West Nile virus in Europe:

Host susceptibility, pathogenesis and intervention studies

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The research described in this thesis was performed at the Erasmus Medical Centre (EMC) in Rotterdam, the Netherlands. Part of the studies were conducted as part of collaborations and funded by the European Community's Seventh Framework Programme (FP7/2007-2013) under the project "VECTORIE", EC grant agreement number 261466.

ISBN/EAN: 978-94-91602-68-9

Cover design: Oil painting by George Lim, 2016. Based on: Mullard, A. *As the crow dies*. 2007. Nature Reviews Microbiology, 5:746-747.

Voor Julien

West Nile virus in Europe:

Host susceptibility, pathogenesis, and intervention studies

West-Nijlvirus in Europa:

Gastheer vatbaarheid, pathogenese, en interventie studies

Proefschrift

ter verkrijging van de graad van doctor aan de

Erasmus Universiteit Rotterdam

op gezag van de

rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 7 september 2016 om 11.30 uur

Stephanie Marsha Lim

geboren te Delft

Erasmus University Rotterdam

Erasmus

Promotiecommissie

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Chapter 1

General Introduction

Partially based on:

West Nile virus: Immunity and Pathogenesis

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Viruses, 2011, 3(6): 811-28

1. General

West Nile virus (WNV) is a neurotropic, arthropod-borne flavivirus with a single-stranded positive-sense RNA genome. It belongs to the Japanese encephalitis antigenic complex of the family *Flaviviridae*, which also includes other important human pathogens such as Japanese encephalitis (JEV), Dengue virus (DENV), and Tick-borne encephalitis virus (TBEV). WNV is maintained in an enzootic cycle between mosquitoes and birds, but can also infect and cause disease in mammals such as horses and humans, which serve as incidental dead-end hosts as viremia in these mammals is generally too low to infect mosquitoes [1].

The WNV genome is approximately 11 kb in length and translates into a single polyprotein, which is cleaved by host and viral proteases into three structural proteins (Envelope E, Pre-membrane/membrane prM/M and Capsid C), and seven non-structural proteins (NS1, NS2a, NS2B, NS3, NS4A, NS4B and NS5) (Figure 1). The E protein binds to the host cell surface receptor mediating viral entry and fusion, C encapsulates the viral RNA, and prM acts as a chaperone for E during intracellular virion assembly by masking and inactivating E to prevent fusion of premature virions. Non-structural proteins are implicated in viral transcription, translation, replication, maturation, and immune evasion [2].

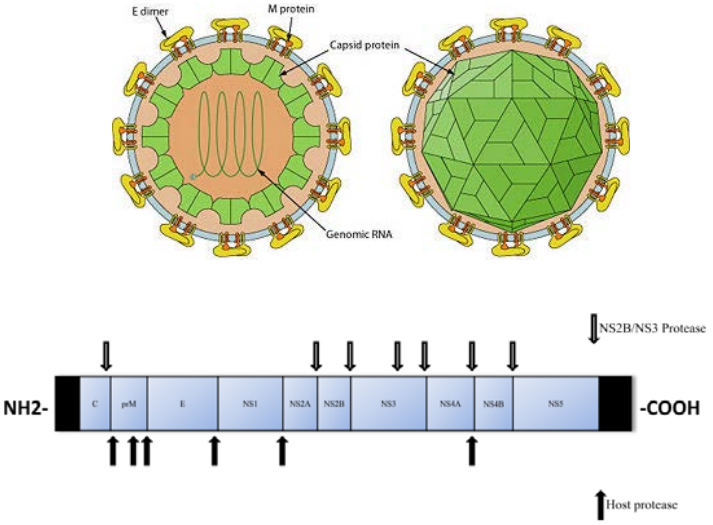

Figure 1. Structure and genome of West Nile virus
(credited to: http://www.expasy.ch/viralzone/all_by_species/24.html, and Valiakos et al., 2013)

Epidemiological studies indicate that frequency and severity of clinical illness increases with age [3, 4]. Infection with WNV remains asymptomatic in the majority of cases while it results in West Nile fever (a mild flu-like illness) in approximately 20 to 30% of infected individuals [3, 5, 6]. Symptoms are of sudden onset and may include malaise,

eye pain, headache, myalgia, gastro-intestinal discomfort and rash [5, 7-10]. In a small percentage (~1%) of cases, WNV-infected patients develop meningitis and/or encephalitis, which may result in acute flaccid paralysis (AFP) [3, 4], and long-term neurological sequelae are common in more than 50% of patients who develop encephalitis [11-15]. This disease manifestation is explained by neuronal damage in several regions of the brain. The fatality rate for hospitalized encephalitic cases is approximately 10%, with increased risk for patients with a compromised immune system, of young and old age, and with underlying conditions such as diabetes mellitus [16].

WNV was first identified in 1937 in a febrile patient in northern Uganda [17] and is currently one of the most geographically spread arboviruses in the world as it can be found on every continent except Antarctica [18]. WNV is endemic in parts of Africa, Europe, the Middle East, Asia [1], and since the turn of the century also in North America. In fact, it is only following its emergence in New York City in the United States in 1999 that WNV gained significant attention in the Americas. In late August of that year, an unusual cluster of encephalitis cases was reported to and investigated by the New York City Department of Health. At the same time, an epizootic among birds associated with a high fatality rate had been noted in and around New York City [19]. Sequencing of the virus isolated from brain tissue of some of these birds, as well as from three human encephalitis cases, all identified the same lineage 1 strain of WNV [20]. In total, 62 human cases were identified during this outbreak, including seven deaths [21], while retrospective extrapolation from a household based study estimated a total of 8200 subclinical infections and 1700 symptomatic infections. This was the first evidence of WNV activity in the Americas, and since the 1999 outbreak WNV spread across the country from east to west within 5 years, and is now reported from all over the Americas [22-24].

WNV can be divided into seven distinct genetic lineages [25], of which lineages 1 and 2 are the most frequently recognized. Lineage 1 can be divided into three subclades: WNV-1a harbors strains from all over the world, including North America and North Africa; the Australian Kunjin virus is part of subclade 1b, and a few Indian strains constitute subclade 1c. The majority of viruses belonging to **lineage 1** are grouped into a cluster called the "European Mediterranean/Kenyan cluster", while those responsible for outbreaks in Israel and in the New World can be grouped into the "Israeli/American cluster." The former cluster is made up of two subclusters, of which one subcluster includes strains isolated in the western Mediterranean basin (WMed; France, Italy, Morocco), while the other includes strains isolated in the eastern Mediterranean basin (EMed; Israel) and southeastern Europe (Romania, Volgograd) [26]. **Lineage 2**, on the other hand, was mainly present in sub-Saharan Africa and Madagascar, where it was considered to be non-pathogenic for humans and horses [27, 28]. More recently, however, more virulent lineage 2 strains have also been observed in South Africa and some European countries, such as Hungary, Austria, Greece, Romania, Italy, and Serbia, where it has demonstrated its capacity to cause severe clinical symptoms in humans, equines and wild birds [29-31].

West Nile virus can be considered an ecological generalist [32] with an exceptionally complex eco-epidemiology, as in North America it has been found to be transmitted by numerous species of mosquitoes and is able to infect a highly diverse vertebrate host range that includes mammals, amphibians, and reptiles [33], while a wide array of bird species act as primary hosts. Despite this wide host range, not all infected hosts can transmit the virus, and only those in which the virus replicates efficiently to reach sufficiently high titers in the blood are able to infect mosquitoes through blood feeding. This is called "host competence" and is a characteristic of each host species that is part of a specific host-virus-vector system [34]. In fact, competent hosts for WNV transmission are found almost exclusively among avian species [35], and a viremia level of $>10^5$ PFU/mL has been determined as the lower limit for efficient infection of feeding *Culex* (*Diptera: Culicidae)* mosquitoes found in the United States [36, 37]. As a result, avian hosts that are competent to transmit the virus to feeding mosquitoes are also often referred to as "amplifying hosts". However, this threshold appears to vary per mosquito species or geographical region, as for example, the threshold titer for *Cx. univittatus* in South Africa was reported to be $<10^{4.0}$ 50% suckling mice lethal doses ($SMLD_{50}$)/mL of blood, and $10^{4.6}$ $SMLD_{50}$/mL of blood for infection of *C. perexiguus* in Egypt [38], while some California populations of *C. tarsalis*, *C. pipiens*, and *C. erythrothorax* can be infected at titers of $<10^{5.0}$ PFU/mL [39]. Even though it is possible that titers below $10^{5.0}$ PFU/mL result in fewer infections, it stresses that for a better insight in the role of different species in transmission, reservoir competence studies should be conducted for a large range of mosquito and bird species from different geographical locations.

Bird mortalities among free ranging birds have been the hallmark of WN disease emergence in North America. Birds of at least 326 different species are known to have died from WNV disease [40], of which bird species belonging to the order Passeriformes have been found to be the most susceptible, with the family *Corvidae* being most affected. In fact, deaths among American crows (*Corvus brachyrynchos*) have been used to track the spread of the virus across many parts of North America [41-43]. In contrast, wild bird mortality events are very infrequent in the Old World, with small, sporadic episodes affecting few individual birds, and are often detected in wildlife rehabilitation centers [44]. Despite the proposition of several hypotheses, an explanation for the differences in wild bird mortality in Europe as compared to North America is still pending.

Among arthropods, WNV replicates in a wide range of mosquitoes, also called "vectors", which are considered to serve as a source of (re-)infection of humans, and are able to sustain a pathogen when it is not infecting humans. WNV has been found to infect up to 59 different species of mosquitoes in the US [45]. Similar to vertebrate hosts, not all infected mosquito vectors are able to transmit the virus efficiently, as the ability of transmission to occur after biting a host is dependent on whether the virus is able to replicate systematically and reach a high enough viral titer in the salivary glands. This so-called "vector competence" applies to each vector species in a particular virus-vector system [34]. *Culex* mosquitoes, and especially the ornithophilic ones (e.g. *Cx. pipiens*), play

an important vector role. However, some non-*Culex* (non-ornithophilic) species, such as *Aedes albopictus*, have been found positive for WNV antigen in a field light trap [46], and were also identified as competent vectors in the lab [47], but their role in transmission is still unclear. More importantly, however, at least 12 mosquito species have been implicated as potential 'bridge' vectors or epidemic vectors, which are considered responsible for transmission to humans [48, 49]. Indirect evidence based on blood-meal analysis and theory suggest that *Cx. pipiens* may serve as both an enzootic and an epidemic 'bridge' vector [50, 51]. However, most mosquito studies have so far been conducted using North American mosquitoes, and it is likely that even within the United States vectors that are most important for transmitting WNV to humans differ per region. As a result, especially for European mosquitoes, research efforts should continue to focus on studying vector competence, as well as feeding preferences and behavior.

Interestingly, other arthropods such as ticks have also been found to be susceptible to WNV infection, as the virus has been isolated from both soft and hard ticks [52-57] and *Ornithodoros moubata* ticks became infected and were able to transmit the virus to rodent hosts in the lab [58]. Even though ticks are unlikely to play a major role as vectors in the transmission of WNV, these findings suggest that some species still have the potential to act as a source of infection ("reservoir host") [58]. As a result, it is likely that this complex eco-epidemiology, which involves hundreds of different vectors and hosts per location, has contributed to the broad geographical range of WNV.

2. West Nile virus in Europe

The first indication of the presence of WNV in Europe was in 1958, when specific WNV antibodies were detected in two Albanians [59]. The first isolations of the virus in Europe were then recorded in 1963 from patients and mosquitoes in the Rhône Delta [60] and from patients and *Hyalomma marginatum* ticks in the Volga Delta [61, 62]. Cases of WN fever were observed in the 1960s in southern France [63], southern Russia [64] and Spain [65], southwestern Romania [66], as well as in the 1970s, 1980s and 1990s in Belarus [67], western Ukraine [68], southeastern Romania [4, 69] and Czech Republic [70]. Despite this, however, before the mid-1990s, WNV was only sporadically detected in humans.

The first major outbreak of WNV, which consisted of a high case fatality rate in humans (~10%), occurred in Romania in 1996 [4, 71]. Since then, the virus has been isolated from horses, humans and mosquitoes, in several Eastern and Western European countries [72, 73]. However, with the exception of an outbreak among horses in Camargue, Southern France in 2000 [74], and a few cases that occurred in Spain [75], significant WNV activity in Europe has mostly been concentrated in Italy and in southeastern and eastern Europe rather than in Western Europe.

Detailed phylogenetic analysis of the lineage 1 viruses that had caused the later outbreaks indicated that those which had been isolated around the western Mediterranean

were caused by a single strain, referred to as the WMed subtype, and that this virus was a single introduction that had overwintered for a number of years [76]. It was speculated that this sub-lineage was transferred between Mediterranean countries by viremic birds, resulting in the initiation of new outbreaks. A second closely related sub-lineage included viruses isolated from Romania and Russia, while a more divergent sub-lineage was responsible for outbreaks in Israel (and North America). It is likely that each sub-lineage represents a separate introduction of WNV into Europe from Africa [31].

WNV lineage 2 was first detected in Europe in 2004 and was isolated from the brain of a goshawk (*Accipiter gentiles*) in Hungary [28]. Retrospectively, a human case of WNV lineage 2 infection was confirmed to have occurred in Russia in the same year [77]. Following surveillance of dead birds of prey, especially goshawks, between 2004 and 2009, led to repeated isolations of the virus across Hungary [30]. Outbreaks of WNV lineage 2 subsequently occurred in a number of other European countries, including Austria, Greece, Romania, Serbia and Italy, of which the outbreak in Greece was particularly severe. The virus was first detected there in 2010 [78] in northern Greece, which, in contrast to the emergence in Hungary, developed into a significant human epidemic. The majority of cases were reported west of the city Thessaloniki, between the rivers Axios and Aliakmonas. In total, 262 patients were recorded, with 65 classified as West Nile fever and 197 as neurological cases, of which 33 died [79]. Subsequent surveillance resulted in the isolation of WNV lineage 2 from mosquitoes, giving rise to the Nea Santa-Greece 2010 strain [80], and from wild resident birds [81]. Retrospective serology in humans suggests that this virus, or at least a closely related one, had been circulating in Greece for several years prior to the first human cases of WN fever [78]. This seropositivity was at a low level of <1%, however, and was not accompanied by reports of disease. Following the 2010 outbreak, further epidemics of WNV lineage 2 infections in Greece occurred during the late summer of both 2011 and 2012 [82, 83]. Sequencing of viruses detected in humans has confirmed that the same virus strain was present in both humans and wildlife in each subsequent year, suggesting endemic persistence of WNV lineage 2 in Greece. So far, this strain appears to cause disease in humans, and infects wild avian species as determined by serological and molecular surveillance [81], but with relatively few confirmed reports of disease in equine species [31] and few confirmed deaths among birds.

WNV lineage 1 has been present in Italy since at least 1998, when the virus was first detected during an outbreak in horses, in which 14 animals had tested positive for WNV, with 6 fatal cases [84]. Subsequent evidence of seroconversions in sentinel horses and chickens was found in different risk areas under serosurveillance in the following years, but no infections in humans were recorded. The first human cases of neuroinvasive disease appeared a decade later affecting eight provinces in three Northern regions of Italy (Emilia Romagna, Veneto, Lombardy) in September-October 2008 [85, 86], where a total of 794 cases of WNV infection in 251 equine stables were detected on the basis of clinical signs and as a result of serological screening [87]. In addition, wild birds captured or found dead, including European magpies (*Pica pica*), carrion crows (*Corvus corone*), rock pigeons

(*Columba livia*) and Eurasian jays (*Garrulus glandarius*) were found positive by RT-PCR [87-89], and nine human cases of WNV, which included four characterized by neuroinvasive disease, were detected in 2008 [90-92]. In 2009, a new epidemic emerged in the same area as that of the 2008 outbreak, but with new foci of infection in Central Italy. Since then, WNV lineage 1 has become endemic in Italy, and is often detected in humans, horses, wild birds, mosquitoes and sentinel chickens, with most WNV epidemic activity localized in the northeastern part of Italy [86].

Interestingly, the first case of a WNV lineage 2 infection in Italy, in the coastal town of Ancona on the Adriatic Sea, was reported in 2011 [93], a year after the first detection in Greece. Shortly after this, six cases of neurological disease resulting from a WNV lineage 2 infection were reported in Sardinia between September and October, 2011 [94], and mosquito surveillance also detected a lineage 2 WNV strain in *Cx. pipiens* mosquito pools and in a collared dove (*Streptopelia decaocto*) in northern Italy [95], where lineage 1 has been circulating since 2008 [86]. WNV lineage 2 in Italy continued to be detected in subsequent years, including in more human cases [86, 95]. The presence of a lineage 2 strain in the same area where a lineage 1 strain is still circulating could potentially create a new scenario with unpredictable consequences [95].

3. Virulence

The virulence of WNV is mostly associated with its capacity to invade the nervous system (neuroinvasiveness), its ability to infect neurons and spread within the CNS (neurotropism), and its tendency to cause disease through infection of the nervous system (neurovirulence). Even though a neurovirulent virus is usually neuroinvasive, it does not always indicate that the virus is neurotropic [96] as it may cause disease by triggering immunopathologic responses. Furthermore, neuroinvasiveness does not always lead to neurovirulence [96] as a virus may infect the brain without causing disease. As a result, the virulence of WNV appears to be quite complex and WNV strains may therefore possess varying degrees of virulence, which may be caused by different underlying mechanisms.

3.1. Molecular basis of virulence

The ability of WNV to persist and cause disease within the host largely depends on its ability to infect target cells and evade recognition by the immune system. Particular biological aspects of WNV facilitate its capacity to cause severe disease. Firstly, WNV is able to productively infect diverse cell populations from many animal species, which suggests the use of multiple and/or well-conserved receptors [97-103]. Secondly, *in vitro*, WNV is cytolytic and induces apoptosis in a variety of cell types, most importantly neurons [104, 105]. There are several cases in which WNV has been found to cause neuronal necrosis *in vivo*, in for example, wolves [106], large falcons [107], raptors [108], squirrels [109], dogs [110], horses [111] and humans [112]. Studies have indicated that individual

WNV proteins may contribute to virus-mediated cytotoxicity and may therefore function as virulence factors. For example, *in vitro* expression of NS3 or capsid protein induced rapid, caspase-dependent apoptosis, and *in vivo* capsid protein expression led to cell death [113, 114]. Further details on mechanisms of cell death will be discussed below, in the section Pathogenesis.

Genetic variation also influences WNV virulence. Several mutations have been found to increase or reduce virus pathogenicity via various mechanisms. Of these, some appear to have a major impact on the replication mechanisms of WNV. Beasley et al. found that mutations in the envelope protein at residues 154 to 156, which abolished the N-linked glycosylation motif (N-Y-S/T), attenuated virus pathogenicity for mice. In fact, it was hypothesized that the enhanced virulence of North American strains of WNV compared to Old World lineage 1 strains was at least partly related to this envelope protein glycosylation [115]. They in particular appear to alter the protein such that it cannot be recognized by oligosaccharyl-transferase, resulting in a loss of glycan [116]. This glycosylation motif is common to various flaviviruses and spatially it is located close to the center of the fusion peptide of domain (D) II (which is one of the three structural domains (DI-III) of the E protein [117, 118]), and has therefore been proposed to increase the stability of the protein [119, 120], including at high temperatures [121]. Beasley et al. therefore hypothesized that the decreased neuroinvasive potential of non-glycosylated variants of WNV is due to their lower stability, which results in less infectious virus in the periphery. This dose of "infectious virus" additionally contains a larger proportion of noninfectious virus, which acts as an inactivated antigen that can significantly stimulate innate and adaptive immune responses. Nonetheless, it is also possible that E glycosylation may have an effect on other aspects of the WNV replication cycle, such as target cell tropism, virion assembly and release, and efficient fusion of E protein with target cell membranes [115].

Glycosylation was also found to be important for virus replication in a bird model as E glycosylated WNV variants inoculated into young chicks were more virulent and replicated to higher viremia titers than non-glycosylated variants. Furthermore, glycosylated variants also showed more heat-stable propagation in mammalian (BHK) and avian (QT6) cells. Interestingly this phenomenon was not seen in mosquito (C6/36) cells [121]. E-protein glycosylation may therefore also be a requirement for efficient transmission of WNV from avian hosts to mosquito vectors.

Contrastingly, however, when Moudy et al. mutated the E protein glycosylation site from NYS to IYS in a full-length clone of the NY99 genotype, which resulted in a virus lacking the glycan at aa154 (WNV-N154I), they found that this mutant replicated less efficiently than WNV-WT in *Culex* mosquito tissues, although the decrease was more profound in *Cx. pipiens* than in *Cx. tarsalis*. Furthermore, following peroral infection, mosquitoes infected with WNV-N154I were less likely to transmit the virus than those infected with the WT [122]. The contradicting results between the studies by Murata et al. and Moudy et al. may be due to the fact that the former generated non-glycosylated variants by specifically mutating aa156 to NYP for one variant, and to NYE for another variant,

while the latter mutated the site from NYS to IYS. It is therefore possible that very specific differences in amino acid sequence have an effect on viral propagation and dissemination in vector mosquitoes.

Interestingly, multiple amino acid changes at the first glycosylation motif in the NS1 protein also attenuated mouse neuroinvasiveness. Specifically, mutants lacking glycosylation at either two or three of the three NS1 glycosylation sites (residues 130, 175, and 203) induced lower viremia and decreased lethality in mice [123]. Additionally mutating all three amino acids of the $NS1_{130-132}$ glycosylation motif (NTT-QQA) gave the most attenuated strain out of several mutants harboring different mutations in the NS1 glycosylation motif [124]. It therefore appears that substituting amino acids in WNV E as well as in NS1 glycosylation sites can result in a virus with a highly attenuated phenotype. A follow-up study using confocal and transmission electron microcroscopy (TEM) found that the lack of attenuation is due to a lack of NS1 glycosylation that blocks efficient replication, maturation, and NS1 secretion from the ER, resulting in changes to the virus-induced ultrastructure [125].

Most of the virulent lineage 1 strains, as well as the recent virulent lineage 2 strains associated with the Greek outbreak possess the N-glycosylation site, which could implicate it as a prerequisite for the efficient circulation and amplification of the virus in a mosquito-avian transmission cycle [81, 126]. However, the role of the NS3 protein in the propagation of virus between birds and mosquitoes can also not be excluded. The NS3 protein contains a serine protease at the N-terminal and the RNA helicase, an NTPase and an RTPase at the C-terminal. Mutations in this protein have so far been found to have the most profound effect on the virulence of WNV in birds. Specifically, the introduction of a T249P in an attenuated Kenyan strain was found to be sufficient to generate a phenotype highly virulent to American crows, while a P249T introduction in the virulent NY99 strain resulted in an attenuated phenotype [127]. The H249P mutation in the Greek strain that caused the major WNV disease outbreak in 2010-2011 in Greece, where it was detected in mosquito pools, corvids and chickens, is considered a contributor to the observed increase in virulence of this lineage 2 strain, as all other lineage 2 strains contain a histidine at this position [126, 128]. It is therefore possible that both the N-glycosylation site and the presence of a proline at NS3-249 play a (simultaneous) role in the emergence of virulent strains of WNV.

The exact mechanism through which the NS3-249P mutation would increase the pathogenicity of WNV is unknown. It is possible that the proline mutation enhances RNA helicase function, resulting in an increased replication rate, leading to high viremia titers that may help surpass the bird viremia threshold required for infection of many mosquito species vectors ($> 10^5$ PFU/mL) [129]. Brault et al. did observe that the helicase activity on the uwinding of short dsRNA by alternative WNV NS3-249 helicase proteins differed between amino acid substitutions (249A, 249D, 249H, 249P, 249T); however, they found that the proline and threonine point mutations separated >80% of the dsRNA strands, while the NS3-249 proteins containing the alanine and histidine residues unwound more of the

dsRNA strands (>95%). As a result, these differences did not correlate well with the *in vivo* effects observed in American crows, where the 249P mutant replicates to higher viremia titers [130]. However, further helicase analysis under more biologically relevant temperatures (< 43ºC) [131] may provide more insight in this matter.

WNV has also evolved certain strategies to avoid and/or attenuate innate and adaptive immune responses. The whole family of *Flaviviridae* is largely resistant to the antiviral effects of IFN as soon as cellular infection is established. In particular the flavivirus nonstructural proteins NS2A, NS2B, NS3, NS4A, NS4B and NS5 have been found to disable IFN-induced responses at multiple stages within the cell by delayed IRF-3 activation and IFN-β gene transcription, as well as by impairing JAK1 and Tyk2 phosphorylation [132-138]. In particular for WNV, the ability to control the host IFN response has been linked to the replication fitness and virulence of lineage 1 and 2 strains, as a pathogenic lineage 1 Texas isolate actively antagonized IFN signaling, whereas an attenuated lineage 2 strain from Madagascar did not have this ability [139]. However, the reduced replication and virulence of the lineage 2 isolate was restored in cells and mice lacking the IFN-α/βR. It therefore appears that inhibition of type I IFN responses are a key feature in the evolution of pathogenic WNV strains. Consistent with this, a mutation (A30P) in the NS2A of the Kunjin subtype WNV strain was found to reduce the ability of the virus to inhibit the IFN response, leading to increased levels of IFN synthesis in weanling mice, as well as attenuated neurovirulence [140]. Interestingly, introducing this mutation into a North American (lineage 1) strain did not lead to significant changes in phenotype, indicating that in many cases the effect of particular mutations can be virus strain-specific [141].

Several other non-structural proteins have been implicated in virulence. For example, a study has shown that substitution of cysteine with serine at position 102 of NS4B (Cys102Ser) leads to a high temperature-sensitive phenotype as well as attenuation of the neuroinvasive and neurovirulent phenotypes in mice [142]. The first 125 amino acids of the N-terminal of the NS4B protein of flaviviruses appear to be sufficient for the inhibition of IFN-α/β signaling [138], and a mutation located in this region of WNV may therefore interfere with the ability of the virus to inhibit IFN signaling.

Another mutation in NS4B, E249G, also attenuated viral pathogenicity, as recombinant E249G virus exhibited smaller plaques, slower growth kinetics, and lower RNA synthesis than the WT *in vitro*, with the greatest difference in rodent cells (C3H/He and BHK-21) and the least difference in mosquito cells (C6/36). In addition, mice experienced no lethality upon inoculation with this virus [143]. A study examining an NS4B-P38G mutation also observed no lethality in a mouse model, as well as a lower level of viremia. Furthermore, mutant NS4B-P38G infected in cultured bone marrow-derived dendritic cells (DCs) exhibited a reduced replication rate but a higher level of innate cytokine production than WT WNV. This was shown to be related to the induction of higher innate and adaptive immune responses in mice, with specifically higher type I IFNs

and IL-1β levels and stronger memory and effector T cell responses. It therefore appears that the NS4B-P38G mutant strain induces faster and higher protective innate and adaptive immune responses in mice, which results in a lower viremia and no lethality [144].

Theoretically, amino acid changes that are considered to have a potential effect on the secondary structure of the proteins include substitutions of hydrophilic to hydrophobic amino acids or vice versa, and also substitutions of cysteine, glycine, and proline residues [145]. A study examining complete genome sequences of lineage 2 WNV strains isolated from patients in South Africa that had suffered mild or severe disease recognized that these kind of substitutions at NS3 (S160A and R298G), NS4A (A79T) and NS5 protein (T614P, M625R, M626R) were present in strains of high virulence [146]. They therefore concluded that mutations in the NS proteins encoding viral replication and protein cleavage mechanisms are the most likely determinants of differences in virulence.

Flaviviruses have a low-fidelity RNA-dependent RNA polymerase that leads to the generation of quasispecies. This is because viral RNA polymerases exhibit characteristically low fidelity mutation rates of approximately 10^{-4} mutations per nucleotide copied, which is much greater than those of nearly all DNA-based viruses and organisms [147-149]. Given the large population sizes observed in both experimental and natural infections, it has been estimated that every possible point mutation and many double mutations are generated during each viral replication cycle and may therefore be present within the population at any given time [150]. Even a defined molecular clone will rapidly transform into a collection of related sequences when introduced into cells. This collection is termed the quasispecies and is organized around a master sequence [151].

WNV has also been demonstrated to exist as quasispecies in nature. WNV was sampled from ten infected birds and ten infected mosquito pools during the peak of the 2003 transmission season in New York State. Analysis of the E and NS1 revealed that WNV infections are derived from a genetically diverse population of genomes, with WNV sequences in mosquitoes being significantly more diverse compared to those in birds. Non-consensus clones obtained from two avian specimens were genetically highly similar, which suggests that WNV genetic diversity may be maintained throughout the enzootic transmission cycle, rather than arising independently during each infection [152].
In a follow-up study, the mutant spectra that arose as a result of 20 serial *in vivo* passages in *Cx. pipiens* and young chickens was examined in order to determine the impact of mosquitoes and birds on intra-host WNV population dynamics. Genetic diversity was found to be greater in mosquito-passaged WNV compared to chicken-passaged WNV. Furthermore, mortality in mice was significantly negatively correlated with the size of the WNV mutant spectrum, in the sense that the more variable mosquito-passed strains were less pathogenic to mice while the genetically more homogenous chicken-passed strains were as virulent as the parental unpassed WNV. Together these studies suggest that mosquitoes serve as sources for WNV genetic diversity, that birds are selective sieves, and that both the consensus sequence and the mutant spectrum contribute to the WNV

phenotype [153]. It is also tempting to speculate that the antigenic variation exerted by quasispecies allows escape from antibody-mediated neutralization [154], as it is possible for strains with mutations at the dominant neutralizing epitope in DIII to emerge [155].

3.2. Host genetic determinants of WNV susceptibility

A small number of genetic risk factors have been linked to increased susceptibility to WNV, and may explain why a certain number of individuals may progress to paralysis, meningitis, and/or encephalitis after WNV infection. In inbred mouse strains, a truncated form of the gene for OAS1b has been mapped to susceptibility to infection by flaviviruses [156, 157]. For humans, a recent study with 33 WNV-infected patients showed an increased frequency of an OAS splice-enhancer site that could give a dominant-negative protein [158]. Furthermore, a study by Lim et al. suggests that a hypomorphic allele of the *OAS1* human ortholog is associated with both symptomatic and asymptomatic WNV infection [159]. However, an actual analogous deletion in humans linked to severe WNV infection has not been identified yet.

Experiments in animals have established that chemokines direct leukocytes to the brain to clear WNV from infected neurons and promote survival, as a genetic deficiency of the chemokine receptor CCR5 or the chemokine CXCL10 in mice was associated with depressed leukocyte trafficking, increased viral burden, and enhanced mortality [160, 161]. Interestingly, a study examining CCR5Δ32 (a relatively common mutant allele of the chemokine receptor CCR5) in WNV-infected cohorts found a greater incidence of CCR5Δ32 homozygosity in symptomatic and lethal WNV cases. In fact, even though only about 1% of the general United States population is homozygous for the CCR5Δ32 allele, 4%-8% of individuals with laboratory-confirmed symptomatic WNV infection were homozygous for the mutant allele. These studies therefore suggest that CCR5 may function as an essential host factor for the resistance of neuroinvasive WNV infection [162]. Ultimately this may also have implications for the use of CCR5 antagonists in HIV therapy, as it could imply that HIV patients are more susceptible to WNV.

4. Pathogenesis

Despite the significance of central nervous system (CNS) pathology in severe disease, the mechanisms by which WNV and other encephalitic flaviviruses cause neurological signs and symptoms *in vivo* have not been completely elucidated. The increased risk for immunosuppressed patients seems to suggest that an intact immune system is essential for the control of WNV infection. Even though peripheral immune responses to WNV can prevent encephalitis, up to 40% of immunocompetent animals infected with a virulent strain of WNV develop lethal neuroinvasive disease [163, 164]. As a result, the involvement of the immune system in the pathogenesis of such cases cannot be excluded.

Most of the knowledge regarding the mechanisms of WNV dissemination and pathogenesis is derived from studies using rodent models. It is believed that after a mosquito bite WNV infects keratinocytes [165] and Langerhans cells (LC), of which the latter migrate to regional lymph nodes where initial replication occurs [166-170]. WNV then spreads systemically to visceral organs, such as the kidney and spleen, where a second round of replication takes place, presumably in epithelial cells and macrophages respectively [171]. WNV can then enter the brain, probably via the blood-brain barrier (BBB) or another unconfirmed mechanism (as discussed below), and cause meningo-encephalitis. The envelope glycoprotein of WNV has been implicated in neuroinvasiveness, particularly DIII of the protein, which constitutes the receptor binding domain [172-174].

It is interesting to note that several other flaviviruses are known to cause neuroinvasive disease, but little is known about the pathogenic mechanisms. Studies of flaviviruses that rarely cause neuroinvasive disease may contribute to a better understanding of the mechanisms involved in viral entry of the brain. For instance, infection with DENV, one of the most important arboviruses of humans, may result in severe systemic disease, manifested as haemorrhagic or shock syndrome [175], but this virus is generally considered non-neurotropic. Recent observations, however, indicate that the clinical profile of DENV infection may be changing, as neurological manifestations are becoming more frequent [176, 177].

4.1. Crossing the Blood-brain-barrier (BBB)

The BBB is a diffusion barrier that impedes the influx of compounds from blood to brain. It is made up of three cellular elements, namely endothelial cells on the capillary basement membrane (BM), astrocyte end-feet that ensheath the vessels, and pericytes (PCs) that are embedded in the BM. Tight junctions (TJs) are present between the cerebral endothelial cells and form the diffusion barrier, which selectively prevents most blood-borne substances from entering the brain. Lastly, the CNS is populated by three different glial cell populations: (1) astrocytes, (2) microglia, and (3) oligodendrocytes, which form the immune system in the brain.

In mice, WNV crosses the BBB and infects the CNS after peak viremia (around day 3) [178]. How WNV is able to enter the CNS is not completely understood, but several mechanisms have been proposed: (i) infection or passive transport through the endothelium or choroid plexus epithelial cells (Wang, 2004, hypothesized only), (ii) infection of olfactory neurons and spread to the olfactory bulb [179-181], (iii) a “Trojan horse” mechanism in which the virus is transported by infected immune cells trafficking to the CNS [99], and (iv) direct axonal retrograde transport from infected peripheral neurons [182, 183]. Even though the cited studies provide indications that such mechanisms may indeed be employed by the virus to enter the brain, the majority of the studies do not provide actual clear evidence in support of these hypotheses, as the majority of the conclusions are based on an over-represented amount of *in vitro* experiments that lack adequate *in vivo* validations

as support. As a result, several gaps remain in the hypothesized routes of WNV neuroinvasion and need to be investigated further; in addition to the investigation of alternative routes of neuroinvasion that have thus far received little attention, such as the role of the arachnoid (meningeal barrier) [184].

In fatal human cases, WNV is most often detected in neurons in the cerebral cortex, thalamus, brainstem, basal ganglia, cerebellum, and spinal cord (mainly anterior horn), and in some cases, infection has been detected in the olfactory bulb and hippocampus [185] (Figure 2). WNV has been detected in the same regions of the brain of experimentally infected mice as in humans, indicating a similar tropism of WNV in humans and mice [184]. Histological analysis of samples from fatal human cases also provided evidence of gliosis, indicating involvement of microglial cells and astrocytes during WNV infection. Overall, *in vitro* experiments have shown that WNV can infect primary neurons, human and mouse neuroblastoma cells [104, 105, 186], human brain cortical astrocytes (HBCA), human brain microvascular endothelial cells (HBMVE) [187, 188], and oligodendrocytes [189], while infection of microglia resulted in low viral yield [187]. So far, animal experiments have only shown infection of neurons by WNV [103, 112, 190, 191] and have provided limited evidence of *in vivo* glial cell infection.

A study performed by Verma et al. using HBMVE cells and HBCA, showed that several matrix metalloproteinases (MMPs), which are produced by monocytes and glia cells and appear to be involved in the migration of leukocytes to the perivascular space as well as migration through the glia limitans [192], were significantly induced in WNV-infected HBCA cells [188]. Incubation of naïve HBMVE cells with the supernatant from WNV-infected HBCA cells resulted in loss of tight junctions. These data provided evidence that astrocytes represent a source of MMP in the brain, which may lead to disruption of the BBB. Degradation of components of the glia limitans is another mechanism facilitating migration of leukocytes into the brain parenchyma. Collagen (a component of glia limitans) could be degraded by extracellular proteases such as the cysteine protease cathepsins K, S, and L [193, 194], whereas conversion of plasminogen into plasmin may lead to degradation of laminin or fibronectin, other important components of the glia limitans [193, 195]. When the integrity of the BBB is compromised, immune cells may enter the brain, thereby contributing to WNV viral clearance and immune mediated damage.

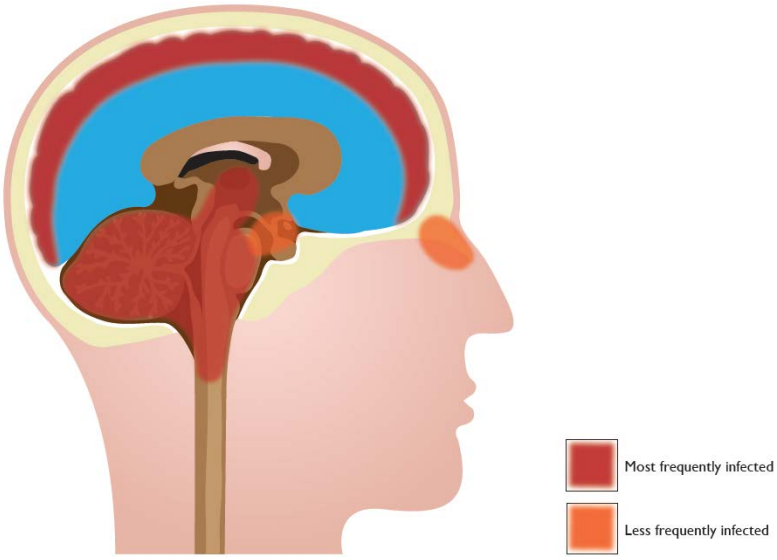

Figure 2. Frequency of infection of several regions of the human brain by West Nile virus (Lim al., 2011)

4.2. Mechanisms of Cell Death upon Infection

Programmed cell death can be considered a defense mechanism of the host in response to pathogenic insults. Pathogens may induce cell death to the host either by direct infection of host cells (e.g. cytolytic viruses) or by releasing toxic products (e.g. bacterial toxins). Cell death has been generally divided into necrosis, which is accidental, uncontrolled cell death resulting in an inflammatory response, and programmed cell death, a regulated and controlled process that has traditionally been considered to be non-inflammatory. Even though programmed cell death is often used as a synonym for apoptosis, it is more accurately described as cell death that is dependent on genetically encoded signals or activities within the dying cell [196, 197]. No cellular activity is required as acute cell breakdown occurs as a result of the direct action of a damaging stimulus, and programmed cell death is therefore only prevented by the absence of this stimulus [196].

Recent studies have revealed several pathways that lead to programmed cell death: apoptosis, pyroptosis, autophagy and oncosis [198]. Apoptosis is mediated by a subset of cysteine-dependent aspartate specific proteases, or caspases, which can be divided into two functional subgroups: initiator caspases (caspase-2, -8, -9, and -10) are mainly involved in activation of the effector caspases-3, -6, and -7, which cleave a variety of cellular substrates. Apoptosis involves nuclear and cytoplasmic condensation and formation of membrane-bound cellular fragments or apoptotic bodies. During viral infection, programmed cell death has an antiviral effect by inducing the death of infected cells. However, cell death can also have pathological effects if it occurs in non-renewing cell populations, such as neurons, or late in the process.

Many studies have shown that WNV induces replication-dependent apoptosis *in vitro*, and it has been hypothesized that virus-induced apoptosis contributes to neuronal death and the pathogenesis of encephalic flaviviruses [104, 114, 199, 200]. Experiments in mice have been performed to analyze the occurrence of apoptosis *in vivo*. Most studies used the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining, which detects DNA fragmentation, to confirm that apoptosis occurred in the CNS of WNV-infected mice.

Recently, evidence has been provided that the cell death of WNV-infected neurons is caspase-3 dependent [201]. It was shown that WNV infection induced caspase-3 activation and apoptosis in the brains of wild-type mice, while congenic caspase-3-/- mice were more resistant to lethal WNV infection. It is interesting to note that no significant differences in the tissue viral burdens or the kinetics of viral spread were found, but decreased neuronal death was observed in the cerebral cortices, brain stems, and cerebella of caspase-3-/- mice. Consistently, primary neurons derived from the CNS of wild-type mice showed caspase-3 activation and induction of apoptosis after WNV infection, and treatment with caspase inhibitors resulted in a significant decrease in virus-induced cell death. Nonetheless, a deficiency in caspase-3 did not completely protect neurons from WNV-mediated death *in vitro* or *in vivo*, indicating that caspase-3-independent pathways also contribute to WNV pathogenesis. For example, it is possible that the activation of non-caspase proteases, such as calphain and cathepsin family proteins, is also triggered during WNV infection [202, 203].

Yang et al. (2002) showed that direct expression of the WNV capsid protein in the striatum of mouse brain or interskeletal muscle caused cell death and inflammation [114]. Similar effects were observed in cultured, SH-SY5Y neuroblastoma cells, which could eventually be attributed to capsid-induced apoptosis occurring via the mitochondrial pathway, involving caspase-9 and caspase-3 activation. These studies suggest a role for the capsid protein of WNV in viral pathogenesis through the induction of the apoptotic cascade. No role for alternative cell death pathways, such as pyroptosis, parthanatos or necroptosis, has yet been described in the pathogenesis of WNV. However, WNV has been shown to induce necrosis *in vitro* in cells exposed to very high viral inocula [204]. More effort should be deployed to define the different cell death pathways involved in the pathogenesis of severe WNV neuroinvasive disease.

The function of caspase-12 in viral immunity has not received much attention. Previously, it was shown that caspase-12 plays a role in endoplasmic reticulum stress-induced apoptosis in response to amyloid toxicity [205]. Wang et al. found that caspase-12-deficient mice had greater mortality, higher viral burden in peripheral (serum, lung, spleen) and neural (brain, cerebellum, spinal cord) tissues, and a defective type I interferon response after WNV challenge compared to wild-type mice [206]. *In vitro* studies of primary neurons and mouse embryonic fibroblasts further demonstrated that caspase-12 positively modulated the production of type I interferon by regulating E3 ubiquitin ligase TRIM25-mediated ubiquitination of RIG-1, which is a critical signaling event for the type I

interferon response to WNV and other viral pathogens. Alternatively, high levels of WNV non-structural (e.g. WNV-NS2A, 2B, 4A, 4B) or glycoprotein (WNV-E) may result in endoplasmic reticulum (ER) stress and unfolded protein response induction, resulting in apoptosis [207].

4.3. Immunopathology

How much cell injury can be attributed to viral cytopathology and how much to the inflammatory response is not known. Infection of neurons with WNV leads to the induction of several cytokines and chemokines, which promote leukocyte invasion into the CNS and neuroinflammation [161, 208]. However, the extent to which this inflammation contributes to disease pathology remains unclear. In particular, the relative contribution of neurons to inflammation is subject of intensive research.

Recently, it has been shown that WNV induced the expression of IL-1β, -6, -8, and tumor necrosis factor (TNF)-α in human neuroblastoma SK-N-SH cells in a dose- and time-dependent manner, which coincided with increase in virus-induced cell death [209]. Treating cells with anti-IL-1β or anti-TNF-α resulted in a significant reduction of the neurotoxic effects of WNV. When naïve astrocytes were treated with UV-inactivated supernatant from WNV-infected SK-N-SH cells, expression of glial fibrillary acidic protein (GFAP) and key inflammatory cytokines were increased. These results suggest that neurons are a source of pro-inflammatory cytokines in WNV-infected brain, and that pro-inflammatory mediators are one of the main factors driving WNV-induced neurotoxicity. Recent studies with Japanese encephalitis virus (JEV; a closely related neurotropic flavivirus) also support a role of TNF-α in cell death, as increased expression of TNF-α receptors in neurons directly resulted in the initiation of death cascade via tumor necrosis factor receptor type 1-associated DEATH domain protein [210].

In animal models of JEV there is some evidence that activation of microglial cells plays a role in the pathogenesis of encephalitis through the action of pro-inflammatory mediators, which induce neuronal cell death [211]. Although reactive gliosis (activation of astrocytes and microglia) has been reported in WNV neuroinvasive disease and is considered a key pathogenic feature [212-214], the extent to which infection of glial cells contributes to WNV-induced neurological disease has never been fully investigated. It is believed that collateral damage is mediated by inflammatory factors that are either neurotoxic or attract leukocytes into the affected area, which results in a detrimental inflammatory milieu.

Murine infection models have provided substantial evidence that Toll-like receptor 3 (TLR3) is involved in WNV immunopathogenesis. Firstly, Wang and colleagues demonstrated that mice deficient in TLR3 experienced a reduced viral load and a reduced production of antiviral and pro-inflammatory cytokines, as well as reduced mortality rate upon intraperitoneal WNV challenge [215]. Specifically, the reduced production of the pro-inflammatory cytokines TNF-α and IL-6 by microglia was associated with their inability to

promote injury of neuron-like cells and primary mouse neurons, whereas wild-type microglia released inflammatory cytokines and induced neurotoxicity. The authors hypothesized that the reduced peripheral inflammatory responses, with in particular TNF-α, which has a detrimental effect on the permeability of the BBB, is in particular responsible for the enhanced survival shown by TLR3-deficient mice. These results suggest that the presence of TLR3 plays an important role in neuroinvasion. A later study, on the other hand, showed the opposite, namely that a lack of TLR3 enhanced viral replication in neurons, both *in vitro* and *in vivo*. As a result, TLR3 may limit WNV infection in a cell-type restricted manner [216]. This is supported by another *in vitro* study, which demonstrated that TLR3 may be dispensable for WNV recognition in certain cell types [217].

Interestingly, observational studies suggest that macrophages from young individuals can down regulate TLR3 following infection with WNV, whereas macrophages of the elderly cannot [218]. Therefore, it has been hypothesized that failure to down regulate TLR3 in infected cells results in production of high levels of pro-inflammatory and vasculogenic cytokines, which may lead to increased BBB permeability. This could partially explain the increased severity of WNV infection observed in older individuals. Nonetheless, the role of TLR in immunopathogenesis remains controversial.

A study conducted by van Marle et al. using fatal cases of human WNV encephalitis suggested that WNV infects both neurons and glia cells, and that infection of these cells, in particular astrocytes, contributed to neuronal death by releasing neurotoxic mediators [208]. This study also showed induction of neuroinflammatory genes, where a subset of these genes was specifically induced by the capsid protein of WNV. Particularly CXCL10, IL-1β and indolamine-2',3'-deoxygenase (IDO) were shown to be over-expressed in astrocytes *ex vivo* and *in vivo*. Interestingly, production of CXCL10 by astrocytes has also been implicated in the neuropathogenesis of other viral infections, such as human immunodeficiency virus [218-222]. Nonetheless, further studies are needed to define the genetic programs associated with neuroprotection or the neurotoxic action of glial cells during WNV infection.

Recently, a paradoxical role for neutrophils in WNV pathogenesis has been described. When Bai et al. investigated the role of chemokines in WNV pathogenesis by infecting macrophages from mice with WNV, they found that expression of CXCL1 and CXCL2, which are two CXC-type chemokines that induce the migration of neutrophils, was dramatically up-regulated [223]. In addition, neutrophils were found to be the most abundant cell type in the peritoneal cavity as early as 12 hours after WNV inoculation. These results suggest that neutrophils are the predominant immune cells that are initially and rapidly recruited to sites of infection with WNV. Contrastingly, however, mice depleted of neutrophils had significantly lower WNV in their blood on day 2 or 3 after infection, and increased survival rates were seen. In contrast, when mice were infected with WNV before the depletion of neutrophils on days 1 and 2 after infection, they showed higher levels of viral load as well as reduced survival rates. The authors concluded that

WNV may replicate in neutrophils and increase WNV load in blood early in infection, but that in the later course of infection these cells contribute to the control of infection. Although these results should be confirmed, it is tempting to speculate that neutrophils play a critical role in WNV replication and dissemination *in vivo*, especially in humans that have neutrophils as the predominant cell type in blood.

5. Adaptive immune responses against severe WNV infection

Most of the neutralizing antibodies against flaviviruses recognize the structural E protein, even though a subset binds to the prM/M [224-227]. Interestingly, antibodies to the NS1 protein, which is not part of the virion, are also protective against WNV *in vivo* [228, 229]. Also antibody responses to the intracellular proteins NS3 and NS5 have been observed during WNV infection [230], but their significance remains uncertain. Most of the potent neutralizing antibodies against WNV recognize the upper lateral surface of DIII that is protruding off the surface of the virion [172, 231, 232]. Even though humans can produce antibodies of this specificity in response to natural infection [233], studies have indicated that the human humoral immune response to WNV infection is actually narrower than anticipated, with the specificity of the antibody focused on determinants around the fusion loop at the tip of DII. In fact, B-cell repertoire analysis of three WNV-infected humans showed that only 8% of WNV-specific B-cell clones produced antibodies to DIII, while almost half produced antibody that bound to determinants in DII, particular the fusion loop [234]. Moreover, functional studies of the polyclonal response of WNV-infected horses and humans revealed that the neutralization activity of sera is actually not dependent upon antibodies directed against the DIII-lateral ridge (lr) epitope [233, 235].

It has been recognized that the elderly and immunocompromised are especially at risk for disseminated WNV infection and for developing fatal encephalitis. Even though there have been several indications, as mentioned earlier, the exact basis for the increased susceptibility in the elderly remains unclear. Nonetheless, experiments with mice have also begun to elucidate the role of the different components of the innate and adaptive immune response in controlling infection, in particular the role of immunoglobulin M (IgM), CD4+ and CD8+ T cells. It is believed that changes in both innate and adaptive immune function converge in the reduced response to vaccination and protection against infection in the elderly [236]. For instance, the decline in thymic output of naïve T cells diminishes responses to novel antigens, such as WNV, while clonal expansions leading to defects in the T cell repertoire are associated with blunted responses of memory T cells to conserved epitopes of the influenza virus [236].

Use of a well-characterized mouse model of WNV infection, which in many respects mimics human disease, showed that mice deficient in the production of secreted IgM (sIgM), but still capable of expressing surface IgM were vulnerable to lethal infection, even after inoculation with low doses of WNV [237]. sIgM-/- mice developed higher levels of

infectious virus in sera compared to wild-type animals. This enhanced viremia correlated with higher WNV burden in the CNS. Consistently, passive transfer of polyclonal anti-WNV IgM or IgG protected sIgM-/- mice against mortality, while administration of comparable amounts of a non-neutralizing monoclonal anti-WNV IgM did not provide any protection. Overall, these results indicate that the induction of a specific, neutralizing IgM response early in the course of WNV infection limits viremia and dissemination of virus into the CNS, resulting in protection against lethal infection. Whether the kinetics of the IgM response to WNV differs between young and the elderly and how it might affect susceptibility to severe WNV infection in humans is not clear.

Furthermore, it has also been demonstrated that mice with a genetic or acquired deficiency in CD4+ T lymphocytes display protracted WNV infection in the CNS, leading to uniform lethality by 50 days after infection [238]. Mice that survived past day 10 had high-level persistent infection in the CNS compared to wild-type mice, even up to 45 days after infection. WNV-specific IgM levels decreased about 20-fold at day 15 post-infection in CD4-deficient mice and IgG levels were about 100 to 1,000-fold lower throughout the course of infection compared to wild-type mice. Furthermore, WNV-specific CD8+ T-cell activation and trafficking to the CNS were markedly compromised at day 15. These results suggest that the main protective role of CD4+ T cells during primary infection of WNV is to assist in antibody responses and to sustain WNV-specific CD8+ T cell responses in the CNS that enable viral clearance.

CD8+ T cells have been shown to directly play a role in controlling WNV infection and preventing severe disease. When mice lacking CD8+ T cells or classical Ia major histocompatibility complex (MHC) antigens were infected with a virulent WNV isolate, higher viral titers were recovered from the CNS and increased mortality rates were recorded [239]. In contrast, absence of CD8+ T cells did not affect the quantitative antibody response and did not alter the kinetics or magnitude of viremia during primary infection. Interestingly, infectious virus could still be recovered from the CNS of CD8+ T cell-deficient mice that survived initial WNV challenge for several weeks. However, in spite of the normal quantitative antibody response, WNV was still able to enter the brain, even though it has been shown that IgM and IgG are important in preventing dissemination of WNV into the CNS [237]. These experiments collectively suggest that WNV-specific antibodies are responsible for reducing viremia and preventing development of severe disease, while CD8+ T cells play an important role in clearing infection from tissues and preventing viral persistence. Whether antibodies can prevent neuroinvasion remains to be determined.

6. Diagnosis of WNV

Even though the majority of people infected with WNV do not suffer from serious disease, the outcome can be quite severe for the young, elderly and immunocompromised. However,

if treated quickly, the outlook for West Nile virus recovery can be quite good. As a result, it is important to diagnose West Nile neuroinvasive disease (WNND) accurately and quickly. In addition, identifying any case of WNV is important for surveillance, as circulation of the virus needs to be identified so that important measures can be taken accordingly. Several diagnostic tools are currently available for the laboratory diagnosis of WNV infections [240], including many nucleic acid based assays (Table 1). When WNND is suspected, the diagnosis is generally made by the detection of viral RNA in serum or cerebrospinal fluid (CSF) samples with real time RT-PCR assays. Detection of the WNV genome in the CSF or serum during the acute stage of neurological involvement is generally considered to be a confirmatory diagnostic parameter [241]. When viral RNA is undetectable, identification of a specific IgM response in either the CSF or serum is accepted as a reliable diagnostic indicator, and the detection of IgG (a four-fold or greater increase in the serum) during the convalescent phase of the infection can subsequently confirm this.

WNV-specific antibodies can be detected by immunofluorescence assay and enzyme immunoassay (incl. indirect IgG, IgM antibody-capture and blocking) [240]; however, an important practical weakness of these techniques is the limited specificity due to the wide-spread immunological cross-reactions among flaviviruses [242]. As a result, a confirmatory plaque reduction neutralization test (PRNT) is generally advisable. Unfortunately, due to the requirement of BSL3 safety conditions [243], only a few laboratories in Europe are able to routinely perform this confirmatory test. The detection of a specific IgG response, usually by enzyme immunoassay, can be valuable in the context of epidemiological studies as the potential evolving circulation of WNV among humans in Europe can be monitored.

As a response to the emergence of WNV, many countries adopted regulations regarding blood safety in blood products for direct transfusion. For example, in the USA, screening for WNV RNA was introduced in 2003 [244]. In Europe, on the other hand, the Commission Directive 2004/33/EC introduced a temporary 28 days deferral of donors after leaving an area with ongoing transmission of WNV to humans. Alternatively, blood donations can be screened using Nucleic Acid Testing (NAT) for WNV [245, 246]. Two commercially licensed tests are available for the screening of WNV RNAs in both blood and organ donors [247, 248], of which the first uses real-time RT-PCR, while the second is based on transcription-mediated amplification technology. A potential concern regarding the sensitivity of these tests in the detection of WNV in Europe, is the well-documented genetic variability of the European strains and the recent emergence of human infections caused by WNV lineage 2 in Europe, which contrasts the more straight-forward North American situation [93, 249-251].

Fortunately, diagnostic tests are continuously advancing towards improved specificity and sensitivity. For example, in an effort to improve the detection of lineage 2 viruses, Linke et al. developed a real-time PCR targeting a conserved region of the 5’-untranslated region (5’-UTR) and part of the capsid gene of lineage 1 and 2 WNV [252], while Eiden et al. developed two quantitative RT-PCRs for the detection of lineage 1 and 2 strains using

primers and probes located in either the 5'-UTR or in NS2a [253]. All assays were able to detect both lineages with high sensitivity.

Technique	Reference
SYBR Green RT-PCR	Papin, 2004; Johnson, 2010
TaqMan RT-PCR	Lanciotti, 2000
TaqMan-MGB RT-PCR	Chao, 2007
Multiplex RT-PCR	Dyer, 2007; Naze, 2009
Molecular beacon RT-PCR	Jimenez-Clavero, 2006
RT-PCR/ESI-MS	Lee, 2005
RT-PCR/LDR	Grant-Klein, 2010
RT-PCR/FRET	Rondini, 2008
RT-LAMP	Parida, 2004
NASBA	Notomi, 2000
Digital PCR	Invitrogen

Table 1 Overview of nucleic acid based assays for WNV detection.

7. Prevention

Within Europe, the quality of data concerning the circulation of WNV among vectors, birds and humans varies between countries. Currently, there are no implemented surveillance methods or health policies for the application of control measures in the event of disease outbreaks [254]. However, as precise identification of viral circulation in vectors and vertebrate hosts within defined geographical areas is essential to defining the risk of WNV transmission via mosquitoes, blood transfusions and organ donations, ECDC recently introduced a web-based publication of WNV-affected areas (available at: http://ecdc.europa.eu/en/healthtopics/west_nile_fever/west-nile-fever-maps/pages/index). On the basis of these risk maps and the local surveillance data, each European country should be able to define the areas and seasons for the implementation of vector control measures, as well as the laboratory screening of blood and organ donations, in order to reduce the risk of WNV transmission to humans.

Currently, three licensed equine vaccines against WNV exist for commercial use, namely a formalin-inactivated, whole West Nile virus, (West Nile Innovator®), another killed virus vaccine (Vetera® WNV vaccine), and a chimeric recombinant canarypoxvirus vaccine (Recombitek® Equine West Nile Virus Vaccine) [241]. In contrast, no licensed vaccination options or other immune prophylaxis for human WNV disease are currently available. A vaccine preparation developed in the USA (a chimeric vaccine based on the backbone of Yellow-Fever 17D human vaccine expressing the prM and E genes of the NY99 strain (ChimeriVax-WN01)) went successfully through a phase II trial a few years

ago; however, the manufacturer decided to stop the development process due to market uncertainty regarding the potential target population that should receive the vaccine [255].

Nonetheless, many vaccines for WNV that employ different kinds of platforms or antigens are at early stages of development. The best-known strategy for vaccination uses purified viral proteins. For example, Martina et al. produced a recombinant domain III (rDIII) of the E protein to vaccinate mice, and compared it to a β-propiolactone (BPL) inactivated WNV vaccine [256]. Neutralizing antibodies against WNV were detected in all mice and cross-neutralizing IgG against JEV was also produced. However, survival rates were lower (80% for WNV and 60% for JEV) in comparison to mice vaccinated with the BPL inactivated WNV (100% for WNV and 80% for JEV). In fact, even though purified viral protein vaccines often provide protection against disease in animal models, multiple injections and/or strong adjuvants are usually required to reach acceptable efficacy. As a result, many other vaccination platforms are often used for the development of vaccines, including for WNV.

One common strategy used in WNV vaccination is the recombinant vector vaccine. For example, Martina et al. evaluated a recombinant influenza virus expressing DIII as a WNV vaccine candidate in a mouse model. Specifically, the WNV DIII was cloned in the N-terminal region of the influenza virus neuraminidase (FLU-NA-DIII), which destroys the functional activity of the influenza protein [257]. Subcutaneous immunization of mice with the vaccine resulted in high virus-neutralizing and WNV-specific IgG ELISA titers and a 100% survival rate. Another study created an adenoviral vaccine vector (CAdVax-WNVII) that expressed WNV proteins C, prM, E and NS1. Despite the proteins being derived from a lineage 2 virus strain, neutralizing antibodies were produced against both lineage 1 and 2 viruses [258]. In fact, with the emergence of pathogenic lineage 2 strains in Europe, it was questioned whether the existing WNV vaccines, which are mainly based on lineage 1 strains, would be able to protect against the new circulating lineage 2 strains of WNV. However, Minke et al. demonstrated that Recombitek® Equine West Nile, a vaccine that expresses the prM/E genes of lineage 1 in a recombinant canarypox virus, was able to protect horses against a neurovirulent lineage 2 WNV isolate [259]. Even though the poxvirus vector Modified Vaccinia Ankara virus (MVA) is a commonly used vaccination platform, it has not yet been utilized in WNV vaccination studies. This platform will be discussed in depth in the Discussion section of this thesis.

Another strategy for WNV vaccination is DNA vaccination [260-264]. Davis et al. were the first to demonstrate that plasmid DNA encoding the WNV membrane and envelope proteins injected intramuscularly into mice and horses was able to provide protection against challenge with WNV, resulting in both a humoral response as well as a strong Th1 response [260]. In fact, this study paved the way for the licensing of the first DNA vaccine for animal use, namely West Nile Innovator® DNA. After this, other administration routes and carriers for the delivery of WNV DNA vaccines were also exploited. For example, Dunn et al. evaluated DNA vaccines with derivatives of the WNV E gene (full length, truncated E or DIII region) that were conjugated to the P28 region of

the complement protein C3d, vaccinated either intramuscularly or by the gene gun route [263], while Chang et al. developed a plasmid DNA (pDNA) that after transfection gave rise to single-round infectious particles (SRIPs) based on WNV [264].

Despite the use of different platforms, a commonly used antigen across platforms is DIII. DIII contains predominantly sub-complex- and type-specific epitopes, of which many induce neutralizing antibodies [118, 265-270]. Many vaccines for WNV based on DIII have demonstrated immunogenicity and efficacy among different platforms [256, 257, 263, 271-275]. For example, Spohn et al. chemically coupled recombinantly expressed DIII protein to VLP derived from bacteriophage AP205 and this conjugate vaccine was found to be more immunogenic in mice than a mixture of corresponding amounts of free DIII and its carrier AP205. Moreover, after three injections mice were also fully protected against a lethal challenge with WNV [273].

Lastly, other vaccination strategies employed for WNV include the use of an attenuated non-epidemic WNV strain of lineage 2, which was found to be effective against a virulent epidemic strain of lineage 1 in mice [276]. Another strategy followed by Mason et al., used a live-attenuated virus, single-cycle WNV (RepliVAX WN) in which the gene encoding the capsid protein was deleted from the WNV genome. This vaccine induced protective immunity in mice [277], hamsters [278] and non-human primates [279]. In addition to the existence of many different types of vaccines in the pre-clinical stages, there are currently no specific therapeutic treatments for WNV infections. Ribavirin [280], interferon-α [281, 282] and WNV-specific immunoglobulin [283, 284] have all been considered as specific treatments for WNV, yet no rigorously conducted clinical trials have been completed. As a result, only preventive or supportive care can currently be administered for WNV disease.

Aims and scope of this thesis

The once limited geographic and host ranges of many vector-borne diseases are rapidly expanding. WNV is currently expanding into Southern and Eastern Europe. As a result, it is necessary to address Europe's need to prepare for vector-borne diseases such as WNV. This includes the pursuit of improved ways to monitor the spread of WNV, to diagnose, treat and also to prevent infections caused by this virus.

The first part of this thesis looks at the virulence and pathogenesis of WNV, which addresses the question whether the strains circulating in Europe differ in virulence. This may help to improve Europe's ability to monitor the spread of WNV infections and provide a basis for diagnostic and prognostic tools and novel intervention strategies. **Chapter 2** compares the neuroinvasive capacity of a selection of WNV strains currently circulating in Europe. **Chapter 3** describes the development and application of a qRT-PCR assay that more accurately quantifies replicating WNV and therefore aids in the assessment of virulence as well as the determination of WNV tropism. **Chapter 4** aims to identify markers of virulence in WNV lineage 2 strains, based on virulence markers known for lineage 1 strains.

The second part of this thesis focuses on the vertebrate host as part of the enzootic cycle of WNV, which is aimed at generating knowledge on avian susceptibility that may lead to important insights necessary for determining the type of surveillance system that may be appropriate for European countries in order to detect and predict emerging WNV outbreaks. In **Chapter 5**, the susceptibility of the European jackdaw is addressed, and in **Chapter 6** the susceptibility of the closely-related Carrion crow.

The third part of this thesis investigates a potential prevention technique that may eventually be useful as part of an intervention strategy in the preparation of Europe against WNV. The severity of WNV neuroinvasive disease, the long-term sequelae reported from a number of cases of infected individuals, as well as the potential negative impact of these infections on the blood transfusion system may collectively justify the development of a safe and effective vaccine against WNV. **Chapter 7** evaluates the effectiveness of a vaccine for lineage 1 and 2 WNV based on the MVA vaccination platform. **Chapter 8** summarizes and discusses the results of this thesis in the context of the rapidly progressing field of WNV research.

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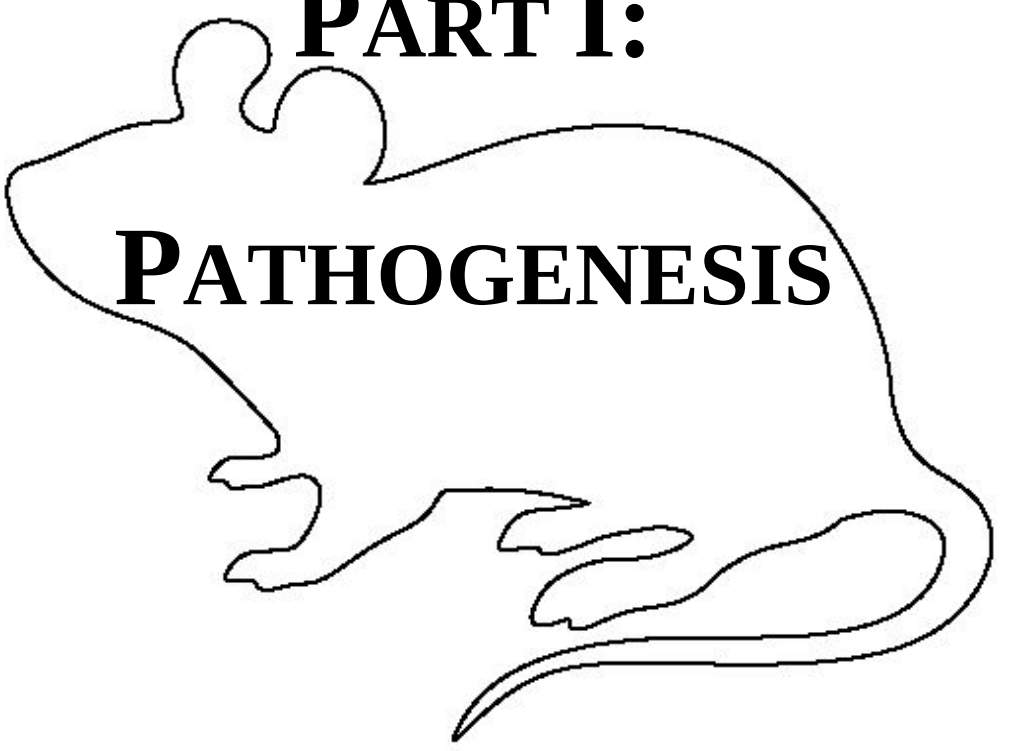
PART I:

PATHOGENESIS



Chapter 2

Characterization of the Mouse Neuroinvasiveness of Selected European Strains of West Nile Virus

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PLoS ONE, 2013, 8(9):e74575

ABSTRACT

West Nile virus (WNV) has caused outbreaks and sporadic infections in Central, Eastern and Mediterranean Europe for over 45 years. Most strains responsible for the European and Mediterranean basin outbreaks are classified as lineage 1. In recent years, WNV strains belonging to lineage 1 and 2 have been causing outbreaks of neuroinvasive disease in humans in countries such as Italy, Hungary and Greece, while mass mortality among birds was not reported. This study characterizes three European strains of WNV isolated in Italy (FIN and Ita09) and Hungary (578/10) in terms of *in vitro* replication kinetics on neuroblastoma cells, LD_{50} values in C57BL/6 mice, median day mortality, cumulative mortality, concentration of virus in the brain and spinal cord, and the response to infection in the brain. Overall, the results indicate that strains circulating in Europe belonging to both lineage 1 and 2 are highly virulent and that Ita09 and 578/10 are more neurovirulent compared to the FIN strain.

INTRODUCTION

West Nile virus (WNV) is a positive-sense single-stranded RNA virus, which belongs to the genus Flavivirus. WNV is transmitted by infected mosquitoes and is maintained in an enzootic cycle between mosquitoes and birds, but can also infect and cause disease in horses and humans, which serve as incidental dead-end hosts. Previously, WNV was considered an Old World virus being endemic in parts of Africa, Europe, the Middle East, and Asia [1]. However, in 1999, WNV emerged in New York City in the United States and has since rapidly spread across North America, Mexico, South America, and the Caribbean [2-4]. No vaccines or specific therapy are currently registered for use in humans.

WNV has caused sporadic outbreaks in Central, Eastern and Mediterranean Europe for over 45 years. Phylogenetically WNV strains are classified into two major lineages. Lineage 1 constitutes strains from North America, Africa, the Middle East, Asia, Australia (Kunjin virus) and Europe. Lineage 2 strains were restricted to sub-Saharan Africa. This genetic classification has been used frequently to classify WNV obtained during outbreaks. In this respect, many strains isolated from patients with neuroinvasive disease have been classified as lineage 1. In 2008, an outbreak affected small numbers of wild birds, horses and humans in eight provinces in three regions of Italy [5,6]. Subsequently, in 2009, a new epidemic was reported in the same region, as well as in other neighboring regions in Italy, with up to 17 confirmed cases of WNV neuroinvasive disease [7]. Several strains of WNV were isolated from human specimens and sequenced. Phylogenetic analyses on the basis of the E and NS3/NS5 revealed that these strains belong to lineage 1, clade 1a, and constitute a distinct group within the western Mediterranean cluster [8]. In 2004, a lineage 2 strain was isolated from birds of prey in Hungary [9], which established itself in the region and largely spread throughout the country and into eastern Austria by 2008 [10]. During this outbreak, cases of human neuroinvasive disease were comparatively rare and rather mild with no deaths reported [10]. Recently, Greece became the focus of a large outbreak in summer-autumn 2010 [11]. Up to October 4th, 2010, 192 cases of neuroinvasive disease in humans, including 32 deaths, had been laboratory diagnosed, all in the elderly. *Culex pipiens* mosquitoes trapped in Nea Santa were found to be positive for WNV RNA, and sequencing of the NS5 gene gave the first indication that this virus belongs to lineage 2, and that it is highly similar to the strain that emerged in Hungary in 2004 [10]. As of November 2012, 237 confirmed human cases have been reported in the European Union (EU), of which 161 cases were in Greece, 50 in Italy, 14 in Romania and 12 in Hungary (http://www.ecdc.europa.eu/en/healthtopics/west_nile_
fever/West-Nile-fever-maps/Pages/index.aspx). These outbreaks were caused by both lineage 1 and 2 strains of WNV.

It is remarkable that many of the outbreaks in humans caused by lineage 1 and 2 were not preceded by massive bird mortality. WNV-induced wild bird mortality has been described in Europe but much less intensively compared to the US. The outbreak in 1998 in Israel and 1999 in New York were the first ones where mortality among birds was reported.

Because of the higher incidence of WNV neuroinvasive disease seen during the US outbreak, it was hypothesized that the introduced strain was more virulent. The complete genomic sequencing of the bird and human virulent IS-98 and NY99 strains of WNV revealed that both isolates belong to the same phylogenetic clade, sharing more than 99.8% nucleotide similarity [12]. We wished to characterize and determine the virulence profile of the European-derived WNV strains.

Virulence for WNV has often been associated with envelope (E) protein glycosylation [13] and glycosylation of the NS1 protein [14]. Other virulence factors described for WNV include tropism, induction of rapid cell death, resistance to interferon, quasispecies generation and up-regulation of MHC class I expression [15]. It is therefore clear that virulence is a multi-factorial process and that many aspects need to be studied in order to elucidate the pathogenic force of viruses. Several parameters can be used to describe virulence. *In vivo* surrogate markers of virulence include immune-interfering properties, lethal dose 50 (LD_{50}), median survival time (ST_{50}), tropism for particular cells or tissues, as well as the viral burden present in infected tissues. In the present study we characterized the virulence of three selected European strains of WNV *in vitro* and also *in vivo* by infection of C57BL/6 mice with different doses of these virus strains. We compared their LD_{50}, ST_{50}, cell tropism and pathology in the brain, as well as the response to infection in the brain.

MATERIALS AND METHODS

Ethics statement

All animal experiments described in this paper have been conducted according to Dutch guidelines for animal experimentation and approved by the Animal Welfare Committee of the Erasmus Medical Centre, Rotterdam, The Netherlands. All efforts were made to minimize animal suffering. The Dutch Animal Experimentation Act (1977) demands that research establishments request a licence from the Ministry of Welfare, Public Health and Cultural Affairs before carrying out any experiment. Research plans must be approved by local ethical review committees that consider the benefit of an experiment and whether this justifies the distress caused to the animals used in the procedure. The pain assessment is prospective and a system of research plan review based on the cost-benefit principle is also in place. A statistical reporting system of all animal experimentation provides the opportunity to count the number of experiments involving pain or distress to the animals with or without pain relief drugs.

Cells and viruses

Vero E6 cells were cultured in DMEM with 10% heat-inactivated fetal bovine serum (HI-FBS), supplemented with 0.75% sodium bicarbonate and 10 mM hepes buffer. C6/36 insect cells were cultured in Leibovitz-15 medium supplemented with 5% HI-FBS, 10% tryptose phosphate broth, 0.75% sodium bicarbonate and 10 mM hepes buffer. All media were supplemented with antibiotics (100 U penicillin, 100 µg/ml streptomycin) and 2 mM L-glutamine. Cell culture reagents were obtained from LONZA (Lonza Benelux BV, Breda, the Netherlands). All cell lines tested negative for mycoplasma using a PCR assay as described [16]. Viruses used in this study and passage history were as follows: two lineage 1 Italian strains, FIN (a kind gift from Dr. Vittorio Sambri, University of Bologna, Italy; P2 on Vero E6) and Ita09 (accession GU011992.2, kindly provided by Dr. Louisa Barzon, University of Padova, Italy; P1 on Vero E6) and the Hungarian lineage 2 strain 578/10 (accession KC496015, a kind gift from Dr. Tamás Bakonyi, Szent István University, Hungary; P2 on Vero E6) isolated from the brain of a horse that died of WNV-neuroinvasive disease. Virus stocks used for this study were prepared by growing the viruses once on C6/36 insect cells and viral titers were determined on Vero E6 cells using the Spearman & Kärber method [17,18] after determining cytopathic effects five days post inoculation.

Sequencing of the envelope gene of WNV strains

Viral RNA was isolated from C6/36 derived viral stocks using the MagnaPure LC robot system and the Total Nucleic acid isolation kit according to the manufacturer's instructions (Roche, Almere, The Netherlands). Primers specific for the E protein were designed using

the Primer Select module of DNASTAR software (DNASTAR, Madison WI, USA) and adjusted manually to obtain highest similarity with NY99 (Table S1). cDNA was synthesized using specific primers and Superscript III RT enzyme (Invitrogen, Breda, The Netherlands) and subsequently PCR-amplified using Taq DNA polymerase (Invitrogen) according to the instructions of the manufacturer. DNA fragments were gel-purified and cloned into the pCR4-TOPO vector (Invitrogen). The cloning reaction products were transformed into *E. coli* (One-Shot Top 10 competent cells; Invitrogen). Positive transformed bacteria were identified by PCR using M13 primers and sequenced using specific primers (Table S1). Five bacterial clones were selected to determine the consensus sequence of the virus stocks. Sequencing was performed in an ABI3130XL sequencer using ABI PRISM Big Dye® Terminator (Applied Biosystems, Bleiswijk, The Netherlands). Sequences were analyzed using the SeqMan module of DNASTAR software and aligned to a reference strain (original sequence of isolate deposited in GenBank) so that the E protein of the different strains was obtained from the consensus sequence of five bacterial colonies. The GenBank sequence for the original FIN isolate had not yet been deposited; therefore a closely related Italian (JF719067) sequence that gave at least 99% identity in BLAST was used as a reference instead.

Sequencing the complete genome of WNV-FIN

RNA was isolated from the WNV-FIN strain (P2 on Vero E6) with the High Pure RNA isolation kit (Roche) according to the instructions of the manufacturer. cDNA was synthesized using random hexamer primers (Invitrogen) or a reverse primer spanning the last 24 nucleotides of the 3'UTR of published WNV sequences, as well as Superscript III RT enzyme (Invitrogen). Fifteen sets of primers spanning the complete genome sequence of WNV were designed in conserved areas. Primers were designed using the PrimerSelect module of DNASTAR software (DNASTAR, Madison WI, USA). Primer sequences are available from the authors upon request. cDNA was amplified using PfuUltra II Fusion HS DNA Polymerase (Stratagene) and DNA fragments were purified from gel and sequenced directly in an ABI3130XL sequencer using the same primers as used for PCR amplification. Sequences were analysed using the SeqMan module of DNASTAR software.

Next generation sequencing (NGS)

Primers were designed for the E gene that allowed five fragments of sizes between 200-400 nucleotides with about 50 nucleotides of overlap to be generated (Table S1). RT-PCR was conducted using random primers (Invitrogen) and Superscript III (Invitrogen), and DNA amplification was performed using the specific primers and PFU polymerase (Invitrogen). Fragments were gel-purified using QIAquick gel extraction kit (Qiagen, Venlo, The Netherlands) and were organized in libraries of equal concentration. Libraries were created for each virus without DNA fragmentation (GS FLX Titanium Rapid Library Preparation, Roche), emPCR (Amplification Method Lib-L) and GS junior sequencing runs were

performed according to instructions of the manufacturer (Roche). Amplicons were sequenced in a blinded fashion using 454 technology. Reads from the GS-FLX sequencing data were sorted by barcode and aligned to reference sequences using CLC Genomics software 4.6.1. Using the alignment, a SNP table was made with a minimum coverage of 10 reads and a minimum variant frequency of 1.0%. Raw nucleotide sequences were filtered, aligned, trimmed and translated using pre-specified criteria applied uniformly so that all the protein E sequences used in the analyses spanned the exo-domain and the transmembrane region.

Replication kinetics of WNV-FIN, Ita09 and 578/10 viruses

The replication kinetics of WNV-FIN, Ita09 and 578/10 were studied *in vitro* by means of a one-step growth experiment using a multiplicity of infection (MOI) of 5. N2a cells were cultured overnight in 96-well flat bottom culture plates (10^5 cells/well) and virus was added. Viruses were allowed to adsorb for one hour at 37 ºC. Cells were subsequently washed three times with serum-free medium to remove virus inoculums, replenished with fresh medium and cultured at 37 ºC for 24 hours. Culture supernatants were collected in triplicate at time points 0 and 6 followed by sampling every 2 hours up to 24 hours, and were subsequently stored in -80 ºC until virus titer determination. Studies of replication kinetics were conducted in parallel to eliminate any confounding effects of host cell culture.

Several parameters were determined using the results of the one-step growth experiment. The approximate eclipse period was defined as the time point before infectious virus was detected in the supernatant. The latent period (LT_{50}) was defined as the time point at which half the number of virus progeny has been released into the environment and was determined by use of curve fitting to the data points by least squares (ordinary) fit. Replication rate (RR) is the slope obtained by the linear regression of the natural logarithm (ln) of the titer against time during the period of exponential growth.

Mouse infection and survival studies

Six-week old (age-matched) female C57BL/6 mice (Harlan Laboratories B.V., Venray, The Netherlands) were inoculated intraperitoneally (i.p.) with several doses of each three virus strains (n=8 for each dose). Mice were euthanized by cervical dislocation under isoflurane anaesthesia when they reached humane end-points (immobility and paralysis), after which the brain was immediately collected for further processing. At 14 days after infection, the end-point for the survival experiment was reached and the survival rate was analyzed, and LD_{50} was calculated according to the Reed & Muench [19] and the Probit method [20]. Mice were maintained in specific pathogen-free conditions, had a 12-hour day-night cycle and were fed *ad libitum*. Serology studies were conducted using enzyme-linked immunosorbent assay (ELISA).

Quantitation of virus in the brain

RNA copy numbers were quantified using a standard curve of *in vitro* transcribed RNA of known quantities. Run-off transcripts were generated from a plasmid containing the sequence of the 3' UTR of WNV-NY99. Plasmid was linearized and run-off transcripts were generated using the Ambion® MaxiScript T7/T3 kit (Invitrogen). The product was digested with DNase to remove residual DNA and the reaction was cleaned up using the Qiagen RNeasy Minikit (Qiagen). *In vitro* transcribed RNA was diluted to a concentration at which DNA was no longer detected. In order to quantify viral burden in the brain, half the brain was weighed and homogenized using a metal bead in 1 mL of DMEM containing antibiotics (100 U penicillin, 100 µg/mL streptomycin). RNA copy numbers in the brain homogenates were determined using qRT-PCR with the Taqman® EZ RT-PCR kit (Applied Biosystems) and primers and probe located on the 3' UTR of WNV (Table S1). Infectious titers in the brain were determined by titration of the brain homogenates on Vero E6 cells and calculation of the $TCID_{50}$.

Immunohistology

Sagittal brain and transverse spinal cord 4-µm thick paraffin sections were processed for streptavidin-biotin-peroxidase immunohistochemistry and immunofluorescence of virus nonstructural protein and cell-type markers. Sections were deparaffinized in xylene, rehydrated in descending concentrations of ethanol and incubated for 10 min in 3% H_2O_2 diluted in PBS to block endogenous peroxidase activity. Antigen exposure was performed by incubation for 15 min at 121 ºC in citrate buffer (0.01 M, pH 6.0). Sections were incubated overnight at 4 ºC with one of the following primary antibodies: goat anti-WNV NS3 (1:100; R&D Systems, Abingdon, UK), rabbit anti-human CD3 (T cell marker; 1:100; Dako, Eindhoven, Netherlands), rabbit anti-GFAP (astrocyte marker, 1:500; ZYMED, Breda, The Netherlands), and rabbit anti-Iba1 (microglial marker, 1:500; WAKO, Ochten, The Netherlands). For immunohistochemistry, primary antibodies were detected with secondary goat anti-rabbit IgG-PO, rabbit anti-goat IgG-PO (Dako) or biotinylated goat anti-polyvalent/streptavidin peroxidase (Thermo Scientific, Etten-Leur, The Netherlands) antibodies. Sections were counterstained with Mayer's hematoxylin and mounted with Kaiser's glycerin-gelatin and analyzed using a light microscope.

For double staining, immunofluorescence sections were incubated with goat anti-WNV NS3, and either rabbit anti-Iba1, rabbit anti-GFAP or rabbit anti-NeuN (neuronal marker, 1:500; Millipore, Amsterdam, The Netherlands) was used. Secondary antibodies directly conjugated to Alexa Fluor 488 (donkey anti-goat) and 555 (donkey anti-rabbit) (Invitrogen) were used. Nuclei were stained with DAPI. Fluorescence staining was analyzed using a Zeiss LSM 700 confocal microscope.

Determination of inflammatory and cell death markers by qRT-PCR

RNA was isolated from brain homogenates of infected mice using the MagnaPure LC system according to the manufacturer's instructions. cDNA was synthesized using oligo dT primer (Invitrogen) and Superscript III enzyme (Invitrogen) according to the instructions of the manufacturer. Primers specific for matrix metalloprotease (MMP)-3, MMP-9, tumour necrosis factor (TNF)-α, neuronal pentraxin (Nptx)-1,-2 and pentraxin-related protein (Ptx)-3 (Applied Biosystems) were used in PCR amplification, and mRNA copy numbers were quantified relative to β-actin using the following formula: $(2^{-quantity})*100000$. Quantity was determined by subtracting the Ct value of β-actin from the Ct value of the specific marker.

Statistical analysis

P-values equal to or less than 0.05 were considered to be statistically significant. Survival curves were analyzed with the Log-rank Test, differences between viral loads and differences in expression of inflammatory and apoptotic markers were assessed using the Mann-Whitney *U* test.

RESULTS

The E protein of different WNV strains is not significantly affected by *in vitro* culture

To determine whether generation of virus stocks, through one extra passage on insect cells, resulted in changes in the consensus sequence of the respective viruses, the E protein of the different virus stocks was sequenced using the Sanger method and sequences were compared to those deposited in GenBank. All virus stocks were 99% identical to the sequences of the low passage isolates deposited in GenBank (Figure S1). There were no amino acid changes found in Ita09 and 578/10 compared to the sequences deposited in Genbank. The FIN isolate, was compared to the highly similar sequence in Genbank because FIN was not sequenced before. A conservative amino acid substitution (histidine to tyrosine) was found at position 371. The lineage 2 strain from Hungary (578/10) differed by 20 amino acid substitutions from the lineage 1 strains. We have determined the complete sequence of the FIN isolate used in this study and the sequence was deposited in GenBank (accession: KF234080).

Differences in amino acid sequence of closely related strains WNV-FIN and Ita09

As the lineage 1 strains FIN and Ita09 are very closely related and completely identical on a nucleotide level in terms of the envelope, the complete genome of FIN was compared with Ita09. The complete genome of the WNV-FIN strain (KF234080) was 99.7% identical to the nucleotide sequence of Ita09 (GU011992.2). Specifically, nine conservative nucleotide substitutions were observed throughout the genome compared to Ita09. In addition, three

non-conservative amino acid differences were observed in NS3, including the proline (Ita09) to threonine (FIN) at position 249, a threonine (Ita09) to isoleucine (FIN) change at position 267 and a histidine (Ita09) to glutamine (FIN) change at position 488.

Dominant virus variants were recovered from viral stocks and the brain of infected mice

Measuring the virulence of individual variants within a virus stock may misrepresent the virulence of a quasispecies. Since the virus population diversity is an important component of virulence, we next characterized the population diversity in our stocks using NGS (deep sequencing). Between 30-47 $\times 10^3$ reads were obtained per sample from the virus stocks. Given the sequence heterogeneity within protein E, the use of strain-specific primer sets with degeneracy or located in conserved regions resulted in efficient amplification. The reads generated during sequencing were aligned using the reference sequences deposited in GenBank. The viral RNA sequences recovered from the brains yielded an average of 23-38 $\times 10^3$ reads per sample. Coverage of the amplicons was heterogenous and ranged from 2,950 to 30,675 reads for RNA sequences recovered from the viral stocks, and 1,079 to 28,362 reads for viral RNA sequences recovered from the brain. After the filtering steps, >99.7% of the original sequences were retained.

In FIN virus stock, 52.4% of the baseline viral population consisted of the T form and 47.6% of the C form at reference position 1176 (Figure 1), leading to a conservative amino acid substitution (H371Y). Furthermore, at position 2118, 92.9% of the variants contained the C form and 7.1% of the minor mutations were of the T form. Several other low-frequency mutations were found in the viral stock, ranging from 1% to 6%. In the brain of FIN-infected animals, all the variants were in similar ratios, while the frequency of the minor variant at position 2118 increased significantly by 26.1%. This variant, C2118T, also resulted in a conservative amino acid substitution (H685Y).

The baseline viral stock of Ita09 consisted of the 62.5% T form and 37.5% of the C form at reference position 2058 (Figure 1). Several other minor variants were found, ranging from 1.1% to 4.8%. For all the variants found in the viral stock, only the predominant variants were detected in the brain of infected animals, with the exception of the 2058 variant where both mutations were found. The viral stock of 578/10 consisted of only one variant at position 2270 with 96% of the T form and 4% of the C form (Figure 1). This variant was found at a similar ratio in the brain. The results indicate that the predominant variants in all the viral stocks were replicating in the brain of infected animals and that minor variants were not preferentially selected. The results from the NGS sequencing also confirmed the consensus sequence that we acquired using the Sanger method.

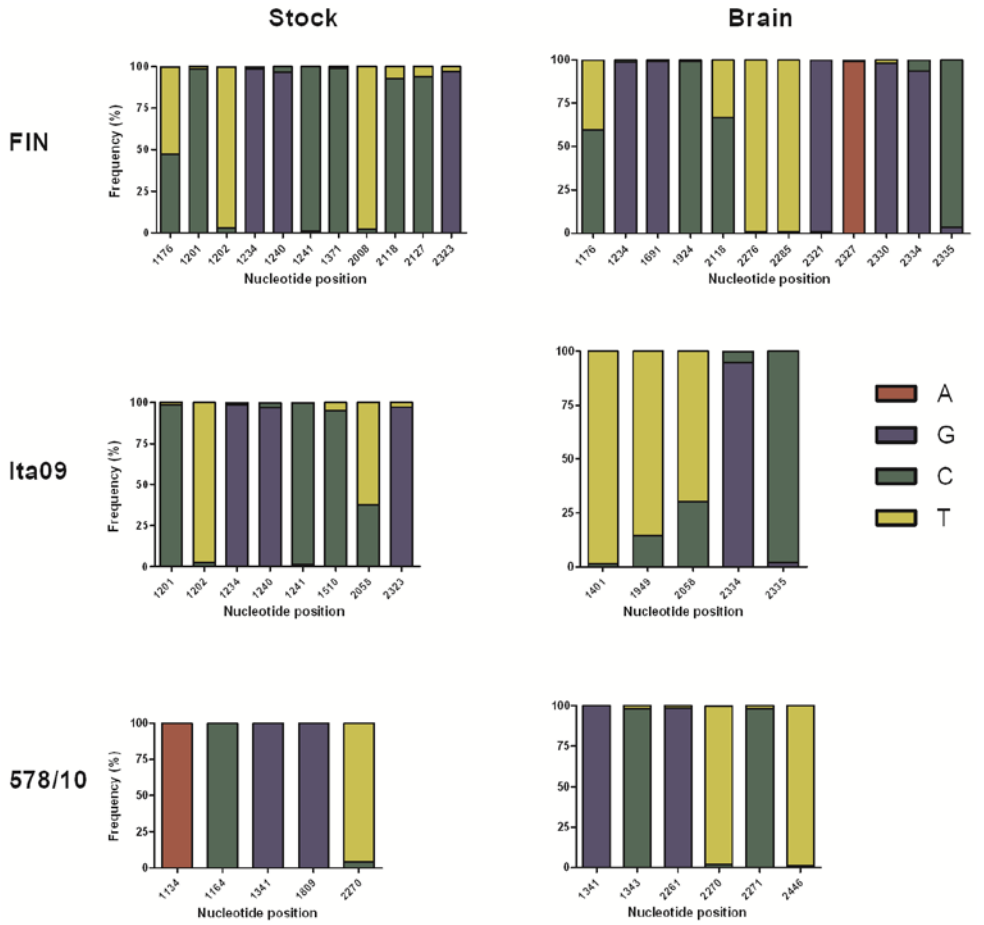


Figure 1. Virus variants recovered from the different WNV stocks and from the brains of infected mice. Sequences of glycoprotein E were obtained using Next Generation Sequencing (NGS) and aligned with reference sequences deposited in GenBank. Variant frequencies are indicated by nucleotide substitution at a particular reference position.

WNV-FIN, Ita09 and 578/10 have similar replication kinetics *in vitro*

As the replicative capacity of a virus is considered a surrogate marker for virulence, we decided to compare the replication kinetics of the different WNV strains using a one-step growth experiment. This approach assumes that virulent strains produce more progeny within the host than the avirulent ones, which in turn leads to higher viral densities and consequently greater virulence levels. The one-step growth curves of the different WNV strains are summarized in Figure 2A and Table 1. Infectious virus production by the three virus strains began at approximately 14 hours post-infection. However, in order to obtain a more accurate estimate of the latent period and facilitate comparisons between the strains,

we calculated the $LT_{50,}$ which is a mathematically more robust determination of the latent period. As shown in Table 1, the RR was found to be 1.69, 1.75 and 1.71 for FIN, Ita09 and 578/10, respectively, indicating similar replication rates for the different virus strains. The LT_{50} was calculated as 16.06, 13.44 and 16.09 hours for FIN, Ita09 and 578/10, respectively. This indicates that Ita09 is released the earliest from the cell, followed by FIN and 578/10. Furthermore, the burst size of Ita09 was ten-fold higher compared to the two other strains.

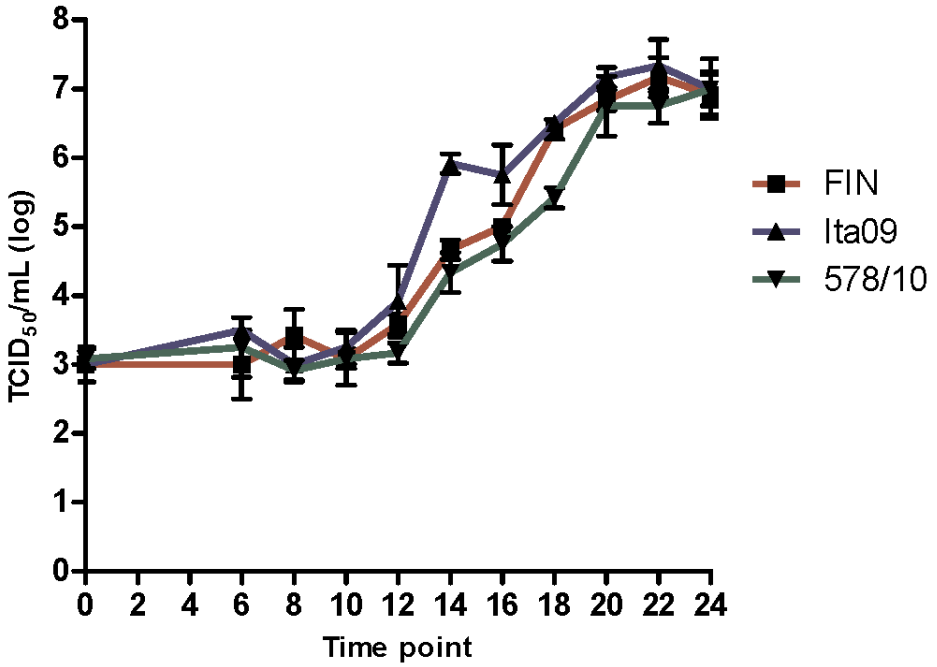


Figure 2. Infectious virus titers recovered from supernatant over 24 hours after infection of N2a cells with different WNV strains at a high (5) MOI. N2a cells were inoculated with WNV-FIN, Ita09 and 578/10 at an MOI of 5 $TCID_{50}$/cell. Experiments were performed in triplicate and data represent mean ± standard deviation.

Virus	Eclipse period (h)	Latent period (h)	LT_{50} (h)	Burst size ($TCID_{50}$)	RR
FIN	12	14	16,06 ± 0,02	$10^{1.1}$	1.69 ± 0.04
Ita09	12	14	13,44 ± 0,72	$10^{2.1}$	1.75 ± 0.05
578/10	12	14	16,09 ± 1,11	$10^{1.2}$	1.71 ± 0.02

LT: latent time, RR: replication rate.

Table 1. Analysis of the replication kinetics of WNV-FIN, Ita09 and 578/10 on N2a cells at high (5) MOI over 24 hours. Experiments were carried out in triplicate and values for LT_{50} and RR are indicated as mean ± standard deviation. The LT_{50} was determined using curve fitting by least squares (ordinary) fit and the replication rate (RR) is the slope obtained by the linear regression of the natural logarithm (ln) of the titer against time during the period of exponential growth.

Neuroinvasive properties of WNV-Ita09, FIN and 578/10 strains

As factors such as mortality rate, *in vivo* tropism, and immune response to infection constitute important components of virulence in the dynamic host environment, we first determined the outcome of infection in 6-week old female C57BL/6 mice following i.p. infection. Mice were infected with three different doses of virus (10^4, 10^2 and 10^1 $TCID_{50}$) and differences in mortality rates were observed between the respective WNV strains (Table 2). The cumulative mortality by 14 days after challenge was higher for 578/10 (91%) than FIN (78%) and Ita09 (74%). However, comparison of the cumulative survival curves of the mice infected with all doses of the various strains revealed no statistically significant differences (Figure 3B; $P = 0.70$). By contrast, significantly more mortality was observed for Ita09 compared to FIN ($P < 0.001$) and 578/10 ($P = 0.001$) for the 10^4 $TCID_{50}$ dose of virus (Figure 3A). In addition, the ST_{50} for Ita09 was shorter (8.5 days) followed by 578/10 (11 days) and FIN (11 days).

The LD_{50} calculated using the Reed & Muench method is in agreement with the probit method, indicating similar LD_{50} values for FIN and Ita09, while considerable lower values were found for the 578/10 strain (Table 3).

WNV strain / dose	First day mortality	Last day mortality	Median day mortality	Total mortality	Mortality (%)	Cumulative mortality (%)
			FIN			
10^1 $TCID_{50}$	9	11		4/8	50	
10^2 $TCID_{50}$	10	12	11	6/7	85.7	78
10^4 $TCID_{50}$	9	11		7/8	87.5	
			Ita09			
10^{-1} $TCID_{50}$*	12	12		1/8	12.5	
10^1 $TCID_{50}$	8	11		5/8	62.5	
10^2 $TCID_{50}$	7	12	8.5	6/8	75	74
10^4 $TCID_{50}$	7	9		7/7	100	
			578/10			
10^{-1} $TCID_{50}$*	8	11		3/8	37.5	
10^1 $TCID_{50}$	9	14		6/8	75	
10^2 $TCID_{50}$	9	11	11	8/8	100	91
10^4 $TCID_{50}$	8	12		7/7	100	

Table 2. Mortality of 6-week old i.p. infected C57BL/6 mice using three different doses of WNV-FIN (n=23), Ita09 (n=23) and 578/10 (n=23). *These groups were only used to calculate the LD_{50} and were not used for determination of median day of mortality and cumulative mortality percentage.

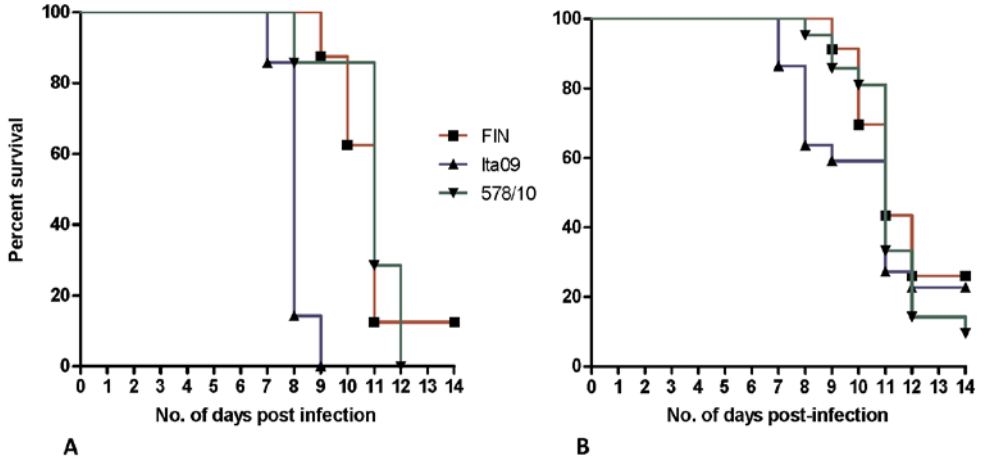


Figure 3. Survival curves of mice inoculated intraperitoneally with different WNV strains. (A) Mice were inoculated i.p. with 10^4 $TCID_{50}$ WNV-FIN (n=8), Ita09 (n=7) and 578/10 (n=7). Significant differences in survival were observed between FIN and Ita09 ($P < 0.001$), and Ita09 and 578/10 ($P = 0.001$). **(B)** Cumulative survival curves of mice inoculated i.p. with doses of 10^1 $TCID_{50}$, 10^2 $TCID_{50}$, and 10^4 $TCID_{50}$ of WNV-FIN (n=23), Ita09 (n=23) and 578/10 (n=23).

No statistical significance was found between the cumulative survival curves (P = 0.70). Mice were euthanized between days 6-14 upon display of clinical signs of disease.

WNV strain	LD_{50} (Reed & Muench)	LD_{50} (Probit)
FIN	$10^{0.98}$ $TCID_{50}$	$10^{0.75}$ $TCID_{50}$
Ita09	$10^{0.43}$ $TCID_{50}$	$10^{0.75}$ $TCID_{50}$
578/10	$10^{-0.42}$ $TCID_{50}$	$10^{-0.14}$ $TCID_{50}$

Table 3. LD_{50} calculated with the Reed & Muench and the Probit method of WNV-FIN (n=23), Ita09 (n=23) and 578/10 (n=23) i.p. infected C57BL/6 mice.

High viral RNA load in brains of mice infected with Ita09 and 578/10 strains

We further investigated the viral burden in the brain of the mice infected with the different strains of WNV and euthanized when the humane end points were reached, within 14 days post infection. RT-PCR analysis of brain homogenates revealed high titers of viral RNA (10^5 -10^9) for all mice. However, mean viral RNA copies were found to be significantly higher in brains of mice infected with Ita09 and 578/10 compared to mice infected with FIN (P = 0.009 and P = 0.02, respectively) (Figure 4A). However, in terms of infectious virus titers ($TCID_{50}$), no significant differences were observed in the brains of these mice (P >0.05 for all) (Figure 4B).

Mice that did not develop signs of WNV neuroinvasive disease by day 20 post infection (n=26) were considered survivors of the infection. Infection was confirmed by the fact that all these animals developed IgG antibodies to WNV (data not shown). Viral RNA was detected in the brain of nine survivors (Figure 4C). Specifically, four mice from the FIN group (one from the 10^4, one from 10^2 and two from the 10^1 $TCID_{50}$ group), and one mouse infected with 10^2 $TCID_{50}$ of Ita09. However, viral RNA titers were considerably lower (10^1-10^5) in these mice compared to those that died from infection. No significant differences were found in the number of RNA copies in the brains of these mice compared to each other (Figure 4C; P >0.05 for all). No viral RNA could be detected in the blood of any of the animals that survived WNV infection (data not shown), indicating that the detected RNA was not spillover from blood.

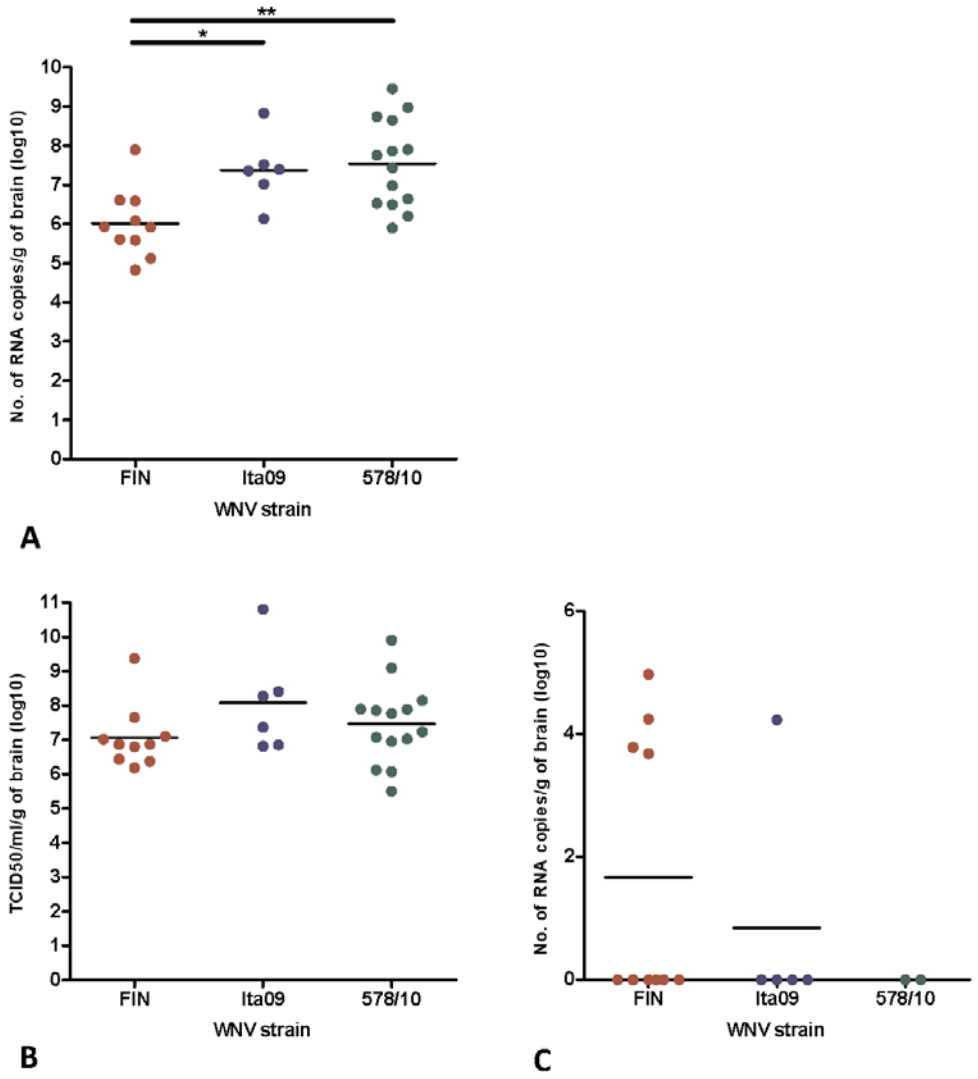


Figure 4. Viral burden in the brains of mice infected with WNV-FIN, Ita09 and 578/10. Mice were euthanized within 6 to 14 days upon reaching humane endpoints and viral burden was measured in terms of **(A)** RNA copies per gram of brain, and **(B)** $TCID_{50}$ per gram of brain. **(C)** Viral RNA copies per gram of brain in mice infected with WNV-FIN, Ita09 and 578/10, and euthanized on day 20 in the absence of clinical signs of disease. * P <0.05, ** P <0.01.

Histopathology and immunohistochemistry

In order to assess if the viruses differ in their tropism for particular regions of the brain, and compare the relative damage caused by the different strains, we performed immunohistochemical staining with anti-WNV-NS3 polyclonal antibody. WNV-NS3 positive cells occurred in brains and spinal cords of all mice that developed neurological

signs. Most positive cells could be identified as neurons, with the antigen being distributed in the cell body and proximal dendrites (Figure 5A). Some positive cells showed features of degenerative processes such as dystrophic neurites, small cell body and pyknotic nuclei (Figure 5B). In addition, labeling was associated with unidentifiable structures likely representing neuronal debris. In order to confirm that positive cells were neurons, we performed double-labeling immunofluorescence with the neuronal marker NeuN (Figure 5C). Most positive cells were positive for NeuN, while no WNV-NS3 positive cell was positive for glial fibrillary acidic protein (GFAP, astrocytes) or Iba1 (microglia), although in many occasions WNV positive cells and debris-like structures were surrounded by Iba1 positive processes, pointing to an intimate relationship with microglia cells (Figure 5D).

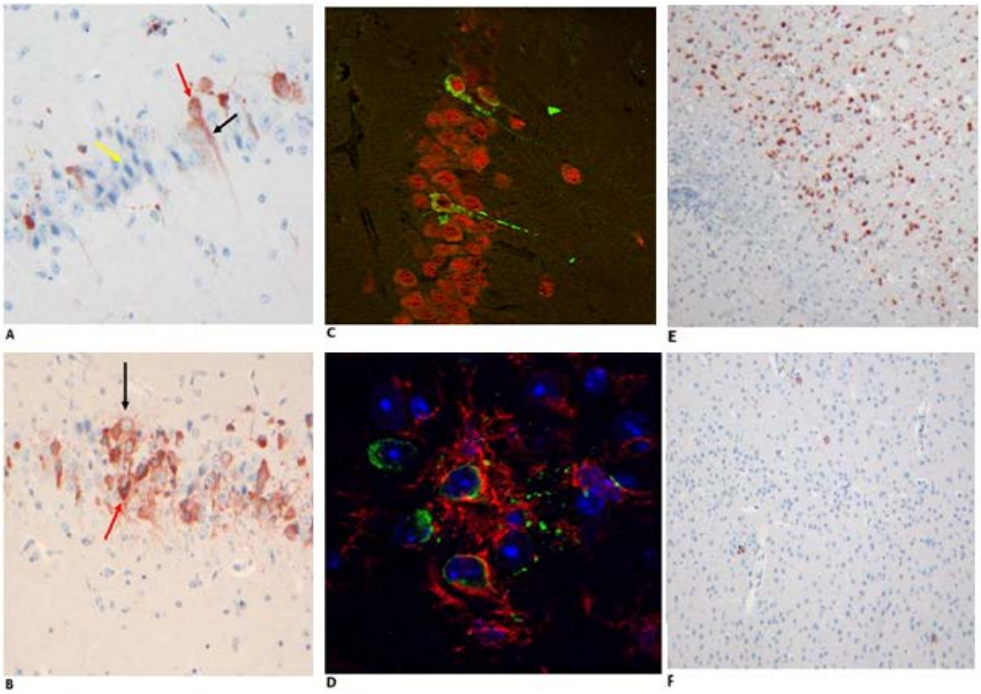
The extent of infection in the brain was determined in terms of infected brain areas (Table S2). In general, positive neurons occurred in all areas of the brain (Table S2) and the spinal cord, although the regional distribution and relative density of positive cells was highly variable between animals injected with the same virus. For instance, animals infected with Ita09 virus showed highly sporadic, moderate amounts, or very frequent NS3-positive neurons in the hippocampal CA1 area (Figure 5A & B). A similar variability was also observed in other brain areas, including the neocortex, striatum and cerebellar cortex (Figure 5E & F), and there was no correlation between the amount of positive cells in different brain areas in the same mouse. Because of the large variability between animals injected with the same viruses, it was difficult to determine whether systematic differences occurred in the amount and distribution of positive cells between animals injected with different viruses. For instance, infection of Purkinje cells in the cerebellum was observed in sections of some mice infected with Ita09 (Figure 5H) and 578/10 but not in mice infected with FIN. However, in view of the potential focal distribution of infected cells, the question whether this difference holds true for all Purkinje cells of FIN-injected mice would require the systematic analysis of the entire cerebellum of all mice, which is beyond the scope of this study. Nevertheless, based on the analyses performed in this study, mice infected with Ita09 and 578/10 showed more positive cells than mice infected with FIN.

Furthermore, antigen distribution and quantity of positive cells in the spinal cord was also highly variable, with WNV positive cells occurring in both dorsal and ventral horns. Although not systematically investigated, spinal motor neurons in the ventral horn were infected more often than dorsal horn cells, despite their relatively low abundance compared to other spinal cord cells (Figure 5G).

Analysis of WNV-NS3 expression in the brains of mice that did not develop clinical disease and that were killed at 20 days post infection revealed no positive cells, which is consistent with the absence or low abundance of virus antigen in the brains of these mice. Positive cells were also not found in the spinal cord of these animals.

To determine whether the presence of virus-positive neurons and neuronal debris correlated with microglia cell activation we stained for Iba1, which is up-regulated in activated microglia cells [21-24]. Increased Iba1 staining, as compared to control mice, was observed in the nervous system of all mice with virus-positive neurons (Figure 5J). In

accord with the variability in distribution and quantity of WNV-NS3 positive neurons, changes in Iba1 staining was also variable between different mice infected with the same virus or different viruses. Remarkably, however, in some cases, areas with high levels of WNV-NS3 positive cells and debris did not show a strong increase in Iba1 positive cells, indicating that a high level of virus-infected cells is not necessarily paralleled by high levels of microglia cell activation. Astrocytosis (as demonstrated by increased GFAP-staining) was seen in all animals and cases ranged from mild to severe for all virus strains (Figure 5I). Astrocytosis and microgliosis were also evident in the brains of the survivor mice.



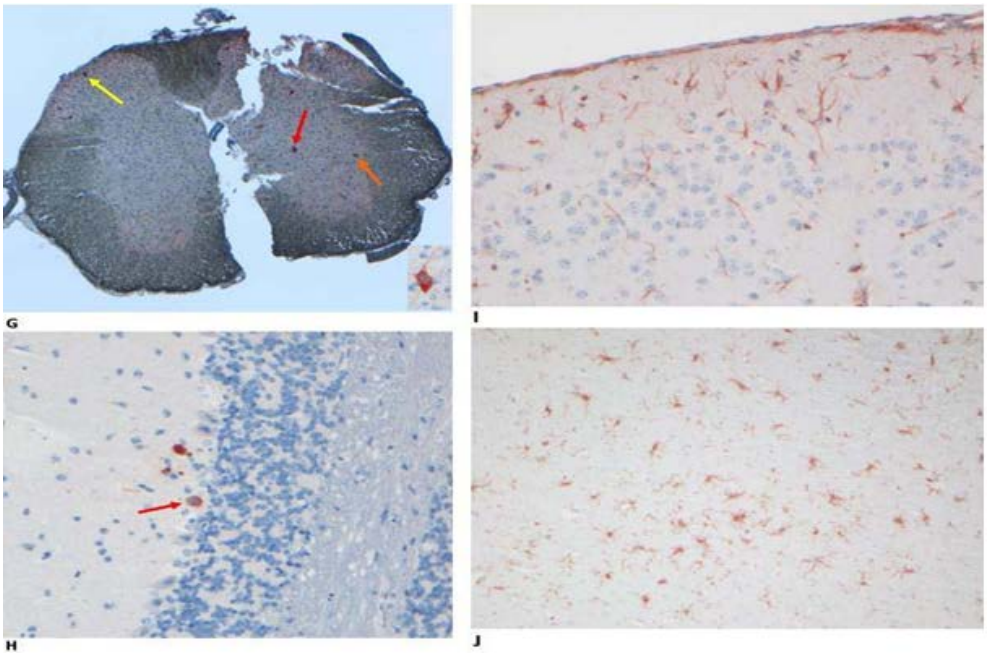


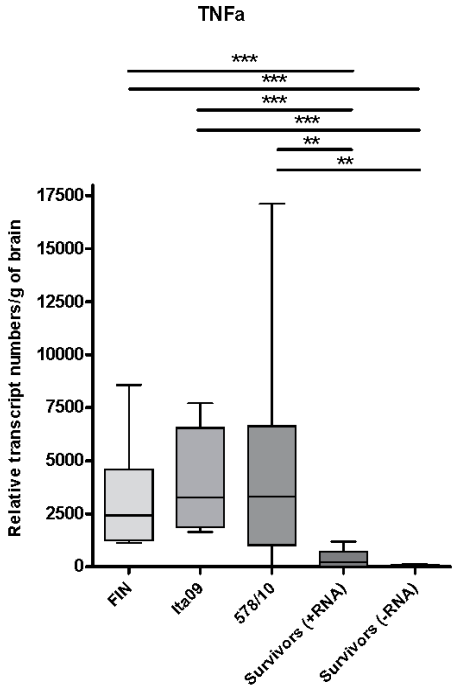
Figure 5. Histopathology of 6-week old C57BL/6 mice infected with WNV-FIN, Ita09 and 578/10 and euthanized upon reaching humane endpoints between days 6-14 p.i. Representative picture of **(A)** Neurons in the hippocampus of a mouse infected with Ita09, stained with anti-WNV NS3 antibody (objective 20×). Antigen is distributed in the cell body (red arrow) and proximal dendrite (black arrow). Some of the infected neurons appear to be in a healthy state with normal nuclear, perikaryal and dendritic morphologies (red/black arrow), while some uninfected neurons appear to be in a moribund state (yellow arrow). **(B)** Neurons in the hippocampus of a mouse infected with Ita09, stained with anti-WNV NS3 antibody (objective 20×). Antigen-expressing neurons appear to be in a different state of health varying from healthy appearance with normal nuclear, perikaryal and dendritic morphologies (black arrow), to dying cells (red arrow). **(C)** Double staining as seen by confocal microscopy (objective 20×) showing neurons (stained with NeuN antibody and Alexa Fluor 555 conjugate; red) infected with Ita09 (stained with anti-NS3 antibody and Alexa Fluor 488 conjugate; green). **(D)** Double staining as seen by confocal microscopy (objective 40×) showing activated microglia (stained with anti-Iba1 antibody and Alexa Fluor 555 conjugate; red) engulfing neurons infected with WNV-Ita09 (stained with anti-NS3 antibody and Alexa Fluor 488 conjugate; green). Nuclei are stained with DAPI (blue). **(E)** Neo-cortical neurons in the brain of a mouse infected with WNV-Ita09, stained with anti-WNV NS3 antibody (objective 10×). **(F)** Neo-cortical neurons in the brain of a mouse infected with WNV-FIN, stained with anti-WNV NS3 antibody (red arrows; objective 10×) **(G)** Spinal cord of a mouse infected with WNV-578/10, stained with anti-WNV NS3 (objective 4×). Infection of motor neurons (red arrow), anterior horn (orange arrow) and posterior horn (yellow arrow) was observed. Insert shows infected motor neuron (objective 20×). **(H)** Purkinje cell in cerebellum of mouse infected with Ita09 (red arrow), stained with anti-WNV NS3 antibody (objective 20×). **(I)** Mild to moderate activation of astrocytes in the cortex of a mouse infected with WNV-578/10, stained with anti-

GFAP (objective 20×). **(J)** Activation of microglia cells in the cortex of the brain of a mouse infected with WNV-FIN, stained with anti-Iba1 (objective 10×).

Finally, we analyzed infiltration of T-cells using anti-CD3 staining (Table S3). Perivascular cuffing by CD3-positive cells was highly variable and was only evident in localized regions in a subset of sections examined, consistent with the large variability of infected areas. Infiltration of the neuropil by T cells (CD3-positive cells) was mainly seen in animals infected with Ita09 in the cerebrum and brainstem (~1 positive cell per high power field [HPF]; objective 40×) and in some of the animals infected with FIN or 578/10 (<1 cell per HPF in the brainstem). Interestingly, infiltration of CD3-positive cells into the cerebrum and brainstem were found in all mice that survived infection with any of the WNV-strains. In comparison to the mice that had died from infection, all mice that survived appeared to have more CD3-postive cells in the brain. Taken together, the IHC studies provide supportive evidence that Ita09 and 578/10 are more virulent than FIN.

Response to WNV infection in brains of mice

To allow for quantitative comparison of the response to infection with different strains of WNV, we performed qRT-PCR using half brain homogenates (Figure 6). The survivor mice were split into two groups based on presence or absence of viral RNA in the brain. We chose for markers that have been associated with viral encephalitis and neurodegenerative diseases. The inflammatory marker TNF-α was increased in the brains of mice infected with FIN (P = 0.002; P <0.0001), Ita09 (P = 0.007; 0.007) and 578/10 (P = 0.006; 0.003) compared to the survivors positive and negative for viral RNA in the brain, respectively. One of the markers involved in the breakdown of extracellular matrix, MMP-3, was significantly up-regulated in the brains of mice infected with FIN (P = 0.004; 0.0009), Ita09 (P = 0.0007; 0.0007) and 578/10 (P = 0.003; 0.002) compared to convalescent mice with and without viral RNA in the brain. In addition, MMP-3 transcript levels were significantly higher in Ita09 compared to those infected with FIN (P = 0.005). MMP-9 transcript was increased in mice infected with FIN (P = 0.02) and 578/10 (P = 0.03) only when compared to the convalescent mice positive for viral RNA in the brain. Nptx-1 (a marker involved in apoptosis) was only up-regulated in the brains of the survivor mice group with viral RNA in the brain compared to the group without viral RNA (P = 0.05). Nptx-2 and Ptx3, inflammatory markers involved in complement activation and complement-mediated clearance of apoptotic cells, were not significantly up-regulated in any of the experimental groups. These data identify some inflammatory markers significantly elevated during infection of the brain with WNV, but none of the examined markers correlate with virulence.


TNFa
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17500
15000
12500
10000
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2500
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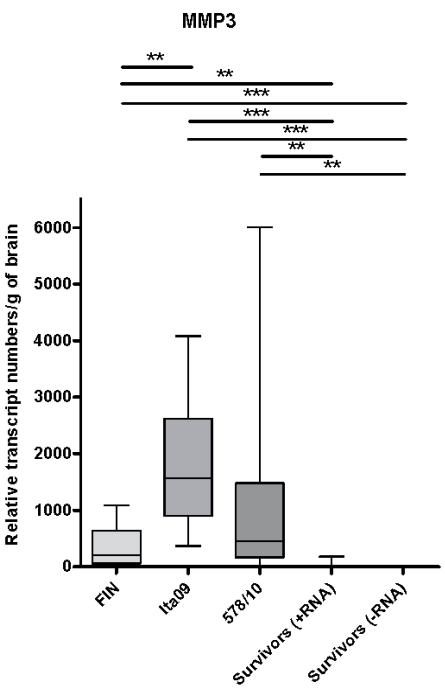
Relative transcript numbers/g of brain
FIN
Ita09
578/10
Survivors (+RNA)
Survivors (-RNA)


MMP3
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Relative transcript numbers/g of brain
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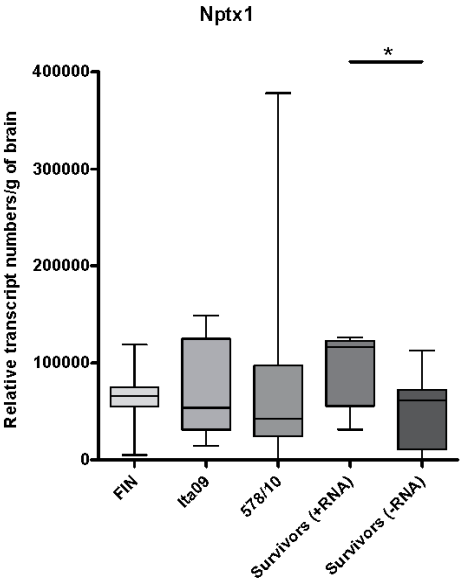
578/10
Survivors (+RNA)
Survivors (-RNA)


Nptx1
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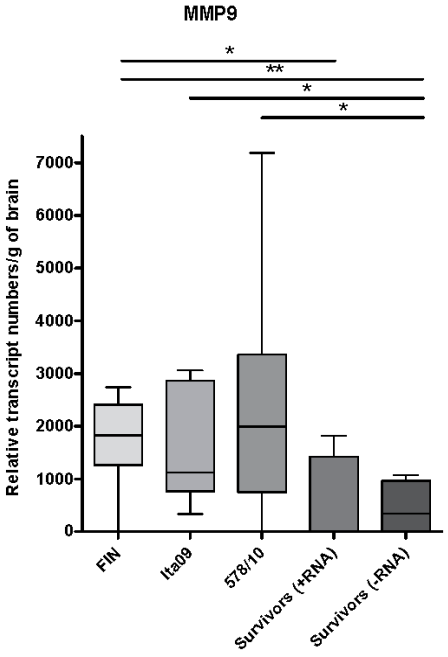
Relative transcript numbers/g of brain
FIN
Ita09
578/10
Survivors (+RNA)
Survivors (-RNA)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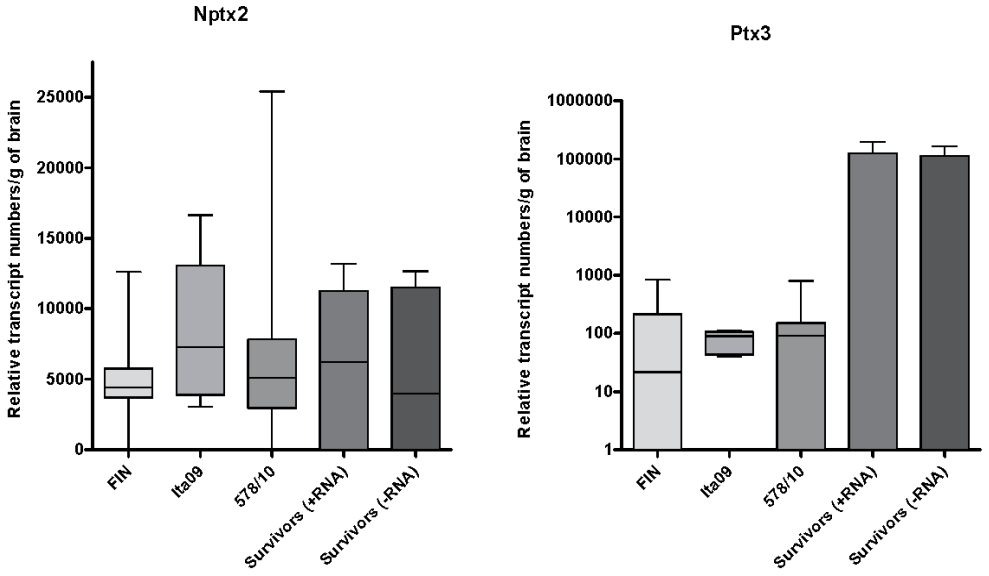

Figure 6. Relative number of RNA transcripts of markers in the brains of mice infected with WNV-FIN, Ita09 and 578/10. Sick animals were euthanized within days 6-14 upon reaching humane endpoints or on day 20 post-infection in the absence of clinical signs. Transcripts were compared with animals that survived WNV infection (day 20 p.i) and that were either positive or negative for viral antigen in the brain. Mean is indicated by a cross and median by a line in the boxes. The box represents the interquartile range. * P <0.05, ** P <0.01, *** P <0.001. Abbreviations: TNF = tumour necrosis factor; MMP = matrix metalloproteinase; Nptx = neural pentraxin; Ptx = pentraxin.

DISCUSSION

In this study we have characterized the European WNV strains FIN, Ita09 (both lineage 1) and 578/10 (lineage 2). Neurovirulence of the three WNV strains were determined by comparing *in vitro* replication kinetics, median day mortality, cumulative mortality, LD_{50}, concentration and distribution of virus in the brain, and the response to infection in the brain.

Despite the fact that WNV has been circulating in Europe for half a century, it is only in the more recent years that this virus has caused considerable outbreaks in humans, horses and to a much lesser extent in wild birds. This is in contrast to the emergence of WNV in North America during which wild birds were heavily affected and significant numbers of human neuroinvasive disease cases with high mortality were reported. It is possible that this increase in severity is a result of the movement of the virus into areas with large immunologically naïve populations that consist of a large proportion of elderly and immunocompromised individuals [25]. However, it has also been suggested that a more virulent strain of the virus was introduced [26]. It is also hypothesized that the viruses currently circulating in Europe differ in their virulence profile compared to the North American strains.

There have been a number of explanations for why viruses are virulent [27,28], and it is clear that virulence is an adaptive process and that it is the result of the trade-offs between virus transmissibility, virus pathogenic force, and recovery potential of the host. In several models it has been shown that changes in virulence are associated with changes in different aspects of the biology of virus-host interaction, suggesting that virulence of a given virus may be affected by a potentially large number of factors (reviewed in [29-33]). Since no single general factor exists that can be used to predict the relative virulence of viruses, we investigated the virulence of European WNV strains by considering a series of parameters. Even though it is possible to investigate virulence by conducting a straight kinetic analysis and examining viral spread in the brain over time, we decided to use survival as an outcome of disease severity. This is because disease can be a direct consequence of viral burden, inflammatory response and injury and death of cells in the CNS, and therefore an appropriate measure of virulence. Following infection, WNV replicates to high levels during the acute phase, after which the virus typically enters the brain and causes meningo-encephalitis before the immune system is able to control the infection. Our aim was to define the virulence profile of European WNV strains by measuring different markers *in vitro* and *in vivo*.

First we determined the population structure (quasispecies) in our virus stocks. We chose to characterize the stock rather than the dominant variant in the stock that was used to infect mice. It has been shown that viral quasispecies is more than just a collection of mutants, but a group of interactive variants, which together contribute to pathogenesis [34]. For instance, it was found that the diversity of the quasispecies of Polio virus correlated with enhanced pathogenesis in mice [34]. Ciota *et al*. [35] have shown that the quasispecies

in WNV populations correspond to substantial phenotypic diversity that differed in relative fitness *in vitro*. We have used NGS to determine the population phenotype of our viral stocks, based on the glycoprotein E gene. This gene was selected because it is the principal receptor that determines tropism for neurons, contains markers of virulence, and it harbors areas that allow monitoring of virus evolution. One advantage of the NGS is the possibility to detect minor variants. In this study, we found that only the dominant variants from the stock were selected in the brain. One important issue concerning the NGS is the ability to distinguish between true variants and variants detected as a result of errors introduced during PCR amplification and/or sequencing. Therefore, within our department, we have determined the error rate threshold specific for this platform. We found that a threshold of 0.026% (*manuscript in preparation*) is sufficient to exclude variants detected as a result of errors. The technical cut-off value of 1% described in this manuscript is therefore well above the error margin attributed to reverse transcription, amplification and sequencing errors. Furthermore, emergence of new variants was also detected in the brain, which could have been a result of mutations arising during the replication cycle. We did not specifically study whether the population structure of our strains contributed to virulence.

The infection cycle of WNV has not been studied extensively *in vitro*, so we first addressed the dynamics of WNV infection *in vitro* in neuronal cells. We found that only adherent cells could be infected and infection of N2a cells in suspension was not successful (data not shown). The reason for this phenomenon is unknown, but may be related to receptor availability on adherent cells. Currently, there is little known about the host receptor for flaviviruses. The replication cycle of WNV can be divided into three phases; (1) dispersal-diffusion-attachment phase, (2) eclipse phase (begins with infection and ends when the virus progeny matures inside the host), and (3) release phase (the virus offspring are released from the infected cell). The total number of progeny released in the supernatant is termed the burst size. Examination of these three stages in virus replication is useful, because the associated growth parameters (eclipse period, latent period, exponential growth rate, and burst) yield plausible hypotheses to account for differences in virulence.

A study has shown that large clusters of matured virus of the Sarafend strain appear at the plasma membrane, as well as in vacuoles, at 10-12 hours post-infection [36]. This is in line with our results where we determined the eclipse period to be at approx. 12 hours p.i. The study further showed that maturation of WNV at the plasma membrane, and therefore budding from infected cells, is the dominant mode of maturation for this virus, but that during the later stage of infection (from 12 hours p.i.) the virus is also released via exocytosis, most likely due to advanced cytopathic effects [36]. As a result, it may be difficult to determine the precise time point of the burst size and we have therefore additionally determined the LT_{50}. Furthermore, analyzing the RR may shed light on the potential of WNV strains to overwhelm the target cells. Virions that are able to overwhelm the system quickly will have an increased chance of colonizing the remaining uninfected cells, an advantage particularly important *in vivo*. We found that Ita09 replicates faster as

evidenced by the LT_{50} and release of up to ten-fold more virus after the first replication cycle.

The increase in morbidity and case-fatality rates caused by North American lineage 1 strains relative to lineage 2 strains led to the hypothesis that lineage 1 strains are highly pathogenic while lineage 2 strains that used to be endemic only to Africa are of low virulence [4]. Conversely, recent outbreaks in South Africa and Europe indicate that lineage 2 strains may also cause severe disease [37]. This observation was also supported by experimental studies in mice showing that differences in virulent WNV strains did not correlate with the phylogenetic lineage, source of isolate, geographic distribution, passage level or year of isolation, and suggest instead that pathogenicity is not genotype specific and that both lineage 1 and 2 are neurovirulent [38,39]. Our results indicate that all the European strains studied are virulent in C57BL/6 mice and that the lineage 1 strain (Ita09) and lineage 2 strain (578/10), which share similar virulence profiles, are slightly more virulent than FIN. Two lineage 1 viruses with 99.7% identity (FIN and Ita09) were found to share a different virulence profile. These viruses differed in terms of three non-conservative amino acids. One of the substitutions present in the Ita09 strain, T249P, is a mutation in the helicase domain of the NS3 protein and has been associated with increased virulence in American crows [40]. This mutation has been found in the more recent Italian WNV isolates from 2008 (15803/08 and 15217/08), while the Italy 1998-equine strain still has the threonine at this position [41]. Interestingly, the 2010 Greek isolate also contains the proline, and may be responsible for the increased virulence of this strain compared to other strains from lineage 2 [10]. However, the virulence properties of this sole substitution in outbred mice remains unclear, and its role in virulence in humans on a population level is questionable. For instance, a WNV strain isolated in 2007 from golden eagles in Spain carrying a T→P mutation did not have increased pathogenicity in mice compared to other strains [42]. Similarly, the WNV-FIN strain used in this study was isolated from a patient with neuroinvasive disease. It is possible that the three amino acids differences between Ita09 and FIN collectively reduced the virulence of FIN.

Mice that had died as a result of infection with Ita09 and 578/10 were found to have a significantly higher number of RNA copies in the brain compared to those infected with FIN. Infectious virus titers, however, were not significantly different. This discrepancy might be explained by the involvement of immature virus particles, which may also play a role in the pathogenesis of WNV [43]. It is therefore possible that there was a higher viral burden in the brains of mice infected with Ita09 and 578/10 as a result of a larger amount of immature virus particles, which may explain the higher virulence observed for these virus strains.

The histopathologic findings observed in the brain and spinal cord samples of the mice that succumbed to the infection were pathognomonic, with moderate to severe infection observed in mice infected with Ita09 and 578/10 strains. In agreement with previous studies, WNV antigen was found in neurons in the spinal cord, cortex, hippocampus and brainstem [15,44]. Interestingly, antigen of Ita09 and 578/10 was found

also in the Purkinje cells of the cerebellum. Infection of Purkinje cells by North American strains of WNV has been demonstrated in hamsters [45-47], birds [48,49], macaques [50] mice [51] and humans [52]. This targeting of Purkinje cells of the cerebellum has been shown to be a unique pathologic finding in WNV encephalitis, unlike the encephalitides caused by other closely related flaviviruses [48,53-57]. The higher frequency and intensity of antigen staining in the central nervous system and the higher mortality observed in the Ita09 and 578/10–infected groups compared with the FIN–infected group (lineage 1), suggest that these two strains are more virulent.

Even though some of the mice that survived infection were positive for viral RNA, IHC staining did not demonstrate antigen in the brain. The intensity of staining found using IHC did roughly correlate with the amount of RNA found in the brain. As RNA titers in the brain of the survivor mice were significantly lower compared to mice that died from infection, it is possible that these low titers, in addition to unsystematic sampling, resulted in the absence of detection of positively stained cells using IHC in brain samples of the survivor mice.

We have also measured the response to infection in the brain as a virulence factor. Although astrocytosis and microgliosis were observed in all mice that died from the infection, we did not find evidence of infection of these cells. It is believed that activated microglia and astrocytes contribute to an excessive inflammatory response, which triggers a process of secondary cell death or functional depression in structurally normal areas distant from, but connected to the original sites involved. We have found that MMP-3 transcript was significantly elevated in all animals that developed severe disease. MMPs are capable of degrading the tight junction proteins of human brain microvascular endothelial cells, thereby compromising the integrity of the blood-brain barrier. Further studies are necessary to address the question of whether MMP-3 is a virulence factor triggered by pathogenic WNV strains.

To our knowledge, this is the first study to characterize pathogenic properties of WNV strains circulating in Europe. We have found that all three European strains of WNV are neurovirulent in C57BL/6 mice; however, the data also suggest that Ita09 and 578/10 show an increased virulence in comparison to FIN. Studies are ongoing to determine the virulence of these strains in European birds and in other outbred animal models.

ACKNOWLEDGMENTS

We would like to thank Tien Nguyen for his assistance with the confocal microscopy. The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/2007 - 2013) under the project "VECTORIE", EC grant agreement number 261466.

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SUPPORTING INFORMATION

Primer	Sequence (5'to 3')	Application
#721modified48R	AGCTCTTGCCGGCTGATGTC	RT-PCR
#722modified51F	AGCTTCAACTGCCTTGGAATGAG	PCR/Sequencing
#723modified57R	TGTCAGCGTGCACGTTCACGGA	PCR/Sequencing
#43WNV.E04F	CGCCAAATTTGCCTGCTCTAC	Sequencing
#44WNV.E05R	AGTTTGAGGAACCACACGCCA	Sequencing
#732WNVII-870f*	CCTCGTTGCAGCTGTCATTG	Sequencing
#733WNVII-1630r*	TCCATGGCAGGTTCAGATCC	Sequencing
WNVE-Deepseq-F1	ATGACAAACGTGCTGACC	454 sequencing
WNVE-Deepseq-R2	GTTCACAGTCCACTGTCACCTCTC	454 sequencing
WNVE-Deepseq-F2	CGCCTTCATACACACTAAAG	454 sequencing
WNVE-Deepseq-R3	AGCCTTTGAACAGACGCCAT	454 sequencing
WNVE-Deepseq-F3	GTAGAGTGAAGATGGAAAAATTGC	454 sequencing
WNVE-Deepseq-R4	GCTGTGTCTCCTAGAGCGGC	454 sequencing
WNVE-Deepseq-F4	ACAACCACCCTCAAAGGA	454 sequencing
WNVE-Deepseq-F1/853-HUN	AACGAGAAAAGAGCTGACCCCG	454 sequencing
WNVE-Deepseq-R2/854-HUN	GCTCACAGTCAACCGTGACCTCAC	454 sequencing
WNVE-Deepseq-F4/859-HUN	CCTTTACCACTACACTCAGAGGAGCT	454 sequencing
WNVE-Deepseq-723-HUN	TGTCAGCATGGACGTTGACCGA	454 sequencing
3'UTR F	CCACCGGAAGTTGAGTAGACG	Taqman

3'UTR R	TTTGGTCACCCAGTCCTCCT	Taqman
3'UTR probe FAM-TAMRA	TGCTGCTGCCTGCGGCTCAACCC	Taqman

Supplementary Table 1. Primers used for sequencing the envelope of WNV-NY99, FIN, Ita09 and 578/10. Primers indicated with * were kindly provided by Dr. Tamás Bakonyi (Szent István University, Hungary) and were used to sequence the envelope of 578/10.

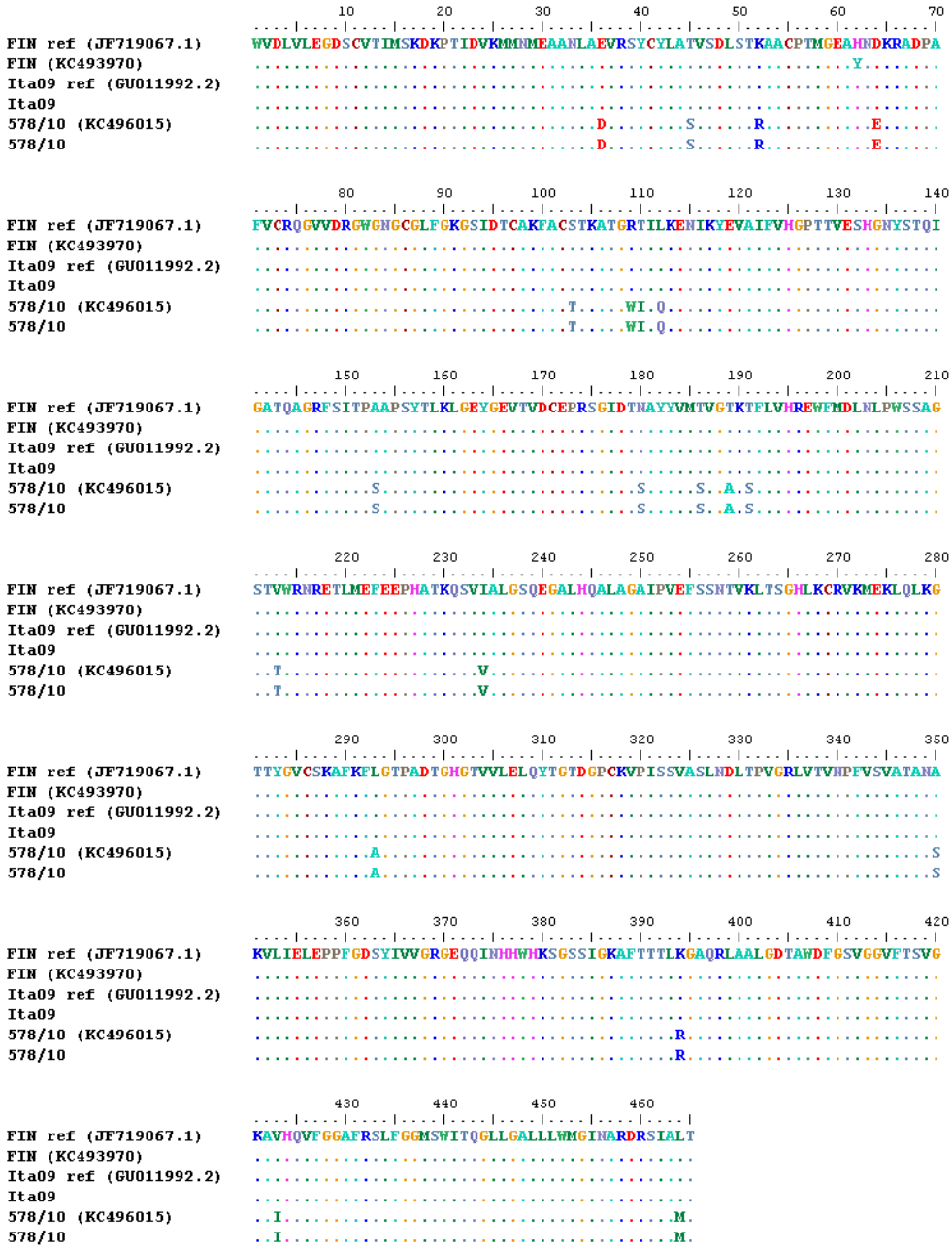
WNV strain	**CTX**	**CNU**	**TH**	**HY**	**HPF**	**MB**	**MY/ Pons**	**CB**
WNV-FIN n=10 (died)	60%	20%	10%	40%	30%	30%	20%	40%
n=9 (survived)								
WNV-Ita09 n=10 (died)	90%	60%	50%	20%	30%	20%	50%	60%
n=6 (survived)								
WNV-578/10 n=18 (died)	78%	50%	39%	39%	33%	44%	44%	17%
n=3 (survived)								

Abbreviations: CTX = cortex; CNU = cerebral nuclei; TH = thalamus; HY = hypothalamus; HPF = hippocampal formation; MB = midbrain; MY = medulla; CB = cerebellum

Supplementary Table 2. Antigen distribution (described in terms of staining of the NS3 protein) in the brains of mice infected with WNV-FIN, Ita09 and 578/10, and either euthanized upon display of clinical signs of disease (between days 6-14) or euthanized on day 20 without showing signs of illness. Percentage indicates the amount of infected mice that are positive for antigen in each particular brain region.

T cell staining (anti-CD3 antibody)						
	Cerebrum		**Brainstem**		**Cerebellum**	
	Infiltration	Perivascular cuffs	Infiltration	Perivascular cuffs	Infiltration	Perivascular cuffs
FIN	<1/ HPF (in 50% of mice)	Neg	<1/ HPF	Neg	Neg	Neg
Survived		1 layer thick in 10% of mice	1 cell per HPF (10% of mice)			
Ita09	1/ HPF (in 50% of mice)		1/HPF			<1/mouse (1 cell layer thick)
Survived	Clusters of positive cells (in 33% of mice)		Clusters of positive cells (in 33% of mice)	2 cell layers thick		
578/10	Neg	Neg	<1/HPF (in 50% of mice)	Neg	Neg	Neg
Survived	<1 cell per HPF (50% of the mice)		<1 cell per HPF (50% of the mice)			

Supplementary Table 3. Detection of CD3 positive cells in the brains of mice infected with WNV-FIN, Ita09 and 578/10, and either euthanized upon display of clinical signs of disease (between days 6-14) or euthanized on day 20 without showing signs of illness. Numbers indicate the number of positive cells; HPF: high power field; objective 40X.



Supplementary Figure S1. The sequences of glycoprotein E of the WNV stock used in this study were determined with the Sanger method. The deduced amino acid sequences were aligned. The sequence of FIN was deposited in GenBank (KC493970). The sequence of Ita09 and 578/10 were compared to GU011992.2 (Ita09) and KC496015 (578/10) and FIN was compared to a highly similar sequence (accession JF719067.1; 99% similar to FIN).

Chapter 3

Development of a strand-specific real-time qRT-PCR for the accurate detection and quantitation of West Nile virus RNA

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J Virol Methods. 2013 Dec;194(1-2):146-53

ABSTRACT

Studying the tropism and replication kinetics of West Nile virus (WNV) in different cell types *in vitro* and in tissues in animal models is important for understanding its pathogenesis. As detection of the negative strand viral RNA is a more reliable indicator of active replication for single-stranded positive-sense RNA viruses, the specificity of qRT-PCR assays currently used for the detection of WNV positive and negative strand RNA was reassessed. It was shown that self- and falsely-primed cDNA was generated during the reverse transcription step in an assay employing unmodified primers and several reverse transcriptases. As a result, a qRT-PCR assay using the thermostable r*Tth* in combination with tagged primers was developed, which greatly improved strand specificity by circumventing the events of self- and false-priming. The reliability of the assay was then addressed *in vitro* using BV-2 microglia cells as well as in C57/BL6 mice. It was possible to follow the kinetics of positive and negative-strand RNA synthesis both *in vitro* and *in vivo*; however, the sensitivity of the assay will need to be optimized in order to detect and quantify negative-strand RNA synthesis in the very early stages of infection. Overall, the strand-specific qRT-PCR assay developed in this study is an effective tool to quantify WNV RNA, reassess viral replication, and study tropism of WNV in the context of WNV pathogenesis.

INTRODUCTION

West Nile virus (WNV) is a neurotropic RNA virus with a positive-sense single-stranded genome that belongs to the genus Flavivirus in the *Flaviviridae* family. The replication of WNV proceeds through a negative strand RNA intermediate [1], synthesized by the virus-encoded RNA-dependent RNA polymerase. The negative strand is used as a template for the synthesis of new single stranded positive-sense RNA molecules [2]. Therefore, detection of this negative strand, which is generally significantly outnumbered by the positive strand, signifies viral replication.

In vitro experiments have shown that cortical astrocytes are susceptible to infection with WNV while replication in microglia cells was not supported [3]. *In vivo*, WNV infects mostly neuronal cells and on occasion has also been shown to infect microglia cells [4]. In general, however, *in vivo* tropism of WNV for astrocytes and microglia cells remains mostly speculative. In order to determine permissibility of these cells to infection by WNV, synthesis of the negative strand can be assessed using strand-specific quantitative RT-PCR. Another issue that could be addressed by determining the presence of negative strand is persistence of WNV, which has been described in several organs and animal species [5-9]. Specific strand detection can therefore be useful in the elucidation of the mechanisms of persistent infection and may contribute to the further understanding of WNV pathogenesis.

Even though qRT-PCR is the method used most widely for the quantitation of viral RNA, its major disadvantage is that it provides limited strand specificity. As a result, standard qRT-PCR cannot determine the absolute quantity of viral RNA copies in a given sample due to the presence of both positive and negative strands of RNA. This lack of strand specificity has been attributed to a combination of factors, including self-priming of the RNA due to secondary hairpin structures [10-15], false priming of the incorrect strand [10, 16-18] and random priming by contaminating endogenous or exogenous nucleic acids [13, 16, 19]. Attempts to overcome these problems include performing RT reactions at high temperatures [20, 21], use of the thermostable RTth enzyme [10, 22-24], use of tagged RT primers [25-28] or a combination of tagged primers and RTth enzyme [29]. It was shown that the approach employing tagged primers in combination with high RT temperature greatly improved the specificity of the RT reactions. So far, quantitation of negative strand RNA for analysis of positive-sense RNA virus replication has been applied for a number of viruses, which include hepatitis A [24, 30], hepatitis C [10, 11, 18, 29, 31], hepatitis E [32], GB virus C [33], dengue [25], O'nyong-nyong and Chikungunya [27], murine norovirus [28] and foot-and-mouth disease virus [34]. Due to the problems associated with false detection of negative strand RNA, conclusions derived from assays not optimized for strand-specific detection of RNA may not be valid [35, 36].

In this study, it was shown that the qRT-PCR system using conventional WNV-specific primers for the detection of positive and negative strand viral RNAs lacks strand-specificity due to amplification of both falsely primed cDNA generated from the incorrect strand, as well as cDNA generated from self-primed RNA, both occurring during the RT

step. As a result, a qRT-PCR system that employs both tagged primers and the thermostable RTth enzyme was developed in order to overcome these problems, which greatly improves the specificity of detection of negative and positive strand viral RNAs for the study of WNV replication.

MATERIALS AND METHODS

Cells and viruses

The mouse microglia cell line BV-2 (kindly provided by Dr. M Leist, University of Konstanz, Germany) was grown in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% heat inactivated fetal bovine serum (HI-FBS). Virus used in this study consisted of the NY99 strain (accession AF196835.2, obtained from the Health Protection Agency, Porton Down, UK; Passage (P) 4 on Vero E6 cells). Virus stock was prepared by growing the P4 virus stock once on C6/36 insect cells. C6/36 insect cells were cultured in Leibovitz-15 medium supplemented with 5% HI-FBS, 10% tryptose phosphate broth, 0.75% sodium bicarbonate and 10 mM hepes buffer. All media were supplemented with antibiotics (100 U penicillin, 100 µg/ml streptomycin) and 2 mM L-glutamine. Cell culture reagents were obtained from Lonza, Breda, The Netherlands.

Synthesis of *in vitro* RNA transcripts

In order to quantify negative and positive strands, run-off transcripts for each strand were generated from a plasmid (pCR®4-TOPO®; Invitrogen, Breda, The Netherlands) containing the sequence of the 3'UTR of WNV. In order to synthesize the positive-sense transcript, plasmid DNA was linearized using the NotI restriction enzyme. For synthesis of the negative-sense transcript, DNA was linearized with the PmeI restriction enzyme. Linearized plasmid DNA was cleaned up using the MinElute Reaction Cleanup kit (Qiagen, Venlo, The Netherlands) and run-off transcripts were generated using the Ambion® MaxiScript T7/T3 kit (Invitrogen). The positive sense RNA was transcribed using the T3 polymerase and the negative sense RNA using the T7 polymerase. The product was subsequently incubated with 2 U of TURBO DNase (Invitrogen) at 37 ºC for 15 min to remove residual DNA and the reaction was cleaned up using the High Pure RNA Isolation kit (Roche Diagnostics, Almere, The Netherlands), which included an additional DNA digestion step using 100 U of DNase I (Roche). The RNA transcripts were eluted in 50 µL and the concentration was determined using spectrophotometer (NanoDrop) readings at wavelength 260 nm. The stocks of *in vitro* transcribed RNA were subsequently diluted to a concentration of 3.7×10^7 copies/µL for the positive sense and 4.5×10^7 copies for the negative sense RNA, a concentration at which DNA was no longer detected using qRT-PCR.

Quantitative RT-PCR of negative and positive strand viral RNAs using unmodified and tagged primers

Primer and probe sets used in this study are specific to the 3'UTR of WNV and are based on previously published primers and probe (Meece et al., 2003), but with an additional TgC triplet added to the probe (Table 1). First, detection of strand-specific RNA was performed using 'unmodified primers'. For detection of positive strand RNA, RT was performed with the R96 primer and for detection of the negative strand RT was done with the F34 primer. Subsequently, PCR amplification was performed using both the F34 and R96 primers added to the reaction after cDNA synthesis. Alternatively, 'tagged primers' were used for the RT reaction. To this end, the R96 and F34 primers were tagged by adding a 32-mer-long sequence of the Grapevine virus A as a tag at the 5'-end of the respective primers (Table 1; tag sequence indicated in bold). This procedure has been reported to prevent the amplification of cDNA products made by false priming of either RNA strand as well as the amplification of cDNA acquired as a result of self-priming [10]. Specific detection of the WNV-positive RNA strand was performed during cDNA synthesis using the Tag-Rev primer, complimentary to the positive-sense strand, and the negative strand was detected using the Tag-Fwd primer, which is complimentary to the negative-sense strand. Subsequently, the positive strand was amplified using the Tag sequence as the reverse primer and WNV F34 as the forward primer. In contrast, the negative strand was amplified using the Tag sequence as a forward primer and WNV R96 as a reverse primer. All RT reactions contained 30 pmol of primer and were carried out for 2 min at 50 ºC and 30 min at 60 ºC using the r*Tth* RT enzyme according to the instructions provided by the TaqMan EZ RT-PCR kit (Applied Biosystems, Bleiswijk, The Netherlands). RT reactions using unmodified primers contained 5 µL of RNA while those using tagged primers contained 7 µL of RNA. The RT function of the r*Tth* enzyme was inactivated for 15 min at 95 ºC. In all cases, PCR amplification consisted of 30 pmol of primer, 15 pmol of WNV 56 probe in a total volume of 50 µL, and cDNA was amplified in 40 amplification cycles of 20 sec at 95 ºC and 1 min at 60 ºC using the r*Tth* enzyme (DNA polymerase function). All reactions were carried out on a 7500 Fast real time PCR System (Applied Biosystems) and analyzed using 7500 Software v.2.0 (Applied Biosystems).

The effect of the transcriptase on false-priming and self-priming was also investigated. To this end, RT-PCRs were carried out using Superscript III (SSIII; Invitrogen), MultiScribe (MS; Applied Biosystems) and Avian Myeloblastosis Virus RT (AMV; Promega). The RT-PCRs for these enzymes were as follows: 15 min at 25 ºC, 45 min 50 ºC, 15 min 70 ºC for SSIII, 30 min at 48 ºC for MS, and 60 min at 42 ºC for AMV. All reactions were succeeded by inactivation of the RT at 95 ºC for 15 min. The RT reactions comprised 10 µL of RNA and 20 pmol of reverse primer. Subsequently, 2.5 µL of the cDNA reaction was added to the TaqMan PCR using primers and probe as described above.

In vitro infection of mouse microglia cells with WNV-NY99

BV-2 cells were cultured overnight in 96-well flat bottom culture plates (10^4 cells/well) and virus was added at MOI 0.5. Virus was allowed to adsorb for one hour at 37 ºC. Cells were subsequently washed three times with serum-free medium to remove virus inoculum, replenished with fresh medium and cultured at 37 ºC for 48 hours. Cells were collected in triplicate at 12, 24, 36 and 48 hours. Culture supernatants were removed and cells were taken in 100 µL of PBS and lysed with 400 µL of lysis buffer (Roche). RNA was then extracted using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche) and an automated nucleic acid robotic workstation (Roche) according to the manufacturer's instructions. RNA was eluted in 50 µL of elution buffer (Roche) and stored at -80 ºC until used. Synthesis of cDNA was initiated with 7 µL of total RNA using the r*Tth* enzyme and tagged primers to detect the presence of both positive and negative strand viral RNA. The $\log_{10}$ titrated *in vitro* RNA transcript standard curve (T3: 3.7×10^7 copies; T7: 4.5×10^7 copies) was reverse transcribed at the same time as the cell culture RNA samples.

Mouse infection with WNV-NY99

Nine-day old female C57BL/6 mice (Harlan Laboratories B.V., Venray, The Netherlands) were inoculated intraperitoneally (i.p.) with 10^5 $TCID_{50}$ of WNV-NY99. Mice were euthanized by cervical dislocation under isoflurane anaesthesia on days 3, 4, 5 and 6 post-inoculation, after which the brain was immediately collected for further processing. Mice were maintained in specific pathogen-free conditions, had a 12-hour day-night cycle and were fed *ad libitum*. Animal experiments were approved by the Animal Ethics Committee of Erasmus Medical Center.

In order to quantify viral RNA copies in the brain, half the brain was weighed and homogenized using a metal bead in 1 mL of DMEM containing antibiotics (100 U penicillin, 100 µg/mL streptomycin) using a tissue homogenizer. 100 µL of brain homogenate was added to 400 µL of lysis buffer (Roche). RNA was then extracted as indicated above and stored at -80 ºC until used. Synthesis of cDNA was initiated with 7 µL of total RNA and brain homogenates were examined for the presence of both positive and negative strand. The $\log_{10}$ titrated *in vitro* RNA transcript standard curve (T3: 3.7×10^7 copies; T7: 4.5×10^7 copies) was reverse transcribed at the same time as the tissue RNA samples. The total number of RNA copies was determined per gram of brain.

RESULTS

Contribution of different reverse transcriptases to self- and false-priming using unmodified and tagged primers

The role of the RT enzyme in self-priming and false-priming using unmodified and tagged primers was investigated (Table 1). To this end, SSIII, MS, AMV, and r*Tth* were used for

the RT reaction and only one concentration of *in vitro* RNA transcript depending on the input RNA for the specific assay. First, the occurrence of self-priming, measured by synthesis of cDNA in the absence of RT specific primers was assessed (Table 2). The RT reaction was followed by PCR amplification using either unmodified or tagged primers. When SSIII, MS, AMV and r*Tth* were used for RT followed by PCR containing unmodified primers, occurrence of self-priming of both strands could be detected. The use of unmodified primers for specific RT could therefore not circumvent self-priming. Self-priming of the negative strand also occurred when tagged primers were used in combination with AMV. However, when the other RT enzymes were used in combination with the tagged primers, self-priming was not observed. This suggests that the tagged primers largely circumvent detection of self-priming, but that this phenomenon is still RT enzyme-dependent.

Secondly, generation of cDNA as a result of false-priming by unmodified or tagged primers using both the positive and negative strand as a template was evaluated (Table 2). In the RT reaction, positive and negative sense RNA transcripts were used in combination with the non-complementary unmodified primers. Following PCR with the corresponding pair of unmodified primers, it was possible to detect the positive strand at a concentration of 3.72×10^7 copies for SSIII and AMV, 1.49×10^7 copies for MS and 1.86×10^7 copies for r*Tth*. Using a positive strand specific RT-PCR, the negative strand was detected at a concentration of 4.54×10^7 copies for SSIII and AMV, 1.82×10^7 for MS and 2.27×10^7 copies for r*Tth*. When tagged primers were used, however, no WNV specific cDNA produced by false-priming was detectable at these concentrations of RNA.

Name	Application	Nucleotide sequence (5' to 3')*
WNV F34	PCR amplification	CCACCGGAAGTTGAGTAGACG
WNV R96	PCR amplification	TTTGGTCACCCAGTCCTCCT
WNV 56	PCR amplification	TGCTGCTGCCTGCGGCTCAACCC (5'FAM-3'TAMRA)
Tag-Fwd	RT primer negative-sense	**TTTGCTAGCTTTAGGACCTACTATATCTACCT**CCA CCGGAAGTTGAGTAGACG
Tag-Rev	RT primer positive-sense	**TTTGCTAGCTTTAGGACCTACTATATCTACCT**TTT GGTCACCCAGTCCTCCT
Tag	PCR amplification	**TTTGCTAGCTTTAGGACCTACTATATCTACCT**

* The non-WNV (tag) sequences are indicated in bold while WNV-specific sequences are in regular font.

Table 1. Nucleotide sequence of primers and probe used for either reverse transcription or quantitative PCR.

			Unmodified primers				Tagged primers			
Enzyme	**RT Reaction (°C)**	**RNase H activity**	**Self-priming**		**False-priming**		**Self-priming**		**False-priming**	
			(+) strand	**(-) strand**	**(+) strand**	**(-) strand**	**(+) strand**	**(-) strand**	**(+) strand**	**(-) strand**
SSIII	50	Reduced	Yes	Yes	Yes	Yes	No	No	No	No
MS	48	Reduced	Yes	Yes	Yes	Yes	No	No	No	No
AMV	42	Yes	Yes	Yes	Yes	Yes	No	yes	No	No
r*Tth*	60	Yes	Yes	Yes	Yes	Yes	No	No	No	No

Table 2. The presence of self- or false-priming events during reverse transcription (RT) of positive and negative strand RNA transcript using four different RT enzymes in combination with unmodified or tagged primers.

Lack of strand-specificity of qRT-PCR using unmodified WNV-specific primers for the detection of positive or negative strand RNAs: contribution of self- and falsely-primed cDNAs to PCR amplification

First the sensitivity of the unmodified primers was addressed. The transcripts were diluted to a concentration of 2.61×10^7 of positive and 3.18×10^7 of negative-sense RNA and serially diluted on a 10-fold scale so that a final concentration of 2.61 and 3.18 copies was reached, respectively. The limit of detection with the unmodified primers was found to be at 26 and 32 copies (10^6 dilutions) of positive strand and negative strand RNA, respectively (Fig. 1A and B).

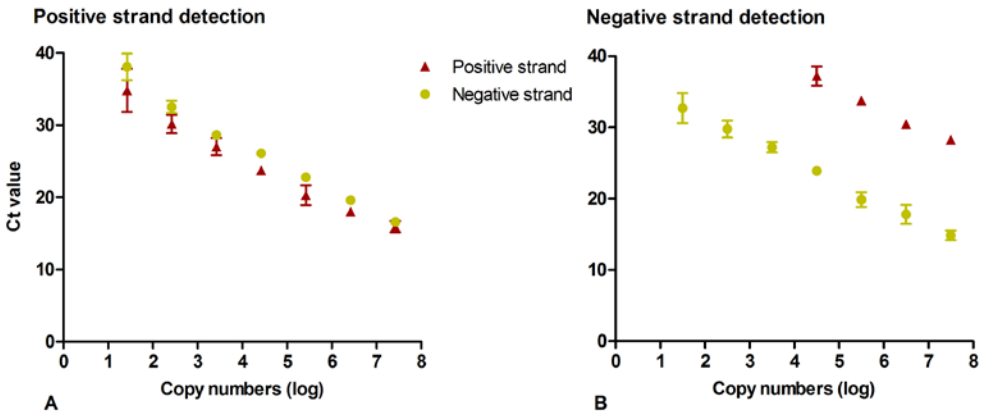

Figure 1. The sensitivity and specificity of the strand-specific qRT-PCR for detection of negative- and positive-sense RNA transcripts using unmodified primers. The strand-specific reverse transcription was initiated with a standard curve of 2.61 x 10^7 copies of the positive strand and 3.18 x 10^7 of the negative strand serially diluted ten-fold up to 2.61 and 3.18 copies, respectively. The log of the RNA copies is plotted against the cycle threshold (C_T). Only data points where amplification occurred are included in the graph. **(A)** Positive and negative strand RNA was detected using positive strand-specific primers. **(B)** Positive and negative strand RNA was detected using negative strand-specific primers. Graphs depict mean ± standard deviation (SD).

Next the contribution of self- and false-priming events during the reverse transcription step of WNV negative and positive strand RNAs using unmodified primers in combination with the r*Tth* enzyme was quantified. WNV-specific RT reactions were performed in the absence of the unmodified strand-specific RT primers (Table 1). cDNAs generated during the RT step were then amplified by PCR using both primers. In the absence of RT specific primer, both strands could be amplified, with a detection limit of 2.61×10^4 copies for the positive strand and a detection limit of 3.18×10^4 copies (10^3 dilutions) for the negative strand, indicating the presence of self-priming for both strands.

Strand-specificity of these primers was also assessed by performing the RT step in the presence of the uncomplimentary (incorrect) strand. The detection limit of the negative strand using the positive strand detection system was 32 copies (10^6 dilution), and the limit of detection for the positive strand was 2.61×10^4 copies (10^3 dilution) using the negative strand detection system (Fig. 1A and B). This indicates occurrence of false-priming of the incorrect strand during strand-specific RT-PCR with a significantly larger contribution in detection of the negative strand.

The RT-PCR was also performed testing both negative and positive-stranded RNA in the presence of unmodified dengue virus (DENV)-specific forward or reverse primer and r*Tth* enzyme. This was followed by PCR amplification using the WNV-specific unmodified F34 and R96 primers. For this purpose, only one concentration of *in vitro* RNA transcript (T3: 1.86×10^7 copies; T7: 2.27×10^7 copies) was used. The positive strand was detected at the indicated RNA concentration with a C*t* value of 34 in the presence of the DENV-specific forward primer. This result confirms the occurrence of self-priming during the RT step.

Validation of the strand-specific tagged primer qRT-PCR for WNV using RNA transcripts

The sensitivity and reproducibility of the strand-specific qRT-PCR assay using tagged primers was evaluated using synthetic positive- and negative-sense RNA transcripts. The transcripts were diluted to a concentration of 2.61×10^7 copies per reaction for the positive strand and 3.18×10^7 for the negative strand. The RNA transcripts were then serially diluted on a ten-fold scale so that an end concentration of 2.61 and 3.18 copies was reached, respectively. Minimum level of detection for either the positive or negative strand varied between 261-2610 and 318-3180 copies, respectively (Fig. 2A, B). In order to determine the sensitivity of the tagged primers more precisely, the RNA transcripts were subsequently diluted on a two-fold scale reaching an end concentration of 25.5 and 31.1 copies. Using this standard curve, the minimum level of detection was found to be around 815 copies for the positive strand and 497 copies for the negative strand.

False priming of the uncomplimentary strand was not detected at any of the dilutions tested. Although detection of the positive and negative strand was fairly similar, detection of the negative strand was more sensitive and less variable. The standard deviation at each dilution was on average 2.88 C_T values for the positive strand, and 0.64 C_T values for the negative strand. The difference in variability is also reflected by the coefficient of variation percentages, where the values for negative strand detection are much lower compared to positive strand detection (Table 3). The displayed PCR efficiency was on average 102% ± 13 for positive strand detection and 109% ± 13 for negative strand detection. The range of the R^2 value for the standard curves constructed from the $\log_{10}$ serially diluted RNA was 0.9-0.999 and 0.957-0.997, respectively. No amplification was observed in the controls lacking RT primer, RNA or reverse transcriptase.

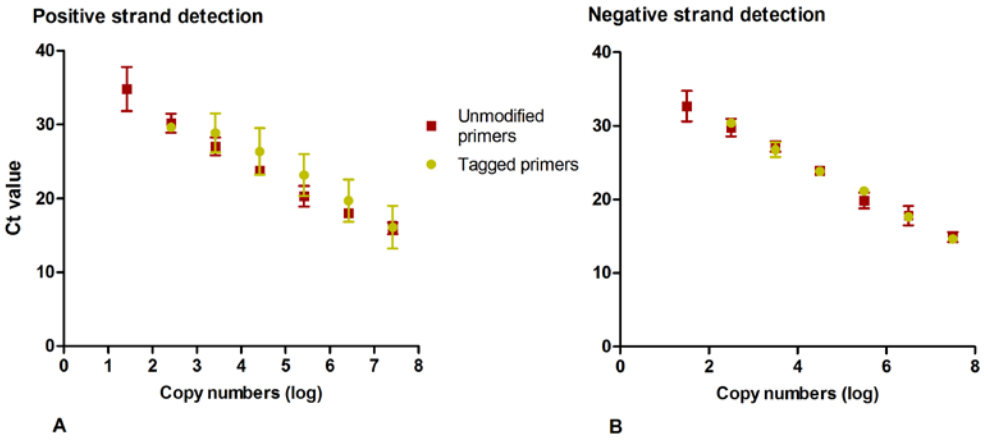

Figure 2. The sensitivity of the strand-specific qRT-PCR for detection of negative- and positive-sense RNA transcripts using unmodified and tagged primers. The strand-specific reverse transcription was initiated with 2.61 x 10^7 copies of the positive strand and 3.18 x 10^7 of the negative strand serially diluted ten-fold up to 2.61 and 3.18 copies, respectively. The log of the RNA copies is plotted against the cycle threshold (C_T). Only data points where amplification occurred are included in the graph. **(A)** Positive strand RNA was detected using positive strand-specific unmodified (square) and tagged (closed circle) primers. **(B)** Negative strand RNA was detected using negative strand-specific unmodified (square) and tagged (closed circle) primers. Graphs depict mean ± SD.

	Copy numbers of positive strand RNA transcript					**Copy numbers of negative strand RNA transcript**				
	10^3	**10^4**	**10^5**	**10^6**	**10^7**	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**
Intra-assay variability (CV%)	6,44	4,04	4,18	2,17	1,69	2,89	1,99	0,73	1,14	1,49
Inter-assay variability (CV%)	6,92	10,5	12,3	16,8	21,4	1,32	1,51	0,97	4,34	1,86

Table 3. Intra- and inter-assay reproducibility of the real-time polymerase chain reaction (PCR) employing tagged primers at different concentrations of positive or negative RNA transcript. CV = coefficient of variation calculated based on the C_T values obtained from seven independent experiments.

The sensitivity of the strand-specific qRT-PCR was also evaluated by testing the tagged primers in the presence of excess complimentary (correct) or uncomplimentary (incorrect) RNA strand (Fig. 3A and B), with the addition of 200 ng of Vero E6 RNA (Fig. 2C and D), or a combination of both. Five dilutions of the positive-sense RNA transcript were made, resulting in 10^7, 10^6, 10^5, 10^4 and 10^3 copies, which were additionally mixed

with 10^7 copies of negative-sense RNA transcript. Alternatively, 200 ng of Vero E6 RNA was added to the reactions. These ratios were then tested using the qRT-PCR for the positive strand. The reverse experiment was also performed where five dilutions of the negative-sense transcript were mixed with 10^7 copies of positive-sense transcript, with and without Vero E6 RNA. It was found that positive and negative strand detection was generally unaltered in the presence of the high numbers of uncomplimentary strand, with a maximum increase of 1.22 C_T value, which occurred at a ratio of 1:10 positive strand:negative strand and a maximum increase of 0.28 C_T value at a ratio of 1:10 negative:positive (Fig. 3A and B).

Furthermore, the addition of 200 ng of Vero E6 RNA also did not change the efficiency of detection of the positive or negative strand as only a maximum increase of 0.71 and 0.07 C_T value was observed, respectively (Fig. 3C and D). The addition of the opposite strand plus Vero E6 RNA led to a maximum increase in C_T value of 1.22 for the positive strand at a ratio of 1:10 (positive:negative) and 0.28 for the negative strand at a ratio of 1:10 (negative:positive). As the described variation in C_T values are within the normal limits of intra-assay variability, these results suggest that the presence of uncomplimentary RNA does not inhibit the accurate quantification of the positive or negative strand of WNV.

Quantitation of viral positive and negative strands of WNV RNA in cell culture

To investigate the susceptibility of the microglia cell line BV-2 to WNV infection and to understand the dynamics of RNA synthesis during virus replication in cell culture, the strand-specific qRT-PCR was used to characterize the kinetics of negative and positive strand synthesis during WNV replication. BV-2 cells were infected at MOI of 0.5 and cells were harvested for RNA extraction every 12 hours post-infection for a total of 48 hours. RNA extracted from the infected cells at the indicated time points were subjected to the strand specific qRT-PCR as described above. The number of RNA copies was determined with the use of a standard curve. It was found that the BV-2 cells were susceptible to infection by WNV as the amount of positive and negative strand RNA as determined by strand-specific qRT-PCR varied over time. Between 0 and 12 hours post-infection, the amount of positive strand increased by approx. 2 $\log_{10}$ RNA copies and then continued to increase steadily up to approx. 6 $\log_{10}$ RNA copies by 48 hours post infection (Fig. 4). In contrast, 3.5 $\log_{10}$ of negative strand RNA was first detected at 24 hours post infection and increased to approx. 4.5 $\log_{10}$ RNA copies within 48 hours.

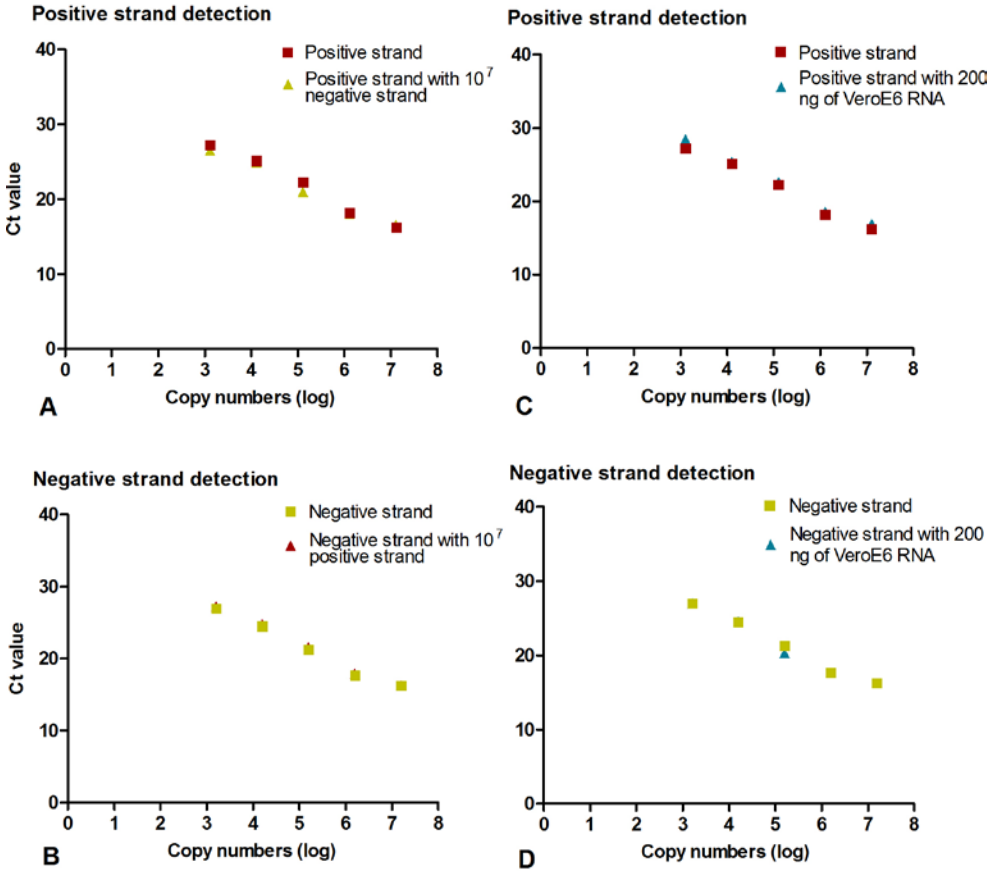

Figure 3. Quantitation of positive and negative-sense RNA transcripts combined with the opposite strand at various ratios or with the addition of 200 ng of Vero E6 RNA. The qRT-PCRs were performed using tagged primer as described in the materials and methods. The log of the RNA copies is plotted against the cycle threshold (C_T). **(A)** Quantification of positive-sense RNA transcript starting at a concentration of 2.61×10^7 copies and diluted ten-fold to 2.61 copies with and without the presence of 3.18×10^7 copies of negative-sense RNA transcript. **(B)** Quantification of negative-sense RNA transcript starting at a concentration of 3.18×10^7 copies and diluted ten-fold to 3.18 copies with and without the presence of 2.61×10^7 copies of positive-sense RNA transcript. **(C)** Quantification of positive-sense RNA transcript starting at a concentration of 2.61×10^7 copies and diluted ten-fold to 2.61 copies with and without the presence of 200 ng of Vero E6 RNA. **(D)** Quantification of negative-sense RNA transcript starting at a concentration of 3.18×10^7 copies and diluted ten-fold to 3.18 copies with and without the presence of 200 ng of Vero E6 RNA.

Quantitation of viral positive and negative strands of WNV RNA in infected mouse brains

In order to understand the dynamics of WNV titers in the brain over the course of infection, this assay was used to characterize the kinetics during replication in brain of C57BL/6 mice. Mice infected with 10^5 $TCID_{50}$ of WNV were sacrificed per groups of five on day 3, 4, 5 and 6 irrespective of clinical symptoms, but no later than when humane endpoints were reached. Brains were harvested for the determination of RNA copy numbers over time. The negative strand increased between days 3 and 4 by about 3.5 log_{10} RNA copies, and then remained constant until day 6 (Fig. 5B), whilst the positive strand steadily increased across days 3 to 6 from approx. 8.3 log_{10} to 9.7 log_{10} RNA copies (Fig. 5A). In general, the amount of positive strand RNA detected in the brain was higher than the amount of negative strand RNA. The ratio of negative to positive strand RNA was found to be on average 1:2.5 x 10^6 on day 3, 1:2220 on day 4, 1:470 on day 5 and 1:800 on day 6. This indicates that the largest amount of positive strand per negative strand was generated on day 3.

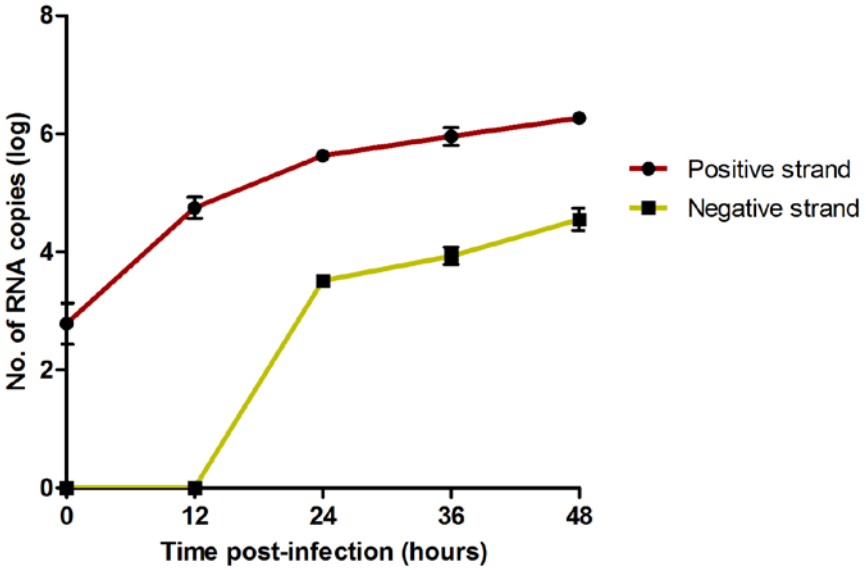

Figure 4. Quantitation of WNV positive and negative-sense RNA during replication in cell culture using tagged primers. BV-2 cells were infected at MOI of 0.5 and samples for RNA isolation were harvested in triplicate at indicated time points post-infection. The production of positive and negative-sense RNA was estimated by extrapolation of standard curves as generated in Fig. 2 and the log of RNA copies recovered from the cells was plotted against time points post-infection.

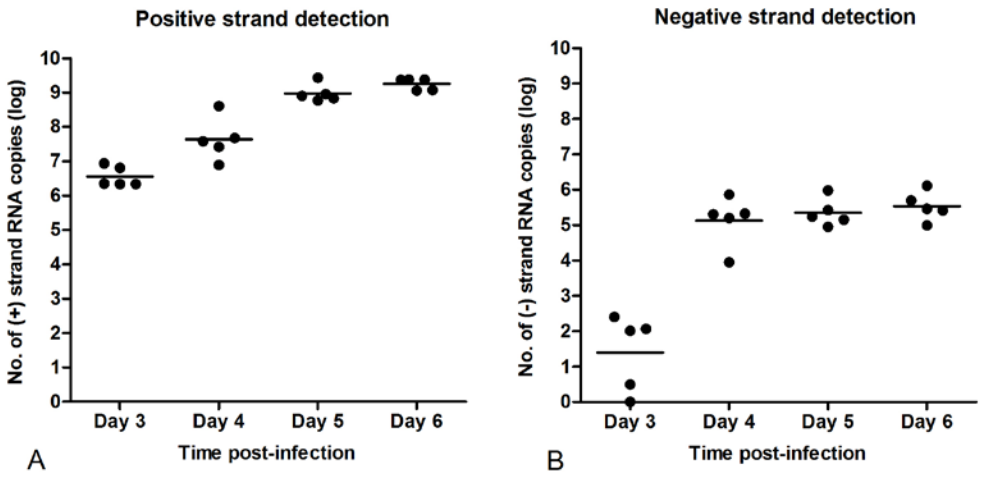

Figure 5. Quantitation of WNV positive and negative-sense RNA in brains of mice infected with 10^5 $TCID_{50}$ of WNV-NY99 (n=5). Mice were euthanized on day 3, 4, 5 and 6 post-infection and brains were collected and homogenized for RNA isolation. The amount of positive and negative-sense RNA was estimated by extrapolation of standard curves as generated in Fig. 2 and calculated per gram of brain. The log of RNA copies was plotted against time points post-infection. **(A)** The amount of positive-sense RNA copies present in the brains of mice between days 3 and 6 post-infection. **(B)** The amount of negative-sense RNA copies present in the brains of mice between days 3 and 6 post-infection.

DISCUSSION

An assay that allows for the specific detection of negative strand viral RNA is a useful tool for studying viral replication and kinetics, in particular for single-stranded positive-sense RNA viruses, which use the negative strand RNAs as a template for the synthesis of positive stranded RNAs [1, 37]. In this study, a strand-specific qRT-PCR using tagged primers and a thermostable rTth has been developed for the accurate quantitation of both positive and negative sense RNA of WNV. It was shown that this qRT-PCR assay is suitable for the study of viral replication in cell culture, but also in experimentally infected mice. The results of both the *in vitro* and *in vivo* study clearly show an increase in both positive- and negative-sense RNA over time.

Many studies have reported the synthesis of non-specific cDNA during an RT reaction, which interfere with strand-specific detection of RNAs [10, 16, 25, 38]. However, recent developments in qRT-PCR assays, such as high temperature reverse transcriptase enzymes and the use of tagged RT primers, have allowed for more accuracy in the detection of specific RNA strands.

In the attempt to develop a strand-specific qRT-PCR for studying WNV replication, the accuracy of a current standard qRT-PCR system using unmodified primers and r*Tth* for the detection of positive or negative strand RNAs of WNV was first investigated. Using synthetic positive and negative RNA transcripts, it was shown that self-primed cDNAs are generated during the reverse transcription step in the absence of primers and that false-priming occurs in the presence of unmodified primers. These self- and falsely-primed cDNAs are subsequently amplified in PCR to amplicons that are indistinguishable from positive or negative-strand specific cDNAs.

To further elucidate these observations, four different reverse transcriptases that function at different temperatures were compared. It was found that both self- and false-priming occurred in the presence of SSIII, AMV, MS and r*Tth*. When these same reactions were carried out in the presence of tagged primers, false-priming was no longer observed. Self-priming was only evident when tagged primers were used in combination with AMV. This suggests that the tagged primers largely circumvent self-priming, but that this phenomenon is also RT enzyme-dependent. This is supported by the fact that very few positive strand RNA was detected when unmodified uncomplimentary primer was used in combination with r*Tth*, as this reaction was carried out at a temperature of 60 ºC. The results in this study are in agreement with published data [29] demonstrating reduced strand-specificity when using tagged primers in combination with AMV or untagged primers in combination with rTth-RT, and further highlight the importance of combining the thermostable rTth enzyme with tagged primers.

As a result, a qRT-PCR assay was developed to overcome self- and false-priming in the detection of WNV-specific RNA that uses rTth enzyme in combination with tagged primers. Even though tagged primers alone have proven to be successful in some studies [25-28], this study shows that a combination of tagged primers with thermostable rTth is

necessary to circumvent these problems for the detection of WNV-specific RNA. This combination greatly improved the specificity of the positive and negative strand-specific qRT-PCR as self-primed and falsely-primed RNAs were no longer detected.

When using tagged primers, it was found that with a $\log_{10}$ titration of the RNA transcripts the minimum level of detection for the positive strand was 2.61×10^3 RNA copies at 67% of the time and 3.18×10^3 copies for the negative-strand at 75% of the time. This assay is therefore approx. 2 $\log_{10}$ less sensitive than the assay using unmodified primers. This extra sensitivity, however, is most likely the result of self- or false-priming, as it was possible to detect both strands of RNA at a minimum concentration of 10^4 copies in the absence of primers, and at 10^4 and 10^1 copies of positive and negative stranded RNA, respectively, in the presence of uncomplimentary primer. This therefore suggests that a large portion of the amplification detected originates from self- and falsely-primed cDNA.

In comparison to the tagged primers that this study has specifically investigated, some other studies investigating tagged primers (using a 10-fold dilution scale) have demonstrated a similar sensitivity in the order of 10^3 copies per reaction [25], or a higher sensitivity of 10^2 [11, 26, 28] or even 10^1 RNA copies per reaction [24]. In another study [34], positive and negative strand of foot-and-mouth disease virus were detected up to a sensitivity of 1×10^2 and 1×10^3 copies/µL, respectively. However, despite the use of tagged primers, the complimentary primer was still able to detect the opposite strand at 1×10^7 copies/µL. This low rate of incorrect strand detection in the presence of high concentrations of target RNA despite the use of tagged primers was also demonstrated in other studies [11, 18, 29]. This therefore suggests that the sensitivity of some tagged primers occurs at the expense of specificity. In this particular assay, however, it was found that in the presence of high concentrations of the opposite strand or cellular RNA, the tagged primers only detected the complimentary strand of RNA, demonstrating the high specificity and effectiveness of these primers.

The positive strand-specific assay was found to exhibit much greater variability than the negative strand-specific assay. This was reflected in the standard deviation of the C_T values as well as in the coefficient of variation percentages. In addition, the displayed efficiency range of the positive strand-specific assay was also greater than that for the negative strand-specific assay. PCR efficiency can be dependent on the assay, the master mix performance, and sample quality. In general, an efficiency range of 90-110% is considered to be acceptable [39]. It is possible that the inter-assay variability for the positive strand was caused by the quality of the synthetic positive-sense RNA transcripts.

The application of the strand-specific assay was tested *in vitro* and *in vivo* in order to study WNV replication. First the strand-specific assay as described above was used to characterize the *in vitro* replication kinetics of WNV in BV-2 microglia cells. This microglia cell line was found to be susceptible to infection. The amount of positive strand increased from 0 to 48 hours. On the other hand, the negative strand was not detectable at 0 hours post-infection and was only detected at 24 hours post-infection. As a full replication cycle of West Nile virus takes approx. 12 hours (unpublished data), it seems unlikely that

no negative strand is yet present at this point in the cell. The largest increase in positive strand was measured between 0 and 12 hours post-infection, indicating that negative strand synthesis must have occurred. As the negative strand-specific assay was found to have a detection limit of 497 copies of RNA, it is possible that negative strand was present in the cell but in amounts that fall below the detection limit of this assay. The lack of detection of negative strand in the first 12 hours of the replication cycle therefore appears to be a limitation of this assay.

Furthermore, the mouse experiment characterizing the positive and negative strand kinetics over the course of six days showed low amounts of negative strand RNA copies on day 3 post-infection for 2 out of 5 mice (in the order of 2 $\log_{10}$ RNA copies). As the ratio of positive to negative strand is highest on this day, it suggests that the largest amount of positive strand per negative strand was produced on this day. However, it is unlikely that the largest amount of positive per negative strand is produced in the brain as early as day 3, as this is usually the first time-point at which virus can be detected in the brain (Halevy et al., 1994; Hunsperger et al., 2006). Instead, it is more likely that most of the positive stranded RNA detected in the brain was the result of contamination with virus that replicated in the periphery instead of *de novo* virus production. Several studies have shown that by day 4-5 after infection, there is no more virus present in the blood [40, 41] (unpublished observation). It is therefore more likely that the largest amount of positive stranded RNA per negative strand is produced in the brain on day 4.

In conclusion, despite the possible limitation of the strand-specific quantitative RT-PCR assay developed in this study, this assay is a useful tool for the evaluation of tropism and replication kinetics of WNV over a longer period of time *in vitro* and *in vivo*.

ACKNOWLEDGEMENTS

This work was supported by the European Community's Seventh Framework Programme (FP7 VECTORIE project number 261466).

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Chapter 4

In Vitro and in Vivo Evaluation of Mutations in the NS Region of Lineage 2 West Nile Virus Associated with Neuroinvasiveness in a Mammalian Model

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ABSTRACT

West Nile virus (WNV) strains may differ significantly in neuroinvasiveness in vertebrate hosts. In contrast to genetic lineage 1 WNVs, molecular determinants of pathogenic lineage 2 strains have not been experimentally confirmed so far. A full-length infectious clone of a neurovirulent WNV lineage 2 strain (578/10; Central Europe) was generated and amino acid substitutions that have been shown to attenuate lineage 1 WNVs were introduced into the nonstructural proteins (NS1 (P250L), NS2A (A30P), NS3 (P249H) NS4B (P38G, C102S, E249G)). The mouse neuroinvasive phenotype of each mutant virus was examined following intraperitoneal inoculation of C57BL/6 mice. Only the NS1-P250L mutation was associated with a significant attenuation of virulence in mice compared to the wild-type. Multiplication kinetics in cell culture revealed significantly lower infectious virus titres for the NS1 mutant compared to the wild-type, as well as significantly lower amounts of positive and negative stranded RNA.

INTRODUCTION

West Nile virus (WNV) is one of the most widely distributed members of the genus *Flavivirus* within the family *Flaviviridae*. The virus can cause general, febrile and severe encephalitic disease in humans and a number of animal species. It possesses a capped and non-polyadenylated positive-sense, single-stranded RNA genome, approximately 11 kb in length, which contains a single open reading frame (ORF) encoding a polyprotein precursor that is co- and post-translationally processed by viral and host cell proteases to three structural proteins (capsid protein C, pre-membrane protein prM/M, envelope protein E) and seven non-structural (NS) proteins (glycoprotein NS1, NS2A, protease cofactor NS2B, protease and helicase NS3, NS4A, NS4B and polymerase NS5).

WNV strains have been divided into at least eight distinct genetic lineages [1–3]. The first WNV strain, isolated in Uganda in 1937 [4], belongs to lineage 2 and this lineage has been confined to sub-Saharan Africa and Madagascar [5]. Until the early 1990s, WNV infections beyond Africa were caused by lineage 1 WNV strains that caused mainly mild clinical symptoms with sporadic encephalitis in humans. Lineage 1 WNV as the causative agent of lethal encephalitis was reported in 1994, in Algeria, followed by a high number of neuroinvasive cases in Romania, in 1996, and more frequent encephalitis cases in several other Eastern and Western European countries [6]. Moreover, in 1999, WNV emerged in the continental United States and rapidly spread throughout North America and also into South America, causing increased mortality among humans and animals as well [7–9].

Lineage 2 strains did not appear to be as pathogenic as lineage 1 isolates, as they generally caused no deaths, and only mild clinical signs of infection [10]. The first proven case of a lineage 2 virus infection with fatal outcome outside of Africa was detected in a goshawk (*Accipiter gentilis*) in Hungary, in 2004 [11,12]. This exotic WNV lineage 2 strain caused subsequent infections, illnesses and deaths in wild birds, horses and humans [11–13], and rapidly spread to Austria [14–16], Greece [17,18] and Italy [19,20]. Based on phylogenetic reconstruction, lineage 2 viruses isolated in Russia [21,22] and Romania [23] cluster together, but are distinct from the Hungarian strain [16].

Several studies have aimed at identifying the molecular markers of virulence in lineage 1 WNV strains [24–27]. A number of lineage 1 viruses of moderate virulence in mice and exhibiting inefficient growth in cell culture have been identified so far [28]. Reverse genetic systems were established to investigate nucleotide and amino acid alterations in the WNV lineage 1 genome that led to decreased neurovirulence and neuroinvasiveness in mice [29,30]. Mutations in the NS protein genes, such as *NS1* [31], *NS2A* [24,26,32], *NS3* [33], *NS4B* [30,34–36] and *NS5* [34], were found to be associated with the attenuation of these viruses.

In contrast to lineage 1 viruses, molecular determinants of pathogenic WNV lineage 2 strains have not been confirmed experimentally. Virulence markers have only been identified *in silico* by analyzing and comparing full genome sequences of highly or less neuroinvasive lineage 2 strains [37], or by comparing those that have emerged and

increased in virulence over time (1937-2011) [38]. The aim of the present study was to investigate the *in vitro* (in cell culture) and *in vivo* (in a mouse model) effects of selected nucleotide substitutions of the NS protein coding genes, known to be attenuating for lineage 1, in a neurovirulent WNV lineage 2 strain (578/10) isolated in Central Europe with the help of reverse genetic methods and site-specific mutagenesis.

MATERIALS AND METHODS

Cells and viruses

Transfection was carried out on baby hamster kidney 21 (BHK-21) cells (ATCC®: CCL-10™) cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% foetal bovine serum (FBS). Virus stocks were propagated and titrated on Vero E6 cells (ATCC®: CRL-1586™) cultured in DMEM supplemented with 0.75% sodium bicarbonate, 10 mM hepes buffer and 10% FBS. All media was supplemented with antibiotics (100 U penicillin, 100 µg/mL streptomycin) and 2 mM L-glutamine. The WNV-578/10 strain (GenBank accession number: KC496015) was originally isolated in Hungary from a horse showing clinical signs of encephalitis, which had died in 2010.

RNA extraction and cDNA synthesis

Total RNA was extracted from virus pellet using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. To obtain long cDNA copies of the viral genome, reverse transcription was performed using the high fidelity SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and gene-specific reverse primers (Table 1). In the first step, 0.5 µL of each primer (10 µM) and 1 µL RNA in 7.5 µL RNase-free water were heated to 65 °C for 5 minutes and then cooled down to 4 °C for 10 minutes. In the second step, 11 µL reaction mixture (2 µL reaction buffer, 4 µL $MgCl_2$, 2 µL dithiothreitol (DTT), 1 µL RNase OUT inhibitor, 1 µL SSIII enzyme and 1 µL dNTP mixture) were added and after 30 minutes at room temperature and the final 20-µL reaction mixture was heated to 50 °C for 90 minutes, followed by 85 °C for 5 minutes, and finally cooled down to 4 °C. Before amplification, cDNA was treated with RNase H (1 µL RNase H was added to 20 µL of the above-mentioned solution and was heated to 37 °C for 20 minutes).

Primer code	Nucleotide sequence (5'→3')	Nucleotide position (5')*
1F†	*GAGCTCGTTTAGTGAACCGTA*GTAGTTCGCCTGTGTGAGC	1
1FRC†	GCTCACACAGGCGAACTACT*ACGGTTCACTAAACGAGCTC*	20
4750F	CACACACTATGGCACACCACTAAGG	4750
5426R	GACATCAGCCTGTGTGTGAGAGTGG	5426
8190F	AGACTGGCTGCACAGAGGACCTAAG	8190
9175R	GGTCTTCATTGAGGAATCCGAGAGC	9175
3'XbaSacIIR‡	*ATCCGCGGTCTAG*AGATCCTGTGTTCTAGCACCACAG	11026

† Bases in italics are part of cytomegalovirus (CMV) promoter. * Primer positions corresponding to the sequence of WNV-578/10 strain. ‡ Bases in italics are the extra restriction enzyme cleavage sites.

Table 1. List of primers used to generate overlapping fragments of the West Nile virus (WNV) genome in order to construct the full-length clone pWNV-578/10.

Sequence analysis

The complete genome of WNV-578/10 was determined by sequencing of overlapping PCR fragments amplified with Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland). Amplification products were directly sequenced with the ABI Prism BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an automated ABI 377 DNA Sequencer (Applied Biosystems). Sequence assembly and comparison were performed with SeqMan and MegAlign programs (Lasergene, Madison, WI, USA), respectively. The full-length WNV clone was sequenced with the ABI Prism BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 310 genetic analyzer (Applied Biosystems), as described in Bakonyi *et al.*, 2004 [39].

Full genome sequencing of WNV clones with the incorporated modifications was carried out by using semiconductor sequencing technology. Briefly, overlapping PCR fragments were generated as described above. Equimolar amounts of the amplicons from each clone were used for preparation of Ion Torrent compatible libraries. Clonal amplification of library DNA (with 200 bp inserts) by emulsion PCR on an Ion One Touch v2 (Life Technologies, Thermo Fisher Scientific, Waltham, MA, United States) equipment was followed by enrichment on an Ion OneTouch ES pipetting robot, and then by sequencing on a 316 Chip using Ion Torrent PGM (Life Technologies). Raw reads were curated and then consensus genomic sequences were assembled using the CLC Genomics Workbench version 7 (www.clcbio.com).

Plasmids and bacteria strains

Plasmid pBeloBAC TGE [40] was kindly provided by L. Enjuanes (*Centro Nacional de Biotecnologia*, Department of Molecular and Cell Biology, Madrid, Spain). The plasmid was propagated in Max Efficiency DH10B competent *Escherichia coli* cells (Invitrogen) that were transformed by heat shock according to the manufacturer's instructions. Modifications, such as deletion of the *XhoI* site downstream of the 3' accessory sequences and insertion of a multiple cloning site containing *SfiI*, *SfoI*, *BstBI*, *ClaI*, *BamHI*, *XhoI*, *AsiSI*, *AvrII* and *SacII*, were described earlier [41]. For large-scale DNA preparation, the BAC vector and recombinant BACs were isolated with the QIAGEN Large Construct Kit (QIAGEN) according to the manufacturer's specifications.

Generation of the full-length clone of WNV-578/10

To amplify long, overlapping PCR products, ranging between 1 and 5.5 kb covering the whole genome in three fragments (Fragment I, II and III), PCRs were performed using genome-specific primers (Figure 1). Long-range PCR assays were implemented using the Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes). The construction of Fragment I was performed in two steps. First, the CMV promoter sequence from the PBeloBAC (with primers 5'SfiIF and 1FRC), as well as the 5' side of 5426 nucleotides of WNV-578/10 strain (with primers 1F and 5426R), were amplified. Primers 1F and 1FRC were reverse complements. In the second step that joined together the two amplicons, a fusion PCR utilizing the overlapping sequences of the above-mentioned primers was performed with Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes). Fragment II was amplified by using primers 4750F and 9175R. For amplification of Fragment III, a specific reverse primer containing two extra restriction sites (*XbaI* and *SacII*) in addition to the 3' conservative end of WNV-578/10 strain (3'XbaSacIIR) and forward primer 8190F was used. Sequences of all the primers used to generate the full-length clone are listed in Table 1. All fragments were separated and purified from 0.8% agarose gel using the QIAquick Gel Extraction kit (Qiagen).

Restriction enzyme cleavage and cloning steps were performed according to standard protocols [42]. Restriction endonucleases and DNA modifying enzymes were purchased from Fermentas (Vilnius, Lithuania) and New England Biolabs (Beverly, MA, USA). The cDNA fragments were ligated with T4 DNA Ligase (Invitrogen Life Technologies). After the digestion of pBeloBac and fragment III with *AvrII* and *SacII* restriction endonucleases, fragment III was cloned into the pBeloBac to obtain WNV-3. Fragment II was digested with *BstBI* and *AvrII* restriction endonucleases, and was cloned into WNV-3 to get WNV-3-2. Fragment I was digested with *SfiI* and *BstBI* restriction endonucleases, and was cloned into WNV-3-2 to obtain the final full-length clone pWNV-578/10. All recombinant plasmids were stable in bacteria, and no toxicity was observed. All the sequences of molecular constructs were confirmed by restriction endonuclease pattern analysis and PCRs.

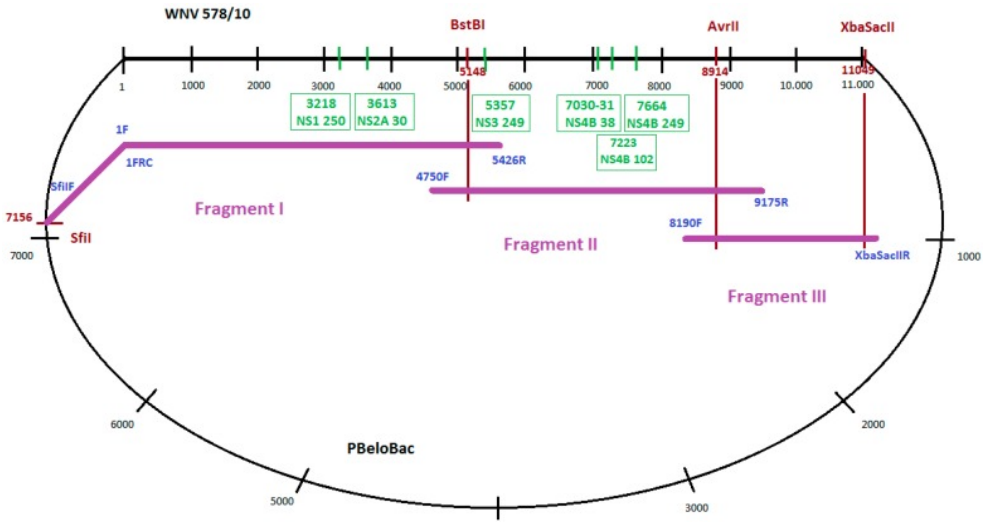


Figure 1. Cloning strategy for the WNV-578/10 genome and positions of the generated mutations. Black numbers represent nucleotide positions in WNV genome/PBeloBac plasmid genome. Red numbers indicate nucleotide positions of restriction enzyme cleavage sites in WNV genome. Green numbers in boxes represent nucleotide positions of inserted mutations. Names of used primers are in blue.

Generation of mutant full-length WNV clones

Point mutations were inserted in the genome of WNV using PCR-based mutagenesis utilizing specific oligonucleotides (forward and reverse, complementary oligos) containing the altered nucleotides (Table 2). Depending on the position of the mutation in the genome, new fragments I or II were synthesized using the mutated complementary oligos in fusion PCRs. In the next step, the original fragment I or fragment II was removed from the pWNV-578/10 clone and was replaced with the newly synthesized fragment I or fragment II, containing the respective mutations in the pWNV-578/10 clone. The generated mutations were as follows: C3218T in NS1 protein gene (P250L), G3613C in NS2A protein gene (A30P), C5357A in NS3 protein gene (P249H), and three mutations in the NS4B protein gene: CC7030-31GG (P38G), G7223C (C102S) and A7664G (E249G) (Figure 1).

Primer code	Nucleotide sequence (5'→3')	Nucleotide Position (5')*
NS1F	CATCACCTTGGCAGGAC***T***C*C*AGAAGCAATCATAA CAGGAGACC	3201
NS1R	GGTCTCCTGTTATGATTGCTTCT*G**A**G*TCCTGCCA AGGTGATG	3201
NS2AF	TTCGCAAGAGGTGGACG***C***C*CC*AAGATCAGCATTC CAGCTATCA	3596
NS2AR	TGATAGCTGGAATGCTGATCTT*GG**G***CGTCCACC TCTTGCGAA	3596
NS3F	GGTACCAAACCTCAGCAGTG*C**A**C*AGAGAGCAC AGTGGAAATGA	5336
NS3R	TCATTTCCACTGTGCTCTCT*G**T**G*CACTGCTGAGG TTTGGTACC	5336
38NS4BF	TTCTTGCTTGATCTGCGG***GG**G*GCTACAGCATGG TCTCTCTAT	7012
38NS4BR	ATAGAGAGACCATGCTGTAGC*C**C**C*CCGCAGATC AAGCAAGAA	7012
102NS4BF	TCAGCTCTCTTGCTGGCGGCCGGG*T**C**C*TGGGGC CAAGTGACCCTG ACTGTGACT	7198
102NS4BR	AGTCACAGTCAGGGTCACTTGGCCCCAG***G**A*CCC GGCCGCCAGCAAGAGAGCTGA	7198
249NS4BF	GGACTCTCATCAAAAACATG***GG**G*AAACCAGGC CTCAAGAG	7643
249NS4BR	CTCTTGAGGCCTGGTTTC*C**C*CATGTTTTTGATGA GAGTCC	7643

* Primer positions corresponding to the sequence of WNV-578/10. Triplets in italics are the loci of mutations, modified nucleotides are in italics and bold.

Table 2. List of primers used to insert point mutations into the genome of WNV-578/10.

Transfection and recovery of the recombinant viruses from the cDNA clones

Before transcription, the CMV-WNV clone was double digested with *SfiI* and *XbaI* restriction endonucleases. To remove the single-stranded nucleotide overhang generated by the digestion, Mung Bean Nuclease (New England Biolabs) was used. In order to get the purified expression cassette, pieces from digestion were separated on a 0.8% agarose gel and the approximately 12 kb cassette was purified with QIAquick Gel Extraction Kit (QIAGEN). Four micrograms of double-stranded cDNA was transfected into BHK-21 cells using Turbofect *in vitro* Transfection Reagent (Thermo Scientific, Waltham, MA USA).

Briefly, 4 µg of plasmid DNA were transfected into an approximately 60% confluent monolayer of BHK-21 cells on a 6-well culture plate after a 20 minute incubation period with the Turbofect reagent. The plates were incubated for 4 h at 37 °C, then the supernatant was removed, and fresh DMEM (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 10% FBS was added. The cells were incubated at 37 °C for 48–96 h until a visible cytopathic effect (CPE) appeared. At this time point, supernatant was harvested, clarified by centrifugation at 1000 × g for 5 min, and stored at –80 °C. To increase infectious virus titres for use in *in vitro* and *in vivo* experiments, virus stocks of the recombinant wild type (WT) and mutant viruses were incubated on 80% monolayers of Vero E6 cells in cell culture flasks. At the point of maximum CPE (4–5 days after inoculation), supernatants were harvested, clarified by centrifugation at 1000 × g for 5 min, and stored at –80 °C. Infectious titres of the virus stocks were determined by $\log_{10}$ titration on Vero E6 cells and calculating the $TCID_{50}$ using the Spearman–Kärber method [43,44] after determination of CPE 5 days p.i.

Subsequent full genome sequencing of each stock was performed by semiconductor sequencing as detailed in Section 2.3.

Multiplication curves in cell culture

Growth curves of the wild-type virus and the mutated clones were generated in Vero E6 cells at 37 °C in two independent experiments. Vero E6 cells were seeded at 10^4 cells/well in flat-bottom 96-well plates, incubated overnight at 37 °C and 80% monolayers were infected in triplicate with 100 µL of virus diluted in serum-free DMEM at multiplicity of infection (MOI) 0.1. Cells were incubated for 1 h at 37 °C and subsequently washed three times with serum-free medium before adding 120 µL of DMEM supplemented with 10% FCS. At 0, 12, 24, 36, 48, 72 and 96 h post-infection (p.i.), supernatants were removed and 70 µL was frozen at –80 °C until used for infectious virus titre determination, while the remaining 50 µL was added to 350 µL of lysis buffer (Roche Diagnostics, Almere, The Netherlands) for RNA extraction. Cells were also collected for RNA extraction in 50 µL of lysis buffer at time points 0, 12, 36 and 48 h p.i. Harvested supernatant infectious virus titres were determined by $\log_{10}$ titration on Vero E6 cells as described previously.

Quantitation of viral RNA titres

In order to quantify viral RNA copies in the supernatant and cells, RNA was extracted using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics) and an automated nucleic acid robotic workstation (Roche Diagnostics) according to the manufacturer's instructions. RNA was eluted in 50 µL of elution buffer and used immediately in quantitative real-time PCR (qRT-PCR). Within *in vivo* experiments, viral RNA copies in half-brains were quantified after weighing and homogenization using a metal bead in DMEM containing antibiotics (100 U penicillin, 100 µg/mL streptomycin) using a tissue homogenizer. One hundred microliters of tissue homogenate was added to

400 µL of lysis buffer (Roche Diagnostics) and RNA was extracted as indicated above. RNA copy numbers were determined in a strand-specific qRT-PCR assay [45] using a standard curve of *in vitro* transcribed RNA of positive and negative strand of known quantities, which were generated from a plasmid (pCR®4-TOPO®; Invitrogen, Breda, The Netherlands) containing the sequence of the 3'UTR of WNV. Plasmid was linearized and run-off transcripts were generated using the Ambion® MaxiScript T7/T3 kit (Invitrogen). The positive sense RNA was transcribed using the T3 polymerase and the negative sense RNA using the T7 polymerase. The product was digested with DNase to remove residual DNA and cleaned-up using the High Pure RNA Isolation kit (Roche Diagnostics). *In vitro* transcribed RNA was diluted to a concentration at which DNA was no longer detected.

Mouse virulence studies

Six-week-old female C57BL/6 mice (Harlan Laboratories B.V., Venray, The Netherlands) were inoculated intraperitoneally (i.p.) with average doses of 10 and 10^4 $TCID_{50}$ per mouse of wild-type infectious clone-derived CMV-WNV as well as the mutant viruses (n = 8 per clone, per dose; Table 3). Mice were observed twice daily and were euthanized by cervical dislocation under isoflurane anesthesia when humane endpoints were reached (immobility and paralysis), after which brains were immediately collected for further processing. At 14 days p.i., the end-point for the survival experiment was reached and all remaining mice were euthanized and brains and kidneys were collected from those that had been infected with the highest viral dose. Mice were maintained in specific pathogen-free conditions, had a 12-hour day-night cycle and were fed *ad libitum*. Animal experiments were approved by the Animal Ethics Committee of Erasmus Medical Centre and carried out under protocol number 122-13-19.

Statistical analysis

Data analysis was performed using GraphPad Prism v5 software statistical analysis. The ANOVA test was used for the comparison of more than two groups. Statistical significance between individual groups was determined using the Mann-Whitney *U* test, and statistical significance was accepted at $p \leq 0.05$.

RESULTS

Comparison of the genome sequences of WNV Lineage 1 (NY99) and lineage 2 (578/10) strains

The complete genome sequence of the 578/10 strain (GenBank accession number KC496015) exhibits 2236 nucleotide (nt) differences (21%) compared to the WNV NY99 strain (accession number AF202541). The nucleotide substitutions are distributed fairly equally within the genome (5'UTR: 0 substitutions (0%), C protein gene: 59 substitutions (16%), preM-M protein gene: 102 substitutions (21%), E protein gene: 309 substitutions (21%), NS1 protein gene: 227 substitutions (22%), NS2A protein gene: 153 substitutions (23%), NS2B protein gene: 82 substitutions (22%), NS3 protein gene: 382 substitutions (21%), NS4A protein gene: 80 substitutions (22%), 2K gene: 13 substitutions (19%), NS4B protein gene: 184 substitutions (25%), NS5 protein gene: 560 substitutions (21%), and 3'UTR: 90 substitutions (20%)). Within the putative polyprotein precursor, 203 amino acid (aa) alterations (6%) are found (C protein: 12 substitutions (10%), preM-M protein: 7 substitutions (5%), E protein: 22 substitutions (5%), NS1 protein: 33 substitutions (10%), NS2A protein: 24 substitutions (11%), NS2B protein: 7 substitutions (6%), NS3 protein: 25 substitutions (5%), NS4A protein: 7 substitutions (6%), 2K protein: 1 substitution (5%), NS4B protein: 19 substitutions (8%), and NS5 protein: 46 substitutions (6%)). Due to this high level of sequence diversity, it is not possible to test the potential effect of each substitution in all combinations. As a result, within this study we only tested the effect (in a neuroinvasive WNV lineage 2 strain) of the nucleotide substitutions that have already been identified as virulence markers in WNV lineage 1 strains.

Rescue of recombinant viruses

In order to investigate whether molecular markers of virulence identified in lineage 1 WNV strains attenuate the Hungarian neuroinvasive lineage 2 WNV-578/10 strain *in vitro* or *in vivo*, infectious cDNA clones encoding each substitution of interest were constructed and transfected into BHK-21 cells. After transcription by CMV promoter, CPE had reached the maximum level at 72–96 h after transfection. Supernatants were harvested and passed on Vero E6 cells, which resulted in the generation of at least 6 $\log_{10}$ $TCID_{50}$/mL virus stocks. When the titre did not reach the desired level, one more passage in Vero E6 cells was performed. All mutations of interest were verified by Sanger sequencing of each stock before *in vitro* and *in vivo* characterization. The NS4B102 mutant recombinant WNV was unable to multiply to a sufficient titre in BHK-21 cells nor after passage in Vero E6 cells, and this mutant clone was therefore excluded from the functional analyses.

Full genome sequence analysis of recombinant virus stocks

To determine whether passaging the virus stocks led to the introduction of additional mutations, the full genome of the wild type and modified clone virus stocks were sequenced. One nucleotide alteration was found in addition to the mutated sites for three mutant clones, of which two out of three nt changes were silent: G to A on locus 627 of the NS1 clone, and A to G on locus 6768 of the NS4B38 clone. One nt alteration in the genome of the NS4B249 clone resulted in a Valine to Isoleucine change in the NS4B protein at locus 188 (G to A on locus 7480). The *in vitro* and *in vivo* characterization was subsequently carried out using the generated clones.

Multiplication kinetics of the recombinant wild type (WT) and mutant WNVs in cell culture

The multiplication kinetics of the mutant WNVs were compared to those of the WT infectious clone-derived WNV-578/10 in Vero E6 cells infected in triplicate at an MOI of 0.1 at 37 °C, sampling every 12 hours. Overall, the infectious virus titers of the clones were significantly different at all time points between 12 h and 96 h p.i. (one way ANOVA; $p < 0.02$). More specifically, infectious virus titres for WT were significantly higher at 24 h p.i. compared to all the mutated viruses; NS1 ($p = 0.005$), NS2A, NS3, NS4B38, and NS4B249 ($p = 0.03$). Moreover, NS1 infectivity titres were found to be significantly lower compared to NS2A ($p = 0.03$) and NS4B38 ($p = 0.03$) (Figure 2). At 36 h p.i., WT infectivity titres were only significantly higher compared to NS1 ($p = 0.002$), NS2A and NS3 (both $p= 0.02$). At this time point, the difference in infectious virus titres between WT and NS1 was the largest, at approx. 2500-fold. In addition, NS1 titres remained significantly lower compared to all the other viral clones (all, $p = 0.03$). At 48 h p.i., WT infectious titres were still significantly higher compared to NS1 ($p = 0.005$), NS2A and NS3 ($p = 0.03$), while NS1 titres were only significantly lower compared to NS2A ($p = 0.04$) and NS4B249 ($p = 0.03$). At 72 and 96 h p.i., WT infectious titres remained only significantly higher compared to NS1 ($p = 0.005$; 0.02, respectively), while NS1 titres were significantly lower compared to all the other WNV strains at 72 h p.i. ($p < 0.04$), and compared to NS4B38 and NS4B249 at 96 h p.i. ($p < 0.04$).

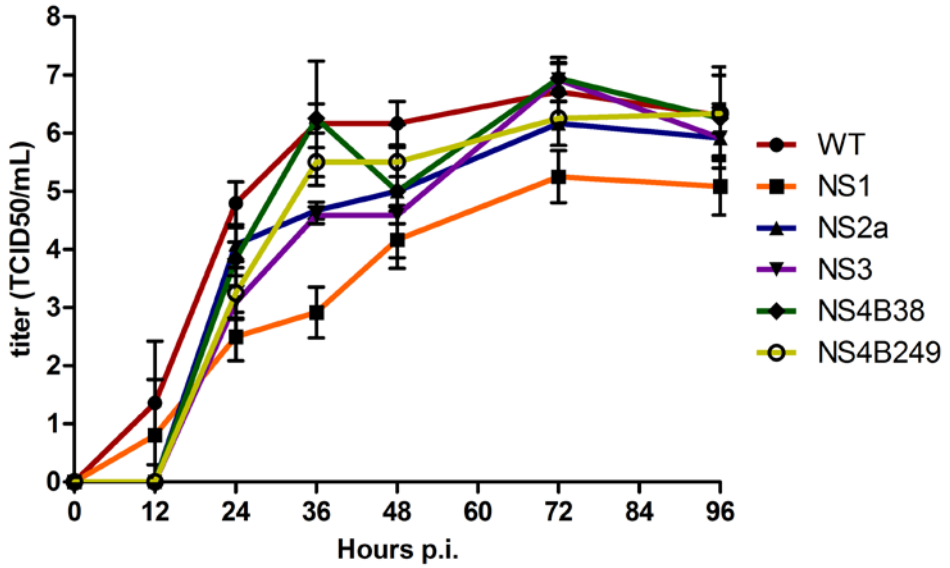


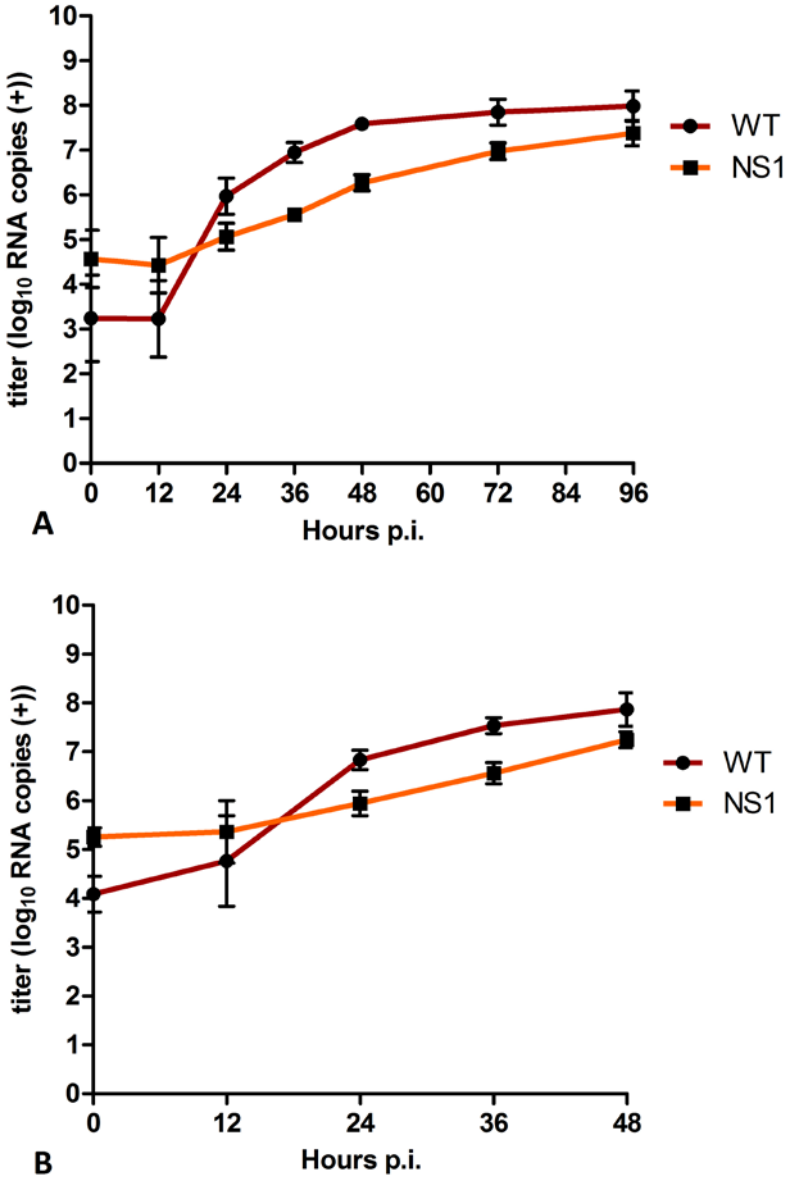
Figure 2. Growth kinetics of infectious virus of the wild type (WT) virus and mutant viruses after triplicate infection of Vero E6 cells at an MOI of 0.1. The titres are given as the mean (log_{10} $TCID_{50}$/mL); error bars represent standard deviation.

Quantification of positive and negative strand RNA for WT and NS1 mutant in vitro

In order to assess whether the *in vitro* replication differences between the WT and NS1 mutant can be attributed to differences in positive and negative strand RNA synthesis, we determined the amount of positive and negative strand RNA copies in the supernatant and cells of the *in vitro* multiplication kinetics experiment using a strand-specific qRT-PCR assay [45]. The amount of positive strand present in the supernatant was more than 1 log_{10} higher for NS1 compared to WT at 12 h p.i., albeit not statistically significant ($p > 0.05$) (Figure 3A). At 24 h p.i., positive strand titres were approx. 0.9 log_{10} RNA copies higher for the WT compared to NS1 ($p = 0.002$). This difference increased to approx. 1.4 log_{10} RNA copies by 36 h p.i. ($p = 0.002$) and 1.3 log_{10} at 48 h p.i. ($p = 0.002$). At t = 72, titre differences had decreased to 0.8 log_{10} ($p = 0.002$) and at 96 h p.i. positive strand titres were only 0.6 log_{10} RNA copies higher for WT compared to NS1, although still significantly different ($p = 0.04$) (Figure 3A).

Positive strand intracellular RNA titres were initially also higher for NS1 compared to WT for the first 12 h p.i. (Figure 3B). However, after 24 h p.i., WT positive strand RNA titres had become approx. 0.9 log_{10} RNA copies higher compared to NS1 ($p = 0.002$), with the most pronounced difference of approx. 1 log_{10} RNA copies after 36 h p.i. ($p = 0.002$). At 48 h p.i., positive strand RNA titres were still significantly higher for the WT compared to NS1 ($p = 0.004$) but the difference had decreased to approx. 0.6 log_{10} RNA copies (Figure 3B).

For intracellular negative strand RNA copies, titres for NS1 were already significantly lower compared to the WT at 12 h p.i. (p = 0.02) (Figure 3C). NS1 negative strand RNA copies remained significantly lower compared to the WT with a difference of approx. 1.2 $\log_{10}$ RNA copies at both 24 and 36 h p.i. (p = 0.002). After 48 h p.i., titre differences decreased to approx. 0.4 $\log_{10}$ RNA copies, at which point the difference was no longer statistically significant (p = 0.06) (Figure 3A).



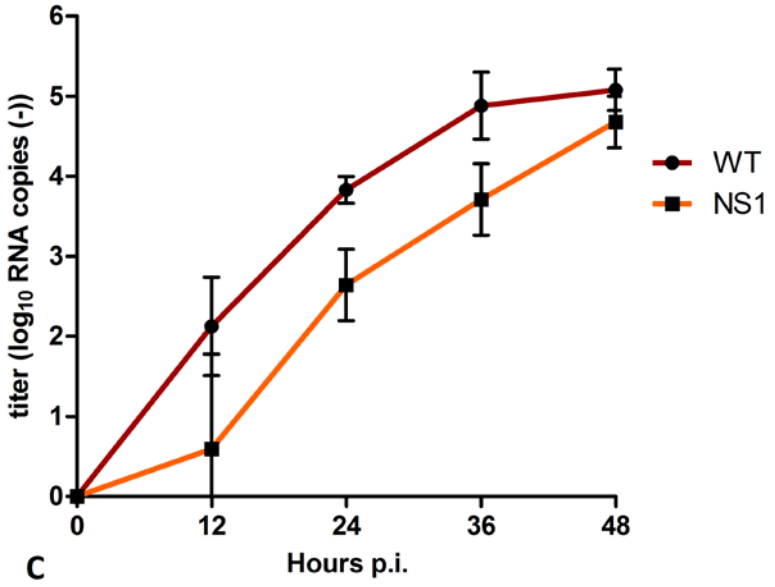


Figure 3. Quantification of **(A)** extracellular positive strand RNA **(B)** intracellular positive strand RNA, and **(C)** negative strand RNA for WT and NS1 mutant after triplicate infection of Vero E6 cells at an MOI of 0.1. Copy numbers are given as the mean of two independent experiments (log_{10} $TCID_{50}$/mL); error bars represent standard deviation.

Mouse neuroinvasiveness of the different mutant WNV strains

The mouse neuroinvasive phenotype of each mutant WNV was examined following i.p. inoculation of six-week-old female C57BL/6 mice. Compared to the WT (100% and 75% mortality at high and low doses, respectively), only the NS1 mutant proved to be significantly attenuating since all mice infected with either the high or low dose of this virus survived the infection (0% mortality for both) (Table 3; Figure 4A and 4B). In contrast, mice infected with the highest dose of the other mutant strains experienced substantial mortality, with rates of 100% (8/8) for NS4B38, 88% (7/8) for NS2A, 75% (6/8) for NS3, and 63% (5/8) for NS4B249, respectively (Figure 4A). Statistical analysis confirmed the attenuation of the NS1 mutant, as significant differences were found between the survival curves of mice infected with the highest dose of NS1 as compared to the WT ($p<0.0001$), NS2A ($p = 0.0004$), NS3 ($p = 0.003$), NS4B38 ($p = 0.0002$) and NS4B249 ($p = 0.008$). On the other hand, the high dose survival curves of the other mutant WNVs showed no significant differences in comparison to the WT, or compared to each other.

Mortality rates of mice infected with the lowest viral dose of the other mutant strains were 75% (6/8) for NS2A, 63% (5/8) for NS4B38, 50% (4/8) for NS4B249 and 38% (3/8) for NS3 (Table 3; Figure 4B). The lower dose survival curves of NS1-infected mice were also significantly different when compared to the WT ($p = 0.002$), NS2A ($p = 0.002$),

NS4B38 (p = 0.009) and NS4B249 (p = 0.03). In contrast, the survival curves of the other mutants were not significantly different compared to the WT, or each other.

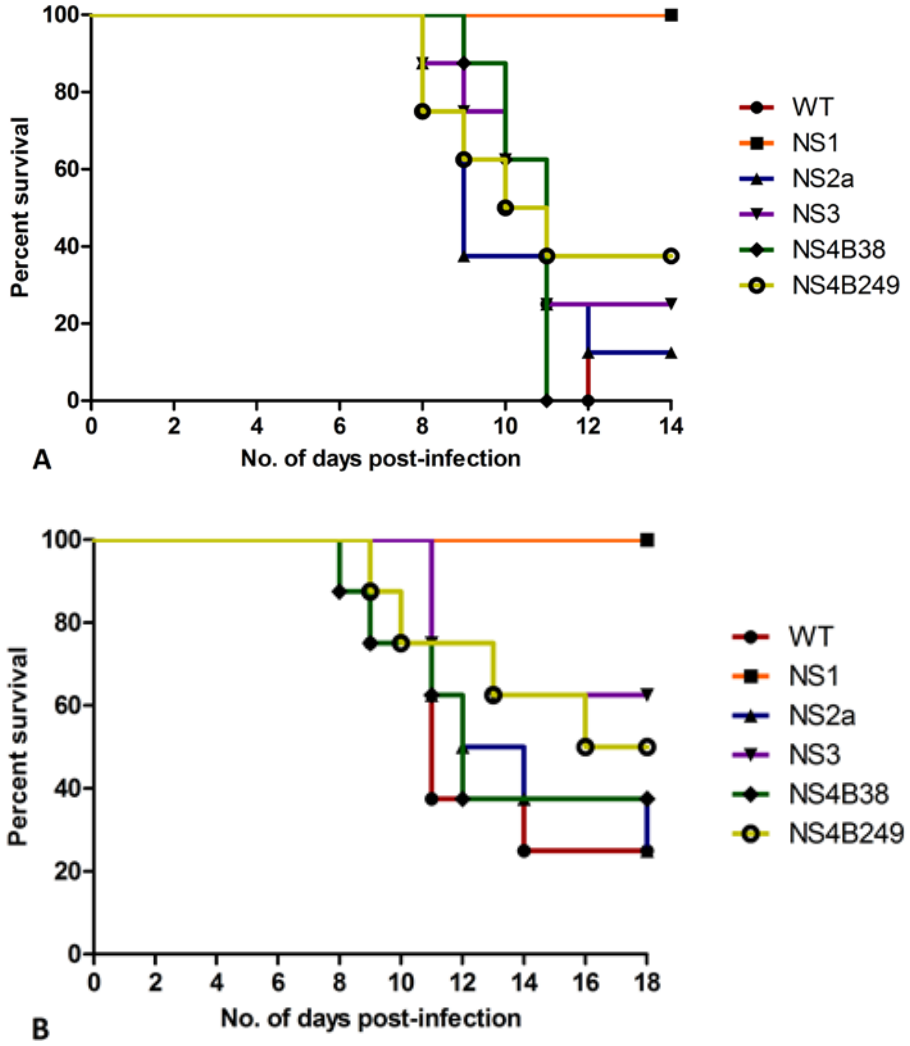


Figure 4. Survival of six-week-old female C57/BL6 mice after i.p. inoculation, with **(A)** high dose (10^4 $TCID_{50}$) and **(B)** low dose (10^1 $TCID_{50}$) of recombinant WNVs.

Virus	Dose ($TCID_{50}$)	Total mortality	Mortality (%)	Median day
WT	10^5	8/8	100	10.5
WT	10^1	6/8	75	11
NS1	10^4	0/8	0	NA
NS1	10^1	0/8	0	NA
NS2a	10^5.5	7/8	88	9
NS2a	10^1	6/8	75	11.5
NS3	10^3	6/8	75	10.5
NS3	10^0	3/8	38	11
NS4B38	10^4	8/8	100	11
NS4B38	10^0	5/8	63	11
NS4B249	10^4	5/8	63	9
NS4B249	10^1	4/8	50	11.5

Table 3. Mortality data of six-week-old female C57BL/6 mice inoculated i.p. with high and low doses of the WT and mutant lineage 2 WNVs.

Sequencing of recombinant WNV in organs of euthanized and survivor mice

All mice euthanized due to illness were found to be positive for viral RNA in the brain (data not shown). To determine whether the mutant strains detected in the brains of mice euthanized due to illness after infection with the highest viral dose had retained the original mutation, viral RNA obtained from two mouse brains per group was sequenced. It was found that the consensus sequences of the virus strains present in the selected mouse brains still contained the original mutation and therefore had not reverted to the wild-type. Additionally, we also determined the presence of persisting virus in the brain and kidney of all mice that had survived infection (euthanized on day 14 p.i.) with the highest dose of the different mutant virus strains (NS1, n=8; NS2A, n=1; NS3, n=2; and NS4B249, n=3) using qRT-PCR. All brains positive for viral RNA were sequenced in order to check for the presence of the original mutation. Out of the eight mice that survived infection with NS1, only one mouse was found to be positive for virus in the brain, which had reverted to the wild-type. For NS2A, the one survivor mouse was found to be positive for viral RNA in the kidney, which still contained the original mutation. Out of the two mice that had survived infection with NS3, and out of the two mice surviving infection with NS4B249, only one mouse in each group was positive for viral RNA in the brain, which was also found to have retained the original mutation.

DISCUSSION

The aim of this study was to generate a full-length infectious clone of the WNV lineage 2 strain 578/10 that has recently been detected in central Europe, in order to improve our understanding on the genetic background of WNV pathogenicity. Infectious cDNA clones are useful tools to investigate genetic determinants of flavivirus virulence, for studying its replication, creating subgenomic replicons, and using gene expression or gene of interest insertions [46–48]. Full-length infectious WNV clones are often constructed by fusion (stitching) polymerase chain reactions or plasmid-based methods [49–52]. Since assembly of full-length flavivirus clones in plasmid vectors have proven to be toxic, or unstable and deleterious for bacterial hosts, on several occasions [53–56], we cloned the full-length genome of WNV-578/10 using a bacterial artificial chromosome (BAC) [57]. This system contains the cytomegalovirus (CMV) immediate-early promoter that allows transcription of WNV RNA in the nucleus by cellular RNA polymerase II, eliminating the step of *in vitro* transcription.

WNVs belonging to lineage 2 were previously considered as agents of low pathogenicity [10]; however, numerous neuroinvasive and highly pathogenic members have recently been identified in horses and humans suffering from severe encephalitis in South Africa [58]. WNV strains belonging to lineage 2 have also been reported in Hungary and surrounding countries since its first proven appearance outside of Africa in 2004. During the last decade, several fatal cases among horses and humans were confirmed as West Nile encephalitis caused by these lineage 2 viruses in the Central European region [15,20]. The changing epidemiology and pathogenicity of WNV outbreaks in Europe highlight our need to further understand how Central European WNV lineage 2 strains differ in their capacity to cause severe disease compared to lineage 1 strains. Furthermore, this study may also provide invaluable information for the development of safe and efficacious vaccines.

Several studies have identified and proven the role of genetic markers in the NS proteins of lineage 1 WNV strains in pathogenicity and virulence studies [24,26,30,31,33–36]. On the other hand, virulence markers of lineage 2 WNV strains have so far only been identified *in silico* by analyzing and comparing full genome sequences [37,38]. Thus far, it is known that flaviviral NS proteins are essential for virus replication; NS1, NS3, NS4A and NS4B can reorganize cellular membranes to generate virus-induced membrane structures (IMS) for site of replication [59]. Furthermore, NS2A [24], NS2B, NS3, NS4A [60], NS4B [61,62] and NS5 [63] play a role in virion assembly and evasion of host innate immune responses by blocking the interferon (IFN) signal transduction pathway [64–66].

The flavivirus nonstructural protein NS1 is a glycoprotein with three conserved *N*-linked glycosylation sites, and has an essential role in viral RNA replication. Normally, NS1 exists as a heat labile homodimer that associates with cellular organelle membranes and is transported to the cell surface [67,68]. Cell surface associated NS1 appears to have an immunomodulatory function via the decrease of the complement activation by different routes [69,70]. NS1 is also secreted by mammalian cells as a soluble hexamer [71,72].

Inclusion of the mutation P250L in a conserved region of the Kunjin virus NS1 gene has been shown to affect the structure of the polypeptide, resulting in the inhibition of dimer formation but still allowing its secretion in the monomeric form. The conformational change resulting from the P250L mutation may have led to the decreased levels of infectious virus titres observed in Vero cells in the early phase of replication (100-fold lower between 12–24 h p.i., compared to the WT); however, this difference gradually decreased and eventually disappeared by 48 h p.i. [73]. Similar to this study, our NS1 mutant also showed a 100-fold decrease in infectious virus; however, this was starting from 24 h p.i. lasting until 48 h p.i., after which the difference was approx. 10-fold until 96 h p.i. We investigated these observations further by quantifying the amount of positive and negative stranded RNA during the first 48 hours of replication in order to determine whether the mutation affected the replication process. We found that the NS1 mutant exhibited significantly slower replication as measured by an approx. 10-fold reduced amount of both negative and positive stranded RNA in the cells, as well as positive stranded RNA in the supernatant.

A role for the importance of NS1 in the process of RNA synthesis has already been described in previous studies. Youn *et al*. found that WNV RNA lacking intact NS1 genes was efficiently translated but did not form canonical replication complexes early after infection, resulting in a failure to replicate viral RNA and consequently significantly lower amounts of positive and negative stranded RNA in the cells [74]. As we also found a reduced amount of negative and positive stranded RNA for the NS1 deletion mutant as compared to the WT during the first 48 hours of infection, it suggests that the P250L mutation potentially affects the activity or the stability of the NS1 protein for the formation of replication complexes during infection.

In the study by Hall *et al.*, 10-fold more virus of the WNV-KUN P250L mutant was required to produce disease in mice. Our results, however, show that this mutation completely abolished the neuroinvasiveness of the lineage 2 WNV-578/10 strain, since no mice died after challenge with either the high or low dose of the virus. This means that in contrast to the Kunjin virus, neuroinvasiveness had decreased at least 10,000 fold for our mutant virus. It is possible that the different mouse model used in the aforementioned study (BALB/c mice) or the different age (18–20 days of age) of the mice explains the discrepancy between our results. However, it can also not be excluded that the silent mutation on locus 627 of the NS1 clone that we identified during full genome sequencing influenced the attenuated phenotype of this mutant. Furthermore, the presence of certain loci in the genome of the lineage 2 virus may have also augmented the attenuating effect of the P250L mutation observed in our study as compared to those involving lineage 1. Studies involving the introduction of this mutation into other, virulent lineage 1 or 2 WNV strains may provide more insight into the importance of this mutation.

Nonetheless, the significantly reduced replication of the NS1 mutant that we observed in cell culture may explain the reduced neuroinvasiveness observed in our study, as it may allow for early control of the immune system followed by elimination of the virus before it

has reached the central nervous system. Even though only one mouse out of eight infected with the NS1 mutant was found to have virus in the brain at day 14 p.i. (albeit lacking the original mutation), we cannot rule out with certainty that the virus had still entered the brain in the majority of the mice but had simply been cleared by day 14 p.i. due to its reduced neurovirulence. Future experiments involving intracranial injections of the NS1 mutant could confirm the reduced neurovirulent or neuroinvasive capacity of the virus.

NS2A is a small, hydrophobic, membrane-associated protein of WNV, believed to anchor the RNA replication complex by intercalating into the endoplasmic reticulum (ER) membrane [75]. The NS2A protein plays an important role in virus assembly and in the inhibition of the cellular antiviral response via the inhibition of IFN-beta promoter-driven transcription [32,76]. In one study, a NY99 mutant virus was less virulent in four-week-old Swiss-Webster outbred mice when the NS2A locus was derived from the Kunjin virus and harbored an introduced A30P mutation [26]. In contrast, Rossi *et al*. found only a slight decrease in virulence of the NS2A A30P mutant NY99 strain compared to the WT virus in five-week-old Swiss-Webster mice [25]. Our results are in line with the latter study, as we observed no significant differences in the replication kinetics of the mutant compared to the wild type virus, and no significant attenuation was detected in mice after challenge.

The full length NS3 protein is a multifunctional enzyme (the N terminal residues encode trypsin-like serine protease, the C terminal residues encode RNA triphosphatase, NTPase and helicase) that possesses various activities in both viral polyprotein processing and RNA replication [77,78]. NS3 is suggested to be involved in virus assembly as well, but in cooperation with NS2A [79]. In corvids, the lineage 1 NY99 virus containing a proline to threonine substitution at the NS3-249 locus was particularly attenuating (100% to 12.5%), while the introduction of a proline at this site in a low virulent strain led to an increase in virulence (31% to 94%), likely related to an increased capacity of the virus to replicate in corvids [33]. This site therefore appears to be a key virulence determinant of the lineage 1 NY99 strain in corvids; however, the influence of this mutation in a mouse model has been minimal [80].

Genetic comparison of the goshawk-Hungary-2004 strain with the closely related lineage 2 Nea Santa-Greece-2010 identified an H249P mutation, which was speculated to play a role in the increased virulent phenotype of this Greek strain [18]. Interestingly, like the Greece-10 strain, our Hungarian lineage 2 WNV-578/10 strain also contains a proline at this position (data not shown). As a result, we investigated whether the NS3-249P mutation may contribute to increased virulence by introducing an NS3-P249H substitution and testing its attenuation in a mouse model. Here, this substitution proved to be slightly attenuating, but this was not statistically significant. Interestingly, infectious virus titres obtained in cell culture were found to be significantly lower for NS3 compared to the wild-type at three time-points. It should be noted, however, that this was also the case for several of the other mutants, such as NS2A, which had not shown any attenuation in our mouse model. It therefore appears that infectious virus titres *in vitro* do not correlate with attenuation *in vivo*. Indeed, in a study by Langevin *et al.*, despite the NS3-P249T mutation

contributing to a 6 $\log_{10}$ PFU/mL lower titre in corvids, there were no significant differences observed in mean peak infectious viral titres on Vero cells at 37 °C for this mutant when compared to the NS3-249P virus [80]. As we were interested in examining the effect of the different mutations in a murine model, no thermosensitivity experiments at temperatures higher than 37 °C were undertaken for any of the clones; however, further studies testing the different mutants in cell culture at a higher temperature (41–44 °C) or in a corvid model might prove to be insightful as well, as in particular mutations at the NS3-249 locus seem to play an important role in the virulence of WNV in bird species.

Flaviviral NS4B is a predominantly helical, hydrophobic, membrane-associated NS protein, which plays a crucial role in blocking host cell antiviral responses. It acts as an interferon antagonist, since expression of NS4B, contributed by the activity of NS4A and NS2A, strongly inhibits the IFN-induced signal transduction cascade by blocking STAT-1 phosphorylation [61]. Amino acid mutations in the coding region of NS4B can alter the inhibitory effect on interferon signaling [62].

The P38 residue of NS4B is predicted to localize to the junction of an ER-luminal region and a transmembrane domain. A study by Welte *et al*. found that the P38G mutation in the NS4B protein induced a lower level of viremia and no lethality in six- to ten-week-old C57BL/6 mice, while inducing higher type 1 IFNs and interleukin (IL)-1 as well as stronger effector and memory T cell responses [35]. A later study by Wicker *et al*. found the NS4B-P38G substitution to be associated with a temperature-sensitive phenotype of the lineage 1 NY99 strain, which involved a significant delay in multiplication in Vero cells at 41 °C, but not at 37 °C, as well as attenuation for neuroinvasiveness with an i.p. LD_{50} value of greater than 10,000 PFU in three- to four-week-old NIH Swiss mice. Importantly, however, two unexpected additional mutations were found at NS4B-T116I and NS3-N480H and actually none of the mutations alone were attenuating in mice [36]. In our study, the P38G mutation did not affect replication in Vero E6 cells and the virus was equally virulent in mice as the WT, where we used the same mouse strain and age as Welte *et al*. Furthermore, full genome sequencing revealed that the NS4B-T116I and NS3-N480H mutations were not present in our NS4B38 clone. Even though it is tempting to speculate that the P38G mutation may therefore not be so important for a lineage 2 WNV strain, we cannot exclude that the presence of other co-mutations, such as the silent mutation at the 6768 locus that we identified during full genome sequencing, or other mutations specifically present in the lineage 1 genetic backbone, are important for the attenuating effect of the NS4B-P38G mutation.

The C102S substitution of the NS4B protein in the NY99 strain has demonstrated thermosensitivity at 41 °C *in vitro* and was found to attenuate mouse neuroinvasiveness and neurovirulence [29]. The same substitution in the 578/10 construct dramatically reduced the replicative ability of the virus *in vitro* as such that no virus could be rescued, and therefore its further *in vitro* and *in vivo* effect could not be assessed. The hypothesized mechanism of C102S attenuation of the NY99 strain was a reduced ability in inhibiting the IFN signaling pathway [29]. However, the C102S mutant 578/10 clone was not able to replicate in BHK-

21 and Vero E6 cells, even though the IFN-α and -β pathways are not functioning in these cell lines. Therefore, probably other factors contributed to the lethal effect of this substitution in the lineage 2 WNV strain.

The NS4B-E249G mutation has been observed in several natural WNV isolates [81,82]. Furthermore, it was shown that a mutant lineage 1 WNV containing the E249G residue replicated at a lower level in C3H/He and BHK-21 cell cultures, but only slightly lower in Vero cell culture. In addition, the E249G mutant lineage 1 WNV was significantly attenuating in six-week-old C3H/HeN mice after footpad inoculation (100% mortality *vs*. 50%) [30]. On the other hand, a study by Rossi *et al*. reported that a WNV lineage 1 virus harboring the NS4B-E249G mutation demonstrated a WT phenotype with foci identical in size to the WT, as well as a similar LD_{50} value in i.p. inoculated five-week-old Swiss-Webster mice [25]. Our results are closer to those obtained by Rossi *et al.* [25], as the E249G mutant lineage 2 WNV propagated to similar titres in Vero E6 cells as the WT and showed reduced mortality in mice (63%) that was not significantly different. It cannot be excluded, however, that the non-conservative mutation found at NS4B188 played an important role in decreasing the attenuated phenotype exerted by the NS4B-E249G mutation.

In summary, our results have shown that in mammalian *in vitro* and *in vivo* models the NS1-P250L mutation contributed to significant attenuation of lineage 2 WNV, while the NS3-H249P and NS4B-E249G mutations conferred a partial, but not statistically significant reduction of virulence in mice. In contrast, the NS2A-A30P and NS4B-P38G were not attenuating at all *in vivo*. Even though it might be possible to conclude that the mutation at the NS1 locus could be an important marker of virulence in lineage 2 WNV strains, the fact that lineage 1 mutation studies as cited herein often showed varying results means that such results should still be addressed with caution. For example, mouse genotype and age may have an important influence on the outcome of mutational studies *in vivo*. The influence of other mutations in the particular genetic backbone used in certain studies may also play an important role. To be specific, the genetic backbone of the lineage 2 virus that we used may play a role in decreasing the attenuation of some of the markers that we have investigated, as well as in increasing the attenuation of the NS1 mutation. As a result, future studies investigating details of the NS1-P250L substitution should be performed in another vertebrate model, and also studies involving the introduction of this particular NS1 mutation into the genome of other lineage 2 viruses, or introducing the entire WNV-578 NS1 locus harboring this particular mutation into a lineage 1 strain, may prove to be insightful.

The infectious clone described in this study provides a useful tool for testing the effect of hypothetical virulence marker loci in *in vitro* and *in vivo* model systems. Within the last decade, virulent lineage 2 WNV strains have emerged in Europe. For example, a descendant of the strain that had emerged in 2004 in Hungary caused an epidemic with an unforeseen large amount of neuroinvasive cases in Greece, in 2010. Another lineage 2 strain (Reb_VLG_07_H, GenBank accession FJ425721) emerged in the Volgograd region

of Russia and caused human neurological cases [21,22]. In 2010, this strain also emerged in south-east Romania [23] and survived for at least three years [83]. As a result, genetic comparisons of different emergent isolates may help to identify and predict potential virulence markers. In this regard, our study has provided more insight into genetic markers that may contribute to the virulence of lineage 2 WNV strains.

ACKNOWLEDGMENTS

The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/2007 - 2013) under the project "VECTORIE", EC grant agreement number 261466. The contents of this publication are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission.

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PART II:

HOST SUSCEPTIBILITY

Chapter 5

Susceptibility of European Jackdaws (*Corvus monedula*) to Experimental Infection with Lineage 1 and 2 West Nile Viruses

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J Gen Virol. 2014 Jun;95(Pt 6):1320-9

ABSTRACT

Mass bird mortality has been observed in North America after the introduction of West Nile virus (WNV), most notably massive die-offs of American crows (*Corvus brachyrhynchos*). In contrast, WNV epidemic activity in Europe has been characterized by very low incidences of bird mortality. As the general susceptibility of European corvids to strains of WNV remains in question, European jackdaws (*Corvus monedula*) were inoculated with WNV strains currently circulating in Greece (Gr-10), Italy (FIN and Ita09) and Hungary (578/10), as well as a North American (NY99) genotype with a demonstrated corvid virulence phenotype. Infection with all strains except WNV-FIN resulted in mortality. Viremia was observed for birds inoculated with all strains and virus was detected in a series of organs upon necropsy. These results suggest that jackdaws could potentially function as a sentinel for following WNV transmission in Europe; however, elicited viremia levels might be too low to allow for efficient transmission of virus to mosquitoes.

INTRODUCTION

West Nile virus (WNV) is a flavivirus (family: *Flaviviridae*) that is maintained in an enzootic cycle between mosquitoes and birds, but may also infect humans and horses, which serve as incidental dead-end hosts. WNV is endemic in many parts of Africa, Australia, the Middle East and Asia, and more recently emerged in North America in 1999, and has since rapidly spread across North America, Mexico, South America, and the Caribbean. Since 2008, WNV has emerged as a serious veterinary and public health problem in central and south-eastern Europe, affecting countries such as Greece [1-3], Italy [4-7], Hungary, Austria [8] and Romania [9].

Birds were considered to be less susceptible to WN disease until high mortality rates were recorded in flocks of young domestic geese in Israel in 1998 [10, 11], after which WNV has been implicated in deaths of members of 326 species of birds in North America [12]. Among the passeriform birds, the family *Corvidae* is ranked as the most highly susceptible species to WNV [13], and particularly deaths among the American crow (*Corvus brachyrhynchos*) have been used to track the spread of the virus in North America [14, 15].

In contrast to the mass bird mortality observed in North America, sporadic isolated death events and low mortality rates have been reported among European birds, even during severe human and equine WN outbreaks [16-19]. Possible explanations for the lack of reporting of bird mortality could be that European birds are less susceptible to WNV-induced disease, or that the North American strain of WNV exhibits an increased capacity for eliciting avian virulence. Other theories include: poor detection of bird carcasses (due to their small size or scavenging) [20], limited monitoring of wild bird mortality, or development of herd immunity due to local transmission spurred by avian migration through WNV endemic areas of the Middle East and Africa [21].

To date, the susceptibility of European jackdaws to WNV isolates circulating in Europe has not yet been addressed. In order to examine the role of a bird that is found ubiquitously across Europe as a natural reservoir for the transmission of WNV disease or as a potential sentinel for WNV activity, European jackdaws were inoculated with four different WNV isolates clinically relevant to Europe, including lineage 1 isolates Ita09 and FIN from Italy, as well as lineage 2 isolates from Greece and Hungary. The NY99 strain was used to assess and compare the susceptibility of the European birds to a strain known to be highly virulent in North American corvids, especially in American crows. Susceptibility was assessed in terms of mortality, median survival time, duration and magnitude of viremia, and dissemination of virus to the different organs.

MATERIALS AND METHODS

Source of Virus and Birds

Five different isolates of WNV were utilized (Table 2): The NY99-4132 strain (lineage 1a) [22] was originally isolated from the brain of an American crow and was passed two times in Vero cells before being used in this study. The lineage 2 Greek strain (Nea Santa-Greece-2010; accession HQ537483.1) was isolated from a pool of *Culex pipiens* mosquitoes [23] and passaged once on Vero cells. Two lineage 1a Italian strains, FIN (provided by Dr. Vittorio Sambri, University of Bologna, Italy; two Vero E6 passages; accession KF234080) and Ita09 (provided by Dr. Luisa Barzon, University of Padova, Italy, one Vero E6 passage; accession GU011992.2) were both isolated from a patient with neuroinvasive disease [4]. The Hungarian lineage 2 strain 578/10 (provided by Dr. Tamás Bakonyi, Szent István University, Hungary, two passages on Vero E6 cells; accession KC496015) was isolated from the brain of a horse that died of WNV-neuroinvasive disease. Virus stocks used in this study (not including the NY99 and Greece-10 strain), were prepared by growing the viruses once in C6/36 insect cells (Table 1).

Jackdaws were captured using walk-in traps in the municipality of Rotterdam, The Netherlands. They were transported to indoor housing where they were kept in groups of seven or eight in isolators under negative pressure. Only seronegative birds were used in this study. All birds were cared for in animal holding facilities at the National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands.

Virus	Strain	Source	Passage history[a]	Location	Genetic lineage
NY99	NY99-4132	American crow (brain)	V2	USA	1a
Greece-10	Nea Santa-Greece-2010	*Culex pipiens*	V1	Greece	2
FIN	FIN	Patient with neuroinvasive disease	V2, C1	Italy	1a
Ita09	Ita09	Patient with neuroinvasive disease	V1, C1	Italy	1a
Hungary	578/10	Horse (brain)	V2, C1	Hungary	2

[a]Viruses were propagated in Vero (V) or C6/36 insect cells (C). Numbers following passage source represent the number of viral passages.

Table 2. West Nile virus strains used for susceptibility studies in European jackdaws.

Detection of Preexisting WNV Antibodies

To confirm that jackdaws had not previously been exposed to WNV, the birds were bled before experimental infection and serum was tested for neutralizing antibodies using tissue culture infectious dose 50 ($TCID_{50}$) neutralization assays. Serum was heat-inactivated at 56

ºC for 30 min, serially diluted two-fold and incubated with an equal volume of virus (strain NY99, originally isolated from a dead Chilean flamingo at the Bronx Zoo in New York, obtained from the Health Protection Agency, Porton Down, UK; P5 on Vero E6 cells; accession AF196835.2) to a final concentration of 100 $TCID_{50}$/0.1 mL. Samples were incubated at 37 ºC for 1 hr and subsequently added to an 80% confluent monolayer of Vero E6 cells in CELLSTAR® 96-well plates (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). Plates were incubated at 37 ºC for 5 days. Samples were read and a 100% reduction in cytopathic effect (CPE) as compared to the serum-negative control was used for the determination of neutralization. Detection of any neutralizing activity to WNV in the serum of any bird precluded its use for experimental inoculation.

Experimental Infection and Sampling Protocol

Jackdaws were subcutaneously inoculated in the thigh region with 2000 $TCID_{50}$ of virus/0.1 mL of Dulbecco's Modified Eagle Medium (DMEM) containing no fetal bovine serum (FBS). Jackdaws were injected with NY99-1432 (n=7), Greece-10 (n=8), FIN (n=7), Ita09 (n=7) and 578/10 (n=7). Blood was collected from all birds (~0.1 mL) at 2-day intervals for a period of 8 days post-infection (dpi). Coagulated blood was centrifuged at 1300 × *g* for 5 min in MiniCollect® vials (Greiner Bio-One) in order to separate serum, which was subsequently stored at -80 ºC. All jackdaws were examined for signs of disease twice daily for 14 days following inoculation and euthanized under isoflurane anesthesia upon display of clinical symptoms. Additionally, two birds per group were euthanized at day 4 post-infection (p.i.) for monitoring the dissemination of virus to the organs at the approximate time of peak viremia, as well as all surviving birds at day 14 p.i.

Necropsies were performed on all euthanized birds and the following tissues were collected: heart, liver, spleen, kidney, bone marrow and brain. A small section of each tissue was collected and subsequently weighed and homogenized using a metal bead in 1 mL of DMEM containing antibiotics (100 U penicillin, 100 µg/mL streptomycin). The remaining portion of the tissues was collected in formalin for use in immunohistochemical staining.

Determination of Viral Loads

To determine viral loads in the serum samples and tissue homogenates, we used quantitative real-time polymerase chain reaction (qRT-PCR) to measure viral RNA titers (serum and tissue), and $TCID_{50}$ titration for the calculation of infectious virus titers (serum only). Briefly, RNA was isolated from 50 µL of serum or 100 µL of homogenized tissue using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Almere, The Netherlands) and an automated nucleic acid robotic workstation (Roche) according to the manufacturer's instructions. RNA was eluted in 100 µL of elution buffer (Roche) and stored at -80 ºC until assayed. RNA copy numbers were quantified as described in Lim et

al. using unmodified primers [24]. The limit of detection of the assay was 0.95 $\log_{10}$ RNA copies.

Infectious titers in the serum were determined by $\log_{10}$ titration of the serum samples on Vero E6 cells and calculating the $TCID_{50}$ using the Spearman & Kärber method [25, 26] after the determination of CPE 5 days p.i. Initial 1:10 dilution of serum resulted in a limit of detection of $10^{1.75}$ $TCID_{50}$/mL.

Immunohistochemistry

Sagittal organ 4-µm thick paraffin sections were processed for peroxidase immunohistochemistry of virus nonstructural protein markers. Sections were deparaffinized in xylene, rehydrated in descending concentrations of ethanol and incubated for 10 min in 3% H_2O_2 diluted in PBS in order to block endogenous peroxidase activity. Antigen exposure was performed by 15 min incubation at 121 ºC in citrate buffer (0.01 M, pH 6.0). Sections were incubated overnight at 4 ºC with primary goat anti-WNV NS3 (1:100; R&D Systems, Abingdon, UK) or goat serum (1:100; Dako, Eindhoven, The Netherlands) for isotype controls, and detected with secondary rabbit anti-goat IgG-PO (Dako) antibody. Sections were counterstained with Mayer's hematoxylin, mounted with Kaiser's glycerin-gelatin and analyzed using a light microscope.

Statistical Analyses

Survival curves were analyzed using the Log-rank (Mantel-Cox) test. All other statistical analyses were performed by Kruskal-Wallis one-way analysis of variance (ANOVA) and any significant difference found was more closely analyzed between the groups using the Mann-Whitney *U* test.

RESULTS

Morbidity and Mortality

During a 9-day period, lethargy, low activity, anorexia and ruffled feathers were observed among some of the jackdaws inoculated with NY99, Greece-10, Ita09 and 578/10. Birds died within 24-48 hours after onset of clinical symptoms. Among the five jackdaws that were inoculated with NY99 and Ita09 and followed for survival, three (60%) died; and of the five inoculated with 578/10, two (40%) died. Among the six jackdaws inoculated with Greece-10, three died (50%). In contrast, all five birds inoculated with FIN survived the infection (Table 1). Comparison of the survival curves of the birds inoculated with the different virus strains revealed a significant difference between the survival curves of jackdaws infected with FIN compared to Ita09 (Figure 1; $P = 0.05$). Median day of death

was 7 dpi for birds that succumbed due to infection with NY99, Ita09 and 578/10, and 8 dpi for birds that died due to infection with Greece-10 (Table 1).

Virus group	Mortality: no. died /N (%)	Median day of death	Mean day onset viremia ± SD	Mean peak viremia[a] viral RNA/mL ± SD	Mean day peak viremia ± SD	Mean duration viremia[b] ± SD
NY99	3/5 (60)	7	2.0 ± 0	6.2 ± 1.9	4.0 ± 0	5.2 ± 1.0
Greece-10	3/6 (50)	8	2.0 ± 0	5.9 ± 1.3	3.3 ± 1.0	6.0 ± 1.2
FIN	0/5 (0)	NA	3.2 ± 1.0	4.0 ± 0.7	4.0 ± 1.3	6.4 ± 0.8
Ita09	3/5 (60)	7	2.8 ± 1.0	4.7 ± 1.5	4.0 ± 1.3	5.2 ± 1.6
578/10	2/5 (40)	7	2.0 ± 0	5.0 ± 1.3	4.0 ± 2.2	6.4 ± 1.5

[a]Viral titers are expressed as $\log_{10}$ RNA copy numbers/mL of sera.
[b]Duration is expressed in days.
NA = not applicable.

Table 1. Clinical profile of five or six European jackdaws infected with West Nile virus strains NY99, Greece-10, FIN, Ita09 and 578/10.

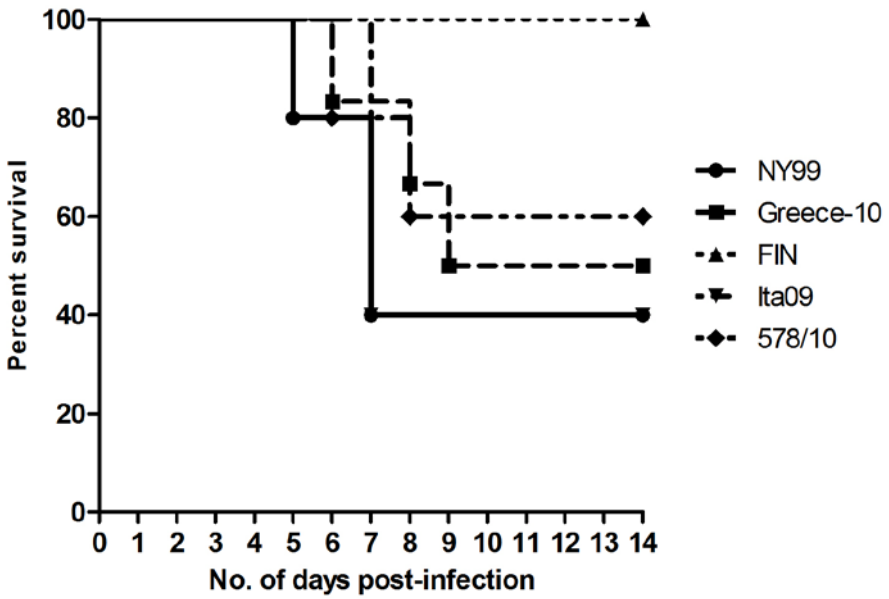

Figure 1. Survivorship of five or six European jackdaws, each inoculated with 2,000 $TCID_{50}$ of West Nile virus strains NY99, Greece-10, FIN, Ita09 or 578/10. Jackdaws were monitored daily for signs of disease up to 14 dpi.

Viremia Profiles

We determined WNV viremia profiles for the jackdaws inoculated with the five different virus strains and found that all birds developed viremia within 96 hours p.i (Table 1; Figure 2). For NY99-infected birds, peak viremia titers ranged from 4.1 $\log_{10}$ to 9.6 $\log_{10}$ RNA copies/mL of serum (mean peak viremia titer = 6.2 $\log_{10}$ RNA copies/mL). Birds infected with the Greece-10 strain had peak viremia titers ranging from 4.2 $\log_{10}$ to 8.4 $\log_{10}$ RNA copies/mL of serum (mean = 5.9 $\log_{10}$ RNA copies/mL). For FIN-infected birds, peak viremia titers ranged from 3.3 $\log_{10}$ to 5.1 $\log_{10}$ RNA copies/mL of serum (mean = 4.0 $\log_{10}$ RNA copies/mL). Ita09-infected birds displayed peak viremia titers ranging from 3.4 $\log_{10}$ to 7.5 $\log_{10}$ RNA copies/mL of serum (mean = 4.7 $\log_{10}$ RNA copies/mL). Among jackdaws infected with the 578/10 strain, peak viremia titers ranged from 3.1 $\log_{10}$ to 6.9 $\log_{10}$ RNA copies/mL of serum (mean = 5.0 $\log_{10}$ RNA copies/mL).

These data demonstrate that birds inoculated with NY99 and Greece-10 had the highest peak viremia titers, followed by 578/10 and Ita09, with the lowest viremia peaks for FIN-infected birds. Mean peak viremia titers were significantly different between Greece-10 and FIN (P = 0.02) and NY99 and FIN (P = 0.03). Onset of viremia occurred significantly later for FIN-infected birds compared to Greece (P = 0.02), NY99 and 578/10 (P = 0.03, for both) (Table 1). The peak of viremia was reached the earliest by birds inoculated with Greece-10, followed by the other viral strains (Table 1), but differences were not statistically significant (P = 0.8). In terms of duration, viremia lasted the longest for FIN and 578/10, followed by Greece-10, NY99 and Ita09 (Table 1), but these differences were also not statistically significant (P = 0.4).

Determination of the viremia profiles in terms of infectious virus ($TCID_{50}$) titers was only successful for a limited set of birds. The infectious titers successfully recorded were for the birds with the highest peak viremia titer (in terms of viral RNA) in each group. These titers were specifically $10^{6.3}$ $TCID_{50}$/mL ($10^{9.6}$ RNA copies) for the NY99-infected bird with the highest peak viremia, $10^{5.3}$ $TCID_{50}$/mL for the Greece-10- and Ita09-infected birds ($10^{8.4}$ and $10^{7.5}$ RNA copies, respectively), and $10^{2.3}$ for the 578/10-infected bird ($10^{6.9}$ RNA copies).

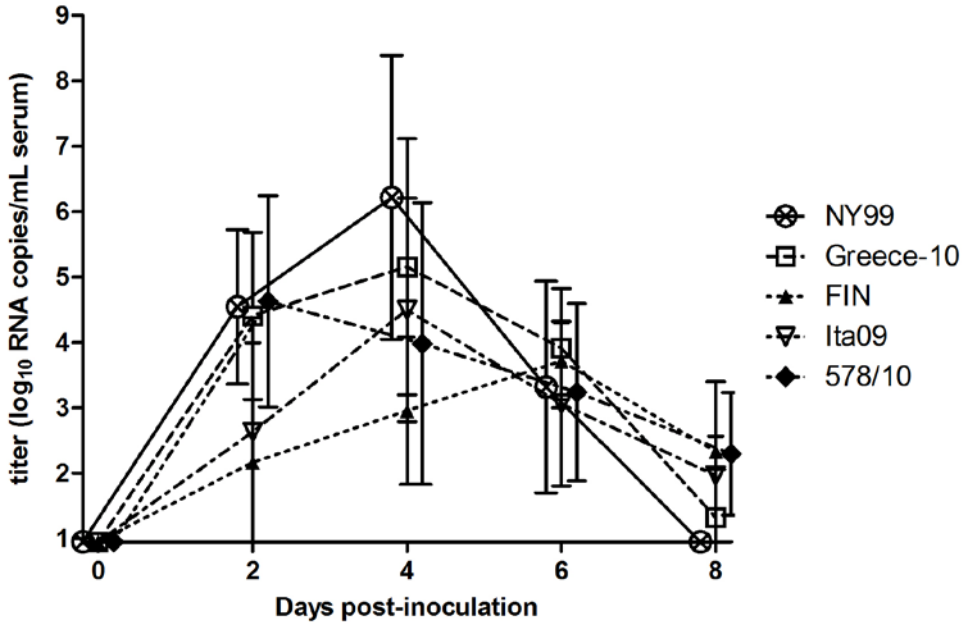

Figure 2. **Viremia profiles for West Nile virus (WNV)-infected European jackdaws after inoculation with 2,000 $TCID_{50}$ of either WNV-NY99 (n=5), Greece-10 (n=6), FIN (n=5), Ita09 (n=5) or 578/10 (n=5).** Viral titers were determined by RNA copy numbers and are represented as geometric means. A detection limit of 0.95 $\log_{10}$ RNA copies/mL of serum was determined. Bars represent standard deviations (SD) of the mean.

Tissue Tropism

Viral loads were determined in the heart, liver, spleen, kidney, bone marrow and brain for all birds. In order to assess the spread of virus to the different organs at the approximate peak of viremia, two birds per group were euthanized on day 4 p.i. Virus was detected in all organs of these birds (Figure 3) with on average the highest viral titers found in the spleen and bone marrow (7.1 and 6.9 $\log_{10}$ RNA copies/g of tissue, respectively), followed by the liver, kidney and heart (5.8, 5.7 and 5.5 $\log_{10}$ RNA copies/g of tissue, respectively). The lowest viral titers were found in the brain (3.8 $\log_{10}$ RNA copies/g of tissue). Between the different virus strains, higher viral RNA titers were found in the organs infected by Greece-10 (7.0 $\log_{10}$ RNA copies/g of tissue), followed by 578/10 (6.4 $\log_{10}$ RNA copies/g), NY99 and Ita09 (5.4 $\log_{10}$ and 5.3 $\log_{10}$ RNA copies/g, respectively), while FIN-infected birds had the lowest tissue viral RNA burden (4.8 $\log_{10}$ RNA copies/g). Viral titers in the organs were significantly different for Greece-10 compared to NY99 (P = 0.05), FIN (P = 0.004) and Ita09 (P = 0.02), and in the organs infected by 578/10 compared to FIN (P = 0.01).

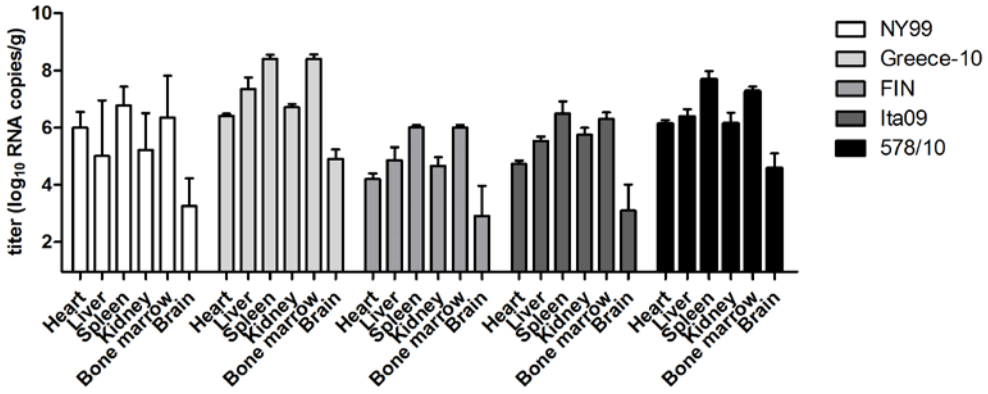

Figure 3. Viral load, as determined by RNA copy numbers, in organs harvested from birds experimentally infected with different West Nile virus strains and euthanized on day 4 (two per group). Viral titers are represented as geometric means. A detection limit of 0.95 $\log_{10}$ RNA copies/g of tissue was determined. Bars represent standard deviations (SD) of the mean.

For birds euthanized due to morbidity, virus was also found in all the organs (Figure 4), with the tendency for the spleen, kidney and heart to contain the highest average viral RNA load (6.7, 6.6 and 6.2 $\log_{10}$ RNA copies/g of tissue, respectively), followed by the liver, brain and bone marrow (5.5, 5.4 and 5.2 $\log_{10}$ RNA copies/g of tissue, respectively). Viral RNA titers were highest in organs of birds infected by Greece-10 (6.2 $\log_{10}$ RNA copies/g), followed by 578/10 and NY99 (6.1 $\log_{10}$ RNA copies/g, for both), with the lowest titers exhibited by Ita09-infected birds (5.4 $\log_{10}$ RNA copies/g). However, these titers were not found to be significantly different ($P = 0.4$).

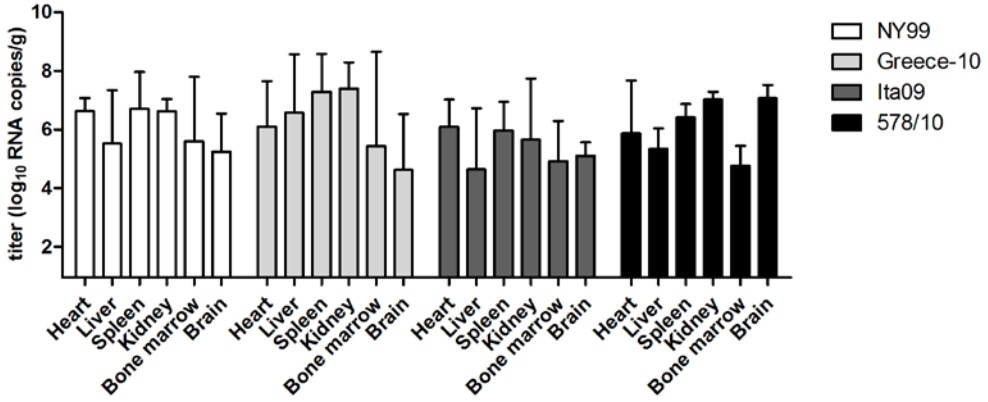

Figure 4. Viral load, as determined by RNA copy numbers, in organs harvested from birds experimentally infected with different West Nile virus strains and euthanized due to morbidity. NY99 (n=3): 1 bird euthanized on day 5 p.i., 2 birds on day 7; Greece-10 (n=3): 1 bird euthanized on day 6, 1 bird on day 8 and 1 bird on day 9; Ita09 (n=3): 3 birds euthanized on day 7; and 578/10 (n=2): 1 bird euthanized on day 6 and 1 bird on day 8. Viral titers are

represented as geometric means. A detection limit of 0.95 $\log_{10}$ RNA copies/g of tissue was determined. Bars represent standard deviations (SD) of the mean.

All birds that had survived infection by day 14 were euthanized and necropsied to determine whether virus could still be found in any of the organs (Figure 5). Viral RNA was detected in all organs of the three Greece-10-infected survivor birds except for in the bone marrow of one bird, while NY99-infected birds had a minimum of three positive organs (out of 6), with the liver being completely negative for both birds. FIN-infected survivor birds had a minimum of two organs (out of 6) positive for viral RNA, while the bone marrow was consistently negative for all five birds. The two birds that survived infection with Ita09 were both positive for viral RNA in five out of six organs, with the liver negative in one bird and the bone marrow in the other. Viral RNA was detected in all organs of the two 578-10-survivor birds except for in the liver of one bird. Between the different virus strains, mean viral RNA titers in the organs were significantly higher in Greece-10-infected birds compared to birds infected with FIN ($P = 0.03$).

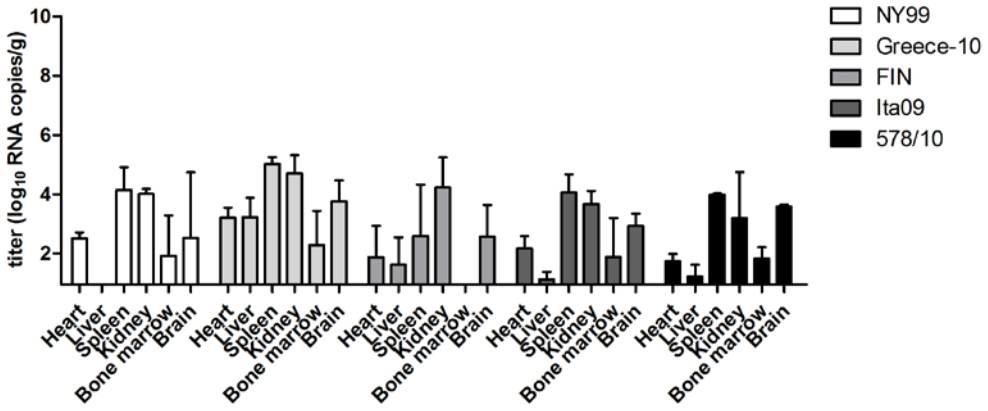

Figure 5. Viral load, as determined by RNA copy numbers, in organs harvested from birds experimentally infected with different West Nile virus strains and euthanized on day 14. NY99: 2 birds euthanized; Greece-10: 3 birds; FIN: 5 birds; Ita09: 2 birds; and 578/10: 3 birds. Viral titers are represented as geometric means. A detection limit of 0.95 $\log_{10}$ RNA copies/g of tissue was determined. Bars represent standard deviations (SD) of the mean.

Overall, average viral titers in the organs of the survivor birds were lower (2.7 $\log_{10}$ RNA copies/g of tissue) compared to the birds necropsied on day 4 (5.8 $\log_{10}$) or after illness/death (5.9 $\log_{10}$), with most virus persisting in the kidney and spleen (4.1 and 3.7 $\log_{10}$ RNA copies/g of tissue, respectively), followed by the brain and heart (3.0 and 2.3 $\log_{10}$ RNA copies/g of tissue, respectively), and the liver and bone marrow (1.7 and 1.6 $\log_{10}$ RNA copies/g of tissue, respectively).

Immunohistochemistry

Sections of organs of birds euthanized on day 4 p.i. were stained with polyclonal anti-WNV NS3 to determine replication of the virus in these tissues and rule out any positive qRT-PCR detection as a result of spillover from blood (Table 3). Tissues most consistently positive for WNV antigen were the kidney (100%), followed by the heart, liver, spleen (90%) and bone marrow (80%), with the brain only positive in 50% of the cases. Antigen was most abundant in the spleen, followed by the kidney, bone marrow, heart and liver (Figure S1). The abundance of viral antigen found in the positive brains was minimal. Overall, organs of birds most often positive, as well as most abundant for viral antigen, were those infected with Greece-10 and 578/10, followed by FIN and Ita09, with the lowest amount of antigen found in the organs of the two NY99-infected birds.

Viral antigen appeared to be slightly more abundant in birds euthanized due to morbidity (Table 4) as compared to the birds euthanized on day 4, with the spleen and kidney consistently positive for viral antigen (100%), followed by the heart and brain (91%), while the bone marrow (64%) and liver (45%) were the least often positive. In terms of antigen abundance, staining was most prominent in the heart, kidney and spleen, while lower amounts of antigen were present in the brain, bone marrow and liver (Figure S1). Between the different virus strains, the abundance of viral antigen in the organs was very similar in Greece-10, 578/10 and Ita09-infected birds, and was also higher than the amount of antigen found in the organs of NY99-infected birds.

Virus strain	Bird no.	Heart	Liver	Spleen	Kidney	Bone marrow	Brain	Total score per bird	Average score per virus strain	No. of positive organs/bird
NY99	6	+/-	+/-	+/-	+/-	+	+/-	7	6.5	6/6
	7	+	+/-	-	+	-	+/-	6		4/6
Greece-10	1	++	-	++	+	++	+/-	12	12.5	5/6
	4	+	+	++	+	++	+/-	13		6/6
FIN	6	+/-	+/-	+	+	+/-	+/-	8	7	6/6
	7	-	+/-	+	+	+/-	-	6		4/6
Ita09	1	+/-	+	+	+	-	-	7	8.5	4/6
	2	+	+	+	+	+	-	10		5/6
578/10	6	+	+	++	+	+	-	11	10.5	5/6
	7	+/-	+	++	+	+	-	10		5/6
Score per organ		15	14	21	19	16	5			
No. of positive birds/organ		9/10	9/10	9/10	10/10	8/10	5/10			

Subjective determinations of the amount of antigen in each organ were made; negative (-), minimal (+/-), moderate (+) or abundant (++). Each determination was given a score from 0 to 3; negative (0), minimal (1), moderate (2) and abundant (3).

Table 3. Immunohistochemical analysis of West Nile virus antigen distribution in European jackdaws euthanized on day 4 p.i.

Virus strain	Bird no.	Heart	Liver	Spleen	Kidney	Bone marrow	Brain	Total score per bird	Average score per virus strain	No. of positive organs/bird
NY99	1	-	+/-	+	+/-	+	-	6		4/6
	3	+	-	+	+	-	+	8	7.7	4/6
	5	++	-	+	+	-	+	9		4/6
Greece-10	5	+	-	+/-	+	-	+	7		4/6
	6	+	++	++	++	++	+/-	15	10.7	6/6
	8	++	-	+	+	+/-	+	10		5/6
Ita09	3	+	+	+	++	+	++	14		6/6
	4	+	+/-	+/-	+	+/-	+/-	8	10	6/6
	5	+	-	+	++	-	+/-	8		4/6
578/10	4	+	+/-	+	+	+/-	++	11	10.5	6/6
	5	++	-	+	+	+/-	+	10		5/6
Score per organ		13	4.5	11	13.5	6	10			
No. of positive birds/organ		10/11	5/11	11/11	11/11	7/11	10/11			

Subjective determinations of the amount of antigen in each organ were made; negative (-), minimal (+/-), moderate (+) or abundant (++). Each determination was given a score from 0 to 3; negative (0), minimal (1), moderate (2) and abundant (3).

Table 4. Immunohistochemical analysis of West Nile virus antigen distribution in European jackdaws euthanized due to morbidity (days 5-9 p.i.).

DISCUSSION

In this study, European jackdaws appear to be susceptible to infection with European strains of WNV from both lineage 1 and 2, as well as the North American strain NY99. However, the same extent of susceptibility as shown by the American crow upon infection with NY99, with 100% mortality rates and production of high viremia titers reaching peaks over 10^7 PFU/mL [22, 27-30], was not observed in this study. Herein, mortality rates ranged between 40-60% for four out of five strains and the highest average peak viremia was approximately 6.2 $\log_{10}$ RNA copies/mL of serum, reached by birds inoculated with NY99.

Viral RNA was detected in all organs on day 4 p.i. and upon the time of euthanasia due to morbidity. Viral RNA titers were observed to be significantly higher on day 4 p.i. in the organs of birds inoculated with Greece-10 compared to NY99, FIN and Ita09, and in the organs of birds inoculated with 578/10 compared to FIN. Even though at this time point spillover of virus from blood most likely resulted in higher viral RNA titers in the organs, immunohistochemistry confirmed that at least the relative differences were the same, as the amount of viral antigen staining confirmed that the two Greece-10- and 578/10-inoculated birds did indeed have the most viral antigen present in their organs as a whole, suggesting that spillover of virus from the circulation did not generally influence these results. Interestingly, Greece-10 and 578/10 are both lineage 2 viruses with 99% identity, possibly indicating a strain-related tropism. Nonetheless, by the time the birds were euthanized due to morbidity (between days 5 to 9), any virus-related significant differences were no longer observed in terms of viral RNA titers, although IHC staining suggested a tendency for viral antigen to be more abundant in organs of Greece-10, 578/10 and Ita09-infected birds.

In a previous study [31], mice inoculated with WNV-FIN displayed a slightly reduced mortality in comparison to the other WNV strains (also used here). In this study, however, the virulence phenotype of FIN has become much more pronounced, as birds inoculated with FIN suffered no mortality and had significantly lower peak viremia titers as well as delayed onset of viremia compared to the other groups. Even though viral virulence generally correlates well with high and prolonged viremia [32], in this case, viremia was not significantly shorter in FIN-infected birds compared to the other virus strains, although onset of viremia did occur significantly later.

Interestingly, the two birds inoculated with FIN and euthanized on day 4 were both positive for virus in the majority of their organs (qRT-PCR and IHC) despite the absence of mortality seen in this group. This, in combination with the presence of viremia observed in all birds of this group, suggests that even in birds that are not susceptible to lethal WNV infection, the virus is able to elicit viremia and disseminate to the organs, including the brain. A similar observation was demonstrated in chickens, which experienced no mortality following infection with WNV, but exhibited titers as high as 5 $\log_{10}$ PFU/mL and virus could also be isolated from several organs, including the spleen and kidney [33].

The introduction of a T249P amino acid substitution (present in North American WNV) in the NS3 helicase of a low virulence strain of WNV has been demonstrated to result in increased virulence in American crows [29]. The virus strains used in this study all contain a proline at this site, with the exception of FIN, which contains a threonine at this position [31] (Table S1). Ita09, which is 99.7% identical to the nucleotide sequence of FIN, did not display the same attenuation in jackdaws as FIN. It is therefore very likely that the attenuated phenotype of FIN demonstrated in this study is likely to be the result of this P249T substitution. Studies are ongoing to test the relevance of the T249P substitution present in the Italian backbone of WNV in both European and American corvids.

It has been shown that a blood titer of 10^6 PFU/mL is required for transmission to feeding mosquitoes [34]. Additionally, viremic titers greater than 10^5 PFU/mL were considered infectious for *Culex pipiens* [35] and *Culex quinquefasciatus* [36]. This suggests that only the NY99-, Greece-10- and Ita09-infected birds that reached peak viremia RNA titers above $10^{7.5}$ RNA copies/mL (which resulted in $10^{5.3}$ $TCID_{50}$/mL upon virus isolation) would be likely to infect feeding mosquitoes, as the lowest cut-off of $10^{5.0}$ PFU/mL will result in approx. $10^{5.2}$ $TCID_{50}$/mL, according to a conversion factor of 1 $TCID_{50}$ to 0.7 PFU [37]. It is possible that the infectious viremia titers of the other samples were below the detection limit of the assay. As the majority of the birds did not attain viremia titers above $10^{7.5}$ RNA copies/mL, it suggests that jackdaws would most likely not serve as efficient amplifying hosts in the transmission cycle of WNV in Europe. However, it is also possible that a higher peak of viremia was missed due to sampling on intervening days. Similar results were observed in a study using a French strain (Fr2000) in Carrion crows [38], where the highest viral RNA titer recorded was equivalent to only 10^4 $TCID_{50}$/mL, which is also below the infectious viral titer required for transmission to feeding mosquitoes.

None of the isolates used in this study have either caused massive die-offs of birds in the field or have spread swiftly across Europe as observed during the North American invasion by WNV [39]. Thus far, experimental studies using European strains of WNV and European birds have shown fairly low mortality rates [38, 40, 41], with the highest mortality rate of 33% observed following inoculation of Carrion crows with the Fr2000 strain [38]. The study presented herein suggests that the susceptibility of European birds could be related to the WNV strain, as much higher mortality was observed in this study with alternative strains compared to the French strain. At the same time, host factors present in the jackdaw or other European avian species could also play an important role in susceptibility, as the mortality induced by the North American strain NY99, known to be highly virulent in corvids, was similar to that exhibited by Ita09 (60%) in jackdaws. This mortality rate, as well as the viremia levels, were also not comparable to those usually seen upon infection of American crows with NY99. This therefore suggests that host factors present in European jackdaws could potentially reduce their susceptibility to WNV.

However, a substantial proportion of the jackdaws did die as a result of infection, which therefore suggests that perhaps some birds in Europe are indeed succumbing to WNV infection in the field, but that the mortality rates of these birds are simply too low to

be detected by the current monitoring systems present in Europe. It is also possible that natural infection via mosquito feeding could result in higher serum viremia titers in jackdaws, or that other ecological factors such as infection due to carcass scavenging by more susceptible birds could play a more important role in the transmission and maintenance of WNV in Europe. Nevertheless, this study shows that jackdaw mortality could potentially be useful for tracking WNV transmission in Europe.

ACKNOWLEDGEMENTS

We thank Vittorio Sambri, Luisa Barzón, Giorgio Palù and Tamás Bakonyi for providing the low-passage isolates used in this study. We would also like to thank Tanja Schouten and Angela Gomersbach for their excellent technical assistance. We thank Jeroen Roose and Peter van Run for their technical assistance with the immunohistochemistry, and Thijs Kuiken for his assistance with the analysis of the histological stainings.

The research leading to these results has received complete funding from the European Community's Seventh Framework Programme (FP7/2007 - 2013) under the project "VECTORIE", EC grant agreement number 261466. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Permission for trapping European jackdaws was obtained from the Ministry of Agriculture (registered under number FF/75A/2011/031). Experimental inoculations were performed under protocol number 122-12-12 with permission obtained from the Animal Ethics Committee of Erasmus Medical Centre. All efforts were made to minimize animal suffering.

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Chapter 6

Susceptibility of Carrion Crows to Experimental Infection with Lineage 1 and 2 West Nile Viruses

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Emerg Infect Dis.2015 Aug;21(8):1357-65

ABSTRACT

West Nile virus (WNV) outbreaks in North America have been characterized by substantial die-offs of American crows (*Corvus brachyrhynchos*). In contrast, a low incidence of bird deaths has been observed during WNV epidemic activity in Europe. To examine the susceptibility of the western European counterpart of American crows, we inoculated carrion crows (*Corvus corone*) with WNV strains isolated in Greece (Gr-10), Italy (FIN and Ita09), and Hungary (578/10) and with the highly virulent North American genotype strain (NY99). We also inoculated American crows with a selection of these strains to examine the strains' virulence in a highly susceptible bird species. Infection with all strains, except WNV FIN, resulted in high rates of death and high-level viremia in both bird species and virus dissemination to several organs. These results suggest that carrion crows are highly susceptible to WNV and may potentially be useful as part of dead bird surveillance for early warning of WNV activity in Europe.

INTRODUCTION

West Nile virus (WNV), a flavivirus (family *Flaviviridae*) transmitted by mosquitoes, uses birds as its primary vertebrate reservoir host. WNV has an extensive geographic range that includes Europe, Africa, the Middle East, southern Asia, and Australia [1]. In 1999, WNV emerged in North America, where it was first detected in New York, New York. The virus subsequently spread rapidly across the continent, becoming the leading cause of arboviral encephalitis in humans and horses [2], and it was associated with deaths among at least 326 bird species [3]. High death rates are most frequently observed among passeriform birds, of which the family *Corvidae* comprises the most highly susceptible species to WNV [4]. In particular, deaths among the American crow (*Corvus brachyrhynchos*) have been used to track the spread of the virus across many parts of North America [5-8].

Since 2008, WNV has been responsible for outbreaks throughout central and southeastern Europe, affecting countries such as Greece, Italy, Hungary, Romania, and Croatia and constituting a serious veterinary and public health problem. Fatalities have been reported among wild birds in Europe, such as eagles [9, 10], sparrow hawks, goshawks, geese, and falcons [11-13]. However, death rates among birds in Europe have been low, and no clustered death events have occurred, even when cases were associated with outbreaks of severe human and equine WNV infections [14-17]. Several theories have been proposed to explain the low death rates among birds in Europe: limited or insufficient monitoring of deaths among wild birds in Europe; development of immunity among birds from infections acquired on wintering grounds [18]; and circulation of WNV strains in Europe with reduced virulence for birds.

Experimental infection of American crows with the North American genotype of WNV (NY99) has shown that the strain has a highly pathogenic phenotype: viremia titers exceeded 9 $\log_{10}$ PFU/mL, and all infected birds died [19-23]. However, the lack of WNV-associated bird deaths in Europe suggests that European birds might not be susceptible to WNV or that WNV strains from Europe are not virulent to birds. Thus, we evaluated the susceptibility of the European equivalent of the American crow, carrion crows (*Corvus corone*), which are ubiquitously present across Europe, by injecting them with selected strains of WNV circulating in Europe and with the prototypic NY99 strain. In addition, we inoculated American crows with a selection of these viruses to assess and compare the virulence of WNV strains from Europe in a bird species known to be highly susceptible to WNV. Susceptibility was assessed in terms of death, survival time, magnitude and duration of viremia, and spread of virus to different organs.

MATERIALS AND METHODS

Source of Virus and Birds

Five different WNV strains were used in this study: lineage 1a strain NY99-4132 (NY-99) [20]; lineage 2 strain Nea Santa-Greece-2010 (Greece-10; GenBank accession no. HQ537483.1) [24]; lineage 1a strain Italy/2009/FIN (FIN; GenBank accession no. KF234080); lineage 1a strain Ita09 (GenBank accession no. GU011992.2) [25]; and lineage 2 strain 578/10 (GenBank accession no. KC496015). Further details about these viruses are provided in Table 1.

Carrion crows were captured by using walk-in traps in the municipality of Rotterdam, the Netherlands, and then transported to indoor housing at the animal holding facilities at the National Institute for Public Health and the Environment, Bilthoven, the Netherlands. After being inoculated with WNV, the crows were cared for in groups of 8 in isolators under negative pressure. Only seronegative birds were used in this study. Seronegativity was determined by using a neutralization assay (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/8/14-0714-Techapp1.pdf).

American crows were captured by using cannon net traps in Bellvue, Colorado, USA; the National Wildlife Diseases Program, Animal and Plant Health Inspection Service, United States Department of Agriculture, assisted with the captures. The crows were banded and transported to Fort Collins, Colorado, where they were housed in 1-m^3 cages (2 birds per cage) at the Colorado State University Animal Disease Laboratory.

Strain	Source	Passage history*	Location	Genetic lineage	Crow species inoculated
NY99-4132	American crow (brain)	V2	United States	1a	Carrion, American
Nea Santa-Greece-2010	*Culex pipiens* mosquito	V1	Greece	2	Carrion
Italy/2009/FIN	Human with neuroinvasive disease	V2, C1	Italy	1a	Carrion, American
Ita09	Human with neuroinvasive disease	V1, C1	Italy	1a	Carrion, American
578/10	Horse (brain)	V2, C1	Hungary	2	Carrion

*Viruses were propagated in Vero (V) or C6/36 insect cells (C). Numbers following passage source represent the number of virus passages.

Table 1. West Nile virus strains used for susceptibility studies in carrion and American crows.

Experimental Infection and Sampling Protocol

Crows were subcutaneously inoculated in the thigh or breast region with 2,000 50% tissue culture infectious doses ($TCID_{50}$) of virus per 0.1 mL of serum-free Dulbecco's Modified Eagle Medium (DMEM) (Lonza Benelux BV, Breda, the Netherlands). Carrion crows (8 per virus) were injected with WNV strain NY99, Greece-10, FIN, Ita09, or 578/10. American crows were inoculated with NY99 (n = 6), FIN (n = 5), or Ita09 (n = 5). Approximately 0.1 mL of blood was collected from carrion crows at 2-day intervals, up to 8 days postinoculation (dpi), and 0.2 mL of blood was collected from American crows at the same time points and added to 0.9 mL of serum-free DMEM. Coagulated blood from carrion crows was centrifuged at 1,300 × *g* for 5 min in MiniCollect vials (Greiner Bio-One, Alphen aan den Rijn, the Netherlands) to separate serum, and coagulated blood from American crows was centrifuged at 3,700 × *g* for 10 min to pellet clotted cells. Serum samples were stored at −80°C until further use.

Crows were examined for clinical signs twice daily for 14 dpi and euthanized under isoflurane anesthesia upon display of clinical signs. In addition, 2 birds per group of the carrion crows were euthanized at 4 dpi.

Necropsies were performed on all euthanized carrion crows; heart, liver, spleen, kidney, bone marrow, and brain samples were collected. A small section of each tissue was collected, weighed, and homogenized by using a metal bead in 1 mL of DMEM containing 100 U penicillin and 100 μg/mL streptomycin. The remaining portion of the tissues was collected in formalin for use in immunohistochemical staining.

Determination of Virus Loads

We used quantitative real-time reverse transcription PCR (qRT-PCR) to determine virus titers in serum and tissue samples and $TCID_{50}$ titration to calculate infectious virus titers in serum only. In brief, RNA was isolated from 50 μL of serum or 100 μL of homogenized tissue by using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Almere, the Netherlands) and a MagNA Pure LC automated nucleic acid robotic workstation (Roche) according to the manufacturer's instructions, and subsequently stored at -80°C. RNA copy numbers were quantified by using unmodified primers as previously described [26]. The limit of detection of the assay was 9 (1.0 $\log_{10}$) RNA copies.

After $\log_{10}$ titration of serum samples on Vero E6 cells, cytopathic effect was determined at 5 dpi and $TCID_{50}$ infectious titers were calculated by using the Spearman–Kärber method [27, 28]. An initial 1:10 dilution of serum resulted in a limit of detection of $10^{1.8}$ $TCID_{50}$/mL.

Immunohistochemistry

Paraffin sections (4-µm thick) of sagittal organ were processed for streptavidin–biotin–peroxidase immunohistochemical detection of nonstructural protein (NS) 3 antigen. Sections were deparaffinized in xylene, rehydrated in descending concentrations of ethanol, and incubated for 10 min in 3% H_2O_2 diluted in PBS to block endogenous peroxidase activity. Antigen exposure was performed by incubation at 121°C for 15 min in citrate buffer (10 mmol/L, pH 6.0). Sections were subsequently incubated overnight at 4°C with polyclonal goat anti-WNV NS3 protease (1:100; R&D Systems, Abingdon, UK) or isotype control (goat serum, 1:100; Dako, Eindhoven, the Netherlands) and then detected with polyclonal rabbit anti-goat IgG/HRP (Dako) antibody. Sections were counterstained with Mayer hematoxylin, mounted with Kaiser glycerin-gelatin, and analyzed by using a light microscope.

Statistical Analyses

Survival curves were analyzed by using the log-rank (Mantel-Cox) test. Statistical analyses between >2 groups were performed by using Kruskal-Wallis 1-way analysis of variance; any significant differences were more closely analyzed between the groups by using the Mann-Whitney U test. A Bonferroni correction was applied to each p value, according to the number of comparisons (corrected p value of 0.05/10 = 0.005 for carrion crow peak viremia and organs of carrion crows euthanized on day 4; corrected p value of 0.05/6 = 0.008 for American crow peak viremia and organs of carrion crows euthanized due to illness). For all comparisons, each group had 6 crows, except for American crow groups that received FIN or Ita09 (n = 5).

RESULTS

Illness and Death

Signs of illness (e.g., lethargy, unresponsiveness, anorexia, and ruffled feathers) were observed among most crows within 9 dpi. All 6 carrion crows inoculated with Greece-10 or Ita09 died, and 5 (83%) of the 6 inoculated with NY99 or 578/10 died. All 6 carrion crows inoculated with strain FIN survived (Table 2). Survival curves of the infected birds showed a significant difference in survival between carrion crows infected with Ita09, Greece-10, NY99, or 578/10 and those infected with FIN (p = 0.005) (Figure 1). The median day of death was 7 dpi for carrion crows that died from infection with NY99, Greece-10, or Ita09 and 8 dpi for birds that died from infection with 578/10. All American crows inoculated with NY99 (n = 6) or Ita09 (n = 5) died, and all 5 crows inoculated with FIN survived (Table 3).

Virus group	No. died/no. total (%)	Day of death, median dpi	Median peak viremia, viral RNA/mL serum (range); no. birds	Mean duration, d, of viremia ± SD; no. birds†	Mean day of peak viremia ± SD; no. birds†	Median peak viremia $TCID_{50}$/mL (range); no. birds‡
NY99-4132	5/6 (83)	7	$10^{8.7}$ ($10^{1.0}$–$10^{10.0}$); 6	5.2 ± 1.0; 5	4.3 ± 0.7; 5	$10^{7.4}$ ($10^{1.8}$–$10^{8.8}$); 6
Nea Santa-Greece-2010	6/6 (100)	7	$10^{10.3}$ ($10^{9.8}$–$10^{11.7}$); 6	5.7 ± 0.7; 6	4.5 ± 0.9; 6	$10^{7.8}$ ($10^{7.3}$–$10^{9.8}$); 6
Italy/2009/FIN	0/6 (0)	NA	$10^{2.7}$ ($10^{1.0}$–$10^{5.9}$); 6	2.7 ± 0.9; 3	6.7 ± 0.9; 3	$10^{1.8}$ ($10^{1.8}$–$10^{2.5}$); 6
Ita09	6/6 (100)	7	$10^{9.7}$ ($10^{8.0}$–$10^{10.0}$); 6	6.0 ± 1.2; 6	4.3 ± 0.7; 6	$10^{7.6}$ ($10^{6.3}$–$10^{8.8}$); 6
578/10	5/6 (83)	8	$10^{8.4}$ ($10^{6.0}$–$10^{10.1}$); 6	5.7 ± 1.8; 6	3.5 ± 0.9; 6	$10^{5.1}$ ($10^{2.8}$–$10^{8.5}$); 6

*dpi, days postinoculation; NA, not applicable; $TCID_{50}$, 50% tissue culture infectious dose.
†Based on viral RNA titers.
‡Viral titers are expressed as $\log_{10}$ $TCID_{50}$/mL of serum.

Table 2. Clinical profile for carrion crows experimentally infected with various West Nile virus strains.

Virus group	No. died/no. total (%)	Median peak viremia, viral RNA/mL serum (range); no. birds	Median peak viremia, $TCID_{50}$/mL serum (range); no. birds†
NY99-4132	6/6 (100)	$10^{9.6}$ ($10^{9.1}$–$10^{10.1}$); 6	$10^{7.2}$ ($10^{4.7}$–$10^{7.2}$); 6
Italy/2009/FIN	0/5 (0)	$10^{1.0}$ ($10^{1.0}$–$10^{6.9}$); 5	$10^{1.8}$ ($10^{1.8}$–$10^{2.7}$); 5
Ita09	5/5 (100)	$10^{8.8}$ ($10^{8.0}$–$10^{9.1}$); 5	$10^{6.7}$ ($10^{6.0}$–$10^{7.5}$); 5

†Virus titers are expressed as $\log_{10}$ 50% tissue culture infectious dose ($TCID_{50}$)/mL of serum.

Table 3. Clinical profile of American crows experimentally infected with West Nile virus strains NY99-4132, Italy/2009/FIN, and Ita09.

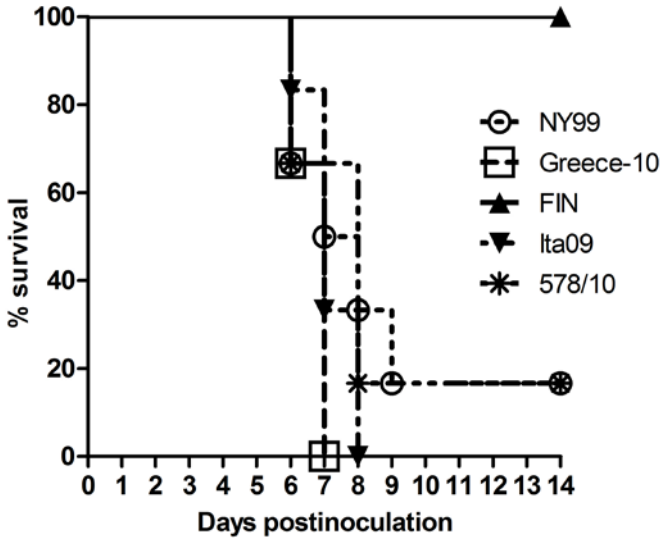

Figure 1. Survival rate for West Nile virus (WNV)–infected carrion crows after inoculation with 2,000 50% tissue culture infectious doses of WNV; each group (n = 6) was inoculated with a different strain. Crows were monitored daily for signs of disease through postinoculation day 14.

Viremia Profiles

WNV viremia profiles were determined in terms of viral RNA (Table 2; Figure 2) and infectious virus titers in serum (Table 2; Figure 3) of infected carrion crows. In strain NY99-infected birds, the median peak viral RNA titer was $10^{8.7}$ RNA copies/mL of serum (range 10^{1}–$10^{10.0}$ [nontransformed values]), and the median peak infectious virus titer was $10^{7.4}$ $TCID_{50}$/mL of serum (range $10^{1.8}$–$10^{8.8}$); these values include 1 bird in which detectable viremia did not develop during the entire course of infection. The median peak viremia titer for Greece-10–infected birds was $10^{10.3}$ RNA copies/mL of serum (range $10^{9.8}$–$10^{11.7}$) and $10^{7.8}$ $TCID_{50}$/mL of serum (range $10^{7.3}$–$10^{9.8}$). FIN-infected birds had median peak viremia titers of $10^{2.7}$ RNA copies/mL of serum (range 10^{1}–$10^{5.9}$) and $10^{1.8}$ $TCID_{50}$/mL of serum (range $10^{1.8}$–$10^{2.5}$); however, viremia was detectable in only 3 of 6 birds, and infectious virus could be isolated from only 1 bird. The median peak viremia titers for Ita09-infected birds were $10^{9.7}$ RNA copies/mL of serum (range $10^{8.0}$–$10^{10.0}$) and $10^{7.6}$ $TCID_{50}$/mL of serum (range $10^{6.3}$–$10^{8.8}$). Birds infected with strain 578/10 had median peak viremia titers of $10^{8.4}$ RNA copies/mL of serum (range $10^{6.0}$–$10^{10.1}$) and $10^{5.1}$ $TCID_{50}$/mL of serum (range $10^{2.8}$–$10^{8.5}$).

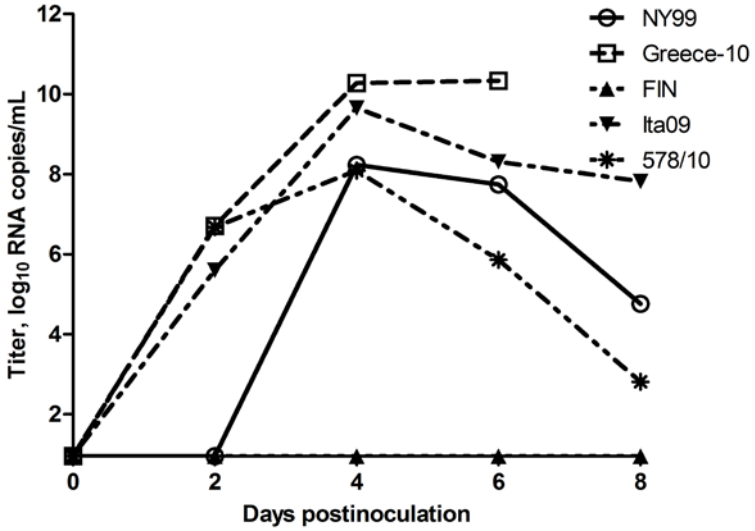

Figure 2. Viral RNA copy numbers for West Nile virus (WNV)–infected carrion crows after inoculation with 2,000 50% tissue culture infectious doses of WNV; each group (n = 6) was inoculated with a different strain. RNA copy numbers are represented as log-transformed medians. The assay had a detection limit of 9 (1.0 $\log_{10}$) RNA copies/mL of serum.

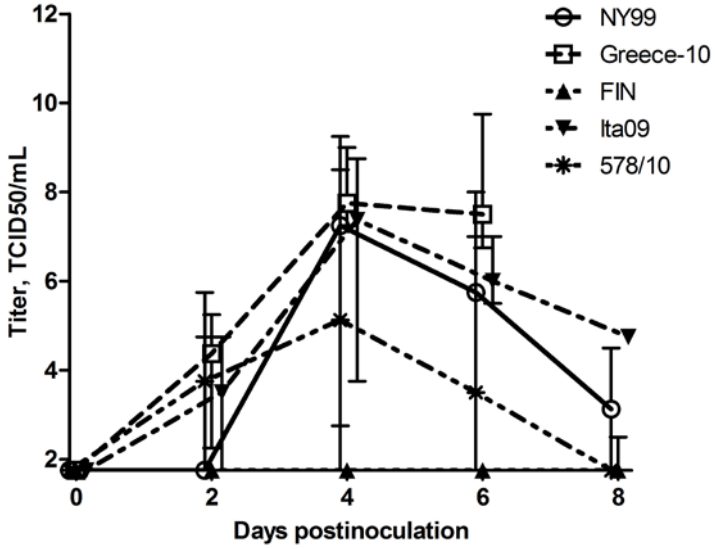

Figure 3. Infectious virus titer profiles for West Nile virus (WNV)–infected carrion crows after inoculation with 2,000 50% tissue culture infectious doses ($TCID_{50}$) of WNV; each group (n = 6) was inoculated with a different strain. Infectious virus titers were determined by $TCID_{50}$ titration and are represented as log-transformed medians; error bars indicate range. The assay had a detection limit of 1.8 $TCID_{50}$/mL.

Strain Greece-10–infected birds had median peak viral RNA titers significantly higher than those for NY99-infected (p = 0.004), FIN-infected (p = 0.005), and 578/10-infected (p = 0.004) birds. Greece-10–infected birds also had median infectious virus titers significantly higher than those for FIN-infected birds (p = 0.003), but FIN-infected birds had RNA and infectious titers lower than those for Greece-10–infected (p = 0.005 and 0.003, respectively), Ita09-infected (p = 0.005 and 0.002, respectively), and 578/10-infected (p = 0.005 and 0.002, respectively) crows.

Viremia profiles were also determined for American crows infected with 3 of the 5 different WNV strains (Table 3). NY99-infected birds had median peak viremia titers of $10^{9.6}$ RNA copies/mL of serum (range $10^{9.1}$–$10^{10.1}$) and $10^{7.2}$ $TCID_{50}$/mL of serum (range $10^{4.7}$–$10^{7.2}$). Detectable viremia developed in only 2 of the 5 FIN-infected birds, resulting in median peak viremia titers of $10^{1.0}$ RNA copies/mL of serum (range 10^{1}–$10^{6.9}$) and $10^{1.8}$ $TCID_{50}$/mL of serum (range $10^{1.8}$–$10^{2.7}$). Median peak viremia titers for Ita09-infected birds were $10^{8.8}$ RNA copies/mL of serum (range $10^{8.0}$–$10^{9.1}$) and $10^{6.7}$ $TCID_{50}$/mL of serum (range $10^{6.0}$-$10^{7.5}$). American crows infected with strain NY99 had the highest median peak viral RNA and infectious virus titers, and FIN-infected birds had the lowest median titers (significant only when compared with each other: p = 0.008 and 0.006, respectively).

Tissue Tropism

Virus loads were determined in the heart, liver, spleen, kidney, bone marrow, and brain of all birds. To assess the spread of virus to the different organs at the approximate peak of viremia, we euthanized 2 birds per group at 4 dpi. Virus was detected in all organs from these birds. On average, the highest viral RNA titers were detected in the liver, followed by the bone marrow, spleen, kidney, and heart; the lowest titers were found in the brain (Figure 4). Between the different virus strains, viral RNA titers were the highest in the organs of birds infected with strain Greece-10 or 578/10, followed by NY99 and Ita09; titers were significantly higher than those for birds infected with strain FIN (p = 0.005 for all). Virus distribution in FIN-infected birds was not consistent; viral RNA was undetectable in the bone marrow and brain of both birds tested on 4 dpi, and for 1 of these birds, viral RNA was also undetectable in the spleen.

Birds euthanized because of illness had virus present in all organs; in most cases, the spleen, liver, and bone marrow contained the highest average viral RNA load, followed by kidney and heart; the lowest average viral RNA titers were in the brain. Viral RNA titers in organs of Greece-10–infected birds were higher than those in organs of birds infected with the other viruses, but this observation was not statistically significant (Figure 5).

The 1 NY99-infected and 3 FIN-infected survivor birds that were free of viremia throughout the 8 days of blood sampling underwent necropsy at 14 dpi. Of interest, virus was present in all organs of the NY99-infected bird (median virus load of $10^{3.1}$ RNA copies/g of tissue) and in at least 3 of the 6 organs from FIN-infected birds (median virus

load of $10^{2.0}$ RNA copies/g of tissue), showing that these birds did undergo productive WNV infection.

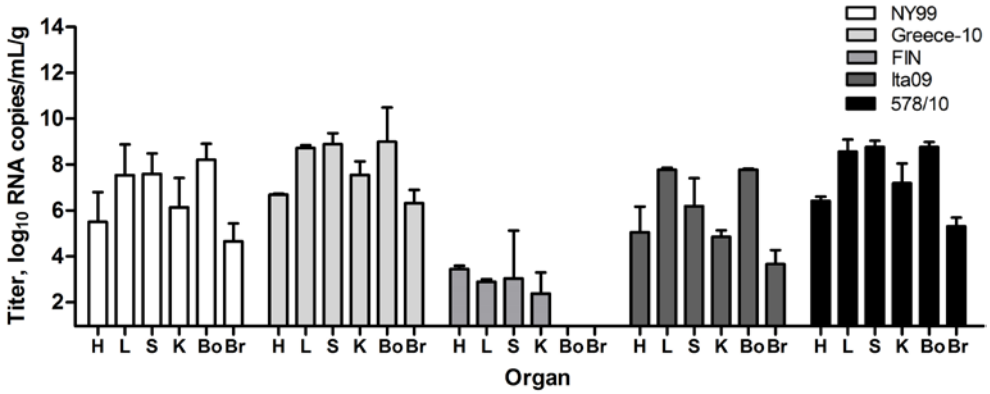

Figure 4. Viral RNA copy numbers in organs from 10 carrion crows (2 per group) euthanized 4 days after being experimentally infected with 1 of 5 different West Nile virus strains (n = 6, per group). Virus titers are represented as log-transformed medians; error bars indicate range. The assay had a detection limit of 9 (1.0 $\log_{10}$) RNA copies/g of tissue. H, heart; L, liver; S, spleen; K, kidney; Bo, bone marrow; Br, brain.

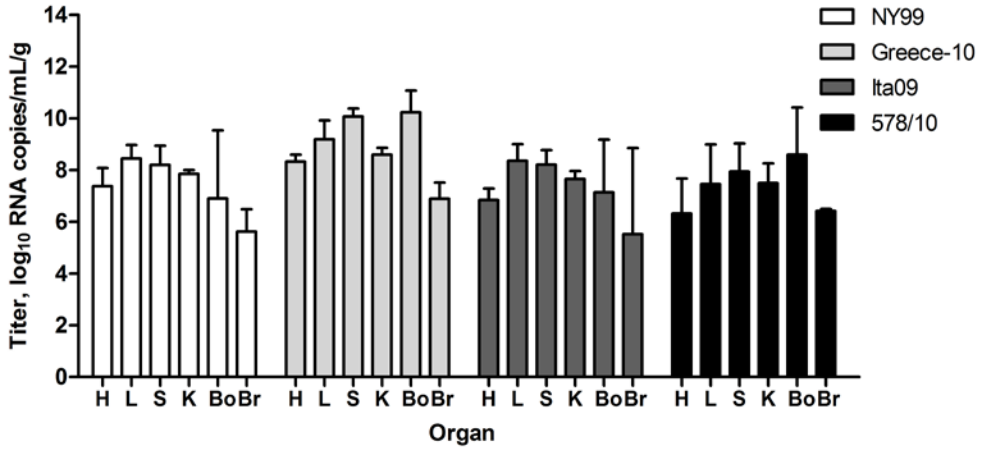

Figure 5. Viral RNA copy numbers in organs from 22 carrion crows euthanized because of illness after being experimentally infected with 1 of 4 different West Nile virus strains (n = 6, per group). Copy numbers are represented as log-transformed medians; error bars indicate range. The assay had a detection limit of 9 (1.0 $\log_{10}$) RNA copies/g of tissue. H, heart; L, liver; S, spleen; K, kidney; Bo, bone marrow; Br, brain.

Immunohistochemistry

Sections of organs from 2 birds necropsied at 4 dpi were stained with polyclonal anti-WNV NS3 to confirm replication of virus in the tissues and to exclude positive qRT-PCR detection due to spillover from blood at the approximate peak of viremia. Tissues most consistently positive for WNV antigen were the liver (80%), kidney (80%), bone marrow (80%), and spleen (78%); tissues least consistently positive for WNV antigen were heart (50%) and brain (10%) (Table 4). However, in terms of virus load, antigen was most abundant in the liver, bone marrow, and spleen. Overall, at 4 dpi, organs of birds most positive and most abundant for viral antigen were those infected by strains 578/10 and Greece-10, followed by NY99 and Ita09. The organs of FIN-infected birds were all negative for virus antigen at this time point.

Virus strain, bird no.	Heart	Liver	Spleen	Kidney	Bone marrow	Brain	Total score per bird	Average score per virus strain	No. positive organs/total no. organs
NY99-4132								9.0	
1	–	++	++	+	++	–	11.0		4/6
7	–	+	+	+/–	+	–	7.0		4/6
Nea Santa-Greece-2010								12.5	
1	+/–	++	++	+	++	+/–	13.0		6/6
7	+/–	++	++	+	++	–	12.0		5/6
Italy/2009/FIN								0	
1	–	–	–	–	–	–	0		0/6
7	–	–	–	–	–	–	0		0/6
Ita09								8.0	
1	+/–	++	++	+/–	+	–	10.0		5/6
7	–	++	ND	+/–	+	–	6.0		3/5
578/10								12.5	
1	++	++	++	+/–	++	–	13.0		5/6
7	+/–	++	++	+	++	–	12.0		5/6
Score per organ	7.0	23.0	20.0	12.0	21.0	1.0			
No. positive birds/total no. birds	5/10	8/10	7/9	8/10	8/10	1/10			

*Subjective determinations of the amount of antigen in each organ were made: negative (–), minimal (+/–), moderate (+), or abundant (++). Each determination was given a score from 0 to 3: negative (0), minimal (1), moderate (2), and abundant (3). ND = not determined. dpi, days postinoculation; ND, not determined.

Table 4. Immunohistochemical analysis of West Nile virus antigen distribution in experimentally infected carrion crows euthanized at 4 dpi*.

DISCUSSION

In this study, we assessed the susceptibility of carrion crows to different strains of WNV. First we demonstrated that carrion crows are susceptible to WNV infection by using the North American strain NY99, which has previously been shown to be highly virulent in American crows [19-23]. In agreement with the findings in those studies, our results showed that infection of carrion crows with NY99 resulted in high viremia titers and death. In addition, virus had disseminated to the organs of infected birds by 4 dpi, further demonstrating the susceptibility of carrion crows to WNV infection, which appears to be very similar to that of American crows.

Next we studied the susceptibility of carrion crows to selected strains of WNV from Europe. We found that carrion crows are highly susceptible to infection with both lineage 1 and 2 WNV strains from Europe. In addition, we showed that susceptibility is strain-dependent. Of the 5 WNV strains tested, 4 led to death for 83%–100% of infected birds and to high viremia titers and abundant antigen in the organs of euthanized birds; however, birds inoculated with FIN did not die from infection, and they had relatively low virus titers in the blood and no viral antigen in the organs at 4 dpi. A previous study describing the inoculation of carrion crows with WNV strains from France (Fr2000) and Israel (Is98) also suggested that carrion crows are susceptible to infection with WNV in a strain-dependent manner [29]. The study showed death rates of 33% (Fr2000) and 100% (Is98) from the 2 strains, and viral RNA loads in serum, oral swab samples, and feathers of Is98-infected birds were higher than those of Fr2000-infected birds [29]. Thus, WNV strains FIN and Fr2000 show a similar attenuation in carrion crows.

To more accurately assess the virulence of WNV strains from Europe, we inoculated American crows, a bird species known to be highly susceptible to WNV, with 2 of the 4 strains from Europe (Ita09 and FIN) and with strain NY99 from North America. Similar to what was seen with carrion crows, American crows infected with Ita09 had high peak viremia titers, and all succumbed to the infection, whereas those infected with FIN had low viremia titers, and all survived infection. Furthermore, it was demonstrated that the Greece-10 strain used in this study was also 100% lethal in American crows (A.C. Brault et al., unpub. data). In fact, American crows infected with Greece-10 (vs. the other strains used in this study) had the highest median peak viremia titers in terms of RNA and infectious virus (data not shown). These results show that in American crows, WNV strains (apart from FIN) from Europe are as virulent as the prototypic NY99 strain from North America.

The fact that susceptibility of birds to WNV can be strain-dependent was clearly demonstrated by the attenuated virulence phenotype of WNV strain FIN in carrion and American crows (this study) and in European jackdaws [30]; FIN-infected crows consistently exhibited an absence of death, lower peak viremia titers, and less dissemination of virus to the organs at the approximate peak of viremia. A previous study showed that the introduction of a P249T amino acid substitution in the NS3 helicase of North American strain NY99 led to a highly attenuated phenotype, whereas a T249P substitution introduced

in a low-virulence WNV strain resulted in a phenotype highly virulent to American crows [22]. Four virus strains used in this study contain a proline at NS3-249, whereas FIN contains a threonine at this position [31]. It is therefore likely that the attenuated phenotype of FIN is a result of this threonine amino acid at NS3-249, a mutation that could be relevant for at least 3 different species of birds in the family *Corvidae*. Studies in North American and European corvids are ongoing in order to test the relevance of the T249P substitution and several other mutations when introduced into the genome of WNV-FIN.

We have shown that bird susceptibility to WNV can be strain-dependent. However, susceptibility is also clearly related to host factors. As a whole, jackdaws were less susceptible than the carrion crows to the same selection of otherwise highly virulent WNV strains, and they had lower death rates and virus loads in blood and organs [30]. Species susceptibility has been shown to differ within various avian families [7], including birds in the family *Corvidae*, of which, for example, the fish crow (*Corvus ossifragus*) was less susceptible to lethal WNV infection [23]. Although the reasons for this varied susceptibility are not well understood, potential contributing factors may include host traits, such as genetic composition, immune response, and physiologic mechanisms [23].

A measure of the potential for transmission of virus to feeding mosquitoes is the level of infectious virus titers produced during viremia. The median peak serum titer of infectious virus was highest in Greece-10–infected carrion crows and lowest in FIN-infected carrion crows. Studies have shown that WNV titers of $>10^5$ PFU/mL were considered infectious for *Culex pipiens* [32] and *Cx. quinquefasciatus* [33] mosquitoes. Considering this cutoff of 10^5 PFU/mL or of $10^{5.2}$ $TCID_{50}$/mL, according to a conversion factor of 1 $TCID_{50}$ to 0.7 PFU [34], infectious titers obtained for carrion crows infected with Greece-10, Ita09, or NY99 would be sufficient for efficient transmission of virus to feeding mosquitoes. Carrion crows infected with strain 578/10 had median peak viremia titers slightly below this threshold ($10^{5.1}$ $TCID_{50}$/mL; Table 2), suggesting that the carrion crow may not be an efficient amplifier for this WNV strain. However, a possible explanation for the apparent low viremia titers in 578/10-infected birds could be that blood sampling was conducted on alternate days, possibly missing higher peak viremia titers of infectious virus. For the American crows, median peak viremia titers for Ita09 (Table 3) were slightly lower than those for carrion crows (Table 2). However, serum samples from American crows underwent 2 repeated freeze–thaw cycles, which could have resulted in the detection of lower infectious virus titers. Nonetheless, these results show that WNV strains from Europe can produce viremia titers in American crows that could be sufficient for efficient transmission to feeding mosquitoes. Nevertheless, reservoir competence studies involving the feeding of European mosquitoes on viremic WNV-infected carrion crows are needed to determine whether the carrion crow could indeed be a potential reservoir host and contributor to the WNV transmission cycle.

We have shown that carrion crows, a species of bird ubiquitously found across Europe, are highly susceptible to WNV strains currently circulating in Europe. These birds could therefore potentially be useful as part of dead bird surveillance in the early detection

of WNV in Europe. Future studies assessing the susceptibility of the closely related hooded crow (*Corvus cornix*) to WNV may also prove to be insightful, as this is the more predominant corvid species in eastern and southeastern Europe, where WNV is more common. The susceptibility of European birds to WNV has been demonstrated in multiple studies [9, 10, 12, 13, 29, 30, 35–38], however, it is peculiar that the number of WNV-associated deaths among birds in Europe is not as extensive as that among birds in North America. Possible explanations may be a lower reporting of bird deaths in Europe as compared with that in the United States or that other ecologic factors, such as mosquito competence, abundance, distribution or behavior, exert a limiting effect on the transmission of WNV in Europe.

ACKNOWLEDGMENTS

We thank Vittorio Sambri, Luisa Barzón, Giorgio Palù, and Tamás Bakonyi for providing the low-passage isolates used in this study. We also thank Tanja Schouten and Angela Gomersbach for their excellent technical assistance; Jeroen Roose and Peter van Run for their technical assistance with the immunohistochemistry; and Thijs Kuiken for his assistance with the analysis of the histological stainings.

The research leading to these results has received complete funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under the project "VECTORIE," European Commission grant agreement number 261466. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Trapping of carrion crows was performed with the assistance of the Gemeentewerken Rotterdam under permission obtained from the Ministry of Agriculture (registered under no. FF/75A/2011/031). Experimental inoculations were performed under protocol number 122-12-12 with permission obtained from the Animal Ethics Committee of Erasmus Medical Centre. All efforts were made to minimize animal suffering. Trapping of American crows was performed with the assistance of Todd Felix under US Fish and Wildlife Scientific Collecting Permit number MB-91672A. Experimental inoculations of crows were performed under Colorado State University IACUC approval number 10-2078A.

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TECHNICAL APPENDIX

Detection of Preexisting West Nile Virus Antibodies

To confirm that Carrion crows had not previously been exposed to WNV, the birds were bled before experimental infection and serum was tested for neutralizing antibodies by using tissue culture infectious dose 50 ($TCID_{50}$) neutralization assays. Serum was heat-inactivated at 56°C for 30 min, serially diluted 2-fold and incubated with an equal volume of virus (strain NY99, originally isolated from a dead Chilean flamingo at the Bronx Zoo in New York, obtained from the Health Protection Agency, Porton Down, UK; P5 on Vero E6 cells; accession AF196835.2) to a final concentration of 100 $TCID_{50}$/0.1 mL. Samples were incubated at 37°C for 1 h and subsequently added to an 80% confluent monolayer of Vero E6 cells in CELLSTAR 96-well plates (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). Plates were incubated at 37°C for 5 days. Samples were read, and a 100% reduction in cytopathic effect, as compared with the serum-negative control, was used for the determination of neutralization. Detection of any neutralizing activity to WNV in the serum of any bird precluded its use for experimental inoculation.

PART III:

INTERVENTION

Chapter 7

Immunogenicity and protective efficacy of recombinant Modified Vaccinia virus Ankara candidate vaccines delivering West Nile virus envelope antigens

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Vaccine. 2016 Apr 7;34(16):1915-26

ABSTRACT

West Nile Virus (WNV) cycles between insects and wild birds, and is transmitted via mosquito vectors to horses and humans, potentially causing severe neuroinvasive disease. Modified Vaccinia virus Ankara (MVA) is an advanced viral vector for developing new recombinant vaccines against infectious diseases and cancer. Here, we generated and evaluated recombinant MVA candidate vaccines that deliver WNV envelope (E) antigens and fulfil all the requirements to proceed to clinical testing in humans. Infections of human and equine cell cultures with recombinant MVA demonstrated efficient synthesis and secretion of WNV envelope proteins in mammalian cells non-permissive for MVA replication. Prime-boost immunizations in BALB/c mice readily induced circulating serum antibodies binding to recombinant WNV E protein and neutralizing WNV in tissue culture infections. Vaccinations in HLA-A2.1-/HLA-DR1-transgenic H-2 class I-/class II-knockout mice elicited WNV E-specific CD8+ T cell responses. Moreover, the MVA-WNV candidate vaccines protected C57BL/6 mice against lineage 1 and lineage 2 WNV infection and induced heterologous neutralizing antibodies. Thus, further studies are warranted to evaluate these recombinant MVA-WNV vaccines in other preclinical models and use them as candidate vaccine in humans.

INTRODUCTION

West Nile virus (WNV) is an arbovirus that continuously circulates between different mosquito species and wild birds, most commonly passerines (particularly crows), as the natural virus reservoir, but it can also infect vertebrate animals, including horses and humans through the bite of a mosquito. The virus is widely distributed in Africa, Europe, the Middle East, Asia and America and can cause neuroinvasive disease with the potential for severe outcomes especially in elderly and immunocompromised humans.

Although not a new disease (Alexander the Great is thought to have died from West Nile fever 2000 years ago), the virus was first isolated in 1937 from a febrile woman in the West Nile district in Uganda [1]. Since then, sporadic WNV outbreaks have periodically occurred in Africa, Asia and Europe [2, 3]. In 1999, the virus suddenly emerged in the United States in the New York City District of Queens, probably introduced by an infected mosquito or bird [4-6]. Subsequently, WNV spread across the North American continent leading to a total of about 41762 human infections and 1765 deaths (http://www.cdc.gov/westnile/statsmaps/). During the last 15 years the virus has increasingly emerged in European countries, resulting in severe disease outbreaks in horses and humans (http://ecdc.europa.eu/en/healthtopics/west_nile_fever/pages/index.aspx).

As a member of the genus Flavivirus in the family of *Flaviviridae*, WNV is characterized by a single-stranded positively-sensed RNA genome of about 11 kb that is processed as a single polyprotein. The polyprotein encodes seven non-structural proteins essential for virus replication and three structural proteins at the amino terminus of the precursor polypeptide, which comprise the capsid protein (C), the membrane protein (M), which is initially expressed as the precursor to M (prM) and the envelope protein (E), which is responsible for the formation of virus particles. The formation and maturation of new WNV virions takes place in the endoplasmic reticulum (ER) and the trans-Golgi-network. The virus life cycle starts in the ER with the synthesis of immature virus particles comprising the prM and E protein. In the trans-Golgi network the prM is proteolytically cleaved into the mature form [4, 7, 8].

Phylogenetic analyses of WNV have identified five different but antigenically related lineages [9-11]. Strains of WNV lineage 1 are mostly associated with severe neuroinvasive disease in humans and animals, while lineage 2 strains have often been associated with relatively mild disease and no deaths in humans. However, very recently a new neuroinvasive strain of lineage 2 arose in Europe [12, 13].

So far there is no effective treatment available for WNV disease, and besides the use of insect repellents, the most effective approach to protect animals against WNV infection is vaccination. Veterinary vaccines already exist for use in horses [14, 15]; however, no WNV vaccine has currently been approved for human use. Preclinical and early-phase clinical trials in humans have been performed with recombinant protein, plasmid DNA, and live-attenuated strain vaccine candidates [16-18].

Given the continuing outbreaks of severe WNV disease in the United States and southeastern Europe, there is a need for a safe and effective human WNV vaccine, especially one that protects at-risk elderly and immunocompromised populations. Another requirement for an effective WNV vaccine includes the induction of cross-protection against different lineages of WNV.

Modified Vaccinia virus Ankara (MVA), a safety-tested and replication-deficient vaccinia virus, is an advanced, well-developed viral vector for constructing new vaccines against infectious diseases and cancer. The WNV E protein has already been shown to be the major target of neutralizing antibodies against flaviviruses due to its essential role in attachment to cell receptors and cell entry. Analysis of the WNV E structure by crystallography has shown three distinct subunits of the protein, ectodamain I, ectodomain II and ectodomain III [19], which are very reminiscent of the structure already identified for dengue virus (DENV) and tick-borne encephalitis virus (TBEV). In WNV, the ectodomain III has been shown to serve as the receptor-binding domain [20]. In this context, ectodomain III-specific antibodies have been demonstrated to neutralize WNV *in vitro* and *in vivo* after passive immunization [21-23].

Here, we constructed recombinant MVA viruses stably expressing the full-length WNV envelope (E) protein. We generated four different versions of the WNV E protein to mimic different forms that are present during the MVA life cycle, and assessed the expression, cellular location, immunogenicity, neutralising antibody response and protective capacity of these MVA-WNV candidate vaccines in different mouse model strains.

MATERIALS AND METHODS

Plasmid constructions

The cDNA sequences encoding the WNV (strain NY99, Genbank accession number AF196835, [4]) envelope target antigens (prM/ME, E_{sol}, E_{TMV}, E_{TMC}) were modified by introducing silent codon alterations to remove G/C runs as well as three termination signals (TTTTTNT) for vaccinia virus early transcription. The altered WNV cDNA sequences were generated by DNA synthesis (Invitrogen Life Technology, Regensburg, Germany). The cDNA prM/ME encodes the WNV NY99 amino acids (aa 128-589). The cDNA E_{sol} represents a truncated version of the WNV NY99 E protein sequence lacking the E transmembrane domain (TM, aa 291-541). The cDNAs E_{TMV} and E_{TMC} contain the WNV–E_{sol} gene sequence fused to the sequence encoding two heterologous transmembrane domains targeting expression of the synthetic fusion protein to the cell surface. E_{TMV} encodes the transmembrane domain of the MVA A56 protein (GenBank No AY603355.1, aa 35-103, TMV= transmembrane domain Vaccinia Virus) whereas E_{TMC} includes the coding sequence for the transmembrane domain of the envelope protein E2 of an

alphavirus: the chikungunya virus (CHIKV; GenBank No AF369024, aa 686-744, TMC= transmembrane domain chikungunya virus). The cDNAs WNV-prM/ME, WNV-E_{sol}, WNV-E_{TMV} and WNV-E_{TMC} were cloned into the MVA vector plasmid pIIIH5red [24] and placed under the transcriptional control of the synthetic vaccinia virus early/late promoter PmH5 [25].

Generation of recombinant MVA viruses

MVA (clonal isolate F6; [26]) virus was propagated on chicken embryo fibroblasts (CEF) prepared from 10-day old chicken embryos (SPF eggs, VALO, Cuxhaven) and served as a backbone virus to construct recombinant MVA expressing the WNV target gene sequences (MVA-WNV) using standard methodology as described previously [24]. Briefly, MVA-WNVs were obtained following transfection of MVA-infected CEF with pIIIH5red-WNV plasmid DNA, clonally isolated in plaque passages on CEF and monitored for the transient co-expression of the red fluorescent marker protein mCherry. MVA-WNV primary stock viruses were grown in CEF and stored at -80°C until further analysis. Quality control experiments were essentially performed as described previously [24]. Genetic identity and genetic stability of the vector viruses were assessed by PCR analysis of genomic viral DNA. Replicative capacities of MVA-WNV were tested in one-step and multiple-step growth experiments in CEF, human HaCat cells [27], and in cultures of equine dermal fibroblasts (generated from primary cultures of horse skin biopsies and generously provided by Cornelia Deeg, Institute of Animal Physiology, LMU, Munich). Viral titres were determined by plaque assays using MVA-specific immunostaining and titrated in plaque forming units (PFU). To generate vaccine preparations, recombinant MVA-WNVs were amplified in CEF, purified by ultracentrifugation through sucrose cushions and reconstituted to vaccine stocks in Tris buffered saline pH 7.4.

Immunostaining of MVA-WNV infected cells

At 48 h, MVA-WNV infected CEF cultures (multiplicity of infection (MOI) 0.01) were fixed at room temperature with 4% paraformaldehyde for 30 min and washed two times with phosphate-buffered saline (PBS). Selected cultures were permeabilized using 0.2% Triton X-100 solution in PBS. Mouse monoclonal antibody against WNV-E (Fa. BioReliance (Rockville, USA) and fluorescent (Alexa) polyclonal rabbit/goat anti-mouse antibodies (Fa. Life Technologies (Darmstadt) were used to stain the cells. Nuclear DNA was stained using 1 µg/ml DAPI (4′,6-diamidino-2-phenylindole) solution (Sigma). A Keyence BZ-X700 fluorescence microscope with a ×20 objective was used for analysis of stained culture.

Western blot analysis of recombinant proteins

Confluent monolayers of different cell lines (CEF, equine fibroblast, HaCaT) were infected at an MOI of 5 with the different recombinant MVA-WNVs (prM/ME, E_{sol}, E_{TMV}, E_{TMC}). Cell lysates or supernatants were prepared at different time points after infection (8, 24, 48 hours post-infection (hpi). Lysates from uninfected cells or wild-type MVA-infected cells served as controls. Polypeptides were separated by SDS-PAGE and electroblotted onto a PVDF membrane. After blocking, membranes were incubated with primary antibodies (anti-WNV E protein, mouse monoclonal, BioReliance, Rockville USA) at 4 °C overnight. After washing, the blots were incubated with secondary antibodies for one hour at room temperature.

WNV challenge viruses

Two isolates of WNV were used for mouse infection studies: one lineage 1a strain, Ita09 (kindly provided by Dr. Luisa Barzon, University of Padova, Italy; one VeroE6 passage; GenBank accession number GU011992.2) isolated from a patient with neuroinvasive disease, and the Hungarian lineage 2 strain, 578/10 (kindly provided by Dr. Tamás Bakonyi, Szent István University, Hungary, two passages on Vero E6 cells, GenBank accession number KC496015), which was isolated from the brain of a horse that died from WNV neuroinvasive disease. The virus stocks were prepared by growing Ita09 once (P1) and 578/10 twice (P2) on *Aedes albopictus* C6/36 cells (ATCC CRL1660).

Immunizations and infection experiments in mice

Female BALB/c mice (6-10 weeks old) were purchased from Charles River Laboratories (Sulzfeld, Germany). Three-week old female C57BL/6 mice were purchased from Harlan Laboratories B.V. (Venray, The Netherlands).

HLA-A2.1-/HLA-DR1-transgenic H-2 class I-/class II-knockout mice (6–8 weeks old) [28] were obtained from Institut Pasteur /Charles River Laboratories (France) and used for immunizations to monitor for HLA-A*0201-restricted CD8+ T cell responses. All vaccinations were performed by intramuscular (i.m.) injection of vaccine suspensions containing 10^8 plaque-forming units (PFU) of recombinant or non-recombinant (WT) MVA vaccine or PBS (mock vaccine) into the quadriceps muscles of the left leg of each animal (BALB/c and C57BL/6 mice). Blood was collected on day 0, 14 and 56. Coagulated blood was centrifuged at 1300 × g for 5 min in MiniCollect vials (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) in order to separate serum, which was subsequently stored at -20 °C until further use. Six weeks after the last immunization (day 56), all animals were challenged by intraperitoneal inoculation (i.p.) of 10^4 $TCID_{50}$ of WNV-Ita09 (n=40) or WNV-578/10 (n=40) in a total volume of 100 µl. Animals were monitored daily for signs of disease and mice were euthanized by cervical dislocation under isoflurane anaesthesia when humane end-points were reached (immobility and paralysis). Additionally, on day 8

after challenge, five mice per group were sacrificed and brains were collected for further processing. At day 14, the end-point of the experiment was reached and all remaining mice were euthanized, and the survival rate was analyzed and compared between the groups.

All mice were maintained under specified pathogen-free conditions, had free access to food and water, and were allowed to adjust to the facilities for one week before vaccination experiments were performed. All animal experiments were handled in compliance with the European and national regulations for animal experimentation (European Directive 2010/63/EU; Animal Welfare Acts in Germany and in the Netherlands).

Serology

To characterize the antibody responses induced by immunization with the different recombinant MVA vaccines, WNV binding antibodies were analyzed by enzyme-linked-immunosorbent assay (ELISA) using recombinant WNV E protein. WNV E protein was expressed in Sf21 insect cells using recombinant baculovirus, as previously described [29]. The sequence encoding the ectodomain of WNV E (NY99 strain) was preceded by a signal sequence derived from the end of WNV M, and the C-terminal transmembrane domain was replaced by a 6xhis purification tag.

96-well ELISA plates (Nunc Maxisorp, Platten, Thermo Scientific) were coated with 0.5 µg recombinant WNV E protein per well. Wells were blocked with PBS containing 1% BSA, 5% sucrose for 1 hour at 37°C. After blocking, 100 µl/well of diluted serum samples were added to top wells, titrated down, and incubated for 1 hour at 37°C. After washing, 100 µl/well of goat anti- mouse linked to horseradish peroxidase (Sigma) was added and plates were incubated for 1 hour at 37°C. Plates were washed and 100 µl/well of TMB substrate solution (3,3,5,5 tetramethylbenzidine, Sigma) was added. The reaction was stopped with 1.8M H_2SO (Sigma) and the plates were read in an ELISA reader using an absorbance at 450 nm. The inflection point of the titration curve was taken as titer value (logarithmic reciprocal titer).

For detection of WNV neutralizing antibodies, virus neutralization assays were performed by using tissue culture infectious dose 50 ($TCID_{50}$). To this end, sera of immunized mice was heat-inactivated at 56 ºC for 30 min, serially diluted two-fold (1:10 to 1:80) and incubated with an equal volume of virus (WNV-Ita09 or WNV-578/10) to a final concentration of 100 $TCID_{50}$/0.1 ml, giving a serum dilution of 1:20 to 1:160. After one hour of incubation at 37 ºC, samples were added to an 80% confluent monolayer of Vero E6 cells in CELLSTAR® 96-well plates (Greiner Bio-One) and subsequently incubated for five days. Neutralizing titres were determined microscopically and a 100% reduction in the cytopathic effect (CPE) as compared to the serum-negative control was used to determine neutralization. Titres were defined as the highest serum dilution still giving 100% inhibition of CPE.

Analysis of T cell responses

Mice were sacrificed eight or 56 days post prime or prime-boost immunizations. A cell suspension was prepared by homogenizing the spleens through 200 µm mesh sieves and red blood cells were removed by adding Red Cell lysis buffer (Sigma). After centrifugation the cell pellet was resolved in RPMI medium supplemented with 10% foetal calf serum, 2 mM L-glutamine and 100 IU/ml penicillin/streptomycin. For detection of WNV specific CD4+ T cell responsess, splenocytes were further processed by using the QuadroMACS Kit (Milteny Biotec GmbH, Bergisch Gladbach, Germany) to deplete CD8+ cells from splenocytes with MACS Micro Beads (Milteny Biotec GmbH, Bergisch Gladbach, Germany). As confirmed by flow cytometry CD8+ T cell depletions resulted in splenocyte suspensions containing CD4+ T cells at 80–95% purity with 0.5% CD8+cells present in the isolate of total CD3+ cells (data not shown). Interferon-γ secreting CD8+ T cells or CD4+ T cells were analyzed by ELISPOT assay (ELISPOTPLUS Kit for mouse IFN-γ, MABTECH, Germany) following the manufacturer's instructions. ELISPOT plates were pre-incubated overnight with the antibody solution and then incubated with the cell suspension that had been stimulated with the WNV-specific peptide SVG9 (SVGGVFTSV; [30]) for detection of WNV CD8+ T cells. Stimulation of the spleen cell suspension with 1 µg/ml recombinant WNV E protein (as used in ELISA) served for analysis of WNV specific CD4+ T cells after depletion of CD8+ T cells. The spots were counted and analyzed by using an automated ELISPOT plate reader and software following the manufacturer's instructions (A.EL.VIS Eli.Scan, A.EL.VIS ELISPOT Analysis Software, Hannover, Germany).

Analysis of WNV loads

To quantify WNV in the brains of mice euthanized on day 8, half the brain was weighed and homogenized using a metal bead in 1 ml of DMEM containing antibiotics (100 U penicillin, 100 µg/ml streptomycin). RNA was isolated from 100 µl of brain homogenate using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Almere, The Netherlands) and an automatic nucleic acid robotic workstation (Roche). RNA was eluted in 50 µl elution buffer (Roche) and RNA copy numbers were determined using a standard curve of *in vitro* transcribed RNA of known quantities (as described previously, Lim et al., 2013 (PMID: 23965252)) and qRT-PCR was performed using the Taqman® EZ RT-PCR kit (Applied Biosystems, Bleiswijk, The Netherlands), an ABI PRISM® 7500 detection instrument (Applied Biosystems) and primers and probe located on the 3' UTR of WNV.

Statistical analysis

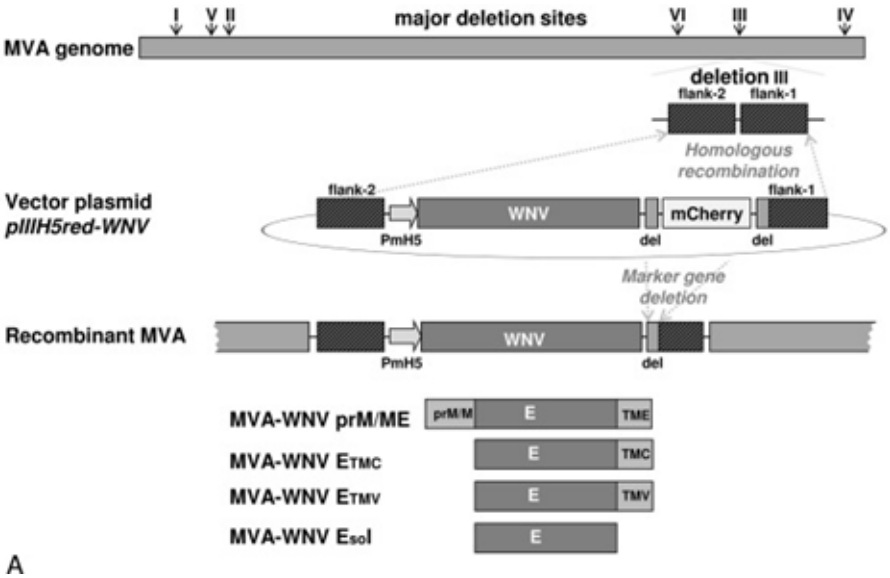
Differences in Kaplan-Meier survival curves between the different groups were assessed using the Log-rank test. Differences in neutralizing antibody titres were assessed using the Mann-Whitney U test and P-values equal to or less than 0.05 were considered to be

statistically significant. All statistical analyses were performed using GraphPad Prism version 5 software (GraphPad software, San Diego, USA).

RESULTS

Construction and isolation of recombinant MVA expressing WNV-E and M genes

To construct recombinant MVA viruses we used the MVA transfer plasmid pIIIH5red, which introduces foreign gene sequences into deletion site III of the MVA genome. The strong synthetic early/late VACV specific promoter PmH5 controls transcription of the recombinant target genes and a marker gene transiently produces the fluorescent protein mCherry. We inserted different versions of WNV gene sequences (WNV-prM/ME, WNV-E_{TMC}, WNV-E_{TMV}, WNV-E_{sol},) into pIIIH5red to generate the vector plasmid pIII5red-WNV derivatives (Figure 1A). Recombinant MVA-WNVs were generated in CEF infected with MVA (clonal isolate F6) and transfected with DNA of the four different pIIIH5red-WNV vector plasmids. The four different MVA-WNVs were isolated in plaque passages by screening for transient co-expression of the fluorescent marker gene mCherry under the transcriptional control of the vaccinia virus late promoter P11. The repetitive sequences (del) served to remove the mCherry marker by intragenomic homologous recombination (marker gene deletion) (Figure 1A). To confirm the genetic integrity and proper insertion of the different WNV genes within the MVA genome we performed PCR analysis of the viral genomic DNA using specific oligonucleotide primers targeting sequences adjacent to the MVA deletion site III. As a control for equal amounts of viral DNA, a second PCR amplified C7L gene sequences in the MVA genome ([31]; Figure 1B).



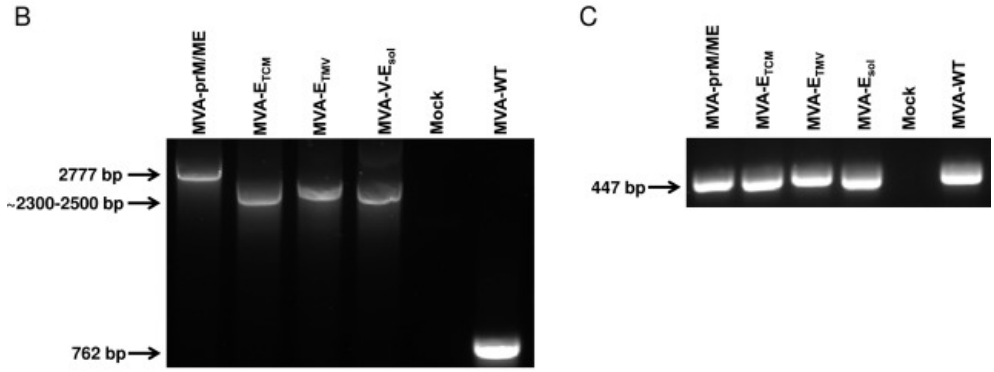


Figure 1. Generation of recombinant MVA-WNV viruses. (A) A schematic representation of the MVA genome with the major deletions sites I-VI shown on the top. Flank-1 and flank-2 refer to MVA DNA sequences at deletion site III in the MVA genome, which target this site for insertion of recombinant genes. MVA vector plasmids pIIIH5red contain recombinant WNV gene sequences under transcriptional control of the vaccinia virus promoter PmH5 and a marker gene sequence for transient expression of the fluorescent protein mCherry. prM/ME encodes the M and E gene sequences including the transmembrane domain (TME), E_{sol} lacks the E transmembrane domain and E_{TMV} and E_{TMC} contain the WNV E gene with substituted heterologous transmembrane domains from MVA or chikungunya virus, (see M&M). The recombinant viruses were isolated in plaque passages screening for red fluorescent cells. Repetitive sequences (del) were designed to remove the mCherry marker by intragenomic homologous recombination (marker gene deletion). **(B, C)** PCR analyses of genomic viral DNA to confirm **(B)** insertion of recombinant genes into deletion III, and **(C)** the genetic integrity of the MVA genome for the C7L gene locus. Genomic template DNA was prepared from recombinant MVA-WNV viruses or non-recombinant MVA (MVA). Oligonucleotides from gene sequences adjacent to deletion III or within the C7L gene were used to amplify specific DNA fragments. PCR products were separated by agarose gel electrophoresis. PCR reactions without template DNA (Mock) served as controls.

Growth of recombinant MVA-WNV viruses in cell culture

Next, we evaluated the *in vitro* growth behaviour of the MVA-WNV viruses (Figure 2A, B). Multiple-step growth analyses were performed with avian cell cultures (CEF and DF-1), which are routinely used to propagate recombinant MVA viruses. We also analyzed the replicative capacity of recombinant MVA-WNVs in cells of mammalian origin. Non-recombinant MVA-WT served as a control virus. In both avian cell lines, the growth of the recombinant WNV viruses was comparable to that of MVA-WT, since all viruses replicated to similar titres within 72 hours, each increasing infectivity by approximately three $\log_{10}$ steps in CEF and DF-1 cells (Figure 2). In the cells of mammalian origin (human HaCat cells and equine fibroblasts, EqF), we could confirm the well-established MVA replication deficiency for the four recombinant MVA-WNVs. After 72 hours, and comparable to non-recombinant MVA-WT virus, amounts of virus recovered remained lower than the titres

used as the infection input. These findings corroborated the expected MVA phenotype and confirmed that the recombinant viruses could be handled under biosafety level 1 conditions.

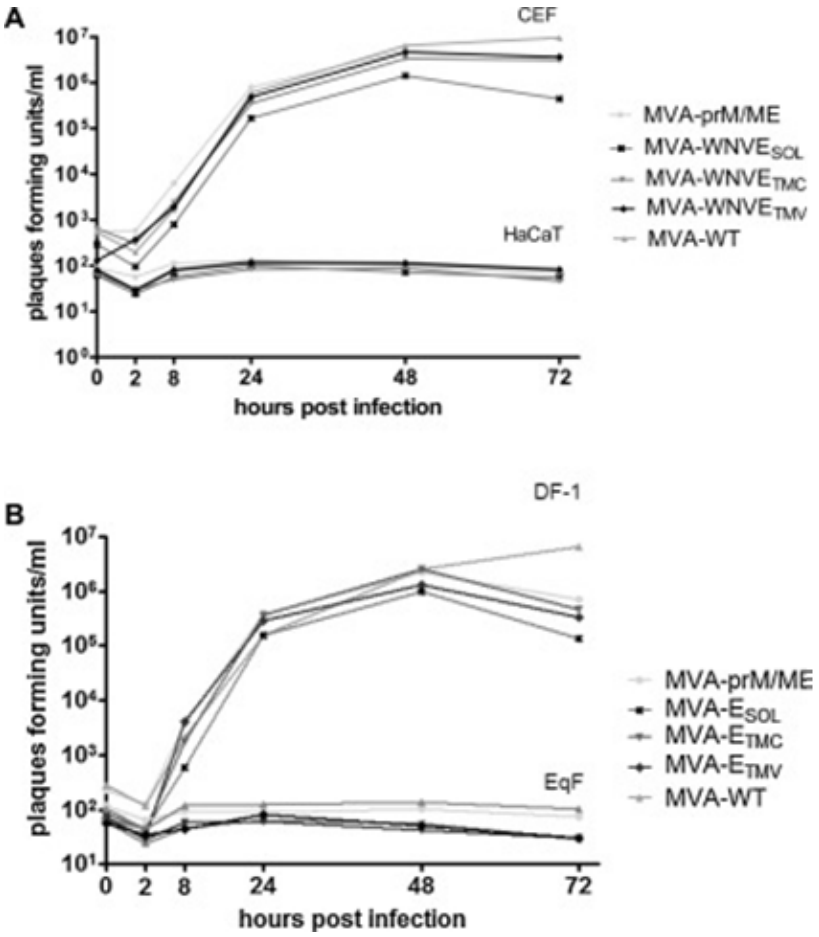


Figure 2. Multiple-step growth analysis of recombinant MVA-WNV viruses. Growth of recombinant viruses MVA-WNV-prME, -E_{sol}, -E_{TMC}, -E_{TMV}, or non-recombinant MVA (MVA-WT) was monitored upon infection (MOI of 0.01) of **(A)** chicken fibroblast cells CEF and HaCat or **(B)** DF-1 and equine fibroblasts EqF.

Characterization of recombinant WNV-E proteins

First, immunofluorescence of MVA-WNV-infected cells was used to assess the synthesis of the WNV proteins. Anti-WNV E antibody followed by fluorescent secondary antibody specifically stained cells infected with recombinant MVA-WNVs (Figure 3). No background staining was observed in control cultures infected with non-recombinant MVA. We observed a reticular pattern upon infection with all recombinant viruses, with a

juxtanuclear accumulation of all E proteins. As anticipated, the E-specific staining in cells infected with MVA-E_{TMV} and MVA-E_{TMC} viruses seemed to include the cell surface, while in MVA-prM/ME or the MVA-E_{sol} infected cells the E proteins appeared to be exclusively located within the cell. To further investigate the distribution patterns we also performed immunostaining of MVA-WNV infected cells without permeabilizing the cells. Here, we specifically detected the E protein only in cells infected with MVA-E_{TMV} and MVA-E_{TMC}, indicating localization of abundant E protein on the cell surface.

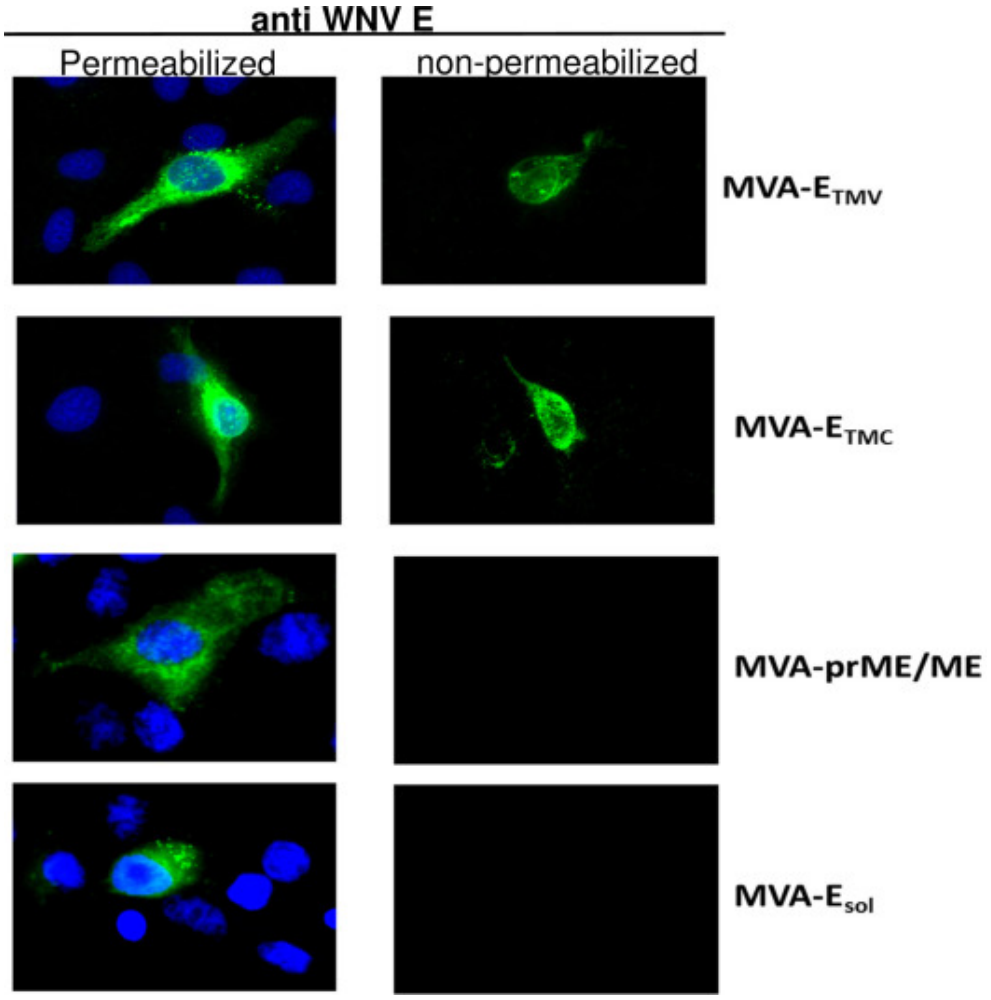
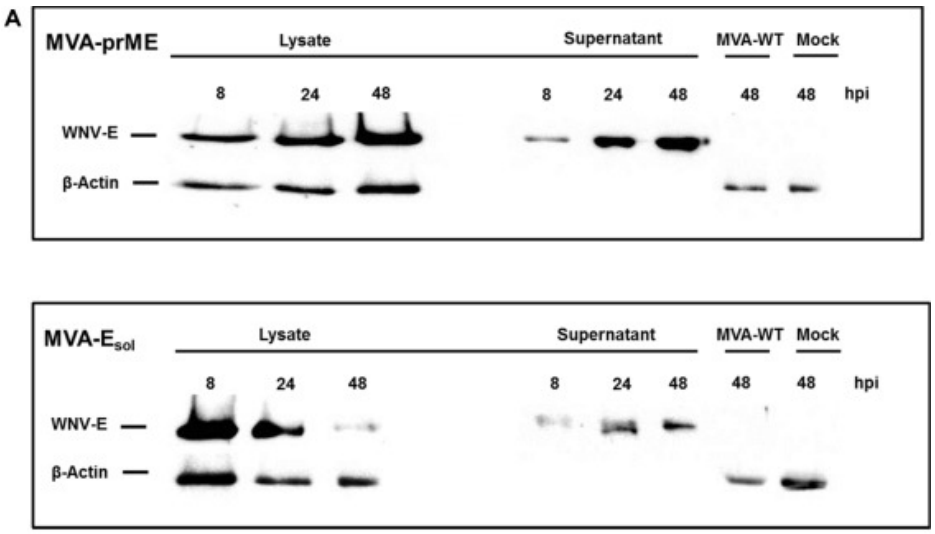
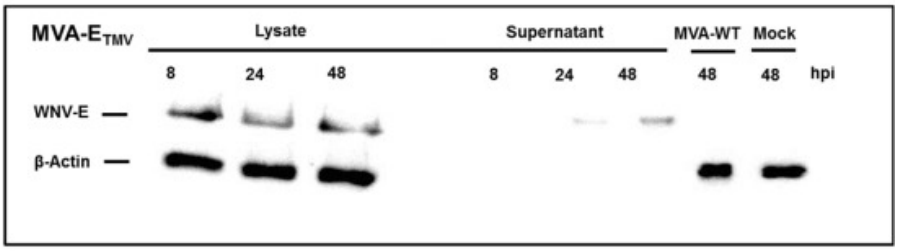


Figure 3. Immunofluorescence staining of cells infected with recombinant MVA-WNV. Virus infected MA-104 cells were fixed with 4% paraformaldehyde and selected samples were treated with 0.2% Triton X-100 for permeabilization. DAPI solution was used to stain nuclear DNA.

To further analyze the synthesis of WNV envelope proteins upon infection with the recombinant MVA-WNV viruses, total cell lysates from infected CEF and HaCat cells were analyzed by Western Blot using WNV-E-specific mouse monoclonal antibodies (Figure 4). We specifically detected a protein with an estimated molecular mass of about 45 kDa in lysates from CEF cells and HaCat cells infected with all the MVA-WNV derivatives. Over the time course of 48 hours, the amounts of E protein remained stable in cell lysates from CEF and HaCat cells infected with MVA-WNV-prM/ME, MVA-E_{TMV} and MVA-E_{TMC}. However, in cell lysates from MVA-E_{sol}-infected cells, the level of WNV E seemed to decline by 48 hours post-infection. To monitor the release of WNV proteins from MVA-infected cells we also analyzed the supernatants of infected CEF and HaCat cell cultures. After MVA-prM/ME or MVA-E_{sol} infection we clearly detected the WNV E specific protein band, indicating the release of soluble WNV E protein or E protein-containing particles from these infected cells. In contrast, we detected only tiny amounts of E proteins in the supernatants from cells infected with MVA-E_{TMV} and MVA-E_{TMC} suggesting that these E proteins remained associated with the infected cells. Comparable results could be detected in EqF (data not shown).

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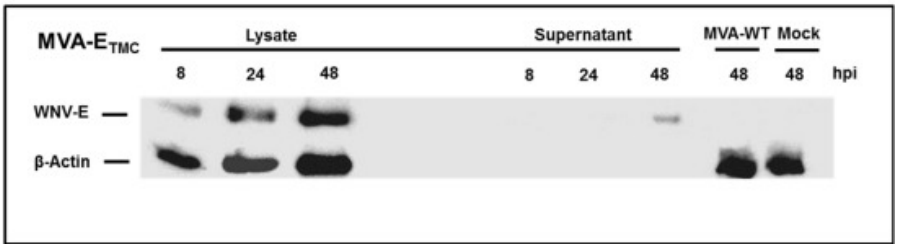
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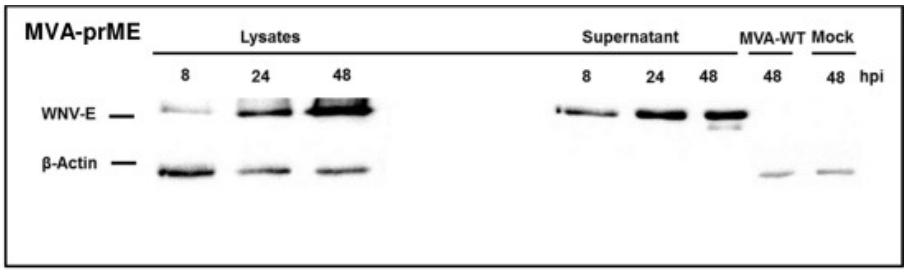
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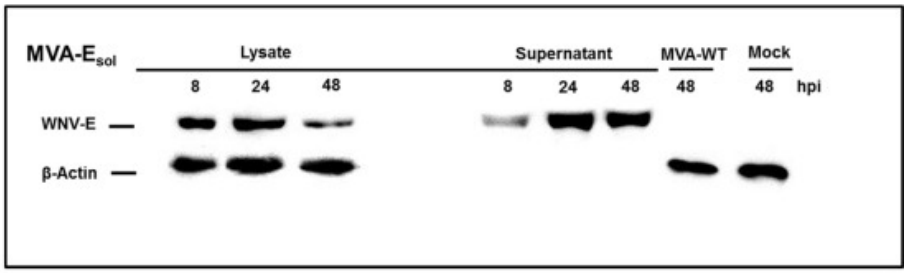
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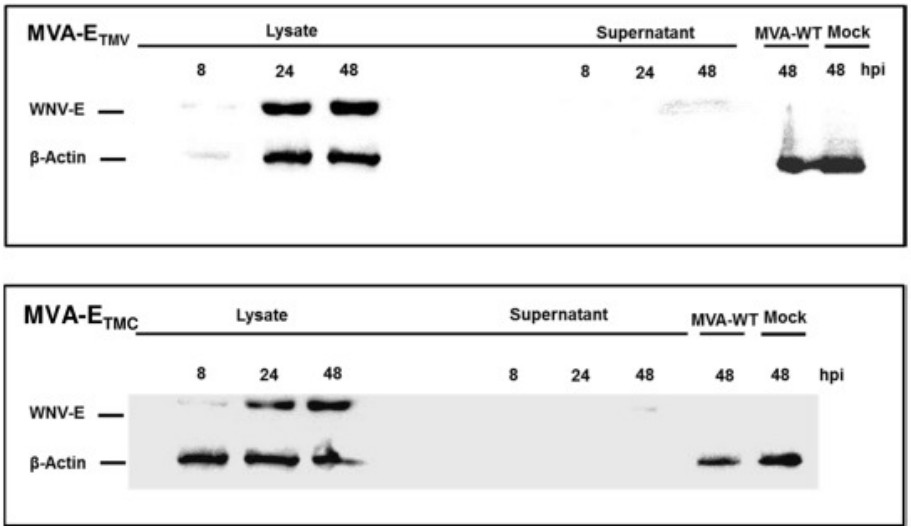


Figure 4. Analysis of recombinant WNV proteins. Recombinant viruses MVA-WNV-prME, -E_{sol}, -E_{TMV} and -E_{TMC} were tested by Western blot analysis using cell lysates and supernatants from infected **(A)** CEF and **(B)** HaCat cells. Polypeptides were separated by SDS-PAGE and tested by immunoblotting using WNV-E specific monoclonal antibodies. Lysates from uninfected cells (Mock) or non-recombinant MVA infected cells (MVA) and Western blot detection of beta actin served as controls. The ~46 kDa WNV E proteins (WNV-E) and 42 kDa polypeptide beta actin (β-Actin) are indicated on the left.

Antibody responses of MVA-WNV vaccines in BALB/c mice

To assess the immunogenicity of the recombinant MVA-WNV candidate vaccines *in vivo*, we vaccinated BALB/c mice in an initial experiment with 10^8 PFU by an intramuscular route at 0 and 3 weeks. Eighteen days after the first immunization (Prime) and ten days after the second immunization (Prime-Boost), serum samples were tested for WNV-binding antibodies by ELISA (Figure 5A). Even a single application of all MVA-WNV vaccines induced significant levels of WNV-specific antibodies in the mice. After booster immunization, all vaccinated animals produced even higher levels of circulating WNV-specific antibodies, with the antibody titres increasing about ten-fold.

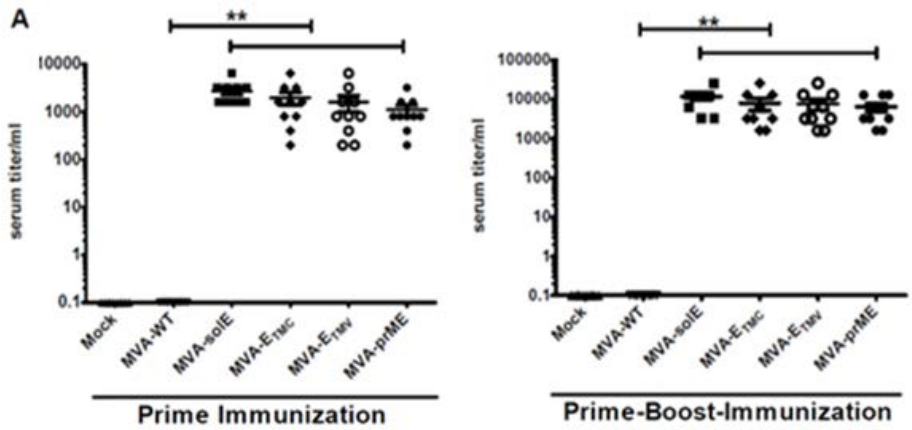
WNV specific CD8+ and CD4+ T cells induced by MVA-WNV vaccines

To assess whether the MVA-WNV recombinant proteins can activate a WNV-specific T cell response, we vaccinated HLA-A2.1-/HLA-DR1-transgenic H-2 class I-/class II-knockout mice using 10^8 PFU of recombinant MVA-WNVs via an intramuscular route in prime and prime-boost immunizations (Figure 5B). We tested splenocytes for WNV E-specific (SVG9 peptide epitope) CD8+ T cells by IFN-γ ELISPOT 8 days after the last immunization. Single intramuscular immunizations induced substantial levels of SVG9-

specific CD8+ T cells (with median absolute numbers of >2,000 IFN-γ SFC/10^6 splenocytes) for all recombinant MVA-WNVs. Of note, the magnitude of CD8+ T cells did not differ significantly when comparing the groups of mice immunized with all recombinant MVA-WNVs. Intramuscular booster immunization harmonized the responses of individually immunized animals in all groups and increased the median levels of WNV-specific CD8+ T cells to about 3,500 to 4,000 IFN-γ secreting SVG9-specific CD8+ T cells/10^6 splenocytes.

At 56 days following a primary immunization with the MVA derivatives, we still found 400 to 800 WNV SVG9 specific IFN-γ-producing T-cells/10^6 splenocytes, suggesting an antigen-specific memory CD8+ T-cell response (Figure 5C). Prime-boost applications confirmed this data demonstrating significantly increased numbers of WNV-SVG9 specific IFN-γ-producing memory T-cells in splenocytes.

In addition, we tested for the activation of WNV-specific CD4+ T cell responses in mice that had been vaccinated twice (day 0, day 21) with the different MVA-WNV candidate vaccines. Eight days after the last vaccination we prepared the splenocytes, depleted CD8+ T cells using MACS Micro Beads and *in vitro* stimulated the remaining spleen cells with purified recombinant E-protein for 48 hours. To measure CD4+ T cell activation we counted IFN-γ secreting cells via ELISPOT assay. Two immunizations by intramuscular application resulted in readily detectable levels of E-specific CD4 + T cell responses (with median absolute numbers of 182 IFN-γ SFC/10^6 splenocytes) for all recombinant MVA-WNV candidate vaccines (Figure 5D). Immunizations with MVA-E_{TMC} or MVA-prME elicited WNV-E-specific CD4+ T cells with means of 23 and 73 IFN-γ SFC/10^6 purified splenocytes. MVA- E_{sol} and MVA-E_{TMV} resulted in activation of higher numbers of WNV E-specific IFN-γ SFC/10^6 purified splenocytes with a mean of 503 for MVA-E_{sol} and 98 for MVA-E_{TMV}.



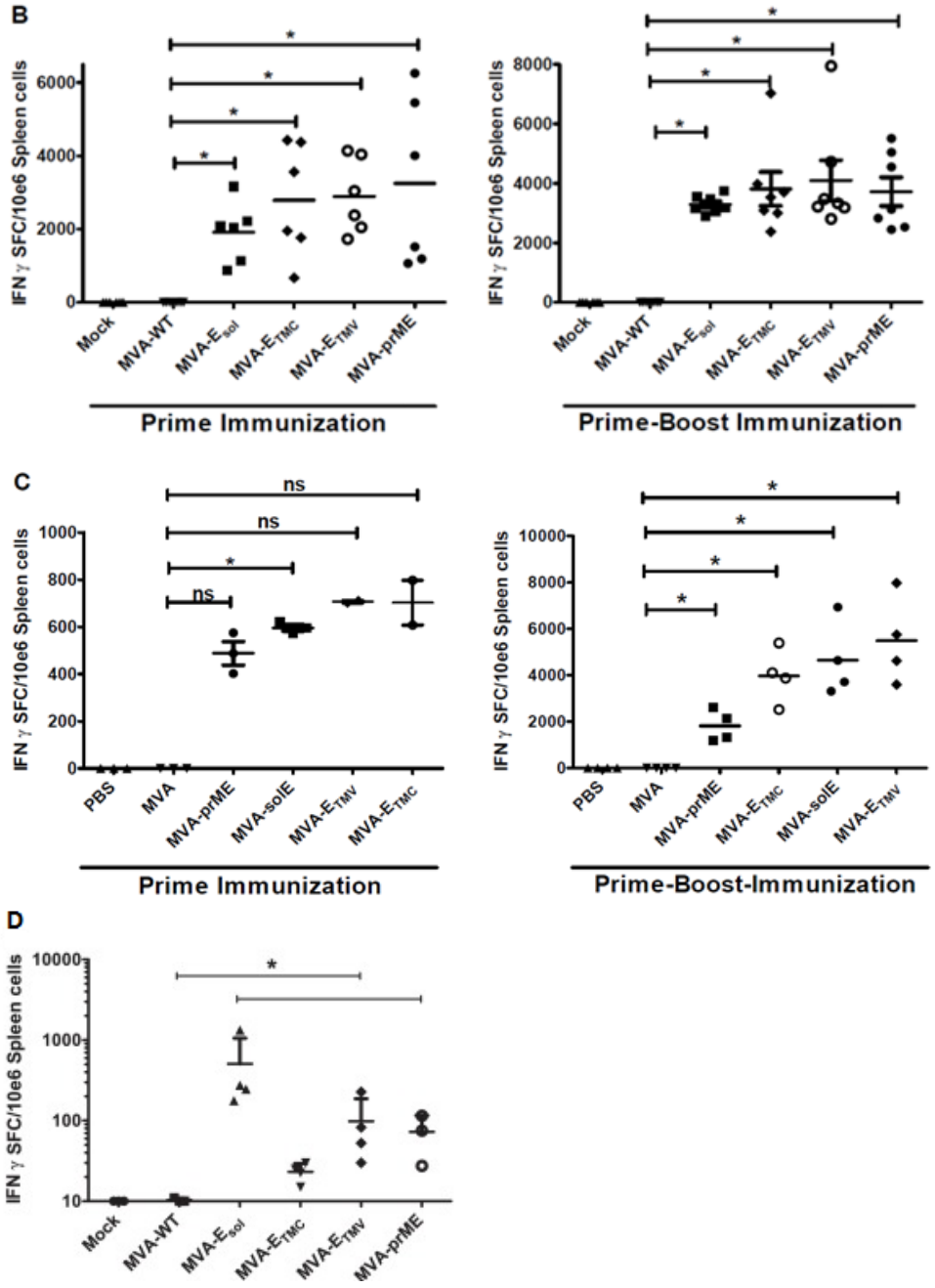


Figure 5. Immunogenicity of recombinant MVA-WNV vaccines. Mice were vaccinated by intramuscular applications of saline (Mock or PBS), non-recombinant MVA (MVA), or the indicated recombinant viruses. Animals received one (prime immunization) or two vaccinations (prime-boost immunization) with 10^8 PFU MVA vaccine. **(A)** WNV E-binding antibodies in BALB/c mice (n=10 per group). Sera were analyzed by ELISA 18 days after the first and 10

days after the second immunization. **(B)** WNV E-specific CD8+ T cell responses in HLA-A*0201 transgenic mice (n=6-7 per group). Splenocytes were prepared at eight days after prime or prime-boost vaccination. SV09 epitope specific, IFN-γ spot forming CD8+ T cells (IFN-γ SFC) were quantified by ELISPOT. **(C)** WNV E-specific CD8+ T cell memory responses in HLA-A*0201 transgenic mice (n=2-4 per group). Splenocytes were prepared at 56 days after prime or prime-boost vaccination. SV09 epitope specific, IFN-γ spot forming CD8+ T cells (IFN-γ SFC) were quantified by ELISPOT. *, $P<0.05$; **, $P<0.01$; ns, no statistically significant difference. **(D)** WNV E-specific CD4+ T cell responses in HLA-DR1 transgenic mice. Splenocytes were prepared at 8 days after prime-boost vaccination. After depletion of CD8+ T cells WNV E-specific, IFN-γ spot forming CD4+ T cells (IFN-γ SFC) were quantified by ELISPOT (Mock, MVA-WT, MVA-prME, n=3; MVA-E_{sol}, MVA-E_{TMV}, MVA-E_{TMC}, n=4). *, $P<0.05$.

Induction of WNV neutralizing antibodies

To monitor the induction of WNV neutralizing antibodies, we vaccinated groups of C57BL/6 mice with 10^8 PFU of the indicated MVA-WNV recombinant viruses (omitting MVA-WMV E_{TMC} because its phenotype resembled MVA-WMV E_{TMV}) by intramuscular inoculation. Two weeks after the initial immunization all mice mounted detectable levels of circulating antibodies that neutralized the heterologous WNV Hungarian lineage 2 strain 578/10 (with average titres >16; Figure 6A). These titres could be further increased after boost immunization on day 21 as measured at day 56 (average titres of 64). Furthermore, these sera also neutralized the homologous WNV lineage 1a strain, Ita09 (Figure 6B). Already two weeks after the primary immunization all mice produced significant levels of neutralizing antibodies in the serum (average titre >64). Booster immunizations given at day 21 further consolidated the neutralizing antibody response to WNV-Ita09 (with average titres of 128).

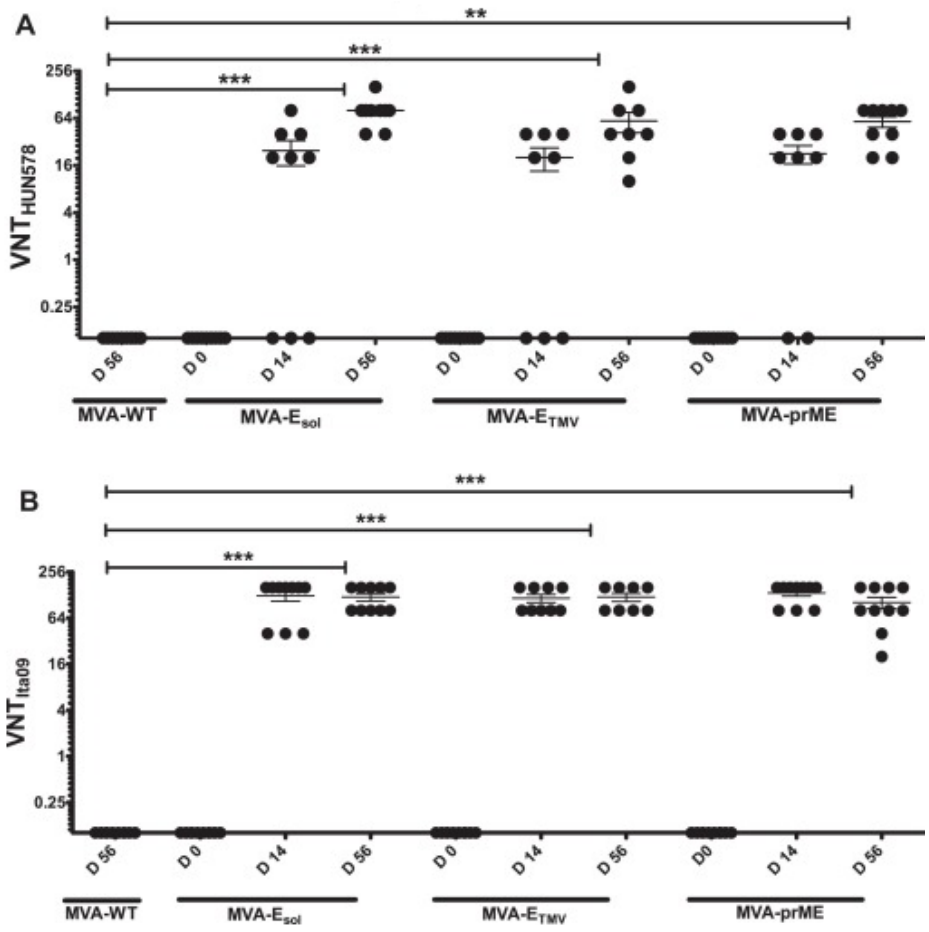


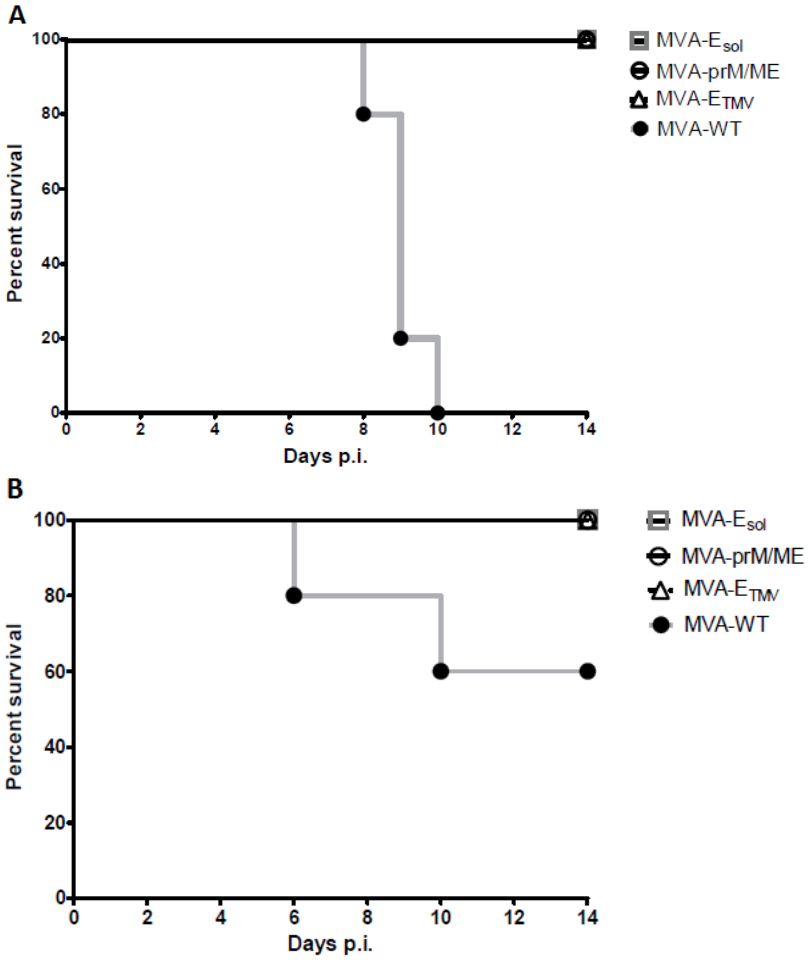
Figure 6. WNV neutralizing antibody responses. C57BL/6 mice (n=8-10 per group) were vaccinated twice (21-day interval) by intramuscular application with non-recombinant MVA (MVA) or the indicated recombinant viruses. We analyzed the WNV neutralizing capacity of mouse serum samples taken at days 0, 14 and 56 after the first immunization. Serum antibodies against **(A)** WNV lineage 2 strain Hungary 578 (HUN578) or **(B)** WNV lineage 1 strain Italy 09 (Ita09) were tested by virus neutralization assays (VNT). Shown are the mean serum antibody titres (log_2) of individual animals. **, P<0.01; ***, P<0.001.

Protection of mice against WNV challenge infection

To evaluate the protective capacity of these MVA-WNV vaccine candidates, C57BL/6 mice vaccinated by an intramuscular route were challenged intraperitoneally with a dose of 10^4 $TCID_{50}$ of either WNV lineage 1a strain Ita09 or WNV Hungarian lineage 2 strain 578/10 (Figure 7). The survival rates of each group were monitored after challenge. Upon challenge with WNV-Ita09, 5/5 (100%) of animals in the mock-vaccinated (MVA-WT) group died, while all mice in the vaccinated groups survived infection (Figure 7A;

p<0.0007, curve; p<0.0001, trend). Upon challenge with WNV-578/10, 2/5 (40%) of the mock-vaccinated animals died from infection, whereas no animals in the vaccinated groups succumbed to the challenge infection (Figure 7B; p<0.1, curve; p<0.056, trend).

To determine whether vaccination prevents virus from entering the brain, five animals per group were sacrificed on day 8 p.i., a time point at which virus has previously been shown to have entered the brain of all susceptible animals (data not shown). All mock-vaccinated (MVA-WT) mice challenged with WNV Ita09 or WNV 578/10 harboured high viral loads in the brain at day 8 p.i. (Figure 7C; mean titre: 4.9 log_{10} RNA copies WNV Ita09/g of brain, or 5.5 log_{10} RNA copies WNV 578/10/g). In sharp contrast, we failed to detect any WNV RNA in the brains of all animals euthanized on day 8 p.i. with the recombinant MVA-WNV vaccines (Figure 7C).



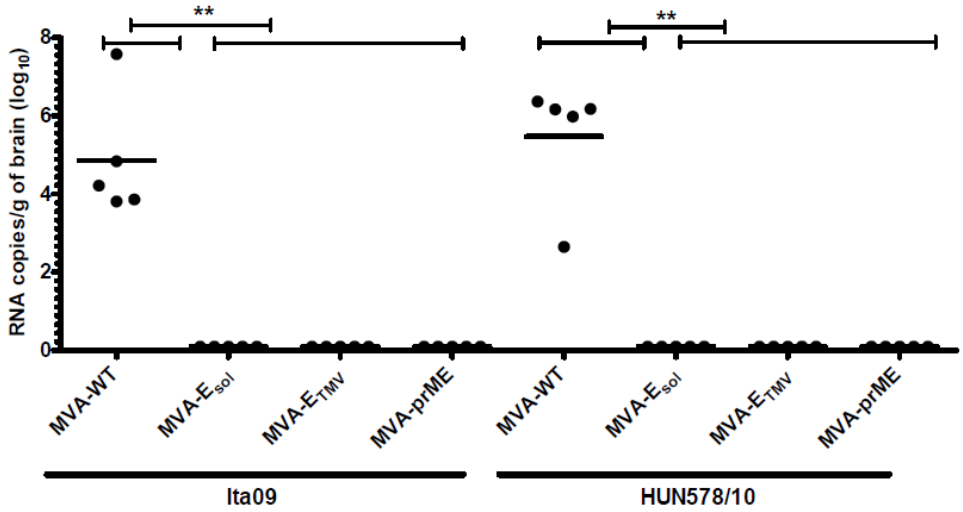


Figure 7. Protection against WNV challenge infection. C57BL/6 mice (n=10 per group) were vaccinated twice (21-day interval) by intramuscular application with non-recombinant MVA (MVA) or recombinant viruses MVA-WNV-E_{sol}, -E_{TMV}, or -prME. Six weeks after the last immunization all animals were challenged by intraperitoneal inoculation of 10^4 $TCID_{50}$ of **(A)** WNV-Ita09, or **(B)** WNV-578/10. **(C)** On day eight after challenge, five mice per group were sacrificed and brains were collected for analysis of virus loads (RNA copies). At the end-point of the experiment (day 14 post challenge) the survival rate was analyzed (A, B). **, $P<0.01$.

DISCUSSION

Ongoing WNV infections in horses and humans, particularly in the United States, demonstrate the continuing need for WNV-specific public health responses (http://www.cdc.gov/westnile/index.html). The availability of a WNV vaccine for humans should greatly reduce the risk of infection in people; however, the commercial development of such a vaccine is expected to be complex [32]. Several requirements must be met for a promising candidate WNV vaccine: (i) it should protect against different WNV strains; (ii) it must be safe and immunogenic in the most important human target population, i.e. the elderly and immunocompromised patients; (iii) it should be suitable for a licensing approach using efficacy testing in animal models because of the low incidence of WNV infections in humans; (iv) it should be suitable for storage as an emergency vaccine (shelf-life), and (v) ideally, the same vaccine technology platform should allow for the development of a vaccine for veterinary use. All these points supported our strategy to evaluate MVA as vector vaccine for delivery of WNV antigens.

Moreover, our motivation to construct a WNV candidate vaccine based on MVA was due to encouraging results with another recombinant MVA vaccine in preclinical and clinical evaluation [33-37]. In these studies, a recombinant MVA expressing the HA of influenza A/Vietnam/1194/04 (H5N1) virus efficiently induced H5 cross-reactive antibodies and prevented virus replication in the upper and lower respiratory tract, as well as the development of severe necrotizing bronchointerstitial pneumonia following challenge infections with homologous and heterologous influenza A/H5N1 viruses. Moreover, the first clinical evaluation in humans demonstrated the safety and immunogenicity of this MVA-H5 influenza candidate vaccine [34, 38].

Here, we focused on the WNV E protein as the immunogen to efficiently activate WNV-specific immune responses. Previous studies on the flavivirus life cycle identified the E protein as an important activator of WNV-specific antibodies [7, 39]. Moreover in a recent study, a Matrix-MTM adjuvanted WNV E protein vaccine successfully protected mice against lethal WNV challenge infection by activating WNV-specific humoral and cellular immune responses. The E protein is a structural protein mainly involved in the attachment of WNV to the receptor of target cells to mediate the cell entry process. Therefore, most of the neutralizing antibodies against flaviviruses recognize the structural E protein, although another population of antibodies exits that also binds to the prM/M protein [40-42].

Efficient vaccine-mediated activation of immune responses can also be optimized by expressing antigens that closely resemble those of the target pathogen during its actual life cycle. Previous studies have identified different modalities of antigen presentation that efficiently activate flavivirus-specific immune responses. For example, secreted E proteins alone in a soluble truncated form lacking the membrane anchor region have been shown to be highly immunogenic when expressed by vaccinia virus vectors or recombinant

baculoviruses [43-45]. Another study demonstrated the immunogenicity and protective capacity of a membrane anchored WNV E protein in BALB/c mice [46].
Another promising approach for vaccine development is the induction of so-called “virus like particles” (VLPs), which are non-infectious virus particles found to be highly immunogenic [47]. For WNV, co-expression of the E and M protein resulted in the production of VLPs that were able to protect mice and horses against neuroinvasive disease upon WNV challenge [18, 48].

In this study, we successfully constructed four recombinant MVA-WNVs delivering the WNV E protein in different forms for antigen presentation to mimic the vaccine optimization strategies described above, i.e. E protein lacking the membrane anchor region, containing heterologous transmembrane domains, or including the M protein encoding sequences. We succeeded in producing all WNV E antigens as stable proteins, alone or in combination with M, and achieved high levels of expression using the strong synthetic early/late vaccinia virus PmH5 [25].

In the case of MVA-E_{sol}, removal of the authentic E membrane anchor sequence resulted in secretion of E antigen from MVA-infected cells and predominant accumulation of E protein in culture supernatants as shown by Western blot analysis. A similar, efficient release of soluble WNV E protein from infected cells has been seen upon expression from recombinant measles virus [49]. In contrast, replacement of the E membrane anchor sequence by the transmembrane domains of the VACV protein A56 (WNV E_{TMV}) or the chikungunya virus E2 protein (WNV E_{TMC}) resulted in stable presentation of WNV E protein antigens on the cell surface. The immunostaining and Western blot analysis data support the idea that these chimeric E antigens undergo cell membrane-associated expression without release from the MVA vector infected cells.

For MVA-prM/ME, the M protein-encoding sequences are maintained in addition to the E gene sequences in order to trigger the synthesis of VLPs [50-52]. Upon cell culture infections, we could detect increasing amounts of WNV E protein in cell lysates as well as in the supernatants, suggesting the assembly of VLPs in the ER, transport via the trans-Golgi, and release of the particles from the MVA infected cells [53, 54].

An important requirement for recombinant MVA candidate vaccines is that the vector viruses can be stably grown and produced in CEF cells at an industrial scale [24]. Our experiments demonstrated the genetic stability of all the vector viruses and comparative growth analyses confirmed that recombinant MVA-WNV replicated in CEF cells as efficiently as the non-recombinant wild-type MVA. Moreover, *in vivo* experiments in mice demonstrated the immunogenicity of all the recombinant MVA-WNV vaccines, including the production of WNV-specific antibodies and CD8+ T cell responses.

The use of HLA*A0201 transgenic mice allowed us to monitor activation of SVG9 epitope-specific IFN-γ secreting CD8+ T cells that mimic an immunodominant T cell specificity also found in humans [55]. In addition, we showed the induction of WNV E-specific CD4+ T cells upon prime-boost immunization in the HLA-DR1 transgenic mice with the MVA-WNV vaccines and using recombinant purified E protein for *in vitro*

stimulation. This data goes well along with WNV E-specific CD4+ T cell responses reported in a recent study by De Filette and coworkers [56]. Further experiments will be needed to characterize the WNV E-specific CD4+ T cell immunity in more detail by monitoring for HLA-DR1 restricted peptide epitopes. Of note, WNV-specific CD4+ T cells are considered essential for the regulation of antibody and CD8+ T cell responses and for protection against lethal WNV infection [57, 58].

In addition, we also demonstrated the maintenance of WNV-specific CD8+ memory T cell responses until 56 days after the last vaccination. These data are of relevance since previous studies have demonstrated that WNV-specific CD8+ T cell responses are critical for protection against neuroinvasive disease [59, 60]. Of note, Brien and coworkers demonstrated that a deficiency in CD8+ T cell immunity may result in severe neurological WNV disease [61].

Furthermore, the different MVA-WNV vaccines also elicited serum antibodies that led to comparable neutralization of the homologous lineage 1 WNV Ita09 and the heterologous Hungarian lineage 2 WNV strain 578/10. Activation of virus neutralizing antibodies is considered to be the most important correlate for protection against flaviviruses [7]. Corroborating the immunogenicity testing, the MVA-E_{TMV}, MVA-E_{sol} and MVA-prM/ME candidate vaccines fully protected mice against lethal challenge infections with homologous and heterologous WNV.

In summary, the immunogenicity and efficacy of the MVA-WNV vaccines appeared at least similar to that described for other WNV candidate vaccines in preclinical testing with regard to the induction of WNV-specific immune responses and the protective capacity against lethal WNV challenge infection [18, 55, 56]. The recombinant MVA-WNV vector viruses we have developed here merit further development as candidate vaccines for potential use in humans and our data strongly support their evaluation in other preclinical models.

ACKNOWLEDGEMENTS

We thank Ursula Klostermeier for expert help in animal studies and Corinne Geertsema for expression and purification of recombinant WNV-E. This work was supported by European Union grants VECTORIE (261466) and FLUNIVAC (602604).

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Chapter 8

Summarizing Discussion

The re-emergence of WNV in Europe in 1990 with continuing introductions into new areas and the genetic and phenotypic diversity of the virus, have led to the intensification of integrated and multidisciplinary research efforts. Where in Europe WNV may emerge and whether it will pose a major threat to Europe is hard to predict. The complexities of its enzootic transmission cycle, which involves mosquitoes and birds as well as related ecological factors, render the geographical spread of WNV unpredictable. More information about the pathogenicity of circulating WNV strains for different reservoir hosts, their potential vector range and ecological factors may at least in part fill these knowledge gaps. This may also shed light on the question why WNV infections in Europe have a much more limited or focal distribution than in the United States.

This thesis therefore aims to address three questions related to the current distribution of WNV in Europe:

1. Are European WNV strains less virulent than their American counterparts?

2. Are European birds less susceptible to WNV than their American counterparts?

3. Does a WNV vaccine protect against both lineage 1 and 2 viruses circulating in Europe?

Part 1: Virulence

Although WNV harbors a single serotype, it exhibits considerable genetic variation within the African continent, which is the source of all the strains found around the globe [1, 2]. While single introduction events in India, Australia, and the Americas respectively, established WNV endemicities, which were followed by spatial categorization of WNV strains (clades 1b and lineage 5 in particular) [1], the genetic diversity of WNV in Europe and the Middle East is most likely the result of independent introductions by migratory birds [1, 3-6].

WNV can be subdivided into at least eight separate lineages, of which lineage 1 and 2 have been responsible for disease outbreaks in humans and equines. Lineage 1 mostly encompasses outbreaks in Africa, Europe, the Middle East, Asia, Oceania (Kunjin strain) and North America, while lineage 2 primarily consists of strains identified in Africa. Lineage 2 strains have historically been considered less pathogenic in humans compared to lineage 1. However, since the bird of prey mortality in 2004 (mostly goshawks, *Accipiter gentilis*) caused by a lineage 2 strain found in central Europe (Hungary), this lineage has caused several epidemics in humans that subsequently occurred in Greece (2010), Romania (2010), Russia (2011), Italy (2011-2013) and Serbia (2012). It therefore appears that the plasticity and adaptive capacity of WNV poses a continuous risk of generating genotypes virulent in vertebrates that may spread outside of Africa [7].

The European strains investigated in this thesis comprise two Italian isolates, FIN and Ita09, and one Hungarian isolate, 578/10. Interestingly, of these strains, only Ita09 has

caused a substantial outbreak in humans, particularly in 2009. The closely related FIN, on the other hand, was obtained from a single case of human neuroinvasive disease, and 578/10 was isolated from a horse presenting with neuroinvasive disease. Although these two strains have displayed their neuroinvasive potential in a single human and a single horse, respectively, the actual virulence of FIN and 578/10 still remains to be determined. As a result, in **Chapter 2** we sought to characterize the neuroinvasive, and indirectly neurovirulent potential, of these European strains of WNV after intraperitoneal (i.p.) injections of C57BL/6 mice, using Ita09 as the more prototypic virulent European strain. We decided to construct a hypothetical virulence profile for these strains based on *in vitro* replication kinetics and fitness, *in vivo* survival (LD_{50}), brain and spinal cord viral load, and response to infection in the brain using markers that play a role in neurodegeneration.

In our study, we found that *in vitro* Ita09 replicated faster than FIN and 578/10, and to higher titers (LT_{50}, burst size). However, the latent period was the same for the three virus strains tested. As a stand-alone *in vitro* experiment, this data suggests that Ita09 would be the more virulent virus. We found that all three virus strains tested were neuroinvasive, as virus was found in the brain of a large proportion of the moribund and surviving mice, and neurovirulent as infection led to neurological disease and in many cases to death. Nonetheless, differences in virulence were observed between the virus strains as 578/10 clearly showed the lowest LD_{50}. Interestingly, *in vivo* Ita09 was found to have a 2.5 day earlier median mortality compared to the other two viruses (day 8.5 compared to day 11). This makes it tempting to speculate that the higher *in vitro* observed replication kinetics of Ita09 is associated with a more rapid onset of disease and mortality. It may be speculated that higher viral titers established more rapidly in the periphery, lead to earlier neuroinvasion and neuronal cell death. However, the one-step growth curve was carried out in neuroblastoma cells only, which makes it difficult to extrapolate the data to events occurring in the periphery before neuroinvasion takes place.

Our *in vitro* and *in vivo* experiments yielded information on neuroinvasive and neurovirulent capacities of the WNV strains tested. Nonetheless, several questions remained. Firstly, we do not specifically know whether the more rapid death of Ita09-infected mice reflected the ability of the virus to enter the brain more quickly, for example, due to higher virus titers that are reached more rapidly in the periphery, or whether it is dependent on the ability of the virus to spread more rapidly in the brain and cause more rapid neuronal cell death (neurovirulence). A large proportion of mice that survived infection with FIN (67%) had viral RNA in the brain, which rather indicates successful neuroinvasion, suggesting that this virus is less neurovirulent than the other two strains. In order to properly assess and compare the neuroinvasive capacity of our virus strains, it would be more instructive to conduct a kinetic experiment in i.p. inoculated mice that are examined and sampled daily comparing virus titers in peripheral organs and brain sections. As we have now confirmed that our viruses are neuroinvasive, it would be interesting to directly compare their neuroinvasive capacities by determining the time required for each

virus strain to gain access to the brain (using qRT-PCR), as well as the initial amount that enters the brain and in which particular areas (using qRT-PCR and immunohistochemistry).

An effective method to investigate neurovirulence is to conduct intracranial (i.c.) inoculations with several doses of virus, followed by observation of the survival as well as a straight kinetic analysis of viral spread by daily sampling of the brain and analyzing the separate sections for virus replication. This method determines neurovirulence, as the more neurovirulent the virus is, the more rapidly it will kill its host.

Initially, alongside FIN, Ita09 and 578/10 we had also included NY99 as a virulent non-European control that has been extensively characterized in different mouse models. We conducted similar experiments for this strain as for the other three strains. We found that *in vitro* infectious NY99 was produced two hours earlier (at 12 hours p.i.) compared to the European strains (Figure 1). In addition, the replication rate of NY99 was found to be 1.97, which is even higher than for Ita09 (1.75). Interestingly, *in vivo*, the cumulative mortality of NY99 (76%) was close to the one of FIN (78%) and Ita09 (74%), while its LD_{50} was found to be the highest out of the four virus strains at $10^{1.43}$. This data gives the indication that *in vitro* NY99 is potentially the more virulent virus; however, *in vivo* the European strains appear to be more virulent. Nonetheless, as the NY99 strain was of a higher passage (P5) than the European strains (P2/3), the comparison with NY99 was eventually not published.

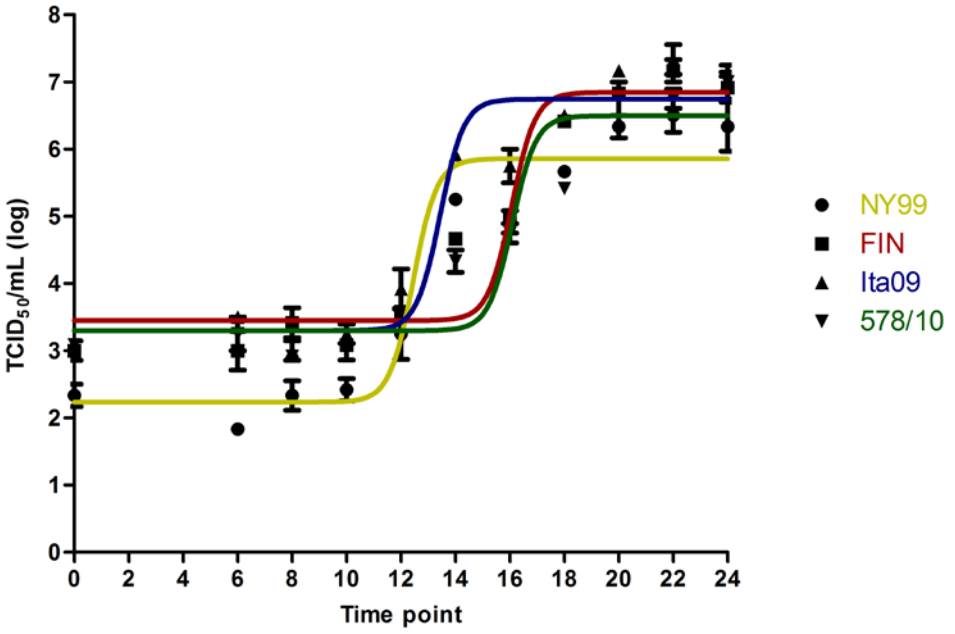

Figure 1. Fitted one-step growth curves of infectious virus titers recovered from the supernatant of N2a cells infected with several WNV strains at a high MOI.

Host tropism determines which cells can be infected by a given pathogen and is a combination of susceptibility, (the cell possesses the surface receptors for binding and entering the cell), and permissiveness (viral replication occurs). Even though it is possible to deduce cell permissiveness by following viral replication and witnessing a substantial increase in RNA or infectious virus titers over time, a more direct approach would be to directly measure negative-stranded RNA. Therefore, in an effort to obtain an additional tool that can aid in the determination and comparison of virulence, in **Chapter 3** we developed a qRT-PCR that specifically measures and quantifies the amount of positive- and negative-stranded RNA of WNV. This is useful for measuring the replication rate more specifically as well as determining the tropism of a virus for particular host cells.

In order to test the sensitivity and applicability of the qRT-PCR, we chose to measure tropism both *in vitro* and *in vivo*. As the tropism of WNV for microglial cells (both *in vitro* and *in vivo*) is still elusive, we decided to investigate its ability to infect a mouse microglial (BV-2) cell line. We found that these cells are susceptible and permissive as we were able to measure an increase in both negative-stranded as well as positive-stranded RNA over 48 hours. To study the *in vivo* relevance of this data, we chose for a set-up in which bulk brains were collected daily from 9-day-old C57BL/6 mice starting at an early time point of infection but during which virus was already detectable in the brain (day 3) up to day 6, which was close to the humane endpoint and could therefore be considered a late stage of disease. We saw that the amount of negative-stranded RNA increased most significantly from day 3 to day 4 (4 $\log_{10}$ RNA copies), which could be an indication of active virus replication in the brain between these two days and suggests that the positive-stranded virus that we found in the brain on day 4 is most likely not derived from the periphery. It is interesting, however, that the increase in negative-strand at this point is much larger than the increase in positive-stranded RNA at the same time point, especially since the ratio of positive- to negative-strand genomic RNA production is 10:1 for flaviviruses [8]. As a result, an increase by 4 $\log_{10}$ RNA copies of negative-stranded RNA between day 3 and 4 should make the amount of positive-stranded RNA detected around this time point about 10-fold as well; instead the difference is only about 1 $\log_{10}$ RNA copies. Nonetheless, the qRT-PCR that we developed proved to be a useful tool for following viral infection over time, and should be useful for future virulence studies.

The non-structural proteins of WNV have been thoroughly studied as possible virulence markers. The conventional way of identifying virulence markers is by comparing a highly virulent strain with an attenuated strain. Many of the studies attempting to identify virulence markers for lineage 1 compared the virulent NY99 strain with the attenuated Kunjin strain. Another method of finding virulence markers has been obtained via cell lines that contain persistently replicating flaviviral replicon, which have been established by selection of cells transfected with replicon RNA expressing an antibiotic resistance gene [9]. These cell lines are stable and useful for complementation analysis of replicase [10], virion assembly [11, 12] and antiviral drug screening [13, 14]. One notable feature of the

flavivirus replicon-containing cells is that the persistent replication of viral RNA does not cause CPE, which may be explained by the lack of viral structural protein expression. Using this system, particular epidemic strains of WNV eventually acquire an accumulation of adaptations in the replicons that favor persistent viral replication [15]. The key, however, is to deduce which adaptive mutations are responsible for the non-cytopathic replication, and especially, how these adaptive substitutions affect WNV replication and virulence. In **Chapter 4,** we investigated whether particular markers of attenuation found in lineage 1 WNV were also attenuating a lineage 2 strain, using a molecular cloning strategy via which we constructed a lineage 2 molecular clone of the Hungarian strain 578/10 harboring lineage 1 attenuating mutations.

In this study we investigated a number of non-structural proteins at which particular mutations proved to be attenuating in lineage 1 NY99, namely NS1-P250L, NS2a-A30P, NS3-H249P, NS4B-P38G, NS4B-C102S, NS4B-E249G, and NS5-A804V. We first examined the neuroinvasive capacity of molecular clones harboring these particular mutations in C57BL/6 mice via i.p. inoculations. Here we found that only the NS1-P250L mutant was significantly attenuated as it resulted in the complete abolishment of neurovirulence. There is a high probability that this mutant was also no longer neuroinvasive, although with the current data we cannot confirm this. To be specific, even though the original mutant could not be retrieved from the brains of all mice 14 days p.i., which therefore suggests that this mutant was not neuroinvasive, we cannot establish with certainty that the virus had never entered the brain, but had simply been cleared by day 14. In light of the data generated in **Chapter 2**, follow-up experiments in which we inject mice i.c. with this particular mutant and assess its neurovirulence, as well as collecting daily brain samples after i.p. inoculation, could be particularly instructive to establish its neuroinvasive capacity.

Even though the other mutations were not significantly attenuating in mice, a slight reduction in mortality (at a high dose that we have previously seen to induce 100% mortality in mice for the original 578/10 isolate [16]) was observed after infection with the NS3 (6/8), NS4B249 (5/8) and NS2a (7/8) mutants. The same attenuating effect for the latter two mutations as was observed in the lineage 1 studies [9, 17] might not have been achieved in our study due to the absence of particular co-mutations that are possibly not present on the lineage 2 backbone and are in fact required to increase the attenuating effect of these mutations in mice. In terms of the NS3 mutation, even though a proline at the NS3-249 locus has been proven to be an important virulence marker in birds, with in particular corvids [18], as we have also highlighted in **Chapter 5** and **Chapter 6**, a proline to histidine mutation has actually been found more recently to not be attenuating in American crows nor in mice, as viremia and mortality for the birds and LD_{50} for the mice were highly similar upon infection with the NS3-249H mutant compared to the parental NS3-249P [19]. Even though the NS3-249H has never been associated with virulence in mice, it is known that the goshawk-Hungary-2004 strain harbors a histidine at the NS3-249 locus and that a large outbreak of human encephalitis in Greece in 2010 with the closely related Greek

strain was in fact associated with a His to Pro substitution at this position [20]. As a result, it is possible that a proline at this position could still be a virulence marker of severe disease for a lineage 2 strain upon infection of humans, and it was therefore still necessary to explore this mutation in a mouse model.

In our study, we measured viral attenuation in terms of replication in cell culture at 37°C and reduction of mortality in mice. However, a few studies investigating virulence markers have also examined plaque size and temperature sensitivity of mutant viruses by conducting multiplication kinetics experiments at 39.5°C or 41°C in comparison to 37°C. Some of these mutants appeared to be temperature-sensitive [21-23], while some were not [24]. As the outcome of these temperature-sensitivity experiments did not directly relate to the presence of an attenuating mutation, in combination with the fact that we have only tested our molecular clones in a mouse model and not in birds for which higher temperatures might be more relevant, we decided not to include temperature-sensitivity experiments in our study.

Nonetheless, our multiplication kinetics studies at 37°C showed that several of the mutants, namely NS1, NS2a and NS3, yielded significantly lower infectious virus titers as compared to the wild-type. However, over the 96 hours, these titers were only consistently significantly lower for the NS1 mutant. This in combination with the fact that we did not see significantly attenuating results *in vivo* for any of the mutants except NS1-P250L, we decided to only further characterize this mutant *in vitro*. In an attempt to elucidate the mechanisms behind the observed attenuating effect of the NS1-P250L mutant, we found the strand-specific qRT-PCR employing tagged primers (Figure 2) developed in **Chapter 4** to be particularly useful for measuring viral genome replication and assessing whether attenuation is caused by an effect exerted during this step of the replication cycle.

Quantifying the amount of positive and negative stranded RNA using the assay described in **Chapter 4**, we found that significantly less positive and negative stranded RNA of the NS1-P250L mutant were synthesized over time. This implied that the given mutation had an effect on the efficiency of replication. Under normal circumstances NS1 exists as a heat labile homodimer that associates with cellular organelle membranes and is transported to the cell surface [25, 26]. Cell surface associated NS1 appears to have an immunomodulatory function via the decrease of the complement activation by different routes [27, 28], but NS1 is also secreted by mammalian cells as a soluble hexamer [29, 30].

Interestingly, NS1 has been shown to be involved in the replication process which was demonstrated in a study examining the ultrastructure of Kunjin virus-infected cells where NS1 was shown to be required for formation of the replication complex and recruitment of other non-structural proteins, such as NS3, to the vesicle packets [31] associated with ER membranes. NS1 also appears to be required in the replication process of other flaviviruses [32], such as dengue virus [33] and yellow fever virus [34], although the precise mechanisms are not completely known.

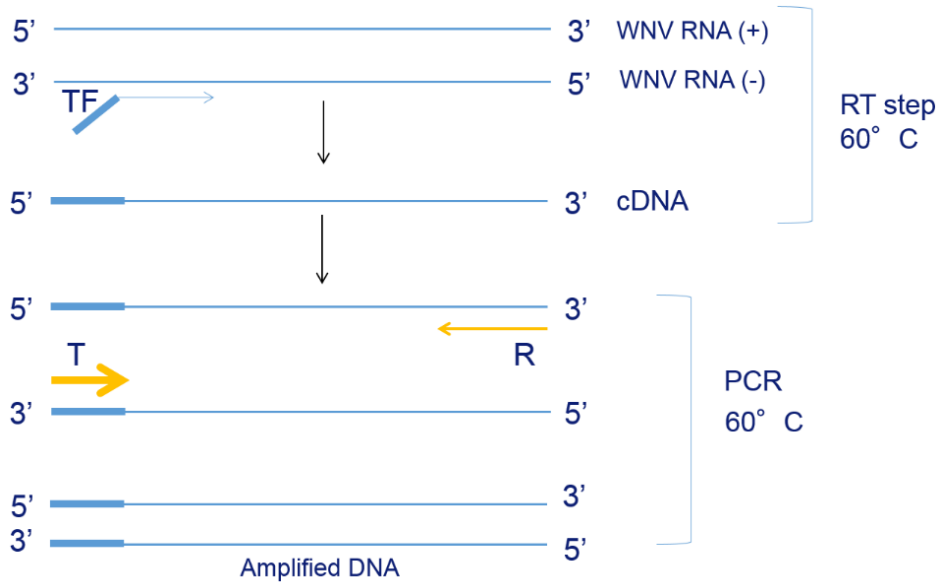

Figure 2. Strand-specific amplification of the negative strand of WNV using tagged primers in qRT-PCR.

Nonetheless, a study by Youn *et al*. further elucidated the role of NS1 as they demonstrated in viral lifecycle experiments that WNV NS1 was not required for virus attachment or input strand translation of infectious viral RNA, but instead was required for replication as WNV RNA lacking intact NS1 genes did not form canonical replication complexes early after infection, resulting in a failure to replicate viral RNA and consequently significantly lower amounts of positive and negative stranded RNA in the cells [35]. We had similar results; however, the reduction in the amount of positive- and negative-stranded RNA was less pronounced as compared to the observations by Youn et al. [35] but could be related to the fact that in their study the entire NS1 gene was deleted, whereas in our study we simply had a proline to leucine mutation.

The Pro to Leu substitution in the NS1 protein has been shown to ablate dimer formation in WNV-KUN [36]. This mutation was specifically discovered by identification of a cDNA clone of KUN virus (FLSD) that replicated efficiently in cell culture but produced and secreted NS1 in monomeric form, after which sequence analysis of the NS1 gene in this FLSD revealed a single amino acid substitution (P250L). Proline is quite a unique amino acid as the last carbon atom of the side chain is bonded to the main nitrogen atom forming a ring structure [36]. As a result, Pro is often found in flexible regions of a polypeptide or gives a bend in the peptide chain [37]. For this reason a Pro to Leu substitution is likely to significantly affect the structure of the surrounding peptide sequence and is therefore consistent with the major structural change indicated by the lack of dimerization of the mutated NS1 [36]. This in turn leads to intracellular trafficking and secretion of monomeric NS1. However, given that the lack of dimerization did not hamper

the distribution of the protein in cells infected with the mutant and that secretion remained relatively efficient, it appears that dimerization may not be an absolute requirement for NS1 function. Nonetheless, the Pro at position 250 in NS1 and a second Pro, six residues upstream, have been found to be strictly conserved in all members of the flavivirus genus (sequenced up to 1999) and lie in a relatively conserved region of the protein as well [36]. As a result, despite the monomeric form of NS1 appearing to be distributed and secreted properly, it is possible that this form of the protein still impacts the stability of the replication complex, consequently affecting the efficiency of the replication process. It remains to be determined whether this mutation also influences the immunomodulatory function of this protein.

Despite our study confirming an already earlier described attenuating effect of the NS1-P250L mutation [36], this mutation proved to be significantly more attenuating in our study: in the study by Hall et al. [36], the impact on replication was not as large (100-fold lower between 12-24 h p.i. compared to the WT) nor was the virus as attenuated *in vivo* (only 10-fold more virus of the WNV-KUN P250L mutant was required to produce disease in mice) as compared to our study. The difference in the mouse model used or the age of the mice (18- to 20-day-old weanling BALB/c mice vs C57BL/6) may explain the discrepancy between these results. However, it is also possible that the presence of certain loci in the genome of lineage 2 viruses augmented the attenuating effect of the P250L mutation previously demonstrated in WNV-KUN. Studies involving the introduction of this mutation into other virulent lineage 1 and 2 WNV strains may provide insight into the importance of this mutation in other lineage 2 viruses, and, if the mutation proves to be only slightly attenuating in lineage 1 viruses, may also confirm that the augmented attenuation seen in our study is related to a co-mutation in the lineage 2 backbone. In fact, it may even be a co-mutation in the NS1, potentially located in the stretch of amino acids comprising the relatively conserved region of the NS1, as described earlier [36].

Even though it appears that we have found a virulence marker in lineage 2 WNVs, with the chosen approach, it is more correct to state that we have identified a marker of attenuation. Virulence markers are most accurately found by comparing emerging virulent isolates with circulating more attenuated strains. In our situation, however, we investigated a mutation that was found in a variant of the attenuated Kunjin virus in cell culture. Whether this variant is also circulating in nature still needs to be determined. It is likely, however, that the majority of emerging virulent strains of WNV have the Pro at the 250 position on the NS1, and this locus has probably not been under such strong selective pressure as the NS3-T249P, which appears to be an evolutionary adaptation driven by avian hosts. In fact, a threonine to proline substitution at this locus has occurred on at least three independent occasions between lineage 1a WNVs, which preceded human WNV outbreaks in Egypt (1950), Romania and Russia (1996) and Israel (1997-98) [19]. To determine whether a leucine to proline mutation has occurred at the NS1-250 position in association with the emergence of more virulent strains of WNV, some in-depth phylogenetic studies of sequenced isolates are required. Only after the identification of strains that harbour the

leucine at this position that are only associated with cases of mild or absent disease, in combination with mouse experiments proving that mutating the leucine to a proline leads to a neuroinvasive phenotype, is it possible to say with more certainty that the NS1-250 is indeed a virulence marker. Therefore, to date it may be more accurate to conclude that we have found a marker of attenuation and that we have confirmed the association between NS1 protein and replication.

To conclude the section on virulence, we have demonstrated that the selected European strains of WNV investigated for this thesis are all neuroinvasive and neurovirulent. In addition, we have found that markers of attenuation in lineage 1 do not consistently produce the same effect in a lineage 2 virus, which confirms the observed difference in virulence of lineage 2 viruses and suggests that different markers of virulence may have resulted in the emergence of more pathogenic lineage 2 viruses, which may not necessarily mimic the emergence of virulent lineage 1 strains. Furthermore, our study has also confirmed the highly virulent phenotype of Ita09 and it is therefore not surprising that this virus strain has caused outbreaks of neurological disease in humans. The virulent phenotype displayed in mice infected with FIN explains why the virus was isolated from a human case of neuroinvasive disease; however, it does not explain the lack of subsequent cases of neuroinvasive disease. This can also be said for 578/10, as this strain was shown to be highly virulent in mice, yet has only caused one isolated case of neuroinvasive disease in a horse in Hungary. These results demonstrate the complexity of the enzootic transmission cycle of WNV and therefore also the importance of a One Health approach. Potentially the answers to the lack of spread of the FIN and 578/10 isolates can be found by studying the reservoir hosts that may be involved in the WNV transmission cycle, as is discussed below.

Part 2: Reservoir Hosts and Vectors

WNV is maintained in an enzootic transmission cycle between birds and mosquitoes. Mosquitoes act as vectors while birds are the amplification hosts. When WNV spreads into new territory, it is often first detected in mosquito pools, subsequently in birds (often marked by bird mortality), which is quickly followed by the first human cases. In fact, in North America, bird mortality, with in particular deaths amongst American crows, were often found to precede cases of WNV in humans, and as a result, bird mortality was used to track the spread of the virus across many parts of the continent [38-41].

In **Chapter 5** and **Chapter 6** we investigated the susceptibility of two bird species ubiquitously found across Europe. We sought to answer the question whether the lack of bird mortality and also the limited distribution of WNV in Europe are related to a lower susceptibility of European versus American bird species to WNV infection. For the experiments we chose the Carrion crow (*Corvus corone*), which is the European counterpart of the American crow, a bird species highly susceptible to WNV in the United States, and we also chose the closely related Western jackdaw (*Corvus monedula*).

Importantly, both bird species are found ubiquitously across northwestern Europe. Although the Hooded crow (*Corvus cornix*) is found in north- and southeastern Europe and may therefore be considered more representative for WNV endemic areas, we had to choose for the Carrion crow for practical reasons. Furthermore, the Hooded crow is genetically so closely related to the Carrion crow (0.28% at the genome level, difference in 40 genes involved in factors such as pigmentation, vision and social dominance behavior [42], which are probably not directly relevant from a virological point of view), that the data generated in experimental infections with Carrion crows are likely to mimic those that would be found in Hooded crows. As a result, we believe that experimental infections of Carrion crows will provide us with more insight on the current distribution of WNV in both northwestern (no WNV activity) and north- and southeastern Europe (focal distribution).

Carrion crow (*Corvus corone*)

Western jackdaw (*Corvus monedula*)

Hooded crow (*Corvus cornix*)

The first study to use a European strain of WNV and the Carrion crow indicated that European strains of WNV have a relatively low virulence to crows, as a strain from France (Fr2000) resulted in only 33% mortality compared to 100% mortality by Is98, a strain closely related to the highly virulent NY99 [43]. This study therefore seemed to suggest that the relatively low virulence of European strains of WNV could be the reason for the observed low bird mortality in Europe. In order to further investigate this hypothesis, it became essential to conduct more experimental bird studies using other strains of WNV circulating in Europe. Importantly, these studies would also include strains that have caused outbreaks amongst humans (Greece-10, Ita09), as well as those that have shown a very limited spread, for example, only isolated from a single human (FIN) or equine (578/10)

case, which would be similar to the French strain (Fr2000) that has only been associated with sporadic equine cases in Camargue, France. Besides it being important to investigate whether other European strains of WNV could be virulent to crows, it was also interesting to examine whether strains that caused large outbreaks in humans also tend to display a higher virulence in crows, which may potentially explain the spread of these viruses throughout a larger area that eventually led to spill over into humans. In fact, the results of **Chapters 5 and 6** strongly indicate that the Western jackdaw and Carrion crow are susceptible to WNV, as mortality (60% in jackdaws, 83% in Carrion crows) was observed after inoculation with NY99, a strain of WNV known to be highly virulent to American crows. We also found that the majority of European strains of WNV are virulent to corvids as three out of four strains induced viremia and mortality in both bird species. In fact, European strains of WNV displayed a similar, if not higher, virulence in the birds than the North American strain. Furthermore, in order to measure the 'absolute' virulence of these European strains of WNV, we conducted parallel studies in the highly susceptible American crow. We found that the European strains were at least as virulent in these birds as the virulent prototype NY99.

Interestingly, mortality and viremia after WNV infection were found to be generally higher in the Carrion crow compared to the Western jackdaw. Despite the close genetic relationship, this suggests that Carrion crows are more susceptible to WNV than Western jackdaws, which could be related to differences in host innate immunity. Nonetheless, similar results were induced after inoculation with the FIN strain, as both Western jackdaws and Carrion crows did not experience mortality nor sustained viremia titers. Here it is tempting to speculate that a possible reason for the lack of more cases of WNV-FIN in either birds, horses or humans, is because the virus was not able to spread beyond infected mosquitoes due to the inability of the virus to be sufficiently amplified in corvid birds. However, more susceptibility studies with WNV-FIN in other bird species, possibly under more nature-mimicking conditions (e.g. infection by mosquitoes), could verify this hypothesis.

Through bird susceptibility studies information can be obtained that may be useful for risk-based approaches and WNV surveillance (Table 1). A risk-based assessment tool such as the Hazard Analysis Critical Control Points (HACCP) analysis [44] has proven to be particularly useful for infectious diseases such as food-borne illnesses as well as the highly pathogenic avian influenza virus (HPAI) strain H5N1. The latter helped to identify the key stages within the poultry trade chain that pose risks for the transmission of HPAI viruses among human and poultry populations [45]. For WNV it was also possible to identify the key stages at which transmission of the virus can take place; however, proposing critical limits and adopting mitigation strategies to limit virus transmission risks proved to be challenging. The WNV transmission cycle is highly complex and also dependent on many extraneous factors pertaining to the environment. For example, it has been hypothesized that WNV can be introduced via mosquitoes by airplane, wind, and boat; and via infected

humans, human-transported birds or other vertebrates, and migratory birds. The most likely and more easily controlled pathway of these can be considered introduction by airplane, as it is theoretically possible to fumigate airplanes. In fact, a study conducted at Amsterdam Schiphol Airport demonstrated that mosquitoes were occasionally found in aircraft cabins, including *Culex quinquefasciatus* [46], which is a competent vector for WNV in North America [47]. However, other introductory pathways, such as infected mosquitoes imported by wind, or infected migratory birds are considerably more difficult to influence. As a result, a passive but probably more realistic approach would be to conduct surveillance, and upon confirmation of WNV circulation, to control human and domestic animal behavior that leads to infection by mosquitoes. Therefore, apart from fumigating airplanes and controlling mosquito populations, it currently seems more realistic to focus the majority of the efforts on WNV surveillance and awareness campaigns.

The information obtained from our bird experiments may help to direct the nature of the surveillance practiced (Table 1). Monitoring susceptible bird species, by e.g. dead bird pathology and virological screening, in combination with measuring viremia and antibody seroprevalence can provide indications about the role of different bird species in the transmission of virus to mosquitoes (amplifier hosts). In addition, viral persistence studies may provide additional information about bird species that can participate in oral transmission (carcass scavenging). Identifying susceptible birds and virulent strains can also help to pinpoint potential risk areas (seroprevalence, amplifier hosts, virulence markers), while duration of viremia can give an indication about duration of infectiousness, and as a result the potential risk posed by a particular migrating viremic bird species.

Our data identified the Carrion crow as an important target for WNV live and dead bird surveillance. In addition, this bird species may transmit WNV as an amplifier host to mosquitoes -although this requires confirmation by reservoir host-mosquito experiments- and may also transmit the virus as a subject of carcass scavenging. In contrast, the lower susceptibility of the Western jackdaw makes the contribution of this bird to maintenance of WNV in the transmission cycle more doubtful, and as a result it is probably more suitable as a sentinel for WNV serosurveillance. On the other hand, the relatively high mortality observed in experimentally infected Carrion crows may render them less appropriate for serosurveillance. Obviously the use of other highly susceptible sentinel birds like domestic chickens may be practiced under circumstances of imminent threat. Nonetheless, to determine the real use of particular bird species as sentinels, proper antibody longevity studies will need to be conducted.

Parameter	Application to WNV surveillance or transmission
Mortality	Dead bird surveillance
Peak viremia titers	Live bird surveillance and potential transmission to mosquitoes
Duration of viremia	Estimation of duration of infectiousness (potential for virus introduction)
Organ viral titers	Oral transmission risk via carcass scavenging
Virus strain-specific virulence	Virulence marker surveillance
Antibody titers	Serosurveillance
Persistence in organs	Oral transmission risk via carcass scavenging

Table 1. The potential application for the data obtained in the bird susceptibility experiments

For virulence marker surveillance, our Western jackdaw and Carrion crow studies have found that a previously described virulence marker identified in the North American strain NY99 [18] is also relevant for Europe. To be more specific, the two closely related Italian strains, FIN and Ita09 (99.7% nucleotide identity) exhibited a contrasting virulence phenotype in both Western jackdaws and Carrion crows. The viral genome revealed the presence of a proline for Ita09 at the NS3-249 locus and for FIN a threonine at this position. In the study by Brault et al., introduction of a T249P in an attenuated Kenyan strain was found to be sufficient to generate a phenotype highly virulent to American crows, while a P249T introduction in the virulent NY99 strain resulted in an attenuated phenotype [18]. This suggests that the different virulence profile exhibited by FIN and Ita09 is related to the presence of the T249P substitution. Interestingly, it has been found that a series of hydrophobic residues (NS3-243-254) within the NS3 helicase are highly conserved among WNVs, with variation observed only at the NS3-249 residue. This residue is positioned at the terminus of the hydrophobic loop and would therefore be in a strategic orientation for direct interaction with alternative viral or host proteins, which may explain the influence of this locus on viral virulence. Furthermore, the positioning of this residue allows for any number of amino acids at this locus, which explains the potential for the variety of lineage-specific amino acid identities observed at this site [19].

In contrast to the importance of this determinant in avian virulence, the NS3-249 substitutions produced no different effect on LD_{50} or neurovirulence in a murine model [19]. The reason why this particular mutation has a dramatically attenuating effect in corvids but not in mice is not very clear. Interestingly, the NS3-249T even appears to have a variable effect in different bird species. To illustrate, this particular locus did not appear to be attenuating in house sparrows (*Passer domesticus*) as inoculation with NY99 (which possesses the proline) and KEN-3829 (known to possess a threonine at NS3-249) resulted

in similar mortality rates (38% vs 50%) [48]. In addition, the Morocco/2003 strain (possesses a threonine at the locus) was more virulent in red-legged partridges (*Alectoris rufa*) compared to the Spain/2007 isolate (possesses a proline) (70% vs 30%) [49].

However, it may be possible to explain this (bird) species-dependent effect in terms of temperature sensitivity. In fact, a study by Kinney et al. found that WNV-KEN-3829 was less efficient than NY99 at replicating at high temperatures (≥43°C), such as those measured in viremic American crows [50]. A follow-up study inoculating American crows and house sparrows with NS3-249 variants found that the NS3-249T mutant was highly attenuating for American crows, as has been demonstrated before, but not for house sparrows, as these birds developed mean peak viremia titers only approx. 10-fold lower than the NS3-249P variant on day 3 p.i. Replication of the different mutants in duck embryo fibroblast (DEF) cells at 44°C demonstrated a consistently temperature-sensitive phenotype for the Thr mutant in comparison to the Pro mutant [19]. These results might be translated into the ability of the aforementioned animal species to develop fever. To be specific, febrile corvids have been shown to develop fever of up to 45°C upon WNV infection [50], while house sparrows have been found to rather develop hypothermia following WNV infection (unpublished data, mentioned in [19]). As a result, this bird species-specific variation in pyrexia could explain the varying results observed with the NS3-249T variant. The fact that mice do not develop fever and can even become hypothermic during infection [51-54] also supports this theory. It would be interesting to see if this theory also holds true for house finches (*Haemorhous mexicanus*), as their body temperature during infection with WNV has been found to average 41.3°C, implying that these birds did not exhibit fever during peak viremia. In fact, several birds were even found to be hypothermic at this point [55]. In addition, monitoring the temperature of red-legged partridges during WNV infection and linking these to the results from the study by Sotelo et al. [49] may provide clues as well.

Many vector competence studies have been conducted using North American (NA) mosquitoes, in which mosquitoes such as *Culex pipiens*, *Cx. restuans* and *Cx. salinarius* were found to be efficient laboratory vectors [47, 56]. In contrast, European mosquito competence studies have only recently been conducted by Fros et al., which have shown that north-west European (NWE) *Culex pipiens* mosquitoes are competent vectors for WNV, as both a lineage 1 (NY99) and a lineage 2 (Greece-10) strain of WNV were able to effectively infect, replicate and disseminate in NWE mosquitoes from The Netherlands, as indicated by infection rate (percentage of infected mosquitoes after feeding on a blood meal containing virus from either WNV-lin2 or WNV-lin1) and transmission rate (indicated by percentage of mosquitoes in which virus was detected in the saliva) [57]. Using mosquitoes from North America (NA) as a control, it was also found that North American mosquitoes were a competent vector for the lineage 1 and 2 isolates; however, significant lower transmission rates were observed for lineage 2 in these mosquitoes compared to NWE mosquitoes. This difference was only apparent after oral injections rather than intrathoracic,

which suggests that WNV-lin2 escapes more effectively from the midgut epithelial cells in mosquitoes from NWE compared to those from NA. The observation that two geographically separated *Culex pipiens* populations have a notably different vector competence for WNV-lin2 suggests that a high degree of genotype-genotype specificity exists in the interaction between virus and vector. This was also noted for isolates collected from different regions in Florida (FL) in the United States that were assessed for vector competence in *Culex pipiens quinquefasciatus*, where transmission rates, body and leg titers appeared to vary between isolates and sometimes even between colonies [58]. This highlights that genetic differences may affect replication rates in mosquitoes and that it is therefore technically important to evaluate vector competence for each different WNV isolate, which in turn may help us understand vector-virus interactions and their role in complex transmission cycles in nature.

The study of Fros et al. [57] demonstrating that mosquitoes in an area of Europe where WNV has not yet been detected are competent to replicate the virus, in combination with our data showing that European birds from the same area (**Chapters 5** and **6)** are susceptible to WNV, indicate that intrinsic factors are not likely to be the limiting factor for the absence of WNV in the northern areas of Europe. Instead, extrinsic environmental factors such as temperature could be more likely players in the current distribution of WNV in Europe. Fros et al. [57] hypothesized that temperature is limiting the vector competence of European mosquitoes for WNV transmission, and tested this by incubating Lin2-infected NWE and NA mosquitoes at three different temperatures, 18, 23 and 28ºC. The first temperature matches the average temperature of the Netherlands, the latter the average temperature during the warmest period in Greece (July and August), which also corresponds to the peak in WNV amplification and transmission [59], and the middle temperature is the intermediate temperature of the two. Higher temperatures significantly increased the percentage of WNV-infected mosquito vectors, for both the NWE and NA mosquitoes. Furthermore, comparison of the spatial arrangement of recent WNV outbreaks in Europe per annum and the corresponding mean temperature during peak transmission season concurs with the hypothesis, as a strong correlation was found between WNV outbreaks and the mean diurnal summer temperature throughout Europe. To be specific, WNV outbreaks have occurred at mean temperatures of 24.6ºC, 25.3ºC and 23.5ºC. Taking the temperatures of locations where individual outbreaks have occurred gives an indication of the average summer temperatures at which there is an elevated risk for WNV activity. Nonetheless, this information does not tell us why the spread and circulation of WNV have still been relatively limited in the south of Europe. Importantly, the mild winters in southern Europe should potentially allow WNV persistence via mosquito overwintering.

Furthermore, we have also not established that viremic European birds, such as the Carrion crow, can transmit the virus to NWE mosquitoes, and that in turn NWE mosquitoes can infect susceptible birds. As a result, reservoir competence studies still need to be conducted using Carrion crows and potentially common blackbirds (*Turdus merula*), if they may be considered the most likely counterpart of the American robin (*Turdus migratorius*;

see below) and susceptibility experiments prove to be successful, in combination with NWE as well as Mediterranean *Culex pipiens*. Even though we have focused the majority of our experiments on northwestern Europe due to the lack of WNV activity in this region, we also do not understand the current distribution of WNV in southern Europe. Several species of wild birds found in Europe, such as eagles [60], sparrow hawks, goshawks, geese, and falcons [61-63] have been shown to be susceptible by analyzing carcasses, while experimental infections of red-legged partridges (*Alectoris rufa*) [49] and house sparrows (*Passer domesticus*) [64, 65] have demonstrated varying (virus strain-dependent) susceptibility. Infection of the red-legged partridge with Morocco/2003 showed the highest mortality (70%) [49]; however, in general no species of bird from southern Europe have shown a similar susceptibility as the Carrion crow, and susceptibility studies in other bird species may be warranted. Even though the Carrion crow is not found in southeastern Europe, the closely-relatedness of the Hooded crow, a bird that is found in southeastern Europe in regions where WNV is often endemic, suggests that our studies may be highly applicable to this area as well. Nonetheless, reservoir competence studies with other susceptible birds indigenous to Europe are warranted.

Despite the relevance of bird susceptibility studies, other factors such as mosquito behavior and density may also be important in explaining the current limited distribution of WNV in Europe. Even though we have shown that Carrion crows are highly susceptible to WNV, an important question that remains is whether they are also the preferred host for a blood meal for competent mosquitoes in Europe. Analyses of data from the mid-Atlantic to Colorado that combined host abundance and mosquito feeding data with host infectiousness indicated that even though introduced house sparrows (*Passer domesticus*) and crows are the more abundant and/or highly infectious species, they actually play a minor role in WNV transmission [66-68]. This is mainly because 30-80% of mosquito feedings by the dominant WNV vectors (*C. pipiens*, *C. restuans*, and *C. tarsalis*) in the US are on American robins (*Turdus migratorius*), despite these birds comprising only 1-20% of the avian communities studied. In contrast, the abundant crows only make up a small fraction of all mosquito blood meals and house sparrows are rarely fed on by mosquitoes relative to their abundance, which results in few bites per individual and inefficient transmission [69].

Furthermore, a European study conducted by Rizzoli et al. specifically looked at the host feeding preference of wild *Cx. pipiens* in a hotspot of virus emergence, namely northern Italy, and found that this mosquito had a clear feeding preference for the common blackbird, both collected from the wild and in the lab [70], suggesting a potential important role for this species in the WNV epidemiology in Europe, and warranting future susceptibility studies and increased monitoring of these birds. Therefore, as mentioned earlier, even though we have demonstrated that Carrion crows are highly susceptible and could therefore function as a sentinel and potentially also as an amplification host, if the majority of the mosquitoes in Europe preferentially feed on other birds, we may be missing important birds that should be incorporated into a surveillance plan. As a result, it may be more strategic to now first determine the geographical abundance of the respective bird and

mosquito species as well as the feeding preferences of NWE and Mediterranean mosquitoes found in areas of WNV activity, for particular bird species. This could be performed with field collections of mosquitoes and subsequent determination of their blood meals, followed by experimental infections (via needle or mosquito bite) of the preferred bird species determined in these studies, in order to determine their susceptibility and potential role as an amplification host.

Moreover, the role of mosquitoes such as *Culex pipiens* and *Culex molestus* hybrids as potential bridge vectors should not be neglected. In fact, a study by Vogels et al. has so far shown that *Culex molestus*, a mosquito with a strong preference for mammalian blood, including that of humans, is more abundant during winter than previously thought, and also displays feeding behavior towards humans. In addition, a large percentage of *molestus* hybridization is occurring in The Netherlands (6-15%), meaning that the potential bridge vector for WNV is present here [71]. More studies on Dutch mosquito populations are required, which should include more extensive mapping of the different mosquito species, as well as examining their blood meal preference, including specific bird species. Studying the feeding behavior of a potentially high-risk mosquito species, such as the bridge vectors, could direct proper surveillance methods and strategies for Europe.

To conclude this section, results obtained from our bird studies combined with published mosquito data tells us that northwestern Europe is an area that could be at risk for the introduction of WNV. In the Netherlands, only reactive surveillance exists due to the associated high costs, and proactive surveillance will probably only be initiated after identification of an animal or human case. It is currently therefore unclear whether WNV is already circulating in the Netherlands. A study conducted by Reusken et al. in which seven potential WNV vector species were collected at a potential high-risk area (Oostvaardersplassen) found no evidence for WNV circulation in mosquitoes [72]. However, more mosquito surveillance studies would be needed to give a better indication of the presence of WNV in the Netherlands. We first propose to conduct serological studies for WNV on wild and migratory birds that are already sampled as part of the avian influenza A surveillance network established by the Erasmus Medical Centre (EMC) in Rotterdam. If serologically positive birds are found we next propose to collect mosquitoes in the areas from which those seropositive birds were collected. In addition, WNV RNA surveillance among mosquitoes should be considered, along with identification of the origins of mosquito blood meals, and this also in combination with future bird susceptibility studies. Even though our bird studies may prove to be useful in reactive or proactive WNV surveillance development for mosquitoes and birds, they have elucidated several remaining research gaps in the complex enzootic transmission cycle of WNV.

Part 3: Vaccine

The neutralizing antibody response against the WNV E protein is most frequently used as the correlate of protection. The E protein is a structural protein involved in the attachment of WNV to the receptor of target cells as well as membrane fusion for mediation of cell entry [73]. Most of the neutralizing antibodies against flaviviruses recognize the E protein, and studies using mouse monoclonal antibodies suggest that those against DIII have a higher neutralizing potency than those against other sites of the molecule [74, 75]. However, another population of antibodies exists that also bind to the prM/M protein [75-77]. Different modalities of antigen presentation have been identified, such as the E protein in a soluble truncated form lacking the membrane anchor region, which was found to be highly immunogenic when expressed by vaccinia virus vectors or recombinant baculoviruses [78-80]. On the other hand, another study demonstrated the immunogenicity and protective capacity of a membrane-anchored WNV protein in BALB/c mice [81]. Lastly, coexpression of the E and M protein has been found to result in the production of virus-like particles (VLPs), which are highly immunogenic non-infectious virus particles [82], that were able to protect mice and horses from neuroinvasive disease after WNV challenge [83, 84].

Although currently different effective WNV vaccines for horses have been developed (for review see [85]), to date, no vaccine for use in humans has been approved after rigorous testing in clinical trials. However, recently the NIH has announced that an experimental vaccine that should protect against West Nile virus will enter human clinical trials. This vaccine is a hydrogen peroxide-inactivated virus that has been shown to protect both young and aged C57BL/6 as well as aged BALB/c mice after intracranial challenge with a heterologous virulent North American WNV strain. In addition, using both wild-type and human HLA-A2 transgenic C57BL/6 and BALB/c adult mice, it was shown that the H_2O_2-WNV-KUNV vaccine also generates a polyfunctional antigen-specific $CD8^+$ T cell response [86].

Nonetheless, one vaccine platform that is being increasingly tested in animal and human clinical trials is based on the orthopox virus vector Modified Vaccinia virus Ankara (MVA), which is a highly attenuated strain of vaccinia virus (VACV), originating from the chorioallantois membrane vaccinia virus Ankara (CVA) via an extensive series of infections in primary chicken embyrofibroblasts (CEF). Propagating this virus through more than 570 CEF passages resulted in a massive loss of genetic information that affected many of the VACV virulence and immune evasion genes [87, 88] and resulted in MVA becoming highly restricted to cells of avian origin and unable to productively replicate in most mammalian cell cultures [88-90]. This growth deficiency is due to a late block in virion assembly, resulting in the production of immature virus particles. As viral early, intermediate, and late gene products are readily synthesized in non-permissive mammalian cells, recombinant MVA has been proposed as an antigen-expressing vector with an excellent safety profile [90] that currently is being tested for many applications, having

shown promising preclinical and clinical results for candidate vaccines against viruses, cancers and even allergies [91-102].

For the production of stable heterologous antigens using MVA as a vector virus, genes are placed under transcriptional control of poxvirus-specific promoters. The subsequent presentation of antigenic peptides by professional antigen presenting cells via the MHC class I pathway leads to the activation of CD8+ T cells [103]. The synthesis of long-lived antigens is an advantageous feature since stable proteins as immunogens are superior at efficiently activating immune responses *in vivo*. This in fact appears to be the case in the induction of antibody responses to typical pathogen surface proteins, such as influenza virus hemagglutinin, as well as for eliciting MHC class I restricted antigen-specific T cells to internal antigens [104-106]. Another feature of MVA that is highly advantageous for future vaccine applications is the notable immunogenicity when compared directly to replication-competent VACV [107, 108]. This observation is most likely related to the particular ability of MVA to induce or upregulate important host responses that activate the immune system early on [109-112].

In **Chapter 7**, we assessed the efficacy of an MVA based candidate vaccine in protecting against WNV infection. We used the WNV E protein as immunogen for activation of the immune response. Therefore we generated four recombinant MVA-WNVs (prM/ME, E_{sol}, E_{TMV}, E_{TMC}) delivering the WNV E protein in different forms in order to mimic previously described vaccine optimization strategies [78-81]. In our study, we found that all wild-type C57BL/6 mice vaccinated with each of the recombinant MVA's exhibited homologous and heterologous neutralizing antibody titers on day 56 post-vaccination and survived subsequent lethal challenge with both lineage 1 and 2 WNVs. In addition, no virus was detected in the brain of mice euthanized on day 8 p.i. Furthermore, vaccinations of HLA-A2.1-/HLA-DR1-transgenic H-2 class I-/class II mice elicited SV9 epitope-specific IFN-γ-secreting CD8+ T cell responses. This immunodominant HLA-A2-restricted epitope that falls within the E protein SVG9 [SVGGVFTSV]) has also been found during WNV infections in humans [113-115] and is conserved between members of the *Flaviviridae* family, including WNV-NY99 and WNV-KUNV. The use of these transgenic mice can give a good indication of whether humans are also likely to elicit protective CD8+ T cell responses upon WNV infection.

It is important to consider that the specificity of an antibody response for a single epitope may not be sufficient to neutralize all epitope (antigenic) variations of a challenge virus strain and sometimes even a number of quasispecies within a single virus stock [116]. This also appeared to be the case for our study, as despite the presence of high homologous neutralizing antibody titers against WNV-Ita09, the heterologous neutralizing antibody titers elicited against WNV-578/10 were approx. 2-fold lower. This is something that we also observed previously using sera from mice that survived infection (collected on day 14 p.i.) with WNV-NY99, FIN and Ita09 versus WNV-578 (data not shown). Such a phenomenon is not unusual, however, as, for example, a rabies vaccination study in which serum titers of human subjects measured *in vitro* against rabies challenge virus strains

found that in the majority of subjects, higher titers were detected against the homologous strain [117]. Several studies have explored the cross-protective efficacy of several WNV vaccine candidates; however, some of these, such as the study by Magnusson et al. did not specifically investigate the cross-protective capacity of their vaccine against their two lineage 1 and 2 viruses via *in vitro* cross-neutralization assays [118]. Nonetheless, lower heterologous neutralizing antibody titers have been described between WNV lineage 1 (Eg101) and several proposed lineage 5 isolates from India due to substantial genetic variation [119]. In addition, it is an even more common phenomenon across flaviviruses, where for example, Martina et al. witnessed lower heterologous neutralizing antibody titers against JEV upon vaccination with WNV-DIII [120]. It therefore appears that genetic variation between lineage 1 Ita09 and lineage 2 578/10 renders these viruses substantially different to the extent that it leads to lower heterologous neutralizing antibody titers.

The peak of vaccine induced IgG antibodies is often reached within 4-6 weeks after primary immunization and is controlled by the intensity of B cell differentiation into plasma cells, and therefore also by the magnitude of germinal center responses, i.e., the quantity and quality of the complex interactions between DC, B cell, follicular helper T cells (Tfh) and follicular dendritic cells (FDCs) interactions. The short life span of these plasma cells results in a rapid decline of antibody titers, but booster exposure to antigen will reactivate the immune memory and result in a rapid (<7 days) increase of IgG antibodies. Short-lived plasma cells will reach and maintain peak antibody levels until approx. 4 weeks later, after which serum antibody titers decline as rapidly as following primary immunization. However, long-lived plasma cells that have reached survival niches in the bone marrow will continue to produce antigen-specific antibodies, which decline with slower kinetics. Given the kinetics of antibody responses, we chose to measure neutralizing antibody titers on day 56, as well as challenging the mice on this day. This allowed us to measure antibody titers after they have reached their postulated peak values, representing a realistic indication of the elicited immune response.

MVA have induced unprecedented frequencies of vaccine-induced antigen-specific T cells during recent human clinical trials. These were observed after priming with Bacille Calmette-Guérian (BCG) and boosting with MVA-85A [101, 121], after priming with natural A virus infection and boosting with MVA-NP+M1 [122]; and priming with a recombinant non-replicating chimpanzee adenovirus vector [123] and boosting with rMVA. These heterologous prime-boost regimens are the only vaccines that induced mean IFN-ɣ ELIspot responses above 1000 spot-forming cells per million peripheral blood mononuclear cells (sfc/million PBMC) in humans, which include some of the highest responses ever measured in humans and well above what has been achieved using individual vectors, such as recombinant adenovirus alone [124, 125] or recombinant canarypox virus (ALVAC) with protein-in-adjuvant [126, 127]. Moreover, our study indicated a specific CD8+ T cell response as prime-boost applications were found to greatly increase the magnitude of WNV-SV9 specific IFN-γ-producing memory T-cells in splenocytes.

Nonetheless, many studies have been published testing the efficacy of various types of vaccines in animal models for the prevention of WNV infection. After the first cases of WNV were detected in New York more than 15 years ago, a large amount of money was invested into understanding and potentially controlling this virus. A lot of progress has been made since then, but interestingly, measures to prevent or treat WNV infection have stalled. In fact, a chimeric vaccine based on the backbone of Yellow-Fever 17D human vaccine expressing the prM and E genes of the NY99 strain (ChimeriVax-WN01) made it through a phase II trial [128] but was suspended when Sanofi Pasteur bought Acambis in 2008. During this year, West Nile cases had dropped from 2006 to 2008, and the company had decided to focus on other priorities, including a dengue vaccine. Clinical trials on treatment, including a monoclonal antibody, interferon, and immunoglobulin, have also been delayed for similar reasons; researchers were not able to enroll enough trial volunteers [129].

As a result, one important question related to vaccine development for WNV is whether there is actually a market. Clinical trials are very costly and the sporadic nature of WNV outbreaks makes it very difficult to get enough volunteers for trials. In addition, the virus usually causes flu-like symptoms and less than 1% of those infected actually develop serious neuroinvasive disease. Due to this, it might only be useful to vaccinate the elderly and perhaps highly exposed groups such as outdoor workers. However, even in this case it is still difficult to do any kind of patient studies when outbreak sites are uncertain [129].

Nonetheless, recent evidence suggests that WNV can cause kidney disease in younger people. A study by Nolan et al. found that in a cohort of 139 people with a mean age of 57 who had tested positive for WNV, 40% had evidence of kidney disease years after infection [130]. If this is indeed the case, then developing an effective vaccine for WNV could become more of a priority, and might also involve vaccinations of younger populations. Whether this will improve the market certainty for a WNV vaccine remains to be determined. As a result, the most cost-effective remedy against WNV infection may continue to be mosquito repellent and screen doors.

This thesis provides invaluable information on the virulence of European WNV strains in corvids as well as in a mammalian model, and demonstrates the efficacy of a vaccine that would be applicable to Europe where both lineage 1 and 2 WNV strains are circulating. In the end, through either surveillance, risk-based approaches or immunization, Europe is now slightly more prepared for WNV outbreaks that may arise in the future.

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Chapter 9

Nederlandse Samenvatting

Het West Nile virus (WNV) is een flavivirus dat de West-Nijl ziekte veroorzaakt. Zoogdieren zoals mensen en paarden kunnen ziek worden van dit virus, en bij een klein percentage van de gevallen kan dit tot dodelijke encefalitis of meningitis leiden. Dit virus wordt door muggen verspreid die geinfecteerd worden met het virus voornamelijk door zich te voeden op geïnfecteerde vogels, die het virus amplificeren. Vogels zijn daarbij ook vaak het slachtoffer van dit virus. WNV was voor het eerst geïsoleerd in 1937 uit een zieke vrouw die woonachtig was in de West Nile provincie van Uganda. Het virus werd echter als apathogeen beschouwd omdat het in de meeste gevallen niet leidde tot klinische symptomen. In 1999 werd het virus voor het eerst gedetecteerd in de Verenigde Staten en dit leidde uiteindelijk tot een uitbraak waarbij veel menselijke slachtoffers vielen. Deze uitbraak ging gepaard met hoge mortaliteit onder vogels, met name de kraaiachtigen. Doordat kraaien in de VS erg gevoelig zijn voor WNV, wordt mortaliteit onder deze vogels vaak gebruikt om WNV activiteit vroegtijdig op te sporen.

Het virus heeft zich uiteindelijk razendsnel weten te verspreiden over de gehele Verenigde Staten binnen een periode van 5 jaar. In Europa is het WNV tot voor kort nooit de oorzaak van uitbraken geweest en waren de meeste ziektegevallen mild. Sinds 1996 heeft het virus echter verscheidene uitbraken veroorzaakt in Europa, in landen zoals Roemenië, Tsjechië, Hongarije, Italië en Griekenland, die gepaard gingen met gevallen van neuroinvasieve ziekte in mensen of paarden. Deze uitbraken hebben echter nooit geleid tot een uitgebreide verspreiding van het virus over Europa en de distributie van het virus is eerder focaal. Omdat de transmissie cyclus van het WNV opgemaakt is uit zowel een vector (de mug) en een gewervelde gastheer (de vogel) is deze uitermate complex, en is het bestuderen van de verspreiding van het virus een uitdaging. Het doel van dit promotieonderzoek was om te onderzoeken waarom de verspreiding van WNV binnen Europa gering is en of dit virus een mogelijk risico vormt voor verdere verspreiding binnen Europa. De volgende vragen kwamen hierbij aan de orde:

1) Zijn de Europese stammen van WNV minder virulent dan de Amerikaanse variant?
2) Zijn vogels in Europa minder gevoelig voor infectie met WNV?
3) Zou een vaccin bescherming kunnen bieden tegen infectie met beiden lineage 1 en lineage 2 virus stammen?

Hoofdstuk 2, 3 en 4 vallen onder het gedeelte “pathogenese” van dit proefschrift. **Hoofdstuk 2** beschrijft de virulentie en het neuroinvasieve vermogen van een selectie Europese stammen van het WNV (twee uit Italië, een uit Hongarije) die gemeten werden aan de hand van een aantal parameters, namelijk *in vitro* replicatie en fitness in neuroblastoma cellen, gevolgd door intraperitoneale infectie in C57BL/6 muizen en meting van *in vivo* mortaliteit (LD_{50}), hoeveelheid virus in de hersenen en ruggenmerg, en respons op infectie in de hersenen met betrekking tot markers die een rol spelen in neurodegeneratie. Alle stammen bleken neuroinvasief en neurovirulent te zijn in deze

muizen. De stam uit Italië die verantwoordelijk is geweest voor een grote uitbraak in 2009, Ita09, vertoonde de hoogste fitness *in vitro*. De stam uit Hongarije, 578/10, was het meest virulent in muizen, met de laagste LD_{50} waarde en grootste hoeveelheid RNA in de hersenen. De stam FIN uit Italië vertoonde de minste lethaliteit in muizen vergeleken met de andere twee stammen. Sequentie-analyse toonde aan dat op nucleotide niveau Ita09 en FIN slechts 99.7% verschillen; echter is Ita09 iets virulenter gebleken dan FIN in onze studie.

Om virulentie beter te kunnen meten, werd in **Hoofdstuk 3** een qRT-PCR opgezet die nauwkeuriger dan bestaande tests het negatief- en positief-strengs RNA van het WNV kan quantificeren. Hiermee zouden we de replicatie snelheid van verschillende virusstammen beter kunnen meten en ook het cel tropisme kunnen bepalen. Om de bruikbaarheid en specificiteit van deze qRT-PCR vast te leggen is deze gebruikt om de toename van het WNV in de microglia cellijn BV-2 te meten over een periode van 48 uur, omdat het tropisme van het WNV voor microglia nog onduidelijk is. Er was een duidelijke toename van negatief- en positief-strengs RNA te zien, wat aangaf dat deze cellijn permissief is voor het WNV. De qRT-PCR is ook toegepast op een muisexperiment waarin 9-dagen oude muizen zijn geinfecteerd met WNV-NY99 en op dag 3, 4, 5 en 6 zijn hiervan de hersenen verwijderd voor RNA-isolatie. Ook hier was een toename van negatief- en positief-strengs RNA te zien in de tijd. Een mogelijk nadeel van deze qRT-PCR zou de gevoeligheid voor het negatief-strengs RNA kunnen zijn, omdat deze varieerde van 10^2 tot 10^3. Deze qRT-PCR zou hierdoor mogelijk niet geschikt kunnen zijn voor het meten van negatief-strengs RNA in de vroege stadia van infectie.

Het doel van **Hoofdstuk 4** was om virulentie markers voor een lineage 2 WNV te identificeren. Deze zijn geselecteerd uit de literatuur aan de hand van *in vitro* en/of *in vivo* attenuatiemarkers die eerder gevonden zijn voor lineage 1. Vijf verschillende moleculaire clones waren ontworpen op basis van de Hongaarse virusstam 578/10, met elk een mutatie in een nonstructureel eiwit (NS1-P250L, NS2A-A30P, NS3-P249H, NS4B-P38G, en NS4B-E249G). Na intraperitoneale infectie van deze mutanten in C57BL/6 muizen bleek de NS1-P250L mutatie hevig attenuerend te zijn (0% mortaliteit). Ook *in vitro* infectie van een Vero E6 cellijn met deze mutant leidde tot significant lagere infectieuse virus titers dan met het wilde-type. Om deze attenuatie beter in kaart te brengen is hier de qRT-PCR die ontwikkeld is in **Hoofdstuk 3** op toegepast. De NS1 mutant bleek significant minder negatief-strengs RNA te produceren waardoor ook de hoeveelheid positief-strengs RNA minder was. Dit zou kunnen bijdragen aan de attenuatie van deze mutant *in vivo*.

Hoofdstuk 5 en 6 vallen onder het gedeelte “gastheer” van dit proefschrift en onderzoeken de vatbaarheid van kraaiachtigen in Nederland voor WNV-infectie. Kauwen (**Hoofdstuk 5**) en zwarte kraaien (**Hoofdstuk 6**) werden geinfecteerd met vijf verschillende stammen, WNV-NY99, Greece-10, FIN, Ita09 en 578/10. Zwarte kraaien bleken gevoeliger te zijn voor het WNV dan de kauwen omdat deze een hogere mortaliteit vertoonden (tussen de 83-100% voor kraaien vergeleken met 30-60% voor kauwen), hogere virustiters in het bloed (ong. 10^3 RNA kopieën/mL) en meer verspreiding van het virus naar de organen (10^2

RNA kopieën/g). Een van de virusstammen, WNV-FIN, veroorzaakte geen mortaliteit in beiden de kauw en de kraai en ook significant lagere virus titers in het bloed. Een alignment van de nauw verwante FIN en Ita09 gaf een marker voor virulentie weer die mogelijk een rol heeft gespeeld in dit experiment; NS3-249. Deze virulentie marker was al eerder geidentificeerd omdat een mutatie in deze locus de virulentie van een geattenueerde stam verhoogde in de Amerikaanse kraai. Deze marker zou mogelijk ook een rol kunnen spelen in de verspreiding van bepaalde stammen van het WNV binnen Europa. De experimenten in **Hoofdstuk 5 en 6** geven de indicatie dat de zwarte kraai gebruikt zou kunnen worden voor WNV surveillance binnen Europa aan de hand van vogelsterfte, terwijl de kauw gebruikt zou kunnen worden als 'signaalvogel'. De daadwerkelijke rol van deze vogels in amplificatie van het WNV is nog niet duidelijk, maar deze studies suggereren dat de zwarte kraai een goede amplificatie gastheer zou kunnen zijn, terwijl de titers die het WNV teweegbrengt in kauwen waarschijnlijk niet hoog genoeg zullen zijn hiervoor.

Het een na laatste hoofdstuk, **Hoofdstuk 7**, valt onder "interventie strategieën". Hierin is de effectiviteit van een MVA-gebaseerd vaccin getest voor bescherming tegen infectie met lineage 1 en 2 WNVs in C57BL/6 muizen. Het WNV envelop eiwit werd gebruikt als basis voor de ontwikkeling van vier recombinante MVAs; een die het membraan en envelop eiwit afschrijft (prM/ME), twee die de envelop met een transmembraan gedeelte afschrijven (van het Vaccinia Ankara virus (E_{TMV}) of het Chikungunya virus (E_{TMC})) en een die een oplosbare versie van het E eiwit afschrijft (E_{sol}). Alle muizen gevaccineerd met een van de vier constructen waren beschermd tegen mortaliteit na infectie met een lethale dosis van beiden WNV-Ita09 en 578/10. Bovendien was virus ook afwezig in de hersenen op dag 8 na infectie van alle gevaccineerde muizen. Op dag 56 na vaccinatie hadden alle gevaccineerde muizen neutralizerende antilichamen in het bloed tegen Ita09, maar ook tegen 578/10, wat heterologe bescherming aangeeft en de bescherming van de muizen tegen infectie met WNV-578/10 verklaart. Echter waren de neutralizerende (heterologe) antilichaam titers wel lager. Verder induceerde vaccinatie van HLA-A2.1-/HLA-DR1-transgene H-2 class I-/class II muizen een SV9 epitoop-specifieke IFN-γ secreterende CD8+ T cel respons. Dit is een immunodominante epitoop dat is gevonden na WNV infectie in mensen en deze muizen geven hierdoor een goede indicatie dat de WNV-MVAs ook een beschermende CD8+ T cel respons in mensen zou kunnen induceren.

Samenvattend heeft het onderzoek in dit proefschrift geleid tot een aantal nieuwe inzichten betreft de aanwezigheid van en de risico's geassocieerd met WNV in Europa. De drie bestudeerde Europese stammen van WNV zijn allen neuroinfasief en neurovirulent in muizen en zouden hierdoor waarschijnlijk een even grote bedreiging kunnen vormen voor de mens als de NY99 stam in Amerika. Dit geldt ook voor het lineage 2 virus uit Hongarije dat bestudeerd is en geeft weer dat lineage 2 stammen van het WNV die mogelijk in de toekomst opkomen ook virulent kunnen zijn. Dit proefschrift heeft ook aangetoond dat twee van de kraaiachtigen die in Nederland voorkomen vatbaar zijn voor infectie met het WNV en dat de zwarte kraai een mogelijke rol zou kunnen spelen in de verspreiding van het virus.

Het WNV zou dus een bedreiging kunnen blijven vormen voor Europa en de resultaten zouden onder andere gebruikt kunnen worden voor het sturen van WNV surveillance methodes binnen Europa. Mocht het WNV een nog groter probleem worden voor Europa in de toekomst, dan zou het kandidaat MVA-vaccin dat bestudeerd is in deze thesis mogelijk verder ontwikkeld kunnen worden aangezien het bescherming heeft geboden tegen infectie van zowel een lineage 1 als 2 virusstam.

Chapter 10

English Summary

West Nile virus (WNV) is a flavivirus that causes West Nile disease and affects mammals such as humans and horses, of which a small percentage develops WN neuroinvasive disease that may lead to fatal encephalitis or meningitis. The virus is transmitted by mosquitoes that obtain the virus from infected birds, which are the amplification hosts. WNV was first isolated in 1937 from a febrile woman in the West Nile province of Uganda. Despite a small number of cases in Africa, the virus was generally considered non-pathogenic as it led to asymptomatic infection in most cases. However, in 1999, the virus was detected for the first time in the United States in New York where it caused a large outbreak of neuroinvasive disease and several deaths. Within 5 years' time, the virus spread across the entire United States. Several outbreaks of WNV in the US were also characterized by a high mortality among birds, in particular corvids. As crows were found to be very susceptible to WNV and bird mortality often preceded human outbreaks, mortality among these birds is often still used to detect and predict WNV activity in the US.

In Europe, WNV has never been the cause of large outbreaks and most clinical cases were of mild disease. However, since the first outbreak in Romania in 1996, WNV has spread and been responsible for cases or outbreaks in countries such as Czech Republic, Hungary, Italy and Greece, which also included cases of neuroinvasive disease in humans or horses. Nonetheless, these outbreaks have never led to an extensive spread of the virus across Europe and its distribution is more focal and contained to the south and eastern parts of the continent. As the enzootic transmission cycle of WNV is comprised of both a vector (mosquito) and a vertebrate amplification host (birds) it is rather complex, and also a challenge to completely understand.

The aim of this thesis was to investigate the limited spread of WNV within Europe and to assess whether this virus could actually present a greater risk to Europe in the future. As a result, the following questions were addressed:

1) Are European strains of WNV less virulent than the American strains?
2) Are birds in Europe less susceptible to infection with WNV?
3) Would a vaccine be able to protect against infection with both lineage 1 and 2 WNVs?

The section "pathogenesis" of this thesis comprises **Chapters 2, 3 and 4. Chapter 2** characterizes the virulence and neuroinvasive potential of a selection of European strains of WNV (two from Italy, one from Hungary) using parameters such as *in vitro* replication and fitness on neuroblastoma cells, followed by intraperitoneal infection of C57BL/6 mice and measuring the mortality (LD_{50}), viral burden in the brain and spinal cord, as well as response to infection in the brain using genetic markers that are known to play a role in neurodegenerative disease. The results indicated that all the WNV strains were neuroinvasive and neurovirulent in the mice. The Ita09 strain, which was responsible for a large outbreak in Italy in 2009, demonstrated the highest fitness *in vitro*. The strain from Hungary, 578/10 was the most virulent in mice as it had the lowest LD_{50} value as well as

the highest viral RNA burden in the brain. The other Italian strain, FIN, showed the lowest mortality in mice compared to the other two strains. Despite a 99.7% similarity at the nucleotide level between these two strains, Ita09 appeared to be slightly more virulent compared to FIN in this study.

In order to improve the ability to measure virulence, a qRT-PCR assay was set-up in **Chapter 3** that more accurately quantifies the negative and positive RNA strands of WNV. By this means, this assay is able to measure the replication rate of WNV as well as determining its cellular tropism. To assess the *in vitro* applicability and utility of this qRT-PCR, it was used to measure the increase in WNV-specific RNA strands over the course of 48 hours in the microglial cell line BV-2, as infection of microglial cells by WNV is elusive. A clear increase in both positive- and negative-stranded RNA was seen over time, which indicated that this cell line is permissive to WNV. The qRT-PCR was also used in a mouse experiment in which 9-day-old mice were infected with NY99, and mouse brains were sampled on days 3, 4, 5, and 6 for RNA isolation. In this experiment an increase in both negative- and positive-stranded RNA was also seen over time, suggesting that this tool might also be appropriate for studying *in vivo* replication. A potential drawback of this assay, however, might be its sensitivity, as in this study the detection limit was found to vary between 10^2-10^3 RNA copies. As a result, this assay might not be useful for measuring negative-stranded RNA in the early stages of infection.

The aim of **Chapter 4** was to identify virulence markers of a lineage 2 strain of WNV. Markers were selected from the literature which have already been shown to attenuate lineage 1 WNV strains, either *in vitro* or *in vivo.* Five different molecular clones were designed based on the Hungarian lineage 2 strain 578/10, each harboring a mutation in a nonstructural protein (NS1-P250L, NS2A-A30P, NS3-P249H, NS4B-P38G, and NS4B-E249G). After intraperitoneal infection of these mutants in C57BL/6 mice, the NS1-P250L mutant appeared to be the most attenuated giving 0% mortality of these mice at both the low and high dose. *In vitro* infection of a Vero E6 cell line with this mutant also showed significantly lower infectious virus titers compared to the wildtype. This directed the focus to studying the replication competence of the NS1 mutant by measuring the amount of positive- and negative-stranded RNA produced in Vero E6 cells in 48 hours using the qRT-PCR developed in **Chapter 3**. It was found that the NS1 mutant produced significantly less positive and negative strands of RNA as compared to the wildtype, which might explain why this mutant was attenuated *in vivo* as well.

Chapters 5 and 6 are part of the section "host" of this thesis, which investigates the susceptibility of corvids to WNV in the Netherlands. Jackdaws (**Chapter 5**) and carrion crows (**Chapter 6**) were experimentally infected with five different strains of WNV, namely NY99, Greece-10, FIN, Ita09 and 578-10, of which Greece-10 is the strain that was responsible for the large outbreak in humans with many cases of neuroinvasive disease in Greece in 2010. Carrion crows appeared to be more susceptible to WNV than the jackdaws as they demonstrated higher mortality (83-100% for crows compared to 30-60% for jackdaws), higher peak viremia titers in the blood (approx. 10^3 RNA copies/mL) and more

dissemination of virus to the organs (10^2 RNA copies/g). However, FIN did not induce any mortality in both the jackdaws and crows, and birds infected with this strain had significantly lower viremia titers as well. An amino acid alignment of FIN and Ita09 identified a differential marker between the two strains, NS3-T249P, which has already been linked to augmented virulence in the American crow. This virulence marker may therefore play a role in the spread of particular strains of WNV within Europe as well. The experiments in **Chapters 5 and 6** indicate that the carrion crow could potentially be used for WNV surveillance within Europe as part of 'dead bird surveillance', while the jackdaw could be used as a sentinel in serosurveillance. Even though the role of these birds in amplification of the virus still needs to be experimentally confirmed, our studies suggest that the carrion crow could be an efficient amplification host while jackdaws might not sustain viremia titers above the threshold required for efficient infection of mosquitoes ($10^{5.2}$ $TCID_{50}$).

The last chapter, **Chapter 7**, is part of the thesis section "intervention strategies". Herein, the effectiveness of an MVA-based vaccine for the protection against infection with both lineage 1 and 2 WNVs is evaluated in C57BL/6 mice. The envelope protein of WNV was used as a basis for the development of four recombinant MVAs; one that transcribes the (pre)-membrane and envelope proteins, two that code for the envelope harboring the transmembrane part of either the wildtype Vaccinia Ankara virus (E_{TMV}) or Chikungunya virus and one that transcribes the soluble part of the E protein (E_{sol}). All constructs protected mice from infection with a lethal dose of either Ita09 (lineage 1) or 578/10 (lineage 2) 56 days after vaccination. In addition, no virus was detected in the brains of vaccinated mice as compared to the control group on day 8 post-infection. Furthermore, neutralizing antibodies against Ita09, but also against 578/10 were found in the blood on day 56 post-vaccination, which may explain the heterologous cross-protection witnessed *in vivo*. However, heterologous neutralizing antibodies titers were found to be lower compared to the homologous titers. Lastly, vaccination of HLA-A2.1-/HLA-DR1-transgenic H-2 class I-/class II mice induced an SV9 epitope-specific IFN-γ-secreting CD8+ T cell response, an immunodominant epitope found after WNV infection in humans, which indicates that the vaccines could also induce a CD8+ T cell-specific response in humans that may be protective against WNV.

In summary, this thesis has led to several insights regarding the presence of WNV in Europe. The WNV strains utilized in our studies were all three found to be neuroinvasive and neurovirulent in mice and European strains may therefore present an equal threat to Europe as the NY99 strain did to the inhabitants of the US. Our studies have also demonstrated the virulence of a lineage 2 strain of WNV, despite the limited amount of cases that have been associated with this strain. This suggests that lineage 2 strains of WNV that may arise in the future in Europe could also be virulent. Furthermore, this thesis has shown that infection of the carrion crow with WNV may play a role in the (future) spread of this virus within Europe and could serve as an indicator of WNV activity. As a result,

WNV could remain a threat for Europe and the results may prove to be useful for directing WNV surveillance methods. Lastly, if WNV becomes a future epidemic burden to Europe, the MVA-based vaccine that we have investigated may be useful in protecting against infection with both lineage 1 and 2 WNVs.

Addenda

ADDENDUM I

About the Author

The author of this thesis was born on October 2nd, 1985 in Delft, the Netherlands. Her primary education was followed in England, Scotland, Oman and Singapore. In Singapore she completed the International Baccalaureate (IB) program at the United World College of South East Asia in 2004. She then moved back to the Netherlands to start her Bachelor's degree of Liberal Arts & Sciences at University College Utrecht (UCU), the honor's college of the University of Utrecht. During this undergraduate degree she also spent one semester abroad at the University of Western Sydney, Australia. She graduated from UCU in 2007 with a Bachelor of Science (with honors), majoring in Biology. She continued her education by enrolling in the Master's degree Immunity and Infection in Utrecht, which included an extra internship at GlaxoSmithKline (GSK) in Stevenage, England. She obtained her Master of Science in 2010 and started her PhD degree in the same year at the department of Viroscience of the Erasmus Medical Centre in Rotterdam under the supervision of Prof.dr. A.D.M.E. Osterhaus and Dr. B.E.E. Martina. The data presented in this thesis is a collection of the main research tracks undertaken during her PhD. She currently works as a post-doc at Artemis One Health Research Foundation in Utrecht.

ADDENDUM II

List of Publications

Volz A, **Lim SM**, Kaserer M, Lülf A, Marr L, Jany S, Deeg CA, Pijlman G, Koraka P, Osterhaus AD, Martina BE, Sutter G. *Immunogenicity and protective efficacy of recombinant Modified Vaccinia virus Ankara candidate vaccines delivering West Nile virus envelope antigens*. 2016.
Vaccine, 34(16):1915-26.

Szentpáli-Gavallér K*****, **Lim SM***, Bálint A, Dencső L, Koraka P, Osterhaus AD, Martina BE[2], Bakonyi T. *In vitro and in vivo evaluation of mutations in the NS region of lineage 2 West Nile virus associated with neuroinvasiveness in a mammalian model*. 2016.
Viruses, 19;8(2): pii: E49.
*authors contributed equally.

Lim SM, Brault AC, van Amerongen G, Bosco-Lauth AM, Romo HE, Sewbalaksing VD, Bowen RA, Osterhaus AD, Koraka P, Martina BE. *Susceptibility of Carrion crows (Corvus corone) to experimental infection with lineage 1 and 2 West Nile viruses.* 2015.
Emerg Infect Dis, 21(8):1357-65.

Fraisier C, Koraka P, Belghazi M, Bakli M, Granjeaud S, Pophillat M, **Lim SM**, Osterhaus AD, Martina BE, Camoin L, Almeras L. *Kinetic analysis of mouse brain proteome alterations following Chikungunya virus infection before and after appearance of clinical symptoms.* 2014.
PloS One, 9(3): e91397.

Lim SM, Brault AC, van Amerongen G, Sewbalaksing VD, Osterhaus AD, Martina BE, Koraka P. *Susceptibility of European jackdaws (Corvus monedula) to experimental infection with lineage 1 and 2 West Nile viruses.* 2014.
J Gen Virol, 95(Pt 6): 1320-9.

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J Virol Methods, 194 (1-2): 146-53.

Fraisier C, Camoin L, **Lim SM**, Bakli M, Belghazi M, Fourquet P, Granjeaud S, Osterhaus AD, Koraka P, Martina BE, Almeras L. *Altered protein networks and cellular pathways in severe West Nile disease in mice.* 2013.
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Lim SM, Koraka P, van Boheemen S, Roose JM, Jaarsma D, van de Vijver DA, Osterhaus AD, Martina BE. *Characterization of the mouse neuroinvasiveness of selected European strains of West Nile virus*. 2013.
PloS One, 8(9): e74575.

Lim SM, Koraka P, Osterhaus AD, Martina BE. *West Nile virus: immunity and pathogenesis.* 2011.
Viruses, 3(6): 811-28.

ADDENDUM III

PhD Portfolio

Name:	Stephanie Lim
Research department:	Viroscience, Erasmus MC
Research school:	Post-graduate Molecular Medicine
PhD period:	November 2010 – 2014
Promotor:	Prof. dr. Albert D.M.E. Osterhaus
Co-promotor:	Dr. Byron E.E. Martina

Education:

2004-2007:	University College Utrecht, The Netherlands Bachelor of Science, with Honors
2007-2010:	Utrecht University, The Netherlands Master of Science, Infection and Immunity

Conferences:

2014: American Society for Virology, Fort Collins, Colorado, USA
Poster presentation
2013: Dutch Annual Virology Symposium, Amsterdam, The Netherlands
Attended
2013: European Society for Virology, Lyon, France
Poster presentation
2011: Vaccine symposium, Utrecht, The Netherlands
Attended

Meetings:

2014: Molecular Medicine Day, Rotterdam, The Netherlands
Poster presentation
2013: VECTORIE 3rd Annual Meeting, Rotterdam, The Netherlands
Oral presentation
2012: VECTORIE 2nd Annual Meeting, Munich, Germany
Oral presentation
2011: VECTORIE 1st Annual Meeting, Budapest, Hungary
Oral presentation
2010: VECTORIE Kick-off Meeting, Rotterdam, The Netherlands
Attended

In-depth Courses:

2011: Certificate Course on Laboratory Animal Science (Article 9) (Utrecht)
2012: Course in Virology (MolMed)
2012: Research Integrity Course (Erasmus MC)
2013: Get Out of Your Lab Days (lectures & workshops) (MolMed)
2013: Basic data analysis on gene expression arrays (BAGE) course (MolMed)
2014: Advances in Comparative Pathology: The Central Nervous System (MolMed)

Teaching activities:

Lab rotation MSc students “Infection and Immunity”
Supervision of MSc thesis student (HO)

ADDENDUM IV

Acknowledgments (Dankwoord)

Een promotietraject is een ware opleiding tot zelfstandig onderzoeker en toch echt een bijzondere ervaring en hier hebben een heleboel mensen uiteindelijk direct of indirect aan bijgedragen. Ik wil nu graag de tijd nemen om deze mensen te bedanken! Ik hoop dat ik niemand ben vergeten.

Ik wil allereerst mijn promotor **Ab** bedanken dat ik op basis van mijn email langs mocht komen voor een gesprek/interview, en dat je mij door de "Ab-filter" hebt laten passeren en dat ik onder begeleiding van Byron mijn promotietraject in jouw lab mocht uitvoeren! Ook heel erg bedankt voor jouw hulp bij het afronden van mijn proefschrift, met name de introductie en discussie! Ik geloofde eerst heilig dat mijn 'wollige' manier van schrijven goed was maar nu heb ik dankzij jouw verbeteringen ook in kunnen zien dat zinnen toch beter zijn als ze kort en bondig zijn! Ik neem dit zeker mee voor de stukken die ik zal schrijven in de toekomst. En ik ben blij dat je uiteindelijk toch 20 van de 29 pagina's van mijn discussie overeind hebt gelaten;) Toch heb ik je het meest onder leuke omstandigheden mee mogen maken, zoals tijdens de VECTORIE meetings (ook al moest je bijna altijd tijdens mijn presentaties weg of gebeld worden) en op de Ee1714 informele kamer borrels (waarschijnlijk kwam je op de geur van de wijn af), waarbij ik vaak van je uitgebreide anecdotes en verhalen heb kunnen genieten, en je mij net zoals vele andere gelukkigen als een "Truusje" labelde (ik denk toch het meest Truusje Traag). Jouw gastvrijheid, zoals op Syros en in Antwerpen, zal me ook altijd bijblijven!

Mijn co-promotor **Byron**; allereerst bedankt dat ik langs mocht komen voor een interview. Ook al wilde ik toen heel graag onderzoek doen naar Dengue en was ik teleurgesteld toen ik hoorde dat er 'alleen' geld was voor een project op West Nile virus, heb je me toch snel weten te overtuigen dat onderzoek naar dit virus misschien zelfs meer zal lonen dan DENV (toen had je al een overtuigende kracht). Het eerste jaar kwam ik moeizaam op gang dus ik wil je vooral bedanken voor jouw geduld, vertrouwen en de nodige gesprekken om mij uiteindelijk op het juiste pad te krijgen. Ik vind het allerfijnste aan jou als begeleider dat je zo toenaderbaar bent en dat je ook echt een mentor rol aanneemt. Je maakte altijd tijd voor mij (ook al heb ik daar de eerste jaren misschien niet altijd optimaal gebruik van gemaakt) om bv een experiment te bespreken, discussie te voeren (later werd dat steeds meer van brainstorm kwaliteit), te troubleshooten, en later ook filosoferen (jouw zelfgeschreven "Philosophical Synopsis" en "The Scientific Process" zijn toch echt uniek!). Ik denk dat ik door deze regelmatige (uiteindelijk bijna dagelijkse) interactie met jou toch het meeste heb geleerd over hoe je als onderzoeker te werk moet gaan. De hoeveelheid dat je hebt geholpen op het lab (dierexperimenten, one-step growth curves) is ook uitermate bijzonder voor een PI ☺ Je was ook altijd beschikbaar voor een peptalk zodat ik weer de zonnige kant van dingen kon zien of in ieder geval kon zien dat ik me aan het aanstellen was. Behalve de bemoedigende woorden kwamen daar af en toe ook de nodige opsoberende, relativerende "Byronisms" bij kijken:
"Ik zie eerlijk gezegd het probleem niet", "Dat is toch niet handig", "Je kan niet alles hebben", "Nee, hebben we niks aan", "Als ik het niet allemaal zelf doe", "Dat is afhankelijk van de vraagstelling", "Dat zou best kunnen".... Ook al heeft het tijdens dit promotietraject toch wel een tijdje geduurd voordat we elkaars handleiding volledig hadden begrepen, ik kijk erop terug als een hele fijne en vooral leerzame opleiding!

I would like to thank **Penelope** for all your time and efforts in getting me started on the VECTORIE project and introduced into the labs, especially the BSL-3 lab and for the animal experiments. You were always available to help me and discuss experiments and you helped me stay on track. I really learned a lot from you. It can be said for sure that the VECTORIE project and the experiments on our side were successful due to your position as a post-doc on this project! Your cheerful spirit and jokes also contributed to a great atmosphere in the group.

Brooke, my beloved paranimph (even though I chose the worst time for you to be paranimph, thanks for being there!), colleague and friend! I could not have imagined doing this PhD without you! I was so excited for your arrival (although I had some necessary doubts;) and as soon as I saw you I knew we would be friends. Having you as my stable lunchbuddy, cookierunner, and partner-in-crime at borrels (we received the necessary comments;), as well as our conversations about taboo subjects (well taboo for other people!;) made me feel secure and happy, and I felt truly lost when you were on maternity leave (4 months is a long time Brooke!!). You and **Robbert** always made me feel welcome at your place and I hope we will keep in touch and remain friends (and that I can remain aunt Steffie) no matter what our location or future plans.

Jeroen, mijn andere paranimf. Ook zonder jou had ik mij de vier promotiejaren niet voor kunnen stellen. We hadden elkaar al snel gevonden in ons gevoel voor humor en we werden snel matties. Je hebt natuurlijk ook veel bijgedragen op het lab door mij aan een aantal technieken te introduceren en laten we vooral het werk dat jij had gedaan met die K** fragmentjes voor de 454 sequencen niet vergeten! Borrels/Sinterkerst samen waren uiteraard onvergetelijk:p Vijf jaar later zijn we in sommige aspecten toch wat beschaafder geworden helaas maar ik hoop dat we nog lang collega-vrienden zullen blijven!

Geert, dit hoor je vast heel vaak maar je bent onmisbaar! Ik vind het echt bijzonder hoe goed je dieren kan hanteren. Je hebt ons geweldig geholpen met sommige muisexperimenten maar jouw bijdrage aan de kraaienstudies vind ik nog het meest benoemenswaardig! Ook deze vogels had je uiteraard snel onder controle en hadden we deze studie zonder jou niet uit kunnen voeren. Hierbij wil ik natuurlijk ook graag de bijdrage van **Tanja Schouten** en **Angela Gomersbach** benoemen die ook veel praktisch werk hebben verricht aan de kraaienstudies. Daarnaast vond ik het uiteraard ook heel gezellig om met jou samen te werken en hebben we (vind ik) veel leuke en grappige gesprekken gehad.

Aaron Brault: I really enjoyed our collaboration and I would like to thank you for your scientific input to our crow studies and your visit to The Netherlands. My visit to your lab at the CDC and getting acquainted with the American crows was definitely one of the highlights of my PhD! You were a great host and hopefully we will have an opporunity to work together again in the future. I would also like to thank **Angela** and **Hannah** for conducting the AMCR experiments with our European viruses and making me feel so welcome during my visit. **Richard Bowen**, thank you for allowing me to use your lab during my visit at the CDC.

VECTORIE members such as **Tamas, Adam, Gorben, Gerd, Asisa, Martina, Lionel, Christophe, Anna-Bella, Karima** and **Anna Papa**, thanks for the great discussions, collaborations and efforts, as well as for making the annual meetings really enjoyable. Ook **Jelke** wil ik bedanken voor de gezelligheid tijdens de meetings, maar vooral tijdens het ASV congres toen ik jullie adoptiekind mocht zijn!

Frank Deege voor alle hulp en schrijfwerk bij VECTORIE. **Martine** en **Stella**, ook bedankt voor wat jullie hebben gedaan voor VECTORIE en de meetings, en natuurlijk ook de gezelligheid in Budapest toen we op pad gingen de laatste dag, en Martine ook nog voor jouw gezelschap in Munchen!

Kati – Thanks for coming to Holland several times to conduct the *in vitro* experiments together! It definitely made it easier and fun and I really enjoyed your company. I still hope to visit you in Budapest sometime!

Marion Koopmans, dank voor het plaatsnemen als lid in de kleine commissie. Ik kijk er naar uit om met jou tijdens mijn verdediging van gedachten te wisselen over arbovirussen maar hopelijk is hier ook nog kans voor verder in de toekomst.
Ernie Gould, thank you for accepting the position as a member of my defense committee and coming to The Netherlands. I remember your contributions as a member of the SAB for VECTORIE during the meeting in Munich as well as our conversation on the way to the metro. I already knew back then that I wanted you to be a member of my defense committee!
Eric van Gorp, bedankt voor het plaatsnemen als secretaris van de commissie van mijn promotie. Als lid van de "exotica" groep heb ik je gelukkig ook veel mee kunnen maken tijdens de 'pizzameetings' en was elk feestje dat je organizeerde een genot om bij te zijn!
Georges Verjans, bedankt voor het plaatsnemen als commissielid van mijn promotie, ik kijk er naar uit om met jou van gedachten te wisselen over o.a. neurotropische virussen!
Willem Takken, bedankt voor het plaatsnemen als commissielid van mijn promotie, ik kijk er naar uit om met jou van gedachten te wisselen over arbovirussen en vectoren en kunnen we dit hopelijk nog vaker doen in de toekomst!

Thijs Kuiken, bedankt voor jouw hulp met de interpretatie van de verschillende orgaan coupes van onze WNV kraaienstudie. Ik vond het geweldig om deze te mogen bekijken met een expert en te leren over welke cellen precies geinfecteerd waren.

Simone, Loubna, Maria, Anouk, **Sabine,** bedankt voor al het werk dat jullie op en voor de afdeling verrichten! Van het boeken van vliegtickets (bedankt Maria dat ik om 17u op vrijdagmiddag dat nog kon regelen bij jou!), organizeren van meetings en afspraken, versturen van belangrijke mails en hulp bij reglementen rondom mijn proefschrift (Loubna en Simone)!

Wim, Sumeyra, Avinash, Fernanda en Ajla, bedankt voor het regelen van alle personeels en financiele zaken!

David: Hier hebben we het laatst in Kopenhagen nog over gehad maar ik wil je er toch weer aan herinneren… Ik ben je allereerst dankbaar voor ons eerste gesprek in de trein in de eerste week van mijn PhD (die je je jammer genoeg niet meer kunt herinneren:) en voor de gezelligheid tijdens borrels en de lunches die we in het begin vaak met zijn drieën doorbrachten (jij, Brooke en ik) en later met meerdere mensen erbij. Uiteraard jouw hulp en bereidheid om mijn statistische vragen te beantwoorden, zeker toen we een lastige reviewer kregen voor de EID paper, waren onmisbaar. Je hebt ook veel tijd gestopt in het moduleren van een van onze virus kinetiek studies waarvoor ik je nogmaals heel erg wil bedanken; data die we nog steeds willen gaan gebruiken in de toekomst!

Petra: Bedankt voor het delen van al jouw kennis en ook dat ik bij jou terecht kon voor vragen, je bent een onmisbaar onderdeel van het lab en de groep! Op de kamer hebben we

ook vele gezellige gesprekken gehad en hebben we vooral de laatste twee jaar van mijn PhD menig kattenverhaal of wel en wee gedeeld.

Tien, jij bent een van de personen door wie ik me heel snel thuis voelde op de afdeling, met name omdat je me gelijk in jullie 'sociale' groep had geadopteerd, incl. de Sinterkerst verkleedgroep en ook omdat je vaak uitetentjes organizeerde (Tai Wu, Sumo). Door jouw attentheid voelde je al snel als een echte vriend. We hebben het iig erg gezellig gehad, en Sinterkerst, Curaçao en Lyon waren zeker de highlights! Ik kan natuurlijk ook niet vergeten hoeveel je me hebt geholpen toen met de confocal microscoop; jij hebt me in feite die hele techniek geleerd! En natuurlijk alle andere lab-gerelateerde vragen waarmee ik je heb lastiggevallen☺. Ik hoop dat we nog contact blijven houden in de toekomst. Ik vind het ook heel leuk dat **Evelyn** er halverwege bij is gekomen en ik wil haar ook bedanken voor de gezelligheid.

Rogier, bedankt dat je op het laatste moment tijd maakte en ons kon helpen met een muizenvaccinatie experiment. Hier is helaas geen paper uitgekomen maar je had ons zeker uit de brand geholpen!

Dennis en Vincent – Bedankt voor alle hulp in het EDC en bij de dierproeven!

Peter van Run: Bedankt voor al jouw hulp op het immunohistochemie lab en de goede aanwijzingen! Dit geldt ook voor **Lonneke, Debby en Judith** die me hebben geholpen waar nodig**. Sarah Getu**, tijdens de immunohistochemie heb je mij ook heel erg geholpen toen ik voor het eerst een protocol ging uitproberen (je liet me veel zien en gaf mij aanwijzingen). Alle dingen die jij regelt en voor elkaar krijgt op de afdeling zijn uiteraard ook benoemenswaardig!

Arno, Maarten, Henk-Jan en Fatiha, dank voor al jullie werk voor onze genomics studie. Hopelijk kunnen we die WNV-CHIKV paper er nog steeds uitpersen!

Ik wil **Robert** bedanken voor al zijn werk in de keuken en op het lab. Stickers, PBS, formulieren, autoclaaf, noem maar op, maar ook grappige chitchat!

Theo, bedankt dat ik dingen bij jou kon vragen en voor het geven van aanwijzingen, met name wat betreft het BSL3 lab ☺ **Patrick**, jij ook bedankt voor alle regelementen en hulp mbt het BSL3 lab!

Frank van Vliet en **Joris Lenstra** voor het organizeren van allerlei leuke en leerzame MolMed cursussen en evenementen.

Ik wil alle PI's, zoals **Guus, Rik, Arno, Ron, Georges, Bart, Rob en Bernadette** ook bedanken voor de goede en motiverende sfeer op de afdeling en alles wat ik van ze geleerd heb (bv via vragen of opmerkingen tijdens de werkbesprekingen, labgerelateerde zaken of simply life lessons).

Cox – Je was een fijne groeps/PhD/kamergenoot! We hadden elkaar vooral gevonden tijdens het uitwisselen van menig PhD frustraties en kattenverhalen!

Fasha, my dearly beloved Indonesian stepbrother! I remember first seeing you in the elevator on your way to your first meeting with Byron and helping you to a coffee during which I of course immediately showed off my Indonesian genes and vocabulary;) It is especially your gentle nature that makes you feel like family and I would like to thank you for your friendship and unfaltering willingness to help me or others out in the lab! I can of course not forget your concern regarding my wellbeing or physical appearance, which often

included helpful suggestions, such as “You look tired today Steffie, you should go home and take some rest”, or “You look better with your hair down.” :p.

Marco, jij bent zeker een van de mensen die mijn PhD ervaring onvergetelijk heeft gemaakt. Je hebt zeker een groot onderdeel uitgemaakt van het fun aspect maar ook bv jouw scherpe opmerkingen tijdens de pizzameetings of discussies waren onmisbaar! Zomaar uit jouw mouw schudde je een heuze ‘speurtocht’ in Antwerpen, dus voor jouw creativiteit heb ik ook altijd bewondering gehad. Dit is inclusief uitdrukkingen die ik nooit meer zal vergeten, zoals ‘Corona boys’, ‘afberen’, ‘paardelam’ en ‘bangboat’!

Lennert – Als geadopteerd lid in de exotic groep, ook jij hebt veel bijgedragen aan de gezelligheid, zoals bij de pizza meetings, Antwerpen, borrels, Sinterkerst en natuurlijk Lyon!

Oanh: Vanaf mijn tweede jaar hebben we elkaar beter leren kennen via de lunches (vooral buiten in de zomer), bbq, borrels en etentjes en werd het steeds gezelliger. Ik vond het superleuk om onderdeel te mogen zijn van je bachelorette en bruiloft, en hopelijk blijven we elkaar zien en mag ik ook veel meemaken van Kaj☺

Do – When you were a student in the exotic group we interacted the most and it was a great time. Your laugh is infectious and your intelligence undeniable! Whenever I had remaining thoughts after a presentation or meeting you were one of the people I enjoyed discussing these with the most. I also remember the chat we once had where you were able to make me see the bright sides of my thesis☺

Bri, thanks for the fun, friendship and positive vibes!

Fleur, op het laatst nog proefschrift leed gedeeld! Maar we hebben gelukkig ook veel lol gehad op Sinterkerst en borrels (met name de voetbal borrel).

Jurre – Eerst kwam je nog verlegen (dat was hoogstwaarschijnlijk mijn verbeelding!) naar boven om te vragen waar Erik was maar al gauw was je student op de 17e. Met Sinterkerst en andere borrels ontdekte ik al snel jouw feestbeestaard en hebben we veel lol gehad! Ik vond het jammer toen je een half jaar weg was omdat je ook veel leven op de afdeling bracht!

Chantal, jij was voor een tijdje mijn buurvrouw op Ee1714. Ik heb geleerd hoe ontzettend druk jij het hebt maar dus ook hoe goed je kunt multitasken. Door jou was ik ook extra op de hoogte op het gebied van MERS en arbovirussen. Natuurlijk hebben we ook veel gezellige kletspraatjes gehad ;) en vond ik het jammer toen je naar een andere kamer moest verhuizen. **Natalie,** ik vond het leuk de paar keer dat je bij ons op de kamer hebt gezeten en de gesprekken die we hebben gehad over arbovirussen, PhD en katten.

Georgina, bedankt voor de leuke gesprekken tijdens etentjes.

Tijdens mijn 4 jaar op de afdeling zijn er toch een aantal mensen gekomen en gegaan en wil ik deze zeker niet vergeten om nog te bedanken voor hun gezelligheid. Ten eerste **Zèli**, jij hebt me ook veel geholpen in het begin toen ik veel vragen had over het lab en door jouw zachtaardige karakter voelde ik me snel thuis in de groep en op de afdeling. Verder was je op Curaçao een hele fijne kamer- en reisgenoot!
Andere ex-exotica of afdelingleden die ik wil bedanken voor de leuke tijd: **Lisette** en **Lauren** (Sinterkerst en Curaçao waren uiteraard highlights!), maar ook **Latoya, Zuwena, Eurydice, Dineke, Fenny, Gaby, Marina, Sander B, Sander v K, Sebastian, Leontine,**

Javier, Samar, Minoushka, Ilona, Erik (lil Haeffie), Justin, Kirsty, Benjamin, Kim, Marine, Freek, Bahar, Monique, Joyce, Pascal, Christel, Stijn, Wessel, en Liz. En **Varsha** wil ik niet alleen bedanken omdat je een leuke groepsgenoot was maar ook omdat je heel veel werk voor de vogel IHC hebt gedaan! **Imke:** Door jouw spontaniteit en behulpzaamheid voelde ik me snel thuis op Ee1714 en ik kan me nog steeds herinneren dat je op mijn eerste werkdag mijn bureau (lees: bijzettafeltje) voor me had schoongemaakt en dat ik je login en wachtwoord mocht gebruiken toen ik nog niet mijn eigen account had. Ik hoop je nog vaker te zien in Utrecht voor een kletspraatje!

De volgende andere mede-collega's wil ik graag bedanken voor hun gezelligheid, zoals bij borrels, etentjes, op het lab of in de trein: **Stefan V, Werner, Ramona, Carolien, Mathilde, Miranda, Claudia, Stalin, Stefan N, Gijs, Stella, Sander, Rachel, Eefje, Lidewij, Johanna, Mart, Rory, Alwin.** Ook collega's die zijn gekomen nadat ik al klaar was, **Wesley, Laura en Stephanie.**

Josanne – Pas na de echte PhD periode kwamen we met elkaar in contact! Maar omdat we ongeveer in dezelfde fase van afronding zaten wat betreft ons proefschrift hebben we veel hierover kunnen bespreken en kon je mij vaak al vertellen over de volgende stap. Ik hoop dat we elkaar nog vaak zullen treffen in Utrecht of Hannover en samen nog veel TBEV besprekingen en hopelijk ook experimenten zullen hebben.

My UCU friends: **Cheryl, Emily, Stijn, Wouter, Xandra, Britt, Frederieke, Niels, Phil, Sietske, Ruben** and **Illy** (UCU-adopted;) (en bijgekomen aanhang zoals **Jurriaan, Peter, Lex, Lisa, Ingunn, Indira, David, Charlotte, Ioannis, Marion**). I am so glad I had you guys for fun, distractions, conversations, drinks and trips and also being able to share PhD or research perils with some of you! We have been friends for at least 10 years (even though we don't feel much older) and I hope we will remain friends for many years to come.

Britta en Nina – Tijdens onze stage bij het EWI heb ik jullie gevonden en hebben we daarna ook nog vele leuke tijden doorgebracht in oa Utrecht, Neede (tentfeest;), Duinrell, Haarlem, Amsterdam. Het feit dat jullie tegelijkertijd bezig waren met jullie PhD (of iig met labwerk) heeft ons uiteraard nog meer bij elkaar gebracht. Ik zou nog steeds willen dat jullie vaker plek hadden in jullie overvolle agenda's;). Britta, ik vind het erg jammer dat je naar Stockholm gaat verhuizen en ik ga je zeker missen, vooral nu we onze HSP gelijkenissen hebben gevonden, maar Nina en ik komen jullie zeker opzoeken!

George and Alanna – Nothing, incl Leiden or my PhD period would have been the same without you guys! I found you on a cold, winter's night in 2011 when I was moody from walking through the snow☺ We immediately became a great quartet and saw each other almost every weekend. Great dinners, experiMentationS, bbqs, beach, the lake, chilling in the park, Science club, movies, Paris, New York, Giethoorn....! Life truly changed when you guys left for the USA and becoming JAGS again is probably now more a dream than ever. Thanks for your friendship!

I would also like to thank other Leiden friends that made Leiden truly feel like home and gave me some great company; **Mira, Daniel, Mia, David, Geoffrey, Marlène, Phat, Stefanie, Youssry, Samira, Job, Marjolein, Eva, Sebastian, Sanjay, Anna, Pim, Miriam, Iris, Yessica, Bas.**

Some French friends such as **Anastasia, Anne-Laure and Niels, and Anais and Rocco** for some wonderful dinners and fun!

Huidige (ex-)Artemis collega's zoals **Leslie, Linda, Susan, Loubiela, Mirelle, Guido**, **Anne, Ruud;** ook al niet direct betrokken geweest bij mijn promotietraject, wel aanwezig tijdens mijn (lange) afrondings phase. Jullie zijn/waren hele fijne collega's! Loubiela en Mirelle, bedaknt voor al jullie werk op het lab, en door jullie te mogen begeleiden heb ik veel geleerd!

Lieve **Lim** familieleden (alfabetisch☺): **Gavin, Gunilla, James, Jasper, Jon, Lance, Tiara, Tita, Twyla** en de kids. En **Goedraad** familieleden, **Pucky, Iris, Ad, Remke, Willem-Jan, Sanne en Thije.** Ik vind het altijd zo fijn om bij jullie te zijn en we hebben altijd zoveel lol. Hopelijk kunnen we elkaar nog vaker zien in de toekomst.☺

Papa en mama: Ik ben zo blij dat jullie in 2011 naar Nederland zijn gekomen! Elkaar maar 2× per jaar zien (mama wel wat vaker natuurlijk) voor 7 jaar lang begon steeds zwaarder te wegen en ik ben blij dat papa het mogelijk gemaakt heeft om overgeplaatst te worden. Nu zien we elkaar 2× per maand en hebben jullie een belangrijke periode eindelijk van "dichtbij" mee kunnen maken (zoals papa altijd voelde dat hij veel dingen heeft gemist). Van bijna pathologisch verlegen tweelingmeisjes hebben jullie ons toch om weten te toveren naar iets redelijk geslaagds;) Bedankt voor alles wat jullie voor ons gedaan hebben ("alles" in 30 jaar is echt heel veel!)! Hopelijk kan ik jullie nog steeds trots maken in de toekomst!

Zusje **Jamie.** Het heeft zeker geholpen dat we samen naar Nederland zijn gekomen en dat we iig elkaar hadden. (Bijvoorbeeld, op UC kon ik altijd mijn kleren bij jou komen strijken als ik me eenzaam voelde;)) Dat we bijna tegelijkertijd begonnen met onze PhD en beiden iets gerelateerd aan de hersenen deden kon eigenlijk niet missen! Vier jaar lang hebben we veel verhalen uitgewisseld en daardoor veel aan elkaar gehad. Het was voor mij altijd een geruststelling en aanmoediging, "als jij iets kan dan kan ik het ook", en ik denk dat dat nog lang zal gelden. Ook al doe je nu geen research meer vind ik het leuk dat het zo goed gaat bij je nieuwe baan en gaan we de komende tijd misschien toch echt een keer iets niet hetzelfde doen☺ Ik hoop dat we nog lang dicht bij elkaar in de buurt zullen blijven!

Chers beaux-parents **Luc et Micheline,** merci de m'avoir toujours accueillie à Lyon ou au chalet et de m'avoir permis de me détendre pendant les vacances avec des randonnées exceptionnelles, de la bonne nourriture et des conversations agréables! **Tom**, even though we should have seen each other more often (for example in France or England), the two times we saw you in London as well as the two times you came to visit us in Leiden were great!

Julien: C'est à mon tour, à présent (en français et presque 6 ans après toi!). Tu m'as suivie jusqu'aux Pays-Bas et ma période de thèse n'aurait jamais été la même sans toi! Tu es mon meilleur ami et mon "roc" et tu m'as toujours soutenue pendant cette période. J'admire ton intelligence et tes connaissances, tu m'as aussi aidée avec certains calculs et graphiques. Ton esprit calme, appaisant, rationnel mais positif, a toujours été très important pour moi, et passer du temps avec toi les soirs et les weekends m'a tellement aidée à me détendre et à oublier le mauvais temps au boulot. Tu m'as bien initiée aux sorties au grand air (les randonnées et l'escalade) et je n'oublierai jamais nos excellentes vacances aux États-Unis et en France! En ce moment nous nous trouvons dans une période très difficile mais j'en suis sûre qu'on va se battre et s'en sortir!