**Local Update**

**Can West Nile Virus Outbreaks be Controlled?**

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West Nile virus fever is a zoonotic disease. In recent years WNV has caused outbreaks of febrile illness in humans and equines in Europe and North America, and in the last year in humans and in geese flocks in Israel. The most serious manifestation of infections is fatal encephalitis in humans, horses, and domestic and wild birds. Bird-feeding mosquito species are the principal vectors of WNV. The main reservoir of the virus in nature seems to be wild birds and domestic fowls. Public attention was drawn to the virus and its control when WNV appeared in New York City in 1999 causing the deaths of several elderly people. So far, no effective vaccine has been developed.

This review summarizes basic information on WNV outbreaks – past and recent, global and local – and on the pathogenesis of the virus, its diagnosis, and possible control measures.

**The infectious agent – West Nile virus**

WNV was first isolated in Uganda in 1937 from the blood of a febrile woman [1]. West Nile virus is a member of the Japanese encephalitis serogroup of the genus flavivirus (which includes also the St. Louis encephalitis virus) of the family Flaviridae. The flavivirus genus is subdivided into nine serogroups [2], encompassing about 60 species that are distributed around the world (another flavivirus endemic in Israel is the Israel turkey meningoencephalitis virus, which belongs to the Ntaya virus group). These viruses can infect a wide range of vertebrates including birds and mammals, and are mostly transmitted by arthropods – namely mosquitoes or ticks [3].

Structurally, the flavivirus’ virion is an enveloped 30–80 nm diameter spherical particle. The genome is a positive-stranded RNA associated with a core protein (nucleocapsid). The envelope is a host-derived membrane that contains the viral envelope protein [4]. This protein bears the group, type and the species-specific neutralization epitopes.

**Transmission cycles**

The virus is maintained in nature by two replication cycles – one in mosquitoes and the other in birds [Figure 1]. Infected mosquitoes carry virus particles in the salivary glands and infect susceptible particles during their blood-meal. The infected birds exhibit a long lasting high level viremia, vital to...
the start of a new infection in feeding mosquitoes. Other incidental hosts, like humans and horses, do not develop high (enough) viremia levels to serve as an effective reservoir. Although most vertebrates – including mammals, amphibian and reptiles – are susceptible hosts to WNV in experimental infections, their natural infection is considered accidental and of no real importance for virus perpetuation in nature [3,5].

An important requirement for the endemization of the virus is its trans-ovarian transmission in mosquitoes that enables its persistence over the winter season. A combination of several factors is needed to start the amplification cycle of the virus, leading to an epidemic. Among them are a high proportion of infected birds, a dense population of bird-feeding mosquitoes, and a susceptible human population.

**Distribution and epidemics**

WNV is widely distributed throughout Africa, the Middle East, parts of Europe and the former USSR, India and Indonesia. Recently WNV was introduced into North America for the first time. In endemic areas most residents are infected during childhood, and up to 60% of the population may be carrying anti-WNV antibodies. In regions where the virus is less active, occasional epidemics occur among people of all ages. There have been several recent epidemics of WNV encephalitis in humans: in Algeria (1994), Rumania (1996–97), the Czech Republic (1997), the Democratic Republic of Congo (1998), Russia (1999), and the USA (1999) [6–13].

**WNV in Israel**

WNV is endemic in Israel. The virus was isolated from both humans [14,15] and mosquitoes [16]. WNV outbreaks occur in Israel periodically, and the outbreaks of the 1950s [15], 1981 [17] and 1999/2000 [18] were documented. In the 1981 outbreak, 12 of 49 positive diagnosed individuals developed encephalitis and 4 of them died [17]. During this summer’s outbreak WNV has already taken the lives of 23 elderly people of the approximately 300 patients diagnosed as WNV positive. A recent survey for the prevalence of anti-WNV antibodies in Israel showed that about 40% of adults aged 45–55 years, but only 7% of adults aged 18–19 years were positive for WNV antibodies [19]. According to these data the majority of Israelis today do not have antibodies against WNV.

Antibodies to WNV were found in domestic and wild animals and fowls in Israel [20,21]. As in other countries [10,11,22], WNV has affected horses in Israel as well. Serological tests in horses manifesting neurological disorders indicated that WNV was the causative agent [21]. The current epidemic is unique in that WNV was introduced into domestic geese flocks [18,23], causing losses to the goose industry.

**The disease in humans**

Infection in humans is usually sub-clinical. The symptomatic infection can vary in severity from temporary fever (resembling a flu-like syndrome) to serious encephalitis. The disease is generally mild in children and more severe in the elderly. The incubation period is usually 1 to 6 days. Onset of the disease is sudden, with fever, severe headache, lymphadenopathy, myalgia and sometimes rash. The duration of the febrile illness is usually 3–6 days. The incidence of central nervous system involvement has not been clearly defined, but this complication appears to be rare. No specific therapy for patients is known and the treatment is mainly supportive [3,7].

**The disease in animals**

Among the domestic animals horses are very susceptible to the virus, which causes severe illness, sometimes resulting in paralysis and death. The mortality rate in horses resulting from the outbreaks in Morocco [10], Italy [11] and the USA [22] was reported to be around 45%. In birds, the mortality rate is high (100% for crows and 40% for geese) [13,18,23–26].

**Pathogenesis**

Flaviviruses exhibit two distinct pathogenic properties that are required for the development of encephalitis. The first is the capacity to establish a lethal infection within the central nervous system. This neurovirulence, an inherent property of most flaviviruses, involves the ability to replicate in the CNS and to cause an acute cytolysis in neurons if introduced directly into the mammalian CNS. The second is neuroinvasiveness, which is the entry into the CNS from peripheral sites. This property is less inherent and is exclusively associated with virulent strains. Thus, most virulent strains of flaviviruses exhibit both properties: neurovirulence and neuro-invasiveness. Natural infections are presumed to involve spreading from peripheral sites to the CNS by viremia, suggesting entry across the blood-brain barrier [27–29].

The pathogenesis of WNV is similar to that of other flaviviruses. Many isolates of WNV were shown to be neuro-invasive in mice, indicating their virulent trait(s) [17,30,31]. In humans, pathologic studies were limited to a very small number of patients with fatal encephalitis and CNS = central nervous system
show lesions of diffuse inflammation and neuronal degeneration. WNV was isolated from the spleen, liver and lung of those who died from WNV complications [32].

**Laboratory diagnosis**

Laboratory diagnosis of WNV can be performed by two different methods: virus isolation during the acute phase of the disease, or detection of specific antibodies. Virus isolation is achieved by inoculating mice (or tissue cultures) with blood obtained from patients (up to 4 days after onset of clinical symptoms) and detecting the replicating virus by immunofluorescence of the infected tissue. Direct determination of the virus in the blood can be done by detection of WNV-specific RNA sequences or by polymerase chain reaction techniques.

Serological diagnosis is based on the comparison of acute and convalescent paired sera by hemaggutination inhibition, complement fixation or neutralization tests [24]. Seroconversion obtained in one of these tests indicates a recent infection. An enzyme-linked immunosorbert assay was developed in our laboratory and used for detection of specific anti-WNV immunoglobulins in human sera [33]. Rapid diagnosis can be made by the detection of IgM-specific antibodies that often appear within a few days of onset of symptoms and persist for 1–2 months.

**Control measures for WNV outbreaks**

There are several ways to control virus spread and minimize its effect on domestic animal and human populations. These include preventive measures to avoid exposure to bird-feeding mosquitoes, prevention of mosquitoes breeding in freshwater reservoirs and untreated sewage ditches and ponds, and immunization of domestic fowls.

**Vector control**

Although the target for eradication is the virus itself, the lack of a vaccine – veterinary or human – makes mosquitoes the obvious target for control measures. Large-scale global campaigns for mosquito eradication have been carried out in connection with another flavivirus, namely dengue virus [34,35]. Although the vector for this virus – *Aedes* Spp. – is different from the vectors for WNV (*Culex* Spp.), some of the conclusions reached following these campaigns may bear relevance to the situation in Israel. Vector control should be based on the surveillance of vector abundance. The traditional larval survey (for the dengue virus vector) was demonstrated to be unsatisfactory. Adult mosquito surveillance could thus reinforce control decisions. Furthermore, since the adult stage is the infective stage, studying the fluctuation of adult abundance and survival as influenced by climate and environmental factors is of great importance. Another aspect to be considered is the behavioral trends of the mosquito populations. In a study conducted in four Central America countries [35], outdoor fumigation was ineffective against *Aedes aegypti*. This was due to the endophilic behavior of the mosquito.

In order to achieve effective control of the vector it is essential to survey and study the current populations of mosquitoes in Israel, their behavior, and their susceptibility to insecticides.

**Vaccine**

In an attempt to develop an anti-WNV vaccine we used an Israeli virus isolate to generate an attenuated variant strain that can be used as a veterinary live vaccine. Our original WN virus, WNI, was isolated in Israel from the blood of a patient during the febrile phase of the disease [15]. In mice the WNI strain is both neurovirulent and neuro-invasive. Flaviviruses were shown to undergo a significant decrease in virulence to mice following either serial passages in mosquito cells

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**Table 1. Neurovirulence and neuro-invasiveness of wild-type and attenuated WNV strains in ICR mice**

<table>
<thead>
<tr>
<th>Virus</th>
<th>PFU/ml*</th>
<th>Mouse LD&lt;sub&gt;50&lt;/sub&gt;/ml</th>
<th>IC/IP</th>
<th>PFU/LD&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MDTD***</th>
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<td></td>
<td></td>
<td></td>
<td>IC</td>
<td>IP</td>
<td>IC</td>
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<tr>
<td>WNI</td>
<td>9.0x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>8.4x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.1x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>4.0</td>
<td>1.1</td>
</tr>
<tr>
<td>WNI-25</td>
<td>1.1x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.1x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>&lt;3.2x10&lt;sup&gt;1&lt;/sup&gt;**</td>
<td>&gt;3.4x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>10.0</td>
</tr>
<tr>
<td>WNI-25A</td>
<td>6.5x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>4.9x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>&lt;3.2**</td>
<td>&gt;1.5x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>13.0</td>
</tr>
</tbody>
</table>

Groups of mice were inoculated IC or IP with serial dilutions of virus stock solutions (1:10 dilution of WNI-25 virus and concentrated WNI-25A stock solutions were used as the highest virus concentration inoculated IP). Deaths were recorded daily and mouse LD<sub>50</sub> (IC and IP) were determined.

* PFU titrations were carried out on Vero cell monolayers
** No virus was recovered from the brains of ICR mice during the period of 5–10 days post-inoculation.
*** Mean days to death (MDTD) following IC inoculation of 10<sup>5</sup> PFU/mouse.
[36] or following selection of escape mutants by using neutralizing monoclonal antibodies [29,37].

We used both methods sequentially to generate an attenuated stable non-invasive WN virus strain that may serve as a candidate for vaccination. *Aedes aegypti* mosquito cell line was persistently infected with WNI. Repeated subcultures of the infected cells resulted in the emergence of a WN virus variant that produced small plaques on BHK (baby hamster kidney) cell monolayers. Since the mouse is the experimental model of choice, for the study of the pathogenicity of flaviviruses in mammals, we have monitored the change in virus virulence in mice. Most of the flaviviruses will cause death upon intracranial inoculation to mice, but only virulent viruses will induce encephalitis and cause death upon peripheral, e.g., intra-peritoneal inoculation, due to their neuro-invasion trait(s). The LD50 ratio IC/IP of a given virus stock is indicative of virulence and is low for virulent viruses. Following attenuation the LD50 IC/IP ratio is much higher. At the 25th sub-culture passage, the virus caused no morbidity or mortality in mice when inoculated intraperitoneally with a dose of 1.1x105 plaque-forming units. This is a reduction of at least 5 orders of magnitude in virulence, since the parental WNI has an IP LD50 of 4.3 PFU [Table 1]. This variant was plaque purified and designated WNI-25. WNI-25 was later reacted with a neutralizing monoclonal antibody and an escape mutant, WNI-25A, was isolated and plaque purified. Schematic presentation of the variants' isolation procedure is shown in Figure 2.

During the attenuation process we followed the change in IC/IP ratio of the different virus stocks. The IC/IP ratio for the parental strain is 4.0, while for the attenuated strain WNI-25 the IC/IP ratio is 3.4x10^5 (or higher) and for the second stage attenuant WNI-25A the ratio is 1.5x10^6 (or higher) [Table 1].

The nucleotides’ sequence of the wild type and the attenuated strains’ envelope protein genes were determined [38]. We found that two changes could be correlated to the attenuation. The first is the addition of a glycosylation site due to a change of tyrosine at position 155 to asparagine. The second is the change of lysine at position 307 to glutamic acid, which occurred during the Mab A selection step.

Live attenuated vaccine can induce protection even after a single inoculation. While this is an advantage over inactivated vaccine preparations, a major concern with live vaccines is the possible reversion to virulence. To study the genetic stability of the attenuated variants, the possible reversion to virulence *in vivo* was tested in two immunocompromised mouse systems. The first is the stress-induced-mouse model [39], the second is the genetically immunocompromised SCID mice. It was found that WNI-25 did revert to virulence while WNI-25A maintained its attenuation despite prolonged high level viremia [40].

The protective potency of WNI-25A was tested in two sensitive animal systems – ICR mice and young geese (20 days old). The immunization scheme was one dose of 10^5 PFU of WNI-25A inoculated peripherally. High titers of anti-WNI specific antibodies were observed in the immunized animals. Three weeks post-inoculation the immunized mice and geese were challenged IC with WNI or WN virus isolated from a naturally infected moribund goose respectively. The challenge dose was 10^3 IC LD50 and in both systems 100% protection was observed.

Both WNI-25 and WNI-25A are non-invasive attenuated variants of WNI that induce neutralizing antibodies, therefore they may be used as a potential veterinary vaccine. However, due to its genetic stability WNI-25A seems to be a more suitable candidate. In the stressed mice model, the virus WNI-25A did not invade the CNS following peripheral inoculation. In the immunocompromised mice (SCID) it did not revert to a WN-like virus even after massive replication. Thus WNI-25A was successfully used as a live vaccine to protect geese against a wild-type virulent WNV strain. It is interesting to note that this wild-type strain is a recent field isolate, almost 50 years apart from our original WNI isolate [15], and closely resembling the WNV isolated during the 1999 New York epidemic [13].

**Perspectives**

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**LD50** = lethal dose 50%, i.e., the amount of virus needed to cause mortality of 50% of inoculated mice

**IC** = intracranial

**IP** = intraperitoneal

**PFU** = plaque-forming unit

**Mab A** = monoclonal antibody

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From sporadic outbreaks restricted to endemic areas in the 1950s, infections caused by WNV have spread globally during the last few years to cause epidemics affecting humans and domestic animals. In addition, environmental and man-made factors have contributed to the increase in the mosquito vector population, which in turn affects virus spread.

The current WNV outbreak in Israel may have been the result of a combination of three factors: a) a rise in the vector population; b) introduction of the virus into a new member of the amplification cycle, namely the goose; and c) susceptibility (low antibodies prevalence) of the human population.

Obviously, the total eradication of WNV is impossible due to the continuous introduction of the virus by migratory birds. Since human encephalitis is relatively rare, the development of a human vaccine may not be commercially feasible [24]. The remaining alternatives to interfere with the amplification cycle are vector control and an anti-WNV veterinary vaccine. Effective vector control should be a continuous effort involving governmental and municipal authorities. Personal protective measures are also of great importance in this respect.

Currently there is no commercially available WNV vaccine [24]. Live attenuated vaccine has the advantage of being effective and relatively inexpensive. It is of interest to note that among flaviviruses a special phenomenon is believed to play some role in viral pathogenesis. Known as ADE (antibody-dependent enhancement), this phenomenon occurs in Dengue virus infection, where antibodies against one subtype enhance rather than neutralize the pathogenicity of a second infection by another subtype [28]. Therefore, the use of an attenuated strain of the same subtype as the epidemic virus as a live vaccine is preferable. This way a specific protection may be achieved rather than cross-protection [23]. We believe that WNI-25A is a good candidate for such a vaccine. It is highly attenuated, genetically stable, induces neutralizing antibodies in mammals (mice) and fowls (geese), and protects both species against intracranial challenge of a wild-type virus.

Another possible use for the attenuated strain is its introduction into the wild bird population, in a strategy similar to the immunization of wild foxes in Europe against rabies. If successful, such an introduction could reduce wild-type WNV circulation in nature.

In conclusion, the combination of both measures, namely, mosquito vector control and a veterinary effective vaccine, is needed to eliminate or reduce WNV outbreaks in Israel.

References
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