river and bird sanctuary with a dense mosquito population. The individual did not recall any illness in the past year. He had not travelled from the area in the past 16 years. Genetic sequencing was not performed [35]. Since 80% of all cases are asymptomatic it is possible that there are other blood donors that have had past infections.

2.3.2 Entomology

WNV has been found in over 65 mosquito species with the *Culex* species of mosquitoes being the predominant amplifying vectors for WNV in North America. *Culex pipiens* is primarily ornithophilic however there are zoophilic forms that can serve as bridge vectors. Bridge vectors are the mosquitoes transmitting the virus from birds to mammals [36].

Culex tarsalis and *Culex nigripalpus* are important bridge vectors since they switch feeding from birds to humans. *Culex quinquefasciatus* and *Culex salinaris* feed indiscriminately on both birds and mammals. The competence of the mosquito vector is critical for the transmission of WNV.

Ochlerotatus trivittatus, one of the most abundant mosquito species in the North and North Central United States, is anthropophilic but will also feed on other mammals and avian species. In a study by Tiawsirisup et al (2005), *Aedes albopictus* was found to be a more efficient vector when fed bloodmeals with high viral titers whereas *Culex pipiens*

and Ochlerotatus trivittatus were more efficient vectors when fed bloodmeals with lower viral titers [36]. After the mosquito ingests a bloodmeal the virus enters the midgut. Transmission appears to depend on the ability of the virus to escape the midgut to the salivary gland and then escape into the saliva [36].

Theilman and Hunter (2006) surveilled the Niagara Peninsula from 2001 to 2004 for *Oclerotatus japonicas*, a mosquito native to south-east Asia. This mosquito was found to breed in containers of standing water with little organic debris and is thought to have been imported to North America in the international used tire trade. *Oc. Japocicus* is known to bite mammals and some birds but do not seem to favour biting humans. Although WNV has been found in this mosquito in the US, it has not been found in Canada. The numbers of *Oc. Japonicas* increased from 2001 to 2004 however the risk as a WNV vector is unknown [37].

Soft ticks (argasid) in Israel have been found to carry WNV; however, their importance as vectors is unknown [38].

In temperate climates infected mosquitoes overwinter by seeking out shelter and enter into a diapausing phase. In the spring the mosquito awakens and serves as a continuing source of the virus. WNV is also transferred vertically, with the virus entering the mosquito egg at the time of fertilization, ensuring infected mosquitoes will emerge in the spring to enter back into the enzootic transmission cycle [2, 39].

2.3.3 Immunopathology and host immune response

2.3.3.1 Pathogenesis

Following a mosquito bite the virus replicates in the dendritic cells of the dermis, disseminates to the regional lymph nodes and then spreads hematogenously creating a

primary viremia. The virus moves through the bloodstream to the spleen and kidney replicating to produce high levels of viremia. The virus then disseminates to the central nervous system (CNS) and targets the cerebellar purkinge cells, thalamus, basal ganglia, and anterior-horn cells of the spinal cord. The ability of the virus to penetrate the blood-brain barrier is dependent on the immunocompetence of the individual. Most cases of neurological disease are found in apparently healthy patients over 50 years of age. Immune senescence is thought to be the cause, however, other risk factors include the presence of coexisting cardiovascular disease and hypertension, diabetes, and other causes of immunosuppression [40]. West Nile Virus upregulates expression of CNS chemokine receptor, CCR5, which creates an inflammatory response and may be a factor in the penetration of the blood-brain barrier. Loss of neuronal cells are due to direct viral damage as well as the results of the inflammatory response. The histopathology appears as diffuse perivascular inflammation with a predominance of CD8+ T cells, microglial nodules and direct neuronal degeneration [4]. Other modes of transport to the CNS include axonal transport, cytokine directed leukocyte diapedesis through the endothelial cells of the blood-brain barrier, viral release through the choroid plexus, and an increase in endothelial cell permeability by tumour necrosis factor alpha [41].

2.3.3.2 Innate immune response

The innate immune response is achieved through the production of interferons. The RNA virus is recognized by receptors, such as Toll-like receptor 3 (TLR3), which results in the activation and expression of interferon expressing genes. Toll-like receptor 3 and the cytoplasmic double-stranded RNA sensors (dsRNA) are nucleic acid sensors that bind RNA and induce macrophages and dendritic cells to produce interferon [42].

Interferons, type I (alpha and beta), type II (gamma) and III (delta), are potent antiviral cytokines produced upon viral invasion. Interferons alpha and beta are produced by most cell types by direct activation of B and T cells. The result is a restriction on the replication and spread of WNV despite the evasive response by the virus. Interferon gamma is produced by T cells, CD8 T cells, and natural killer (NK) cells and serves also to limit viral dissemination by increasing cell surface expression of major histocompatibility complex (MHC) class I molecules [41].

Complement is a group of serum proteins and surface molecules that recognize antigen and initiates clearance from the body. Complement activates direct opsonisation, chemotaxis, and modulation of B and T cell functions. WNV activates complement and initiates this immune clearance process [41].

Cellular innate immunity involves macrophage uptake of WNV and activation of interferon production of dendritic cells. [41].

2.3.3.3 Adaptive immune response

The adaptive immune response to a viral infection involves both a humoral and a cellular response. The humoral response is the production of antibody against the virus from B lymphocytes. The cellular response is the production of cytotoxic T lymphocytes called CD4+ and CD8+ cells.

Peak viremia occurs from 3 days before and 1 day after the onset of clinical illnesses. This rapid decline in viremia from the serum and peripheral organs is due to macrophage clearance and production of immunoglobulin M (IgM). Viremia in immunosuppressed patients can continue for several months. Immunoglobulin G (IgG) is not produced until

later in the infection. IgG is produced after viral clearance and after the virus has invaded the CNS (fig. 4).

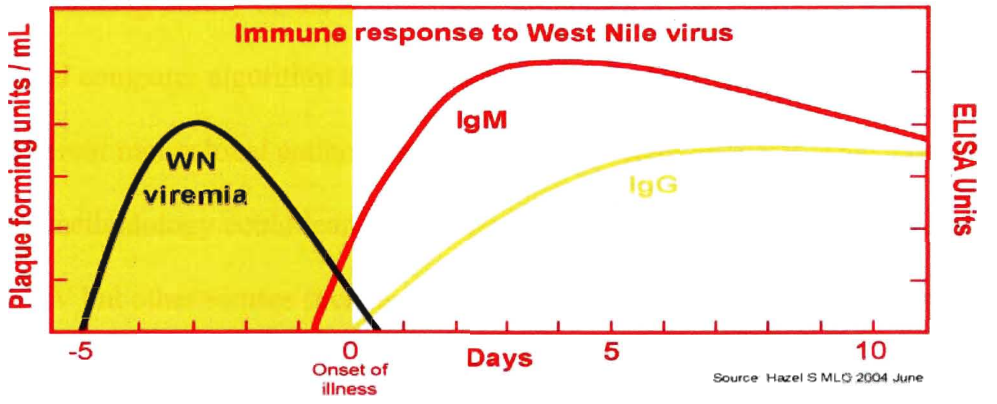


Fig. 4. Immune response to West Nile Virus. Hazel et al Panbio Inc. Australia (2004) [43].

The E protein of the virus elicits production of neutralizing antibodies targeted to all 3 domains of the E protein. The antibodies produced against domain III prevent viral fusion. Studies by Samuel and Diamond (2006) indicate that only partial epitope saturation by the neutralizing antibody is necessary to neutralize the virus. Most neutralizing antibodies bind the viral structural proteins. Nonstructural viral protein 1 (NS1) is a cofactor in replication and inhibits complement activation helping the virus evade the host immune defence. Neutralizing antibodies to NS1 have been found to limit the ability of the virus to replicate [44].

The epitope specificity of a humoral response has been unknown until recently.Work by Oliphant *et al.* discovered that domain II of the E protein appeared to be immunodominant rather than domain III. These investigators found that the majority of E specific monoclonal antibodies were reactive in other regions of the E protein other than

domain III and in particular favoured domain II [45]. Previous work by Throsby *et al.* also indicated the immunodominance of domain II of the E protein and the weak neutralizing ability of the monoclonal antibodies to the epitopes of this domain [46]. A novel computer algorithm developed by Denisova *et al.* was able to identify epitopes to 5 different monoclonal antibodies against the E protein of WNV. This work is important as the methodology could lead to the development of epitope specific vaccines, not only for WNV but other viruses such as HIV [47].

The T cell response targets a limited number of conserved epitopes of the E protein [48] WNV infected cells are recognized by cytotoxic T cells via the expressed major histocompatibility molecule (MHC) and release proinflammatory cytokines and lyse the cell through the delivery of perforin and granzyme. B and T cell memory cells are produced once the infection is cleared [41].

Clearance of the virus from the CNS requires release of chemokines from circulating leukocytes, astrocytes, and microglial cells which attract CD8+ T cells to the site of infection. Brien *et al.* (2007) demonstrated that the transfer of CD8+ T cells into alymphoid mice protected them against a lethal WNV challenge. It was shown that the hierarchy of epitopes was maintained in the memory responses and concluded that CD8+ T cells can protect against a WNV challenge single-handedly [49, 50]. McMurtrey *et al.* reported the discovery of several (1 in particular) conserved and dominant WNV epitopes. These epitopes are presented by the class 1 human leukocyte antigen (HLA) to the cellular immune system and allow recognition of WNV infected cells by cytotoxic T lymphocytes [51].

Interferon assists in clearance by mediating a noncytolytic clearance of the virus from the infected neurons. The virulence of WNV is based on its ability to use multiple and well conserved receptors in a variety of species as well as to induce apoptosis. WNV also can evade the innate and adaptive immune response. The NS proteins are able to modulate interferon responses by delaying transcription of the interferon gene [41, 52]. WNV also elicits the production of the proinflammatory cytokine macrophage migration inhibiting factor (MIF). Increased levels of MIF can be found in the serum and cerebrospinal fluid (CSF) of patients infected with WNV. Macrophage migration inhibiting factor has been found to enhance neuroinvasion by compromising the integrity of the blood- brain barrier [53]. The following figure summarizes, although simplistically, the innate and adaptive immune response (fig.5).

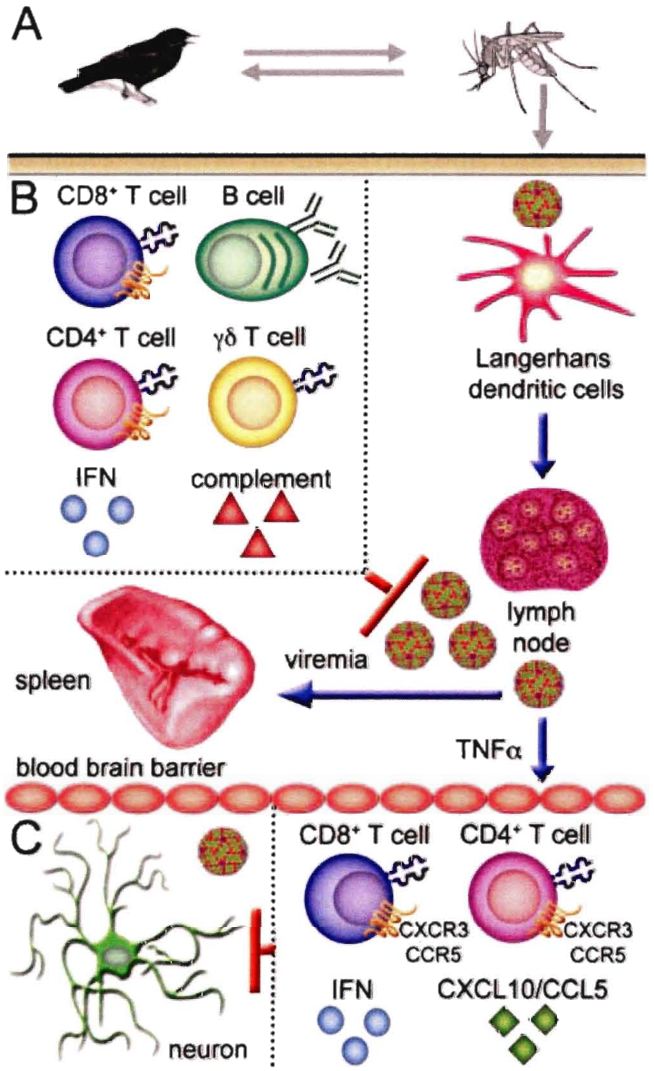


Fig. 5. WNV dissemination and immune system control. Samuel and Diamond (2006) [41].

(A)WNV is maintained in nature in an enzootic mosquito-bird-mosquito transmission cycle.
(B) Following *Culex* mosquito inoculation, WNV replicates in skin Langerhans dendritic cells, which traffic the virus to the lymph node, where further replication ensues. Following induction of a primary viremia, WNV spreads to other peripheral organs. Several aspects of the innate and adaptive immune response limit WNV replication in the periphery. IFN-α/β acts as an antiviral agent that restricts viral translation and replication soon after infection. B cells and antibody (primarily IgM) modulate viral levels in serum and prevent early CNS seeding, while complement is required for efficient priming of humoral and cellular immune responses. IFN-γ-secreting T cells control viral replication through direct antiviral mechanisms and contribute to the generation of adaptive immune responses. CD4 and CD8 T cells participate in viral clearance from peripheral tissues.
(C) Following replication in the periphery, WNV spreads to the CNS possibly through TNF-α-mediated changes in BBB permeability. Neurons are the primary target of WNV in the brain and spinal cord. IFN-α/β is required to control WNV infection in the CNS and may prolong neuronal survival. The chemokines CXCL10 and CCL5 and their cognate ligands CXCR3 and CCR5 aid in recruiting CD4 and CD8 T cells and monocytes to the CNS, where they function to clear virus from infected tissues.

2.3.4 Clinical Presentation of West Nile Virus Infection

Currently, WNV infection in humans presents asymptomatically in approximately 80% of cases. General flu-like symptoms can occur in 20% of those infected. Neurological disease occurs in less than 1% of all those infected with the virus. The incubation period is variable between 2 and 14 days after exposure. The clinical picture changed during 1996 with the development of a more virulent strain of the virus resulting in an increased incidence of neurological disease and death, particularly evident with the epidemic in North America compared to cases occurring in the Old World. Death is due to neuronal dysfunction, cerebral edema, and respiratory failure [4, 5].

2.3.4.1 West Nile fever

Symptoms of West Nile fever include pyrexia (>39° C), lethargy, myalgia, arthralgia, roseolar or maculopapular skin rash involving the neck, trunk, arms or legs, lymphadenopathy and headache. Gastrointestinal signs can also arise including vomiting and diarrhea. Symptoms usually resolve within a few days and the individual will probably not have considered a WNV etiology. Sometimes the infection can travel hematogenously to other internal organs causing myocarditis, hepatitis, pancreatitis, orchitis, chorioretinitis, vitritis, and uveitis [4, 5, 40].

2.3.4.2 West Nile neurological syndrome

Neuroinvasion can lead to meningitis, encephalitis, acute flaccid paralysis and other neurological signs. People at risk of neuroinvasion include those with chronic disease such as cardiovascular disease, diabetes, cancer, and alcoholism. Those individuals receiving immunosuppressive medication or having immunosuppressive disease are at an increased risk. Almost every case of neuroinvasive disease is in an individual over 50

years of age. Immune senescence is thought to be the factor creating increased accessibility of the virus to the central nervous system [4, 5, 40]. There may also be genetic reasons explaining the onset of neurological disease in older people and for the very few cases of such disease in the people of Mexico and South America.

Meningitis is characterized by a stiff neck with headache, photophobia and fever. The other signs of West Nile fever may or may not precede the onset of meningitis. Most patients recover and need no further assistance. If the virus penetrates the blood-brain barrier and gains access to the parenchyma of the brain, then encephalitis will ensue. The virus will attack in various locations of the brain but it has predilection for the cerebellum, substantia nigra in the brainstem, and the basal ganglia.

Symptoms include disorientation, tremors, ataxia, involuntary movements, parkinsonism, seizures, and altered levels of consciousness. Persistent signs of muscle weakness, memory loss, depression, and fatigue affect 30-60% of those after recovery from encephalitis. Pathological lesions include viral proliferation in the neuronal and glial cells resulting in necrosis and cell death, diffuse perivascular inflammation (large number of B lymphocytes present) and microglial nodules composed of a large number of CD8+ T cells. Case fatality rate for those with neurological disease ranges from 4-14% [4, 5, 40, 54].

2.3.4.3 Acute flaccid paralysis

Acute flaccid paralysis (AFP) can develop in 10% of all the neurological cases and may occur in the absence of the typical viral prodrome. Asymmetric paralysis can occur within hours of onset and is characterized by dark limbs with no motor function. Sensory pathways are left intact thus creating intense muscular pain. The recovery is poor leaving

more than half the patients with some degree of permanent disability. AFP needs to be differentiated from polioencephalomyelitis and Guillain-Barré Syndrome (GBS). Respiratory failure can occur in 50-60 % of the cases. Cranial nerve deficits with dysphagia and facial weakness, is less common. The virus attacks the anterior motor horn neurons of the spinal cord as polio virus does. It is not clear whether the virus enters the anterior motor neurons directly or whether the damage is from inflammation and immune reaction. [54, 55].

2.4 Diagnosis of West Nile Virus infection

2.4.1 Clinical diagnosis

Diagnosis is based on clinical suspicion of adults over 50 years of age who develop unexplained neurological signs in the late summer or fall. The presence of other WNV cases in the area or travel to an area with WNV supports the diagnosis [4].

2.4.2 Clinical pathological parameters

Hematology reveals anemia with lymphocytopenia in half the patients and leukocytosis in the other half. The total serum protein is increased. Cerebrospinal fluid (CSF) analysis has a consistent finding of pleocytosis (30-100 cells/ul) with lymphocytes predominating and elevated protein concentration of 80-105 mg/dl [56, 57].

2.4.3 Laboratory diagnosis

The gold standard of laboratory testing is virus culture. However, the viremic phase has usually ceased by the onset of clinical signs. Other antigen tests are also of limited use because of the short viremic phase as well. Antibody testing is the first choice in serological evaluation for WNV. Of all the antibody tests available, Enzyme Linked

Immunosorbent Assay (ELISA) is used the most frequently. Reverse ELISA tests are under development and may have improved specificity with less cross-reaction with other flaviviruses. Hemagglutination Inhibition and Complement Fixation tests were used in the past but have poor sensitivity, poor specificity, are more labour intensive and are now rarely used. Immunofluorescent antibody tests have good sensitivity and specificity and are a choice if a fluorescent microscope is available. Microsphere immunoassays are currently under development and are showing promise with improved sensitivity with multiple flavivirus identification. The Plaque Reduction Neutralization Test (PRNT) is currently used to confirm probable results from other tests [58].

2.4.3.1 Enzyme linked immunosorbent assay

Most cases are in need of serological diagnosis due to the large number of symptoms being nonspecific. Enzyme linked immunosorbent assay (ELISA) is the first test usually performed as it is inexpensive, reproducible, rapid, and employs the use of non-infectious agents. These serological assays are based on anti E antibody detection in the serum but have poor specificity due to cross-reaction from neutralizing antibodies from other flaviviruses due to infection or vaccination. The diagnostic antigen in ELISA's is from the suckling mouse brain which contains highly conserved immunodominant E glycoprotein epitopes responsible for eliciting the cross-reactive serum antibody. The initial test usually performed is for the presence of Immunoglobulin M (IgM) in CSF. Immunoglobulin A (IgA) was evaluated in a study by Nixon and Prince (2006) and results indicated that IgA levels paralleled IgM levels in the CSF. If CSF cannot be obtained then a serum sample is used. It is necessary to obtain an acute and convalescent sample in order to confirm the disease. If only an acute sample is processed then other

flaviviruses in the area should be tested for as well. If the highest titre is for WNV then a presumptive diagnosis of WNV is made. The original antigenic sin is the term applied to the highest titre if several flaviviruses are being evaluated simultaneously [4, 59]. Roberson et al (2007) demonstrated the use of viral-like particle (VLP) antigens in place of the suckling mouse brain antigens. The use of these VLP antigens resulted in less cross-reaction thus yielding higher specificity and sensitivity of the assay. The other advantage is safety since VLP's do not contain RNA and thus are not infectious to personnel preparing the antigen [60].

IgM antibodies can persist in the serum for up to 500 days. Therefore the ELISA test cannot differentiate between a recently acquired infection or an infection acquired the previous year. Immunoglobulin G (IgG) avidity testing can be performed to differentiate between recent and past exposures. The avidity refers to the net antigen binding force by the antibodies. New infections will have weak avidity and mature infections will have high avidity. When used in conjunction with IgM testing a presumptive diagnosis of WNV can be made. Avidity testing requires a database of acute and convalescent titres to establish a cutoff value. [61].

2.4.3.2 Plaque Reduction Neutralization Test

For confirmatory results all positive ELISA IgM samples should be sent for the PRNT [59, 62]. Serum samples are incubated with live WNV virus and then cultured on vero cells. Plaques, which are visible areas of killed vero cells, are observed. If there are antibodies present in the serum they will bind to the WNV envelope proteins and neutralize the ability of the virus to infect the vero cells. The number of plaques will be reduced if antibody is present. The end point is determined when the highest serum

dilution achieves 50-90% plaque reduction depending on laboratory protocol. If there are other flaviviruses in the area then PRNT for the other flaviviruses should be run in parallel with the WNV PRNT. Acute and convalescent samples need to be run with the PRNT to demonstrate a four-fold increase in titre to confirm the infection. High PRNT results are associated with WNV-specific IgG rather than IgM [58]. In Canada, due to the use of live virus material all PRNT must be performed in a class III laboratory [62, 63].

2.4.4 Public Health Agency of Canada (2007) diagnostic criteria

2.4.4.1 WNV non-neurological syndrome

The Public Health Agency of Canada defines the clinical criteria for WNV non-neurological syndrome to include the following: history of exposure and at least 2 of fever, myalgia, arthralgia, headache, fatigue, lymphadenopathy, or maculopapular rash. A suspect non-neurological case must include the clinical criteria in the absence of diagnostic results and in the absence of any other obvious cause. A probable non-neurological case must include the clinical criteria and one of the below probable diagnostic test criteria in Table 1. A confirmed non-neurological case must include the clinical criteria and one of the below confirmed diagnostic test criteria in Table 2 [64]

2.4.4.2 WNV neurological syndrome

The Public Health Agency of Canada defines the clinical criteria for WNV neurological syndrome to include the following: history of exposure and onset of fever with the recent onset of at least one of encephalitis, meningitis, acute flaccid paralysis, movement disorders, Parkinsonism or some other neurological manifestation. A suspect case is the inclusion of the clinical criteria with the absence of diagnostic test criteria and in the

absence of any other obvious cause. A probable neurological case includes the clinical criteria and one of the below probable case diagnostic test criteria found in Table 1. A confirmed neurological case includes the clinical criteria and one of the below confirmed test criteria found in Table 2 [64].

2.4.4.3 WNV asymptomatic infection

A probable asymptomatic case must include the below probable diagnostic test criteria in Table 1 in the absence of clinical signs. A confirmed asymptomatic case must include the below confirmed diagnostic test criteria in Table 2 in the absence of clinical signs [64].

2.4.4.4 Probable case diagnostic test criteria

Table 1. Probable case diagnostic test criteria for Canada. (Public Health Agency of Canada,2007) [64].

	IgM	**IgG**	**HI***	**PRNT****
Option 1	Positive			
Option 2		Seroconversion		
Option 3		Positive		Positive
Option 4			Single titre greater than 1:320	
Option 5			4 fold increase between acute and convalescent samples	

*Hemagglutination Inhibition. **Plaque Reduction Neutralization Test

2.4.4.5 Confirmed case diagnostic test criteria

Table 2. Confirmed case diagnostic test criteria for Canada. (Public Health Agency of Canada,2007) [64].

	IgM	IgG	HI*	PRNT**	Virus Isolate	Virus genome sequence
Option 1				4 fold increase between acute and convalescent samples		
Option 2					Positive isolation on culture	
Option 3						Demonstration of genome from blood, CSF, or tissue
Option4	Positive			Positive		
Option 5		Serocon version		Positive		
Option 6			4 fold increase between acute and convalescent samples			

*Hemagglutination Inhibition. **Plaque Reduction Neutralization Test

2.4.5 Antigen tests

Nucleic amplification tests are not of much value due to the short viremic period. The patient will usually present after the viremic state thus all antigen tests will be negative. Virus isolation and Real Time RT PCR are also of little value in a clinical setting although they are used for blood donor screening as well as research.

2.4.6 Histopathology

Inflammatory lesions in the CNS are described as perivascular lymphomonocytic infiltrates with infected neurons found in the cerebellum, brainstem, cerebral cortex, hippocampus, and the ventral motor horns of the spinal cord. Multifocal glial-microglial nodules, neuronal necrosis, and rare neuronophagia have also been described [65]. Virus particles have been detected in the blood as early as day 1 and in the dermal dendritic cells from day 1 to 4. By day 4 the highest levels of viral replication have been achieved in the spleen and kidney. Virus has been found in the CNS as early as day 4 and in the highest levels by day 9 and 10. Cytokine and chemokine production, which are upregulated in WNV infections creating inflammation, precede the development of the histopathologic lesions. Early production of monocyte chemoattractant protein (MCP-5), interferon gamma inducible protein 10 (IP-10), and monokine induced gamma interferon (MIG) precede the production of interferon gamma (IFN-gamma) and tumour necrosis factor alpha (TNF-alpha). This cascade of cytokine and chemokine production create the necessary inflammation so that WNV can penetrate the blood brain barrier and then create the inflammatory and necrotic histopathological lesions characteristic of WNV infection [65].

2.5 Treatment of West Nile Virus

2.5.1 Clinical supportive treatment

There are no current specific treatments for WNV infection. In WNV endemic areas all patients hospitalized for acute onset of neurological symptoms must be suspected as having neurological WNV. This must be differentiated from other causes of acute meningoencephalitis including Guillain Barré syndrome, herpesvirus infection and

bacterial meningoencephalitis. Supportive and symptomatic treatment with hydrating fluids, anticonvulsants, antipyretics, anti-inflammatories, respiratory support, prevention of secondary bacterial infection, and management of cerebral edema assist with patient recovery but do not enhance viral clearance.

2.5.2 Anti-viral treatment

The natural history of WNV involves a very short period of viremia and progression of the virus into the central nervous system (CNS). Any antiviral therapy needs to be targeted towards the cells of the CNS. Development of antiviral agents has only been shown to be effective in a laboratory setting or with animal models. Several antiviral medications are in clinical trials.

2.5.2.1 Small Interfering RNA

Some recent studies have worked with delivering small interfering RNA (siRNA) into cells to make them refractory to WNV replication. In a study by Yang et al (2008), the siRNA was delivered into human neuroblastoma cells by using a retrovirus as a vector. The siRNA was shown to interfere with viral protein synthesis and inhibit viral replication [66]. Kumar et al (2006) had previously demonstrated viral replication inhibition using a lentivirus as a vector to deliver WNV specific siRNA [67]. Neither study indicated that the potential therapeutic use of siRNA will be able to clear the virus from the CNS.

2.5.2.2 Interferon

Further studies are also investigating ways to block the activity of interferon antagonism by viral proteins. Interferon alpha 2b, a cytokine, enhances the efforts of the innate

immune system by inhibiting viral replication. Interferons work on cells before they are infected. Interferons bind to the surface of these noninfected cells triggering the phosphorylation of tyrosine kinase which initiates an intracellular transduction cascade involving several STAT proteins. These proteins are activators of transcription which move into the nucleus and interact with DNA leading to the production of cytoplasmic RNAases that inhibit intracellular viral replication upon infection [52, 68]. Clinical trials are in process for evaluating the effectiveness of interferons against WNV neurological disease in humans [2].

2.5.2.3 Neutralizing antibodies

Neutralizing antibodies have been shown in the laboratory to protect mice and hamsters from a lethal challenge with WNV. In a study by Ben-Nathan *et al.* (2003) immunoglobulin preparations having a high titre of anti-WNV antibodies demonstrated the need for timely administration after the exposure to WNV. The later in the course of the disease that the immunoglobulin is received, the less chance of survival [69]. Oliphant *et al.* (2005) used a humanized monoclonal antibody against the WNV envelope protein in mice challenged with WNV. The preparation demonstrated efficacy as late as day five of the infection [70]. Use of monoclonal antibodies are in phase I/II clinical trials [2].

2.6 Control of disease and exposure

2.6.1 Vaccination

Currently there are four licensed vaccines for horses and one vaccine licensed for geese.

2.6.1.1 Human vaccines

There are no vaccines approved as yet for humans, although the following four vaccines described are underway in clinical trials [2]. An attenuated vaccine produced by Crucell, requires multiple doses to achieve immunity and is in phase I clinical trials. This attenuated vaccine has mutations in the structural genes which interfere with virus replication or in the capsid gene preventing release of the virus particle from the cell. The National Institute of Allergic and Infectious Diseases (NIAID) and the National Institutes of Health (NIH) have developed a chimeric vaccine with the PrM and E genes of WNV with a flavivirus (yellow fever 17D) backbone as well as subunit recombinant vaccine administered by direct inoculation or by way of DNA plasmids or virus vectors expressing WNV genes. These are both in phase I clinical trials. A fourth vaccine produced by Acambis is also a chimeric vaccine and is in phase II clinical trials [2].

Chimeric vaccines are live attenuated vaccines able to generate good host immunity and unlikely to cause sufficient viremia that would allow mosquitoes to become infected. The West Nile Virus/Dengue 4 chimeric vaccine tested by Hanley *et al.* (2005) demonstrated this attenuation in both *Culex tarsalis* for WNV and *Aedes aegyti* for Dengue. It was not attenuated in *Aedes albopictus* for either WNV or Dengue. Attenuation can occur with the inability of the virus to replicate immediately after the bite, inability to reach the midgut of the mosquito, inability to escape the midgut and reach the salivary glands of

the mosquito, or inability of the virus to escape the salivary gland and enter into the saliva [71].

2.6.1.2 Equine vaccines

The first equine vaccine was introduced in 2001 and used widely in North America in 2002. The vaccine appeared to be very successful as the number of equine fatalities decreased in the US from 15000 in 2002 to 5100 in 2003. This vaccine is an attenuated vaccine requiring two initial intramuscular doses 3 weeks apart with annual boosters. A recombinant canarypox vaccine became available in 2003 [40].

2.6.2 Control of human exposure

Prevention and control of WNV include reducing exposure and contact with the mosquito vector, reducing the mosquito population, and continued surveillance of mosquito pools. Since *Culex* mosquitoes bite between dusk and dawn, avoiding outdoor activities during these hours would seem intuitive. However, if exposure to biting mosquitoes is anticipated, then covering with long sleeves and long pants is advised and using repellents such as N,N-diethyl-methyl-toluamide (DEET), is effective for preventing mosquito bites. The American Academy of Pediatrics advise the use of no more than 10% DEET on children [5].

2.6.3 Environmental control of mosquitoes

Control of mosquito populations is approached by identifying breeding sites of the *Culex* species of mosquito that are serving as the bridge vectors and biting both birds and people. These sites should be mapped and surveilled, and targeted spraying of mosquito habitat should occur early in the year to disrupt the enzootic transmission cycle. Spraying

with insect growth regulators such as methoprene is advised to prevent development of the larvae into adult mosquitoes. Both biting and oviposition behaviours occur 2 hours after sunset, so spraying targeted areas at this time is most effective [72]. Methoprene is not associated with any serious side effects. Using adulticide sprays, such as the pyrethroids, should be reserved for WNV outbreaks. Pyrethroids are associated with cholinergic symptoms such as nausea, vomiting, diarrhea, dizziness, headaches, seizures and pulmonary edema [5]

Mosquito control can also be addressed by elimination of the larval habitats. Removing standing water and organic material from roof gutters, wading pools, old tires, and other areas around the home will help reduce the number of breeding sites and thus effectively reduce the mosquito population. Biopesticides including toxins from *Bacillus thruingiensis israelensis* and *Bacillus sphaericus* have been shown to have good larvicidal activity against *Culex* species of mosquitoes [73].

New methods of mosquito control with the use of mass traps using attractants such as heat have met with some success in the southern US states [74]. Other simple techniques at interfering with the larval habitat have been suggested including the use of floating layers of polystyrene beads. These beads form a barrier over the surface of the water preventing the larvae from surfacing to breathe [75].

2.7 Surveillance

Transmission of WNV relies on adequate numbers of mosquitoes and passerine birds and is enhanced by high summer temperatures and low precipitation. Active surveillance in areas experiencing the above conditions has been the cornerstone to predicting a WNV outbreak.

2.7.1 Mosquito pool surveillance

Sampling of mosquito pools for the presence of WNV indicates the presence of the virus in a given geographical area however this alone cannot be used to predict an outbreak. Early season detection, when infection rates are low, requires a large sample of mosquito pools for adequate power of detection [76].

2.7.2 Dead bird surveillance

Surveillance for dead birds has been a cornerstone for predicting WNV outbreaks in humans since avian deaths precede human and equine WNV mortalities. Roberts *et al.* (2006) demonstrated a strong positive association between avian risk of WNV death and equine WNV risk, although vaccination can be a confounder or effect modifier. Human population density can also be a confounder since more dead birds will be found and reported in areas of greater population densities. Detection bias is another concern since people may be more likely to submit dead birds if they are aware of the presence of WNV in their area of residence. On the other hand relying on public participation has been shown to lose effectiveness over time in endemic areas [2]. Public education regarding the presence of positive mosquito pools and instructions on how to avoid mosquito exposure is important in WNV endemic areas.

2.7.3 Syndromic surveillance

Syndromic surveillance includes monitoring hospital discharge meningoencephalitis diagnoses of unknown etiology, laboratory requests and testing of CSF, and monitoring neurological disease in horses. This surveillance system was used in the Netherlands from 1999-2004 but did not reveal an endemic WNV problem in either humans or horses [77].

2.7.4. Temperature and rainfall surveillance

Weather variations have also been established as predictors of WNV transmission. Higher than normal summer temperatures, particularly the minimum temperature during this period of the year has been found to be a good indicator for an increase in the number of mosquitoes. The Romanian (1996) and the New York City (1999) epidemics occurred after a sustained heatwave in the summer [78]. High temperatures increase the mosquito population as well as shortening the extrinsic incubation period resulting in increased vector competence. A period of drought, followed by heavy rains, also increases the population and competence of mosquitoes. Drought is thought to result in standing pools of water being richer in organic matter which *Culex* species requires for breeding. Drought also encourages birds to congregate into smaller areas increasing their contact with mosquitoes and enhancing the enzootic transmission cycle. Israel has had several epidemics throughout the last half of the twentieth century in which there were warmer summers than in earlier years. The summers of 1998, 1999, and 2000 were extremely hot and are considered to be a factor in the epidemic of 2000 in Israel. Paz (2006) has determined that recent outbreaks occurred 3-9 weeks after a sustained intense heatwave [78]. It is of interest that the outbreak in Saskatchewan in 2007 followed a dry spring and hot summer to the degree not previously seen since WNV was introduced into the province in 2003 [79].

2.7.5 Wetland surveillance

Wetland area conservation has been seen to have a positive correlation with WNV transmission. Diversity in the host amplifier species and the passerine: non-passerine ratio can influence the amplification and transmission of WNV. There are over 130

species of passerine birds that can amplify and transmit WNV but the non-passerine species interrupt the transmission of WNV. Conserving a large wetland suitable to support a large diversity of birds, particularly allowing the increase in numbers of non-passerine birds, reduces the transmission of WNV. On the contrary wetland conservation does not have a positive correlation with mosquito density and transmission of WNV [80].

2.8 Epidemiology

2.8.1 Global epidemiology

In 1937, the first case of WNV was diagnosed in a woman living in Uganda. The virus was isolated from a blood sample taken during the febrile period. No antibodies were found in this sample from the febrile period however 3 months later antibodies were detected in the convalescent sample [8]. The first virologically proven epidemic of WNV occurred in Israel in 1951. A total of 123 cases were found with no fatalities. The virus at this time caused disease in children with half of the reported cases in children less than 6 years of age [7, 81]. Those children under 3 years of age were most febrile however all recovered with no neurological progression. In Egypt, an ecological study was conducted between 1951 and 1954 with serological evaluation and experimental infection of humans (now would be viewed unethical), equines, birds and mosquitoes which revealed the enzootic cycle of WNV. It was also determined that 60% of the population along the Nile River demonstrated the presence of WNV antibodies [81].

Between 1951 and 1996 there were sporadic epidemics with only rare neurological cases and very few deaths. In 1957, another epidemic occurred in Israel resulting in an unrecorded number of children and adults having West Nile fever, with 12 elderly

individuals developing severe meningoencephalitis. Four of these neurological patients died [7]. These were the first deaths definitively attributed to have occurred from neurological invasion of WNV. In 1962, an outbreak occurred in the Camargue region of France causing neurological disease in 80 horses, characterized by ataxia and weakness, with deaths occurring in 25-30% of them. This epidemic lasted until 1965 with a total of thirteen human reported cases and 1 death. From 1965 to 1996 there were few reported outbreaks, the most significant occurring in South Africa in 1974 and an outbreak in Israel in 1980. Another outbreak was recorded in Algeria in 1994 with 50 neurological cases and 8 deaths [6, 81]. In Morocco in 1996, 94 horses were diagnosed with WNV neurological disease and 42 died. In 1997, an outbreak in Tunisia claimed 5 lives out of 111 positive cases. Fourteen horses in Tuscany contracted WNV in 1998 and 6 of these were fatal [81]. In 1996 a series of epidemics began to occur throughout Europe and then in 1999 WNV crossed the Atlantic Ocean and appeared in New York City. Within 3 years the virus moved across both Canada and the United States causing an unprecedented number of avian, equine, and human neurological cases and deaths. The increase in virulence is thought to be due to a mutated viral genome and naive host populations.

Since its discovery, WNV has appeared in unpredictable epidemic and epizootic patterns. All cases diagnosed are seasonal and in temperate climate zones with cases emerging from August through October. Above average summer temperatures with less than usual precipitation appear to favour the increase in the number of mosquitoes. As water bodies begin to dry, the organic content increases, which favours the development of larvae. In urban areas mosquitoes will go indoors looking for standing water in basements, bringing

the mosquito in closer proximity to its host. Higher temperatures also favour the extrinsic incubation period for the WNV within the mosquito, enhancing transmission rates.

2.8.2 West Nile Virus in Europe

2.8.2.1 Romania 1996

The epidemic in Romania in 1996 occurred in the city of Bucharest, along the Danube River, where 393 cases of meningoencephalitis occurred with 17 deaths ensuing. This was the largest epidemic globally to date, involving a record number of neurological cases. From 1997 to 2000, there were 39 further recorded cases with 5 deaths [82]. Seroprevalence surveys indicated that the overall infection rate in Bucharest was 10% during the epidemic year of 1996 [83]. There was no recognized WNV disease in either birds or horses. This is not unusual when compared with other WNV epidemics/epizootics that have occurred elsewhere in the world. WNV behaves very unpredictably affecting different species with different degrees of severity. Some of the reasons contributing to this Romanian epidemic include poverty, weather, vector competence, and avian competence. During this period in Romania, the urban dwellings known as blockhouses were found to be in severe deteriorated condition, with basements often flooded with water or sewage creating a favourable larval habitat for *Culex pipiens*. Family homes were also in close proximity to their poultry sheds, bringing the mosquitoes in close approximation with the amplifying hosts. This particular summer also lacked precipitation creating summer drought conditions. It is also suspected that migratory birds introduced a strain of WNV into the Bucharest area for the first time. It is possible that the strain may have been previously present but had been absent for some time, creating a naive population.

2.8.2.2 Volgograd Russia 1999

Volgograd City and Volzskii City are located along opposite banks of the Volga River in southern Russia. In the summer of 1999, a WNV epidemic occurred with 394 confirmed neurological cases and 40 deaths. Sixty percent of the deaths were in individuals over 60 years of age. Ninety-five percent of these cases originated from the urban area. There was also 428 individuals suspected of West Nile fever leading to the suspicion that 5% of the population had been infected with WNV [84].

Genomic sequences from non-survivors indicated that the Volgograd strain of WNV was 99.6% homologous with the Bucharest strain from 1996. Sporadic cases of WNV had been seen in individuals living in the Volga delta area. The outbreak in 1999 was unexpected and was not diagnosed until very late in the season. Initially meningococcal meningitis had been suspected however most cases historically occur in February to March in Russia. These WNV meningoencephalitis cases had arisen in late summer and fall indicating that the meningococcal organism was not likely the causative etiological agent. As well, meningococcal meningitis historically causes high mortality in young children, while with this Russian outbreak the higher number of lethal cases involved people over 60 years of age. As in the Bucharest outbreak, mosquitoes were found in 85% of the dwellings in Volgograd. Also concordant with the Bucharest outbreak was the presence of a dry hot summer causing mosquitoes to seek places such as damp basements bringing the mosquitoes in close approximation to its hosts.

The epidemics in Bucharest and Volgograd emphasize the common features that favour an urban epidemic such as close proximity to a large body of water, urban dwellings in disrepair harbouring standing water, and high temperatures [82-84].

2.8.2.3 Israel 1999-2000

In 1998, Israel experienced an outbreak of WNV in migratory birds and farmed geese. Serological studies revealed an 86% seropositive result among those individuals who had contact with the geese, 28% in people along migratory bird flyways, and 27% of the general population. Israel is located along many migratory bird flyways from Europe to Africa. Two fatalities occurred in 1999 and then in 2000, 439 people were confirmed with WNV with 29 fatalities. Phylogenetic studies revealed WNV positive individuals living in the central region of Israel homologous to a 1999 New York flamingo isolate. West Nile Virus positive individuals living in the northern region of Israel were found homologous to a 1997 Romanian mosquito isolate [85, 86]. This particular outbreak was found in all highly populated areas of Israel in contrast to previous outbreaks which were more contained. It is speculated that migratory birds introduced the Romanian strain into Israel geese, which were highly susceptible and served as very efficient amplifying hosts. Also of interest was the presence of a dry hot summer in 2000 in Israel, which was also evident in Romania in 1996 and in Russia in 1999 [85].

In September of 1998, migrating white storks that had not previously flown over Israel were forced into a different flyway by strong, hot westerly winds. They were forced to land in Eilat, a town in southern Israel. Serological analysis revealed the presence of WNV neutralizing antibodies. Sequence analysis was homologous to the isolate from a goose which had died later in 1998. It is assumed that the storks were infected from seropositive storks from Germany and subsequently spread WNV into the naive goose population of Israel [87].

West Nile Virus has been endemic in Israel for decades however the epidemic of 2000 produced more neurological cases and deaths than all previous epidemics combined. It also involved all parts of the country, both urban and rural areas. The reasons for WNV re-emerging in Israel with increased virulence are unclear. One theory is that of waning herd immunity since there had not been on outbreak since 1980 [88]. WNV epidemiology has demonstrated a consistent appearance with increased neuroinvasiveness and a high case-fatality rate since 1996. The epidemics in Romania, Russia, and Israel have all been affected by a virus with increased virulence. It is also of interest that long term death rates were found to be increased in WNV survivors. Prognostic factors included older age, male sex, diabetes and dementia. The reasons are unclear [89].

2.8.2.4 Camargue France 2000

The Camargue is a conservation wetland in the south of France, which includes a dry area to the north and west of the wetlands. The Camargue, located in the Rhone delta, is home to a large diversity of avian species. The area serves as a breeding area, migration stop-over, or wintering ground. These birds are often migrating from the African continent. Migrating birds introduce WNV into the delta where the virus is amplified in other resident birds. Spreading birds then disperse the disease from the wetlands to the dry areas. There are 111 species of passerine and shore birds that visit the Camargue for breeding or a stop-over. Eighty-seven of these are likely responsible for the ongoing re-introduction of new strains of WNV into the Camargue. Eighteen species have been identified as spreading the virus to the dry areas to the north. In 2000, in the dry area of the Camargue, 76 horses were diagnosed with WNV and 21 of the cases were fatal. No human cases were reported [90]. The results of a serosurvey in horses in 2000 suggest

that WNV is not endemic in the Camargue but occurs in sporadic outbreaks with long silent periods [91].

2.8.3 West Nile Virus in USA and Canada

2.8.3.1 New York City 1999

In early August of 1999 a large number of crows were found dead around the grounds of the Bronx Zoo. By late August some of the birds at the zoo began to die, including three Chilean flamingos. Zoo veterinarians submitted samples to the National Veterinary Laboratory in Ames, Iowa. A flavivirus was identified and the sample was forwarded to the Center for Disease Control (CDC) in Atlanta, Georgia. The CDC isolated the virus on culture and identified it as WNV. Concurrently 5 patients with symptoms of encephalitis, between the ages of 57 and 87, were admitted to a hospital in Flushing, New York. Four of the five patients developed acute flaccid paralysis. What was initially thought to be St. Louis encephalitis turned out to be WNV [9, 10]. Sequence analysis identified the virus as homologous by 99.8% to the strain currently causing an epizootic in geese in Israel. Called NY99, the WNV had undergone a single amino acid substitution creating a new strain with increased virulence. It is speculated that the mutation arose in Israel before arriving in New York City. The actual transport of the virus likely occurred in the mosquito by plane or perhaps in a smuggled bird. Armed with enhanced virulence and a naive population of birds, people and horses, WNV moved across the continent to become endemic in 48 states and 5 provinces within 4 years [9].

2.8.3.2 Westward spread across USA and Canada

A typical pattern has developed in North America over the last several years. After the introduction of WNV into a new geographic area and successful overwintering, an

explosive epidemic is seen to occur. Following this, a low endemic situation is established with intermittent outbreaks. Since its introduction into the United States, St. Louis encephalitis has been reported at record low levels. This is thought to be due to a cross protective effect from WNV. If an individual survives a WNV infection then that individual will also be protected against St. Louis encephalitis. The reverse has not been found to be true [10].

By the end of 1999, WNV spread through New York State, Connecticut, New Jersey, and Maryland. During the year 2000, WNV had been detected in 12 Atlantic coast states with 21 human reported cases and 2 deaths. By 2001 WNV was found in 21 states and had moved across the border into Ontario. Human cases in Ontario were not detected until 2002 with 66 reported cases and 9 deaths. In 2002 the virus reached California with 4156 cases being reported in the United States with 2946 neurological cases and 284 deaths (10%). Also in 2002, 14751 horses were reported with WNV in the US and the case-fatality rate was almost 30%. [92]. The epidemic/epizootic was the largest flavivirus encephalitis outbreak ever recorded in the western hemisphere [93]. The number of positive reported cases from 2002 to 2006 in Canada and the United States are indicated in Table 3.

Table. 3. West Nile Virus reported human clinical cases in Canada and the United States, 2002-2007 [11, 17].

	2002	2003	*2004*	2005	2006	2007	2008
Canada[1]	66	1495	25	229	151	2215	36
US[2]	4156	9862	2539	3000	4261	3630	918

[1]Public Health Agency of Canada, 2008; [2]Center for Disease Control, 2008.

In Texas in 2003, a distinctly different strain of WNV was identified. This new genotype, WN02, displaced the original strain NY99. This new genotype appears to be have an increased transmission efficiency in the *Culex* mosquito [2]. The new genotype only differed from NY99 by 0.27% [93]. The change occurred so rapidly that the decreased prevalence of NY99 was not immediately detected. The extrinsic incubation time for WN02 in *Culex pipiens* was decreased by 2 days, explaining how this strain displaced NY99. Less incubation time leads to an increase in vector capacity. Recently, another strain has been identified and termed the 'North American' strain differing only 0.18% for NY99 and 0.55% from the Texas strain [94]. However in 2007, epidemic numbers re-emerged in Canada. This situation is not different than the patterns established in Europe. The numbers in Canada and in the United States for 2007 and 2008 are depicted in the maps below (fig.6 -9). The numbers have markedly decreased in 2008 in both Canada and the United States[11, 17]. See Table 4 below for numbers in Canada from 2002-2008.

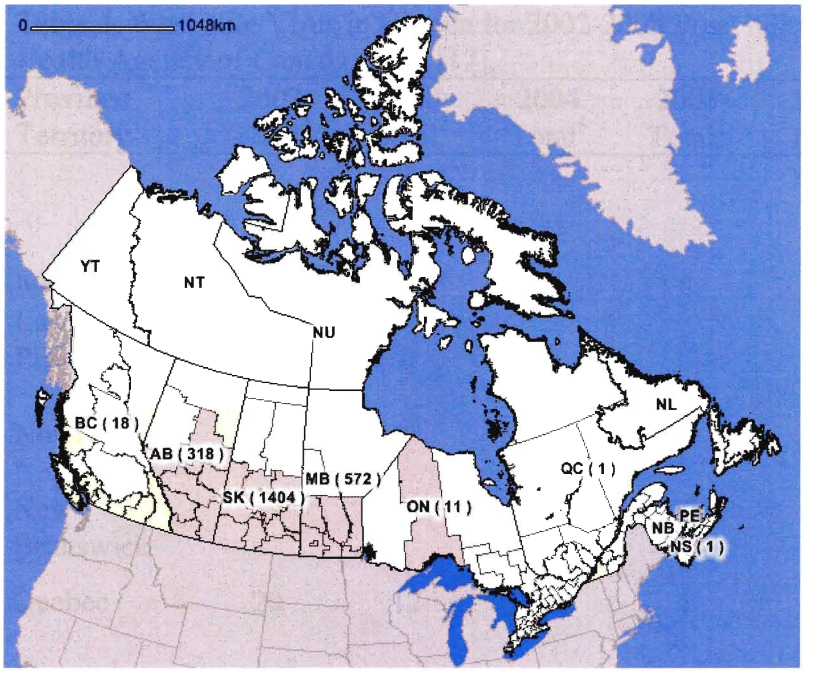


Fig.6. Human West Nile Virus clinical cases in Canada: 2007. Public Health Agency of Canada (PHAC) 2007 [17].

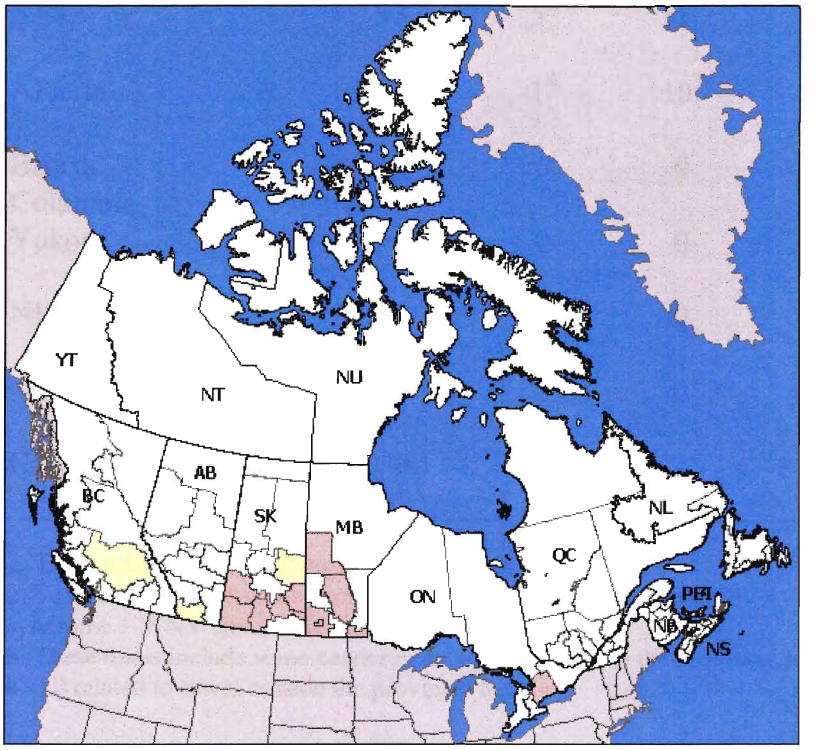


Fig.7. Human West Nile Virus clinical cases in Canada: 2008 Reported to the PHAC as of Oct 18, 2008 [17]. (See Table 4)

Table 4. West Nile Virus in Canada for 2002-2008.West Nile Virus Monitor: Public Health Agency of Canada, 2008 [17].

Province Territory	2002 Total[2]	2003 Total[2]	2004 Total[6]	2005 Total[2]	2006 Total[2]	2007 Total[2]	2008 Total[2]
NFLD and Lab.	0	0	0	0	0	0	0
PEI	0	0	0	1[4]	0	0	0
Nova Scotia	0	2[4]	0	1[4]	0	1[4]	0
New Brunswick	0	1[4]	0	1[4]	0	0	0
Quebec	20	17	3[3]	4	1	2[4]	2
Ontario[1]	394[3]	89[3]	13[3]	95[3]	42[3]	12[3]	3
Manitoba	0	142[3]	3	55	50	578	12
Sask.	0	937[3]	5[3]	58[3]	19[3]	1285[3]	17[4]
Alberta[1]	0	272[3]	1[4]	10[3]	39[3]	318[3]	1[4]
British Columbia	0	20[5]	0	0	0	19[4]	1
Yukon	0	1[4]	0	0	0	0	0
NWT	0	0	0	0	0	0	0
Nunavut	0	0	0	0	0	0	0
Canada	414	1481	25	225	151	2215	36

1. These totals include both probable and confirmed WNV cases.
2. Total clinical cases is the sum of WNV neurological syndrome + WNV Non- neurological syndrome + WNV unclassified/unspecified.
3. These totals include some cases related to travel outside the province/ territory.
4. All related to travel outside the province/ territory.

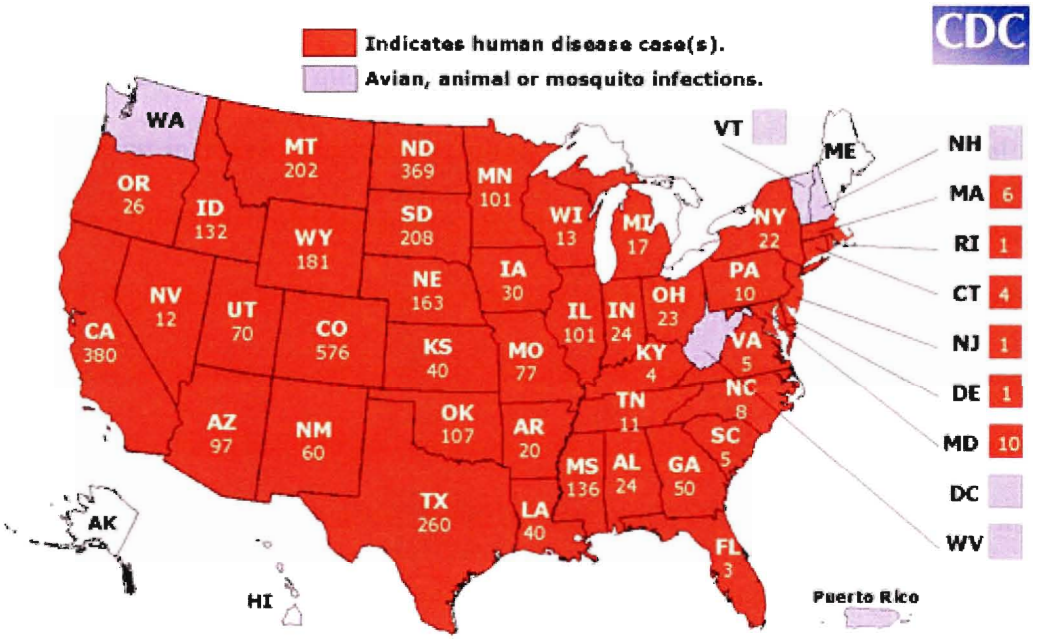


Fig.8. West Nile Virus infections in the USA: 2007. Center for Disease Control [11].

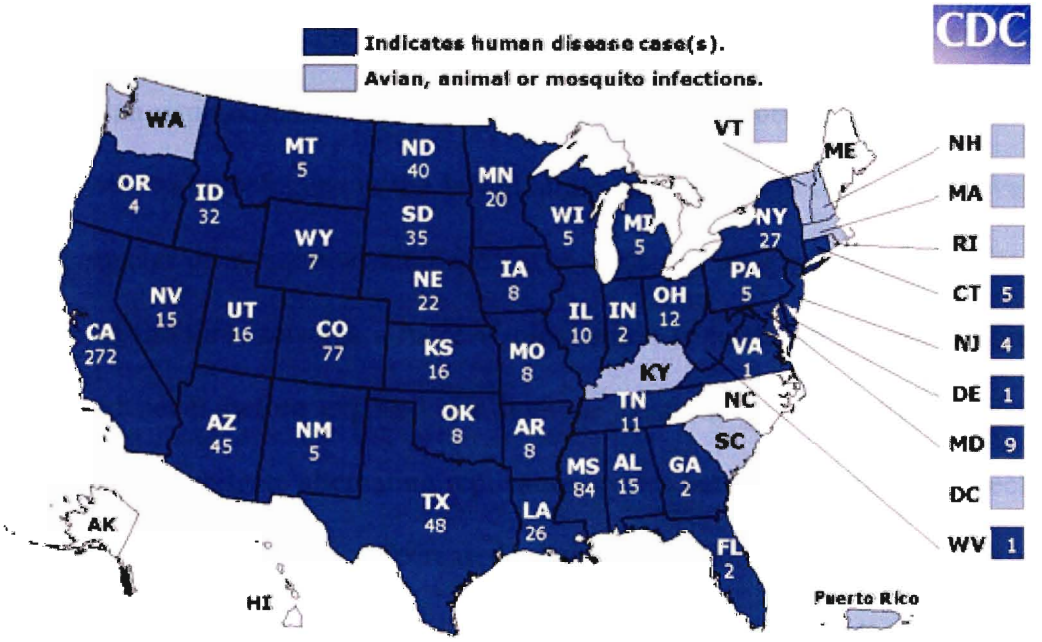


Fig.9. West Nile infections in the USA: 2008. Center for Disease Control [11].

2.8.4 Mechanisms of disease emergence

The overall encompassing question is how WNV has emerged in Canada and the United States with an increased virulence resulting in higher numbers of neurological disease and death in humans, birds, and horses. Examination of the epidemiological triangle with respect to WNV transmission indicates the complex interactions that take place between the virus, its hosts, and the environment. Changes in transmission cycles, host range, and evolution of the virus have all played a role. For instance, a single amino acid mutation in the envelope gene V159A that was found in 2002 in the US has also been found in strains circulating through Europe. Since there have been increased numbers in both neuroinvasive disease and in mortality since 1994 worldwide a mutation such as that mentioned above could account for enhanced virulence [2, 4]. Increases in reported cases of neurological disease during outbreaks started in Romania in 1996 followed by Russia and Israel. Equine epizootics occurred during these same years but in different countries including France, Tunisia, and Israel. The virus found its way to New York City in 1999 and caused an unprecedented avian epizootic and deaths in corvids. Although the NY99 virus was 99.8% homologous to the Israeli strain the host range increased to eventually infect 61 species of mosquitoes, 300 species of birds and 30 species of non-avian hosts. Evolution of the virus towards a more virulent strain is enhanced by new geographical territory and never before exposed non-immune hosts. Is has been hypothesized that viruses that have obligate alternating replication cycles between an arthropod vector and an amplifying host will have a decreased adaptation potential. However this is not the case for West Nile Virus. To date, West Nile Virus has demonstrated a propensity to mutate and cause an inconsistent pattern of epidemics in Canada and the United States.

Although the virus appears to have established itself and become endemic in the US, it has not yet given enough evidence in Canada whether it will become endemic causing constant low levels of transmission or become epidemic causing annual or intermittent outbreaks. As long as birds remain as efficient amplifying hosts then epidemics will likely continue, however, if a resistance develops in the avian population, then virulence may decline [93]. Other amplifying hosts have been identified in Canada and in the United States such as the squirrel and alligator which indicates the ability of this RNA virus to expand and adapt to new hosts [2]. Other risk factors have been identified such as urbanization. Brown *et al.* (2008) demonstrated in a study that urbanization enhances environmental conditions for mosquitoes that are ornithophilic and bridge vectors [95]. *Culex sp* are the predominant vectors and have been found to reproduce the most efficiently in standing containers of organically rich water which are frequently found in urban areas. The predominant species of mosquito present will also influence the risk of disease. Lindsay *et al.* (2008) have found that *Culex tarsalis* and *culex quinquefasciatus* are predominant species in the Great Plain states and mid-southern states, respectively and are associated with high incidence of WNV nonneurological disease [96]. Along with an appropriate vector population and urbanization there must be an adequate population of amplifying hosts.

2.9 West Nile virus in Saskatchewan

West Nile Virus first made its appearance in Saskatchewan in 2003 with 937 reported human cases. The incidence in the following years then decreased until 2007 when a record number of 1285 cases were reported. This was the largest number for any province since the appearance of WNV in Canada in 2001. Although there have been 17 species of

mosquitoes found to carry WNV in Saskatchewan only 3 of these have a high vector capacity to transmit WNV. The principal transmitter of WNV in Saskatchewan is *Culex tarsalis* which acts as both ornithophilic as well as a bridge vector. The female take more than one blood meal and there are several generations produced per season. As well as overwintering, vertical transmission has also been observed. All of the above factors enhance the competence of *Culex tarsalis* as a vector for transmission to humans. *Culex tarsalis* is not a vector in Ontario which may help explain the difference in transmission rates between Saskatchewan and Ontario. *Culex restuans* is present in Saskatchewan as it is in Ontario although it has only been found in small numbers and is primarily ornithophilic. *Culiseta inornata* has been identified in Saskatchewan as a competent transmitter to horses but not humans. Although *Aedes vexans* is the most abundant mosquito in Saskatchewan it rarely feeds on birds and is not a common vector for WNV transmission. The more southern areas of Saskatchewan have higher numbers of *Culex tarsalis* than further north due to higher temperatures and higher wetland densities and this has a positive correlation with the number of WNV cases, more being reported from the southern areas of the province [97]. *Culex tarsalis* appears to be a more competent vector than *Culex pipiens* if just the vectors are used as predictors for WNV epidemics when considering the number of reported cases in Saskatchewan and Ontario. However, *Culex pipiens* has proved to be a very competent vector in the northeastern states of the United States. Other predictors must be considered including avian amplification and their interaction with the resident mosquito vectors as well as environmental conditions [97].

2.10 West Nile Virus in the Niagara Peninsula

Surveillance and control programs for WNV were instituted in Niagara in 2001. The first reported human case of WNV in Niagara was in 2002. This was concurrent with a large mortality of crows in Niagara and a report of a high mortality rate of owls at the Owl Foundation in Vineland, Ontario. Mortality rate in the owls varied among the species, with the older and larger owls more susceptible. A large infestation of the louse fly, *Icosta Americana,* were found on all the dead owls. Of all the flies tested for WNV, 88.9% were found to contain WNV RNA. Of 125 owl deaths, 98 were tested with RT-PCR for WNV and 80 (81.6%) were positive. All the affected owls were housed outside and also susceptible to mosquito bites. It is unknown whether WNV was transmitted by the louse or if the louse was an incidental host [98].

Since the first case was reported there have been 30 confirmed cases of WNV infection in humans, 47 birds, and 33 mosquito pools, with the last reported human case of WNV in Niagara was in 2006. One mosquito pool and 2 birds were found positive in 2007 and 14 birds been found positive to date in 2008.

There are several species of mosquitoes found in Niagara but only *Culex pipiens, Culex restuans,* and *Culex salinarus* have been found positive for WNV. Of these three, only *Culex salinarus* is a bridge vector feeding on both birds and mammals. The larval habitat for *Culex pipiens* and *Culex resutans* is in artificial containers of water with decaying matter such as catch basins and gutters making an urban area more conducive for amplification of these mosquito populations. *Culex salinarus* has been found to have larval habitats in bodies of natural water that have decaying vegetation making rural areas as well as urban areas more conducive for amplification of this species of mosquito. The

population peak for *Culex pipiens* is in early summer and for *Culex restuans* in late summer. *Culex salinarus* appear to have a consistent population thoughout the summer but dependent on environmental conditions. These facts lead to some important questions. Amplification is dependent on both a competent mosquito population as well as a competent local avian population. If the ornithophilic mosquitoes and those acting as bridge vectors are located in different geographic areas, can the bridge vector become sufficiently infected to transmit the virus to humans? It would become dependent on the infected birds moving from the urban areas of *Culex pipiens* and *Culex restuans* in the early summer to the less urbanized areas of *Culex salinarus* in the later summer in order for *Culex salinarus* to have a sufficient number of mosquitoes to transmit to humans. However the majority of the Niagara human population lives in urban or suburban areas. This question may help explain why there has been such a low transmission of WNV to humans in the Niagara Region (Table 5).

Table 5. West Nile Virus reported cases for the Niagara Peninsula 2002-2008. Niagara Regional Public Health Department, 2008 [18].

Year-to-Year Comparisons of WNV Surveillance in Niagara Region							
	2002	2003	2004	2005	2006	2007	2008
Human Cases	18	5	1	3	3	0	0
Positive Birds	11	8	4	5	5	1	14
Pos.Mosq. Pools	15	0	0	10	7	1	0
Positve Horses	12	2	0	1	1	0	0

Despite initial low transmission the Niagara Regional Department of Public Health developed a yearly three tier mosquito control program in 2002. The first tier is aimed at reducing the source population. Public education campaigns with production of literature and web page encouraged residents to eliminate the urban larval habitat. This involved removing standing water in artificial containers, particularly those with decaying vegetative matter. Personal protection was also encouraged with covering of the entire body with clothing at dawn and dusk when these mosquitoes are biting as well as using mosquito repellents such as DEET. The Public Health Department also places 21 mosquito traps across Niagara every year to monitor the mosquito population for the species that are present and the presence of WNV. The public is encouraged to report dead bird sightings to the Public Health Department and the health inspectors will decide whether to pick the dead bird up for evaluation of the presence of WNV. The bird cannot be decomposed and must be from the corvid family of birds. All birds are sent to the Canadian Cooperative Wildlife Health Centres in Guelph, Ontario or Saskatoon, Saskatchewan. According to the Public Health Agency of Canada's website, after 5 birds have been diagnosed with WNV no further birds are submitted. This appears to have been adopted procedure after 2003 since more than 5 birds were reported positive in 2002 and 2003. For 2008, the submissions have continued past the cutoff of 5 positives and there are 14 positive birds to date.

Tier two is aimed at mosquito larval control in catch basins and artificial and natural bodies of water. Methoprene, an insect growth inhibitor, has been placed in catch basins throughout the region in 4 cycles from June through August. The catch basins are labeled with coloured indicators informing the public of placement of methoprene pellets in the

catch basins. Sensitive bodies of water are treated with biological larvicides such as *Bacillus thuringiensis israelensis (*Bti*),* a bacterial spore that destroys the mosquito larvae's intestinal lining when consumed. This biological larvicide is only active in the alkaline gut of the mosquito and is of no risk to humans, birds or other mammals. It is a low impact pesticide and will not damage the environment. However, it may kill other insects besides mosquitoes and this could potentially impact the ecosystem. All public use of larvicidal agents must be registered with the Pest Management Regulatory Agency Health Canada and applied by a licenced exterminator. A commercial product, known as Aquabac which contains Bti, is available to use around the household for mosquito larval control.

The third tier involves the spraying of Malathion, an organophosphate insecticide, for adulticide activity. The use of Malathion is determined by the public health department if it appears that a WNV epidemic is developing. The public must be informed by two different forms of media. Application of sprays to the environment must be done at dawn and dusk with appropriate environmental conditions. It is not effective to spray during rain or fog although a light wind will enhance thorough coverage. Adulticides have not been used in Niagara. Natural mosquito predators can be considered to decrease the adult mosquito population. Purple martins and dragonflies both ingest adult mosquitoes but are active during daylight hours when the *Culex* species of mosquitoes carrying WNV are quiet. Bats are active at the same time as the *Culex* species of mosquitoes however increasing the bat population may increase the risk of rabies transmission since bats are the natural reservoir host for the rabies virus.

The Niagara Regional Health Department continues to encourage residents to take preventive measures against WNV as the virus is still present in the area [18].

The number of recorded cases of WNV in humans, birds, and mosquitoes in Niagara never reached epidemic proportions as in other parts of Canada such as Saskatchewan. Since horses are now vaccinated and dead bird sightings are few the following study has been developed to evaluate a population of agricultural workers in the Niagara Region for the presence of anti-WNV antibodies. The study population selected is the migrant agricultural worker. These men and women spend most of their working and leisure hours outdoors and are a substantial risk of mosquito bites. There has been no previous study examining this population of people in the Niagara Region. Since the workers originate from Mexico, a Dengue endemic country, presence of anti-Dengue antibodies will also be assessed since both WNV and Dengue are flaviviruses and can cross-react with most antibody tests.

2.11 West Nile Virus in Mexico

West Nile Virus was first serologically detected in horses and birds in Mexico in 2002. However the widespread transmission that has been seen in both Canada and the USA has not occurred in Mexico or the rest of Latin America. Reported cases in humans curiously have been very rare. Explanations for this apparent low rate of transmission of WNV throughout Mexico and Latin America will be discussed below.

2.11.1 Avian WNV isolates

Since the introduction of WNV into Canada and the USA, the morbidity and mortality in birds has been dramatically increased from that previously observed in Europe, Asia, Africa, and Australia. Corvids in particular have died in large numbers throughout

Canada and the USA and smaller birds such as the sparrow have been found to amplify and transmit the virus for longer periods of time than corvids. This same pattern has not been observed in Mexico. There appears to be more than one entry of WNV into Mexico. West Nile Virus was first isolated in Mexico from a raven in the southern state of Tabasco in 2003. Nucleotide sequence of the premembrane (PrM) and envelope (E) structural protein genes were found to be divergent from the strains circulating in the northern Mexican states along the Texas border. In this raven from Tabasco, an E-156 proline residue, which ablates the glycosylation site, was not found in the variants in the northern Mexican states [14, 99]. Loss of the E protein glycosylation is associated with attenuation of the virus and thus reduced neurovirulence [100]. It is likely that the virus isolated in Tabasco came from the Caribbean or the southeastern United States as the Mississippi migratory bird flyways cross these states and the Yucatan Peninsula [14, 100]. Note the map in Fig. 10 depicts the presence of WNV in Mexico compared with that in the USA for 2006. The pattern in birds in Mexico is of a clustering nature and not widespread as seen in the USA. Of the 943 birds tested for WNV, only 117 were found positive. The positives were found, in order of decreasing number of reports, in the states of Chihuahua, Oaxaca, Sinaloa, Nuevo Leon, Baja California Sur, Sonora, Baja California, and Taumaulipas [101]. Hidalgo-Martinez *et al.* (2008) conducted a serological study in two zoos in Tabasco. Using ELISA serology, the prevalence of anti-WNV antibodies found in a variety of species of birds was 29.5% which is close to that reported in the United States at 34%. However PCR on these samples did not detect the presence of the virus. The study demonstrates the presence of WNV in Mexico in birds and could be an indicator for possible transmission to people [102].

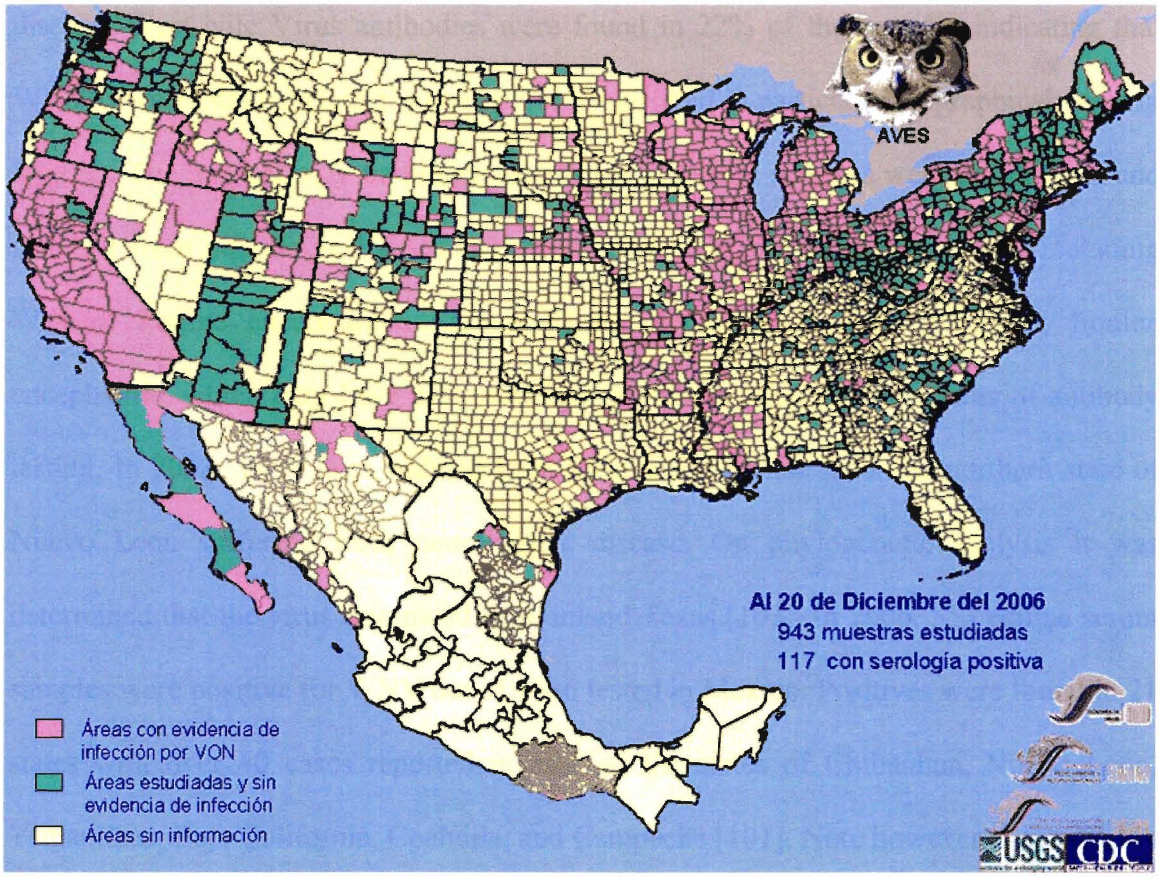


Fig.10. Prevalence and location of WNV positive birds in Mexico in 2006 compared to the United States. Mexican Ministry of Health, Mexican Ministry of Agriculture, United States Geological Survey (USGS), and the Center for Disease Control (CDC), 2006 [101]. Translation: pink are areas with evidence of WNV infection; green are areas studied without evidence of infection, yellow are areas without any information. As of December 20, 2006 943 samples were tested with 117 found serologically positive.

2.11.2 Equine WNV isolates

Serosurveys have been performed in horses since 2002 to monitor for WNV. A small study with 24 equine serum samples from the northern state of Coahuila, adjacent to the Texan border, was conducted in 2002 and 62.5% of the samples demonstrated WNV antibodies [15]. Another serosurvey was conducted with 441 equine serum samples from 14 states in Mexico from 2002-2003. The samples were from horses with neurological

disease. West Nile Virus antibodies were found in 22% of the samples indicating that WNV is well established in the equine but is clustered in particular geographical areas in Mexico. The number may be higher than actual since the samples were not random and were all from neurological cases [13]. There are other flaviviruses in horses including Western Equine encephalitis, Eastern Equine encephalitis, and Venezuelan Equine encephalitis, which may have cross-reacting properties affecting the results of antibody testing. In 2003, WNV was isolated from a euthanized horse from the northern state of Nuevo Leon suffering from neurological disease. On phylogenetic analysis it was determined that the virus originated from inland Texas [103]. In 2006, 553 equine serum samples were positive for WNV out of 1586 tested in Mexico. Positives were found in 21 states with over 40 cases reported in each of the states of Chihuahua, Nuevo Leon, Tamaulipas, Baja California, Coahuila, and Campeche [101]. Note however in the map in Fig. 11 the lack of wide spread transmission as compared to the transmission in the United States.

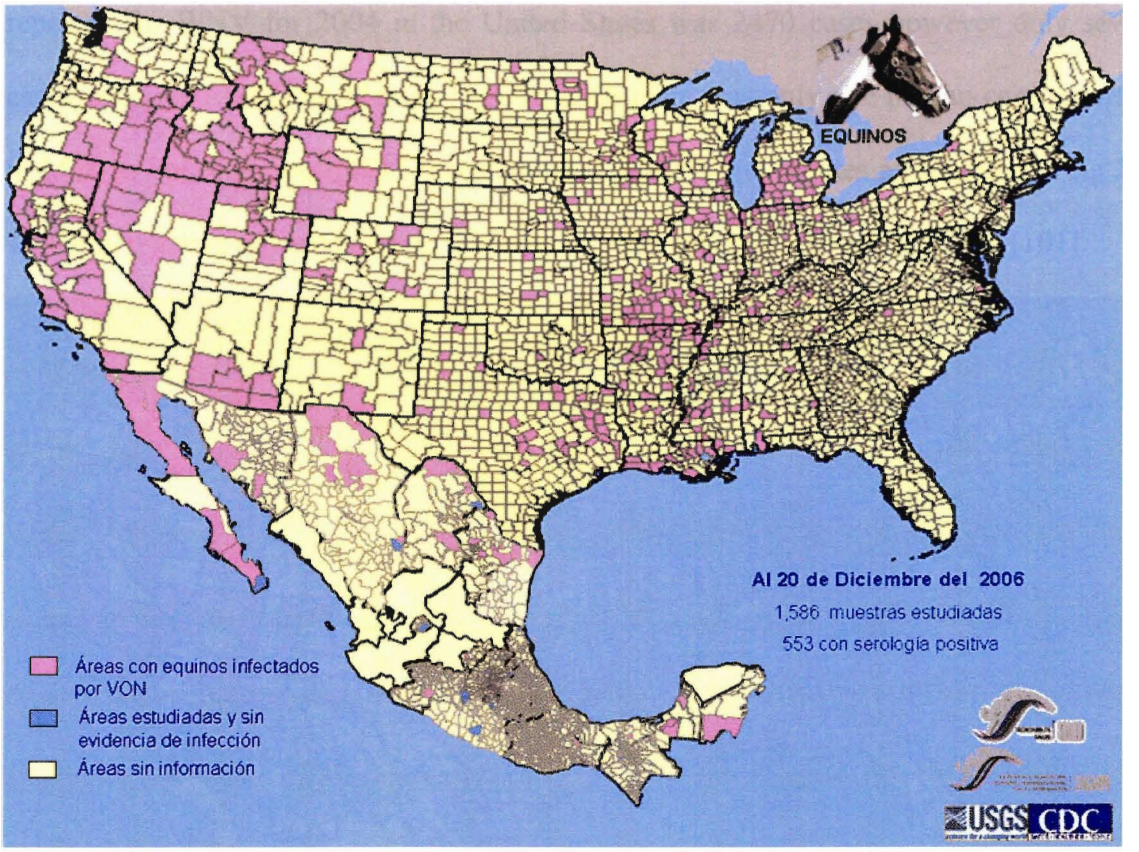


Fig.11. Prevalence and location of WNV positive horses in Mexico in 2006 compared with the United States. Mexican Ministry of Health, Mexican Ministry of Agriculture, United States Geological Survey (USGS), Center for Disease Control (CDC) 2006 [101]. Translation: pink are areas with horses infected with WNV infection; green are areas studied without evidence of infection, yellow area areas without any information. As of December 20, 2006 1,586 samples were tested with 553 found serologically positive.

2.11.3 Human WNV isolates

The first WNV human isolate was from a 62 year old woman from the state of Sonora in 2004. The woman first visited a hospital with symptoms of WNV fever. A presumptive diagnosis of Dengue was made and blood was drawn for serology and culture. Hemagglutination Inhibition was negative for Dengue, WNV, and St. Louis encephalitis. RT-PCR was negative for Dengue and the blood was cultured in vero cells. RNA was extracted from the culture and RT-PCR was performed and identified a WNV strain similar to that circulating in the western states of USA [16]. The human incidence

reported for WNV for 2004 in the United States was 2470 cases however only seven cases were reported from Mexico [14]. In 2006 there was only one human case reported in Mexico but there were 4015 reported cases in the United States as depicted in fig 12. The only positive reported case in a human in 2006 was in the state of Oaxaca [101].

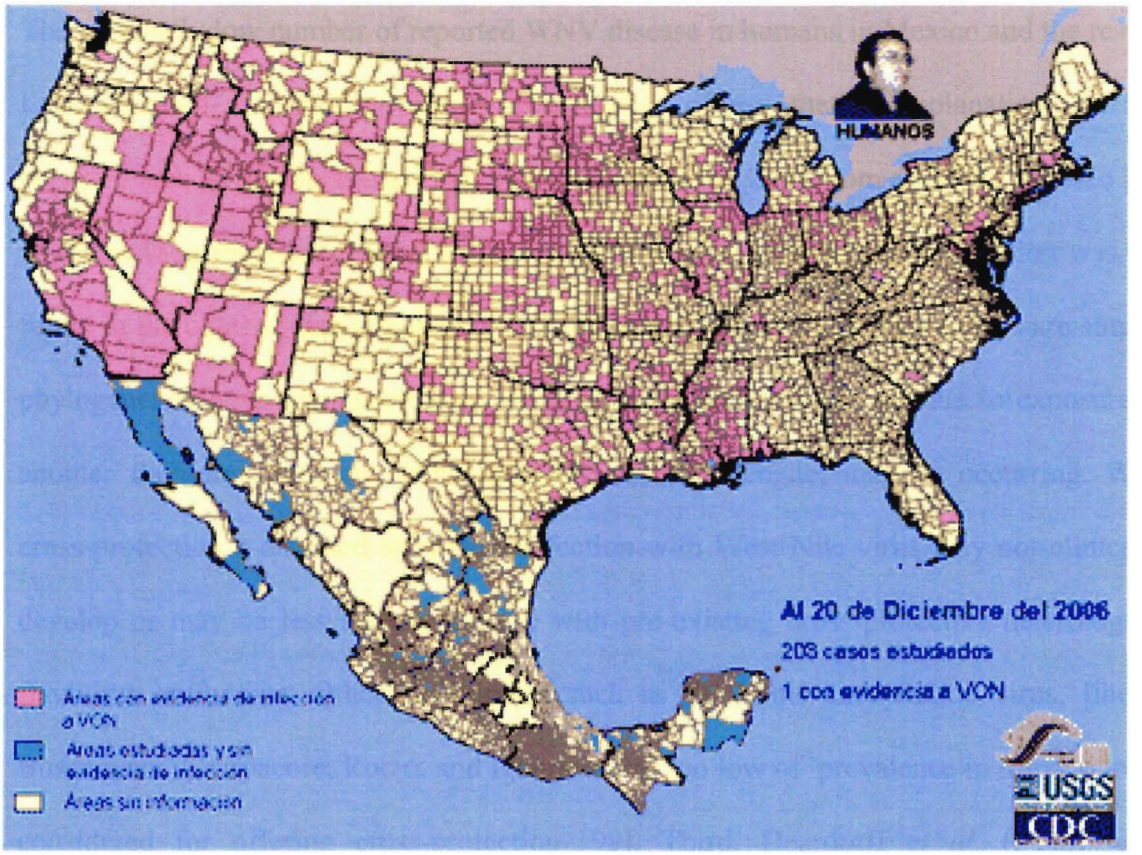


Fig.12. Prevalence and location of WNV in humans in Mexico for 2006 compared with the United States. Mexican Ministry of Health, Mexican Ministry of Agriculture, United States Geological Survey (USGS), Center for Disease Control (CDC) 2006 [101]. Translation: pink are areas with individuals infected with WNV infection; green are areas studied without evidence of infection, yellow area areas without any information. As of December 20, 2006 203 samples were tested with 1 person found serologically positive.

Fernandez *et al.* (2007) conducted a serological study for the presence of anti-WNV antibodies and anti-Dengue antibodies in humans visiting walk-in clinics and hospitals in the 3 northern states of Mexico that border Texas. Of the 237 people tested, 40 % were positive for ELISA WNV IgG, 0% positive for ELISA WNV IgM, and 79% were

positive for ELISA Dengue IgG antibodies. This study indicates the difficulty in diagnosing WNV in a country such as Mexico where there are other flaviviruses circulating such as Dengue. There is cross-reaction on ELISA between the antibodies for the different flavivirus antigens [104].

The extremely low number of reported WNV disease in humans in Mexico and the rest of Latin America are puzzling. There are a number of hypothetical explanations for this apparent lack of transmission. First, the isolate found in 2003 from a bird in Tabasco had an attenuating mutation leading to decreased virulence. However, this mutation was not found in the other strains circulating in Mexico. Second, since all flaviviruses are similar phylogenetically, a cross-protective effect from the immune system due to exposure to another flavivirus endemic within Mexico, namely Dengue, may be occurring. With cross-protection a diseased state from infection with West Nile virus may not clinically develop or may be less severe in those with pre-existing cross-protective heterologous flavivirus antibodies. Other flaviviruses such as St. Louis encephalitis virus, Ilheus, Bussuquara, Cacipacore, Rocio, and Iguape are of too low of prevalence in Mexico to be considered for offering cross-protection [99]. Third, Deardorff *et al.* (2006) have suggested that insufficient surveillance for WNV due to limited availability of resources and funds or misdiagnosis as Dengue could lead to gross under-reporting and hence an apparent lack of transmission of WNV [14]. Fourth, the competence of the native mosquito species and the interaction of the vector with the amplifying host may be different in Latin America and have an effect on transmission. The abundance of WNV infected mosquitoes are directly related to the risk of human disease. Abundance of mosquitoes may be diluted with increased biodiversity as seen in Mexico. The relative

supply of ornithophilic mosquitoes versus the supply of bridging vectors will play a role in both the amplification and the transmission of WNV to other species. Certain species of *Culex* have preferred avian sources of blood. The availability of preferred amplifying hosts as well as the ability of the amplifying host to survive a high and prolonged viremia will affect the transmission. Fifth, migration patterns through Mexico and Latin America are different than in the United States and Canada and may not be as conducive for the spread of WNV.

Finally, Mexico and the rest of Latin America have a different climate which may affect vector and host competence and interaction between the vector and the host. Availability of larval habitat is important for the ongoing transmission of WNV within the mosquito population [99]. All of the above could explain the lack of transmission in Mexico and the rest of Latin America however all are conjecture and there is nothing proven. It still remains a perplexing phenomenon to explain. [12, 16, 92].

CHAPTER THREE: METHODOLOGY

This West Nile Virus study was developed with assistance from the tuberculosis study conducted by co-investigator Angela Duarte, MSc, and supervisor Ana Sanchez, PhD. The same participants from the tuberculosis study were approached to participate in the WNV study. Since the present study was performed concurrently with the tuberculosis study, logistic costs were absorbed by the latter. Extra costs (i.e., lab reagents) were paid by Dr. Sanchez' Professional Development Fund.

3.1 Research Objectives

3.1.1 General Objective

To advance the understanding of WNV transmission in Niagara Region, the present study aimed to establish whether or not active WNV transmission could be detected in a mosquito exposed human population residing in Niagara during the 2007 season.

3.1.2 Specific Objectives

In a study population considered high for WNV exposure (i.e., originating from a country with low human WNV infection and with an occupation that allows for an increased exposure to mosquito bites than in the general population), the present study aimed to:

1. Conduct a seroepidemiological investigation to examine the presence of anti-WNV acute and memory antibodies as a proxy for natural infection in serum samples of healthy Mexican adults working in Niagara Region for the 2007 agricultural season.

1.a. Since Dengue virus, a related flavivirus endemic to Mexico, is highly cross-reactive with WNV, serological studies were extended to rule out potential false positive results to WNV by examining participants' samples for the presence of anti-Dengue memory antibodies

2. Analyze risk factors that might be associated with WNV and DEN seropositivity.

3. Assess the awareness of study participants about WNV infection as well as their understanding of the mode of transmission and clinical importance of the infection

3.2 Research Ethics

3.2.1 Research Ethics Board approval

A research ethics application was accepted and approved by the Brock University Research Ethics Board (REB) File # 06-363 Mergl (Appendix A-C). The application provided the rationale for the study and the risks and benefits for the participants. The application provided details regarding the collection of data and blood samples and ensured methods of maintaining confidentiality.

3.2.2 Biosafety

The investigators have all received Biosafety Awareness Training administered by the Office of Environment, Health and Safety, Brock University. The investigators adhered to Brock University's Policy on Safety and Liability for Field Research. A written protocol was prepared describing blood collection technique as well as methods to handle accidental spills, needle sticks, and disposal of biohazard materials. Gloves were worn

during blood collection and handling of all blood collection equipment. All investigators wore clothing indicating their presence from Brock University as well as name tags.

3.2.3 Biosafety level II laboratory

Dr. Ana Sanchez' laboratory located in MC C307A is certified as a Biosafety level II laboratory (Appendix D). Proper laboratory protocol and safety precautions were followed for all investigators working in the laboratory. Proper clothing, gloves and eye-ware were worn during all procedures.

3.3 Research design

This was a cross-sectional study among the migrant Mexican agricultural workers of the Niagara Peninsula using a convenience sample of volunteers. The group was chosen as a high risk population for exposure to WNV since most of their daylight and evening hours are spent out of doors. Bender et al (2006) have suggested that agricultural workers are an index group for many emerging infectious diseases [105].

High risk populations are used to investigate the epidemiology and ecology of an infectious agent for the purpose of disease monitoring. By detecting the presence or evidence of an infectious agent early there can be a rapid response to prevent larger and longer outbreaks of disease. With low or near zero prevalence other conventional surveillance procedures may not detect the index cases. With prevalence near zero the necessary sample size may be so large that it approaches the size of the population. However, use of a high risk population (that being a population at higher risk of exposure) permits a smaller sample size, presuming they have a higher infection rate [106].

Supporting factors for using the Mexican workers as our sample population include the following. First, as previously explained in the background section (p. 61 to 62), WNV transmission to humans is negligible in Mexico therefore any anti-WNV antibodies detected in research participants will most likely have been developed in response to exposure to the virus in Canada. Second, agricultural workers spend more time outdoors than the general population and are at higher risk of mosquito bites which increases probability of mosquito-borne infections. Third, the study population is believed to be susceptible to WNV infection (i.e., there have been no genetic studies to prove that the population is resistant to WNV infection). Finally, the study population individuals are by requirement healthy and assumed immunologically competent and are able to generate an immunological response.

3.3.1 Study period

The study was conducted from July to October 2007, the year with highest number of cases recorded in Canada, with the blood samples drawn during the months of August and September, the period in which WNV transmission occurs in Canada.

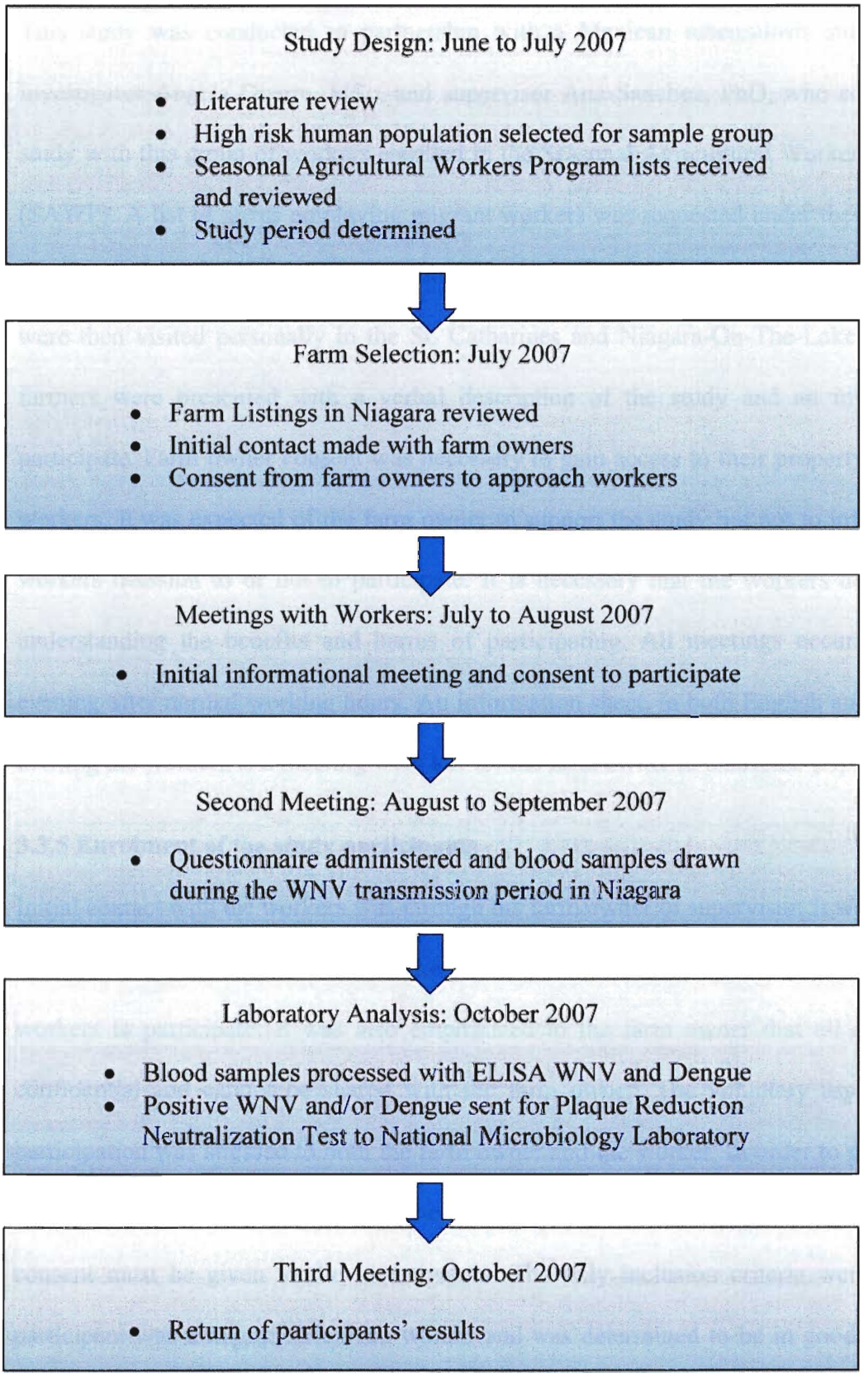
3.3.2 Sample size

The sample size for this study was dependent and limited to the samples from the tuberculosis study conducted by Angela Duarte, MSc, and Ana Sanchez, PhD. The sample was thus a convenience sample and any study results will be at best preliminary. Since there are no previous WNV studies of this particular group of individuals, it was difficult to determine a sample size that would be representative of the group in Niagara.

Formulas exist that assist in calculating the necessary sample for the study. However, those formulas require known parameters such as a priori prevalence estimate of the existing disease with confidence intervals, maximum tolerable error, and the population size [106]. Since there are no WNV prevalence studies in Niagara either in the general population or in this particular group of Mexican migrant workers, a cross-sectional study was ideal to obtain preliminary data, even though using a convenience sample is an important limitation.

Before providing the details of the methodology, the following flowchart depicts the steps taken to implement the study.

3.3.3 Flowchart for methodology of WNV serosurvey July to October 2007



3.3.4 Study population selection

This study was conducted in partnership with a Mexican tuberculosis study by co-investigator Angela Duarte, MSc, and supervisor Ana Sanchez, PhD, who conducted a study with this group of workers enrolled in the Seasonal Agricultural Workers Program (SAWP). A list of farms employing migrant workers was requested under the Municipal Freedom of Information and Protection of Privacy Act in the Niagara Peninsula. Farms were then visited personally in the St. Catharines and Niagara-On-The-Lake areas and farmers were presented with a verbal description of the study and an invitation to participate. Farm owner consent was necessary to gain access to their property and their workers. It was expected of the farm owner to support the study but not to influence the workers decision to or not to participate. It is necessary that the workers decide after understanding the benefits and harms of participating. All meetings occurred in the evening after normal working hours. An information sheet, in both English and Spanish, inviting the workers to a meeting were left for the farm owner to distribute (Appendix E).

3.3.5 Enrolment of the study participants

Initial contact with the workers was through the farm owner or supervisor. It was stressed to the farm owner to inform the workers of the study and not encourage or require the workers to participate. It was also emphasized to the farm owner that all results are confidential and cannot be shared with the farm owner. The voluntary aspect of the participation was stressed to both the farm owner and the worker. In order to participate, the worker must fully understand the study's risks and benefits and signed written consent must be given to the investigators. The only inclusion criteria were that the participant was a migrant Mexican worker and was determined to be in good health by

the usual screening tests performed before leaving Mexico. There was no need to exclude individuals not in their first year in Canada since the immunological response to the WNV antigen will result in production of IgM, indicating a new immunological response. The limiting factor would be the fact that IgM can last up to 500 days so the exposure may have been the previous year [4, 5]. However, since there is almost negligible human detection in Mexico, the antibody response would be the result of exposure in Niagara, unless other travel occurred.

3.4 Meetings

There were three meetings conducted with each group: the initial information meeting, followed by the meeting to conduct the questionnaire and collect the blood sample, and then a final meeting to distribute the results. All verbal explanations and interviewing were done by co-investigator Angela Duarte, MSc, supervisor Ana Sanchez, PhD, and other Spanish speaking assistants. The farm owner or supervisor organized the time and provided a space for the investigators to meet with the workers. The workers were given an explanation of the study in Spanish with some literature they could understand (Appendix F) as well as the requirements for voluntary participation, confidentiality, risks and benefits. A written invitation in Spanish to participate was read to each interested person (Appendix G). The consent form in Spanish was read and explained and if the participant chose to consent, the form was signed and a copy was provided for the participant (Appendix H). During the second meeting, the questionnaire was administered in a private area with the investigator explaining and recording each answer. Afterwards a blood sample was collected in a serum separator tube. The final meeting was conducted

to give the participants their results in a private area with an explanation of any positive findings.

3.5 Questionnaire

3.5.1 Description of the questionnaire

The questionnaire (Appendix I) was designed by Ron Mergl, DVM, co-investigator Angela Duarte, MSc, and supervisor Ana Sanchez, PhD and was based on known risk factors for the transmission of WNV. The questionnaire was entirely bilingual. That is, each question was typed both in English and in Spanish. The components are explained under the following headings:

1. Demographics: name, age, sex, address and contact phone number if available
2. Length of time and location of participation in the agricultural workers program
3. Living conditions on the farms in Niagara: includes condition of the home as well as the number of people in the home and sharing a bedroom.
4. Socio-demographic data from their home in Mexico: includes city and state in Mexico as well as the condition of the housing. Also includes any animals that are owned and kept on the property.
5. Participant's evaluation of his/her own health.
6. Knowledge of WNV and presence of associated symptoms.
7. Working conditions, mosquito exposure, and recognition of dead birds.

Each questionnaire was assigned a 6 digit code to help ensure confidentiality and that code was recorded in the top right corner. The first 2 digits apply to the town; the second 2 digits apply to the farm and the last 2 digits to the individual.

3.5.2 Administration of the questionnaire

Each participant was treated to a private session with an investigator to ensure confidentiality. The participant was assured of the privacy of the recorded answers. The questions were asked verbally in Spanish and then recorded in Spanish and English on the document by the investigators. Each answer was repeated back to the participant to ensure accuracy. The participant was encouraged to ask questions to resolve any apprehensions or doubts he or she may have. The research code was assigned and this number was used to identify the blood sample as well as all further recording of results and information.

3.5.3 Blood collection

The blood was collected by Angela Duarte, MSc, a certified phlebotomist, from the median cubital vein of the forearm. The skin was disinfected with 70% ethanol and a tourniquet was applied above the puncture site. A sterile blood collecting needle was introduced into the vein and a vacutainer serum separator tube attached and 3 ml of blood collected. The needle was then withdrawn and a sterile pad applied to the puncture site. The participant was instructed to keep pressure on the pad and bend the elbow for a few minutes to allow the site to coagulate. A band-aid was then applied and the participant informed of any expected problems that may occur with the site such as bruising. The blood samples and all biohazard waste were transported to the laboratory in MC C307A at Brock University by research co-investigator Angela Duarte, MSc, who received Transportation of Dangerous Goods (TDG) training. The tubes were centrifuged at 3000 rpm for 10 minutes. The serum was aspirated by a pipette and aliquots of 1ml placed in serum storage tubes, identified with the participant's research number, and frozen.

3.6 Antibody determinations

Antibody testing involved a profile of anti-WNV antibodies including IgM, IgG and avidity testing for the IgG. Antibody testing for anti-Dengue antibodies was also run since the participants may have been exposed to Dengue while in Mexico. All laboratory work was performed by Ron Mergl, DVM. Commercial Enzyme-Linked Immunosorbent Assay (ELISA) was chosen to evaluate the serum for anti-WNV and anti-Dengue antibodies. A Capture IgM ELISA for WNV was chosen to evaluate for an acute WNV antibody response and an Indirect IgG ELISA for WNV was chosen to look for memory antibody. The Indirect IgG ELISA test for Dengue was chosen since the participants originated from a Dengue endemic country and may have anti-Dengue antibodies in memory which could crossreact with the WNV antibody tests. The ELISA test kits were purchased from Panbio (Australia) on recommendation from researchers using the tests for Dengue antibody at the National University in Tegucigalpa, Honduras (Ivette de Rivera, personal communication). Panbio tests are standardized for clinical use (i.e., as an aid in the diagnosis of patients with symptoms consistent with WNV). The tests have not been used for seroprevalence studies and the interpretation guidelines were established for clinical cases. In addition to doing antibody determination, positive WNV samples were confirmed by Plaque Reduction Neutralization Test (PRNT).

3.7 WNV IgM Capture ELISA

The WNV IgM Capture ELISA detects IgM antibodies to WNV in serum. Panbio reports a sensitivity of 96.7% (CI 88.7-99.6%) and a serological specificity of 85.5% (CI 75.0-92.8%) using PRNT as the gold standard but states that all positive results must be confirmed by the PRNT or by using current CDC guidelines for diagnosis of the disease

[107]. In brief, the principle of this test is as follows: microwell test strips provided with the kit are coated with sheep anti-human IgM antibodies adhered to the polystyrene surface of the well. When positive serum is added, the IgM antibodies bind with the anti-human IgM antibodies. When the WNV antigen/monoclonal antibody complex is added, it then binds to the complex. A colour substrate is added and identifies the presence of the positive complex with a colour change, with the optical density evaluated by a spectrophotometer [107]. For details of the ELISA procedure see Appendix J.

3.8 WNV IgG Indirect ELISA

The WNV IgG Indirect ELISA is for the qualitative presumptive detection of IgG antibodies to WNV in serum. Panbio reports a sensitivity of 79.0% (CI 69.7-86.5%) and a specificity of 90.5% (CI 85.6-94.2%) using PRNT as the gold standard but states that all positive results must be confirmed by the PRNT or by using current CDC guidelines for diagnosis of the disease. There is cross-reactivity with other flavivirus antibodies as well as to antibodies to cytomegalovirus, Epstein-Barr virus, and rheumatoid factor. The principle of this test is as follows: the polystyrene surfaces of the microwells are coated with WNV antigen. Serum antibodies combine with this antigen and when horse radish peroxidase (HRP) conjugated anti-human IgG is added, it binds to the serum IgG. When tetramethylbenzidine (TMB) is added, HRP, present in the positive complex, hydrolyzes the TMB and produces a colour change, with the optical density evaluated by a spectrophotometer [108]. For details of the ELISA procedure see Appendix K.

3.9 WNV IgG Avidity

In addition to running an Indirect IgG WNV ELISA an avidity test of IgG WNV was used. Avidity refers to the antigen binding force of IgG antibodies. Low avidity

antibodies are produced in acute infections and develop into high avidity antibodies as time progresses. The IgG avidity test is useful in differentiating recent from past infections. The avidity reagent is a protein denaturing solution which removes low avidity antibodies and changes the IgG absorbance indicating past infections only as positives. Since the participants are from Mexico, they may have been exposed to Dengue virus and have circulating IgG antibodies against Dengue. If this were the case, a marked cross-reactivity will be observed between WNV and Dengue IgG ELISAs, making it impossible to determine the true result. In the absence of further confirmatory tests, the avidity test may be helpful in establishing the true result. An assumption is made that if the participants have been exposed to Dengue and if it is their first time in Canada, then the antibodies detected with the ELISA will more likely be anti-Dengue antibodies. Therefore they would show high IgG avidity. If they have only been exposed to WNV in the current year, they will have low avidity antibodies. Naturally the avidity test will only be useful for first time participants. A low avidity index, that is $< 40\%$, indicates a recent infection, therefore more likely WNV. A high avidity index, that is $\geq$ 40%, indicates a past infection, therefore more likely Dengue [109]. A high avidity indicates a primary WNV of at least 40 days or an anamnestic response from a previous flavivirus infection [61]. It is also important to include the fact that these antibody tests are being performed in the absence of clinical signs. For details of the IgG Avidity ELISA procedure see Appendix L.

3.10 Dengue IgG Indirect ELISA

ELISA's for Dengue were performed to investigate the cross-reactivity between WNV and Dengue antibodies. Since the participants are coming from a Dengue endemic

country, the IgG Indirect ELISA was selected to look at the possible presence of past Dengue infections and the presence of IgG Dengue antibodies. Panbio reports a sensitivity of 97.9% (CI 92.5-99.7%) and a specificity of 100% (CI 96.6-100%) against Hemagglutination Inhibition (HI). The principle of this test is as follows: the polystyrene surfaces of the microwells are coated with Dengue antigen. Serum antibodies combine with this antigen and when HRP conjugated anti-human IgG is added, it binds to the serum IgG. When TMB is added, HRP, present in the positive complex, hydrolyzes the TMB and produces a colour change, with the optical density evaluated by a spectrophotometer [110]. For details on the procedure for IgG Dengue see Appendix M.

3.11 Plaque Reduction Neutralization Test

As mentioned earlier, positive WNV ELISA samples were confirmed with the PRNT, which measures the ability of neutralizing antibodies to neutralize a virus and is the most viral specific test for the flaviviruses. This test can only be performed in a Level III biosafety laboratory due to the use of live virus. Our positive samples were transported to McMaster University and then to the National Microbiology Laboratory (NML) in Winnipeg Manitoba. All samples were re-tested with WNV IgM Capture ELISA, WNV IgG Indirect ELISA, Dengue IgG Indirect ELISA, as well as Dengue IgM Capture ELISA. PRNT were performed in parallel for both WNV and Dengue. The principle of this test is as follows: sample sera are serially diluted and incubated with live WNV or Dengue virus and cultured on vero cells. After a period of incubation an overlay of culture medium is added to prevent virus diffusion beyond plaque formation. After further incubation plaques are counted and compared to the starting concentration of the virus to determine the percent reduction in the number of plaques. Since the

concentration of the virus is a constant, the highest dilution reducing the number of plaques by 50% is reported as the endpoint [111, 112]. For details on the PRNT see Appendix N.

3.12 Interpretation of antibody determination

Table 6. Combined interpretation of Panbio ELISA tests for WNV infection. Panbio Australia 2005 [108]

	IgM positive	IgM negative
IgG positive	Presumptive diagnosis of current or past WNV infection or other flavivirus infection	Presumptive diagnosis of past WNV infection or other flavivirus infection or exposure
IgG negative	Presumptive diagnosis of current WNV infection or other flavivirus infection	Presumptive negative diagnosis

3.13 Return of the participants results

The final meeting on each farm was to give each of the participants their results in private. All results were recorded on an information results card and handed to the participant (Appendix O). Farm owners' participation was also acknowledged with a Certificate of Recognition (Appendix P). These certificates were distributed personally and the farm owners were thanked for their cooperation.

3.14 Data analysis

3.14.1 Data management

All data from the questionnaires and laboratory results were recorded into an Excel spreadsheet using Microsoft Office 2007 using the participant's research code. All information was checked for accuracy. Data was checked for errors and missing values and then entered into STATA version 10.0 (StatCorp. 2005, USA) for analysis.

3.14.2 Statistical analyses

For Objective No. 1 and 1a (*determine the presence of antibodies to WNV and Dengue virus*), the presence of antibodies was reported as seroprevalence proportions (# positive samples out of the total analyzed) with 95% confidence intervals. When possible, predictive values for ELISA were calculated.

For Objective No.2 (*analyze risk factors that might be associated with seropositivity*), logistic regression was used to identify risk factors for seropositivity. Odds ratios (ORs) and their 95% confidence intervals were calculated. Factors associated with seropositivity in univariate analyses were considered with a p value <0.15 for incorporation into a multivariate regression model. Previous research studies conducted to investigate risk factors for Dengue have utilized a p value of < 0.15 in order to control confounding in the model for multivariate analysis, particularly when the sample size is small [113-116]. Those factors that had a p value <0.15 in univariate analysis were selected for multivariate analyses by backwards selection and the risk factors that resulted with a p value of < 0.05 were considered statistically significant.

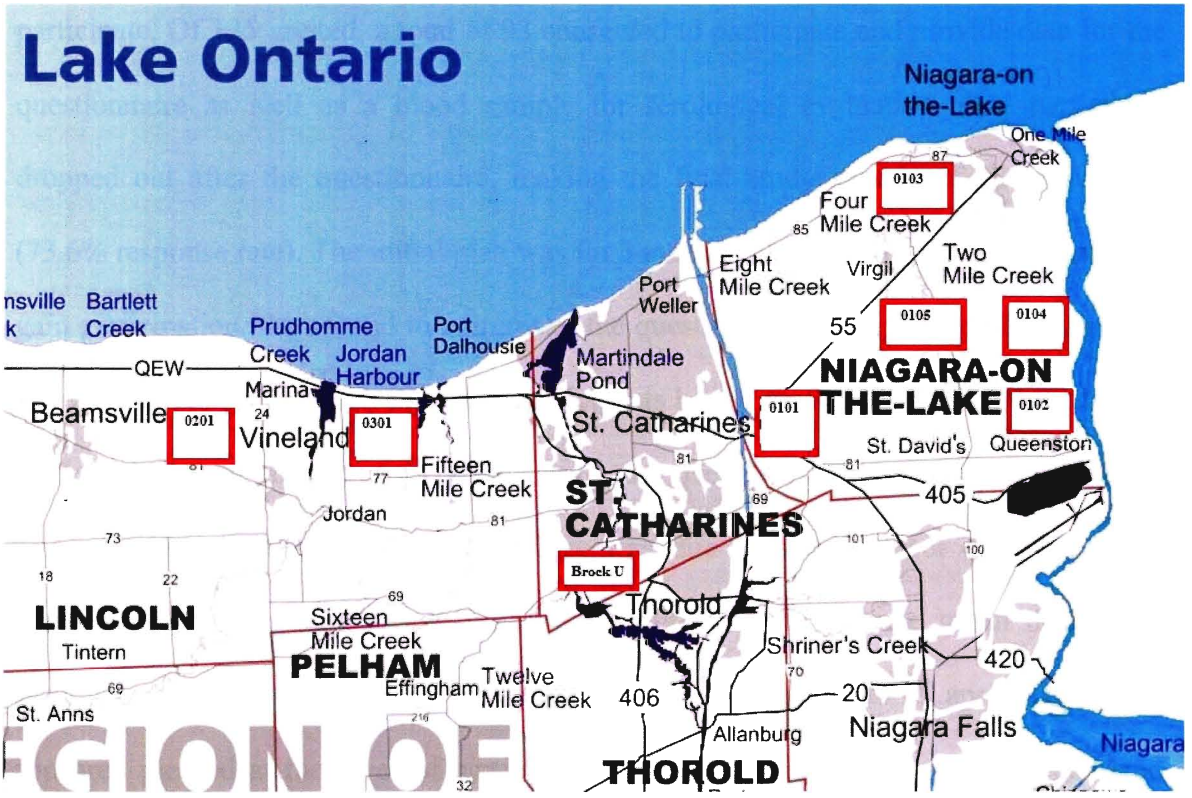
For Objective No.3 (Assess the awareness of study participants about WNV infection as well as their understanding of the mode of transmission and clinical importance of the infection), a descriptive analysis was performed and data was presented as percentage of individuals possessing the characteristics in question out of the total number of participants.

CHAPTER FOUR: RESULTS

This West Nile Virus (WNV) seroprevalence study was conducted during the WNV transmission season of 2007 among the Mexican agricultural workers of the Niagara Region. Since there is no public registry of farms operating in the Region, nor is it public domain how many employ Mexican workers, an attempt was made to get such registry information through the Niagara Regional Municipality. The information from the Niagara Region gave only the addresses where the workers lived but not the location of the farm of employment. In all, Niagara had 378 housing facilities registered to 229 farms in the cities and towns of Beamsville, Grimbsy, Jordan, Jordan Station, Lincoln, Niagara Falls, Niagara-On-The-Lake, Pelham, Smithville, St. Anns, St. Catharines, Vineland, and Wainfleet for the 2007 season.

Since it was necessary to obtain consent first from the farm owners to approach his or her workers on their land, the initial contact needed to be with the farm owner. Due to the need for many farm visits and time constraints of the WNV transmission season, only farms in the nearby cities were eligible for invitation. Letters of WNV information and invitation to participate were prepared for 132 farms in the cities and towns of Niagara-On-The-Lake, St. Catharines, Jordan Station, and Vineland. Letters were personally delivered to 54 (41%) farm owners during several field trips occurring between June 15, 2007 and July 15, 2007. Letters could not be delivered to 78 out of the 132 farms due to invalid addresses, empty houses or lack of a principal office or home of the farm owner. During the initial conversation with farm owners, the study was presented, discussed and questions were answered. Out of the 54 farms owners visited, 12 (22.2%) agreed during the first visit; their number of employees ranging from 2-80. The remainder wanted more

time for consideration and were given a contact at Brock University should they decide to participate. None of the owners would give specific reasons for not wanting to participate beyond the general statement that the workers were too busy with long working hours. Some appeared reluctant but it was not evident as to why. These were subjective observations by this researcher. Only two farms owners contacted Brock later during the study period but it was too late to accommodate them in the study. Out of 12 farms, five (two farms had two sites) were selected to proceed with initially, due to their larger size and proximity to Brock University. This gave an overall participation proportion of 5/54 or 9.3%. We did not contact the remaining seven farms due to time constraints of the WNV transmission season. Map 1 indicates the location of the farms selected for the study. All farms are reasonably close to water sources and thus mosquito habitat.



Map 1. Location of participating farms in the Niagara Region (Niagara Peninsula Conservation Authority)

Further, the largest five farms were selected, based on the need for several visits, the short WNV transmission season, and the departure dates of the workers leaving the Niagara Region for their homes in Mexico in September or October. Two of the five farms had two locations, giving a total of seven locations for the study. Four farm owners agreed that the researchers could approach all their workers and one requested preselecting the workers to be approached for invitation. That particular farm supervisor chose 10 workers to participate but did not reveal his reasons for the selection. In total, 125 Mexican workers were invited to the study (as mentioned earlier, the WNV study was an additional study of that being undertaken by Angela Duarte, MSc and supervised by Ana Sanchez, PhD and therefore was limited to their enrolled participants).

An initial information meeting was planned for each farm and the workers were invited to participate. Of 125 invited, a total of 93 consented to participate and provide data for the questionnaire as well as a blood sample for serological evaluation. One participant dropped out after the questionnaire, making the final study sample of 92 individuals (73.6% response rate). The initial plan was for 3 meetings: the first for information and to gain participation, the second to administer the questionnaire and take the blood sample, and the third to give results. Since the participants had to meet with us after their normal working hours on six out of the seven farms, the questionnaires and the blood samples were obtained during the first visit. This was due to a high level of interest and convenience for the participants. The results with discussion were given during the second meeting. Farm owners were recognized with a certificate of appreciation and appeared pleased at the level of participation, particularly since the meetings occurred after normal working hours. They were not surveyed for interest in future studies.

4.1 Characteristics of research participants

Of 125 invited, a total of 92 persons (73.6%) completed the study. Most participants were male (78.3%) and their age ranged from 22 to 65 years (mean 38.5±9.5). The workers originated from 17 Mexican States, most commonly from Mexico State (32.6%), Tlaxcala (12.0%), Puebla (7.6%) and Guanauato (6.5%). More than one-half of the participants (52.0%) had completed primary school and 61.9% worked as farmers in their home country. See Table 7 for a list of other characteristics.

Living conditions of research participants in Mexico and Canada

The WNV questionnaire (App.I) also collected data regarding the living conditions and type of housing both in Mexico and Canada. The housing condition questions were developed based on the standard measurement of living conditions and access to basic services suggested by the Government of Mexico. For this study, housing conditions were classified from I to V based on the presence or absence of indoor running water, indoor flushing toilet, indoor electricity, and concrete (tile or cement) floor in entire house. Type I included all the 4 amenities whereas type II to V included only three, two, one, or none respectively [117]. Most of the workers lived in type I and II housing in Mexico (94%). The majority of workers (80%) also lived at the same address for more than 20 years indicating permanency in their geographic area. The number of persons per house as well as their ages can be viewed in Table 7.

Almost all the workers lived in the equivalent of type I housing in Niagara (97%). Only 3 workers lived in a trailer adjacent to a house and with all the basic services. There were 4-16 workers per house and 1-6 people per bedroom. See Table 8 for details per farm.

Table 7. Demographic characteristics of study participants (n=92)

Characteristic	n=92 (% of sample)
Sex	
Male	72 (78.3%)
Female	20 (21.7%)
Age (yrs)	
22-31	24 (26.1%)
32-37	22 (23.9%)
38-43	22 (23.9%)
44-65	24 (26.1%)
Education	
None or Primary (completed)	48 (52.2%)
Secondary (completed) or Technical	43 (46.7%)
Missing	1 (1.1%)
Mexican State of origin	
Mexico	30 (32.6%)
Tlaxcala	11 (11.9%)
Puebla	7 (7.6%)
Guanajuanto	6 (6.5%)
Oaxaca	4 (4.4%)
Queretaro	4 (4.4%)
Veracruz	4 (4.4%)
Yucatan	4 (4.4%)
Other	22 (23.8%)
Occupation in Mexico	
Farmer	57 (61.9%)
Construction labour	8 (8.7%)
Merchant	4 (4.4%)
Other (e.g. housewife, fisherman)	23 (25.0%)
Type of Housing in Mexico[1]	
Type I	65 (70.6%)
Type II	21 (22.8%)
Type III	4 (4.4%)
Type IV	1 (1.1%)
Missing	1 (1.1%)
Number of people per household in Mexico	
3	12 (13.0%)
4	16 (17.4%)
5	25 (27.2%)
6	14 (15.2%)
7	10 (10.9%)
≥8	15 (16.3%)

[1]Type of housed stratified by presence of indoor water, indoor flushing toilet, electricity, concrete or tile floor. Definition: Type I- all 4; Type II- any 3; Type III- any 2; Type IV-any 1;Type V-none

Table 8. Number of workers per home and bedroom in each farm in Canada

	Farm number						
# workers	0101	0102	0103	0104	0105	0201	0301
# workers per house	14	16	4/8/14[1]	10/11[1]	7/11[1]	6/14[1]	8/10[1]
# workers per bedroom	2	2-3	2-4	2-6	1-5	2-6	1-4
# workers participating	12	8	13	21	11	12	15

[1]Each number represents the number of people per house with multiple houses on a farm

Self-reported health status of research participants

All individuals entering into the seasonal agricultural program in Canada must pass a physical exam. Workers were asked to report their health on a three point scale from good to fair to poor. We found 81% workers reporting overall good health with no underlying medical conditions that could affect an immunological response. The remainder reported fair health; the problems being experienced were minor or chronic such as headache and backache. History of Dengue infection was assessed due to the potential cross-reactivity of the Dengue virus and WNV, but only 4/92 (4.3%) of the workers recalled having had a history of Dengue infection.

Dietary habits, body weight, or body mass index were not examined, as they were not relevant to the study. However, some lifestyle choices that negatively affect health such as excessive alcohol consumption and smoking, which may affect behavior (more time outside with mosquitoes), were examined with 63% of the workers consuming alcohol to

varying degrees [fourteen out of 92 workers (15%) reported consuming 3 servings 3 times a week of more] and 30% of the workers reported smoking.

Permanency in the Seasonal Agricultural Workers Program

The majority of participants (73%) were return workers to Canada with a range of 2-22 seasons (mean 6.7). Nineteen workers (21%) were first time workers for the 2007 season. Seventy-eight per cent of the workers reported that they have remained in Ontario. Of the remaining 22% working in other provinces, 70% reported working in Quebec. Within Ontario, movement between farms was frequently reported with 39% working in other cities.

4.2 West Nile Virus point seroprevalence

Enzyme Linked Immunosorbent Assays (ELISA), (Panbio Inc, Australia), were utilized to evaluate the serum samples for the presence of anti-WNV and anti-Dengue antibodies. Both an IgM capture and an IgG Indirect ELISA were used for the detection of anti-WNV antibodies and an IgG Indirect ELISA was used for the detection of anti-Dengue antibodies. Anti-Dengue antibodies were assessed because this infection is prevalent in Mexico and the established cross-reactivity between flavivirus antibodies on ELISA. Avidity testing with the WNV IgG positive samples was also performed to attempt to differentiate the positive WNV IgG from the positive Dengue IgG samples. All samples which were positive for either anti-WNV antibodies or anti-Dengue antibodies were forwarded to the National Microbiological Laboratory (NML) in Winnipeg, Manitoba for confirmation with the Plaque Reduction Neutralization Test (PRNT).

4.2.1 Antibody determination

As depicted in Table 9 below, out of 92 samples, two (2.2%) were positive for WNV IgM, 19 (20.7%) were positive for WNV IgG, and 16 (17.4%) were positive for Dengue IgG. Should these results be interpreted literally, without further confirmation, it could have been inferred that there were a couple of recent WNV exposures and that WNV and Dengue exposure in the past was frequent among these workers (with point prevalences of 20.7% and 17.4%, respectively). However, of the 19 positive for WNV IgG, 15 were concurrently positive for IgG Dengue suggesting crossreaction of the ELISA results between WNV and Dengue antibodies. In order to understand the magnitude of this IgG cross-reactivity, data were organized in the contingency table shown in Table 10 below. Since 15 individuals were positive for both WNV IgG and Dengue IgG there was no way to differentiate if workers had been exposed to both viruses or to just one of them. It is widely established that sera positive to Dengue will also be positive to other flaviviruses such as WNV (i.e., heterologous antibodies). The next step chosen to elucidate this cross-reactivity was performance of a WNV IgG avidity test.

Table 9. Antibody reactivity for WNV and Dengue virus in a commercial ELISA in the study population (n=92)

	WNV IgM+	WNV IgG+	Dengue IgG+
Positive	2 (2.2%)	19 (20.7%)	16 (17.4%)
Negative	90 (97.8%)	73 (79.3%)	76 (82.6%)
Totals	92	92	92

Table 10. Cross-reactivity for WNV and Dengue antibodies with a commercial ELISA (n=92)

	Dengue IgG		
WNV IgG	Positive	Negative	Total
Positive	15 (16.3%)	4 (4.3%)	19 (20.7%)
Negative	1 (1.1%)	72 (78.3%)	73 (79.3%)
Total	16 (17.4%)	76 (82.6%)	92

4.2.2 Avidity test

The WNV IgG Avidity ELISA attempts to differentiate between recent and past infections. Early infections produce low avidity IgG antibodies as opposed to high avidity antibodies of past infections. The lab test involves the addition of an avidity reagent which removes low avidity antibodies thus only antibodies of high avidity will be revealed by the test. As said earlier, this test was used as an attempt to differentiate those individuals with both positive findings for WNV IgG and Dengue IgG. An assumption is that a past infection would likely be a Dengue infection acquired in Mexico and a recent infection would likely be WNV acquired in Canada. The avidity test would naturally be only useful for those individuals working and residing in Canada for their first time because return workers may have been infected with WNV in Canada in a previous year. As depicted in Table 11 below, 19 individuals found positive for WNV IgG, 6 could be considered recent infections and 13 past infections. Of those 6 recent infections only 1 was a first time worker in Canada which would likely indicate WNV exposure. However, this individual originated from the state of Yucatan, which is endemic for Dengue and was later found PRNT positive for Dengue and negative for WNV. Of the 13 persons

considered with past infection results, 6 were in Canada for their first time so they were likely Dengue.

Table 11. West Nile Virus IgG avidity results in individuals positive for WNV IgG (n=19)

Worker entry into Canada	WNV IgG low avidity recent infection	WNV IgG high avidity past infection	Total
First time in Canada	1	6	7
Return worker/resident	5	7	12
Total	6	13	19

IgG avidity was not performed for Dengue because it was assumed that if any of the workers had anti-Dengue antibodies, they would have high avidity results from at least the last season spent in Mexico (there is no Dengue transmission in Canada).

Since the 12 return workers could have antibodies to either or both WNV and Dengue, the avidity test did not clarify the cross-reactivity in any of the cases (i.e., 5/12 (42%) were interpreted as "recent" and 7/12 (58%) were interpreted as "past" infections). If the avidity test was not performed in this study, the interpretation of the results would be the same. In other words the avidity test was of no assistance in interpreting the cross-reactivity between WNV and Dengue ELISA results. Therefore further confirmation was required. The next step needed to differentiate and confirm for the presence of the true antibodies in our 15 participants with both anti-WNV and anti-Dengue antibodies was to send the samples for confirmation with the Plaque Reduction Neutralization Test (PRNT).

4.2.3 Plaque Reduction Neutralization Test

Samples that demonstrated antibodies to either WNV or Dengue were forwarded to the NML for confirmation with the PRNT. A total of 22 samples were sent for confirmation with PRNT. Of those 22, two were positive for IgM WNV, 19 for IgG WNV, 16 for IgG Dengue, and 15 were positive for both WNV and DEN IgG antibodies. The PRNT results yielded 0 positive results for WNV (Table 12).

Table 12. WNV confirmation of 22 ELISA positive samples with PRNT

	WNV PRNT		
WNV ELISA[1]	Positive	Negative	Totals
Positive	0(a)	21(b)	21
Negative	0(c)	1(d)	1
Totals	0	22	22

[1]Positive ELISA results include those that are positive for WNV IgG or WNV IgM.
Sensitivity a/a+c; Specificity d/b+d; Positive predictive value a/a+b; Negative predictive value d/c+d

Since only ELISA positive samples were sent for confirmation with PRNT only some of the ELISA test parameters could be estimated as indicated below:

- WNV ELISA sensitivity = 0/0 = undefined number
- WNV ELISA specificity = 1/22= 4.5%
- WNV ELISA positive predictive value = 0/21= 0%
- WNV ELISA negative predictive value = 1/1 = 100%

Conversely, Panbio's ELISAs for Dengue were much closer to the PRNT results: 13 out of 16 (81.3%) ELISA positives were true positives and 3 (18.8%) were false positives (Table 13).

Table 13. Dengue confirmation of 22 ELISA positive samples with PRNT

	Dengue PRNT		
Dengue IgG ELISA	Positive	Negative	Total
Positive	13	3	16
Negative	0	6	6
Total	13	9	22

For Dengue the ELISA test parameters are as follows:

- Dengue ELISA sensitivity = 13/13 = 100%
- Dengue ELISA specificity = 6/9 = 66.7%
- Dengue ELISA positive predictive value = 13/16 = 81.3%
- Dengue ELISA negative predictive value = 6/6 = 100%

As mentioned above, the Panbio ELISAs positive for WNV resulted negative in the PRNT. This degree of cross-reactivity is certainly perplexing and warrants further investigation in order to identify potential factors influencing cross-reactivity or at least predictors of it. With this in mind, WNV false positive results were aggregated and are presented in Table 14 and Map 2 below.

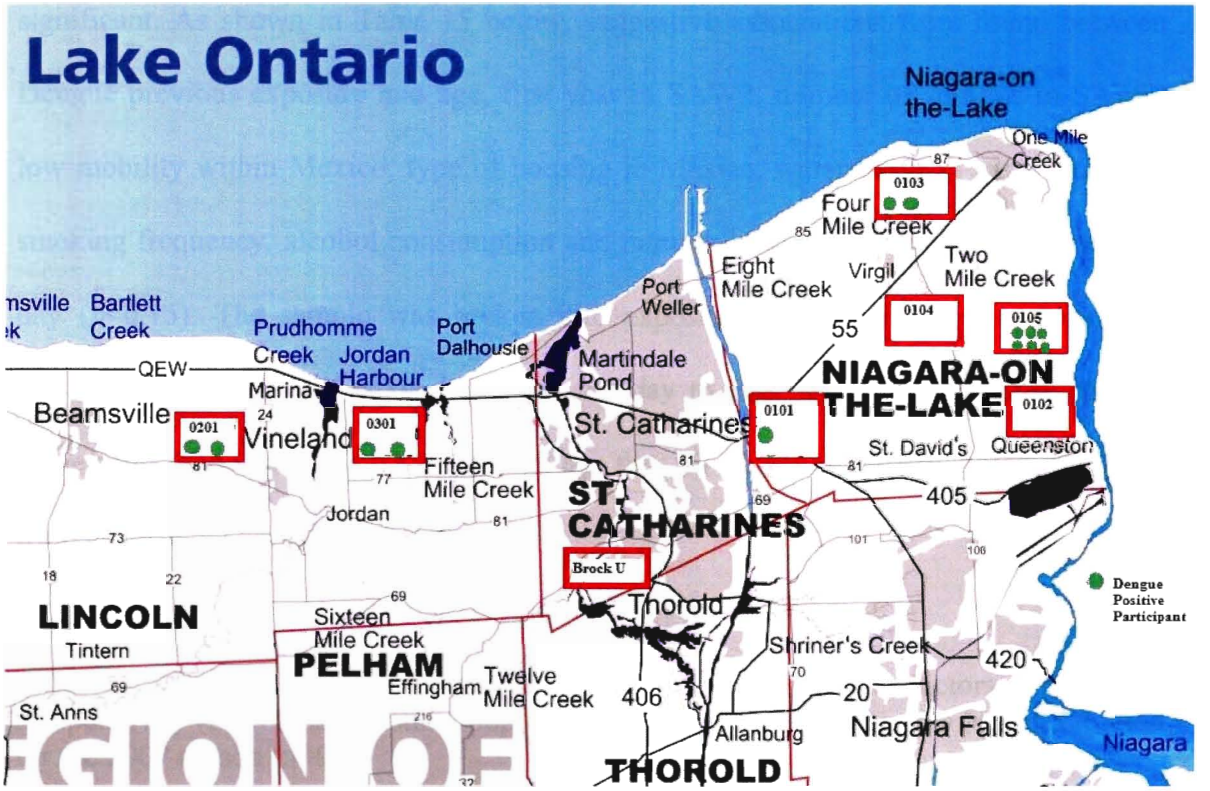
Table 14 below shows that the 19 samples from individuals positive for WNV ELISA IgG originated from 11 different states, without predominance of a particular state. The table also includes those individuals positive for WNV IgM and those positive for

Dengue IgG. The lack of commonalities among the six WNV IgG ELISA+/DEN PRNT negative would appear to indicate another factor(s) was present eliciting the cross-reactivity. Four individuals were men, two originated from Dengue non-endemic states (Puebla and Guanajuato), 5/6 of them were return workers and they all worked in different farms in Niagara Region. However, no conclusions can be drawn since there is a lack of power. Of the 13 WNV ELISA+/Den PRNT positive, all were from Dengue endemic states.

Table 14. Participants with positive ELISA results and their confirmatory PRNT (n=22)

State of Origin	Sex/Age	Dengue Endemic Mx State	Years in SAWP	WNV IgM ELISA	WNV IgG ELISA	DEN IgG ELISA	WNV PRNT	DEN PRNT	Interpretation
Chiapas	M/33	Yes	First	Neg	Pos	Pos	Neg	Pos	WN unexp /DEN hx
Chiapas	M/38	Yes	First	Neg	Pos	Pos	Neg	Pos	WN unexp /DEN hx
Yucatan	M/29	Yes	First	Neg	Pos	Pos	Neg	Pos	WN unexp /DEN hx
Yucatan	M/26	Yes	First	Neg	Pos	Pos	Neg	Pos	WN unexp /DEN hx
Yucatan	M/22	Yes	First	Neg	Pos	Pos	Neg	Pos[1]	WN unexp /DEN hx
San Luis	F/29	Yes	First	Neg	Pos	Pos	Neg	Pos[1]	WN unexp /DEN hx
Guerrero	M/50	Yes	7	Neg	Pos	Pos	Neg	Pos	WN unexp /DEN hx
Mexico	M/26	Yes	5	Neg	Pos	Pos	Neg	Pos	WN unexp /DEN hx
Oaxaca	M/37	Yes	6	Neg	Pos	Pos	Neg	Pos	WN unexp /DEN hx
Veracruz	M/34	Yes	9	Neg	Pos	Pos	Neg	Pos	WN unexp /DEN hx
Yucatan	M/29	Yes	5	Neg	Pos	Pos	Neg	Pos	WN unexp /DEN hx
Tlaxcala	M/38	Yes	8	Neg	Pos	Pos	Neg	Pos	WN unexp /DEN hx
Veracruz	F/42	Yes	8	Neg	Pos	Pos	Neg	Pos	WN unexp /DEN hx
Nayarit	F/24	Yes	First	Neg	Pos	Pos	Neg	Neg	WN & DEN unexp
Guerrero	M/35	Yes	5	Neg	Pos	Pos	Neg	Neg[1]	WN & DEN unexp
Morelos	F/38	Yes	4	Neg	Neg	Pos	Neg	Neg	WN & DEN unexp
Veracruz	M/35	Yes	4	Neg	Pos	Neg	Neg	Neg	WN & DEN unexp
Guanajau	M/50	No	22	Neg	Pos	Neg	Neg	Neg	WN & DEN unexp
Guerrero	M/43	Yes	3	Neg	Pos	Neg	Neg	Neg	WN & DEN unexp
Puebla	F/33	No	8	Neg	Pos	Neg	Neg	Neg	WN & DEN unexp
Mexico	M/36	Yes	5	Pos	Neg	Neg	Neg	Neg	WN & DEN unexp
Michoaca	M/41	No	6	Pos	Neg	Neg	Neg	Neg	WN & DEN unexp

[1]These participants recalled having suffered from Dengue
WN unexp: no previous exposure to West Nile virus; DEN unexp: no previous exposure to Dengue; DEN hx: Dengue infection history; Pos=positive; Neg=negative



Map 2. Dengue positive PRNT participants. Positive individuals are represented as green dots within the farms (Niagara Regional Conservation Authority)

4.2.4 Risk factors for Dengue exposure/infection

Risk factors from the study questionnaire for Dengue PRNT positivity (as a proxy for Dengue exposure) were examined using regression analysis. First, a univariate regression analysis was performed and risk factors associated with an outcome at a p value< 0.15 were selected for inclusion in the multivariate analysis. This study was an exploratory hypothesis generating study. A p value of 0.15 instead of 0.05 was chosen to control confounding more effectively and not to exclude possible associations that might have otherwise been missed [118].Other studies in Dengue research have utilized a similar p value of 0.15 for univariate analysis [113-116]. Second, the multivariate analysis was performed and variables with the traditional value of p<0.05 were considered to be

significant. As shown in Table 15 below, suggestive associations were found between Dengue previous exposure and age, first year in SAWP, number of seasons in SAWP, low mobility within Mexico, type of housing in Mexico, water basin in yard, smoking, smoking frequency, alcohol consumption and number of alcoholic drinks consumed per day ($p<0.15$). The sample was broken into exposure sections for age, seasons, low mobility, and number of alcoholic drinks per day to look for a dose-response pattern. None were found apart from a trend with age thus all variables were dichotomized apart from age which was left continuous.

Table 15. Univariate logistic regression analysis to identify risk factors for Dengue seropositivity (PRNT) in the study population of Mexican workers in the Niagara Region for the 2007 season (n=92)

Variable	Number	% with Dengue antibodies (PRNT)	OR (95% CI)	$p < 0.15$
Sex				
Female	20	2/20 (5%)	1.00	
Male	72	11/72 (15%)	1.62 (0.33-8.00)	0.55
Age (years)				
22-31	22	6/22 (23%)	1.00	
32-37	24	3/24 (13%)	0.47 (0.10-2.18)	0.34
38-43	24	3 /24 (13%)	0.47 (0.10-2.18)	0.34
44-65	22	1/22 (5%)	0.13 (0.01-1.18)	0.07
Age: risk per year of age	92		0.92 (0.85-1.00)	0.05*
Level of Education				
Primary education	48	5/48 (10%)	1.00	
Higher than primary ed.	43	7/43 (16%)	1.67 (0.49-5.72)	0.41
First year in SAWP in Canada				
More than 1st year	73	7/73 (10%)	1.00	
First year in Canada	19	6/19 (32%)	4.35 (1.26-15.06)	0.02*

Number of seasons in SAWP in Canada (seasons)				
1	19	6/19 (31%)	1.00	
2-4	24	1/24 (4%)	0.09 (0.01-0.87)	0.04
5-8	28	6/28 (21%)	0.59 (0.16-2.21)	0.44
9-22	21	0/21 (0%)	dropped	
Seasons 1	19	6/19 (31%)	1.00	
Seasons more than 1	73	7/73 (9%)	0.23 (0.07-0.80)	0.02*
Mobility within Mexico-lived in same home for years				
1-25	21	5/21 (23%)	1.00	
26-33	24	6/24 (25%)	1.07 (0.27-4.17)	0.93
34-41	24	2/24 (8%)	0.29 (0.05-1.69)	0.17
42-65	23	0/23 (0%)	dropped	
Low mobility 25 yrs and less	21	5/21 (24%)	1.00	
Low mobility more than 25 yrs	71	8/71 (11%)	0.41 (0.12-1.41)	0.15*
Type of Mexican home				
Type I only	64	12/64 (19%)	1.00	
Type II-V	26	1/26 (4%)	0.18 (0.02-1.46)	0.11*
Water basin Mexican yard				
No	22	7/22 (32%)	1.00	
Yes	70	6/70 (9%)	0.20 (0.06-0.69)	0.01*
Smoking				
No	64	6/64 (9%)	1.00	
Yes	28	7/28 (25%)	3.22 (0.97-10.69)	0.06*
Number of cigarettes smoked per day				
No smoking	64	6/64 (9%)	1.00	
Smoke more than 1	28	7/28 (25%)	3.40 (1.20-9.64)	0.02*
Alcohol consumed				
No	34	2/34 (6%)	1.00	
Yes	58	11/58 (19%)	3.74 (0.78-18.03)	0.10*
Number of alcoholic drinks consumed per week				
No drinking	34	2/34 (6%)	1.00	
1 per week	24	5/24 (21%)	4.20 (0.74-23.90)	0.10
2 per week	16	1/16 (6%)	1.07 (0.09-12.70)	0.96
3 or more per week	18	5/18 (28%)	6.15 (1.05-35.80)	0.04
Number of alcohol drinks 0	34	2/34 (6%)	1.00	0.10*
Number of alcohol drinks more than 0	58	11/58 (19%)	3.74 (0.78-18.03)	0.10

*associations with $p<0.15$

As shown in Table 16 below, the multivariate analysis showed statistical associations between Dengue exposure and first year in SAWP (p = 0.006), type of Mexican home (p=0.029), and a water basin in the yard (p=0.004). Participants in their first year in the SAWP program seemed to be at an increased risk for Dengue exposure. On the other hand, a home of lower socioeconomic condition in Mexico appeared to reduce the risk of Dengue exposure. The presence of a water basin in the yard also suggested a decreased risk of exposure to Dengue.

Table 16. Multivariate logistic regression analysis to identify risk factors for Dengue seropositivity(PRNT) in the study population of Mexican workers in the Niagara Region for the 2007 season using the suggested associations found with univariate analysis

Variable	OR (95% CI)	$p < 0.05$
First year in SAWP		
More than 1st year	1.00	
First year	9.16 (1.90-44.30)	0.006
Type of Mexican home		
Type I	1.00	
Type II-V	0.06 (0.01-0.75)	0.029
Water basin Mexican yard		
No	1.00	
Yes	0.11(0.03-0.50)	0.004

4.3 Awareness of West Nile Virus

Of the 92 participants, 21 (22.8%) indicated that they had heard or had some knowledge of WNV. Of those with some knowledge, three-quarters (76.2%) knew that the infection was transmitted by mosquitoes, almost half (47.6%) associated the infection with a fever, and approximately two thirds (62.0%) had some knowledge of the symptoms caused by

WNV infection (see Table 18 below). Only 2/92 (2.2%) participants indicated that they used repellent to protect themselves from mosquito bites. It is important to note that none of the participants knew that all 3 symptoms of fever, pain and fatigue occur simultaneously with a WNV infection.

Table 17. Awareness of West Nile Virus among study participants (n=92)

Awareness of WNV	**Yes**	**No**
General awareness of WNV n=92	21 (22.8%)	71 (77.2%)
Knowledge of symptoms and transmission n=21		
Symptoms n=21		
Fever	10 (47.5%)	11(52.4%)
Pain	1 (4.8%)	20 (95.2%)
Fatigue	1 (4.8%)	20 (95.2%)
Fever, pain and fatigue	0 (0%)	100 (100%)
Blindness	1 (4.8%)	20 (95.2%)
None of the above	8 (38.1%)	13 (61.9%)
Transmitted n=21		
Mosquito	16 (76.2%)	5 (23.8%)
Water	2 (9.5%)	19 (90.5%)
Food	1 (4.8%)	20 (95.2%)
Unknown	2 (9.5%)	19 (90.5%)

CHAPTER 5: DISCUSSION

It has been 10 years since West Nile Virus arrived in North America. In Canada, viral activity commenced in 2001 and the first human case occurred in Ontario the following year. Along with public awareness campaigns, Public Health Departments across the country rapidly implemented active surveillance strategies, control programs, and made available screening and confirmatory laboratory diagnosis for the infection. Clinical reports of WNV fever and WNV neurological disease continued to increase throughout 2002-2003, but the downward trend experienced in the period 2004-2006 suggested that the epidemic could have been reaching its maximum potential. This was not the case across Canada, as human cases in 2007 reached record numbers (n=2,215, >50% more cases than previously documented). This alarming increase, however, plummeted in 2008, when only 36 cases were reported across the country. In the present year, 2009, seven human clinical cases of WNV have been reported to the Public Health Agency of Canada [17].

Despite these upward and downward WNV transmission patterns in the country, the prevalence of the disease in humans in Niagara never developed to the degree experienced in other areas of Canada. Only 30 human cases have occurred in Niagara Region since 2002 with the last case reported in 2006 [18]. There is, to date, no clear explanation as to why despite having the apparent environmental conditions for transmission and viral activity in birds and mosquitoes, so few human WNV cases have occurred in Niagara. Similarly, there is no prediction model that can accurately indicate whether or not this pattern will continue leading eventually to the nil levels prior to 2001

(i.e., that WNV transmission will die out in the Region). If this were the case, does the neglible risk to the human population warrant continued costly disease surveillance? This is certainly a difficult question to answer. On the other hand, lessons learned from countries where WNV has re-emerged dramatically after quiet periods suggest that public health preparedness and active surveillance remain the best decision [78, 81-85, 88, 91].

This is the case of the Regional Municipality of Niagara where dead bird surveillance and virus determination in mosquito pools are still done. This is a well-established practice in North America, as sudden deaths in large numbers of birds, particularly corvids, is still the best sentinel for predicting an outbreak [119]. In fact, a Canadian study by Elliott et al (2005) reported a peak in dead corvids approximately 5 weeks before most of the human cases were detected, thus providing further support to this practice [120]. Additional surveillance methodology using strategically placed chicken flocks and horses (before introduction of the equine WNV vaccine) was tested early on in Canada but both methods underperformed compared to the results obtained from dead bird evaluation and mosquito population surveillance [121].

Human surveillance through anti-WNV antibody determination of a representative segment of the population has never been attempted in Niagara Region. Regardless of the lack of clinical cases, WNV transmission can still be occurring as research studies determined that the majority of WNV infections (> 80%) are asymptomatic [4, 5, 40]. A human seroprevalence study for WNV could help to elucidate at least the question if transmission is still occurring in the Region. Therefore, the aim of the present MSc project was to conduct an investigation in persons considered at high risk for mosquito bites and with low probability of past WNV exposure. As such, this study aimed to

investigate WNV antibodies as an indicator of transmission in a group of Mexican agricultural workers in Niagara. This group met the two criteria set for the study: due to their prolonged outdoor activities they were deemed as high risk of exposure for mosquito bites and their originating from Mexico minimized the probability of previous exposure to WNV.

In analyzing published literature, however, it became clear at the onset of the study, that WNV serology in humans is plagued with problems due to the notorious cross-reactivity of flaviviruses in general. Moreover, the fact the study sample was originally from Mexico added a high degree of certainty of previous exposure to Dengue virus and thus of cross-reactivity. Delays in commencing the study added yet another complicating factor: since the workers had been already in Niagara for some time before enrolment in the study, no baseline serology could be established and was therefore not attempted. In an ideal situation, seroconversion while in Niagara would prove local transmission. If this was not feasible and a single-sample approach was needed, first time workers should be enrolled at entry. Since neither of these was viable, a mix of first-time and return workers were enrolled and serological tests were planned carefully, as follows:

a) Testing for WNV IgM would permit us to discern, with 96% sensitivity and 85% specificity, a very recent infection by WNV

b) Testing IgG for both WNV and Dengue would allow us to determine the cross-reactivity

c) WNV IgG avidity determination would help to elucidate cross-reactive samples and conclude which cases could be deemed as "probable cases" (the epidemiological history would enable us to strengthen the conclusions)

d) PRNT confirmation of positive samples would allow us to establish definitive diagnoses and compare PRNTs with ELISAs results to assess the latter's performance in a seroprevalence study

5.1 Serological tests and cross-reactivity issues

As evidenced by our results, however, antibody determination with Panbio's ELISAs resulted in such high degree of cross-reactivity that any credible interpretion was impossible. The serological standard, PRNT, revealed that all of the samples determined WNV-positive by ELISA were in fact, false positives. As mentioned earlier, cross-reactivity is a well acknowledged issue in flavivirus serology, as false positivity can be due to multiple causes, the most common being exposure to other flavivirus such as Dengue virus, St. Louis encephalitis virus and Eastern equine encephalitis virus; exposure to other viruses as Varicella-Zoster virus, Cytomegalovirus, Epstein-Barr virus, Enterovirus; or even physiological factors such as rheumatoid factor, and anti-nuclear antibodies. Several studies have reported cross-reactivity but no study has reported to date the universal cross-reactivity observed in the present work [16, 122-127]. A Canadian study undertaken by Loeb *et al* in 2005, the ELISA screening test employed (from CDC) performed considerably better than the one used in the present study: 100% sensitivity and 99% specificity and negative and positive predictive values of 100% and 76%, respectively (n=1505; 60 ELISA positives of which 46 were PRNT positive) [125]. It is important to mention that the quality and reproducibility of the ELISAs done at

Brock were at par with those done at the reference laboratory in Winnipeg, as they repeated ELISAs titres (using kits from a different manufacturer) obtaining almost identical results as the ones found with Panbio's (results not shown).

Our study findings emphasize the superiority of PRNT over Panbio's commercial kit. Had we relied on the ELISA methodology alone, based on the two IgM positive individuals, two recent cases of WNV exposure would have been concluded. Similarly, erroneous conclusions would have been reached in regards to WNV exposure, due to the extreme cross-reactivity between the WNV and DEN IgG results.

5.2 Study population suitability

Before analyzing study results, it is pertinent to discuss whether or not the study population was indeed at increased risk for mosquito bites. Our sample of farm workers spent time outdoors from dawn to dusk, both working and socializing, most of them averaging 12-16 hours a day/6 days a week outdoors. This is by far, longer than other outdoor non-agricultural workers in Niagara [128, 129]. Another reason why these workers may have been more suitable for a WNV study is that despite the fact that they were aware of the presence of mosquitoes, only 2% used repellent such as DEET (however all participants reported and were observed wearing long pants and long sleeves). Loeb *et al* (2005), in the Oakville study mentioned above, reported that to obtain protection of approximately 50% study participants needed to practice 2 or more personal protective behaviors. Risk of infection did not decrease for persons practicing only one protective behaviour [125]. Extrapoloating Loeb's *et al*. findings to the present study, we can conclude that our participants were indeed at higher risk for mosquito bites. Even though the use of pesticides by some farms may have exerted a protective effect

against mosquito bites it is likely that the length of time spent outdoors diluted this environmental effect.

We believe that this group of agricultural workers provided an improved study design and are indeed a valuable population for vector-borne infections surveillance. In fact, Bender *et al*. suggest that agricultural workers are good as sentinels for some infectious diseases. Farm workers are in close contact with animals and animal products and may be the first to acquire new or re-emerging diseases [105]. With respect to WNV it is the close proximity to the mosquito vector that places this group of people at a higher risk. A study of farm workers in Veneto Region, Italy, aimed to investigate the prevalence of human WNV on farms where WNV positive horses were found. A seroprevalence of approximately 2% (WNV PRNT positive) was found among the workers indicating successful transmission to humans as well as to the horses had occurred, although the workers were asymptomatic [130]. This study shows that the level of asymptomatic WNV infections may be underestimated.

5.3 Objective 1: Conduct a seroepidemiological investigation for WNV and Dengue virus

Failure to prove WNV transmission

Our small sample size does not allow for conclusive interpretation of our findings. There could be a chance –albeit small- that with a larger sample size we would have different findings. However, our negative findings in regards to WNV exposure by these research participants are likely accurate (point seroprevalence 0/92 [95% CI 0%-3.9%]) as they are supported by the small number of cases of human disease observed in the Niagara Region. Even with proven intense enzootic transmission, negative human seroprevalence

is not an unusual finding in the literature. A human serosurvey done by McCarthy *et al.* (2001) in an area of intense transmission in the United States found zero prevalence as well, concluding that an intense epizootic can occur in an area without presenting a high risk for infection to humans [131]. In Ontario, a human surveillance study conducted in 2000, reported no cases of WNV infection either [123].

Conversely, in 2005, Loeb *et al.* conducted a study in Oakville, Ontario and found an overall 3.1% (95% CI 2.2%–4.0%) of seroprevalence in 1,505 persons tested [125]. If we were to assume an expected prevalence at Oakville's lower CI limit (2.2%), and setting the worst acceptable frequency at 0.5%, for a population of 400,000 in Niagara we would have needed a sample size of 286 participants to reach a 95% level of confidence (CDC, 2002, Epi-Info Stats-Calc). Thus, with a sample size of 92 persons in the present study, we cannot conclude a total absence of WNV circulation in humans in Niagara, but we can speculate that if this circulation occurs, it would still be very low, within zero and 3.9%.

Instead of discouraging public health actions, low or nil seroprevalence for WNV in a specific area should be interpreted as a matter of concern, for an immunologically naïve population is more susceptible to outbreaks than one where transmission has reached a consistent pattern [78, 88]. For 2009 in the Niagara Region, no humans have been reported as yet for WNV positivity, however, 1 mosquito pool and 2 birds have been reported as positive for WNV [18] indicating that the human population may remain immunologically susceptible.

In analyzing outbreaks elsewhere in Canada, it is difficult to explain why WNV transmission remains so low in Niagara. Perhaps the local populations of *Culex pipiens*

and *Culex restuans* are more ornithophilic thus resulting in relatively few human cases in Niagara over the years. It could also be possible that the population density and degree of urbanization in Niagara is not able to sustain mosquito-human transmission. Several studies have determined urbanization a risk factor for the transmission of WNV [2, 90, 131]. The population in Niagara is spread over a wide geographic area but as urbanization increases in Niagara, along with age, there may be an increased risk for transmission of WNV.

Dengue exposure in the study participants

A secondary objective of the present study was the determination of Dengue serum antibodies in the research participants. Although this was done primarily to rule out cross-reactivity, the results obtained are in themselves, worthy of discussion. Firstly, Panbio's ELISA performed much better for Dengue than for WNV (13 out of 16 [81.3%] ELISA positives were true positives and 3 [18.8%] were false positives). Secondly, of 92 workers tested, 13 had confirmed evidence of Dengue virus infection. This amounted to 14.1.% of point seroprevalence (95% CI 7.7%-23.0%). Other studies in Mexico report a wide range of seroprevalence data, depending on the type of population and the geographical location studied. For instance, a household survey done by Brunkard *et al.* in Matamoros, Northern Mexico in 2004, reported a seroprevalence of 7.3% for recent infection and 78.0% for past infection (for both sexes aged 15 -75+ years) [132]. Conversely, a new study by Rodriguez *et al* (2009) in Northern Mexico examined a population of febrile patients, encephalitic patients, and healthy blood donors for the presence of WNV and Dengue antibodies. The study found that none of the patients had an acute WNV infection as per CDC's guidelines, but 4.0% demonstrated antibodies to

WN virus in the PRNT. Additionally, of 800 asymptomatic blood donors tested, 16 (2.0%) were positive for Dengue antibodies [126]. These studies show diametrically different results and demonstrate the heterogeneity of seroprevalence patterns, as well as illustrate the difficulty in obtaining national averages. The study population of the present study, on the other hand, was composed of workers from 17 different Mexican states and might be perhaps a more moderate assessment of the overall Dengue virus exposure in a Mexican study group.

In the literature search performed for the purpose of this thesis, no studies on the prevalence of Dengue in migrant workers or rural workers in Mexico were found. Thus, the present study provides for the first time data previously not available.

The present work also provides interesting data as to asymptomatic Dengue infections. Of all the workers with antibodies against Dengue virus, only 1/13 (7.7%) recalled experiencing the disease. This suggests that similar to WNV infection, there is a greater prevalence of asymptomatic infection compared to those presenting clinical symptomology [133]. Other studies have found supporting data: a seroprevalence study in asymptomatic Costa Rican children in 2003 found a seroprevalence of 36.9% in the coastal areas and 2.9% in the inland areas [134] and another longitudinal study of healthy individuals in West Jakarta reported close to 50.0% Dengue infections being asymptomatic [135].These studies, although not in Mexico, still provide further support that in areas endemic for Dengue, the number of asymptomatic individuals may be of concern. This is critically important for the individuals since a primary infection is believed to increase the risk of Dengue hemorrhagic fever or Dengue shock syndrome upon secondary infections with a different virus type [134, 135]. When living in endemic

areas- like our research participants- persons with previous Dengue history should pay particularly attention to exercising Dengue prevention behaviours. From our findings of the predominance of asymptomatic Dengue, we can draw two important conclusions: a) Dengue clinical history is not reliable as most infections present without symptoms, and b) previous asymptomatic infections (as well as symptomatic) may confound the diagnosis of WNV infections. Therefore, when testing for any particular virus of this family, other circulating flaviviruses should be tested as well. Moreover, travel history and potential exposure should be taken into consideration when recording the epidemiological history of the suspect case.

5.4 Objective 2. Analyses of risk factors associated with seropositivity

Risk Factors for Dengue Exposure

Since all our study PRNT results were negative for WNV it was not possible to ascertain variables that are associated with infection. However it was possible to do this for Dengue seropositivity. After identifying suggested associations through univariate regression analysis, these variables were analyzed in a multivariate model. Three variables were found to be significantly associated with Dengue seropositivity. First, the first year in the SAWP program increased the odds of Dengue exposure. It is intuitive that if an individual is spending the entire year in a country endemic for Dengue, in this case Mexico, then it would certainly increase the exposure to Dengue infected mosquitoes. Second, the Mexican home with fewer amenities (as previously described) decreased the odds of Dengue exposure. This is counter-intuitive as one would assume that homes belonging to people of a lower socioeconomic status (SES) would be associated with mosquito habitat (i.e., lack of running water and stagant water sitting in

barrels). This does not appear to be the case. It is important to note that the particular grading system of the Mexican home used in this study does not take into account parameters for preventing mosquitoes in the home. Another consideration is that these workers are only living in Mexico for part of the year, and during that time may be working in other areas of Mexico and not living in their family home. These results may be spurious and warrant further research and confirmation.

Third, the presence of a water basin in the yard decreased the odds of Dengue exposure. The presence of a water basin indicates running water in the home and thus less opportunity to utilize household containers to store water. The water basin is used for laundry washing and the water is changed and not left to stagnate with organic matter as opposed to rain barrels in which water may sit for prolonged periods of time. In this regard, a water basin with regular running water reduces the probability of mosquito breeding habitats. This finding may be also associated with higher SES as laundry basins are usually found in people's homes with higher incomes. Water storage in open containers such as rain barrels has been consistently found associated with Dengue transmission, as evidenced in a study by Brunkard and colleagues in Mexico [132].

Is Dengue previous exposure affecting our WNV findings?

In the light of our WNV negative results, the analysis of our Dengue serological data led us to two related and yet different interesting theories described in the literature. The first is the possibility that pre-existing anti-Dengue antibodies may have protected our research participants from establishing an infection with WNV. The second theory, of an even more speculative nature, the possibility that individuals with pre-exisiting anti-

Dengue antibodies may have responded to a WNV infection with the production of more anti-DEN antibodies intead of producing specific anti-WNV antibodies.

The first theory is supported by the the scarce number of reports of human WNV disease in tropical and subtropical areas in the Americas, even from countries with established WNV surveillance systems such as Mexico. This has led to the hypothesis that transmission of WNV in those areas may be modulated by the presence of partially protective heterotypic antibodies [99, 104]. Although, there are several flaviviruses circulating in South America and the Caribbean, Dengue is by far the most predominant. In fact, a number of recently published papers point to this extreme [16, 126]. The presence of WNV circulating in mosquitoes, birds, and horses has been previously established in the northern parts of Mexico [13-16, 100, 103, 104] as well as in Chiapas (the southernmost state in Mexico) [124] however transmissions to humans is rare. More recently, a paper by Garza-Rodriguez *et al* (2009) suggests that the high prevalence of antibodies against Dengue virus in Northern Mexico may very well be the reason the incidence of WNV is so limited [126]. If this were the case, our study sample would not be appropriate to assess WNV transmission in Niagara. A paired-sample examination of antibodies in the research participants would have been useful in ruling out this potential protective effect and future studies should take this into consideration.

The second theory is supported by the concept of the 'original antigenic sin' [136, 137] and is particularly attractive when trying to explain our negative results. This theory would make it plausible to reject our findings and argue that there may be local transmission in Niagara but our research population had a biological bias that prevented us from detecting WNV antibodies. Unfortunately, this theory remains controversial and

our small sample size does not allow for such speculations (considering that the Dengue-negative portion of the research group supposedly not affected by this immunological interference was too small to find any positives).

5.5 Objective 3. Assessing awareness of WNV transmission

Only 23% (21/92) of the workers had some previous awareness of WNV and only 2% practiced protective behaviour from mosquitoes (use of DEET). All workers reported and were observed wearing long sleeves and pants which have been demonstrated to be protective against vector-borne diseases. This behaviour however was not related to a conscious behaviour of mosquito bite avoidance but rather required to prevent the physical hazards associated to the nature of their jobs. Conversely, in a study by Averett et al.(2005), in Kansas, 97% of the general population had heard of WNV although only 41% of the Spanish speaking population reported this same awareness [138]. As in our participants, knowledge and awareness did not translate into the same level of protective behaviour. In the Kansas study, 89% indicated knowledge of one or more protective behaviour, but only 21% reported using DEET. A study of Canadian First Nations (n=248), in 2003, indicated an overall awareness of WNV of 95% and 34 % reported using DEET [139]. In a study to determine retention and compliance with public health messaging in Oakville in 2003 researchers found that 99% of the individuals surveyed had heard of WNV and 61% reported undertaking 2 or more personal protective behaviours against mosquito bites [120]. The fact that only 2% of our study population used DEET suggests a lack of awareness for using mosquito repellent however other reasons such as cost or fear of applying chemicals to the skin cannot be ruled out.

Of the participants that responded to having some awareness of the existence of WNV in Canada, when enquired about the infection's mode of transmission, a good proportion (76%) correctly identified that WNV is transmitted by mosquito bites. Conversely, the First Nations study mentioned above indicated that 90% of the participants had knowledge of mosquito transmsission [139]. Other workers in our study believed that the virus was transmitted by water (10%), food (5%) or they did not know (10%), demonstrating that there are many misconceptions about this disease transmission among the group. Similarly, even when workers affirmed knowing about WNV existence, their knowledge of the disease symptomology was inaccurate. Fifty percent recognized fever as a symptom of WNV infection, less than 5% were aware of any other symptom and none was aware that fever, pain, and fatigue occurred together as a triad of symptoms.The identified lack of understanding and awareness about WNV among the workers leads to the assumption that there is not enough health education provided for them prior to leaving Mexico, or that if there is, that the messages are not being retained by the workers. As part of this study, the participants were presented with informative sessions and handouts written in plain Spanish, about transmission of WNV and symptomology. We did not formally evaluate increases in knowledge and awareness or measure any behavior change -as this was not an objective of the study- but it was subjectively observed during conversation at the last meeting that workers had a better understanding of WNV. Workers also reported transmitting the messages to colleagues that were not enrolled in the study. As these workers are fundamental to Niagara Region's economy, it would be desirable that their employers, community associations and the public health department combined their efforts in implementing health promotion

activities with this population. Further promotion of WNV prevention is beneficial since it is not possible to predict the future transmission of WNV. A low cost intervention could be in the form of conducting health promotion and health education sessions as well providing them literature in Spanish in regards to important health issues they may encounter in Canada. Increasing these workers' awareness and encouraging protective behaviors is fundamental at both the individual and societal level.

5.6 Study findings implications for public health

The Public Health Agency of Canada (PHAC) recommends that all index cases positive for WNV IgM be confirmed with the PRNT. After an index case has been reported in a health region, PHAC recommends that for surveillance purposes all clinical cases following the index case fulfilling the probable diagnostic criteria (i.e., positive for ELISA WNV IgM) can be confirmed without the PRNT [17]. Our results show that IgM results can sometimes be unreliable. As well, recent literature shows that IgM determination by ELISA is not without problems. In September 2008, the Center for Disease Control (CDC) investigated a commercially available WNV IgM ELISA kit from Panbio Inc. (Australia) due to reports of a high number of false positive results. The CDC found false positive IgM in 72% of 166 samples tested [140, 141]. Since this test has failed in clinical studies, performance in epidemiological studies would also likely be poor. The two samples in our study that were solely positive for WNV IgM ELISA were negative for all other ELISA and PRNT serology.

It would be important for public health to evaluate the tests that are being used for WNV diagnosis, and if possible conduct studies to determine the extent of the problem with false positivity for IgM [3, 124, 142-146]. Moreover, it would be beneficial if the Public

Health algorithm for diagnosis of WNV [17] considered the epidemiological history of the individual as well as the probability of exposure, and at the same time recommended performing serological tests, preferably PRNT, for all potential circulating flaviviruses. From the Mexican Public Health standpoint, knowing if these workers are at an increased risk for WNV infection is paramount. Although the possibility of these workers being viremic enough to introduce the virus to Mexico is minimal (as humans do not develop sufficient viremia to sustain vectorial transmission), other concerns such as blood donation and pregnancy remain. Having resided in a WNV endemic area could be an important factor when screeing blood donors in Mexico. Just as in Canada, prior exposure to Dengue would confound WNV diagnosis, Dengue diagnosis in Mexico could possibly in turn be confounded by WNV exposure. This illustrates that infectious diseases, emerging and re-emerging, and are becoming a globalized issue [1].

5.7 Study Limitations

This study used a small non-randomized convenience sample to conduct a preliminary investigation. The biases intrinsic to our study population and the lack of statistical power do not allow for a conclusive interpretation of our findings. Since no WNV serosurveillance studies had been done with this particular population the researchers felt that although the results may not be generalizable, the present work could serve as a pilot trial as well as a feasiblity study in which to base further research.

Future studies set to demonstrate WNV in the Niagara Region could make use of a longitudinal design with a representative sample of the population.

5.8 Study Strengths

Having access to PRNT confirmatory testing through PRNT at the National Microbiology Laboratory (thanks to Dr Mark Loeb, McMaster University) we were able to elucidate the high degree of cross-reactivity observed in the studied samples. Without the PRNT, the results of this study would have been of little value.

CHAPTER 6: CONCLUSIONS, RECOMMENDATIONS AND FUTURE RESEARCH DIRECTIONS

6.1 Conclusions

1. Our study demonstrated the limitations of ELISA antibody testing for WNV in individuals that are exposed to multiple flaviviruses and the need to utilize the confirmatory PRNT for both clinical diagnosis and surveillance purposes.

2. A study of an infectious disease (or any other biomedical study) in the Mexican migrant agricultural population in the Niagara Region has never been attempted. The challenges with enrolling participants and conducting the study culminated with the collection of baseline data that is now available for further research The base of farm owners that did participate can be used to network to others to gain support for other research projects.

3. Awareness of WNV is limited among this group of workers and those who understood the disease and its transmission did not utilize precautions. Greater attention is needed to inform the migrant worker of the potential risk to their health from diseases that may not be present in their native country.

4. Mexican migrant workers are potentially at risk for WNV transmission while in Canada.

5. This study did not prove WNV transmission in the study population and thus no predictive conclusions can be made. This study did not measure or evaluate the

geography, climate, or species of birds and mosquitoes, however, the Niagara Region is postioned geographically near many water sources, has the appropriate climate for mosquito breeding habitat, appropriate vectors are present, and is home to many migratory passerine birds, thus making this area vulnerable for further transmission of WNV to the human population.

6.2 Recommendations *(These recommendations are not necessarily based on this study's results but also are a result of the review of the current literature)*

1. All positive ELISA findings, whether diagnostic or surveillance, should be confirmed with the PRNT

2. We suggest re-examination of the PHAC's diagnostic case criteria in regards to the interpretation of WNV serological testing as well as the inclusion of epidemiological history in the diagnostic algorithm.

3. Continued surveillance in birds and mosquitoes in the Niagara Region is encouraged. Mosquito surveillance for population estimates, species and the minimum infection rate of WNV in the mosquito pools should be performed. Maintaining control on the mosquito population in known habitat areas should occur in parallel with surveillance.

4. Partnerships of the Niagara Regional Health Department and other research institutions will help facilitate surveillance and enhance the development of new knowledge that can be applied to infectious disease control.

5. In Niagara, in particular, designing a study involving Mexican migrant workers is challenging due to the barriers these workers experience including language and

access to adequate health care. By providing a setting for accessible health care other information can be collected by public health professionals (that would otherwise be logistically difficult to collect and not cost-feasible) to evaluate the population health of this group of agricultural workers. Gaining information regarding susceptibility to infectious disease is not only important to the health of the individual worker, but to the country of Mexico and the scientific community.

6.3 Suggested directions for future research

1. Historically, WNV has not established a predictable pattern of disease transmission and hence it has been impossible to predict an outbreak of WNV, beyond observation of death in corvids in North America prior to a human outbreak. Spatial risk assessment for WNV based on mosquito abundance, bird populations, and human population data through the use of Geographic Information Systems (GIS) can help identify areas in the Niagara Region at greatest risk for WNV transmission to humans. Temperature, rainfall, vegetation cover, and wetland data are important factors to incorporate in a spatial map for predicting mosquito abundance. British Columbia has been free of human transmission of WNV (apart from imported cases), up to this year in which 1 human case has been confirmed.This province has developed GIS to work as an early warning system for a potential outbreak of WNV in humans [147].

2. Although the present seroprevalence study could not prove transmission of WNV to humans among the sample population, the virus is still present in the mosquito and bird population (as of 2009) [18] which means that it may be present, yet

undetected, in other small mammals so far identified. Even if undetected in the mosquito population the virus could be reintroduced by migratory birds. Therefore, proactive measures with mathematical modelling using GIS could be a consideration for the Niagara Region. Niagara's climate is conducive for an increase in the enzootic level of transmission which could lead to an increase in human exposure to mosquitoes.

3. As previously mentioned, it has been suggested that surveillance of healthy human groups that are highly exposed to vectors and animals, as is the case of as agricultural workers, may be valuable when monitoring for emerging or re-emerging disease [105]. For WNV, however (in which cross-reactivity with related flavivirus is a serious issue) perhaps regional permanent residents with different degrees of exposure to mosquito bites would be a better indicator of local transmission than foreign agricultural workers. In any case, surveillance of a resident human population would still require the confirmatory PRNT, as it did in our migrant worker population.

4. Development of WNV and other flavivirus confirmatory tests that do not require a high level of biocontainment should be a priority to effectively monitor the patterns of WNV transmission. Such tests would prove extremely necessary should WNV finally spread to developing countries where laboratory technology is not as available as in industrialized nations.

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APPENDICES

Appendix A: Research Ethics Board Application

Reviewer Disposition
(For REB Use Only) ▶ File # :_____ Reviewers: _____ Due Date: _____

Decision: □Accepted as is □Approval Pending Revision □Clarification Required □Resubmission □Full Review □Withhold Approval

Brock University Research Ethics Board (REB)

Application for Ethical Review of Research Involving Human Participants

Please refer to the documents "Brock University Research Ethics Guidelines", which can be found at http://www.brocku.ca/researchservices/, prior to completion and submission of this application.

If you have questions about or require assistance with the completion of this form, please contact the Research Ethics Office at (905) 688-5550 ext. 3035, or reb@brocku.ca.

Return your completed application and all accompanying material **in triplicate** to the **Research Ethics Office** in **MacKenzie Chown D250A.**
Please ensure all necessary items are attached prior to submission.
otherwise your application will not be processed (see checklist below).

No research with human participants shall commence prior to receiving approval from the research ethics board.

DOCUMENT CHECKLIST **3 complete sets of the following documents (one original + 2 copies)** Please Note: Handwritten Applications will *not* be accepted.	**✓ if applicable**
Recruitment Materials • Letter of invitation • Verbal script • Telephone script • Advertisements (newspapers, posters, SONA) • Electronic correspondence guide	 [X] [ ] [ ] [ ] [ ]
Consent Materials • Consent form • Assent form for minors • Parental/3rd party consent • Transcriber confidentiality agreement	 [X] [ ] [ ] [ ]
Data Gathering Instruments • Questionnaires • Interview guides • Tests	 [X] [ ] [ ]
Feedback Letter	[ ]
Letter of Approval for research from cooperating organizations, school board(s), or other institutions	[ ]
Any previously approved protocol to which you refer	[X]
Request for use of human tissue sample in research Please Note: this form is required for all research projects involving human tissue, bodily fluids, etc.	[X] REB # 06-268]
Signed Application Form	[X]

Office of Research Services

SIGNATURES

Principal Investigator:

Please indicate that you have read and fully understand all ethics obligations by checking the box beside each statement.

[x] I have read Section III:8 of Brock University's Faculty Handbook pertaining to Research Ethics and agree to comply with the policies and procedures outlined therein.
[x] I will report any serious adverse events (SAE) to the Research Ethics Board (REB).
[x] Any additions or changes in research procedures after approval has been granted will be submitted to the REB.
[x] I agree to request a renewal of approval for any project continuing beyond the expected date of completion or for more than one year.
[x] I will submit a final report to the Office of Research Services once the research has been completed.
[x] I take full responsibility for ensuring that all other investigators involved in this research follow the protocol as outlined in this application.

Signature _____ Date: ____June 15, 2007__

Co-Investigators:

Signature _____ Date: ____ June 15, 2007__

Signature _____ Date: ____June 15, 2007__

Signature _____ Date: _ _____

Faculty Supervisor:

Please indicate that you have read and fully understand the obligations as faculty supervisor listed below by checking the box beside each statement.

[x] I agree to provide the proper supervision of this study to ensure that the rights and welfare of all human participants are protected.

[x] I will ensure a request for renewal of a proposal is submitted if the study continues beyond the expected date of completion or for more than one year.
[x] I will ensure that a final report is submitted to the Office of Research Services.
[x] I have read and approved the application and proposal.

Signature _____ Date: __ June 15, 2007__

SECTION A – GENERAL INFORMATION

1. **Title of the Research Project: West Nile Virus Exposure in the Mexican Migrant Workers of the Niagara Peninsula**

2. **Investigator Information:**

	Name	Position (e.g., faculty, student, etc)	Dept.	Phone No.	E-Mail
Principal Investigator	Ron Mergl	Graduate Student	Community Health Sciences	905-354-3827	rm80jq@brocku.ca
Co-Investigator	Angela Duarte	Graduate Student	Community Health Sciences	905-688-5550 x 3882	angela.duarte@brocku.ca
Faculty Supervisor	Ana Sanchez	Associate Professor	Community Health Sciences	905-688-5550 x 3882	ana.sanchez@brocku.ca

3. **Proposed Date (dd/mm/yyyy)** (a) of commencement: June 2007 (b) of completion: November 2007

4. **Indicate the location**(s) where the research will be conducted:

Brock University [x]
Community Site [x] Specify Agricultural Farms employing Mexican
Grow Support Centre [ ] workers
School Board [ ] Specify
Hospital [ ] Specify
Other [ ] Specify

5. **Other Ethics Clearance/Permission:**

(a) Is this a multi-centered study?
[ ] **Yes** [x] **No**
(b) Has any other University Research Ethics Board approved this research?
[ ] **Yes** [x] **No**

If **YES**, there is no need to provide further details about the protocol **at this time,** provided that **all** of the following information is provided:

Title of the project approved elsewhere:
Name of the Other Institution:
Name of the Other Board:
Date of the Decision:
A contact name and phone number for the other Board:

Please provide a copy of the application to the other institution together with all accompanying materials, as well as a copy of the clearance certificate / approval.

If **NO**, will any other Research Ethics Board be asked for approval?
[ ] **Yes** [x] **No**
Specify University/College

(d) Has any other person(s) or institutions granted permission to conduct this research?
[ ] **Yes** [x] **No**
Specify (e.g.,. school boards, community organizations, proprietors)

6. **Level of the Research:**

[ ] Undergraduate [**X**] Masters Thesis/Project [ ] Ph.D.
[ ] Post Doctorate [ ] Faculty Research [ ] Administration
[ ] Undergraduate Course Assignment [ ] Graduate Course Assignment [ ] Other (specify)
(specify course) (specify course)

7. **Funding of the Project:**

(a) Is this project currently being funded [ ] **Yes** [x] **No**
(b) If **No**, is funding being sought [ ] **Yes** [x] **No**

If Applicable:
(c) Period of Funding (dd/mm/yyyy): From: To:

(d) Agency or Sponsor (funded or applied for)

[ ] CIHR [ ] NSERC [ ] SSHRC [ ] Other (specify):

(e) Funding / Agency File # (not your personal PIN)

8. **Conflict of Interest:**
(a) Will the researcher(s), members of the research team, and/or their partners or immediate family members receive any personal benefits related to this study – Examples include financial remuneration, patent and ownership, employment, consultancies, board membership, share ownership, stock options. Do not include
conference and travel expense coverage, possible academic promotion, or other benefits which are integral to the general conduct of research.
[ ] **Yes** [x] **No**
If **Yes**, please describe the benefits below.

Not applicable

(b) Describe any restrictions regarding access to or disclosure of information (during or at the end of the study) that the sponsor has placed on the investigator(s).

None

SECTION B – SUMMARY OF THE PROPOSED RESEARCH

9. **Rationale:**

Briefly describe the purpose and background rationale for the proposed project, as well as the hypothesis(es)/research question(s) to be examined.

West Nile virus exposure in the Mexican Migrant workers of the Niagara Peninsula

West Nile virus (*Flavivirus: Flaviviridae;* WNV) is an emerging infectious agent that has established itself successfully in the United States since 1999 and in Canada since 2001.
In the United States and in Canada, WNV is causing large numbers of human cases with neurological disease and death, and even greater amounts of milder disease characterized mainly by fever and rash. In the US, WNV is presently the leading cause of arboviral encephalitis.
Even though the numbers of human cases of WNV infection in Canada remain much lower than in the US, the predictions are that cases will increase as the virus adapts itself in this new ecosystem.
Across Canada, from 2002 to 2006, the number of reported clinical cases (both probable and confirmed WNV) for each year was respectively: 414 (394 in Ontario), 1481 (89 in ON), 25 (13 in ON), 225 (95 in ON) and 151 (42 in ON). These totals are the sum of WNV Neurological Syndrome + WNV Non-Neurological Syndrome + WNV Unclassified/Unspecified. Clearly the number of cases peaked in 2003 and tapered off since then. But infectious diseases show temporal patterns and just as the US have recently experienced a dramatic increase of 42% (4,261 cases of WNV disease in 2006, compared with 3,000 cases in 2005), a resurgence of WNV human infection in Canada could be in the making.
Luckily, as much as 80% of people who become infected show no signs and symptoms. The remaining 20% may experience mild symptoms (fever headache, body aches, or skin rash) out of which less than 1% will present with severe symptoms and life-threatening neurological illness. Naturally the most important aspect of the WNV infection is the neurological disease, but it is important to remember that even in the absence of severe disease infected persons may still be reservoirs for mosquitoes or other humans through blood transmission or congenitally. The Centers for Disease Control estimates that for every case of severe WNV there are about 140 cases of WNV infection either asymptomatic or not diagnosed.

Until 2001 WNV was known in Canada as a potential threat to visitors of endemic countries in Africa but nowadays, Canadians are aware that the pathogen can be acquired locally; in their own backyards, literally. Additionally, visitors and temporary workers to Canada must be aware of the existence of this infectious disease and observe preventive measures to reduce the risk of infection.
The single most important factor for acquiring WNV is exposure to mosquito bites during outdoor activities. In the absence of an effective human vaccine, prevention of WNV disease depends on community-level mosquito control and promotion of personal protection against mosquito bites, such as use of repellents and avoiding outdoor exposure when mosquitoes are most active (usually from dusk to dawn). Similarly, intact window screens or air conditioning can reduce mosquito exposure in homes. Numbers of mosquitoes can be reduced by removing

or emptying water from larval habitats such as flower pots, buckets, gutters, and barrels around human dwellings.

Agricultural workers in the Niagara Region come to the area exactly during the WNV season (May to October) and spend many hours outdoors both during their work hours as well as for social activities. It is very important to investigate the rate of exposure of these workers to WNV during their stay in Canada not only to implement measures to protect them individually but also to estimate the potential for these workers of bringing the virus back to their countries. Although WNV has been isolated in wild birds and horses in Latin America, human clinical cases are not being reported despite several investigations. In Mexico, several species of birds have been found antibody-positives but the literature shows only one locally-acquired human case in 2005, and one blood unit out of almost 4,000 investigated. This means -at least for Mexico- that the human exposure is at this point in time at a very low -almost negligible- level.

For this reason the present study is of utmost scientific and public health importance: in the event one of the Mexican workers became infected in Canada and went back home with a significant level of viruses in their blood, they could initiate a chain of transmission, if not to mosquitoes (so far mammals do not appear to allow sufficient viremia for mosquito infection) at least to other humans: transmission through blood transfusion from apparently healthy donors has occurred in North America, and a few cases of congenital (intrauterine) transmission or through human milk have been reported as well.

The objective of the present study is to assess the level of exposure of Mexican Migrant workers to West Nile Virus during their stay in the Niagara Region by measuring serum antibodies which indicate exposure to the pathogen. Also, the level of awareness and knowledge regarding this infection will be measured to suggest and help implement preventive measures to decrease the likelihood of mosquito bites and therefore of infection.

Methodology

Study Population: the study will be done in conjunction with the tuberculosis study (REB file # 06-268 Duarte et al) to access the same population (same sample size, enrolment process, etc). With the participant's consent, general demographic information will be shared as well as an aliquot of a blood sample taken for the TB testing. In addition, a specific short questionnaire will be administered to the participants to record history of past or present possible WNV infection symptoms, exposure to mosquito bites and knowledge and awareness regarding the infection.
Lab testing: to assess West Nile virus infection/exposure, serum from participants will be tested for IgM antibodies against the virus (by ELISA). IgM antibodies are usually indicative of a recent West Nile virus infection but since anti-West Nile virus IgM can persist for 1 year or longer a single positive test will not necessarily be associated with the participant current illness, especially in the absence of illness. Additionally, since cross-reactivity with other flaviviruses circulating in Mexico may occur (e.g., Dengue virus), samples will be tested for other viruses in order to rule out false positives. For this, some samples (anonymized) may be sent to Honduras or a specialized Canadian lab to be assessed for Dengue (or the PI Ron Mergl will take them down there and do the testing himself after proper training).
Questionnaire: Participants' general demographic information will be collected in the TB study and consenting individuals who consent to this study will provide consent for the researchers to access that information. So for the WNV study, only relevant information to WNV will be

additionally asked from the participants. This data collection instrument will be attached at the end of the TB questionnaire and the enquiry process will follow the same procedure as the TB study.
Educational Activities: will be done in conjunction with the TB study.
Ethics, workplace safety and biosafety: All research activities will be in compliance with the Canadian Tricouncil research ethics policies for human subjects. Similarly, all field and laboratory work will follow work safety and biosafety precautions and regulations.

10. **Methods:**

Are any of the following procedures or methods involved in this study? Check **all** that apply.

[ ] Questionnaire (mail)
[ ] Questionnaire (email/web)
[**x**] Questionnaire (in person)
[ ] Interview(s) (telephone)
[ ] Interview(s) (in person)
[ ] Secondary Data
[ ] Computer-administered tasks
[ ] Focus Groups
[ ] Journals
[ ] Audio/video taping (specify)
[ ] Observations
[**x**] Invasive physiological measurements (e.g., venipuncture, muscle biopsies)
[ ] Non-invasive physical measurement (e.g., exercise, heart rate, blood pressure)
[**X**] Analysis of human tissue, body fluids, etc. (Request for Use of Human Tissue Sample attached)
[ ] Other: (specify)

***NOTE: Request for Use of Human Tissue Sample was submitted and approved for TB study (REB: Duarte et al 06-268)**

Describe sequentially, and in detail, all of the methods involved in this study and all procedures in which the research participants will be involved (e.g., paper and pencil tasks, interviews, questionnaires, physical assessments, physiological tests, time requirements, etc.)
Attach a copy of all questionnaire(s), interview guides, or other test instruments.

1. Questionnaires: The West Nile study is being done in conjunction with the latent tuberculosis study so the questionnaire for WNV study will be a supplement to that questionnaire. The Co-investigator and Supervisor of the TB study are competent in Spanish and will assist the Principal Investigator with both verbal and written communication. The questions will be asked verbally, in private, and then recorded in Spanish. The questionnaire will have 2 components: the demographics and the West Nile virus related questions. The demographic section will not have to be repeated as the information can be drawn from the tuberculosis study.
2. A blood sample to investigate antibodies will be necessary but further blood will not have to be drawn as the tests can be performed from the same serum samples drawn for the tuberculosis study.
2. Educational Program: All questions regarding West Nile virus will be answered and a further program will be developed to offer information regarding other infectious diseases. This education program will be carried out in conjunction with the tuberculosis study.

11. **Professional Expertise/Qualifications:**

Does this procedure require professional expertise/recognized qualifications (e.g., registration as a clinical psychologist, first aid certification)?

[X] **Yes** specify: Phlebotomy [x] **No**

If **YES**, indicate whether you, your supervisor, or any members of your research team have the professional expertise/recognized qualifications required?

[X] **Yes** [ ] **No** **Note: see** (REB: Duarte et al 06-268)

12. **Participants:**
Describe the number of participants and any required demographic characteristics (e.g., age, gender).

The number of participants will be restricted to the number included in the tuberculosis study, since the same blood samples will be used. The WNV population sample would be therefore a 'convenience' sample. Since t his is the first research study of this nature in the Region, the present study is consider a pilot or preliminary study to explore if further research is warranted.

13. **Recruitment:**

Describe how and from what sources the participants will be recruited, including any relationship between the investigator(s), sponsor(s) and participant(s) (e.g., family member, instructor-student; manager-employee).
Attach a copy of any poster(s), advertisement(s) and/or letter(s) to be used for recruitment.

We will only be using the recruits from the tuberculosis study.

14. **Compensation:** **Yes** **No**

(a) Will participants receive compensation for participation? [x] [ ]

(b) If yes, please provide details.

The compensation for the tuberculosis study will also cover the extra West Nile virus participants

SECTION C – DESCRIPTION OF THE RISKS AND BENEFITS OF THE PROPOSED RESEARCH

15. **Possible Risks:**

1. Indicate if the participants might experience any of the following risks:

a) Physical risks (including any bodily contact, physical stress, or administration of any substance)? [**X**] **Yes** [ ] **No**

b) Psychological risks (including feeling demeaned, embarrassed worried or upset, emotional stress)? [**X**] **Yes** [ ] **No**

c) Social risks (including possible loss of status, privacy, and / or reputation)? [ ] **Yes** [**X**] **No**

d) Are any possible risks to participants greater than those that the participants might encounter in their everyday life? [**X**] **Yes** [ ] **No**

e) Is there any deception involved? [ ] **Yes** [**X**] **No**

f) Is there potential for participants to feel coerced into contributing to this research (e.g., because of regular contact between them and the researcher)? [ ] **Yes** [**X**] **No**

2. If you answered **Yes** to any of 1a – 1f above, please explain the risk.

1a. Related to collection of blood samples., but since the samples will be taken from the LTBI study, the risks and minimizing measures are addressed in the respective protocol (REB: Duarte et al 06-268)

1b. Concerns about the tests being positive. Information regarding the signs and symptoms as well as the risk factors will be discussed. If the antibody test is positive the participants will be explained that this is a past infection of no chronic consequences to them and there are no risks of transmission from them to others. The participants will be reassured that this is a private research project and does not involve Citizenship and Immigration Canada.

1d. The workers have participated in previous medical questionnaires to receive access to Canada. However, it is not a daily event. The fact that all communication will be in Spanish will put the workers at ease and facilitate accuracy.

3. Describe how the risks will be managed and include the availability of appropriate medical or clinical expertise, qualified persons. Explain why less risky alternative approaches could not be used.

Again, this study is using the demographic questionnaire and the blood sample from the tuberculosis study. The only risks would be anxiety regarding a positive result for West Nile virus. This will be handled by providing education regarding this disease.
The risk management for the invasive procedure (blood collection) is explained in (REB: Duarte et al 06-268)

16. **Possible Benefits:**

Discuss any potential direct benefits to the participants from their involvement in the project. Comment on the (potential) benefits to the scientific community/society that would justify involvement of participants in this study.

Benefit to the Participants

Knowing serological status (tests results) for WNV and Dengue will provide valuable information to the worker. If results are positive for WNV they will be aware that they had the infection but are now protected. The same is true for Classic Dengue; however a seropositive person should take maximum precautions to avoid a second infection due to the increased risk of developing hemorrhagic fever. Clinically, should they have another viral-fever-like illness they will know whether WNV and/or Dengue can be ruled out or not.
Providing knowledge of the West Nile virus in Niagara in order to prevent exposure to this disease would be the main benefit to the participants. An understanding of vector transmitted diseases is necessary so that the workers will be diligent at preventing mosquito bites. The worker will be familiar with yellow fever and dengue fever but may not be aware that the West Nile is a similar virus.

Benefit to the farm owners and SAWP

Knowing the exposure status of their workers will indicate to the farm owners their own potential exposure and need for vector control. The health of their workers at large is directly proportional to the productivity of the farm.

Benefit to the Niagara Region

If positive individuals are found then further studies regarding the prevalence of West Nile virus are justified

Benefit to the Scientific Community

To the researchers' knowledge this is the first study of West Nile virus prevalence in migrant workers in the Niagara Region. Tracking West Nile virus is very difficult in sentinel groups. Birds are the most obvious, but the location a dead bird contracted the virus is usually unknown. Most horses in the Niagara Region will have been vaccinated thus are not an accurate sentinel group. Most poultry is housed and not exposed to mosquitoes. Of any group (human or animal) the migrant worker is most vulnerable since they spend most of their working hours outside and around irrigation waters.

SECTION D – THE INFORMED CONSENT PROCESS

17. **The Consent Process:**

Describe the process that the investigator(s) will be using to obtain informed consent. Include a description of who will be obtaining the informed consent. If there will be no written consent form, explain why not.

For information about the required elements in the letter of invitation and the consent form, as well as samples, please refer to:
http://www.brocku.ca/researchservices/Certification&Polices/Certification&Polices_App_Guidelines.html

If applicable, attach a copy of the Letter of Invitation, the Consent Form, the content of any telephone script, and any other material that will be utilized in the informed consent process.

Participants will be provided with a letter of invitation and informed consent document after they have received clear information, in Spanish, regarding West Nile virus and the health risks associated with this disease. The educational sessions and explanations of the studies will be given concurrently with the tuberculosis study to respect the time of the workers. The intermediary, as per in-council guidelines, will be the same person as the tuberculosis study.

Miss Janet Maclaughlan, a doctoral candidate from the University of Toronto, is a familiar face to the Mexican workers as she has been working with them regarding issues in social justice. Participants who qualify and consent for the TB study (REB: Duarte et al 06-268) will be consulted if they would like to participate in the WNV study. Only participants who agree to the first study will be consulted for enrollment. However, a participant may choose to participate in the TB study only. The reverse will not be possible as the PI (Ron Mergl) is not proficient in Spanish and he needs the assistance of the researchers from the TB study.

18. **Consent by an authorized party:**

If the participants are minors or for other reasons are not competent to consent, describe the proposed alternative source of consent, including any permission form to be provided to the person(s) providing the alternative consent.

Not applicable

19. **Alternatives to prior individual consent:**

If obtaining individual participant consent prior to commencement of the research project is not appropriate
for this research, please explain and provide details for a proposed alternative consent process.

Not applicable

20. **Feedback to Participants:**

Explain what feedback/ information will be provided to the participants after participation in the project. Include, for example, a more complete description of the purpose of the research, and access to the results of the research. Also, describe the method and timing for delivering the feedback.

The participants will receive their results in private. Also an aggregate report will be presented at a final educational session

21. **Participant withdrawal:**

a) Describe how the participants will be informed of their right to withdraw from the project. Outline the procedures that will be followed to allow the participants to exercise this right.

All participants will be made aware that they can withdraw at any time during the study.

b) Indicate what will be done with the participant's data and any consequences that withdrawal might

have on the participant, including any effect that withdrawal may have on participant compensation.

All information and samples will be destroyed immediately upon the participant withdrawing from the study.

SECTION E – CONFIDENTIALITY & ANONYMITY

Confidentiality: information revealed by participants that holds the expectation of privacy. This means that all data collected will not be shared with anyone except the researchers listed on this application.

Anonymity of data: information revealed by participants will not have any distinctive character or recognition factor, such that information can be matched **(even by the researcher)** to individual participants. Any information collected using audio-taping, video recording, or interview cannot be considered anonymous. **Please note that this refers to the anonymity of the data itself and not the reporting of results.**

22. Given the definitions above, in the student project(s):

a) Will the data be treated as confidential? **[x] Yes** **[ ] No**

b) Are the data anonymous? **[ ] Yes [x] No**

c) Describe any **personal identifiers** that will be collected during the course of the research (e.g., participant
names, initials, addresses, birth dates, student numbers, organizational names and titles etc.). Indicate how personal identifiers will be secured and if they will be **retained** once data collection is complete.

Personal identifiers are described in the TB study. A code will be assigned to each participant allowing the information to be recorded electronically with only the Principal Researcher and Supervisor knowing the codes. All paper documents will be locked in Dr. Sanchez' office. Electronic information will be password protected and only recorded in the computers of the Principal Researcher and Supervisor.

d) If any personal identifiers will be **retained** once data collection is complete, provide a comprehensive rationale explaining why it is necessary to retain this information, **including the retention of master**
lists that link participant identifiers with unique study codes and de-identified data.

In order to give the participants results, the personal identifiers must be retained until the study is complete.

e) State who will have access to the data.

The data will only be accessible to the researchers.

f) Describe the procedures to be used to ensure anonymity of participants and/or confidentiality of data **both during the conduct of the research and in the release of its findings.**

The original questionnaires will be the only document to contain personal identifiers. All electronic data will have codes only. The participant will be given results in private and not released in any other means. The report will only contain aggregate results of the study.

g) If participant anonymity and/or confidentiality is not appropriate to this research project, explain, in detail, how all participants will be advised that data will not be anonymous or confidential.

All information and samples are confidential.

h) Explain how written records, video/audio tapes, and questionnaires will be secured, and provide details of their final disposal or storage, including how long they will be secured and the disposal method to be used.

All paper data will be locked in Dr. Sanchez' office and all electronic data will only be available by security code to the researchers. All blood samples will be coded and stored in the laboratory.

SECTION F -- SECONDARY USE OF DATA

23. a) Is it your intention to reanalyze the data for purposes other than described in this application?

[ ] **Yes** [x] **No**

b) Is it your intention to allow the study and data to be reanalyzed by colleagues, students, or other researchers outside of the original research purposes? If this is the case, explain how you will allow your participants the opportunity to choose to participate in a study where their data would be distributed to others (state how you will contact participants to obtain their re-consent)

No

If there are no plans to reanalyze the data for secondary purposes and, yet, you wish to keep the data indefinitely, please explain why.

The data will be destroyed after the study is completed and published.

SECTION G -- MONITORING ONGOING RESEARCH

24. **Annual Review and Serious Adverse Events (SAE):**

a) Minimum review requires the completion of a "Renewal/Project Completed" form at least annually.

Indicate whether any additional monitoring or review would be appropriate for this project.

It is the investigator's responsibility to notify the REB using the "Renewal/Project Completed" form, when the project is completed or if it is cancelled.
http://www.brocku.ca/researchservices/Forms/Forms.html

The enrollment and contact with participants will only last one season (less than 1 year)

***Serious adverse events** (unanticipated negative consequences or results affecting participants) **must be reported** to the Research Ethics Officer and the REB Chair, **as soon as possible** and, in any event, no more than 3 days subsequent to their occurrence.

25. **COMMENTS**

If you experience any problems or have any questions about the Ethics Review Process at Brock University, please feel free to contact the Research Ethics Office at (905) 688-5550 ext 3035, or reb@brocku.ca

Appendix B: Brock University Research Ethics Acknowledgement of Receipt

FROM: Linda Rose-Krasnor, Chair
Research Ethics Board (REB)

TO: Ana Sanchez, Community Health Sciences
Ron MERGL
Angela Duarte

FILE: 06-363 - MERGL

DATE: June 21, 2007

The Brock University Research Ethics Board has received the research proposal:

West Nile Virus Exposure in the Mexican Migrant Workers of the Niagara Peninsula

Initial screening of your proposal has been completed. Your proposal has been submitted for an **Expedited Review**.

If a reviewer of a proposal submitted for expedited review decides that a full review is warranted, that proposal will be reviewed at the next REB meeting. We will be in touch by Email when the reviewers have made their recommendations (approximately 15-20 working days).

Thank you for submitting your proposal.

Please remember that no research with Human Participants shall commence prior to receiving clearance from this committee.

LRK/bb

Brenda Brewster, Research Ethics Assistant
Office of Research Ethics, MC D250A
Brock University
Office of Research Services
500 Glenridge Avenue
St. Catharines, Ontario, Canada L2S 3A1
phone: (905)688-5550, ext. 3035 fax: (905)688-0748
email: reb@brocku.ca
http://www.brocku.ca/researchservices/ethics/humanethics/

Appendix C: Brock University Research Ethics Clearance

DATE: July 13, 2007

FROM: Michelle McGinn, Chair
Research Ethics Board (REB)

TO: Ana Sanchez, Community Health Sciences
Ron MERGL, Angela Duarte

FILE: 06-363 MERGL

TITLE: West Nile Virus Exposure in the Mexican Migrant Workers of the Niagara Peninsula

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The Brock University Research Ethics Board has reviewed the above research proposal.

DECISION: Accepted as is.

This project has received ethics clearance for the period of July 13, 2007 to November 30, 2007 subject to full REB ratification at the Research Ethics Board's next scheduled meeting. The clearance period may be extended upon request. ***The study may now proceed.***

Please note that the Research Ethics Board (REB) requires that you adhere to the protocol as last reviewed and cleared by the REB. During the course of research no deviations from, or changes to, the protocol, recruitment, or consent form may be initiated without prior written clearance from the REB. The Board must provide clearance for any modifications before they can be implemented. If you wish to modify your research project, please refer to http://www.brocku.ca/researchservices/forms to complete the appropriate form **Revision or Modification to an Ongoing Application.**

Adverse or unexpected events must be reported to the REB as soon as possible with an indication of how these events affect, in the view of the Principal Investigator, the safety of the participants and the continuation of the protocol.

If research participants are in the care of a health facility, at a school, or other institution or community organization, it is the responsibility of the Principal Investigator to ensure that the ethical guidelines and clearance of those facilities or institutions are obtained and filed with the REB prior to the initiation of any research protocols.

The Tri-Council Policy Statement requires that ongoing research be monitored. A Final Report is required for all projects upon completion of the project. Researchers with projects lasting more than one year are required to submit a Continuing Review Report annually. The Office of Research Services will contact you when this form ***Continuing Review/Final Report*** is required.

Please quote your REB file number on all future correspondence.

MM/bb

Brenda Brewster, Research Ethics Assistant
Office of Research Ethics, MC D250A
Brock University
Office of Research Services
500 Glenridge Avenue
St. Catharines, Ontario, Canada L2S 3A1
phone: (905)688-5550, ext. 3035 fax: (905)688-0748
email: reb@brocku.ca
http://www.brocku.ca/researchservices/ethics/humanethics/

Appendix D: Laboratory Biosafety Operating Permit

Approval for Biosafety level II laboratory from the Office of Environment, Health and Safety (EH&S) of Brock University

Biosafety Operating Permit

Brock University

Permit Holder: **Prof. Ana Sanchez**
Department of Community Health and Sciences.
BL2 Facility: **MC-C307A**
Brock Permit: 2007-002-BIO
Valid: 01/09/2007 – 31/08/2008

Biological Material	Containment Level (Explanation)	Safety Precautions
Human Blood (Field)	Universal Precautions for the handling of bodily fluids. Clinical standard.	Universal Precautions: http://0-www.cdc.gov.pugwash.lib.warwick.ac.uk/mmwrhtml/00001450.htm
Human Blood (Labwork)	BL2 Bodily fluids can contain infectious materials and therefore are treated as low level pathogens	PHAC Biosafety Level 2 (BL2) facility and precautions: http://www.phac-aspc.gc.ca/publicat/lbg-ldmbl-04/index.html SOP followed (Brock University Research Ethics Clearance 06-268-Duarte et al.)

This permit is issued by Brock University's Biosafety Officer in accordance with Brock University Policy and is based on criteria established in PHAC's "Laboratory Biosafety Guidelines, 3rd edition, 2004.

I accept the prescribed safety precautions:

Permit Holder: Prof. Ana Sanchez

Permit Approvals:

Chair Community Health Sciences: Prof. Tony Bogaert

Research and Academic Safety Officer: Andy Prankevicius

Appendix E: Circular Information Sheet of Invitation for Mexican Workers

(English)

Circular of invitation for Mexican workers
English Version

Brock University

Mexican Friend,

You are invited to protect your health
From infectious diseases

Come and participate in a research studies from Brock University to detect if you have been exposed to:

WEST NILE VIRUS
TUBERCULOUS BACTERIA

We will administered a questionnaire, and we will do some lab test
You will have your results and a

In addition, you will have special information
About some infectious diseases, useful for you and your family

Don't miss this opportunity

Where:
When:

Your supervisor will give you permission and we will bring delicious sandwiches!!!

Appendix E: Circular Information Sheet of Invitation for the Mexican Workers (Spanish)

Circular of invitation for Mexican workers
Spanish Version

Brock University

Amigo Mexicano.

Estas invitado a proteger tu salud de las enfermedades infecciosas

Participa en un estudio de investigacion realizado por la universidad de brock para saber si te has expuesto a:

VIRUS DEL NILO OCCIDENTAL Y
LA BACTERIA DE LA TUBERCULOSIS

Llenaras un cuestionario, te haremos unas pruebas de laboratorio, tendras tus resultados y una consejeria,

ADEMAS RECIBIRAS EDUCACION ESPECIAL SOBRE COMO PROTEGERTE A TI Y A TU FAMILIA

NO TE PIERDAS LA REUNION INFORMATIVA

CUANDO:
DONDE:

TIENES PERMISO DE TU JEFE Y HABRAN BOCADILLOS!!

Appendix F: Information for the Mexicans on West Nile Virus Infection

Appendix G: Letter of Invitation for the Mexican Workers (English Version)

LETTER OF INVITATION

TO PARTICIPATE IN A RESEARCH STUDY

"WEST NILE VIRUS EXPOSURE IN THE MEXICAN MIGRANT WORKERS OF THE NIAGARA PENINSULA"

Investigator Principal: Ron Mergl BSc, DVM, Estudiante de Post-grado. Departamento de Ciencias de la Salud Comunitaria, Univesidad de Brock

Co-Investigator: Angela Duarte, BSc Graduate Estudiante de Post-grado. Departamento de Ciencias de la Salud Comunitaria, Univesidad de Brock

Faculty Supervisor: Ana L. Sanchez, PhD, Profesora Asociadoa. Departamento de Ciencias de la Salud Comunitaria, Univesidad de Brock

I, Ron Mergl, BSc, DVM Graduate student, and my supervisor Dr. Ana Sanchez, PhD, from the Department of Community Health Sciences, Brock University invite you to participate in a research project about West Nile Virus in Mexican agricultural workers that are in the Niagara Region this year (2007) through the Seasonal Agricultural Workers Program (SAWP).

The purpose of this research project is to evaluate West Nile Virus exposure in a group of Mexican workers who agree to volunteer. The study is to determine if the Mexican workers are being exposed to West Nile Virus through mosquito bites while in Canada.

The expected duration of the research study is one year, but you are asked for at least 3 encounters with us in for the study; these 3 meetings will be in private but we would also like to implement additional group sessions as explained below:

1. During the first meeting, called "informational meeting", we will explain the purpose of the study, what will be involved, an explanation of benefits and risks, management of information (anonymity and confidentiality), the laboratory tests that will be done and what their results mean. We will also explain things about the virus and the infection it causes and what are the best ways to protect yourself against infection. At the end of the meeting and following a period of questions and answers, we will ask for your voluntary participation. If you agree, together we will set a date and place for the next meeting (preferable at your place and after work at your convenience). This meeting may take one hour approximately.

2. During the second meeting, called "the data collection meeting" we will administer in a private and in Spanish a questionnaire to you in which you will be asked questions about your health and living conditions both in Canada and in Mexico; also questions to

see how much you know about the West Nile virus and the infection it causes. At the end of the meeting and with your permission we will draw a blood sample from you, which will be collected in 2 tests tubes. The entire process can take 30-40 minutes.

3. In the third meeting, called "Report meeting" we will come back with the results of your tests and in a private short session we will explain their meaning to you. Also we will provide more information or counseling as to how best protect yourself against WNV. This meeting may take 15 minutes.

4. Additionally, if you and your coworkers would be interested, we would like to arrange group gatherings to develop educational activities about WNV, Tuberculosis and other infectious diseases.

This research study has several benefits:

TO YOU: because you will get a complete screening for West Nile Virus; you will know if you are protected or susceptible and if so, what are the best ways to prevent infection or if it happens how to recognize it and seek medical care. You will also get much information and health education from the several activities planned within the study. The educational component will give you important knowledge about infectious diseases and West Nile Virus and will promote healthy behaviours for the future.

TO MEXICO: because so far WNV is not affecting humans so far. If you were infected in Canada, there is a possibility you could bring it back. The knowledge generated by this study could help the Mexican health authorities to monitor for WNV transmission patterns.

THE SCIENTIFIC COMMUNITY: from your participation because you will be collaborating to answer important questions about WNV transmission and its frequency in the Niagara Peninsula.

IF YOU HAVE ANY PERTINENT QUESTIONS ABOUT YOUR RIGHTS OR BENEFITS AS A RESEARCH PARTICIPANT, PLEASE CONTACT THE BROCK UNIVERSITY RESEARCH ETHICS OFFICER (905 688-5550 EXT 3035, reb@brocku.ca) OR IN SPANISH WITH MISS JANET MACLAUGHLAN, CELL PHONE # _____

IF YOU HAVE ANY QUESTIONS ABOUT THE STUDY OR ABOUT WEST NILE VIRUS A, PLEASE FEEL FREE TO CONTACT ME.

Thank you!

Ron Mergl , BSc,DVM **Ana L. Sanchez, PhD**

Graduate student Associate Professor

Department of Community Health Sciences Department of Community Health Sciences

Brock University Brock University

905 6885550 ext. 3882 905 685550 ext. 4388

rm80jq@brocku.ca ana.sanchez@brocku.ca

This study has been reviewed and received ethics clearance through Brock University's Research Ethics Board file # 03-363 MERGL

Appendix G: Letter of Invitation for Mexican Workers (Spanish Version)

CARTA DE INVITACION

PARA PARTICIPAR EN EL ESTUDIO DE INVESTIGACION

"EXPOSICION AL VIRUS DEL NILO OCCIDENTAL EN TRABAJADORES AGRICULTORES MIGRANTES MEXICANOS EN LA PENINSULA DEL NIAGARA"

Investigador Principal: Ron Mergl BSc, DVM, Estudiante de Post-grado. Departamento de Ciencias de la Salud Comunitaria, Univesidad de Brock

Co-Investigator: Angela Duarte, BSc Graduate Estudiante de Post-grado. Departamento de Ciencias de la Salud Comunitaria, Univesidad de Brock

Supervisora Academica: Ana L. Sanchez, PhD, Profesora Asociadoa. Departamento de Ciencias de la Salud Comunitaria, Univesidad de Brock

Yo, Ron Mergl, estudiante de post-grado de la Maestria en Ciencias y mi supervisora Dra. Ana Sanchez, profesora del Departamento de Ciencias de la Salud Comunitaria, Univesidad de Brock por este medio lo INVITAMOS A PARTICIPAR en un □tudio de investigacion sobre el virus del Nilo Occidental que podria estar infectando a trabajadores agricultores migrantes Mexicanos que estan en la Region del Niagara este ano de 2007 a traves del programa temporal de trabajadores agricolas [Seasonal Agricultural Workers Program (SAWP)].

El objetivo de este □tudio de investigacion es □tudio□ssi exposicion o infeccion al virus del Nilo Occidental en un grupo de trabajadores mexicanos del SAWP que decida participar voluntariamente. El □tudio tratara de □tudio□ssi si los trabajadores estan siendo infectados por medio de picaduras de mosquito mientras trabajan esta temporada en Canada.

La duracion de todo el □tudio es de un año pero a cada participante le pediriamos que se reuna con nosotros y en privado al menos tres veces. Tambien queremos tener Estas tres □tudio□s seran en privado pero tambien nos gustaria hacer reunions de grupo como se explica a continuacion:

1. Durante la primera □tudio□, llamada "□tudio□ □tudio□ssio", explicaremos el propósito del □tudio y lo que implicada, se explicaran las ventajas y los riesgos, como se manejara la información (anonimato y confidencialidad), los pruebas de laboratorio que serán hechos y qué significan sus resultados. También explicaremos cosas sobre el virus y la infección que causa y cuáles son las mejores maneras de protegerse contra la infección. Al final de la □tudio□ y después de un □tudio de preguntas y de respuestas, pediremos su participación voluntaria. Si esta de acuerdo, juntos fijaremos una fecha y un

lugar para la □tudio□ siguiente (□tudio□ssi en donde vive después de horas de trabajo, su conveniencia). Esta reunion puede durar una hora mas o menos.

2. Durante la segunda □tudio□, llamada "□tudio□ de la recolección de datos" usaremos un cuestionario para hacerle preguntas sobre su salud y condiciones vida en Canadá y en México; también habran preguntas □tudio□s cuánto sabe sobre el virus del Nilo Occidental y la enfermedad que causa. Al final del cuestionario

y con su permiso le tomaremos una muestra de la sangre que □tud recogida en 2 tubos de laboratorio. El proceso entero puede tomar 30-40 minutos.

3. En la tercera □tudio□, llamada la "□tudio□ de reporte" volveremos con los resultados de sus pruebas y en una □tudio□ corta y privada explicaremos su significado. También proporcionaremos más información o consejos de como □tudi protegerse contra WNV. Esta reunion puede durar 15 minutos.

4. Además, □tudi y tus compañeros de trabajo estuvieran interesados, quisiéramos arreglar □tudio□s del grupo para desarrollar actividades educativas sobre virus del Nilo Occidental, tuberculosis y otras enfermedades infecciosas.

Este □tudio de investigación tiene varias ventajas:

PARA USTED: Porque le vamos a hacer □tudio□ sobre el virus del Nilo Occidental; usted sabra si tiene proteccion o no contra el. Tambien sabra como protegerse □tudi para que no le de la infeccion o que hacer en el caso de que se infecte. Tambien usted obtendra bastante informacion y educacion para la salud. El □tudio□ss educacional le dara conocimientos importantes sobre esta infeccion, y otras, y como □tudio□ habitos saludables para su □tudi salud.

PARA MEXICO: porque aun virus del Nilo Occidental no esta afectando personas en Mexico como lo hace en Canada. Si usted fuera infectado mientras esta en Canada, podria llevar el virus a Mexico. Los resultadoes de este □tudio le pueden ayudar a las autoridades de salud en mexico a como vigilar el comportamiento de □tudio□ssion de este virus.

PARA LA COMUNIDAD CIENTIFICA: porque gracias a su participacion usted ayudara a dar respuesta a preguntas importantes sobre la transmission y frecuencia del virus del Nilo Occidental en la region del Niagara.

SI TIENE ALGUNA PREGUNTA O DUDA EN RELACION A SUS DERECHOS O BENEFICIOS DE ESTE ESTUDIO POR FAVOR NO DUDE EN LLAMAR A LA OFICINA DE ETICA PARA LA INVESTIGACION EN LA UNIVERSIDAD DE BROCK AL TEL. 905 688-5550 ext 3035, reb@brocku.ca) O EN ESPANOL CON LA SENORITA JANET MACLAUGHLAN A SU TELEFONO CELLULAR #

_____

SI TIENE ALGUNA PREGUNTA SOBRE EL ESTUDIO O EL VIRUS DEL NILO OCCIDENTAL POR FAVOR NO DUDE EN CONTACTARME

Gracias!

Ron Mergl, DMV Ana L. Sanchez, PhD Estudiante de Maestria Profesora Asociada

Departamento de Ciencias de la Salud Comunitaria Departamento de Ciencias de la Salud Comunitaria Univesidad de Brock Univesidad de Brock

905 6885550 ext. 3882 905 685550 ext. 4388

rm80jq@brocku.ca ana.sanchez@brocku.ca

Este □tudio ha recibido aprobacion por el Comite de Etica de la Universidad de Brock, expediente # 03-363 MERGL et al

Appendix H: Consent Form (English Version)

INFORMED CONSENT

TO PARTICIPATE AS A VOLUNTEER IN THE RESEACH STUDY

"WEST NILE VIRUS EXPOSURE IN THE MEXICAN MIGRANT WORKERS OF THE NIAGARA PENINSULA"

Principal Investigator: Ron Mergl BSc, DVM, Graduate student, Department of Community Health Sciences, Brock University

Co-Investigator: Angela Duarte, BSc Graduate student, Department of Community Health Sciences, Brock University

Faculty Supervisor: Ana L. Sanchez, PhD, Professor associated, Department of Community Health Sciences, Brock U.

This study has been reviewed and received ethics clearance through Brock University's Research Ethics Board file # 06-363 MERGL et al & file # 06-268 DUARTE et al for the Tuberculosis study from which some information will be drawn and a portion of the blood sample will be shared.

Name of the participant: _____ Code _____

I have been explained in detail the specific aspects of the study by the researchers.

I understand that my participation is **voluntary**; that I can refuse to participate with no penalty and that I may stop my participation at any time.

I understand that I will participate in a study to see if I have been infected by the West Nile virus and that the study will also provide me with extensive information about this infectious disease and ways to protect myself while I am in Canada.

I understand that I will be asked some questions (personal information and West Nile Virus related) of a questionnaire and that it will be administered by a Spanish-speaker researcher. I also understand that my general demographic information and a portion of my blood sample will be obtained from the latent TB study conducted by Angela Duarte.

I understand that my blood will be tested for immunity against West Nile Virus.

I understand that if I ever got infected by other similar microbes carried by mosquitoes such as Dengue virus my results can be uncertain, in which case, my sample without any personal identifier can be sent to another laboratory in Canada, Honduras or Mexico.

I understand that all information will be kept confidential in a secure location and that my personal information will be protected from any person other than the researchers.

_____ have read and understand this information

_____ agree freely to participate

_____

Signature

Date: _____ _____, **2007**

Month Day

IF YOU HAVE ANY PERTINENT QUESTIONS ABOUT YOUR RIGHTS OR BENEFITS AS A RESEARCH PARTICIPANT, PLEASE CONTACT THE BROCK UNIVERSITY RESEARCH ETHICS OFFICER (905 688-5550 EXT 3035, reb@brocku.ca) OR IN SPANISH WITH MISS JANET MACLAUGHLAN, CELL PHONE # _____

IF YOU HAVE ANY QUESTIONS ABOUT THE STUDY OR ABOUT WEST NILE VIRUS A, PLEASE FEEL FREE TO CONTACT ME.

Thank you!

Ron Mergl, DMV Ana L. Sanchez, PhD

Graduate student Associate Professor

Department of Community Health Sciences Department of Community Health Sciences

Brock University Brock University

905 6885550 ext. 3882 905 685550 ext. 4388

rm80jq@brocku.ca ana.sanchez@brocku.ca

Copy to participant
Original to researchers

Appendix H: Consent Form (Spanish Version)

CONSENTIMIENTO INFORMADO

PARA PARTICIPAR COMO VOLUNTARIO EN EL ESTUDIO DE INVESTIGACION

"EXPOSICION AL VIRUS DEL NILO OCCIDENTAL EN TRABAJADORES AGRICULTORES MIGRANTES MEXICANOS EN LA PENINSULA DEL NIAGARA"

Investigador Principal: Ron Mergl BSc, DVM, Estudiante de Post-grado. Departamento de Ciencias de la Salud Comunitaria, Univesidad de Brock

Co-Investigator: Angela Duarte, BSc Graduate Estudiante de Post-grado. Departamento de Ciencias de la Salud Comunitaria, Univesidad de Brock

Supervisora Academica: Ana L. Sanchez, PhD, Profesora Asociadoa. Departamento de Ciencias de la Salud Comunitaria, Univesidad de Brock

Este estudio ha recibido aprobacion por el Comite de Etica de la Universidad de Brock, expediente # 03-363 MERGL et al (y expediente # 06-268 DUARTE et para el studio de tuberculosis con el cual se compartira cierta informacion general y del cual se obtendra una porcion de la muestra de sangre)

Nombre del participante: _____ Codigo _____

Los investigadores me he explicado detalladamente todos los aspectos específicos del estudio.

Entiendo que mi participación es voluntaria; que puedo rechazar participar sin penalidad alguna y que me puedo retirar del estudio en cualquier momento.

Entiendo que participaré en un estudio para ver si he sido expuesto o infectado a el virus del Nilo Occidental, y que el estudio también me proveerá información extensa sobre esta y otras enfermedades infecciosas para protegerme mientras estoy en Canadá.

Entiendo que se me haran preguntas de caracter personal y relacionadas al virus del Nilo Occidental mediante un cuestionario el cual sera aplicado en Espanol. Tambien entiendo que cierta parte de mi informacion y un poco de mi muestra de sangre seran obtenidos del estudio de tuberculosis conducido por by Angela Duarte.

Entiendo que mi sangre sera examinada para ver si tengo inmunidadad contra el virus del Nilo Occidental (si he sido expuesto o infectado).

Entiendo de haber padecido infeccion por otros microbios parecidos transmitidos por mosquitos las pruebas pueden ser inciertas, y en tal caso mi muestra de sangre puede ser enviada a otros laboratorios en Canada, Honduras o Mexico

que de haberme infectado alguna vez pueden hacer que los resultados de la prueba para el virus del Nilo sean inciertos y que para confirmar que para confirmar resultados

Consentimiento para participar en un estudio de investigacion sobre el virus del Nilo Occidental de la Universidad de Brock

Entiendo que toda mi información será mantenida confidencial una localización segura y que mi información personal será protegida contra cualquier persona con excepción de los investigadores.

_____ He leído y entiendo esta información

_____ ACEPTO PARTICIPAR VOLUNTARIAMENTE

_____

Firma

Fecha: _____ _____, **2007**

Mes Dia

SI TIENE ALGUNA PREGUNTA O DUDA EN RELACION A SUS DERECHOS O BENEFICIOS DE ESTE ESTUDIO POR FAVOR NO DUDE EN LLAMAR A LA OFICINA DE ETICA PARA LA INVESTIGACION EN LA UNIVERSIDAD DE BROCK AL TELEFONO 905 688-5550 extension 3035, reb@brocku.ca) O EN ESPANOL CON LA SENORITA JANET MACLAUGHLAN A SU TELEFONO CELLULAR # _____

SI TIENE ALGUNA PREGUNTA SOBRE EL ESTUDIO O EL VIRUS DEL NILO OCCIDENTAL POR FAVOR NO DUDE EN CONTACTARME

Gracias!

Ron Mergl, DMV Ana L. Sanchez, PhD

Estudiante de Maestria Profesora Asociada

Departamento de Ciencias de la Salud Comunitaria Departamento de Ciencias de la Salud Comunitaria Univesidad de Brock Univesidad de Brock

905 6885550 ext. 3882 905 685550 ext. 4388

rm80jq@brocku.ca ana.sanchez@brocku.ca

Copia para el participante

Original para el Investigador

Appendix I: Questionnaire

Brock University

"WEST NILE VIRUS EXPOSURE IN THE MEXICAN MIGRANT WORKERS OF THE NIAGARA PENINSULA"

"EXPOSICIÓN AL VIRUS DEL NILO OCCIDENTAL EN TRABAJADORES AGRICULTORES MIGRANTES MEXICANOS EN LA PENÍNSULA DEL NIAGARA"

Research code/Código

Date: _____ Code of interviewer: _____ WNV participation: **Y / N**
Fecha del Nilo — Nombre del entrevistador — Participacion en el estudio de Virus

Name of the participant: _____
Nombre del participante

Age: _____ Edad — Date of Birth: _____ Fecha de nacimiento — Sex: _____ Sexo

Address: _____ Telephone: _____
Dirección de la finca — Teléfono

Please answer the following questions.
Por favor responda las siguientes preguntas

Seasonal Agricultural Workers Program

1. Is this your first with the Seasonal Agricultural Workers Program (SAWP)?
Es ésta la primera vez que viene a Canadá con el programa de trabajadores agricolas?
Y / N _____

IF YES JUMP TO QUESTION # 10

2. What was the first year you came to Canada with the Seasonal Agricultural Workers Program?
En qué año vino por primera vez a Canadá con el programa de TAT? _____

3. How many times have you worked in Canada?
Cuántas temporadas ha trabajado en Canadá? _____ (#)

4. Have you ever worked in provinces other than Ontario?
Ha trabajado en otras provincias fuera de Ontario? **Y / N** _____

IF YES,

5. If yes, which one?
Si contestó que sí, cual (es):
Quebec ( ) British Columbia ( ) Alberta ( ) Manitoba ( )
Others:_____

6. Have you worked in cities other than the Niagara Region?
Ha trabajado en que otras ciudades de esta region? **Y / N** _____
Other: _____

7. Have you worked in other farms in the Niagara Region beside the current one?
Ha trabajado en otras fincas aquí en Niagara? **Y / N** _____

IF YES

9. If yes, how many/Cuántas _____

10. When did you arrive in Canada this year?
En qué fecha llegó a Canadá este año? _____(exact date: mm/dd)

LIVING CONDITIONS WHLE IN CANADA/ CONDIONES DE VIDA EN CANADÁ

10. While in Canada, where do you live?
Mientras está en Canada, dónde vive?

- Farm house ( )
  Casa en la finca, hacienda

- Trailer ( )
  Carro-casa
- Other (please specify): _____
  Otro (Por favor especifique)


11. Including yourself, how many persons live in the same house or facility?
Incluyéndolo a usted, cuantas personas viven en esta casa? _____ # people


12. How many people sleep in the same room/quarters with you? _____ # people
Incluyéndolo a usted, cuantas personas duermen en la misma habitación o área?


13. Are there any windows in the room you sleep in?
Hay ventanas en el dormitorio? **Y/N** _____

IF NO JUMP TO QUESTION # 15

IF YES


14. If yes, are the windows covered by screens without any holes?
Si contesto que sí. están esas ventanas protegidas con mosquitero?
Y/N _____


15. Are there any standing containers of water sitting next to your house?
Hay agua estancada en los alrededores de su casa? **Y/N** _____

GEOGRAPHICAL RESIDENCE/MOBILITY / RESIDENCIA Y MOBILIDAD

16. Where were you born (city and state in Mexico)?
Cómo se llama el lugar donde nació? (guiarse por el mapa)
_____
State: _____

17. What Mexican city and state are you coming from?
De qué ciudad y Estado de México viene?
_____
State: _____

18. For how long have you lived in your current address in Mexico?
Por cuánto tiempo ha vivido en su actual dirección en México? _____
(años)

19. What countries have you traveled to outside of Mexico in the last 8 years?
A que otros paises ha viajado fuera de México en los últimos 8 años?
_____
_____

20. If you have visited the United States since 1999 which states have you visited?
Si ha visitado los EEUU desde 1999, a qué estado (S) ha ido?
_____

SOCIO-DEMOGRAPHIC DATA IN MEXICO / DATOS SOCIODEMOGRÁFICOS EN MEXICO

21. Including yourself, how many people live permanently or for more than 6 months in the same household with you in Mexico?
Incluyéndolo a usted, cuántas personas viven con Ud en su casa en México?

Children (0-12) _____ Teenagers (13-19)_____ Adults 1+_____
Niños Adolescentes Adultos

22. In your home in Mexico, do you have the following
En su casa de México tiene lo siguiente?

Indoor Potable water (agua potable adentro) **Y / N**

Indoor flushing toilet (Servicio sanitario) **Y / N**

Indoor electricity (electricidad en toda la casa) **Y / N**

Floor made of cement or tiles in <u>whole</u> house (piso de cemento o cerámica/loza en <u>toda</u> la casa)**Y/ N**

Screens on the windows of your house? (mosquitero en las ventanas) **Y / N**

Water basin in the yard (Tanque abierto de recolección de agua o lavadero) **Y / N**

Do you collect rainwater in barrels? (recoge agua de lluvia en barriles/canecas) **Y / N**

A body of water near your house? (Hay agua estancada alrededor de la casa) **Y / N**

One horse or more (tiene caballos) **Y / N**

Poultry flocks (tiene aves de corral como gallinas, pollos, gallos) **Y / N**

Any wild birds kept in confinement (aves silvestres enjauladas como loros, guacamayas,?) **Y / N**

23. What type of job do you have in Mexico?
Cúal es su ocupación u oficio mientras está en México?
_____

24. What is the highest school grade you successfully <u>completed</u>? (check as many as apply)
Qué nivel de educación tiene COMPLETA?

- Elementary/ Primaria _____
- High School/ Secundaria _____
- Technical/Técnica (oficio) _____
- Other/otro_____

25. How do you consider your health in general?
Cómo considera su salud en general en este momento? (solo una)

- Excellent/Excelente_____
- Good/Buena _____
- Fair/Mas o menos _____
- Poor/Mala _____

26. If other than excellent, what health problems do you think you have?
Si respondió menos que excelente, qué problemas de salud tiene?

_____

_____

WEST NILE VIRUS– KNOWLEDGE & AWARENESS
CONOCIMIENTO / CONCIENTIZACION SOBRE VNO

60. Before this study, did you ever heard of West Nile Virus Fever?
Antes de participar en este studio, había oido hablar de la Fiebre del virus del Nilo Occidental? **Y / N**

IF NO JUMP TO QUESTION # 66

IF YES,

61. What symptoms of West Nile Virus do you know?
Qué sintomas conoce que produce el virus del Nilo Occidental?

_____

_____

62. How do you think West Nile Virus is transmitted?
Cómo cree que se transmite el Virus del Nilo Occidental?
Don't know ( ) Water ( ) Food ( ) Air ( ) Mosquitos ( ) Other:
_____

63. If you developed West Nile Virus, how serious do you think it would be?
Cree que la fiebre del Virus del Nilo occidental es mortal sin atención médica?
Y / N Don't Know

HISTORY OF WEST NILE VIRUS EXPOSURE (HISTORIA Y EXPOSICIÓN AL VNO)

64. Have you ever been diagnosed as having West Nile Virus?
Ha sido diagnosticado alguna vez con Fiebre del virus del Nilo Occidental aca en Canada? **Y / N Don't know ( )**

IF <u>NO/DON'T KNOW</u> JUMP TO QUESTION # 66

IF YES,
65. In what year or how old were you?
En qué año o que edad tenia? _____ **Don't know ( )**

66. Have you experienced any of the following symptoms in last 2 weeks and in the last 12 months or since 2002?

Ha tenido alguno de los siguientes síntomas en las últimas 2 semanas/últimos 12 meses/desde 2002?

	Last 2 weeks Últimas 2 semanas	Last 12 months Últimos 12 meses		Since 2002 Desde 2002	
	CAN	CAN	MEX	CAN	MEX
• High fever? • Fiebre elevada de más de 38 grados?					
• Fever with headache **and** stiff neck? • Fiebre con dolor de cabeza y el cuello rígido, duro o tieso?					
• Skin rash over the trunk of the body? • Sarpullido o ronchas en el pecho y espalda?					
• Weak or painful muscles not related to your work? • Debilidad muscular o dolor que no tiene relación con su trabajo?					
• Nausea and/or vomiting accompanying any of the above symptoms? • Nausea y vómito con los otros síntomas mencionados arriba?					
• Muscle tremors or convulsions? • Convulsiones o ataques					
• Encephalitis or meningitis • Meningitis o encefalitis?					

67. Have you been bitten by mosquitoes while in Canada this year?

Lo han picado mosquitos desde que vino a Canadá este año? **Y/N**

Todos los dias ( ) Por la noche ( ) Fines de semana ( )

68. Do you use mosquito repellent?

Se pone o aplica repelente para insectos o moquitos? **Y/N**

IF YES,

69. If yes, can you provide the name?

Si es así, se acuerda qué marca? _____

70. Do you work around standing containers of water or irrigation ditches?

Trabaja alrededor de agua estancada o canales de irrigación? **Y/N**

71. When you are working are your arms and legs covered by clothing?
Usa camisa manga larga y pantalon largo mientras trabaja? **Y/N**

72. Do you wear a hat for work?
Usa sombrero o gorra durante su trabajo en el campo? **Y/N**


73. Have you seen mosquitoes inside your house?
Ha visto mosquitos dentro de la casa? **Y/N** (By researcher) **Y/N** (By participant)

74. Have you seen any dead wild birds (Crows or Blue Jays) on the farm?
Ha visto pajaros muertos (cuervos o azulejos) en la finca? **Y/N**

75. Have you handled any dead wild birds (Crows or Blue Jays) this year?
Ha tocado algún pájaro muerto este año en Canadá? **Y/N**

76. Have you taken any ticks off your body this year?
Se ha encontrado garrapatas en el cuerpo este año en Canadá? **Y/N**

OTHER MEDICAL HISTORY

77. Have you ever been diagnosed with Dengue Fever?
Le ha dado Dengue? (le han dicho en la clínica o usted cree?) **Y/N Don't know ( )**

78. Have you been vaccinated against Yellow Fever?
Se ha puesto la vacuna contra Fiebre Amarilla? **Y/N Don't know ( )**

79. Have you donated blood in the last year?
Ha donado sangre en el últimos 12 meses? **Y/N**

80. Have you received blood by transfusion in the last 5 years?
Le han hecho una transfusión de sangre en los últimos 5 años? **Y/N**

IF YES,

81. If yes, where in Mexico or other country?
Dónde? En México o en otro país? _____

82. Are you planning to donate blood when you return home?
Planea donar sangre cuando vuelva a México? **Y/N**

83. What other infectious diseases would you like to know more of (how to prevent and get treated for)?

_____

Sobre qué otras enfermedades infeccionsas le gustaria saber mas (sobre como prevenir o tener tratamiento)?

THANK YOU!!!

CODE: _____

FOR RESEARCH USE ONLY

Venipuncture performed by (initials) _____

Blood with heparin for TB study

Blood without anticoagulant

Record of any Special Observations or complications: -

_____

RESEARCHER WHO COMPLETED THIS INTERVIEW:

_____

RESEARCHER WHO CHECKED FOR INFORMATION **COMPLETNESS**

Appendix J: Procedure for the WNV IgM Capture ELISA

A diagram for this procedure is shown in Fig. 13.

- All frozen serum samples for analysis are removed from the freezer and allowed to thaw and equilibrate to room temperature.
- Microwell strips are removed from the foil packet and placed into the strip holder.
- An additional five microwells are required for positive control, negative control, and 3 cut-off calibrators. All controls are human serum with preservatives. The controls must meet specifications provided. If not, it indicates reagent failure.
- All participant serum samples and controls are diluted by using 10 µl of serum or control and adding 1000 µl of the serum diluent provided. The serum diluent is tris buffered saline with 0.1% Proclin as preservative.
- Before proceeding, the antigen/monoclonal antibody tracer must be mixed. An amount of 0.5 ml of WNV antigen (NY99 strain) is required for each test strip. An equal volume of horseradish peroxidise (HRP) conjugated monoclonal antibody tracer is added to the WNV antigen and this is incubated at room temperature for 1 hour. This monoclonal antibody is an antibody to the E glycoprotein of the virus.
- Within 10 minutes of completing the antigen/monoclonal antibody mix, 100 µl of diluted serum or control are pipetted into each well and covered and incubated at 37 ° C for 1 hour.
- The wells are then removed from the incubator and washed 6 times in the automated washer using the prepared buffer. Concentrated wash buffer is provided and is diluted using 1 part buffer with 19 parts of distilled water. The buffer is

phosphate buffered saline with preservative. The wells must then be inverted on an absorbent surface and tapped firmly.

- The antigen/monoclonal antibody tracer is added, pipetting 100 µl into each well, covering and incubating at 37 ° C for 1 hour.
- The wells are washed 6 times in the automated washer with phosphate buffered saline
- Tetramethylbenzidine (TMB), the colour substrate, is added, pipetting 100 µl of TMB into each well and incubating for 10 minutes at room temperature. A blue colour will develop in the positive samples when the HRP hydrolyzes the TMB.
- A stop solution of phosphoric acid is added by pipetting 100 µl into each well. The positive samples will turn yellow.
- Using a dual length spectrophotometer, the optical density is read at 450nm with a reference filter set between 600-650nm. The reference filter is necessary to subtract out the absorbance from background materials [107].
- The cut-off value is determined by the average of the absorbance of the 3 cut-off samples.
- The sample absorbance is then divided by the cut-off absorbance and the index value is achieved.
- The reference values for index levels were determined from a US population of 304 samples with 235 negative samples and 69 positive samples. If the index value for a sample is < 0.9 then the result is negative. If the result is 0.9 to 1.1 then the result is equivocal. If the result is > 1.1 then the result is positive.

- Equivocal samples must be repeated in duplicate and remain equivocal if duplicate repeats are not the same.
- A positive result indicates the likely presence of IgM, however cross-reactivity is well documented with other flaviviruses and rheumatoid factor [107]. As well, IgM for WNV can persist for up to 500 days so the presence of IgM does not necessarily indicate an acute infection [58].

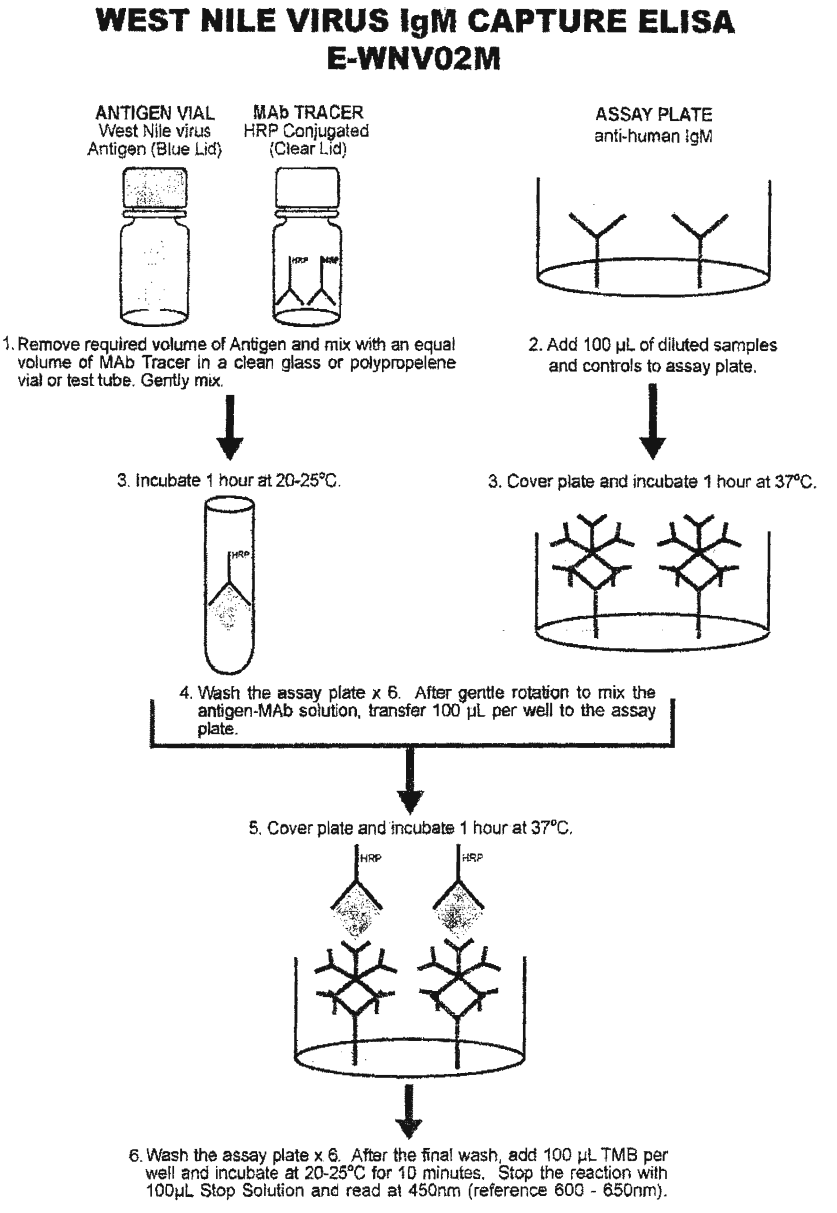


Fig.13. Procedure for the WNV IgM Capture ELISA. Panbio, Australia 2005.

Appendix K: Procedure for the WNV IgG Indirect ELISA

- All frozen serum samples for analysis are removed from the freezer and allowed to thaw and equilibrate to room temperature.
- Microwells are removed from the foil packet and placed into the strip holder.
- An additional five microwells are required for positive control, negative control, and 3 cut-off calibrators. All controls are human serum with preservatives. The controls must meet specifications provided. If not it indicates reagent failure.
- All participant serum samples and controls are diluted by using 10 µl of serum or control and adding 1000 µl of the serum diluent provided. The serum diluent is tris buffered saline with .1% Proclin as preservative.
- 100 µl of diluted serum is added into each well and incubated for 30 minutes at 37 ° C.
- The microwells are washed 6 times in the automated washer, removed and inverted and tapped to remove any retained buffer.
- 100 µl of HRP conjugated anti-human IgG is added into each well, covered and incubated for 30 minutes at 37 ° C.
- The microwells are washed 6 times in the automated washer, removed and inverted and tapped to remove any retained buffer.
- 100 µl TMB is added into each well and incubated for 10 minutes at room temperature. The positive samples will turn to a blue colour as the HRP hydrolyzes the TMB substrate.
- A stop solution of phosphoric acid is added by pipetting 100 µl into each well. The positive samples will turn yellow.

- Using a dual length spectrophotometer, the absorbance is read at 450nm with a reference filter set between 600-650nm. The reference filter is necessary to subtract out the absorbance from background materials [108].
- The cut-off value is determined by the average of the absorbance of the 3 cut-off samples.
- The sample absorbance is then divided by the cut-off absorbance and the index value is achieved.
    - The cut-off has been achieved from a study with 379 positive and 593 negative samples from 3 different trials within the USA. An index value < 1.4 indicates a negative result. An equivocal result is 1.4 to 1.6 and a positive result is > 1.6.
    - A negative results means the test did not detect IgG antibodies however the accuracy of the result is dependent on the sensitivity of the assay.
    - An equivocal result must be repeated in duplicate and if the repeated tests do not agree then the IgG cannot be determined.
    - A positive sample indicates the likely presence of WNV IgG or another flavivirus. The test must be confirmed with PRNT or by using current CDC guidelines for diagnosis of the disease [108].

Appendix L: Procedure for the IgG Avidity ELISA

- All frozen serum samples for analysis are removed from the freezer and allowed to thaw and equilibrate to room temperature.
- Microwells are removed from the foil packet and placed into the strip holder.
- An additional five microwells are required for positive control, negative control, and 3 cut-off calibrators. All controls are human serum with preservatives. The controls must meet specifications provided. If not, it indicates reagent failure.
- All participant serum samples and controls are diluted by using 10 µl of serum or control and adding 1000 µl of the serum diluent provided. The serum diluent is tris buffered saline with 0.1% Proclin as preservative.
- 100ul of diluted serum is added into each well and incubated for 30 minutes at 37 ° C.
- The microwells are washed in an automated washer 2 times with buffered saline, inverted and tapped to remove excess buffer.
- 100 µl of avidity solution is added into each well except the control wells.
- The microwells are incubated for 5 minutes at room temperature.
- The microwells are washed in the automated washer 6 times, inverted and tapped to remove excess buffer.
- 100 µl of HRP conjugated anti-human IgG is added into each well, covered and incubated for 30 minutes at 37 ° C.
- The microwells are washed 6 times in the automated washer, removed and invert and tapped to remove any retained buffer.

- 100 µl TMB is added into each well and incubate for 10 minutes at room temperature. The positive samples will turn to a blue colour as the HRP hydrolyzes the TMB substrate.
- A stop solution of phosphoric acid is added by pipetting 100 µl into each well. The positive samples will turn yellow.
- Using a dual length spectrophotometer, the absorbance is read at 450nm with a reference filter set between 600-650nm. The reference filter is necessary to subtract out the absorbance from background materials [109].
- The absorbance is measured and is divided by the absorbance reading from the standard IgG indirect ELISA for each sample. The result is referred to as the avidity index.
- Cut-offs for determining acute versus past infections with the avidity test need to be validated in a laboratory by comparing values for acute and convalescent samples [109].

Appendix M: Procedure for the Dengue IgG Indirect ELISA

- All frozen serum samples for analysis are removed from the freezer and allowed to thaw and equilibrate to room temperature.
- Microwells are removed from the foil packet and placed into the strip holder.
- An additional five microwells are required for positive control, negative control, and 3 cut-off calibrators. All controls are human serum with preservatives. The controls must meet specifications provided. If not, it indicates reagent failure.
- All participant serum samples and controls are diluted by using 10 µl of serum or control and adding 1000 µl of the serum diluents provided. The serum diluent is tris buffered saline with 0.1% Proclin as preservative.
- 100 µl of diluted serum is added into each well and incubate for 30 minutes at 37 ° C.
- The microwells are washed 6 times in the automated washer, removed and inverted and tapped to remove any retained buffer.
- 100ul of HRP conjugated anti-human IgG is added into each well and cover and incubate for 30 minutes at 37 ° C.
- The microwells are washed 6 times in the automated washer, removed and inverted and tapped to remove any retained buffer.
- 100 µl TMB is added into each well and incubate for 10 minutes at room temperature. The positive samples will turn to a blue colour as the HRP hydrolyzes the TMB substrate.
- A stop solution of phosphoric acid is added by pipetting 100 µl into each well. The positive samples will turn yellow.

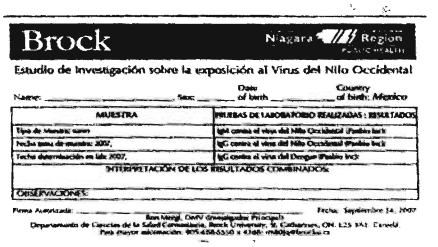
- Using a dual length spectrophotometer, read the absorbance at 450nm with a reference filter set between 600-650nm. The reference filter is necessary to subtract out the absorbance from background materials [110].
- The cut-off value is determined by the average of the absorbance of the 3 cut-off samples.
- The sample absorbance is then divided by the cut-off absorbance and the index value is achieved.
- A negative result is < 0.9, an equivocal result is 0.9 to 1.1 and a positive result is > 1.1.
- A negative result indicates that there were no anti-Dengue antibodies detected however it is dependent on the sensitivity of the assay. An equivocal result should be repeated in duplicate but if the repeated samples do not agree the sample must be tested with a different test.
- A positive result indicates either a past or recent Dengue or other flavivirus infection [110].

Appendix N: Procedure for the PRNT

- The NML follows the guidelines established by the World Health Organization (WHO) for the PRNT.
- All sera is heat inactivated by placing the samples in a water bath of 56° C for 30 minutes.
- Serial dilutions are prepared for the test sera as well as for negative and positive controls.
- a series of 6 tubes is used for each sample.
- 450 µl of a standard serum diluent is added to the first tube and 250 µl is added to the remaining 5 tubes.
- 50 µl of heat-inactivated serum is added to the first tube making a dilution of 1:10.
- 250 µl from the first tube is then added to the next tube. This serial dilution process continues with the final serum dilution being 1:20. Further dilutions can be made up to 1:160.
- An equal volume of thawed virus stock with a known concentration of plaque forming units is added to each of the serially diluted tubes of serum.
- The tubes are all incubated at 37° C for 1 hour.
- 200 µl of the virus-serum mixture is added to a Vero-cell monolayer in a culture plate.

- The plates are incubated at 37° for 1 hour in a 5% CO2 incubator and are rocked at regular intervals to maintain moisture.
- The inoculums is then removed and 2.5ml of agarose-containing overlay medium is added.
- The plates are then allowed to solidify for 15-20 minutes at room temperature.
- The plates are then incubated upside down at 37° C in 5% CO2 incubator for 5 days.
- A second overlay with vital stain neutral red is added to enhance visualization of the plaques.
- The plates are then allowed to solidify for 15-20 minutes at room temperature.
- The plates are then incubated upside down at 37° C in 5% CO2 incubator for 18-48 hours.
- The plaques are then counted with the highest dilution that results in a $\leq$ 50% of input plaque count being recorded as the end-point titre.
- Results are interpreted as follows: $\leq$ 1:20 the results is negative, between 1:20 and 1:40 the result is equivocal, and $>$ 1:40 the result is positive for WNV neutralizing antibodies [111, 112].

Appendix O: Personal Result Card



Brock — Niagara Region Public Health

Estudio de Investigación sobre la exposición al Virus del Nilo Occidental

Name: _____ Sex: _____ Date of birth _____ Country of birth: *Mexico*

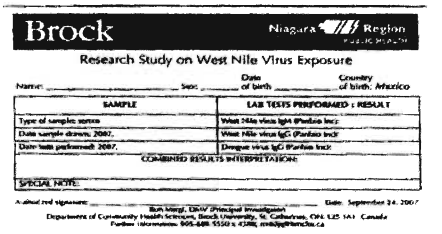
MUESTRA	PRUEBAS DE LABORATORIO REALIZADAS : RESULTADOS
Tipo de Muestra: suero	IgM contra el virus del Nilo Occidental (Panbio Inc):
Fecha toma de muestra: 2007,	IgG contra el virus del Nilo Occidental (Panbio Inc):
Fecha determinación en lab: 2007,	IgG contra el virus del Dengue (Panbio Inc):
INTERPRETACIÓN DE LOS RESULTADOS COMBINADOS:	
OBSERVACIONES:	

Firma Autorizada: _____ Fecha: Septiembre 24, 2007

Ron Mergl, DMV (Investigador Principal)

Departamento de Ciencias de la Salud Comunitaria, Brock University, St. Catharines, ON. L2S 3A1 Canadá.

Para mayor información: 905.688.5550 x 4388; rmb@gbrock.ca



Brock — Niagara Region Public Health

Research Study on West Nile Virus Exposure

Name: _____ Sex: _____ Date of birth _____ Country of birth: *Mexico*

SAMPLE	LAB TESTS PERFORMED : RESULT
Type of sample: serum	West Nile virus IgM (Panbio Inc):
Date sample drawn: 2007,	West Nile virus IgG (Panbio Inc):
Date test performed: 2007,	Dengue virus IgG (Panbio Inc):
COMBINED RESULTS INTERPRETATION:	
SPECIAL NOTE:	

Authorized signature: _____ Date: September 24, 2007

Ron Mergl, DMV (Principal Investigator)

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Further information: 905-688-5550 x 4388; rmb@gbrock.ca

Appendix P: Certificate of Recognition for the Participating Farms

The Office of the Associate Vice-president of Research and International Development

&

The Department of Community Health Sciences

Award this Certificate of Recognition to:

Name of the establishment

For their Valuable Contribution to the Advancement of Research in Infectious Diseases

Presented this 30th of September, 2007

Michael Owen, PhD	Ana L. Sánchez, PhD
Associate Vice-President	Associate Professor
Research and International Development	Dept. Community Health Sciences