DISSERTATION

# THE PATHOGENESIS OF WEST NILE VIRUS IN DOGS, CATS, AND HOUSE SPARROWS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

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ABSTRACT OF DISSERTATION

THE PATHOGENESIS OF WEST NILE VIRUS IN DOGS, CATS, AND HOUSE SPARROWS

In the 1990s, a more aggressive and neurovirulent form of West Nile virus (WNV, family *Flaviviridae*, genus *Flavivirus*, species *West Nile virus*) emerged that has caused morbidity and mortality in human beings, horses, and most severely, in a large number of avian species. The unexpected enhancement of virulence, rapid global spread, and ready establishment of the virus in a diversity of ecosystems underscore the need to modify and enhance the body of basic knowledge about this zoonotic agent. This work describes what is known about WNV and explores the replication of this virus in three animal species: companion animals, dogs and cats, of potential import due to their close association with human beings; and house sparrows, important avian hosts of WNV.

Domestic dogs and cats were infected by mosquito bite and evaluated as hosts for WNV. Each of four dogs developed a viremia of low magnitude and short duration, but did not display signs of disease. Twenty-one of 23 cats became viremic, with peak titers ranging from $10^{1.0}$-$10^{4.2}$ plaque forming units/ml. Several of the cats showed mild, non-neurologic signs of disease. During the course of infection, WNV was not isolated from saliva of any of the dogs nor cats tested. An additional group of four cats were exposed to WNV orally, via ingestion of infected mice. Two cats consumed an infected mouse on three consecutive days and two cats consumed a



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single infected mouse. All of these cats developed viremia with magnitude and duration similar to that seen in cats infected by mosquito bite, but none of the four showed clinical signs. These results suggest that dogs and cats are readily infected by WNV. The efficiency of oral transmission observed with cats suggests that infected prey animals may serve as an important source of infection to carnivores. The magnitude of viremia found in dogs and cats indicates that neither species is likely to function as an epidemiologically-significant amplifying host, although the peak viremia observed in some cats may be high enough to infect mosquitoes at low efficiency.

*Passer domesticus*, the house sparrow, has been identified as being likely to have key importance in the amplification and maintenance of WNV in the United States. In order to gain understanding of the pathogenesis of WNV in this significant host, virus isolation was performed at regular intervals on a battery of tissues obtained from experimentally infected birds. WNV rapidly established disseminated infection with wide tissue tropism. All tissues studied were infected in at least some of the birds. Skin was the tissue from which virus was most consistently isolated, and was also the first in which virus was detected – as early as 12 hours postinfection. Other tissues frequently containing virus included blood, heart, lung, liver, kidney, and testis.

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# I. Review of the Literature

## I.A. Historical Overview

It has been hypothesized that the most common arboviral infection of human beings is caused by West Nile virus (WNV, family *Flaviviridae*, genus *Flavivirus*, species *West Nile virus*), which was first isolated in Uganda in 1937. In an initial surge of research, much was elucidated regarding the ecology and enzootic range of WNV. Study ebbed considerably within a few decades, perhaps because the clinical disease produced by the virus was characterized as an inapparent or mild febrile infection with severe manifestations occurring only rarely. However, in the mid-to-late 1990s, a more aggressive and neurovirulent form of WNV emerged; some of these outbreaks were in areas not previously documented to have encountered the agent. Most dramatically, WNV was detected in North America late in the summer of 1999: a transoceanic expansion of the virus already thought to have the largest geographic range of any arbovirus. The strain of WNV imported into the United States has caused morbidity and mortality in human beings, horses, and most severely, in a large number of avian species. The establishment and rapid spread of WNV in North America has prompted a deluge of research into the ecology, pathogenesis, immunology, and molecular genetics of this once-foreign zoonotic agent.

* * * * *

West Nile virus was first isolated from the blood of a febrile woman in the West Nile district of Uganda in 1937 by a team attempting to obtain isolates of yellow fever virus. Although the infected woman repeatedly denied feeling ill, researchers suspected otherwise, and performed a



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fairly extensive laboratory investigation of the novel agent in her blood that was neurotropic and fatal in mice. The subsequent publication by Smithburn *et al.* named the pathogen "West Nile virus" in accord with the locale from which it was obtained and declared it to be a virus of 21-31μm (*sic*) that was antigenically related to Japanese encephalitis virus. The group discovered that WNV caused fever and encephalitis in rhesus monkeys, but the clinical manifestation in human beings was not yet known (235).

West Nile virus was not again isolated until 13 years had elapsed, in a region near Cairo, Egypt - over 2000 miles distant to the site of its initial discovery. At this time, the Rockefeller Foundation had enacted a large program for the study of viral diseases; among those funded were the team working in Egypt (178, 272). While performing a poliomyelitis antibody serosurvey, they isolated WNV from the blood of three Egyptian children, none of whom had appreciable illness. This group not only repeated and confirmed much of the work performed by Smithburn and colleagues, but also performed an epidemiologic investigation of antibody prevalence. The high incidence of antibodies in the local population, including complement fixing antibodies in very young children, indicated that WNV was highly endemic in the region. The absence of virus in throat swabs or stool samples from experimentally infected primates led them to conjecture that "a biting insect acts as a vector in the transmission of the infectious agent," (172). Indeed, Philip and Smadel had published a report seven years previously that demonstrated mosquito transmission of WNV: *Aedes albopictus* mosquitoes multiplied virus after feeding upon experimentally infected hamsters; naïve hamsters were subsequently infected when these mosquitoes fed upon them (203). Within a few years, a Japanese investigator performed a somewhat similar study suggesting vector competence of mosquitoes from two other genera (123).

Another team working in Egypt conducted an immense investigation that provided an



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excellent springboard for future work. Initiated in 1951, the study spanned several years and resulted in multiple publications including a comprehensive summary printed in 1956 (250). These works tackled subjects as diverse as pattern of human antibody response, seropositivity rates in humans, crows, and other animals in the Nile River Valley, experimental infection of several species, and attempted virus isolation and viral transmission from a large number of vertebrates and insects. The group was the first to describe clinical WNV infection in human beings: "a self-limited, non-fatal febrile disease rarely associated with ...manifestations of encephalitis." The group correctly hypothesized that WNV was enzootic in the Nile River Valley and, as had been determined for other encephalitogenic viruses, was maintained in a bird-mosquito cycle. This theory was strongly supported by the isolation of WNV from both wild-caught mosquitoes (249) and birds (270), and by high WNV seropositivity and laboratory-demonstrated host suitability of several native avian species (271).

Israel became another focus of research following a disease outbreak in 1950, the first epidemic attributed to WNV. The clinical attack rate was quite high, with over 600 cases in a regional population of around 1000 individuals. The first cases appeared in mid-summer; within a month the epidemic was full-blown, and peaked in late August or early September (125). The following year, again in late summer, the small Israeli agricultural settlement of Maayan Zvi suffered a "self-limited and non-fatal" disease. WNV was identified as the etiologic agent by both serology and virus isolation. Morbidity was again very high, particularly in children and adolescents (14). WNV was repeatedly isolated from patients during a series of outbreaks occurring in Israel over the next decade. Certain aspects were consistent, such as the late summer seasonality of cases and unusual abundance of mosquitoes in years with outbreaks, while others were baffling. For example, primarily children were afflicted in some outbreaks while more uniform age distributions were seen in other regions or years. Further puzzling was the



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epidemic manifestation in Israel of the virus that was "hyperendemic" yet "silent" in nearby Egypt (71, 73, 125).

Laboratory confirmation of WNV in Israel was not obtained until the outbreaks in the early 1950s, so researchers could only speculate as to whether WNV was newly introduced, hitting hard a naïve population (72) or had been active in the region previously, as tentatively evidenced by a disease outbreak in late summer of 1941 reported to have a similar clinical presentation (71, 266). The repeated incidence of human WNV infections in Israel in the 1950s through the early 1960s led to the publication of comprehensive reports of the clinical course of, and humoral immune response to, the disease now termed "West Nile fever" (WNF) (71, 72, 152, 211, 243). The conjecture that "WNV may infect the human central nervous system, but its virulence is of a low order" (71) was most likely correct, but the "severe headache" seen more commonly in adults (14) and "light transitory meningeal involvement" (71) presaged more severe neurologic manifestations.

In the summer 1957 WNV outbreak in Israel, a significant addition was recognized in the already "wide clinical spectrum of infection" (73): meningoencephalitis. Within the Shomron region, residents of three different areas suffered neurologic disease. Two of the three disease foci had a very low incidence of neurologic involvement without age predilection, while at the third site, roughly 25% of cases, all elderly, were reported to have severe meningoencephalitis (243). A low incidence of central nervous system (CNS) disease in conjunction with WNV infection, estimated at less than one in every 100 cases (73), continued to be reported in subsequent years (211). However, in their 1981 report of meningoencephalitis in three young Israeli adults, Flatau *et al.* reviewed the literature and postulated that "central nervous system involvement in WNF was underdiagnosed," (62).

Although WNV isolations were made in many countries in central and southern Africa (69,



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165), South Africa was the primary focus of research. McIntosh and Jupp declared that "it is probable infections by Sindbis or West Nile viruses were responsible for" outbreaks in 1954-1956 (165). An ensuing serosurvey revealed that the incidence of neutralizing antibodies in residents did not exceed 3.2% for either suspected agent in any of three ecologic zones (236). This indicates that the recent outbreak had most likely been caused by other agent/s, and underscores the ambiguity of diagnosis of viral fevers on clinical grounds. In 1958, WNV was isolated from a bird and from a man who was briefly ill (126). The prevalence of the virus in South Africa, particularly the inland plateau, further increased by the end of that decade; along with Sindbis virus (SIN, family *Togaviridae*, genus *Alphavirus*, species *Sindbis virus*), WNV was soon considered to be enzootic (159). In 1982, infection with WNV was declared to be one of the most common arboviral infections of human beings in southern Africa (165).

A number of non-fatal illnesses have been attributed annually to either WNV or SIN in South Africa, with epidemics or small outbreaks of the two disease agents occurring infrequently, but often simultaneously (108, 110, 159, 163). Such a co-epidemic, which was dominated by WNV, occurred in Cape Province, South Africa in 1974. That human infections were estimated to have numbered in the tens of thousands, with only one incident of encephalitis and no fatalities, distinguishes this outbreak as the largest of WNV to manifest in the fairly mild form of WNF (27, 89, 163).

As with Israel and South Africa, the first laboratory confirmation of WNV in India was obtained in the 1950s. WNV has received a moderate amount of research attention in India and Pakistan. The virus has been isolated in India with regularity from a number of sources, most frequently from human beings and mosquitoes (272). The considerable geographic overlap and high activity of both WNV and immunologically-related Japanese encephalitis virus (JEV, family *Flaviviridae*, genus *Flavivirus*, species *Japanese encephalitis virus*) make difficult the



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interpretation of serologic data. Small outbreaks of encephalitis are not uncommon, but are generally attributed to JEV; reports of WNV infection in the region are limited (66, 67). However, when formal diagnostic work has been performed at disease foci, it has revealed that a minority of patients are infected with WNV (67, 68, 118). In addition to cross-protection caused by immunologic overlap with JEV, the circulation of more mild strains of WNV likely contributes to the limited incidence of disease caused by WNV in India.

Kunjin virus (KUNV), enzootic in Australasia, has enough serologic disparity from prototype WNV isolates that it was long thought to be a distinct agent. More advanced molecular techniques prompted the reclassification of KUNV as an antigenic subtype of WNV (223). KUNV is considered to have low-level, widespread enzootic status in a bird-mosquito cycle on the Australian continent, but to only sporadically cause clinical disease in humans (20, 85, 155, 205). In fact, Hawkes asserts that KUNV appears to remain enzootic even at times when closely-related Murray Valley encephalitis virus breaks into epidemics (85). A 1974 outbreak of febrile and encephalitic disease in human beings, preceded by "remarkable... abnormal... climactic conditions" was a "possible exception" (85, 155): KUNV was isolated three times more frequently from birds and mosquitoes than was Murray Valley encephalitis virus (MVEV, family *Flaviviridae*, genus *Flavivirus*, species *Murray Valley encephalitis virus)*, and retrospective serologic research indicated that some of the non-fatal human cases in the outbreak were probably attributable to KUNV infection (155).

Like Australia, France has encountered small outbreaks and sporadic cases of encephalitis. WNV activity in France is unique in that it has historically affected horses to a far greater extent than human beings. Early work in Egypt revealed that equidae were susceptible to experimental inoculation (250), and described isolated instances of illness in a police horse (224) and a single debilitated pigeon (270) attributed to WNV. However, the 1962 equine outbreak in France was



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the first report of an epizootic caused by the virus, and is the first diagnosed occurrence of WNV in any species in France. Retrospectively, WNV may have been the etiologic agent of the human illness "summer fever" seen frequently in the French Mediterranean (83) and in epidemic form in 1942 (216). In 1962, 30% of the horses of the Camargue region (100) had serologic evidence of WNV infection. Horses suffered often-fatal neurologic illness (100) and a few human cases of encephalitis occurred that summer and the following. "Most sera" from these patients had antibody that neutralized WNV, but paired samples were not obtained, so definitive diagnoses were not made (83). WNV was eventually isolated in France in 1964 and 1965, from mosquitoes, from two entomologists suffering "summer fever" (83, 216), and from a foal with neurologic disease (100). Although complicated by the co-circulation of viruses serologically cross-reactive with WNV (83, 216), several serostudies of human beings, horses, and other animals, in combination with the lack of clinical reports after 1965, indicate that the regionally-intense WNV activity in France was probably due to a brief incursion rather than the existence or establishment of an enzootic state (83, 100, 143, 178, 216).

WNF is "one of earliest recognized arthropod-borne viral diseases of man," and it has been suggested that the incidence of human infection with WNV is higher than that of any other arbovirus (88). The ability of WNV to establish enzootic transmission cycles in a wide range of climates, combined with brief incursions into many areas, has resulted in an extensive geographic range. Sporadic cases and small outbreaks caused by WNV continued to be reported in a number of regions (2, 62, 68, 69, 108, 115, 118, 180), but overall the vigorous WNV activity seen in the 1950s and 1960s seemed to be declining as the twentieth century progressed.



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## I.B. Recent West Nile Virus Outbreaks: emergence of aggressive strains with increased neurovirulence

At the commencement of the 1990s, West Nile virus had an extensive enzootic range spanning Africa, the Middle East, and much of India and Australasia; reports of the virus also occasionally surfaced in many European countries. Although widespread and the cause of common infection, WNV was not a pathogen of much concern: even the epidemics of WNF with high clinical attack rates were rarely associated with severe disease or mortality. Further, serologic and clinical evidence pointed to a considerable waning of WNV activity in many regions (143, 178, 180). However, in the mid-to-late 1990s, typically-mild WNF began to present as a more aggressive, neurovirulent disease.

Experts are not in agreement as to the "turning point" of WNV epidemiology: the first large, urban outbreak in Romania in 1996, the first report of large-scale avian mortality in Israel in 1998, and the first detection of the virus in the Americas in 1999 were certainly all unique manifestations and significant occurrences. A less frequently mentioned but perhaps quite noteworthy event was a small 1994 outbreak of WNV in Algeria. In a 2002 review article, Murgue *et al.* declare north Africa (Tunisia, Morocco, Algeria, Egypt) to be the "emergent zone" for WNV outbreaks (180); it is certainly possible that the disease focus in Algeria was related to subsequent outbreaks, rather than being an isolated incident. Reminiscent of early findings in Egypt and the Maayan Zvi outbreak in Israel, the predominance of Algerian patients were children. However, in contrast to the subclinical or comparatively mild illnesses common in the past, a high proportion of neurologic involvement, including encephalitis, coma, and death, was seen in those afflicted in Algeria (142, 178, 180, 266).

The year 1996 marks the first large outbreak of WNV in Europe. Romanians suffered over 800 clinical cases, with unusually high incidence of both CNS complications and mortality (34, 89, 95, 221, 258). The outbreak was also unique in that it was concentrated in Bucharest, with an



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estimated 43,000 - 96,000 infections (258), thus distinguishing it both as the first to occur predominantly in an urban area, and as an even larger outbreak than the previous record epidemic in South Africa (180, 206). Interpretation of serologic work is complicated by the circulation of other flaviviruses in the region (180). Opinions differ as to whether WNV long circulated at low levels in Romania (94) or if serologic evidence of WNV in the 1970s (50) was caused by an incursion of the virus that did not result of the establishment of the virus in the region. Thus, some feel that WNV was reintroduced into a largely naïve population prior to the 1996 epidemic (34, 221, 248), helping explain the severity of the outbreak. The overwhelming majority of clinical infections occurred in late summer and early fall of 1996, but modest numbers of cases were also diagnosed in subsequent years (180).

The 1996 human epidemic in Romania was not the only surprising manifestation of WNV that year. Although it had not been recorded since the mid-1960's in France, disease in horses, often with neurologic involvement, surfaced in Morocco (251). Typical for WNV, this epizootic broke in late summer and persisted through early fall, affecting 94 horses with a 44% mortality rate (128, 178, 180). Reminiscent of the 1962 outbreak in France, illness was almost exclusively limited to equidae: only one human case of encephalitis was reported (178, 180). Subsequent years brought other moderately-sized equine outbreaks: in Israel and Italy in 1998, and in the south of France in 2000 (28, 52, 128, 178, 179, 180). Equine disease was first diagnosed in the New World in New York in 1999 and has been a prominent feature of WNV in the Americas.

Despite the emergence of virus strains that have higher rates of severe morbidity and even mortality, the mild form of WNV infection, WNF, still crops up. Outbreaks occurred in 1998 in the Democratic Republic of Congo (189), and in the Czech Republic in the fall of 1997 (92). The Czech outbreak was the first report of human WNV cases in Central Europe. Notably, that same year, "emergent zone" country Tunisia experienced an outbreak with features typical of the



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"new" WNV: very high incidence of meningitis and meningoencephalitis, with morbidity and mortality rates increasing dramatically in the elderly (178, 180).

Illnesses resulting from WNV infection reported in Tunisia were limited to human beings, whereas from 1997 to 1999 in Israel, disease induced by WNV was recognized exclusively in animals. In addition to the aforementioned 1998 equine outbreak, bird mortality, particularly in storks and domestic geese, was attributed to the virus (150, 180). Migratory storks were the suspected source of this strain of WNV (191) that resulted in the first report of large-scale avian mortality associated with WNV. Small, uninvestigated episodes of pigeon illness in Egypt (270) and deaths in experimentally infected birds (271) perhaps provided a foreshadow, but that most of the experimental birds died shortly after inoculation arouses suspicion that WNV was not solely responsible for their demise. Further, potentially avian-pathogenic strains seen in the mid-twentieth century seem to have been limited and sporadic, whereas those isolated more recently in Israel induced bird mortality that affected a large geographic area for a number of years.

Meanwhile, the 1998 Israeli avian virus – or a very close relative – had spread across the globe to North America. A small outbreak of encephalitis in Queens, New York in the late summer of 1999 was initially attributed to enzootic Saint Louis encephalitis virus (SLEV, family *Flaviviridae*, genus *Flavivirus*, species *Saint Louis encephalitis virus*), with Guillain Barré syndrome as a differential diagnosis (238). Many of the diagnostic techniques employed - serology, immunohistochemistry of brain tissue, and clinical presentation (232) - were confounded by the fact that, like WNV, SLEV is also an encephalitogenic member of the JEV serocomplex. However, things did not quite add up: PCR tests for SLEV were negative (232), an abnormally high number of encephalitis cases in horses were appearing, and unusual mortality was noted in wild and zoo-kept birds (21, 153, 257). While geese in Israel were being culled and vaccinated for confirmed WNV infection (191), the agent in New York was cautiously diagnosed



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as a "Kunjin/West Nile–like virus" (21). It was hoped that the foreign invader from considerably more temperate climes would not overwinter in New York, but it emerged the following spring and subsequently continues to spread and establish enzootic transmission cycles throughout North America. The highly avian-pathogenic strain seen in Israel first in 1997 and again the following three years (150) has not again been documented in that region.

The year 1999 brought not only the arrival of a foreign pathogen in New York, but also an uprising of a formerly benign one in Eastern Europe. Several strains of WNV had been isolated from southern Russia and western Siberia beginning in the 1960s, but human seropositivity rates were historically low and clinical disease was merely sporadic (207). It is thus not surprising that the epidemic in the Volgograd region that resulted in the hospitalization of over 800 patients (206) - most of them suffering neurologic disease - was not expediently attributed to WNV (207). As with disease manifestation in Romania and New York, the Russian outbreak had unusually high rates of neurologic disease and fatalities, particularly in the elderly, and was primarily urban (89, 206): 95% of patients were city dwellers. A small study revealed a human seropositivity rate of 58%, indicating quite high levels of viral activity (180). Smaller, concurrent outbreaks occurred in the Astrakhan and Krasnodar regions (207), as well as in Moravia, Czech Republic. The outbreak in Moravia had a very high clinical attack rate with apparent: inapparent infections at 1:1.6, and differed considerably from the other disease foci in 1999 in that no encephalitis cases were reported (95).

Russia suffered only a small outbreak the following year, 2000 (207), but the people of Israel were less fortunate: their former foe hit fairly hard. Although limited avian disease attributed to WNV had occurred annually since 1997 in Israel, only a very few human cases were diagnosed during those years (180). It was not until the year 2000 that the sylvatic cycle of the virus spilled over into urban populations (150, 266). Illness recurred with force in people, and to



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a lesser extent birds, throughout the country that had experienced so many outbreaks in the 1950s. At least two strains of WNV infected the human population that summer and fall (91), resulting in over 400 laboratory confirmed cases (180, 266) in which were seen the suddenly-typical trend of the virus to cause mortality, with a positive correlation seen between case fatality rates and age of patient (91, 266).

Israel's series of WNV outbreaks in the 1950s provide an exception to the epidemiologic "rule" seen in the Old World: that epidemics or epizootics wax and wane swiftly, with only few cases the following year or two. Experts have repeatedly attributed the causation of this pattern to unusual environmental conditions in certain years that foster sudden increased viral activity. This may well be true, but there have been a range of climatic conditions in the New World since WNV was first detected in 1999, yet the virus has spread significantly each vector season. Further, the virus has caused disease in birds, horses, and human beings residing in myriad ecological zones. At the time of writing, WNV has been documented in almost every state in the USA, stretching coast to coast; in every province of Canada, with viral activity extending nearly to her western shore; and most recently, in Mexico (18, 147). Certainly, the history of this aggressive form of West Nile virus is yet an open book.

## I.C. The Birds and the Bugs: West Nile virus ecology

### I.C.i. Mosquito vectors

Impressively, not long after the discovery of West Nile virus, its bird-mosquito transmission ecology was elucidated and substantiated by investigative work. In 1943, only six years after the initial isolation of the virus, Philip and Smadel published their investigation of the hypothesis that like other neurotropic agents, WNV might be transmitted by mosquitoes (203). In their study, *Aedes albopictus* mosquitoes were allowed to take bloodmeals from WNV-viremic



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hamsters; the mosquitoes subsequently became infected. Following a range of incubation periods after the "donor meal", it was demonstrated that these mosquitoes had replicated virus and were able to transmit the virus to naïve hamsters. In 1950, a similar report was published in which *Armigeres obturbans* and three species from the mosquito genus *Culex* infected with WNV by feeding on a suspension of virus in sugar water were able to transmit mice via bloodfeed (123). Five years later, Work *et al.* mimicked the natural cycle of WNV by feeding Culex mosquitoes on viremic birds and later refeeding those mosquitoes on naïve birds. They found that the bite of only one mosquito is needed to infect avian hosts, and that hooded crows and house sparrows in particular were efficient at transmitting virus to engorging mosquitoes (271).

**I.C.i.a. Infection of mosquitoes**

Work *et al.* were also the first group to discuss thresholds of viremia necessary to infect feeding mosquitoes. Much of the experimental vector work that followed addressed this issue in a number of mosquito species, as an integral consideration in their potential as vectors of WNV. Many groups also examined the kinetics of and optimal environmental conditions for WNV replication in mosquito hosts. Results from these numerous studies vary substantially and thus must be regarded with some caution. Explanations for the variation include differences in strain and passage history of virus tested, use of mosquitoes of different strains within species and with captivity histories ranging from wild-caught to long-established laboratory colonies, lack of standardized and accurate virologic techniques, and a tendency to store samples at -20°C, a temperature at which viruses are now known to gradually lose viability. The variability due to strain differences is illustrated in a study published by Hayes in 1980. This work examined the susceptibility of several stocks of *Culex tritaeniorhynchus* mosquitoes to Egyptian and Indian isolates of WNV: up to four-fold differences were seen among various mosquito-virus combinations (86).



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Vector competence studies of mosquitoes for WNV commonly examined different methods of inoculating mosquitoes and the subsequent ability of mosquitoes thus infected to replicate and/or transmit the virus. Experiments on members of the *Culex* and *Aedes* genera predominate the literature, but a handful of other mosquitoes have also been examined. Study results of threshold virus concentrations needed to infect mosquitoes via various routes are summarized in Figure I. Oral infection via virus suspensions in sugar water or ex vivo blood generally appear to be less efficient than "natural" feeds on infected chicks or other animals. Although intrathoracic inoculation is not relevant to the natural cycle of WNV, mosquitoes are quite susceptible to this parenteral route and can be consistently infected in high proportions (86), making this technique quite desirable for laboratory work.

Many studies have examined the course of infection of WNV in mosquitoes. WNV is detectable immediately following a bloodmeal containing sufficient quantities of virus, but titers soon drop (86, 123). As virus begins to replicate within the mosquito, titers begin to climb, again detectable around two days postinfection (86, 97, 123) and peaking in most species between days six and nine (86, 97, 123). *Culex vishnui* is reported to reach maximum titers very quickly, by day three (97). Further, intrathoracic inoculation may produce an earlier peak than *per os* infection (86, 96). Titers as high as 8 $\log_{10}$ pfu of virus per insect have often been measured in *Aedes albopictus* (23, 96, 263) but most species harbor 5 to 7 logs of WNV (86, 96, 97). Study results often conflict, but overall it appears that apex levels are often maintained in plateau for a variable number of days before beginning a very gradual decline. High-titer virus can be detected in mosquitoes for at least several weeks after experimental feeding (49, 96, 123, 263) and evokes no known pathogenic effects (154); infection is in fact assumed to persist for the life of the mosquito (49, 250).



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| | | Infecting Method | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | viremic bird or rodent | | | oral via viral suspension | | | intrathoracic | | | |
| infection rate | | 10-30% | 31-70% | >70% | 10-30% | 31-70% | >70% | 10-30% | 31-70% | >70% | references |
| *Culex* species | *antennatus* | | 2.5 | | | | | | | | 96 |
| | *fusocephalus* | | | | | 3.0 | | | | | 4 |
| | *neavei* | | 4.4 | | | | | | | | 109 |
| | *pipiens complex* | 5.2, 1.0, 5.2 | | 7.2, 3.0, 7.2 | 4.0 | | | | | | 102, 107, 259, 260 |
| | *pseudovishnui* | | | | | 3.8 | | | | | 4 |
| | *quinquefasciatus* | 1.4 | | 2.6, 3.8 | >4.3 | | | | | | 4, 107 |
| | *theileri* | <2.4 | | ≤2.4 | 1.5 | 2.5 | 4.5 | | | | 104, 107 |
| | *tritaeniorrhyncus* | | ≥5.0 | 2.0 | | 1.9, 2.4 | 2.9, 3.4 | | 1.2 | | 4, 86, 97 |
| | *univittatus* | 1.7 | 2.5, 4.0 | | | 4.3 | | | | | 4, 96, 97, 103 |
| | *vishnui* | | 4.0 | | | | | | | | 97 |
| *Aedes* species | *atropalpus* | | | 7.2 | | | | | | | 260 |
| | *aegypti* | 7.2 | | | | | | | | | 260 |
| | *albopictus* | | | 7.2 | 4.3 | | | | 1.9 | | 4, 260 |
| | *caspius* | | | | | 4.3 | | | | | 4 |
| | *dentatus* | >5.5 | | | | | | | | | 106 |
| | *unidentatus* | 5.5 | | | | | | | | | 106 |
| | *vexans* | >5.2 | 7.2, 7.2 | | | | | | | | 259, 260 |

Figure I. Comparison of experimental studies of the susceptibility of mosquitoes from the Culex and Aedes genera to West Nile virus (log10/ml) via various methods of inoculation.

### I.C.i.b. Transmission studies

Studies have confirmed that once infection is established, mosquitoes transmit the virus both during feeding on vertebrate hosts and when infected insects are consumed by some but not all species of birds (131, 141). Comparisons of the ability of various species of mosquitoes, particularly those from the *Aedes* and *Culex* genera, to transmit WNV are usually performed by subjecting chicks or mice to bloodfeed by infected mosquitoes, then determining whether or not these vertebrates became infected as a result. Most investigators routinely incubate mosquitoes for one to three weeks before attempting transmission, but transfer of WNV to naïve vertebrates has been documented to occur as early as five days after an infective blood meal (49, 96) and two (96) or three (263) days post intrathoracic inoculation. Once infected, mosquitoes may be capable of transmitting WNV for the remainder of their lifespan; in the laboratory it has been shown to occur as late as 62 days after the initial infective bloodmeal (263).

The mosquito species that have been tested have supported replication of WNV, and it has been demonstrated that many are also capable of transmitting the virus. Certainly, there is considerable variation in the efficiency at which various species transmit WNV in the laboratory. The ability of WNV to develop a disseminated infection is likely of great importance in vector competence (259). Assessing the role a given species may play in the ecology of the virus involves many other factors; a 2002 publication by Turell *et al.* attempts to do so by compiling field and experimental data from a number of *Aedes*, *Culex*, and *Ochlerotatus* species. In addition to vector competence, relative abundance, feeding preference, field virus isolations, and known involvement with other arboviruses were among the factors considered in a semi-quantitative analysis of the potential importance of these mosquitoes in WNV ecology in North America (261). Members of the *Culex* genus were incriminated as the chief vectors of maintenance for the enzootic cycle, whereas some species of *Aedes* and *Ochlerotatus* mosquitoes



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were implicated as sources of infection for mammals. The origins of the mosquito colonies were not specified, but that this study utilized unpassaged or once-passaged WNV of a single strain from North America and modern virologic techniques corrects some of the pitfalls of older works and makes it a superior reference for New World ecology.

### I.C.i.c. Mosquito epidemiology

Mosquitoes selected for vector competence studies tend to be those thought to be most likely to have a high level of involvement in WNV ecology. Influential data include incidence of virus isolation from, and regional abundance of the individual species. Worldwide, mosquitoes of the genus *Culex* are thought to be the chief vectors involved in the maintenance and amplification of WNV (93, 94, 128, 261). Of the *Culex* mosquitoes, *Culex univittatus* has not only been shown to have excellent vector competence in laboratory studies, but is also thought to play a key ecologic role for WNV throughout the Middle East, southern Africa, and on the inland plateau in South Africa (73, 101, 103, 108, 109, 110, 125, 160, 163, 165, 213) with other *Culex* species (*tritaeniorhynchus*, *antennatus*, *theileri*, *pipiens* complex, and *neavei*) likely of lesser significance (73, 101, 109, 110, 125, 163, 263). Indeed, it has been demonstrated that in both Egypt and South Africa, intensity of viral activity correlates with population density of *Culex univittatus* mosquitoes (110, 250). Further, this orthinophilic mosquito is also thought to be the chief vector of JEV (61, 81), a close relative of WNV.

*Culex* mosquitoes are principal vectors in the maintenance and amplification of WNV across the globe: *Culex vishnui* in India (263), abundant *Cx. tritaeniorhynchus* in Pakistan (4, 215) and species of the *Cx. pipiens* complex in both India and Pakistan (88), *Cx. modestus* in France (83, 179); in Australia, *Cx. annulirostris* looks to be the principal insect species in which KUNV



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circulates (85, 155). The "highly orthinophilic" species of the *Cx. pipiens* complex have been implicated as the chief vectors in the urban outbreaks in Romania (207, 221, 258) and New York (13, 27, 94, 134, 135, 181, 259). In the the eastern United States *Cx. restuans* may also be of considerable importance (153). In the less-urbanized western states, *Cx. tarsalis*, *nigripalpus*, and *pipiens* complex, the major vectors of related arbovirus SLEV (148) may also be proven to have primary involvement in WNV ecology.

Mosquitoes that feed on birds as well as mammals, and can replicate and transmit WNV, are referred to as "bridging vectors." Such vectors expose incidental hosts to this virus that thrives in a bird-mosquito cycle. Mosquitoes of the genus *Aedes*, which tend to have promiscuous feeding habits, often yield WNV in isolation studies (32, 134, 153, 164, 165, 180) and are those most often implicated as having substantial involvement as bridging vectors (135, 261, 263). Additionally, *Aedes* species have been considered to be of great involvement in two outbreaks of WNF (92, 189).

Despite low feeding rates on mammals, the great abundance of certain mosquitoes implicates them as common bridging vectors. In South Africa, populous *Culex univittatus* and *theileri* were demonstrated to have low feeding rates on human beings, yet are suspected to be the principal source of human WNV infection (4, 163). Although not always abundant, feeding preferences alone strongly implicate members of the the *Cx. pipiens* complex (88, 102) and *Cx. quinquefasciatus* (215) as major vectors of transmission to human beings. In New York, *Cx. salinarius* is suspected to transmit many WNV infections to people (135). There is evidence that transmission of WNV from birds to domestic quadrupeds in Egypt may often be through *Cx. univittatus*, (250), to cattle in India and Pakistan via *Cx. tritaeniorhynchus* (88), horses in the eastern United States from mosquitoes in the genera *Aedes* and *Ochlerotatus* (135), and horses in France via *Cx. modestus* (179). Due to their nonspecific feeding habits on people, other



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mammals, birds, and reptiles, *Cx. modestus* and other mosquitoes with promiscuous feeding habits likely transmit WNV to a wide variety of species (88).

Certainly, entomologic epidemiology is difficult to determine. Results obtained from independent investigations on the natural feeding preferences of certain mosquito species have differed, possibly due to strain variation (88). WNV infection rates in mosquito populations are generally extremely low, and sometimes no virus isolations are obtained, even during outbreak years. Further, various methods of mosquito collection bias study populations, causing over- and underrepresentation of many species (261). Some mosquitoes known to be very common as larvae, including those in the *Aedes* genus, are very difficult to capture as adults (261). Jupp explained that an avian-baited trap used in a study was not suitable for capture of *Cx. fatigans* (101). Also, light and $CO_2$ traps bias toward night-active mosquitoes such as *Cx. annulirostris* (155), traps utilizing dry ice favor mosquitoes in the genera *Aedes* and *Ochlerotatus*, and gravid traps primarily attract *Culex* mosquitoes (261). The amount of organic material used to bait a trap is an additional variable influencing the species of mosquitoes that will be attracted. Lastly, WNV is found in extremely diverse habitats, each of which likely supports an altered or unique cycle of WNV. In fact, Marshall *et al.* reported high variability of isolation of KUNV and MVEV from mosquitoes within even small regions: traps located only feet from one another, one on the shoreline and one over water, yielded dramatically different rates of infected mosquitoes (155).

### I.C.ii. Avian hosts

As with the mosquito vectors of the virus, WNV's avian amplifying hosts appear to be very numerous and to vary with geography. WNV and KUNV isolations and detection of antibody, both of which vary annually and increase in later summer months, have been documented in wild



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and domesticated birds ranging from the American robin to the yellow warbler. Virus isolations have been made from the brain, spleen, serum, and/or liver of several species. In the Old World, the first WNV isolation from a bird was in 1953 from a feral pigeon and soon thereafter from the hooded crow (270). Subsequent isolations have been obtained from the crombec warbler (126), barred warbler (264), turtle dove (58, 187), knight wheatear, black-headed gull, lapwing (58), thrush (256), squacco heron (64), vasa parrot, chicken, yellow oriole (223), domestic goose (150, 191), stork, white-eyed gull, collared dove, and rosella (150). In the United States WNV detection has been predominantly in American crows – up to 89% of virus positives have been from this species each year (153) – but also from over 150 other species (131) including the red-tailed hawk (65) and swan goose (129).

Serosurveys of birds (5, 87, 113, 129, 150, 160, 163, 187, 188, 221, 270), and experimental infections with WNV or KUNV (20, 107, 131, 141, 161, 170, 229, 246, 247, 271) demonstrate that a very wide range of avian species - possibly all – are permissive hosts of WNV infection, but are not equally susceptible. Viremia levels of some birds are not likely to efficiently infect feeding mosquitoes, thus such species are probably of little significance in the ecology of WNV. Similar findings are reported for both serosurveys and laboratory infections conducted to study SLEV in birds (145, 146, 167, 168, 242). Widely distributed in the Americas, this virus shares many antigenic and ecologic characteristics with WNV.

Determination of which birds are the most important amplifying hosts of WNV in a given region is challenging. This is generally estimated using relative seropositivity rates of local birds, but as with mosquitoes, sampling is difficult: some species, such as starlings, are quite common yet underrepresented in studies due to the difficulty of their capture. Further, anti-WNV antibodies may be artificially elevated in areas where immunologically related viruses co-circulate. Serosurveys in which naturally exposed wild birds were re-captured, and reports in



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which birds were held in captivity for long periods following experimental infection have revealed that detectable antibodies to both WNV and SLEV may be short-lived in birds (88, 229), particularly Passerines (150, 160, 161, 166). Finally, some birds infected experimentally with WNV or close relative JEV failed to generate a detectable humoral response after viremia (81, 161).

Many avian species replicate WNV to titers necessary to infect feeding mosquitoes, but by combining seroprevalence rates in nature, relative abundance, and, when known, magnitude and duration of viremia, birds in the Passeriform order look to be key WNV amplifying hosts throughout much of the geographic range of the virus. Of the Passeriform birds, the most important hosts of WNV are thought to belong to the Corvid and Passerid families - particularly crows (5, 55, 56, 131, 250, 271), and sparrows (55, 87, 113, 129, 131, 160, 163, 188, 250, 271). The ubiquity (129, 145, 163, 271) of these species in both rural and urban settings and their ability to support high-level replication of virus (131, 161, 271 make them excellent candidates for amplification of virus in many parts of the world. Further, the European house sparrow, *Passer domesticus*, is considered to be the chief vertebrate host of closely related SLEV (19, 145, 146, 166, 167, 168). As with the relative importance of insect vectors, data indicate that bird involvement is subject to geographic variation. Certainly, many other avian species have been identified as possibly having considerable importance in WNV ecology; for example, in wetland ecosystems of Australia (20) and India (218), herons may be the primary amplifying hosts.

### I.C.iii. Other hosts and vectors

Serologic studies, experimental infections, and field virus isolations indicate that WNV is an unusually promiscuous virus, capable of replicating in a large number of animal and insect species. The hypothesis that WNV circulates in a bird-mosquito cycle was generated very early



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in the study of the virus, and overwhelming supporting evidence has been gathered. However, it is not unlikely that the cycle of the virus has greater complexity, with other animals and insects contributing to a minor or perhaps even significant extent to WNV ecology. Naturally-occurring seropositive status has been detected in, or virus isolations obtained from, a diversity of wild and domesticated animals including bats (5, 117, 153, 250, 262), camels (119, 192, 250, 256), dogs (17, 34, 94, 129, 165, J. Kile personal communication), hedgehogs (120), various species of rats (5, 87, 94, 120, 188) and cattle (5, 24, 50, 87, 94, 111, 192). Viral pathogenesis and vertebrate host relations and range are listed among the high priority research topics in the USA (33).

Studies of the clinically significant hosts, human beings (or of other primates utilized as models), equidae, and most recently birds, indicate that the two former species are incidental or "dead end" hosts of WNV. They do not shed virus in saliva, nasal secretions, urine, stool, nor from skin lesions, and rarely produce viremia of adequate magnitude to infect feeding mosquitoes (2, 14, 23, 72, 90, 159, 172, 185, 217, 224, 235, 239, 240). A handful of prospective experimental studies have been performed on varying mammalian species and again evidence that these animals do not likely contribute significantly to the ecology of the virus, and suffer little to no clinical disease as a result of infection. WNV in mammals will be discussed in more depth in sections K and L of this chapter; additionally, the 2002 review by McLean *et al.*, "West Nile virus in livestock and wildlife," is an excellent source of information.

In that same paper, the authors state of WNV that "this virus has one of the broadest host and vector ranges" (170): even reptiles and amphibians are thought to be among the vertebrate hosts. This may seem surprising, however, reptiles have considerable phylogenetic similitude with birds, the amplifying hosts of the virus. Antibodies to WNV have been found in a turtle (188) and in grass snakes (94). WNV has been isolated from frogs (94), and experimentally inoculated lake frogs were susceptible to viral infection and transmitted WNV to mosquitoes



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(132, 133). WNV was isolated from some affected animals in recent, small outbreaks of alligator mortality in the southern United States (173). Titers of virus suggested that alligators might serve as amplifying hosts of WNV, and studies to determine the host capability of this species are currently underway (M. Bunning, personal communication).

Lastly, mosquitoes are not the only possible vectors of WNV. Arthropod borne flaviviruses have been loosely grouped by host and antigenic properties as being tick-borne, mosquito-borne, or bat or small rodent-associated; WNV belongs to the largest, penultimate grouping (45). Individual flaviviruses tend to replicate well in either tick or mosquito cell cultures, but not in both (154). Collectively, ecological and laboratory-derived evidence that mosquitoes are the primary vector of WNV is persuasive, but other vectors may certainly play a minor role.

In a 1956 publication by Hurlbut, WNV was reported to replicate in a diversity of intrathoracically inoculated arthropods, of which mosquitoes and hard and soft tick species were subsequently able to transmit virus to vertebrates. When inoculated via bloodfeed, several types of arthropods acquired viral infection, but only mosquitoes were able to transmit (96). Later work showed that ticks infected as nymphs were also able to transmit WNV when engorging (255). Ticks collected in field studies have yielded isolates of West Nile virus (17, 94), and a WNV-like virus isolated from ticks was lost in the Suez Crisis of 1956 before definitive typing could be performed (272). Additionally, WNV's close relative SLEV has been isolated from ticks (168) and may have a transmission cycle involving offshore marine birds, presumably transmitted by nonmosquito vectors (242). That mice exposed to ectoparasites from bird nests seroconverted to WNV and other arthropod-borne pathogens (234) suggests other birds, particularly nestlings, may also be infected with viruses in this manner.



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### I.C.iv. Overwintering

The possibility that WNV may be harbored in ticks is among the theories as to how the virus persists during cold-weather months, when mosquito vectors are not active. Much of the traditional enzootic range of the virus has at least some mosquito activity year-round (96, 101, 250), yet other affected areas, including most of North America, have distinct, seasonal mosquito activity. With few exceptions in certain individual study subjects, viremia lasts only a matter of days in vertebrates, thus it is not likely that vertebrates are involved in WNV overwintering. It is possible that reservoir hosts exist that have not yet been identified, but evidence suggests that WNV overwinters in hibernating mosquitoes.

Both SLEV (148) and WNV (38, 181) have been detected in mosquitoes collected during winter months. Studies subjecting experimentally infected mosquitoes to simulated seasonal conditions (39, 48, 250), as well those examining naturally infected specimens collected in the field (38, 181) are in agreement that WNV is at very low levels, around the threshold of detectability by various methods, when the insects are incubated at cold temperatures, but that titers quickly rise in response to warmer conditions. Cornel *et al.* demonstrated that mosquitoes experimentally infected with WNV then induced to hibernate were capable of both transmitting virus to birds and of reproduction after "spring re-emergence" (39). Another report documents that infection with KUNV did not alter the fecundity of females (252).

Limited experimental work has demonstrated that WNV can be transmitted vertically to the offspring of parenterally and orally infected mosquitoes of several species (10, 174, 252, 260) but not to the progeny of ticks (250). Reports vary as to the frequency of this phenomenon in mosquitoes infected with WNV, but it is perhaps around 1% (10) – a considerably lower rate than seen with other groups of arboviruses. For example, Tesh reported the transovarial



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transmission rate of WNV and other flaviviruses to be roughly two orders of magnitude lower than that of LaCrosse, another encephalitogenic mosquito-borne virus (family *Bunyaviridae*) (252).

### I.C.v. Environmental considerations

Enzootic on five continents, West Nile virus has definitely proven to be "adaptable to a broad range of environmental conditions," (88). Outbreaks of disease caused by the virus have appeared in diverse settings, from isolated military camps to highly urban areas. The level of preexisting immunity in the amplifying and clinically important hosts of a region almost certainly impact the magnitude of an outbreak, yet by no means do epidemics occur only in areas with no or low prior exposure to WNV. The triggers for these often temporally and geographically isolated upwellings of viral activity are poorly understood. Geographic and climatic features of regions with intense WNV activity are truly variable, ranging from marshes or coastal plains and river deltas (14, 29, 52, 71, 100, 112, 150, 179, 218, 266) to irrigated regions or riverside communities (206, 236, 250, 270) to semidesert or arid environments (52, 88, 109, 110, 165). Many reports describe unusually high levels of spring or early summer precipitation followed by hot temperatures preceding the outbreak (39, 92, 108, 155, 163, 165, 189) yet paradoxically, regions that normally have such weather patterns may not have high viral activity (163). More perplexing is that other epidemics have been triggered by drought conditions, including those in the French Mediterranean (83) and more recently in Bucharest, New York, and Volgograd (89, 221).

Several outbreak reports mention heightened populations of mosquitoes (92, 125, 163, 189, 258). Laboratory and field data suggest that temperature and access to water sources are key factors of mosquito ecology and viral replication. A several-year study of SLEV in Florida,



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including a year in which an outbreak occurred, found that lengthy spring droughts followed by a rise in the water table fostered intense viral activity (230). The authors theorized that droughts concentrate birds at dwindling water sources, increasing their exposure to mosquitoes - thus facilitating the amplification cycle of SLEV (42), and possibly also those of WNV and eastern equine encephalitis virus (230). That direct contact of some species of birds has been demonstrated as a mode of transmission for both WNV and eastern equine encephalitis virus in laboratory settings (131, 169) strengthens the theory that high concentrations of birds in an area would intensify viral activity. Platonov postulated that arid summers enhance human exposure to WNV because dry conditions encourage mosquitoes to oviposit in urban water sources (207). In their 2002 publication, Turell *et al.* conclude that container-breeding mosquito species are generally better vectors of WNV than those species associated with flooding (261). Even in drought years, the flower pots, bird baths, discarded tires, and the like in those areas inhabited by people afford ample hatching sites.

The year of the initial outbreak of WNV in New York city, 1999, had "one of the warmest summers on record" (261). Epidemics occur in late summer and early fall, the hottest time of year, and several have been associated with higher than average temperatures (39, 108, 163). As discussed previously, WNV replication is inhibited by cold and enhanced at warmer environmental temperatures. A number of studies have examined the effect of incubation conditions on WNV replication in mosquitoes, and some also researched temperature effects on transmission. WNV multiplies most rapidly in mosquitoes at 28 to 32°C but can replicate in, and be transmitted to vertebrates from, mosquitoes subjected to temperatures ranging from 9 to 33°C, with daily means of 12 to 23°C (96). Dohm and Turell report that viral replication and dissemination are dramatically inhibited at 10°C (48); at the other end of the spectrum, mosquito longevity decreases as temperature increases (39). The ability of mosquitoes to transmit WNV is



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directly proportional to the viral titer of the mosquito, and peak titers are reached more quickly at warmer temperatures (39). Significant but by no means extreme decreases in efficiency of transmission are seen in mosquitoes housed at temperatures in the mid-teens when compared with those at 26°C (48, 105) or 30°C (39). Regarding vertical transfer of WNV, the efficiency of transovarial transmission is constant between 20 to 32°C (252) and infected mosquito eggs are stable for three months at 28°C, but the viability of infected larvae may be more sensitive to temperature (10).

It is inevitable that the ecology of West Nile virus varies among the myriad ecosystems within its expansive range. Presence and relative abundance of potential vectors and amplifying hosts vary with geography, climate, and time. Globally, locations and precipitating conditions of epidemics and epizootics are variable and even contradictory. However, when regions are assessed on a small scale, it is almost certain that these diverse factors converge to create local environments that simultaneously foster thriving populations of susceptible birds and aviophilic, transmission-competent mosquitoes.

**I.D. Coping with West Nile Virus**

Shortly after the initial detection of West Nile virus in the United States, federal agencies convened and compiled strategies for surveillance, diagnosis, prevention, control, infrastructure, data sharing, and research priorities (33, 78). Although several arboviruses posing significant health threats have long been enzootic in the United States, surveillance programs were independent and sparse, and other facets including diagnosis and reporting lacked standardization. Although "management" could not eliminate WNV nor impede its spread, the development of ArboNET, a cooperative surveillance program, has been a very positive outcome.



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Further, the improvements in capabilities, intercommunication, and standardization of diagnostic laboratories, a vital first step in the WNV protocol, should provide significant benefit for the detection and management of not only WNV, but also other infectious agents.

### I.D.i. Surveillance strategies

Historically, a menagerie including mice (256), dogs (70, 127, 193, 265), pigs (66), a number of species of mosquitoes, and wild or captive birds (44, 103, 128, 129, 153, 164, 176, 188) have been used or suggested for use as sentinels for the detection of activity of WNV. In the United States, the active surveillance programs utilize mosquitoes and birds. Perhaps due to low infection rates (92, 155, 181, 221, 263), testing mosquitoes lacks efficiency (153), but has provided warning prior to some outbreaks (13, 135). Traditional arbovirus surveillance programs using captive birds (most often chickens) as sentinels are being eliminated due to funding restrictions and doubts as to their sensitivity for WNV (153). The literature contains many suggestions for, and some attempts and analyses of, programs utilizing serotesting of wild birds, particularly house sparrows, to monitor SLEV activity. Authors of a recent study tracking seropositivity in free-ranging house sparrows in New York City concluded that it was not an efficient surveillance method for WNV (44).

Testing bird carcasses for infectious WNV or for viral RNA is quickly becoming the strategy of choice (55, 169). That bird die-offs have been associated with both eastern equine encephalitis outbreaks (169) as well as the recent appearance of African Usutu virus in Austria (267) signifies that the utility of testing bird carcasses is not limited to WNV detection. The density and the occurrence of carcasses of crows and other birds containing virus are not only sensitive and early indicators of the presence of WNV activity in a given region, but also correlate well with its intensity. Data compiled from 20 states revealed that in 2000, the first



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human WNV cases were preceded by detection of virus in bird carcasses by as many as three months, and by elevations in densities of dead birds by weeks (55, 56, 153).

The final arm of WNV monitoring is enhanced passive surveillance by medical professionals in their human and equine patients. Outbreaks in several regions have been limited to equidae, but when both human and horse cases occur, the latter have been reported to precede human illnesses by only a matter of days (135). Monitoring of equine populations thus probably lacks the temporal sensitivity desirable for active surveillance. However, vigilant analysis of the clinical impact of WNV in horses and people is crucial for directing and evaluating control measures.

**I.D.ii. Diagnosis**

A number of diagnostic techniques may be used in clinical and field specimens to detect infectious WNV, viral genetic material or protein products, or humoral immune response to infection. These methods are reviewed in several recent articles (27, 128, 202, 238) and will be discussed here only in brief. From tissue, infectious virus can be isolated in cell culture and resultant plaques definitively identified using a number of techniques including immunofluorescence (13). Viral RNA can be detected by in situ hybridization (245) or reverse transcriptase polymerase chain reaction (RT-PCR) (13, 21, 209), or quantified using a realtime version of the RT-PCR (13, 139, 231). Finally, viral antigen can be identified using immunohistochemistry or fluorescent antibody (13, 209, 232).

When WNV infection of mammals has progressed to the stages of morbidity or mortality, virus and viral antigen have generally begun to clear from, or are no longer present in most tissues. Low viral load in many tissues and neutralization or steric hindrance by antibody further jeopardize the success of these techniques. Therefore, virus isolation and antigen detection using



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antemortem samples from mammals are rarely successful, but are more reliable postmortem since tissues from the CNS can be obtained (210, 232, 237, 238, 258). RT-PCR tests, particularly those employing realtime technology, are generally (but not unanimously) thought to have excellent specificity and sensitivity. Advantageously, these tests can detect evidence of infection for a limited time after infectious virus is no longer present. They are becoming invaluable diagnostic tools for samples of a number of species - particularly avian and mosquito - and tissue origins (13, 130, 139, 170, 231).

Although paired serology has traditionally been considered to be the most definitive diagnostic test after virus isolation, the specificity of serologic methods for the quantification of antibody against WNV are often confounded by WNV's immunologic cross reactivity with other circulating flaviviruses. This difficulty is often exacerbated in samples from people and other animals whom have experienced multiple flaviviral infections (2, 5, 31, 99, 124, 253, 272). Plaque reduction neutralization and isotype class-specific IgM tests are considered to have the greatest specificity of any tests of humoral components (31, 210, 225, 248, 269); methods more prone to cross-reactivity such as hemagglutination inhibition (16, 31, 64, 124, 253) are rarely used today. Measurement of neutralizing antibody via plaque reduction looks to be a reliable method to confirm infection in horses (210), but can only be performed in laboratories with biosafety level three facilities. IgG enzyme-linked immunosorbent assays (ELISAs) have been developed (54, 60, 225, 248), but due to superior specificity, IgM ELISA is the most commonly utilized diagnostic technique for sera and cerebrospinal fluid (CSF) samples from both horses and human beings (194, 202, 253, 266).

Although tests of IgM antibody are less prone to cross-reactivity with other flavirviruses than are other methods of serodiagnosis (99, 248, 253), they are by no means infallible. IgM is considered to be short-lived, yet can persist over a year following flaviviral infections (202, C.



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Calisher personal communication). The potential longevity of the IgM response (25), combined with the lack of pathognomonic clinical signs for WNV infection, suggest that reliance on a single positive IgM test result may lead to erroneous diagnoses of WNV infection in patients with resolved WNV infections suffering disease of alternative etiologies. In recognition of this phenomenon, single-sample IgM findings in human and horse sera are considered "probable" cases (194, 202) while those in human CSF are considered confirmatory (202). However, the origin of antibody in CSF it is not known, and may not be uniform among cases. Perhaps due to the added expense and inconvenience associated with obtaining and testing paired samples, in field situations single IgM tests are often considered adequate for diagnosis in horses with compatible clinical signs.

**I.D.iii. Therapy and prevention**

Treatments for WNV infection are limited. Evidence for the efficacy of many treatments, including ribavirin, interferon alpha, and brefaldin A (6, 238), is limited to *in vitro* or animal model studies or is even anecdotal, such as is the case with hyperimmune serum (233). Clinical disease is managed with supportive care and symptomatic treatment (210, 237, 238). WNV infection of the CNS is thought to incite a considerable amount of immune pathology, thus steroidal and nonsteroidal antiinflammatory agents are often employed. However, a study of corticosteroid use in patients suffering JEV infection did not demonstrate benefit (238).

Certainly, prophylaxis is advisable for infections such as that with WNV that are difficult to treat and are potentially fatal. The primary preventive modalities consist of minimizing vector exposure, reduction of vector populations, and vaccination. Simple recommendations for avoidance of mosquito bites via alterations in lifestyle and use of mosquito repellents are outlined in the publication "Guidelines for surveillance, prevention, and control of West Nile virus



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infection – United States" (33). As previously mentioned, vector eradication efforts are directed by surveillance; mosquito larvicides are utilized to prevent outbreaks, while adulticide sprays are employed in efforts to curb those outbreak foci that do establish (27, 78).

An inactivated and adjuvanted vaccine for horses was licensed early in 2003. As dictated by the achievement of only marginal success with past attempts to protect from flaviviral diseases with similar formulations (170, 197), the equine vaccine has had many documented failures in horses (210) as well as in a hamster model (254). Although side effects may be encountered from vaccination with live attenuated flaviviruses, a reasonable level of protection from later challenge with homologous virus has often been demonstrated (149, 197, 212, 254). Vaccination with inactivated heterologous flaviviruses tends to be less efficacious (82, 170, 253). It is of note that passive immunity was shown to be highly protective in hamster and mouse models, but a number of shortcomings including brief duration of immunity (47, 254) make application of this modality improbable in real-world settings. At the time of writing, promising products of more sophisticated formulations are under development for a number of species including human beings, horses, and birds (43, R. Bowen personal communication).

### I.E. Phylogeny

The species *West Nile virus* is in the family *Flaviviridae*, genus *Flavivirus*, and by serologic cross-reactivity is categorized as a member of the JEV antigenic complex of flaviviruses (26, 31, 45). Within the WNV species there exist a number of strains or subtypes, as identified by degree of serologic, amino acid, or nucleotide sequence identity. WNV subtypes will be discussed here cursorily; further detail is available in an excellent review published in 2002 by Scherret *et al.*, "Phylogeny and molecular epidemiology of West Nile and Kunjin viruses" (223).



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Serologic techniques have long been used to differentiate WNV isolates. Early works demonstrated that not only are there antigenic variations of WNV, but that considerably divergent strains can cocirculate within a region (15, 91, 138, 180). Strains are often indistinguishable by plaque reduction tests (221), while hemagglutination inhibition and monoclonal antibody studies are often more revealing (138, 157, 180, 223). Of potential importance when reviewing laboratory studies of WNV is that the Sarafend strain of WNV, used with fair frequency in works predating the mid-1990s, has been found to be somewhat aberrant. This strain has few sequence differences from other WNV isolates, but monoclonal antibody work has revealed that Sarafend lacks "several WNV-specific epitopes on the envelope protein" (223). One set of investigators also declared the protoype WNV isolate, Egypt 101, to be in a unique topotype based on their work with monoclonal antibodies and differential restriction enzyme digests of cDNA (157).

Analyses reveal few correlations between the genetic makeup of isolates and their species of origin, geographic location, or year of isolation (15, 53, 209). Some biologists use this somewhat random temporal and geographic distribution of genetically similar isolates as evidence for the popular hypothesis that migratory birds are primarily responsible for movement of WNV (15, 150, 180, 221). Phylogenetic trees constructed for WNV via antigenic, amino acid, or nucleotide sequence are in fairly good agreement but do differ among and within each method employed.

Most studies at the nucleic acid level utilize regions within the gene for the envelope protein (91, 138, 179, 206, 221), which is thought to be of considerable importance in tropism, and host immune response to WNV infection. One of the largest and most recent phylogenetic studies was published in 1999 by Lanciotti *et al.* As with many previous works (15, 223), this analysis of 33 WNV and seven KUNV specimens resulted in a tree with two branches designated lineages I and II; the most closely-related isolates form subgroups termed "clades." Lineage II viruses



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were all isolated from Africa and seem to have been restricted to enzootic circulation; lineage I encompasses viruses from diverse origins including Africa and contains all of the isolates within the study that had been obtained during outbreaks of disease in human beings or horses. All WNV specimens associated with recent outbreaks were placed in lineage Ia, and all but one clustered into a tight clade in Lanciotti's phylogenetic tree (138). Isolates not included in Lanciotti's original study, several from Volgograd and one from a later outbreak in France, were analyzed in a 2002 contributory report by Lanciotti as well as by other groups, and were likewise determined to be lineage Ia viruses (140, 179, 206). A 1998 WNV isolate from Israel that also exhibited marked avian virulence appears to be the closest relative of the North American strain of WNV (138, 179, 206).

Also included in the 1999 publication by Lanciotti *et. al.* was a sequence comparison of a much larger region of the genome of 14 WNV isolates, including nine from the eastern United States. Very little divergence was identified among the US samples and the presumed parental Israeli isolate: a maximum of two substitutions over the 1278-nucleotide span studied, or <0.2% (138). The subsequent Lanciotti study examined full-length genomic nucleotide and amino sequences of the Israeli stork strain with 1999 and 2000 field isolates from the United States revealed no more than 0.3% divergence from the Israeli virus. The US strains were markedly homologous with variation of 0.2% or less in nucleotide sequence and at most 0.1% at the amino acid level (140). More recently, Beasley *et al.* compared nucleotide sequences from the prM and E coding regions. The group found at most 0.6% variation between an isolate obtained from a flamingo in New York in 1999 and those harvested in Texas in 2002 (12). The surprisingly high degree of homology maintained by WNV as it has spread across the continent is also reported for some KUNV isolates with considerable geographic and temporal separation (223).



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## I.F. Viral Properties and Replication Strategy

WNV stocks were initially maintained *in vivo*, and despite the skepticism of peers, Bhatt succeeded in propagating WNV and JEV in chick embryo and monkey kidney cell cultures (272). That vital first step was eventually followed by a revolution in cellular and molecular biology, and a considerable amount is now known about formerly mysterious "filterable agents" including WNV.

WNV has a spherical structure with a diameter of approximately 500 Å (46, 177). The outermost region is a lipid bilayer rich in viral envelope (E) and membrane (M) proteins. Heterodimers of E and M proteins form a herringbone pattern that creates icosahedral symmetry in mature virions (177, 274). Deep to this envelope is the nucleocapsid or core, measuring 20-30nm and composed of the viral capsid protein (C) surrounding and complexed with the genetic material of the virion. In a 1959 publication Ada *et al.* extracted the material from MVEV, and found it to be infectious (3). The group cautiously referred to it as "'RNA'" and indeed, flaviviruses have positive-sense RNA genomes.

The WNV genome is nearly 10,700 nucleotides in length. The 5' terminus is capped and has a noncoding or untranslated region (UTR) of about 100 nucleotides (27). Some regions of the 5' UTR are conserved within the *Flavivirus* genus (46) - particularly so within antigenic groups - and may be involved in secondary structure (154, 171). The structural proteins, C, pre-membrane (prM), and E are the first coding elements in the single reading frame encoding a polyprotein of over 3400 amino acids. Roughly 75% of the coding sequence is dedicated to the seven following nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (36, 226). The 3' terminus of the genome has another UTR and lacks a poly-A tail (154). The 3'



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UTR, approximately 600 nucleotides in length (27), contains regions conserved amongst the mosquito-borne flaviviruses, one of which may interact with the 5' UTR to circularize the genome (154, 171).

Little is known about the functions of the nonstructural proteins of flaviviruses, particularly NS2A, NS2B, NS4A, and NS4B (171). NS1 may be involved in early viral replication, and because it is expressed on the surface of and secreted from the host cell, may also be a target for host immune responses (154, 171). Both NS2B and NS3 are thought to serve as a protease in the cleavage of the viral polyprotein (171). NS3 also appears to bind nucleotide triphosphates (NTPs), and may have additional functions as a helicase and NTPase (154, 171). Ng *et al.* found that NS3 associates with cellular microtubules, and hypothesized that it may act as an anchoring substrate in the transport of progeny RNA (184). NS5, the largest and most highly conserved of the nonstructural proteins, is an RNA-dependent RNA polymerase that may have methyltransferase activity for the capping of nascent RNA (154, 171).

WNV has wide species and tissue tropisms (114). The mechanism of WNV binding to host cells has not been elucidated; it has been proposed that the viral E protein and cellular heparin sulfate residues may be involved with binding (46). WNV is generally thought to enter host cells via uptake in coated pit vesicles (46, 75). However, work with closely-related JEV and dengue-2 virus demonstrated "no evidence of viral entry by receptor-mediated endocytosis," but instead "direct penetration" in insect cell lines and *ex vivo* human monocytes (84, 154). Soon after entering the host cell, the tertiary structure of E is thought to reorganize to expose a "fusion segment". When virus is contained in endocytic or prelysosomal vesicles, the change in the conformation of E is attributed to a lowering of pH (75, 77, 274). The exact mechanism is not known, but the fusion of the virion with the vesicle triggers the uncoating and release of the viral RNA into the cytoplasm of the host cell 15-30 minutes after initial entry (77). The capped,



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positive sense RNA of WNV is then translated by host machinery. Replication is thought to be semiconservative, and unlike that of alphaviruses, which are also positive-stranded RNA viruses, flaviviral synthesis of the negative strand does not seem to be regulated (154).

The polyprotein arising from translation of WNV's RNA must be cleaved into individual components. Even during translation, host signalase, a cleavage enzyme on the lumenal face of the endoplasmic reticulum, liberates the structural proteins C, prM, and E. These are then thought to anchor to the membrane of the endoplasmic reticulum (154). Host furin or furin-like protease and viral NS3-NS2B complex, highly conserved among flaviviruses, cleave the remainder of the polyprotein, possibly within the lumen of the endoplasmic reticulum (36, 154, 274).

Within seven hours of WNV infection, the rough endoplasmic reticulum, particularly that near the nucleus, begins to proliferate and form vesicles. Thread-like structures visible in the vesicles by electron microscopy are assumed to be progeny RNA strands. Virion maturation begins around 10 hours postinfection and is complete at approximately 30 hours (184). As particles assemble they acquire an envelope from the membrane of the rough endoplasmic reticulum, which is embedded with viral prM and E heterodimers (46, 154). The newly-formed West Nile virions then proceed through the host pathways for exocytosis. Near or at the time of exocytosis from post-Golgi vesicles, much of the prM is cleaved by host furin (46), which results in the loss of the amino terminus of prM. The carboxy terminal, now termed the M protein, remains embedded in the membrane, but dissociates from the heterodimer formed with E (268). Low efficiency of this cleavage of prM may reduce viral infectivity (46, 154).

The effects of WNV infection on the host cell vary among cell types and viral strains. For example, the pathway of release of progeny virions of WNV Sarafend is thought to be divergent from that seen in other strains (46). Umrigar *et al.* demonstrated considerable variation in plaque



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phenotype induced in Vero cell culture by six strains of WNV (262). Many cells infected with WNV become vacuolated and hypertrophic (46) and suffer mitochondrial damage, lysosomal proliferation, distention of inclusion bodies, and fragmentation of reticular membranes (154). WNV replicates to high levels in some types of arthropod cells without inducing known damage. The changes in response to infection consist of benign hypertrophy with proliferation of the cytoplasmic membrane, and syncytia often form in mosquito cells (154).

**I.G. Immunology**

The immune response to WNV has not been very well characterized in any species, but surprisingly little work has been done in birds. Although birds have long been known to be the amplifying hosts for WNV and many other viruses, the literature seems to be devoid of any information beyond characterization of antibody formation following WNV infection. In fact, many aspects of avian immunology have received little research attention, making difficult current efforts to develop vaccines and other control strategies for those birds thought to either be important amplifying hosts of, or endangered by, WNV in North America. The bulk of work performed in WNV immunology concerns mammalian responses to infection, and will be summarized here.

E is the most abundant of the WNV proteins, and is also thought to be both the primary antigenic determinant (27, 46, 154, 171). Although prM and NS1 are also targeted, humoral responses are chiefly directed against E (154, 171). Neutralizing and hemagglutination inhibiting antibodies interfere with the ability of E to bind to host cells (46, 154). Antibodies against E may also inhibit infection slightly later in the process, by impeding the conformational change of E that results in the release of viral RNA from host prelysosomal vesicles. Virions thus impaired are soon degraded in the increasingly-low pH environment, and infection is aborted (76). In



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mouse models, B cell activity was found to be of considerably more importance in limiting the severity of WNV infection than that of T cells (47).

WNV infection induces cell-mediated immunity, but unfortunately many researchers have utilized the likely-aberrant Sarafend strain of WNV to study this process. Studies indicate that class I and II MHC are upregulated following WNV infection (144). It must be noted that the activity of host interferon in response to the viral infection likely contributes, perhaps significantly, to the increased production of class I MHC (144). Work in *ex vivo* systems has demonstrated sensitive, targeted lysis of WNV-infected cells by cytotoxic T cells; type 2 T helper cells also appear to be stimulated (121, 122, 144).

The role of innate immunity in WNV infection is certainly considered to be consequential, but is very poorly understood. A dramatic illustration of the influence of innate immunity on WNV infection has been documented in mice. It has long been known that resistance to WNV infection in some strains of mice was conferred by an autosomal dominant gene (57), only recently determined to encode 2',5'-oligoadenylate synthetase, an interferon-induced enzyme that impedes viral replication (220). Mice from 15 unrelated strains were studied, and those mice that were susceptible to WNV infections had one of two nonsense mutations resulting in truncated and presumably inactive 2',5'-oligoadenylate synthetase (156). Any number of innate immune components could have considerable but yet -undiscovered influence on the outcome of WNV infection in various species and individuals.

### I.H. Immune Enhancement

The acquired host immune response may not always counteract infection with WNV. Antibody-dependent enhancement (ADE) is a well-documented phenomenon in infections of dengue virus (DENV, family *Flaviviridae*, genus *Flavivirus*, species *Dengue virus*), a close



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relative of WNV that is also a Group B arboviruses. ADE occurs in DENV infections of those individuals previously infected with, and having antibodies against, another of the four serotypes of the virus. Importantly, unlike DENV, WNV lacks distinct serotypes, and that ADE can occur in WNV infection of any species is merely speculative (69, 85). However, several studies have provided convincing evidence that the presence of subneutralizing levels of antibody does increase WNV replication in macrophages *in vitro* (30, 59, 74, 75, 198, 199, 200, 204). Some of these experiments utilized monoclonal antibodies specific for WNV while others used polyclonal antisera to WNV or other flaviviruses. ADE seems to occur only in macrophage-like cells, and only when WNV is incubated with antibody specific for those flaviviruses also in the JEV serocomplex (59, 199, 204). ADE in macrophages has primarily been attributed to the binding of IgG-opsonized but still-infective virus particles to Fc receptors, facilitating viral access to permissive host cells (199). Low-levels of IgM have also been demonstrated to promote infection of macrophages with WNV; this is thought to involve binding to at least one complement receptor, CR3 (30). ADE of IgG-opsonized WNV can elevate viral infection as much as 50 to 100 -fold over control systems lacking antibody (198), whereas subneutralizing IgM exerts a much milder effect, with only five to 10 -fold increases (30).

**I.I. Neuroinvasion and Neurovirulence**

The E protein is considered to be the primary virulence factor of WNV, acutely influencing neuroinvasion and neurovirulence (27, 46, 171). This seems logical given the role of E in the binding of WNV to host cells and its importance as a target for host immune defenses. Attenuation during cell culture passage can dramatically affect *in vivo* but generally not *in vitro* viral replication (222), and is "consistently associated with amino acid changes in protein E," (171). However, research attempts have yielded equivocal results *in vivo*. For example, a



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glycosylation site at amino acid residues 154-156 is fairly well-conserved among flaviviruses and has been associated with neuroinvasiveness in mice (222, 223). Many KUNV and WNV isolates lack the complete motif required for glycosylation (222, 223). Unfortunately, both natural reversion and site-directed mutagenesis that restore glycosylation have failed to induce definitive change in neuroinvasion (35, 222, 223). In contrast, removal of other E protein glycosylation sites produced attenuation (35). A considerably more broad approach used indices of binding to mouse brain receptor preparations (MRP) in an attempt to identify WNV isolates of low and high affinity for the CNS. However, when these viruses were tested further, MRP affinity, genotype, $LD_{50}$, and neuroinvasiveness in infected mice were not found to have any correlations (11). Interestingly, a non-lethal, non-neuroinvasive strain of WNV entered the brains of and induced mortality in many of the mice that were exposed to inhalation anesthetics shortly after viral inoculation (116).

There is "clear evidence" that the ability of WNV to enter the nervous system, neuroinvasiveness, is a separate phenotype from neurovirulence, its ability to initiate pathologic changes in the nervous system (171). It has been suggested that neurovirulence may be affected by mutations in the 3' UTR and the nonstructural proteins, but as with neuroinvasiveness, the E protein is thought to be the primary determinant (171). The loss of neurovirulence is thought to indicate a higher degree of attenuation than the ablation of only neuroinvasion (171). Studies have demonstrated loss of neuroinvasiveness without significant decline in neurovirulence, yet "existing data show that neurovirulence and neuroinvasiveness determinants map to identical regions of protein E," (171). Most likely, these characteristics of WNV are interrelated to some degree, and are influenced by myriad factors.

Many groups have attempted to determine the pathophysiology of CNS invasion of WNV and other encephalitogenic viruses, yet this too remains enigmatic. Many possibilities have been



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proposed, including invasion via passive diffusion or transcytosis during high-level viremia, infection of vascular endothelium, diapedesis of WNV-infected white blood cells into CNS parenchyma, retrograde migration through peripheral nerves, and disruption of the blood-brain barrier by preexsisting conditions, host cytokine response, or virus-induced alterations of cellular junctions. Some of these theories have been deemed to be unlikely and have essentially been dismissed, for example CNS entry via ascending infection initiated in peripheral nerves (46). Evidence exists in support of other theories, for example that WNV may enter neural tissues via infected leukocytes, "Trojan horse"-style. WNV has been isolated from peripheral blood leukocytes in naturally-occuring infections (118). Upregulation of ICAM, VCAM, and E selectin in response to WNV infection has been documented in vitro (238), suggesting that diapedesis of leukocytes into the CNS may indeed be a plausible explanation.

As with so many other facets of WNV research, strain variation among viral isolates is most certainly a confounding factor in the study of the pathogenesis of WNV invasion of the CNS. Intradermal inoculation of the 1998 Israeli stork isolate induces lethality in mice at a dosage over two orders of magnitude lower than that needed using a 1990 Senegalese strain (46). The authors of a recent publication concluded that the simultaneous appearance of WNV in both the brains and spinal cords of mice indicated hematogenous entry into the CNS (47). Another group suggests that peripherally inoculated WNV follows an olfactory pathway into the murine CNS (186). Recent studies of the pathogenesis of peripherally inoculated SIN and intranasally administered vesicular stomatitis viruses in mice likewise concluded that the olfactory route may be the mechanism of entry into the CNS for those agents (37, 40).

Hamsters are considered to be superior to mice as a rodent model for the pathogenesis of WNV in human beings. Hamster studies of SLEV have revealed the route of entry into the CNS to be via the olfactory neuroepithelium and bulbs (175). However, in a similar experiment using



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WNV, virus was first detected in the basal ganglia and brain stem, suggesting virus may have been delivered hematogenously. In fact, viral antigen was not detected in the olfactory bulbs of any hamster at any point in the study (273). The often-diffuse distribution in CNS tissues of "higher" vertebrates including horses, cattle, human beings, and non-human primates supports hematogenous seeding of viral infection (79, 195, 238). However, all of those species develop fairly low-level viremias that are often brief in duration. The bloodstream may well be important for the transport of WNV to the CNS of these animals during the viremic phase of disease, but additional events, such as infection of vascular endothelial cells, are probably necessary for neuroinvasion. Certainly, WNV replicates in endothelial cells *in vitro* (114) and flaviviral antigen has been "localized in cerebral microvascular endothelial cells coincident with invasion of the CNS," (51). The concept of virions entering the CNS via diffusion is thought to be likely only with high concentrations of virus in the bloodstream, such as occurs in young mice and many species of birds (175).

**I.J. Persistent Infections**

It is generally thought that WNV is cleared fairly expediently from the tissues following infection in vertebrates. Despite enthusiastic and very broadly-defined claims of "persistent" WNV infections in a recent review by Kuno (137), very little convincing evidence exists that WNV establishes persistent, or even long-lived, infection in vertebrate hosts. Since WNV infection in mosquitoes is thought to be life-long, it is not far-fetched to speculate that persistent infections might be possible in vertebrate hosts. In the early 1950s, Bernkopf *et al.* propagated WNV in embryonating eggs and noted that lesions produced in yolk sacs altered with passage number (14). Further, noncytopathic persistent infections were "readily" established within 16



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weeks in a prospective experiment involving passage of WNV in a murine cell line (22). Affected cultures remained positive for WNV antigen by fluorescent antibody testing in the absence of cytopathic effects or production of infectious virus particles. The viral RNA harvested seemed to be truncated, and efforts to restore an infectious phenotype were unsuccessful (22). However, these findings must be interpreted in light of the limitations of the *ex vivo* and *in vitro* systems employed.

Results from the preponderance of *in vivo* pathogenesis studies indicate that normally-functioning immune systems efficiently eliminate WNV from the tissues of mammalian hosts that survive infection. The importance of the immune response in the resolution of infection is demonstrated by the protracted viremia and increased incidence of severe disease observed during experimental WNV infections in human patients with lymphomatous cancers when compared with infected patients suffering solid tumors (240). Another group reports the isolation of WNV from the brain tissue of monkeys, most or all of which had been treated with an immunosuppressant, as late as 167 days postinfection. Though capable of producing plaques in some but not all cell cultures, the isolates were non-pathogenic in mice (208).

Several persuasive reports do indicate slow clearance or long-lived infections of WNV in vertebrates. WNV was still present in the skin of some aviremic birds two weeks after inoculation (131). Further, severe neurologic signs have appeared in horses at 22 (195) days postinfection. The immune-privileged status of the CNS might make resolution of infections in these tissues considerably slower. Recent work by Xiao *et al.* demonstrated that very low titers of WNV could be isolated from the brains of some convalescent hamsters as late as 52 days postinoculation (273). Although these isolates produced plaques in cell culture, no efforts to determine their virulence *in vivo* were documented. A Russian publication reports that WNV could be isolated from a number of tissues of experimentally-infected pigeons 100 or more days



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after inoculation (229), but only the abstract is available in English, making it very difficult to evaluate the work.

## I.K. Clinical Manifestations of West Nile Virus Infection

### I.K.i. Birds

Of the clinically important hosts of WNV, birds suffer the greatest morbidity and mortality. WNV has long been known to be pathogenic in very young birds, but the newly emerged strains in Israel and the United States induce disease in adult birds of many species. WNV was associated with fatalities in over 150 species of birds in its first four years of known circulation in the United States (131). Interestingly, despite the "nonrestricted tropism" of WNV in birds (245), many birds show little evidence of disease prior to succumbing to infection (131, 247). Death usually follows within hours of the onset of any clinical signs that may be observed, which include lethargy, inappetance, dehydration, emaciation, ruffled feathers, pale beak, dyspnea and open-beak breathing, weakness, recumbency, ataxia, circling, and unusual posture or head carriage (131, 150, 245, 247, 270, A. Davis, personal communication).

Mortality rates vary considerably with species and age of the bird as well as the strain of WNV. American crows have had particularly high mortality rates in the field (13, 153, 169, Eisdon EID 63 01) and those infected experimentally with a New York 1999 WNV isolate have uniformly suffered fatal infections or required humane euthanasia (131). Other birds, particularly Galliformes and Psittaciformes, are comparatively refractory to WNV infection as adults and incur very limited morbidity and mortality (131, 141).

The profound effect WNV strain variation can have on pathogenesis was demonstrated by Work *et al.* in one of only two reports (20, 271) published prior to the Israeli outbreak that describe high levels of mortality in birds. The group measured viremia of over 9 $\log_{10}$ units in



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hooded crows experimentally infected with third-passage WNV, but titers decreased by six orders of magnitude in birds inoculated with that same isolate after six additional passages in mice (271). In that same report, only one of nine young pigeons developed a detectable viremic response following experimental infection with one isolate of WNV, while a strain of the virus that had been isolated from a naturally infected sick pigeon produced viremia of low magnitude but several days' duration (271).

Even those strains of WNV thought to be nonpathogenic in adult birds are fatal to young hatchlings (250). The first isolation of WNV from a bird was obtained in a juvenile (270), and epizootics in Israel have also involved young birds (150, 191). Experimental studies involving the inoculation of several species of birds with KUNV and WNV have demonstrated that severity of infection decreases with advancing age (20, 250, 263). Neither the mechanism of the acquisition of disease resistance with age, nor those changes that have made WNV highly pathogenic in birds, are presently known.

**I.K.ii. Horses**

In the United States, equids are second to birds in frequency of clinical disease from WNV infection. Most exposed horses, perhaps 90-99%, experience subclinical infections. Those that do develop clinical disease may either exhibit a mild course resembling human WNF, with nonspecific signs such as lethargy, or may develop neurologic disease. Clinical presentation is contingent on the extent of lesions in the spinal cord, meninges, and various regions of the brain. None, some, or all of these may incur variable amounts of damage. Nutritional status and overall health are though to be of importance in susceptibility and outcome, but there is no sex, age, or breed bias associated with disease caused by the virus in horses (28, 29, 100, 179, 194, 195, 210, 251). Reports of natural infection in donkeys and mules are rare (179, 251), and little



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experimental work has been done on these species. Two of six experimentally infected donkeys showed only a febrile response while the remainder of the donkeys (224), and two mules in an independent experiment (250) all had subclinical infections. It is not clear whether the low incidence is proportional to their comparatively smaller populations, or whether these equids are less susceptible to WNV than are horses.

Initial presentation of horses suffering CNS infection is not always indicative of neurologic disease; symptoms include colic, hematuria, low-grad fever, and lameness (79, 179, 210, 224, 237, 251). The first neurologic signs often appear at the peak or decline of the febrile response, and commonly manifest as incoordination or hindquarter ataxia, or may be more focal, *i.e.* fasciculations or ptosis. Disease may then resolve or progress, usually rapidly, to include severe symptoms such as abnormal behavior, sensory hyper- or hyposensitivity, forequarter involvement, recumbency, and seizures (28, 79, 100, 179, 194, 195, 210, 237, 251). There have been reports of recurrence of neurologic symptoms in horses that appeared to have recovered from infection (195, 210). Approximately 60-70% of horses treated for WNV infection survive (28, 100, 195, 237, 251). Most recover somewhat slowly but uneventfully, although sequellae have been reported and can render the animal unsuitable for its original usage (210).

**I.K.iii. Human and nonhuman primates**

Despite receiving the majority of media attention, human beings suffer disease from WNV infection relatively infrequently. Estimates vary, but around 20% of those people infected experience the mild, nonspecific WNF form of disease, while fewer than 1% have more severe manifestations suggestive of CNS infection (202). Experimental studies in nonhuman primates, predominantly Rhesus monkeys, have most often inoculated WNV by the intracranial route. Although this results in a much lower incidence of subclinical or mild infections, disease in these



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animals is very consistent with that seen in human beings (90, 151, 172, 182, 197, 208, 235). Human illness from WNV is often reported to involve sudden onset of symptoms that include fever, fatigue, myalgia, rash on trunk and limbs, flushed face, lymph node enlargement, gastrointestinal upset, and frontal or retro-orbital headache that may be exacerbated by movement of the globes (14, 71, 80, 83, 92, 115, 126, 152, 159, 163, 185, 189, 205, 206, 211, 227, 240, 243, 258). As seen in horses, the anatomic location and extent of CNS lesions are variable in primates, thus so are the resultant symptoms. Diagnostic criteria have recently been defined for meningitis, encephalitis, and acute flaccid paralysis resulting from WNV infection (228). Disorientation, severe headache, extreme weakness, tremors, myoclonus, paralysis, respiratory failure, and coma are among the symptoms that accompany severe CNS infections (62, 63, 69, 189, 207, 211, 219, 227, 228, 232, 240, 258). Recovery is often protracted, even in patients with mild symptoms. Human beings (63, 152, 211, 219, 227) and experimentally infected monkeys (172, 208) surviving severe WNV infections sometimes suffer debilitating sequellae. Historically, infections with WNV were most commonly diagnosed in children (14, 67, 93, 178, 180). In contrast, even mild infections with the newly emerged, more neurovirulent strains of WNV seen in children very infrequently, and morbidity and mortality are instead heavily skewed towards elderly patients (89, 178, 180, 206, 266).

### I.K.iv. Rodents and rabbits

WNV has been isolated from wild mice (256), but they, like most other outbred rodents, are generally thought to be refractory to infection (156). However, certain strains of laboratory mice, particularly when young, are very susceptible to the virus (57) and are the most frequently utilized animal model in WNV research. Symptoms vary, but disease usually progresses rapidly once illness becomes apparent. Initial signs include hyperexcitability and ruffled coat; within



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hours mice become hypoactive and weak, assume a huddled posture, and may have tremors, convulsions, or limb paralysis before becoming moribund and succumbing to infection (14, 47, 57, 134, 214, 235). The course of disease from WNV infection in hamsters is very similar to that described for WNV in mice (185, 273). That hamsters can incur fatal encephalitis from WNV infection, but do so at a much lower rate than mice (273) is among the justifications used by those who regard them as being a superior model for WNV encephalitis in human beings (273). Rodents that exhibit clinical illness as a result of WNV infection generally die, but hamsters that recover from severe disease caused by the virus have been reported to have neurologic sequellae (273). Additionally, naturally-occurring anti-WNV antibody has been detected in wild rats and other rodents, and rabbits (5). Intracranial inoculation of virus has had variable results in cotton rats, from seroconversion in the absence of disease to fatal infection (14, 172), but laboratory strains of rats are no longer susceptible to WNV infection after two or three weeks of age (8, 57). Guinea pigs and laboratory rabbits were found to be "highly refractory" to disease following "massive doses" of WNV administered intracranially and via other routes (14, 71, 172, 235).

### I.K.v. Other animals

Disease caused by WNV infection in other species may well be underdiagnosed, but is most certainly rare. Numerous reports have arisen suggesting WNV-induced disease in a variety of animals, but are often purely anecdotal, or are supported only by demonstration of antibody in a single sample. This diagnostic technique is of little value since serologic studies have long indicated that WNV infection is common in a diversity of species in which naturally-occurring clinical disease from the virus has not been recognized, including cattle, sheep, goats, swine, and dogs (5, 18, 41, 50, 87, 94, 111, 192, 250).

Samples from cattle, goats, a dog, and other animals suffering neurologic disease during the



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2000 WNV epizootic in France were submitted for serology and in no case did testing indicate that WNV infection was involved (179). It must be noted that it appears these sera were evaluated using an equine capture ELISA test, which is species-specific. A handful of reports contain sufficient evidence that infectious WNV was present at the time of death or euthanasia in alligators (173), a cat (128), and several dogs (M. Bunning and D. O'Leary, personal communication) exhibiting symptoms of neurologic dysfunction. WNV-infected mosquitoes were used in an early experimental study to inoculate a water buffalo and small numbers of sheep, but did not induce disease (250). Intracerebral inoculation of two hedgehogs failed to induce encephalitis (235). However, clinical abnormalities have been documented in some studies following needle infection with WNV. Barnard and Voges measured fever in one of several infected ewes but not in any of the lambs (9), and two of three dogs were suspected by Blackburn *et al.* to have incurred mild myopathies following exposure to WNV (17). Lastly, nonspecific signs of disease were seen in several calves infected with KUNV, and one intracranially inoculated calf in that study suffered tremors and ultimately became moribund and was euthanized (244).

Some of the newly emerged strains of WNV have dramatically enhanced virulence for birds, and a similar phenomenon could occur in other species. Moreover, effects of WNV infection of species unique to the New World certainly cannot be predicted. Thus, vigilance and open-mindedness are warranted regarding the possibility of WNV-induced disease in those species not previously known to exhibit clinical evidence of infection with the virus.



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## I.L. Pathogenesis and Pathology of West Nile Virus Infection

### I.L.i. Human and nonhuman primates

Human infection with WNV predominantly occurs via mosquito inoculation, but laboratory-acquired infections resulting from aerosol (185) and percutaneous (7) exposure have also been reported. The estimated four day incubation period of the infection prior to onset of symptoms (14, 71, 83) has made it difficult to characterize early WNV pathogenesis. The combination of field data (72, 83, 152, 163) with experimental needle inoculations of terminal cancer patients (239, 240) and nonhuman primates (90, 172, 208, 217, 235) facilitates the creation of a reasonably clear picture. Viremia is generally present within 24 hours of exposure and often resolves by day four. It may fluctuate such that it is not detectable at some timepoints but later "reappears." Those titers quantified in human beings are around $10^2$ pfu/ml serum, below the level thought to be sufficient to infect feeding mosquitoes. In contrast, titers of up to $10^{4.5}$ pfu/ml serum have been observed in monkeys, and transmission to *Aedes* mosquitoes from viremic lemurs was demonstrated in one study (217). WNV is sometimes isolated from cerebrospinal fluid, but multiple attempts have failed to identify virus in throat swabs, urine, or stool from any primate species (14, 172, 239, 240). Indeed, no evidence exists that the virus has ever been spread by direct contact to health care providers or family members of affected individuals.

The distribution and viral load of WNV in most primate tissues have not been determined. Certainly, blood and CNS samples have been demonstrated to contain viral antigen, RNA, or infectious particles. Other tissues are generally believed to have only low levels of virus, but a small number of patients have been reported to suffer hepatitis (69), myocarditis (1), or pancreatitis (201) that appeared to be a result of WNV infection. The aforementioned study involving inoculation of terminal cancer patients with WNV in hopes of viral oncolysis



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demonstrated widespread virus infection of tissues in those patients dying early in the study. Viral quantification was not performed on any tissues, but the spleen, lymph nodes, lungs, and liver were most frequently identified as positive for WNV (240). Notably, WNV was not isolated from any patients succumbing four weeks or later after inoculation (183), suggesting that infection was cleared from even these severely debilitated individuals.

Even fatal infections with WNV may not produce grossly evident lesions in any tissue (183, 219). Those associated with WNV infection are usually limited to the CNS, although cardiac tissue has also been reported to appear abnormal (183, 206). Brain and spinal cord tissue have been described as edematous and congested; perivascular hemorrhage may also be evident (206, 235).

Despite the fact that WNV may be found in tissues lacking microscopic findings (183), study by light microscopy is far more revealing than gross examination. A number of techniques may be employed, but most commonly, hematoxylin and eosin staining is used to examine the architecture of tissues, and immunohistochemistry or in situ hybridization are used to identify the location of viral antigen or RNA. It is of note that the presence of host humoral immune elements may interfere with techniques used to identify WNV or viral antigen, decreasing sensitivity and possibly generating false negatives. Virus also truly may have cleared from host tissues, even those in the CNS, at the time of death.

WNV pathology in the CNS has been examined fairly extensively, and certain aspects are well-established. The lesions produced by WNV in the CNS are often distributed diffusely (172, 208, 243), but the most severe and consistent pathology from the virus is in the cerebellar cortex, thalamus, grey matter of the spinal cord, and brain stem – particularly the medulla oblongata (46, 151, 182, 183, 208, 219). Clinically, it appears that the anterior horns of the spinal cord are very frequently affected (63, 227, 228) and are often reported to have lesions (182, 208, 228). The



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meninges, cerebrum, and white matter of the spinal cord are among other regions that frequently have evidence of damage. Neither WNV nor lesions associated with infection have been observed in peripheral neurons, further discounting the theory that the virus invades the CNS by retrograde migration.

In contrast to many other encephalitides, infection with WNV produces minor to moderate lesions in the CNS. Perivascular cuffing, primarily with lymphocytes, is a common feature, but vasculitis is absent and leukocyte infiltration into the parenchyma is scattered and multifocal, such that inflammation is most frequently characterized as mild or moderate (151, 206, 219, 232). Antigen of WNV has been visualized in lymphocytes and scattered neurons and glial cells (232). Affected neurons usually undergo degeneration (151, 183, 206, 232) while glial cells become hypertrophied and vacuolated (151), often in foci with lymphocytes and histiocytes that are termed nodules (219, 183). Gliosis has been referred to as a "prominent feature" of WNV encephalitis (151). Cerebellar Purkinje cells are consistently shown to be infected and are often severely affected (151, 208). The adjacent cerebellar molecular layer is often involved, albeit more mildly (151). Microscopic lesions were observed in experimental animals sacrificed several weeks after recovery from WNV, particularly in the spinal cord and cerebellar cortex. Some areas of leukocyte infiltration were still present, abnormal neurons were evident, and it appeared that glial cells had proliferated in areas that had incurred neuronal necrosis (151).

**I.L.ii. Horses**

The pathogenesis and pathology of WNV in horses are remarkably similar to those observed in human beings. Previous work has been performed using needle inoculation of Old World strains of WNV, and a recent study by Bunning *et al.* exposed horses to a New York WNV



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isolate via mosquito feed (23). These works are corroborated by data from the field and collectively have led to a more complete characterization of the early events in WNV infection than has been possible in human beings. Most, but not all, exposed equids develop viremia after exposure to WNV (23, 224, 250). Bunning *et al.* first detected virus in the bloodstream as early as 12 hours, but more commonly on day two or three postinfection. The viremia in horses tends to fluctuate, similar to that reported in experimentally infected human cancer patients. Equine viremia peaks at roughly day four or five, and was not observed in any horse beyond six and a half days after inoculation (23). Previous equine studies expressed viremia qualitatively or semiquantitatively, whereas viremia was quantified in the Bunning study. The highest virus yield from blood was only 460 pfu/ml serum, and WNV was not isolated from oral and fecal samples from horses at any point in the study (23), supporting the long-held theory that horses are generally incidental hosts of WNV, unable to transmit infection. Finally, as is suspected to occur in most infected people, illness was not observed by Bunning *et al.* in any horse until day eight, after the resolution of viremia (23). Finally, as seen in people dysfunction of visceral organs concurrent with WNV infection is reported only rarely in horses (179, 224).

The pathologic examinations of WNV-infected horses are so like those in human beings that to describe findings would be redundant. Summarily, horses suffer myelitis or encephalomyelitis, with variable involvement of the meninges. Upon microscopic examination, the cerebellum, brain stem, and grey matter of the spinal cord are those regions most consistently identified as being affected by WNV (23, 28, 29, 79, 195, 210, 224, 237). Cerebellar Purkinje cells are often damaged, as are the ventral horns of the spinal cord, structures homologous to the anterior horns of primates (28, 29, 79, 195). Publications emphasize that the clinical signs in infected horses are consistent with dysfunction of those CNS regions subsequently discovered to have histopathologic changes in individual animals (28, 210, 237). The single difference



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apparent from the literature is that horses are often reported to have grossly congested lungs (79, 251). However, this could certainly be a result of the prolonged recumbency of many of these animals prior to euthanasia or death.

### I.L.iii. Rodents and rabbits

Rodents are of course widely exploited for virology research, thus it is logical that the bulk of pathogenesis and histopathologic studies of WNV in rodents involve common laboratory species. However, the potential of wild rodents to play a substantial role in WNV ecology prompted McIntosh to perform a small study on six species. Only one animal was found to have circulating WNV for a brief period of time. Disappointingly, results are somewhat equivocal, perhaps because sera were stored at -20°C (158).

Rabbits and guinea pigs have been inoculated by a gamut of routes ranging from corneal scarification to intranasal distillation, but viremia has not been reported (14, 71, 172, 235). Very young rats attain WNV titers in excess of eight logs, but as they age, they quickly become refractory to even intracranial inoculation (8, 14, 57). Hamsters inconsistently develop systemic infection when inoculated intraperitoneally (273) or via aerosol (185), but are quite susceptible to intracranial administration of WNV (71). Many strains of laboratory mice are very sensitive to infection with WNV by most every route and virus replication has been found to be widespread in the tissues by day five postinfection (47, 136, 186, 190). Intracranial and intraperitoneal injections are the most reliable methods for producing systemic infection in younger mice (262), while peripheral inoculation tends to produce only peripheral infection in adult mice (71, 200, 262). Mice have been infected by intranasal (14, 186, 235), intratracheal, and aerosol (186) exposure to WNV, and results have conflicted in two studies examining the effects of *per os* inoculation (14, 190) Mice seem to escape WNV-induced disease only when exposed by topical



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instillation of the cornea and skin (190), and do not spread infection amongst themselves by direct contact (235).

Little information is available concerning gross changes resulting from WNV infection. Meningeal hemorrhage, and edema and hyperemia of the brain are grossly evident in mice (134, 172). Most reports do not mention gross visceral abnormalities from WNV, while some state that there are none (235, 241). Kramer and Bernard observed thymic atrophy, splenomegaly, and intestinal distention in mice infected intraperitoneally with a strain of WNV isolated in New York in the year 2000 (134).

Far more data have been published regarding microscopic lesions from WNV, especially those occurring in the CNS. Peripherally, mice may exhibit myocarditis, congestion and edema in the lungs, focal degeneration of hepatocytes, and lymphonecrosis of the spleen and lymph nodes (134). Multifocal splenic necrosis in some hamsters was observed by Xiao *et al.* (273). Regardless of the route of exposure, severe infections of WNV look to have similar histopathology in the CNS of mice and hamsters. Many lesions are consistent with those seen in other mammals, including extensive involvement of the brainstem, vascular congestion and perivascular lymphocytic cuffing, lesions in the anterior (*sic*) horns and other regions of the spinal cord, and degeneration of Purkinje cells and other neurons (71, 186, 190, 241, 273). However, the CNS of WNV-infected mice and hamsters may have a higher degree of vascular congestion and perivascular and parenchymatous inflammation than are typically reported in other hosts. Additionally, the pyramidal cells of the cerebral cortex are often found to be severely affected in mice (235, 241), which may contribute to the paralysis observed so frequently with WNV infection in this species (14, 47, 57, 235, 241).



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### I.L.iv. Other mammals

Few, small studies have been published on the experimental inoculation of various species with WNV. Despite seroprevalence rates of over 20% in sheep (250) and 70% in water buffalo (250), viremia was not detectable in either species following inoculation via WNV-infected mosquitoes. A later study reported viremia at only a single timepoint in one of several infected ewes and lambs (9). The development of viremia was not reported in infections of two hedgehogs and neither animal developed encephalitis (235). Of three dogs each inoculated both intravenously and subcutaneously with large doses of WNV, one developed a low-level viremia that peaked at $10^{2.8}$ pfu/ml serum. Viremia was first detected at day one postinoculation and was sustained through day seven. However, the authors qualified that this dog was later found to have an underlying condition that may have been immunosuppressive (17). Perhaps due to lack of clinical disease in the study subjects, histopathologic examination either was not performed, or results were not reported, in any of these studies.

Cattle are often found to be seropositive for WNV, with rates as high as 36% reported in Pakistan (87). Experimental infections have been performed in calves previously inoculated with JEV. No circulating WNV was detected in any calf, nor were any signs of disease observed, but all animals did develop a slight humoral response to WNV, and antibody titers against JEV were elevated (98). Nine naïve calves were inoculated with KUNV by a number of routes in another experiment. No viremia was detected in those calves tested, and five of the animals failed to seroconvert. Several developed nasal discharge and diarrhea, both of which are nonspecific, common symptoms in calves. One animal that had been inoculated intracerebrally with KUNV developed overt neurologic disease and required euthanasia. Histopathology was performed on the brains and livers of all calves, eight of which were described as having mild, non-purulent



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encephalitis. Similarities exist between lesions in the calves and those in horses and human beings, including perivascular cuffing, gliosis, and degeneration of Purkinje cells. However, brainstem involvement is a prominent feature in other species, but lesions in the calf brains were "largely restricted to the cerebral hemispheres." Further, the one spinal cord examined had no gross or microscopic abnormalities. Interestingly, small foci of degeneration and inflammatory cell infiltration were consistently observed in liver tissue (244).

### I.L.v. Birds

WNV infection has been studied in only a handful of the tremendous number of species of birds. The response to WNV has so much variation amongst and within even the modest number of species examined that summation is difficult. WNV infection has been detected in nearly all birds inoculated by the bite of infected mosquito (131, 141, 271) or by needle inoculation (20, 141, 161, 162, 247, 271). Far fewer species develop systemic infection after exposure *per os* or via direct contact with other infected birds of the same species in laboratory studies (131, 141, 247). Both of these modes of transmission, especially direct contact, are suspected to have relevance to the natural exposure of some species of birds to WNV (65, 131, 169). In those birds that become viremic, WNV is often detected in the bloodstream very rapidly, within 24 hours of initial exposure. In contrast to the fluctuant character of West Nile viremia described in many mammalian species, the viremias reported in birds remain fairly constant until being cleared by approximately day six, or until the death of the bird (20, 131, 141, 161, 271). That birds have dramatic differences in titers of viremia following WNV infection is demonstrated elegantly in a 2003 publication by Komar *et al.* Viremia profiles were generated for birds from ten phylogenetic orders inoculated with one of two strains of WNV isolated in New York in 1999, and the greatest differences in peak titer are nearly eight orders of magnitude (131). Further,



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WNV has been detected in oral and cloacal swabs as well as excreta from many species of infected birds (130, 131, 141, 247), and some birds have been reported to have external hemorrhaging after death (131). Thus, any bird suspected of having WNV infection should be handled cautiously both ante- and postmortem.

WNV has been isolated, often at high levels, from a battery of tissues harvested from infected birds, and as mentioned, has been described as having a "nonrestricted tropism in affected birds," (245). Brain, heart, liver, skin, spleen, and kidney are among those tissues that most consistently yield infectious WNV (134, 196, 245, 247, 270). Despite the prolific replication of WNV throughout the tissues of many species of birds, very few reports mention gross lesions. Those observed include enlargement of the gallbladder, thymic and cloacal atrophy in young birds, pale lungs, and hemorrhage of the brain, meninges, calvarium, periarticular cavities, and, as petechiae, of the splenic capsule (65, 245, 247).

Predictably, microscopic lesions of WNV-infected birds are widely scattered. Although most visceral structures are affected by WNV (131, 245), the brain was described as a "significant target of infection" in many birds incurring natural infection in New York (245). Paradoxically, despite high mortality rates from WNV, corvid birds may have less severe CNS lesions than other species (245). Avian CNS lesions are consistent with those described for WNV infection in other species, including involvement of the brainstem and severe effects on Purkinje cells (65, 245). WNV infection in birds is decidedly unique in that the peripheral nervous system may be involved (245). Visceral organs most damaged by WNV infection in birds include the pancreas, kidney, heart, lungs, and those from the gastrointestinal tract (131, 134, 136, 245, 247). Many vital structures harbor and may be damaged by the virus, including adrenal cortical cells and pancreatic acinar and islet cells (245), all which have delicate endocrine functions. The extensive distribution of lesions induced by WNV make it difficult to predict



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which of the many lesions may contribute most significantly to the death of those birds that succumb to infection, but either multiple organ system failure or the loss of autonomic functions due to the extensive infection of the brainstem are likely to be the ultimate cause of death.

* * * * *

Largely overshadowed by other disease agents for sixty years after its initial isolation, WNV has suddenly gained regard as a newsworthy, emerging pathogen. The unexpected enhancement of virulence would not be so significant had the virus not undergone rapid global spread, nor if it were more easily controlled. Additionally, the new, more neurovirulent strains of virus have readily established in a diversity of ecosystems. Together, these elements underscore the need to modify and enhance the body of basic knowledge about this zoonotic agent. Towards that end, the ensuing chapters explore the replication of WNV in animal species: companion animals, of potential import due to their close contact with human beings; and house sparrows, key vertebrates in the maintenance and amplification of WNV.



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## II. Experiment I: Infection of Cats and Dogs with West Nile Virus

### II.A. Abstract

Domestic dogs and cats were infected by mosquito bite and evaluated as hosts for West Nile virus (WNV). Each of four dogs developed a viremia of low magnitude and short duration, but did not display signs of disease. Twenty-one of 23 cats became viremic, with peak titers ranging from $10^{1.0}$ to $10^{4.2}$ plaque forming units per ml. Several of the cats showed mild, non-neurologic signs of disease. During the course of infection, WNV was not isolated from saliva of any of the dogs or cats tested. An additional group of four cats were exposed to WNV orally, via ingestion of infected mice. Two cats consumed an infected mouse on three consecutive days and two cats consumed a single infected mouse. All of these cats developed viremia with a magnitude and duration similar to that seen in cats infected by mosquito bite, but none of the four showed clinical signs. These results suggest that dogs and cats are readily infected by WNV. The high efficiency of oral transmission observed with cats suggests that infected prey animals may serve as an important source of infection to carnivores. The magnitude of viremia found in dogs and cats indicates that neither species is likely to function as an epidemiologically-significant amplifying host, although the peak viremia observed in some cats may be high enough to infect mosquitoes at low efficiency.



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## II.B. Introduction

The appearance of the West Nile virus (WNV) in New York in 1999 and the subsequent establishment and spread of the virus have aroused considerable public anxiety about the potential of companion animals to become infected, show morbidity and mortality, and transmit WNV to other species, including human beings. Little information is available concerning the susceptibility of dogs and cats to WNV infection. Approximately one-third of a large sample of dogs from a WNV-endemic region of South Africa had neutralizing antibody to WNV, and viremia was detected in one of three dogs inoculated with a large dose of WNV (4). Recently, WNV was isolated from the brain of a cat with seizures (13), but nothing is known about the natural history of WNV infection in this species. Most dogs and cats spend at least some time out of doors and thus risk exposure from hematophagous insect vectors. Also, as carnivores, they may be exposed to WNV through ingestion of infected small mammals and birds, which are known to have large quantities of WNV in blood and tissue during the course of infection (13, 21). Considering that there are an estimated 68 million companion dogs and 73 million pet cats in the United States alone (2), it was important to evaluate their response to WNV infection, assess their potential to serve as amplifying hosts for this virus, and obtain some estimate of the clinical consequences of infection.

## II.C. Materials and Methods

### II.C.i. Animals and examinations

Four young adult female, crossbred hounds (Harlan, Indianapolis, IN) and eight adult, female domestic, crossbred cats (Liberty Laboratories, Waverly, NY) were used for a pilot study. An additional 8 adult altered female crossbred cats (Liberty Laboratories, Waverly, NY) and 11 adult European shorthair cats (4 intact females and 7 intact males; Liberty Laboratories, Waverly,



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NY) were later obtained for use in a study of WNV pathogenesis and as non-immunized controls in a feline WNV vaccine challenge study, respectively. All were purchased as specific pathogen-free animals. Each of the cats was negative for antibodies to feline immunodeficiency virus and negative for feline leukemia virus antigen in serum. Animals were gang housed by species and sex under ABSL3 conditions with *ad libitum* access to food and water. They were examined clinically by a veterinarian twice daily for the duration of the study, but did not receive formal neurologic evaluations. Body temperature and general appearance were recorded twice daily from days -0.5 to 7 (pilot study dogs and cats, pathogenesis cats) or 9 (orally inoculated cats), with day 0 being the day of infection. Cats in the vaccine challenge study were clinically evaluated twice daily on days 0 to 14, while body temperature was measured twice daily from days 1 to 5 and once on days 0 and 10 to 14 postinfection. With few exceptions, blood was collected by venipuncture twice daily from days -1 (dogs) or 0 (cats) to 7, at 2 and 3 weeks postinfection, and at the time of euthanasia. Blood was drawn once daily on days 0 to 10 and 14 from the 11 animals in the vaccine study.

For the initial 4 dogs and 4 cats exposed to WNV via mosquito bites for the pilot study, blood was collected into EDTA tubes for hematology once daily from days -1 (dogs) or 0 (cats) to 10, and on days 14 and 21. Blood from the eight cats in the pathogenesis study was also drawn into EDTA tubes from days 0 to 7 or until the day of euthanasia if sacrificed prior to day 7 postinfection. Hematologic parameters (RBC, WBC, platelet counts, plus relative and absolute mononuclear and granulocyte counts) were evaluated using a QBC-V analyzer (Clay-Adams). Saliva was collected from the initial group of mosquito-inoculated dogs and cats once daily from days 0 to 7 by swabbing the oral cavity with a cotton swab premoistened with BA-1 medium (M-199 salts, 1% bovine serum albumin, 250 mg/L sodium bicarbonate, 100 U penicillin G/ml, 100 ug/ml streptomycin, 1 μg/ml amphotericin B in 50 mM Tris, pH 7.6).



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Dogs were euthanized on day 49 and cats were euthanized between 2 and 49 days postinfection: day 49 for 4 cats in the pilot study, two cats each at days 2, 4, 6, or 8 for the pathogenesis study, and day 14 for those cats in the vaccine trial. All animals were euthanized by pentobarbital overdose, necropsied, and their carcasses incinerated within the containment facility.

Hematologic and body temperature data were analyzed by repeated measures analysis of variance, and compared to a baseline using the differences of least squares means statistical test (Proc Mixed, SAS Statistical Software). Baseline values were obtained on day 0 (body temperature) or represent the mean of values obtained on days 0 and 21 (hematologic data) for dogs and cats in the pilot study; day 0 for the cats in the pathogenesis study; and the mean of values on day 0 and 14 from cats in the vaccine study (body temperature data only). Correlation analysis (Proc Corr, SAS Statistical Software) was used to evaluate the relationship between the number of mosquitoes that engorged during feeding with time of onset of viremia, peak viremia titer, and duration of viremia. Data from all cats inoculated with mosquitoes were used in these analyses except those sacrificed during the period of viremia. Correlation analyses were also preformed for orally inoculated cats to examine the strength of association of number of mice consumed with onset and peak of viremia.

**II.C.ii. Infection by mosquito feeding**

Four dogs and four cats, and at later dates additional groups of 8 and 11 cats, were exposed to WNV through the bites of infective *Aedes albopictus* mosquitoes. The mosquitoes were from a colony strain from Lake Charles, LA, which had been maintained in the insectary for several years with periodic additions of field-collected stock. Mosquitoes were reared in an insectary maintained at 26.7° C, approximately 80% relative humidity and a light:dark cycle of 16:8 hours.



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Larvae were fed liver powder and rabbit chow *ad libitum*. Cohorts of adult female *Ae. albopictus* 3 to 5 days of age were inoculated intrathoracically with roughly 200-500 Vero pfu of the NY99 strain of WNV, isolated originally from an infected crow. Inoculated mosquitoes were placed in 3.8 liter cages, given 5% sucrose for maintenance, and incubated for 12 days under the insectary conditions described above. On the afternoon prior to feeding on dogs or cats, mosquitoes were transferred to cylindrical cartons with netting on both ends, denied sucrose solution, and held overnight with a pad moistened lightly with water on their cages.

Cats were lightly anesthetized with ketamine and dogs were sedated with xylazine during mosquito feeding. Prior to feeding, a 10 - 15 cm diameter area of fur over the thorax was clipped using a #40 blade. Feeding was accomplished by holding the mosquito cage against the clipped area of skin for approximately 5 minutes for dogs and usually 10 but as many as 30 minutes for cats, as needed. Individual lots of 9 to 19 mosquitoes were given the opportunity to feed on each animal, then transported to a secure laboratory. They were chilled and sorted on wet ice. Three mosquitoes from each animal's lot (all engorged mosquitoes when $\leq$3 had fed) were ground individually in BA-1 using a mixer mill. Samples were then clarified in a refrigerated microcentrifuge (5000 x g for 5 min) and tested for virus by plaque assay.

**II.C.iii. Oral exposure to West Nile virus**

Four cats were exposed to WNV via ingestion of mice previously infected by IP inoculation of approximately 100 pfu of WNV (NY99), incubated for 5 to 8 days, and euthanized by $CO_2$ inhalation immediately prior to their presentation to the cats. To estimate the minimal virus load of the mice, cohorts of the mice fed to cats were euthanized at the same time, frozen at -70° C, and their brains later assayed for virus by plaque assay. Feeding was accomplished with the cats housed individually, by leaving the infected mouse on a plate overnight. In most but not all



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cases, the mouse was observed to have been consumed within 1 hour of presentation. If a cat had not consumed the mouse by the following morning it was not considered an exposure, the mouse was discarded and another mouse presented the following evening. Two cats were fed one infected mouse each on three successive days, and two other cats were each fed a single infected mouse.

### II.C.iv. Plaque and neutralization assays

Sera, oral swabs, mosquitoes, and mouse brain homogenates were tested for virus concentration by plaque assay. Briefly, serial 10-fold dilutions of the samples were made in BA-1 medium and 0.1 ml volumes were inoculated onto monolayers of Vero cells in 6-well tissue culture plates. The plates were incubated at 37° C in an atmosphere containing 5% $CO_2$, then overlaid with 3 ml of 0.5% agarose in M-199 medium supplemented with 5% fetal bovine serum and antibiotics. After 48 hours, a second 3 ml overlay containing 0.004% neutral red was added. Plaques were scored on days 3 -5 of incubation. Selected serum samples were tested for antibodies to WNV using a 90% plaque-reduction neutralization test as described previously (6).

## II.D. Results

### II.D.i. Mosquitoes

The number of mosquitoes that fed on pilot study animals, dogs 1 to 4 and cats 1 to 4, ranged from 8 to 11. The quantity of WNV present in the three mosquitoes assayed from each animal after feeding ranged from $10^{7.4}$ -$10^{7.9}$ pfu per mosquito. Between 4 and 9 mosquitoes fed on cats in the pathogenesis study; these mosquitoes were not titrated for virus content. In the vaccine trial, 0 to 4 mosquitoes fed on each cat. WNV titers of three mosquitoes that had been given the opportunity to feed on each cat were $10^{3.3}$ -$10^{9.1}$ pfu per mosquito by plaque assay. In



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no study was it possible to determine the extent of mosquito probing activity not accompanied by engorgement.

**II.D.ii. Mosquito-inoculated dogs**

Viremia was detected in each of the four dogs between 12 and 60 hours following exposure to WNV (Figure II). The quantity of virus in serum was low and fluctuated, and viremia was not detected past day 4.5. The peak viremia detected in the dogs ranged from $10^{1.6}$ to $10^{2.2}$ pfu/ml. Virus was not isolated from any of the oral swab samples. Clinical signs of disease were not observed in any of the infected dogs. They ate readily and remained energetic and afebrile, with the exception of dog 4, which had a slightly elevated body temperature 12 hours after mosquito feeding (Figure IV). Dog 4 was in proestrus and estrus during the challenge period. Statistical analysis of canine hematologic changes revealed a decrease in white cell count from days 2 through 7 and in hematocrit from days 4 through 9. However, the least squares means for those parameters were within the normal clinical reference range throughout the sampling period, suggesting that hematologic analysis is not clinically predictive. At necropsy on day 50, gross abnormalities were not observed in any dog.

Figure II. WNV titers in the sera of dogs following bites of virus-infected mosquitoes.

| Dog | # of engorged mosquitoes | WNV virus titer ($log_{10}$ pfu/ml serum) at days post exposure | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 | 4.0 | 4.5 | 5.0 | 5.5 | 6.0 | 6.5 | 7.0 |
| 1 | 10 | <1 | 1.6 | <1 | <1 | <1 | 1.0 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| 2 | 10 | <1 | <1 | 2.0 | 2.0 | <1 | 2.2 | 2.0 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| 3 | 11 | <1 | <1 | <1 | <1 | <1 | 1.0 | 1.6 | 1.6 | 1.0 | 1.0 | <1 | <1 | <1 | <1 | <1 |
| 4 | 9 | <1 | <1 | 1.6 | 1.6 | <1 | 1.6 | 1.6 | 1.6 | 1.0 | 1.0 | <1 | <1 | <1 | <1 | <1 |

**II.D.iii. Mosquito-inoculated cats, pilot group**

An average of nine (range 8 to 10) WNV-infected mosquitoes fed on each of the four cats in this group. All cats thus exposed to the virus developed viremia within 12 to 36 hours (mean 24



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hours; Figure III). The peak titers of virus in serum ranged from $10^{3.0}$ to $10^{4.0}$ pfu/ml, and virus was not isolated from any samples collected beyond 4.5 days after mosquito feeding. Three of the four cats (numbers 1, 2, and 4) displayed both lethargy and a fluctuant febrile response (Figure IV), but specific neurologic signs were not observed in any of the cats. Statistically significant ($p < .05$) elevations of rectal temperature occurred from days 1 through 6. A number of isolated significant deviations from baseline in hematologic parameters were identified, but meaningful trends were not observed. Cat 4 was anemic at the time of challenge (hematocrit 21%), became more severely anemic during the first week after challenge (hematocrit as low as 13%), then rebounded by days 9 to 14 (hematocrit 18 to 22). WNV was not isolated from any of the oral swab samples. At necropsy on day 50, gross abnormalities were seen in cat 2 (pale, reticulated liver) and cat 4 (hydrocephalus and a nodular, fibrous spleen). These were considered incidental findings, unrelated to WNV infection; hydrocephalus in the absence of overt neurologic signs is not uncommon in cats (D. Baker, personal communication).

**II.D.iv. Mosquito-inoculated cats, pathogenesis study**

Each of the eight cats in the pathogenesis study developed viremia following exposure via engorgement by an average of six WNV-infected mosquitoes (range 4 to 9; Figure III). Onset of viremia was detected between 24 and 60 hours postinfection (mean time of onset 35 hours, mode 24 hours). Several cats were sacrificed prior to the resolution of viremia, thus it was not possible to accurately determine duration and magnitude of viremia for these animals. Of available data, peak titers ranged from $10^{1.7}$ – $10^{4.2}$ pfu/ml serum and occurred between days 1 and 6.5 postinfection, with a biphasic peak in cat 8. Fever and lethargy in 2 cats were the only clinical abnormalities observed. Statistically significant ($p < 0.05$) increases in rectal temperature occurred most days 3 -7.5 postinfection; however only one reading (103.1°F), from cat 9 on day



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5.5, exceeded the clinical reference range. Cat 11 was observed to be subdued and dehydrated on days 1 through 3; a concurrent but not clinically substantial increase in its packed cell volume (maximum reading 41.3%) supported the latter observation. No other cats were observed to exhibit any symptoms of clinical illness. A small number of isolated significant deviations from baseline in hematologic parameters were identified by statistical analysis, but meaningful trends were not observed. No gross changes were observed at necropsy in any cat.

**II.D.v. Mosquito-inoculated cats, vaccine challenge study**

For reasons that were not possible to elucidate, mosquitoes were reluctant to feed on the cats in the vaccine challenge study: a mean of two mosquitoes (range 0 to 4) fed on each cat. Viremia was detected in nine (82%) of 11 nonimmunized cats (Figure III). There was considerable variability in time of initial detection (mean 61 hours, range day 2 -5) and duration (<24 hours to 5 days, mean 63 hours) of observed viremias. The viremic cats in this experiment had peak titers ranging from $10^{1.0}$ -$10^{2.4}$ which occurred on days 3 to 6 postinfection (mode day 5). The precision of these data is affected by the fact that cats in this study were bled only once daily as opposed to the twice daily protocol used in other experiments.

No neurologic signs were observed in any cat, but one cat, v19, was lethargic for the first 4 days postinoculation; it is of note that viremia was not detected at any time in cat v19. Several cats had body temperatures beyond the clinical reference range: cat v6 (102.6°F) on day 3.0, cats v5 (103.0°F) and v16 (102.8°F) on day 5.0, and cat v5 on day 9.0 (102.7°F). Cat v16 had a temperature reading of 102.6°F on both days 0 and 14, yet did not have a reading in excess of the clinical reference range at any other point in the study. Statistical tests did not reveal any significant deviations from baseline temperature for the group on any day.

Neutralizing antibody was assessed in sera collected on day 14 postinoculation. Neither of



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the two aviremic cats had developed detectable neutralizing antibody by that time, nor did four of the nine cats in which viremia was detected. Of the five cats that were found to have seroconverted, two cats, v12 and v29, also had low antibody titers on day 7 postinfection (titers of 5 at 80% plaque reduction, but <5 at 90% plaque reduction). When neutralizing antibody was again measured for day 14 postinfection, antibody was no longer detected in cat v12 (titer <5), while cats v5, v6, v29, and v34 had titers ranging from 10 to 40 at the 80% level of plaque reduction, and from 5 -20 at 90%. During necropsies performed after sacrifice at day 14 postinfection, the liver of cat v1 was observed to be pale.

Figure III. WNV titers in sera of cats following bites of virus-infected mosquitoes.

| | | | WNV virus titer ($log_{10}$ pfu/ml serum) at days post exposure | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cat | Study | # fed[a] | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 | 4.0 | 4.5 | 5.0 | 5.5 | 6.0 | 6.5 | 7.0 | 7.5 | 8.0 | 14.0 |
| 1 | pilot | 9 | <1 | 1.6 | 2.3 | 2.9 | 2.4 | 2.8 | 2.7 | **3.2** | 2.0 | 2.0 | <1 | <1 | <1 | <1 | <1 | -[b] | - | - |
| 2 | pilot | 10 | <1 | <1 | 2.1 | 2.3 | 2.4 | 2.7 | 2.8 | **3.0** | 2.6 | 1.0 | <1 | <1 | <1 | <1 | <1 | - | - | - |
| 3 | pilot | 8 | <1 | <1 | <1 | 1.0 | 1.6 | 1.9 | 2.0 | 2.8 | **3.4** | 3.0 | <1 | <1 | <1 | <1 | <1 | - | - | - |
| 4 | pilot | 9 | <1 | <1 | 1.0 | 2.5 | 3.2 | 3.2 | **4.0** | 3.2 | 3.0 | 1.0 | <1 | <1 | <1 | <1 | <1 | - | - | - |
| 5 | path. | 6 | <1 | <1 | 1.0 | <1 | <1 | **2.2** | E[c] | | | | | | | | | | | |
| 6 | path. | 6 | <1 | <1 | <1 | 1.0 | <1 | **1.8** | E | | | | | | | | | | | |
| 7 | path. | 4 | <1 | <1 | <1 | <1 | <1 | 1.8 | **2.2** | 1.7 | <1 | <1 | <1 | <1 | <1 | <1 | E | | | |
| 8 | path. | 6 | <1 | <1 | **1.7** | <1 | <1 | <1 | 1.0 | <1 | <1 | 1.0 | <1 | 1.0 | <1 | **1.7** | E | | | |
| 9 | path. | 6 | <1 | <1 | 2.0 | 1.7 | 1.0 | 2.6 | 2.6 | 3.2 | 3.7 | **4.2** | 3.1 | 3.0 | 1.0 | 1.0 | - | - | <1 | E |
| 10 | path. | 9 | <1 | <1 | <1 | <1 | 1.0 | 1.7 | 2.0 | 1.0 | 2.9 | 3.0 | **3.4** | 3.3 | 1.8 | 1.7 | - | - | <1 | E |
| 11 | path. | 9 | <1 | <1 | <1 | 1.7 | 1.7 | 1.7 | **2.0** | 1.0 | 1.8 | E | | | | | | | | |
| 12 | path. | 5 | <1 | <1 | 1.0 | 2.0 | 1.0 | 2.4 | 2.2 | 2.7 | **2.9** | E | | | | | | | | |
| v1 | vacc. | 2 | <1 | - | <1 | - | <1 | - | **1.0** | - | <1 | - | **1.0** | - | <1 | - | <1 | - | <1 | <1 |
| v2 | vacc. | 4 | <1 | - | <1 | - | <1 | - | <1 | - | <1 | - | <1 | - | <1 | - | <1 | - | <1 | <1 |
| v5 | vacc. | 1 | <1 | - | <1 | - | <1 | - | <1 | - | <1 | - | **1.0** | - | <1 | - | <1 | - | <1 | <1 |
| v6 | vacc. | 2 | <1 | - | <1 | - | <1 | - | <1 | - | 1.3 | - | 1.3 | - | **1.5** | - | <1 | - | <1 | <1 |
| v12 | vacc. | 1 | <1 | - | <1 | - | <1 | - | <1 | - | 1.3 | - | **1.6** | - | 1.3 | - | <1 | - | <1 | <1 |
| v14 | vacc. | 2 | <1 | - | <1 | - | 1.5 | - | **1.7** | - | **1.7** | - | <1 | - | <1 | - | <1 | - | <1 | <1 |
| v16 | vacc. | 3 | <1 | - | <1 | - | 1.3 | - | <1 | - | 1.3 | - | **1.5** | - | 1.0 | - | <1 | - | <1 | <1 |
| v19 | vacc. | 3 | <1 | - | <1 | - | <1 | - | <1 | - | <1 | - | <1 | - | <1 | - | <1 | - | <1 | <1 |
| v29 | vacc. | 0 | <1 | - | <1 | - | <1 | - | 1.5 | - | 2.3 | - | **2.4** | - | 1.3 | - | <1 | - | <1 | <1 |
| v34 | vacc. | 2 | <1 | - | <1 | - | <1 | - | 1.0 | - | 2.0 | - | **2.1** | - | 1.3 | - | <1 | - | <1 | <1 |
| v35 | vacc. | 2 | <1 | - | <1 | - | 1.3 | - | 1.0 | - | **1.7** | - | <1 | - | <1 | - | <1 | - | <1 | <1 |

Peak titers are in bold text.

[a] The number or mosquitoes that engorged on each cat

[b] Not done

[c] Cat euthanized prior to this sampling



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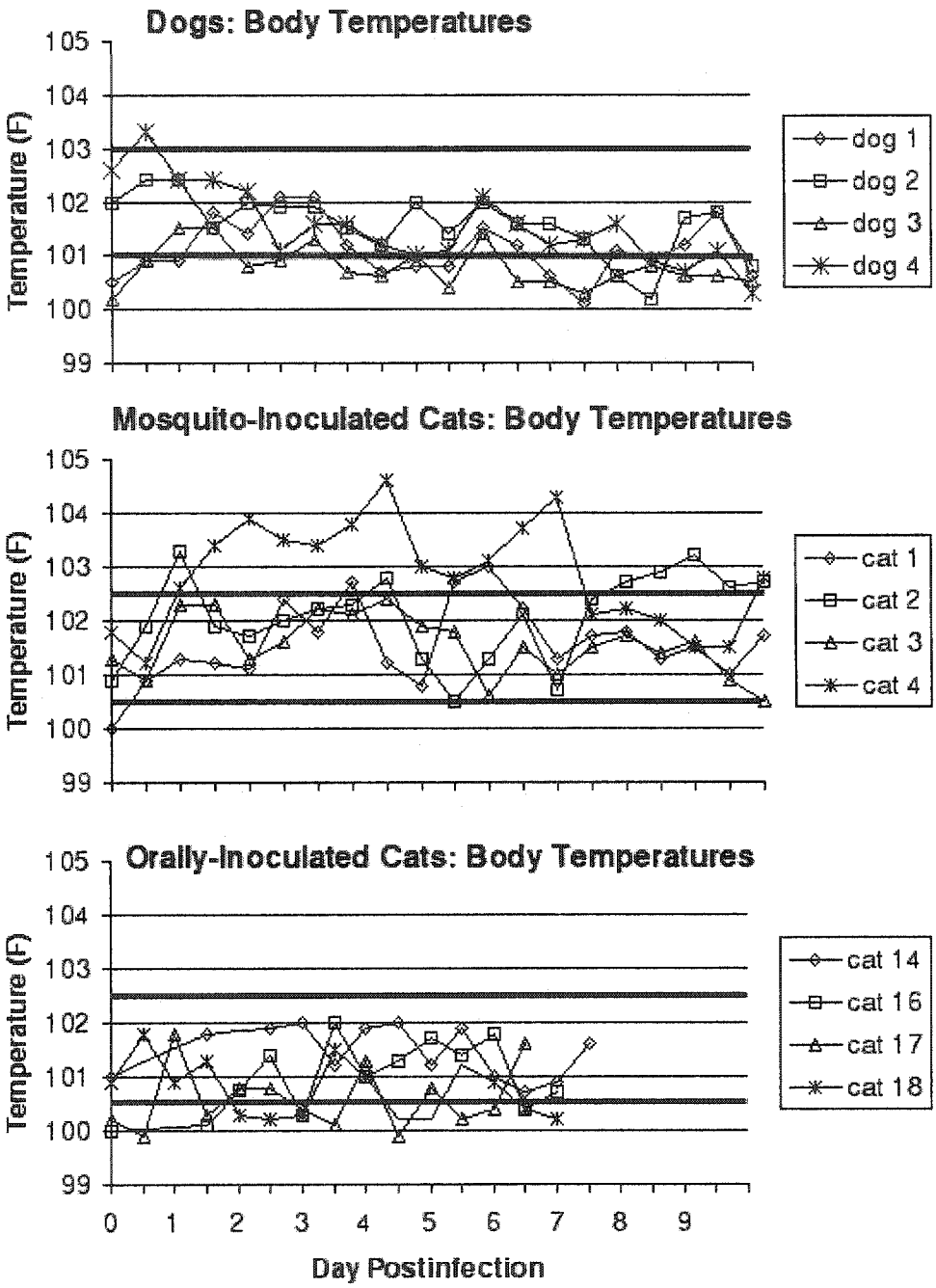
Figure IV. Body temperatures during the course of experimental West Nile virus infection. Reference ranges for clinically normal dogs and cats, indicated by heavy lines, were considered to be 101.0 to 103.0 F and 100.5 to 102.5 F, respectively.



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### II.D.vi. Statistical analysis, mosquito-induced WNV viremia in cats

Statistical tests of data from all groups of mosquito-inoculated cats revealed significant correlation ($p < 0.0001$) of number of engorged mosquitoes with day of onset of viremia. The parameters correlated inversely – higher numbers of feeding mosquitoes induced viremia at shorter intervals postinfection. For the remaining analyses it was necessary to remove from consideration cats sacrificed prior to the resolution of viremia. The number of engorged mosquitoes had a statistically significant correlation with the peak titer of the resultant viremia ($p<0.002$), but not with peak titer of viremia ($p=0.107$) or duration of viremia ($p=0.053$).

### II.D.vii. Orally inoculated cats

Clinical signs, including pyrexia (Figure IV) and neurologic abnormalities, were absent throughout the study period in cats infected with WNV via ingestion of three (n=2) or one (n=2) infected mice. Cats ingesting three mice (cats 14 and 16) developed viremia within 24 hours of ingesting the first mouse (these cats were not bled at 12 hours), and viremia resolved by day 6 (Figure V). The peak viremia in these two animals was $10^{3.7}$ and $10^{3.9}$ pfu/ml, respectively. Cohort mice of those fed at the first of the three feedings yielded $10^{9.5}$ and $10^{9.7}$ pfu per gram of brain. Cats 17 and 18 each ingested a single mouse, which were euthanized approximately two days earlier in the course of their infection than those fed to cats 14 and 16, and virus titration of their cohorts revealed $10^{5.3}$ and $10^{6.5}$ pfu per gram of brain. Cats fed a single mouse had viremia of similar duration but delayed onset (day 4 and 2.5 for cats 17 and 18 respectively), and lower peak titer ($10^{2.5}$ and $10^{2.2}$ pfu/ml serum) than occurred in the cats ingesting mice with higher virus content (Figure V). Statistical analysis revealed a significant correlation between number of mice consumed and peak titer ($p=0.015$) but not with mice consumed and onset of viremia ($p=0.096$).



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Three of the four cats fed infected mice developed neutralizing antibody titers to WNV of at least 10 by day 14 post-exposure (range 10 to 40, 90% neutralization). Cat 18, which had a neutralizing titer <10 on day 14, had a titer of 40 on day 21. At necropsy, both cats ingesting a single mouse were observed to have grossly evident prescapular lymph node enlargement, and cats 14 and 17 also had hyperplasia of intestinal lymphatic follicles.

**Figure V. WNV titers in sera of cats following ingestion of virus-infected mice.**

| Cat | # mice consumed | WNV virus titer ($log_{10}$ pfu/ml serum) at days post exposure | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 | 4.0 | 4.5 | 5.0 | 5.5 | 6.0 | 6.5 | 7.0 | 7.5 | 8.0 | 8.5 | 9.0 |
| 14 | 3 | <1 | - | 1.0 | 3.2 | 2.9 | 3.2 | 3.4 | 3.7 | 3.6 | 3.2 | <1 | <1 | <1 | <1 | <1 | - | - | - | - |
| 16 | 3 | <1 | - | 2.0 | 2.7 | 2.8 | 3.4 | 3.6 | 3.9 | 3.6 | 2.7 | <1 | 1.0 | <1 | <1 | <1 | - | - | - | - |
| 17 | 1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | 1.8 | 1.0 | 1.6 | 2.1 | 2.5 | 2.3 | 1.8 | 1.0 | 1.3 | <1 | <1 |
| 18 | 1 | <1 | <1 | <1 | <1 | <1 | 1.8 | 1.3 | 1.5 | 1.0 | 1.6 | 2.2 | 1.5 | 1.3 | <1 | <1 | <1 | <1 | <1 | <1 |

* Not done

## II.E. Discussion

Little attention has been paid to dogs and cats as hosts for WNV and neither species is a recognized natural host for this virus. However, considering the large populations of these animals in North America and their close association with humans, it is important to understand their clinical response to infection and the likelihood that they might serve as amplifying hosts. Dog and cat owners have also expressed concern about the potential effects of WNV infection on their pets. Roughly one in three dogs from a WNV-endemic region of South Africa was found to have neutralizing antibody to WNV (4), and sera from over 40% of dogs in Romania were considered positive for hemagglutination inhibiting antibody (10). More recently it was reported that 10 of 139 dogs and none of 10 cats sampled from New York City during the fall of 1999 had WNV-neutralizing antibody (14); the number of these animals, particularly the cats, that spent



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significant time out of doors was not known. A considerably larger sampling in Louisiana in 2002 revealed overall seropositivity of 26% in dogs and 9% in cats, with rates in stray animals only slightly higher than those in pets (J. Kile, personal communication). WNV was isolated from the brain of a cat from New York that displayed neurologic disease (13) and a small number of clinical cases of WNV infection in dogs were reported to the Centers for Disease Control and Prevention in 2002 (D. O'Leary, M. Bunning, personal communication).

*Ae. albopictus* mosquitoes were used to challenge the animals in these experiments because they are known to be capable of transmitting WNV by bite (1, 23) and in order to reproduce a natural route of infection. WNV has been isolated from *Ae. albopictus* in the USA (7, 16, 17) and this mosquito species is thought to be of considerable importance in the transmission of WNV from birds to mammals (27).

None of the four dogs infected by mosquito bite showed clinical signs of disease, and although each became viremic, the quantity of virus in blood was low and fluctuated considerably. These results are similar to those described by Blackburn and coworkers, who found clinical signs in none, and viremia in one of three dogs inoculated by subcutaneous and intravenous routes with a South African strain of WNV (4). Collectively, these observations and the paucity of clinical reports of disease in dogs attributed to the virus suggest that WNV infection in dogs is typically subclinical in nature.

A majority of cats (21/23, 91%) exposed to WNV by feeding of infected mosquitoes subsequently developed detectable viremia, but very few developed clinical disease. During the first week following challenge, some cats exhibited mild, nonspecific signs of disease including lethargy and modest decreases in appetite, none to the extent that would typically alarm pet owners. Some cats had isolated instances of temperature readings above the clinical reference range, with periods of distinctly elevated body temperature occurring in only two of the cats



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(numbers 2 and 4). Cat 4 had a preexisting anemia which may have contributed to the course of disease.

Two cats, v2 and v19, both bitten by several mosquitoes (four and three, respectively), did not develop detectable viremic or serologic responses following challenge. Conversely, although no mosquitoes had visibly engorged on cat v29, this animal became viremic and later seroconverted. It is possible that one or more mosquitoes took modest bloodmeals that were not detected during sorting, or that the cat became infected by probing of WNV-infected mosquitoes, the saliva of which has been demonstrated to contain this virus (6).

Few of the cats (five of 11) in the vaccine challenge group developed a measurable neutralizing antibody response to inoculation with WNV by day 14. It is likely that a significant humoral response to infection was progressing, but was not yet at detectable levels. This phenomenon was observed in the antibody response of orally inoculated cat 18, which was determined to have a titer <10 at day 14 that rose to 40 on day 21 postinfection.

The oral transmission experiments conducted with cats were designed to determine whether carnivores are likely to become infected with WNV through consumption of infected birds or small mammals, which often contain high quantities of virus. Transmission of a vaccine strain of Japanese encephalitis virus to mice by instillation of virus into the oral cavity has previously been reported (24), as has the infection of baby hamsters suckling WNV-infected dams (25). Efficient transmission to adult mice by oral instillation of WNV has been reported (21), but two similar attempts resulted in a twofold lower infection rate (3) and failure to establish detectable infections (20). Chimpanzees orally inoculated with WNV did not shed virus nor seroconvert (19), but a infant human developed IgM antibodies after exposure to breast milk with evidence of WNV by TaqMan PCR (22). Further, isolation of WNV from a red-tailed hawk found dead in New York during midwinter was suggested to be due to predation, although the source of an



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infected prey animal at that time of year was not clear (8). Since that time, controlled studies have demonstrated oral transmission of WNV in some but not all species of birds tested (15, 18). Finally, the tick-borne encephalitis flavivirus has been isolated from the milk of certain experimentally infected mammals (11), and milk-borne transmission of the agent from sheep to humans of has been reported (5, 29). Cats and dogs have been shown to be susceptible to oral infections with arboviruses from other families (9, 28); however, the current report provides the first experimental demonstration of oral transmission of a flavivirus using methods that approximate a natural type of exposure.

Systemic infection was established in each of four cats following consumption of one or three WNV-infected mice, indicating that *per os* transmission is quite efficient. Currently it is not known whether oral transmission of WNV results simply from oral exposure to virus, depends upon inoculation through the oral mucosa by small splinters of bone, or proceeds through some other pathway. Nonetheless, it seems likely that transmission of WNV by predation or scavenging may provide another important route of WNV transmission in mammals and birds.

Peak titers in orally exposed cats ranged from $10^{2.2}$ -$10^{3.9}$ while those in mosquito inoculated cats were between $10^{1.0}$ -$10^{4.2}$. A statistically significant correlation was seen between the number of mosquitoes engorging on cats, or infected mice consumed by cats, and the peak magnitude of resultant viremia. Peak titers sustained for short periods in some cats following infection by either route may be adequate to infect feeding mosquitoes. In real-world settings, cats would not be likely to suffer the quantity of bites from infected mosquitoes necessary in this study to produce such titers. Of presumably greater biological relevance is exposure of cats to WNV infected prey. Studies indicate that common species of birds replicate WNV to titers comparable to those measured in the mice fed to cats in this experiment (15).



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The magnitude of WNV viremia necessary to efficiently infect feeding mosquitoes varies with mosquito species. Based on experiments using viremic chicks as a blood source, Jupp estimated 10% infection thresholds for South African strains of *Cx. univittatus*, *Cx. pipiens*, *Cx. quinquefasciatus*, and *Cx. theileri* of $<10^{2.7}$, $10^{2.7}$, $10^{2.7}$ and $<10^{4.1}$ adult-mouse $LD_{50}$/ml, respectively (12). More recently, Turell and coworkers fed a variety of mosquito species from the eastern USA on viremic chicks (26). Infection rates in these experiments ranged from 0% to 17% for mosquitoes ingesting blood with a WNV titer of $10^{5.2}$ Vero cell pfu/ml, and from 0% to 92% for mosquitoes that fed on chicks circulating $10^{7.0\ (\pm\ 0.3)}$ pfu/ml, respectively. In the current study, we did not test the ability of any species of mosquito to become infected by feeding on viremic dogs or cats. The low serum virus titers reported here and by Blackburn and colleagues (4) suggest that dogs are very unlikely to serve as amplifying hosts for WNV. In contrast, the peak viremias observed in a few of the mosquito-fed and orally exposed cats suggest that they may support infection of mosquitoes, albeit with low efficiency relative to many avian hosts.



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## III. Experiment II: Early West Nile Virus Pathogenesis in *Passer domesticus*

### III.A. Abstract

The emergence and spread of West Nile virus (WNV) in North America has had dramatic effects, causing significant morbidity and mortality in birds and horses, and to a lesser extent in human beings. Only limited information existed regarding WNV ecology from regions in which it was historically enzootic, and the variant imported into North America behaved unpredictably, exhibiting avian pathogenicity and increased neurovirulence in some mammals. *Passer domesticus*, the house sparrow, was identified as being likely to have key importance in the amplification and maintenance of WNV in the United States. In order to gain understanding of the pathogenesis of WNV in this significant host, virus isolation was performed at regular intervals on a battery of tissues obtained from experimentally infected birds. WNV rapidly established disseminated infection with wide tissue tropism. All tissues studied were infected in at least some of the birds. Skin was the tissue from which virus was most consistently isolated, and was also the first in which virus was detected – as early as 12 hours postinfection. Other tissues frequently containing virus included blood, heart, lung, liver, kidney, and testis.

### III.B. Introduction

The avian-pathogenic strain of WNV introduced in North America has garnered a considerable amount of research attention since its initial detection in New York in 1999. Morbidity and mortality have been estimated, end-stage pathology examined, and viremia



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characterized in a number of avian species. A 1963 publication by Kundin investigated early WNV pathogenesis in experimentally inoculated hatchling chicks (11). However, the literature is virtually devoid of similar studies of the newly-emerged strains of WNV that are exhibiting enhanced neurovirulence in avian and human hosts.

Many avian species replicate WNV to titers necessary to infect feeding mosquitoes, but by combining seroprevalence rates in nature, relative abundance, and, when known, magnitude and duration of viremia, birds in the Passeriform order are thought to be key WNV amplifying hosts throughout much of the geographic range of the virus (7). Of the Passeriform birds, the most important hosts of WNV are thought to belong to the Corvid and Passerid families - particularly crows (1, 3, 10, 20, 21) and sparrows (3, 5, 6, 8, 10, 13, 15, 17, 20, 21). The ubiquity (8, 12, 15, 21) of these species in both rural and urban settings and their ability to support high-level replication of virus (10, 14, 21) make them excellent candidates for amplification of virus in many parts of the world. In the United States, crows and other corvid birds are suffering the most marked morbidity and mortality (3), but the European house sparrow, *Passer domesticus,* is considered to to be the chief vertebrate host of both WNV (8, 10) and closely-related Saint Louis encephalitis virus (16).

Efforts to "manage" arboviral outbreaks in the United States primarily utilize mosquito control strategies (4). However, these tactics have not prevented WNV from spreading significantly and becoming enzootic, nor from infecting and inciting morbidity and mortality in hundreds of thousands of clinically important hosts, including human beings, horses, and endangered or otherwise valuable birds. A basic understanding of the pathogenesis of WNV in birds is vital for the development of control and prevention efforts directed towards avian hosts.



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## III.C. Materials and Methods

### III.C.i. Animals and examinations

Twenty-two wild adult and juvenile house sparrows were captured by mist netting in a semi-rural housing development east of Fort Collins, Colorado. All sparrows were negative for antibodies to WNV by 50% plaque reduction neutralization test. Birds were leg banded for identification and caged in groups of 5 to 6 and housed under ABSL3 conditions with *ad libitum* access to food and water. They were examined clinically by a veterinarian twice daily for the duration of the study, but did not receive formal neurologic evaluations. General appearance was recorded twice daily from days -2 to 5. Blood was collected by jugular venipuncture immediately prior to challenge, and again at the time of euthanasia.

Sparrows were euthanized at regular intervals following challenge. A single uninoculated bird was euthanized for use as a control. Groups of three sparrows each were sacrificed at twelve hour intervals, 0.5, 1.0, 1.5, 2.0, and 2.5 days postinfection; thereafter, sparrows were euthanized in pairs on days 3, 4, and 5. All animals were euthanized by pentobarbital overdose. Immediately following sacrifice, saliva was collected by swabbing the oral cavity with a cotton swab premoistened with BA-1 medium (M-199 salts, 1% bovine serum albumin, 250 mg/L sodium bicarbonate, 100 U penicillin G/ml, 100 μg/ml streptomycin, 1 μg/ml amphotericin B in 50 mM Tris, pH 7.6). Postmortem examinations were performed, and tissues were collected for virus isolation. Sera, oral swabs, and tissues were stored at -80°C. Animal carcasses were incinerated within the containment facility.



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### III.C.ii. Infection with WNV

Twenty-one house sparrows were exposed to WNV via subcutaneous inoculation with 1100 pfu WNV-NY 99-4132. This strain of WNV was originally isolated from the brain of a naturally-infected crow in Vero cells and since that time had been passaged once each in C6/36 and BHK cells.

### III.C.iii. Plaque and neutralization assays

Sera, oral swabs, and tissue homogenates were tested for virus concentration by plaque assay. Briefly, serial 10-fold dilutions of the samples were made in BA-1 medium and 0.1 ml volumes were inoculated onto monolayers of Vero cells in 6-well tissue culture plates. The plates were incubated at 37° C in an atmosphere containing 5% $CO_2$, then overlaid with 3 ml of 0.5% agarose in M-199 medium supplemented with 5% fetal bovine serum and antibiotics. After 48 hours, a second 3 ml overlay containing 0.004% neutral red was added. Plaques were scored on days 3 and 5 of incubation. Serum samples were tested for antibodies to WNV using a 90% plaque-reduction neutralization test as described previously (2).

## III.D. Results

### III.D.i. Serum virus isolation

WNV was detected in the serum of inoculated birds from days 1 to 4 postinfection (Figure VI). No virus was isolated from sera of any birds euthanized at 12 hours postinfection, but all 3 birds had substantial viremia by 24 hours postinfection (range $10^{2.6}$ - $10^{4.6}$ pfu/ml serum). Virus continued to be present in the sera of all birds until 5 days postinoculation; neither bird sacrificed at that time had detectable circulating WNV. No clear peak was observed, but the highest titers occurred on days 2, 2.5, and 3 postinoculation.



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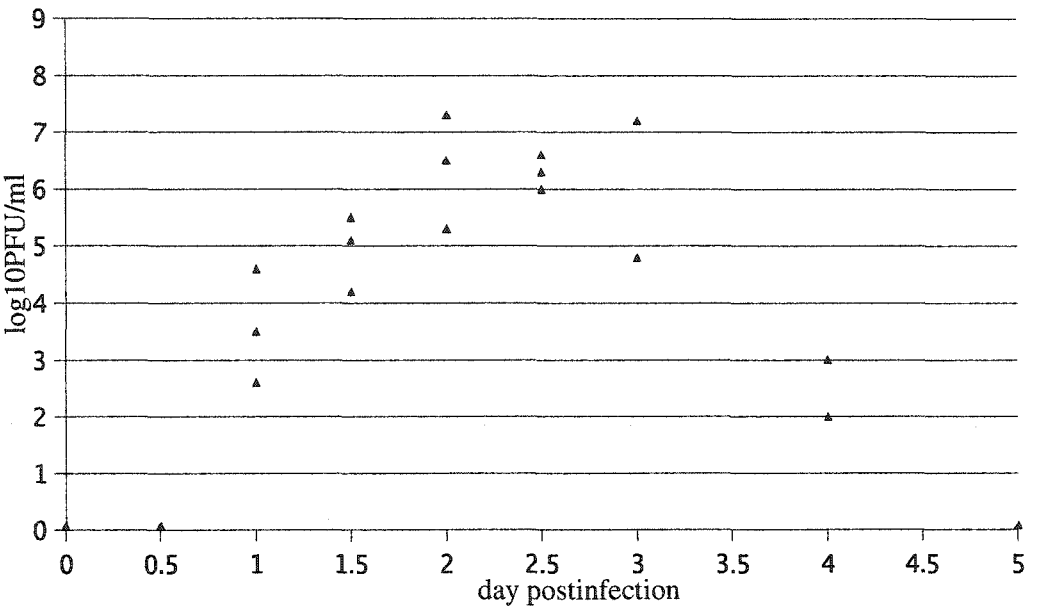
Figure VI. WNV titers in sera of sparrows following subcutaneous inoculation.




**III.D.ii. Virus isolation from the oral cavity**

WNV was isolated from swabs of the oral cavities of a minority of WNV-inoculated birds inconsistently, but with increasing frequency in later stages of infection (Figure VII). Virus was first detected from one of three swabs on day 2.5 postinoculation. With the exception of one of two samples obtained five days after challenge, all subsequent oral swab samples yielded virus. Levels of infectious WNV particles in oral samples varied considerably, with titers ranging from $10^{0.6}$ - $10^{4.2}$ pfu per swab.

**III.D.iii. Organ virus isolation**

WNV was isolated from at least one tissue from all but one bird challenged with WNV (Figure VII). Skin was the first tissue from which virus was isolated, with titers over $10^{4.0}$ pfu/gm



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detected at 12 hours postinoculation, prior to the onset of viremia. WNV was isolated from all skin samples harvested at or beyond 24 hours postinoculation (range $10^{4.3}$ - $10^{10}$ pfu/gm tissue). Skin was the most consistently infected organ (20/21 birds), followed by kidney (17/21). Virus was first detected in kidney samples of two of the three birds euthanized on day 1 postinfection and was present in all ensuing samples (range $10^{3.0}$ - $10^{7.0}$ pfu/gm tissue). Heart (13/21) and lung (16/21) were also infected in most birds. WNV was first isolated from both tissues at 24 hours postinoculation and was present in most subsequent samples of heart and all additional samples of lung. In those tissues found to be positive, WNV titers ranged from $10^{1.8}$ - $10^{5.2}$ pfu/gm of heart tissue and $10^{1.9}$ - $10^{6.0}$ pfu/gm lung. WNV was first detected in two of three liver samples at 1.5 days postinfection and was present in all samples thereafter at titers from $10^{2.8}$ – $10^{6.1}$ pfu/gm tissue. Similarly, WNV was first detected in testis at day 1.5 postinfection and was present in all subsequent samples harvested from male birds. Titers of WNV in positive testes ranged from $10^{2.2}$ - $10^{5.6}$ pfu/gm. Infection of gastrointestinal tissues was inconsistent, appearing in some birds between days 1 and 3 postinfection (range $10^{2.2}$ - $10^{6.0}$ pfu/gm duodenum and $10^{3.7}$ - $10^{5.5}$ pfu/gm large intestine). All three birds sacrificed at day 2.5 postinoculation had evidence of gastrointestinal infection, with five of six total gastrointestinal samples harvested at that timepoint found to be positive for WNV. Likewise, infection of tissues from the central nervous system was not uniform. Virus was detected as early as 24 hours postinfection in brain and 36 hours postinfection in caudal spinal cord. WNV was isolated from one or both of these tissues from most birds for the remainder of the study. Those samples found positive had virus titers of $10^{2.0}$ – $10^{3.9}$ pfu/gm brain and $10^{2.6}$ – $10^{5.7}$pfu/gm caudal spinal cord.



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Figure VII. WNV titers in tissues of sparrows following subcutaneous inoculation.

| Bird | Day | WNV titer ($log_{10}$ pfu/ml serum, $log_{10}$ pfu/swab, $log_{10}$ pfu/gm tissue) at days post exposure | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | serum | oral swab | skin | kidney | heart | lung | liver | duodenum | large gut | brain | spinal cord | testis |
| 3 | 0.5 | <1[a] | <0.6 | 4.3 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| 4 | | <1 | <0.6 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | □[b] |
| 6 | | <1 | <0.6 | 4.9 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | □ |
| 9 | 1.0 | 3.5 | <0.6 | 5.6 | 3.4 | 2.8 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| 11 | | 4.6 | <0.6 | 6.9 | 3.0 | 1.8 | 3.8 | <1 | 2.2 | <1 | 2.0 | <1 | <1 |
| 12 | | 2.6 | <0.6 | 4.3 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | □ |
| 7 | 1.5 | 5.5 | <0.6 | 6.7 | 4.6 | <1 | 4.0 | 2.8 | 3.4 | 3.7 | 3.0 | 2.9 | 2.2 |
| 8 | | 4.2 | <0.6 | 6.9 | 3.0 | 1.8 | 2.8 | <1 | <1 | <1 | <1 | <1 | <1 |
| 10 | | 5.1 | <0.6 | 6.6 | 4.6 | <1 | 1.9 | 3.6 | <1 | <1 | <1 | <1 | <1 |
| 1 | 2.0 | 6.5 | <0.6 | 7.5 | 5.8 | <1 | 5.3 | 5.4 | 4.5 | <1 | 2.0 | 3.1 | □ |
| 2 | | 5.3 | <0.6 | 5.4[c] | 4.4 | 3.8 | 4.5 | 3.0 | <1 | <1 | <1 | <1 | 3.2 |
| 5 | | 7.3 | <0.6 | 8.0 | 6.9 | 3.5 | 5.3 | 5.7 | <1 | <1 | <1 | 4.5 | 3.9 |
| 15 | 2.5 | 6.3 | 3.2 | 7.6 | 6.1 | 4.2 | 6.0 | 4.0 | <1 | 5.5 | 3.5 | 3.9 | 4.8 |
| 17 | | 6.0 | <0.6 | 5.8 | 5.8 | <1 | 4.2 | 4.3 | 2.2 | 3.5 | 3.5 | 4.4 | □ |
| 19 | | 6.6 | <0.6 | 7.4 | 6.0 | 3.1 | 5.4 | 4.5 | 5.5 | 5.5 | <1 | 4.5 | □ |
| 14 | 3.0 | 7.2 | 4.2 | 8.0 | 7.0 | 5.2 | 6.1 | 6.0 | <1 | <1 | 5.0 | 5.7 | □ |
| 16 | | 4.8 | 1.1 | 7.5 | 5.5 | 2.8 | 2.9 | 3.6 | 6.0 | <1 | 2.0 | 2.9 | 4.8 |
| 18 | 4.0 | 2.0 | 0.6 | 6.9 | 5.1 | 3.3 | 3.9 | 4.0 | <1 | <1 | 3.0 | 3.6 | □ |
| 20 | | 3.0 | 3.9 | 10.0 | 4.7 | 3.4 | 3.5 | 3.6 | <1 | <1 | <1 | <1 | 3.9 |
| 21 | 5.0 | <1 | <0.6 | 8.0 | 6.3 | 4.4 | 4.4 | 6.1 | <1 | <1 | 3.9 | 5.0 | □ |
| 22 | | <1 | 3.0 | 5.1 | 5.0 | 4.2 | 5.5 | 5.8 | <1 | <1 | <1 | 4.5 | 5.6 |

[a] below the level of detection for this assay. Sensitivity was limited to approximately $10^{1.0}$ pfu/ml serum; $10^{0.6}$ pfu/oral swab; $10^{1.8}$ pfu/gm heart; $10^{1.9}$ pfu/gm lung; $10^{2.0}$ pfu/gm brain, liver, and kidney; $10^{2.2}$ pfu/gm duodenum, large gut, skin, and testis; and $10^{2.6}$ pfu/gm caudal spinal cord.

[b] these birds were female; ovaries were not collected for virus isolation

[c] this sample was mistakenly left at room temperature for several hours following harvest

### III.D.iv. Clinical and gross findings

Clinical signs of disease including ruffled feathers, abnormal posture, ataxia, and dyspnea were not observed in any bird at any time during the study. Sparrows remained active, continued to perch, and exhibited normal social behavior. Birds may have been easier to capture on days 4



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and 5, but this could not be definitively determined. The presence of food in the crop and/or ventriculus and proventriculus of most birds at the time of sacrifice indicated that they were not anorectic. Minimal gross changes were observed: the liver of bird 8 was somewhat pale and enlarged with white, multifocal spots present on the surface (day 1.5), pectoral muscle of bird 16 was dark in color (day 3).

### III.E. Discussion

Most studies of WNV pathology in birds have evaluated only limited aspects of early disease such as viremic response, or have concerned birds that incurred fatal infections. As a result, very little is currently known about the establishment and spread of WNV infection in avian hosts, information that may be of significant importance in the development of preventives such as vaccinations. The primary objective of this study was to examine the tissue distribution of an avian-pathogenic WNV isolate from New York in *Passer domesticus*, the house sparrow, an avian amplifying host thought to be of primary importance in the ecology of WNV.

These data demonstrated that WNV rapidly establishes widespread infection in the house sparrow. It seems likely that the pathogenesis from exposure to a low dose of WNV by the subcutaneous route would be representative of that in birds inoculated by the bite of WNV -infected mosquitoes, thought to be the most prevalent route of exposure in nature. In this experiment, exposure by the bite of *Aedes albopictus* infected with WNV was attempted, but unfortunately the mosquitoes neither probed in or engorged upon the sparrows. Subcutaneous inoculation of a low dose of WNV (1100 pfu/bird) by needle was instead employed as a model.

WNV was detected in all tissues and fluids tested from at least some birds, representing every major organ system save the endocrine system. WNV antigen was frequently identified by immunohistochemistry in pancreatic acinar cells and presumed steroid-producing cells in adrenal



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histosections from a variety of naturally-infected birds from new York tested by Steele *et al.* (19). Thus, endocrine organs may also be involved in WNV infection of house sparrows.

It has been conjectured that the skin is the initial site of replication for WNV in human beings (18). Indeed, skin was the first of the samples from which WNV was isolated in this study. Within 12 hours of infection, WNV had replicated in the skin of two of three birds to titers exceeding the inoculum dose by over an order of magnitude. Of those tissues tested, skin was the most frequently infected, even more so than blood. Virus was isolated from all skin samples harvested at least 24 hours after infection at fairly high titers (range $10^{4.3}$- $10^{10}$ pfu/gm tissue). Komar *et. al.* reported the isolation of WNV from skin samples of two of three surviving birds sacrificed later in the disease process, days 8 and 10 following experimental inoculation by the bite of WNV-infected *Culex tritaeniorhynchus* mosquitoes (10). Collectively, these findings suggest that skin may be an excellent indicator of WNV infections in sparrows for use in dead bird surveillance. Furthermore, samples of skin can be obtained quite easily and do not involve penetration of the body cavity or central nervous system, minimizing risk of exposure to laboratory workers.

WNV was isolated from intestinal tissues and oral swabs only sporadically. Komar *et al.* saw similar variability in WNV positivity and content in cloacal swabs harvested from mosquito-inoculated house sparrows. These data imply that intestinal and salivary organs are not consistently infected in this species of bird. Further, cloacal and oral swabs from crows have been suggested for use in dead bird surveillance (9), but from house sparrows these samples likely lack the positive and negative predictive values desirable for surveillance.

The presence of WNV in the central nervous system (CNS) was detected as early as 24 hours postinfection, and occurred with some regularity in those samples harvested at later timepoints. It is of note that of the 13 birds in which CNS involvement was detected, virus was



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isolated from both the brain and caudal spinal cord of nine individuals. The remaining four birds had measurable levels of virus in only brain (n=1) or caudal spinal cord (n=3) tissue samples. The lack of a consistent pattern in the distribution of WNV in the CNS indicates the unlikelihood of a single anatomic region serving as a point of viral entry into the CNS of house sparrows.

No morbidity or mortality occurred in any bird in this experiment, whereas three of six WNV -infected house sparrows died on days 3, 4, and 5 postinfection in the study by Komar *et al.* (10). If our study had been extended beyond day 5.0, it would be expected that some birds would succumb to the extensive infections we detected at euthanasia. The virus isolation data suggest that the infection may have been running more severe or mild courses in certain birds. For example, at 12 hours postinfection, bird 4 lacked detectable virus in skin whereas cohort birds had significant titers, and bird 11 was the only bird to suffer invasion of the CNS at day 1.

It is evident that virus isolation data are not sufficient to thoroughly characterize the events of early pathogenesis, and investigation of these samples by routine light microscopy and immunohistochemistry are anticipated. Further, the consistency and magnitude of WNV infection in the skin of house sparrows belies that the infection process in this tissue warrants intensive study. It has been hypothesized that WNV develops fairly long-lived infections in the skin of birds, and that high titers of WNV in skin might be adequate to infect feeding mosquitoes in aviremic birds (10). That the virus was observed to replicate aggressively in the skin of house sparrows indicates that vaccination able to induce immunity in this organ might prevent the establishment of WNV infection in these important amplifying hosts.



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## IV. Concluding Remarks

The importation of formerly-exotic WNV into the United States came as a surprise. Within only a few years, infections with both monkeypox virus and the etiologic agent of severe acute respiratory syndrome, a coronavirus, were detected in residents of the continental United States. The appearance of these foreign disease agents brought to life the warning disease experts have made for decades: the advent of ever-increasing globalization and world travel continue to expand the opportunities for, and heighten the probabilities of the transport of pathogens to distant locations. The emergence of both known pathogens in new locations, and of those not previously recognized in any area, should not be regarded as being theoretical, but rather as inevitable.

WNV was previously considered to be a tropical disease agent, yet has spread with alarming rapidity in North America and has readily established enzootic transmission cycles in a diversity of geographic and climactic conditions. Additionally, strains of WNV causing recent epizootics in both the New and Old World have greater neuroinvasiveness than previously documented for the virus. Variants circulating in Israel and North America inflict dramatic morbidity and mortality in a wide range of birds, also considered to be a virtually unrecognized manifestation of WNV infection. Together these findings underscore the dynamic nature of infectious agents and the necessity of obtaining a foundation of scientific understanding, of preemptively developing management strategies, and of constant vigilance by those in the medical and agricultural professions.



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The work I performed under the mentorship of, and alongside Dr. Richard A. Bowen DVM, MS, Ph.D. was primarily an attempt to contribute to the basic understanding of the neurovirulent, avian pathogenic strain of WNV that has emerged, and become enzootic, in North America. Shortly after the detection of WNV in New York, the Centers for Disease Control and Prevention and the United States Department of Agriculture organized a meeting to develop strategies for the surveillance, management, and research of the virus. Among the most pressing research aims were to "determine virus, vector, and vertebrate host relationships." Experiment I, Experimental Infection of Dogs and Cats with WNV, addressed this objective in companion animals. The close interaction of pet dogs and cats with human beings presented the possibility of interspecies transmission of this zoonotic virus, the large and widespread population of pet and feral animals in the continental United States brought concern they might have involvement in the ecology of WNV, and tremendous amounts of public concern arose regarding the effects and associated risks of this virus in their pets. Another research priority specified was to "study viral pathogenesis in humans and birds." When house sparrows were identified as having considerable or even primary importance as amplifying hosts of WNV in the United States, we designed Experiment II, Early WNV Pathogenesis in *Passer domesticus,* to obtain very basic yet heretofore unknown information.

I have also been fortunate to have had the opportunity to be involved, albeit more peripherally, in a considerable variety of additional studies not described in this dissertation. Perhaps most importantly, through my studies I have obtained an understanding of the necessity of communication, cooperation, and collaboration among veterinary and human medical professionals, public health officials, and basic scientists. The disciplines pursued by these groups certainly do not stand alone from a scientific standpoint; in terms of awareness of and preparedness for emerging infectious disease, they must not remain distinct in practice.



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## V. Acknowledgements

A large number of people have been instrumental in each of these projects. Carl Mitchell, Michel Bunning, Chad McFadden, Brent Davis, Jeff Chang, Paul Gordy, Max TeeHee, and Melissa Rouge were involved with the dog and cat work. Thanks are due to Nick Panella, who was essential in the sparrow project. Finally, I am appreciative of the help of Jennifer MacLeay, Ann Wagner, Stargate Farm, and Ann Westhoff in the equine WNV vaccine study.

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## VI. Appendix A: Characterization of neutralizing antibody response in equids following vaccination with a conventional killed West Nile vaccine

To: examine the magnitude and duration of the neutralizing antibody response of previously nonimmunized horses vaccinated with an inactivated, adjuvanted West Nile vaccine.

Ten horses, designated group A, were bled by jugular venipuncture immediately prior to intramuscular vaccination with West Nile virus vaccine (Fort Dodge Animal Health) and at 1 and 2 weeks thereafter. Group A horses received a booster vaccination between 22-29 days after the first does of vaccine, as per the manufacturer's recommendation, and were bled at the time of the booster and at 14-18 days and 4 weeks after the booster. An additional 20 horses, group B, received a booster injection 28 days after initial vaccination. Group B horses were bled at the time of the booster and at 1 month (4 weeks), and 3 and 6 months after. Sera from both groups were stored at –70C prior to assay for neutralizing antibody (Nt Ab) content via 90% plaque reduction neutralization test (PRNT) using West Nile virus (WNV) strain New York 1999 in Vero cell cultures.

One of 10 group A horses had detectable Nt Ab one week after vaccination, 3/10 at two weeks, 3/10 at the time of booster vaccination, and 8/10 at 2 and 4 weeks following the booster (Figure VIII). Of group B horses, 4/20 had detectable Nt Ab at the time of booster vaccination, 13/20 at 4 weeks after the booster, and 9/19 at 13 weeks (Figure VIII).

Twenty-one of 30 total horses had detectable Nt Ab 4 weeks following booster vaccination with West Nile virus vaccine. At 3 months post-booster, 9/19 had detectable Nt Ab, with decreased titers in 8/9. At 6 months post booster, 7/17 horses had detectable Nt Ab, with further



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decline in titers in 4/7. These data suggest that frequent vaccination with West Nile Virus Vaccine may be necessary for protection of horses.

Figure VIII. WNV titers in sera of horses following administration of West Nile Virus Vaccine.

| Horse | | Titer at time | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Post 1° vaccination | | | Post booster | | | |
| | | 0 | 1 week | 2 weeks | Booster | 2 weeks | 4 weeks | 3 months | 6 months |
| Group A | A1 | <5 | 40 | 160 | 80 | 160 | 80 | *[a] | * |
| | A2 | <5 | <5 | 5 | 5 | 640 | 320 | * | * |
| | A3 | <5 | <5 | <5 | <5 | <5 | <5 | * | * |
| | A4 | <5 | <5 | <5 | <5 | 160 | 80 | * | * |
| | A5 | <5 | <5 | <5 | <5 | 5 | 5 | * | * |
| | A6 | <5 | <5 | <5 | <5 | 5 | 5 | * | * |
| | A7 | <5 | <5 | <5 | <5 | <5 | <5 | * | * |
| | A8 | <5 | <5 | <5 | <5 | 80 | 160 | * | * |
| | A9 | <5 | <5 | 40 | 40 | 160 | 320 | * | * |
| | A10 | <5 | <5 | <5 | <5 | 20 | 20 | * | * |
| Group B | B1 | * | * | * | <5 | * | 5 | <5 | s[b] |
| | B2 | * | * | * | <5 | * | <5 | <5 | <5 |
| | B3 | * | * | * | 40 | * | 80 | 20 | 10 |
| | B4 | * | * | * | 160 | * | 160 | s | |
| | B5 | * | * | * | <5 | * | <5 | <5 | s |
| | B6 | * | * | * | <5 | * | <5 | <5 | <5 |
| | B7 | * | * | * | <5 | * | 160 | 80 | 20 |
| | B8 | * | * | * | <5 | * | 160 | 80 | 20 |
| | B9 | * | * | * | <5 | * | 40 | 5 | <5 |
| | B10 | * | * | * | <5 | * | <5 | <5 | <5 |
| | B11 | * | * | * | 5 | * | 40 | 10 | 10 |
| | B12 | * | * | * | <5 | * | 20 | 5 | 5 |
| | B13 | * | * | * | <5 | * | <5 | <5 | <5 |
| | B14 | * | * | * | 5 | * | 5 | 5 | <5 |
| | B15 | * | * | * | <5 | * | 40 | 20 | 20 |
| | B16 | * | * | * | <5 | * | <5 | <5 | <5 |
| | B17 | * | * | * | <5 | * | 5 | <5 | <5 |
| | B18 | * | * | * | <5 | * | 10 | <5 | <5 |
| | B19 | * | * | * | <5 | * | <5 | <5 | <5 |
| | B20 | * | * | * | <5 | * | 40 | 20 | 5 |

[a] sample not taken

[b] horse sold prior to this sampling



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## VII. Appendix B: Functional Characterization of Bovine, Canine, and Human Mx Proteins

Mx proteins are GTPases whose production is induced by type I interferons. They have been found in a phylogenetically broad range of vertebrates, including fish, birds, and mammals. Some members of the Mx family of proteins have been demonstrated to confer strong antiviral resistance *in vitro* and/or *in vivo*. Surprisingly little work has been done to investigate the mechanism of action or antiviral spectrum of these innate immune components.

Our laboratory has identified and sequenced two canine and three bovine Mx genes. Two of the bovine Mx genes, bovine Mx1 and Mx1a, were determined to be alleles of one another. Significant differences in nucleotide and presumptive amino acid sequences suggest that differences in antiviral potency or spectrum may exist. Thus, inheritance of Mx genes may influence natural disease resistance in cattle. Virtually nothing is known about the third bovine Mx gene, designated bovine Mx2, and two canine genes, canine Mx1 and Mx2.

In contrast, human proteins MxA and MxB have received a modest amount of research attention. It appears that MxA has fairly broad antiviral activity against negative sense RNA viruses, while MxB has been devoid of effect against any of the viruses studied. Human MxA was selected for further characterization, and for comparison of its activity to that of the canine and bovine Mx proteins.

Constitutive expression of Mx proteins has proven problematic in our laboratory and those of other investigators. Thus, a transient transfection system using Lipofectamine (GibcoBRL)



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was employed to obtain high-level expression of Mx proteins in Vero cells. As with all transient expression systems, only a fraction of the treated cells show expression of the exogenous gene, resulting in a mixed population of Mx expressing and non-expressing cells. Sparse monolayers of these mixed Vero cells were infected with the following viruses: vesicular stomatitis New Jersey, influenza A, Sindbis, Cache Valley, and bluetongue. Cells were incubated with virus suspensions in DMEM for 30 minutes at 37C to permit viral adsorption, then rinsed twice with PBS and incubated for an additional time at 37C with Dulbecco's modified Eagle's medium supplemeted with 5% FBS. The total incubation time from initial exposure to virus until fixation was: 6 hours for bluetongue and vesicular stomatitis viruses, 7 hours for Cache Valley virus, and 22 hours for bluetongue virus. Incubation periods were designed to terminate infection at an active stage, but prior to death of infected host cells. Following incubation, cells were fixed with paraformaldehyde and permeablilized with triton-X 100. Finally, differential indirect or direct immunostaining of viral antigen and Mx was employed with antibodies labeled with fluorescein or rhodamine. Monolayers thus transfected, infected, fixed, and stained were observed by fluorescent microscopy. Cells were classified as Mx positive or negative, and positive or negative for viral antigen, creating a total of four categories. For each experiment, at least 50 and up to 100 Mx-positive cells, and roughly 1000 Mx negative cells were counted. Those cells equivocal for staining of either Mx or viral antigen were not included.

If Mx protein interferes with viral infection steps up to and including translation of viral proteins, infected cells expressing Mx would be expected to have only low fluorescence under the appropriate filter for detection of immunostaining of viral antigen. Overall infection rates of nontransfected cells varied between 42 and 96% among experiments. The same proportion of cells expressing Mx would be expected to have been exposed to infectious virus as those not expressing Mx. Thus, proportions of infected populations of cells not expressing Mx protein and



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Mx-expressing cells were compared to appreciate the effect of the presence of Mx on viral protein expression (Figures X – XIV). For those cases in which Mx lacked effect on the expression of viral antigen, the data points lie along a line extending from the abscissa with a slope of one. For those situations in which Mx-expressing cells appeared to have a decreased incidence of expression of viral antigen, the slope of the line along which the data points lie is less than one. In no experiment did expression of Mx seem to potentiate the expression of viral antigen, however, such data points would lie along a line with a slope greater than one. Results are summarized qualitatively in Figure IX.

Figure IX. Qualitative measurement of effects of Mx proteins on the expression of viral antigen.

| | virus | | | | |
|---|---|---|---|---|---|
| Mx protein | Sindbis | bluetongue | Cache Valley | influenza A | vesicular stomatitis-NJ |
| human MxA | 1 | 1 | 1 | 2 | 3 |
| bovine Mx1 | 2 | 1 | 1 | 2 | 3 |
| bovine Mx1a | 1 | 1 | 1 | 2 | 3 |
| bovine Mx2 | 3 | 2 | nt | 2 | 3 |
| canine Mx1 | 1 | nt | nt | 1 | 3 |
| canine Mx2 | nt | nt | nt | 1 | 3 |

1 no or mild inhibition
2 moderate inhibition
3 marked inhibition
nt not tested

These experiments reveal that Mx proteins from dogs and cattle have antiviral activity, as has been reported for proteins from other species. Inhibition of protein expression from negative sense influenza A and vesicular stomatitis viruses was observed for all Mx proteins tested, with the exception of canine Mx2. It has long been assumed that Mx proteins do not impede replication of positive-sense RNA viruses, but these experiments suggest that at least one Mx, bovine Mx2, interferes with the expression of viral proteins from at least certain positive-sense viruses, thus also presumably with production of infectious progeny.



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Figure X. The effect of Mx proteins on the expression of Sindbis virus antigen.

| Mx protein | % Mx+, viral Ag+ | % Mx-, viral Ag+ | sample size Mx+ cells |
|---|---|---|---|
| Human MxA | 67 | 72 | 100 |
| | 57 | 55 | 53 |
| Bovine Mx1 | 67 | 77 | 100 |
| | 33 | 51 | 100 |
| Bovine Mx1a | 66 | 74 | 100 |
| | 54 | 57 | 100 |
| Bovine Mx2 | 28 | 78 | 100 |
| | 11 | 42 | 61 |
| Canine Mx1 | 51 | 54 | 100 |

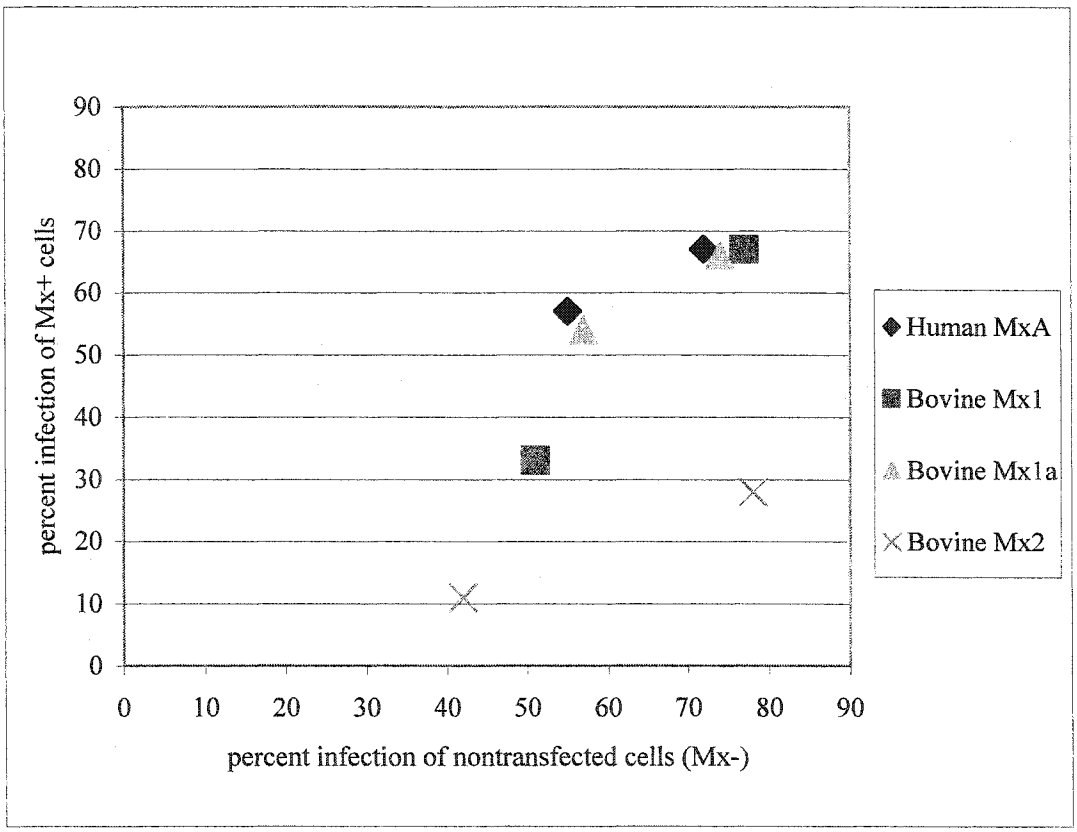




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Figure XI. The effect of Mx proteins on the expression of bluetongue virus antigen.

| Mx protein | % Mx+, viral Ag+ | % Mx-, viral Ag+ | sample size Mx+ cells |
|---|---|---|---|
| Human A | 90 | 81 | 100 |
| | 52 | 52 | 100 |
| Bovine Mx1a | 86 | 80 | 100 |
| | 63 | 53 | 100 |
| Bovine Mx1 | 72 | 73 | 100 |
| | 58 | 60 | 100 |
| Bovine 2 | 44 | 76 | 100 |
| | 46 | 53 | 68 |
| | 37 | 73 | 100 |
| | 34 | 52 | 100 |

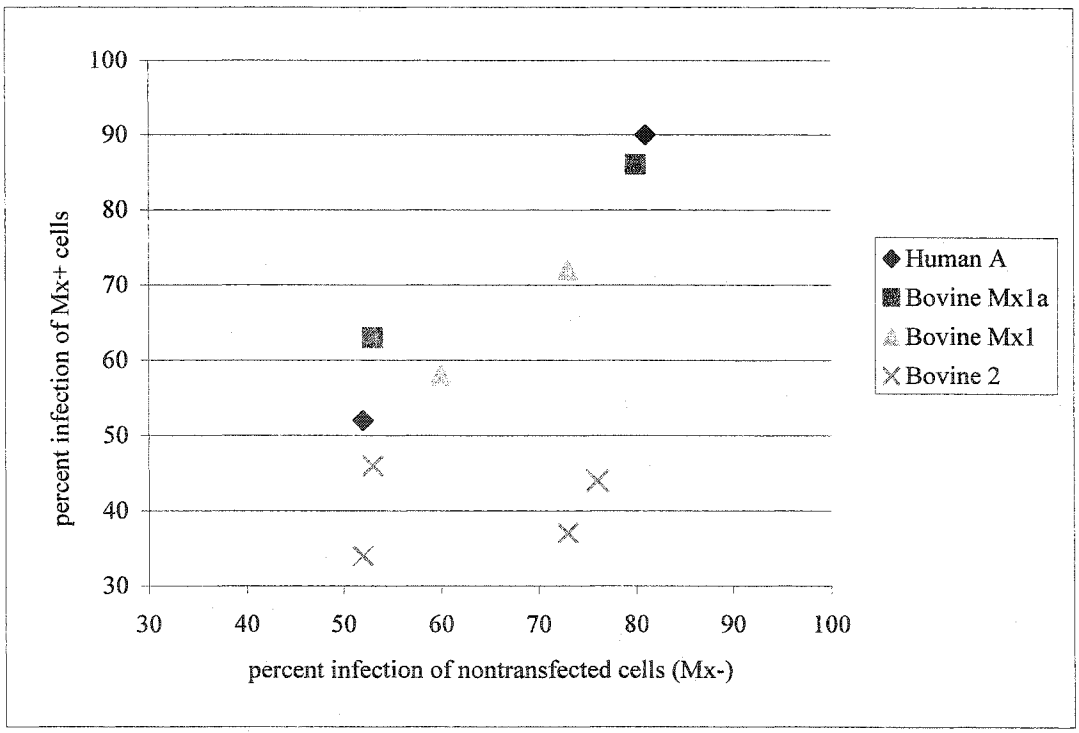




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Figure XII. The effect of Mx proteins on the expression of Cache Valley virus antigen.

| Mx protein | % Mx+, viral Ag+ | % Mx-, viral Ag+ | sample size Mx+ cells |
|---|---|---|---|
| Human MxA | 51 | 43 | 100 |
| | 48 | 52 | 100 |
| Bovine Mx1 | 32 | 43 | 100 |
| | 39 | 44 | 100 |
| | 67 | 72 | 100 |
| Bovine Mx1a | 43 | 48 | 100 |
| | 13 | 44 | 100 |
| | 52 | 69 | 58 |
| | 27 | 45 | 100 |

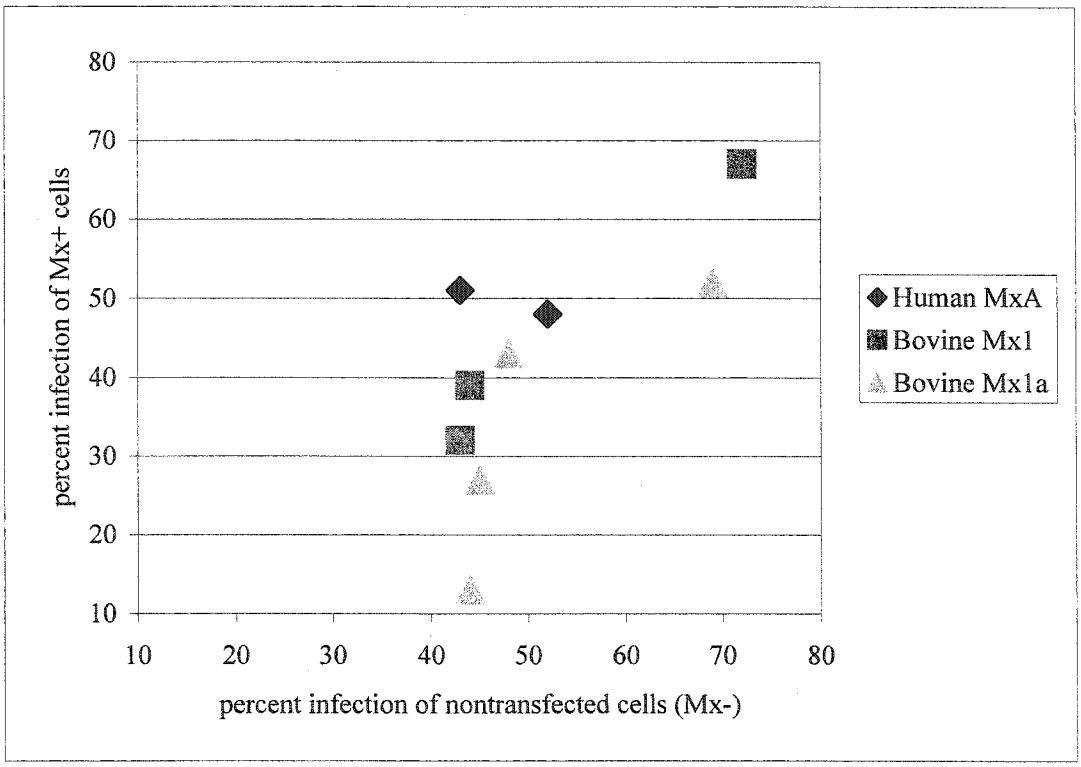




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Figure XIII. The effect of Mx proteins on the expression of influenza A virus antigen.

| Mx protein | % Mx+, viral Ag+ | % Mx-, viral Ag+ | sample size Mx+ cells |
|---|---|---|---|
| Human MxA | 25 | 53 | 76 |
| | 20 | 79 | 100 |
| | 3 | 46 | 100 |
| Bovine Mx1 | 51 | 71 | 89 |
| | 21 | 48 | 100 |
| Bovine Mx1a | 14 | 48 | 100 |
| | 18 | 46 | 100 |
| Bovine Mx2 | 25 | 46 | 57 |
| | 8 | 70 | 100 |
| | 15 | 82 | 100 |
| Canine Mx1 | 56 | 61 | 100 |
| | 57 | 85 | 100 |
| Canine Mx2 | 48 | 52 | 100 |
| | 38 | 42 | 100 |

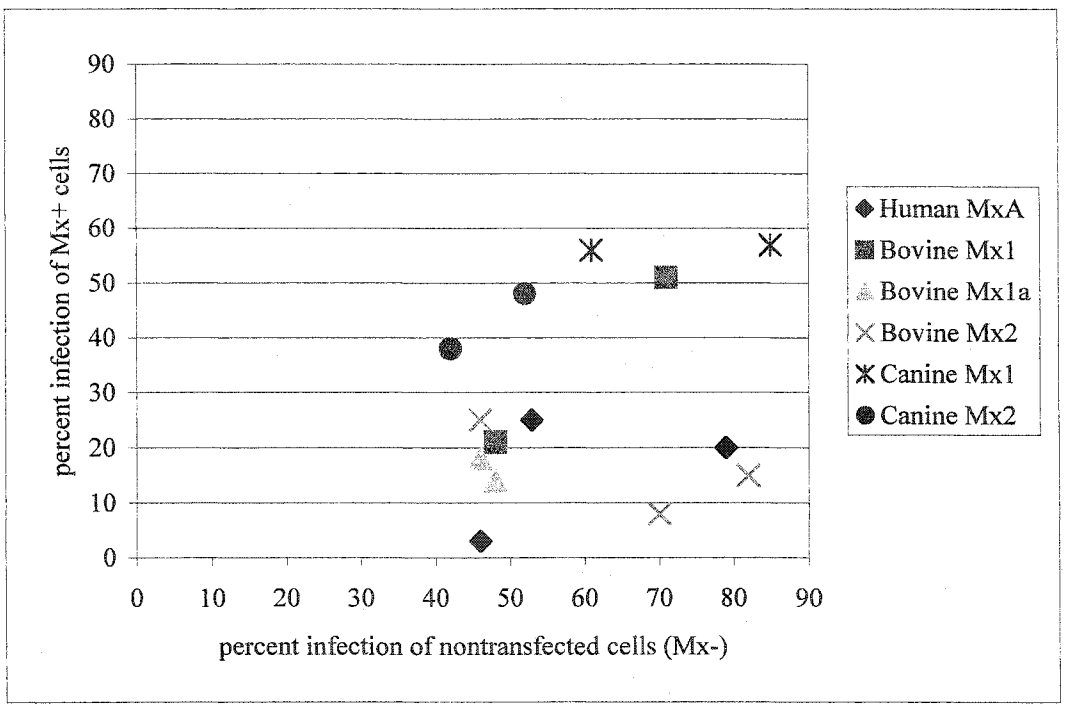




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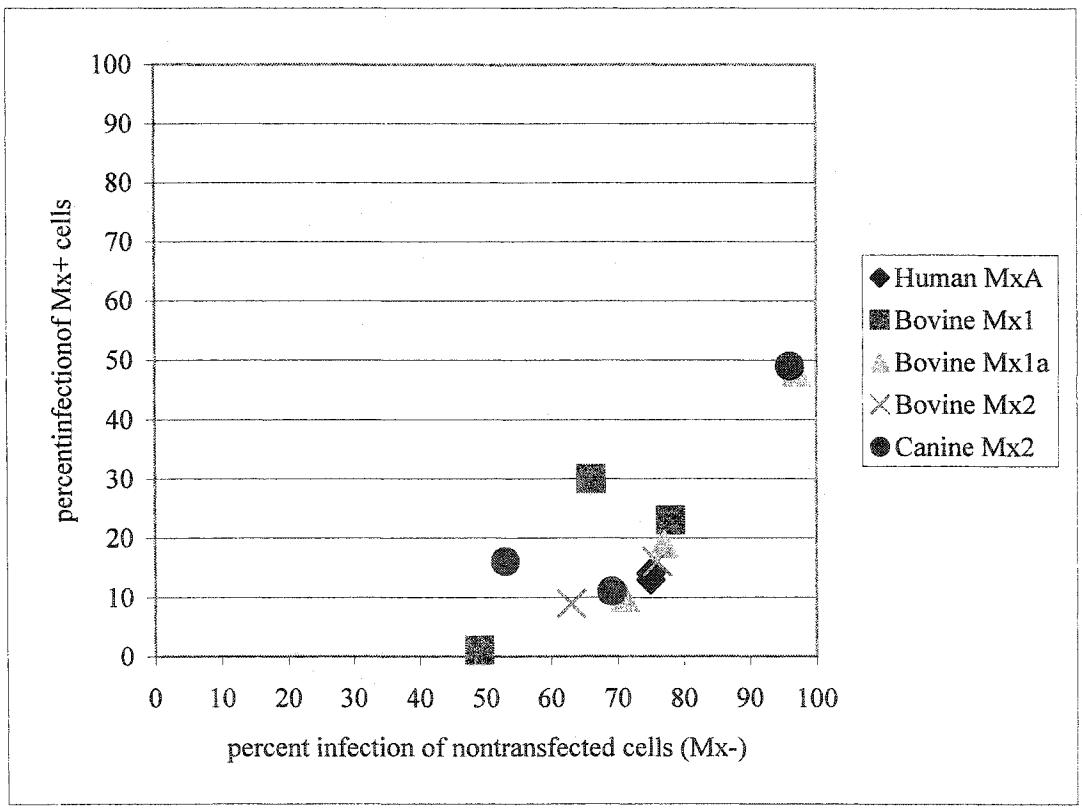
Figure XIV. The effect of Mx proteins on the expression of vesicular stomatitis New Jersey antigen.

| Mx protein | % Mx+, viral Ag+ | % Mx-, viral Ag+ | sample size Mx+ cells |
|---|---|---|---|
| Human MxA | 14 | 75 | 100 |
| | 13 | 75 | 100 |
| Bovine Mx1 | 23 | 78 | 100 |
| | 30 | 66 | 77 |
| | 1 | 49 | 100 |
| Bovine Mx1a | 19 | 77 | 100 |
| | 10 | 71 | 100 |
| | 48 | 97 | 100 |
| Bovine Mx2 | 16 | 76 | 50 |
| | 9 | 63 | 69 |
| Canine Mx2 | 11 | 69 | 100 |
| | 49 | 96 | 63 |
| | 16 | 53 | 95 |



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