# Doctoral School of Veterinary Science

# Study of the presence and the virulence of West Nile Virus in Hungary

PhD thesis

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# I. Introduction

West West Nile virus (WNV) is one of the most widely distributed mosquito-borne flaviviruses (Rossi et al.2010; Weissenbock et al. 2010).

WNV have been detected in birds and mammals since 2003 in Hungary, moreover, the first proven case of a lineage 2 virus infection with fatal outcome outside of Africa was detected in 2004 (Bakonyi et al. 2006). The investigation of these european lineage 2 strains became relevant, as these viruses appeared to be as pathogenic as lineage 1 isolates. In general, the enzootic cycle of WNV is maintained between various reservoir bird species, which amplify the virus and the biological vectors, which are mainly

mosquitoes feeding on birds that occasionally transmit WNV to mammals. In Europe, the principal vectors are *Culex* species and *Coquillettidia richiardii* (Hayes és mtsai 1989; Higgs és mtsai 2004). The establishment and spread of WNV in Hungary (and Europe) requires the presence of both amplifying bird species and transmitting arthropod vectors.

Several studies have aimed at identifying the molecular markers of virulence in lineage 1 WNV strains (Liu és mtsai 2006; Rossi és mtsai 2007; Audsley és mtsai 2011; Donadieu és mtsai 2013). 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 (Wicker és

mtsai 2006; Puig-Basagioti és mtsai 2007; Audsley és mtsai 2011; Wicker és mtsai 2012). In contrast, molecular determinants of pathogenic WNV lineage 2 strains have not been confirmed experimentally. Virulence markers have only been identified *in silico* by analysing and comparing full genome sequences of highly or less neuroinvasive lineage 2 strains.

The aim of this study was the investigation of WNV ecology and epidemiology in Hungary, furthermore the genetic comparison of Hungarian WNV strains, attached to the animal and human health risks. On behalf of this, one part of the study aimed to identify by active and passive monitoring the spectrum of potential mosquito

vectors and wild birds involved in the transmission of WNV in Hungary. The other part of the work aimed 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.

## 2. Materials and methods

### 2.1. Monitoring of WNV in Hungary

We performed active and passive monitoring systems.

The basis of the passive monitoring was the samples of vertebrates (showed clinical

signs of encephalitis or dead) sent to the laboratories of Veterinary Diagnostic Directorate, collected by the staff of national parks, cured at zoos or bird-escape stations. Between 2009 and 2015, we examined 79, 335, 241, 336, 354, 83, and 54 bird carcasses, respectively. The dead wild birds sent to the Veterinary Diagnostic Directorate were tested to other flaviviruses beyond WNV. In a smaller part, IgM antibodies against WNV were checked from samples of horses suffering in encephalitis.

The active monitoring consisted of collection of mosquitoes in 24 collection-site of 5 geographical region in Hungary in 2011 and 2012. Each year the main season of collection was from May to September

corresponding to the maximum annual activity of mosquito vectors, and supplemented with winter-collection on certain sites. During the two years, 23,193 mosquitoes in 645 pools were screened for WNV virus presence. It meant a 2011 collection of 11,728 mosquitoes assembled into 362 pools representing 24 different species and a 2012 collection of another 11,465 mosquitoes in 283 pools comprising 18 different species. Mosquitoes were identified with stereomicroscope, grouped by species, sex, collection site and date, and finally pooled with a maximum size of 50 individuals. The samples of vertebrates were examined individually. After homogenisation, the viral RNA was extracted and the nucleic acid of WNV was scanned with different RT-PCRs (reverse transcriptior

polymerase chain reaction). Blood samples of horses sent to the laboratories of Veterinary Diagnostic Directorate were tested with IgM ELISA kit.

## 2.2. Cloning, mutagenesis, *in vitro* and *in vivo* investigation of pathogenicity of Hungarian lineage 2 WNV strain

The WNV-578/10 strain (GenBank accession number: KC496015) originated from a horse suffered in encephalitis and finally euthanized at Department and Clinic of Equine Medicine of University of Veterinary Science. The isolated virus showed high similarity with the WNV lineage 2 strains circulating in Hungary since 2004.

After propagation and concentration of the virus, extraction of RNA and synthesis of single-strand cDNA (complementer DNA) were performed with reverse transcription and genome-specific primers. The generation of full-length infectious clone was carried out

with two parallel methods. In the first case, the full-length double-stranded cDNA was established with a cascade of fusion PCRs. In the second case, traditional cloning steps were used. Briefly, the cDNA containing the whole genome of WNV was built into a low copy-number plasmid. Point mutations were inserted in the genome of WNV using PCR-based mutagenesis. Then the given part of the genome was removed from the pWNV-578/10 clone and was replaced with the newly synthesized fragment containing the respective mutations. 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 NS4B protein gene: CC7030-31GG (P38G)

G7223C (C102S) and A7664G (E249G).

Transfection was carried out on baby hamster kidney 21 (BHK-21) cells and virus stocks were propagated and titrated on Vero E6 cells. Before the *in vitro* and *in vivo* experiments, the complete genome of all clone-derivated viruses were determined by sequencing.

Growth curves of the wild-type virus and the mutated clones were generated in Vero E6 cells at 37°C and at multiplicity of infection (MOI) 0.1. .). Every 12 hours post infection supernatants were removed for infectious virus titre determination, as well as for RNA extraction. At given time points cells were also collected for RNA extraction. RNA copy numbers were determined in a strand-specific

qRT-PCR assay.

In the *in vivo* experiments, 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). From all dead mice (and at the end-point all remaining euthanized mice) brains and kidneys (from those that had been infected with the highest viral dose) were collected. Animal experiments were approved by the Animal Ethics Committee of Erasmus Medical Centre and carried out under protocol number 122-13-19.

## 3. Results

## 3.1. Monitoring of WNV in Hungary

In each year of the study, RNA of WNV was detected in wild bird carcasses (32 in total; and 16, 6, 3, 2, 3, 1, 1 by year, respectively). Regarding the IgM antibodies against WNV, 74 out of the 416 examined horse blood sample proved to be positive. Furthermore, during the passive monitoring, RNA of other flavivirus (Usutu virus) was found in 5 blackbird (*Turdus merula*) and a fieldfare (*Turdus pilaris*).

During the 2-year active monitoring in 2011-2012, three mosquito pools were found positive with RT-PCR for WNV RNA: one pool of *Ochlerotatus annulipes* collected in Fényeslitke in June, 2011, one pool of *Coquillettidia richiardii* collected in Debrecen,

Fancsika-tó in July, 2011 and one pool of *Culex pipiens* captured near red-footed falcon colonies at Kardoskút in September, 2011. All mosquitoes collected in 2012, as well as all male mosquitoes tested negative for the virus.

We calculated the minimal infection rate (MIR = the proportion of infected mosquitoes per 1000 mosquitoes) in 2011, when WNV was identified in mosquitoes in Hungary. It was 0.25 regarding all collected mosquitoes, and when calculating by mosquito species, MIR was 2.03 in *Ochlerotatus annulipes*, 0.63 in *Coquillettidia richiardii* and 2.70 in *Culex pipiens*.

**3.2. Cloning, mutagenesis, *in vitro* and *in vivo* investigation of pathogenicity of**

## Hungarian lineage 2 WNV strain

The complete genome sequence of the 578/10 strain exhibits 2236 nucleotide (nt) differences (21%) compared to the WNV NY99 strain (GenBank accession number AF202541), which difference is in accordance with the usual variance between lineage 1 and 2 virus strains.

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 the *in vivo* transcription, the titer of NS1 mutant virus remained $10^{4,6}$

$TCID_{50}$/mL instead of the desired 6 $log_{10}$ $TCID_{50}$/mL because with each passage we lost the mutation. 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.

When sequencing the clone-derived virus stocks, 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).

Regarding the *in vitro* multiplication kinetics overall, the infectious virus titres of the clones were significantly different at all time points between 12 h and 96 h p.i. (1way ANOVA; $p<0.02$). More specifically, statistica comparison of infectious virus titres to each other, infectious virus titres for WT were significantly higher at 24 h p.i. compared to all the mutated viruses, but 72 h p.i. only compared to NS1 mutant virus. Moreover, NS1 infectivity titres were found to be significantly lower compared to WT and other mutant viruses in all time points. 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. The difference in the amount of positive strand RNA present in the supernatant of WT and NS1 increased till 36 h p.i., later the difference decreased but still remained significant at 96 h p.i. Positive strand RNA titres of WT had become approx. 0.9 log10 RNA copies higher compared to NS1 at 24 h p.i. with the most pronounced difference at 36 h p.i. After the difference had decreased but were still significant till the last time point. For intracellular negative strand RNA copies, titres for NS1 were already significantly lower

compared to the WT at 12 h p.i. and remained significantly lower till 48 h p.i. when the statistically significant difference disappeared.

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). In contrast, mice infected with the highest or lowest dose of the other mutant strains experienced substantial mortality. 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

and all other mutant viruses. The lower dose survival curves of NS1-infected mice were also significantly different when compared to the WT, NS2A, NS4B38.

All dead mice or euthanized due to illness were found to be positive for viral RNA in the brain and virus strain 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. 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, one survivor mouse of infection with NS3, and one survivor mouse of infection with NS4B249 were positive for viral RNA in the brain, and all were found to have retained the original mutation.

# 4. Discussion

## 4.1. Monitoring of WNV in Hungary

Monitoring of WNV in Hungary is opportune as in the last 15 years WNV caused several disease in this country. The bird species in which WNV RNA was detected showed high diversity, but the susceptibility of *Accipitrinae* gained verification with the 14 goshawk-originated isolates out of the total 32. In Europe, the house sparrow is

supposed to play an important role in the epidemiology of WNV. Our result can support that hypothesis as we found the virus in the mentioned species. The susceptibility of blackbird to other flaviviruses is known (eg. Usutu virus) (Bakonyi et al. 2017), it seems that blackbirds also have important part in the transmission of WNV. In the years of the passive monitoring we amplified both USUV and WNV RNAs in that species.

During the two-year active monitoring, we found 3 WNV positive mosquito pools of species that are widespread throughout Europe. Moreover these species are able to overwinter in continental climate. Two species out of the three (*Culex pipiens* and *Coquillettidia richiardii*) are considered the

principal mosquito vectors of WNV in Europe (Higgs és mtsai 2004; Reiter és mtsai 2010). *Culex pipiens* is essentially ornithophilic, *Coquillettidia richiardii* and *Ochlerotatus annulipes* feed mainly on mammals and occasionally on birds, they are known to be bridge vectors of WNV between birds and mammals (Kulasekera és mtsai 2001; Becker és mtsai 2003).

The estimated prevalence of WNV infection in mosquitoes in Hungary was comparable to that reported by other studies from Europe. MIR calculations of different studies need to be evaluated with care as the time, the method and environmental factors may have huge influence on the results. Variation in vector competence is known not

only between species, but between mosquito populations from different geographical locations as well (Richards et al. 2014). Its more likely that the temperature limits the vector competence of European mosquitoes for WNV transmission (Fros et al. 2015). The appearance and the ability to get endemic of new mosquito-borne viruses in an unaffected region requires competent arthropod vectors and amplifying (wild bird) hosts on the spot, beyond the suitable climate for the maintenance of the transmission cycle. Based on the detection of WNV in every year in wild birds, in horses and in humans in Hungary (and in the surrounding countries), we can declare that WNV is endemic in Centra Europe.

## 4.2. Cloning, mutagenesis, *in vitro* and *in vivo* investigation of pathogenicity of Hungarian lineage 2 WNV strain

WNVs belonging to lineage 2 were previously considered as agents of low pathogenicity, however, numerous neuroinvasive and highly pathogenic members have recently been identified in horses and humans as well.

In this study, 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 with reverse genetic methods.

It is known that flaviviral NS proteins

have several different functions. The flavivirus nonstructural protein NS1 has an essential role in viral RNA replication, moreover it is immunomodulating. Inclusion of the mutation P250L in a conserved region of lineage 1 WNV strain (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 (Hall et al. 1999). 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 (Youn et al. 2013). In our study, the NS1 mutant virus showed significant attenuation *in*

*vitro* and *in vivo*. 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, 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. It is possible that the different mouse model used in the aforementioned study or the different age of the mice explains the discrepancy between our results. However, it can also not be excluded that the silent mutation 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.

NS2A is a small, hydrophobic, membrane-associated protein of WNV and plays an important role in virus assembly and in the inhibition of the cellular antiviral response. The attenuating effect of A30P mutation in WNV lineage 1 strains has variable evaluation (Rossi et al. 2007; Audsley et al. 2011). In our study NS2A mutant virus neither *in vitro* nor *in vivo* showed significant attenuation.

The full length NS3 protein is a multifunctional enzyme that possesses various activities in both viral poliprotein processing

and RNA replication. The proline at locus 249 of this protein 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. 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. 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.

Flaviviral NS4B acts as an interferon antagonist, contributed by the activity of NS4A and NS2A. Different studies of WNV lineage 1 viruses found that the P38G mutation in the NS4B protein induced a lower level of viraemia and no lethality in 6-10-week-old mice and *in vitro* thermosensitivity (Welte 2011, Wicker 2012). 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. 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 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 *in vitro* and was found to attenuate mouse neuroinvasiveness and neurovirulence. 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.

The NS4B-E249G mutation has been

observed in several natural WNV isolates. The effect on virulence of E249G mutation is different in the studies of lineage 1 WNV strains (Puig-Basagioti és mtsai 2007, Ross és mtsai 2007). The mutant lineage 2 WNV propagated to similar titres in Vero E6 cells as the WT and showed reduced mortality in mice that was not significantly different.

To summarize the conclusion, genetic backbone of lineage 1 and 2 WNV strains are similar, the influence on virulence of given mutations can be highly variable.

## 5. New scientific outcomes

1. Setting up passive monitoring system of WNV in Hungary, which meant the collection and the analysis of mosquito vectors in 2011 and 2012. Detection of WNV in *Ochlerotatus annulipes, Coquillettidia richiardii* és *Culex pipiens* mosquito species.
2. Determination of the full genome sequence of the Hungarian lineage 2 WNV strain WNV- 578/10.
3. Generation of the full-length infectious clone of WNV-578/10, modifying its genome with the help of reverse genetic methods and site-specific mutagenesis, during what introduction of point mutations into the nonstructural proteins ((NS1

(P250L), NS2A (A30P), NS3 (P249H NS4B (P38G, C102S, E249G)).

4. *In vitro* (in Vero E6 cells) and *in vivo* (in mice) study of virulence of wild type and mutant viruses, and identification one mutation that contributed to significant attenuation of lineage 2 WNV (NS1-P250L), one mutation that abolished completely the reproduction of the virus (NS4B-C102S) and four mutations with moderate (or mild) influence on the virulence (NS2A-A30P, NS3-P249H, NS4B P38G és NS4B-E249G ) in the genome o WNV-578/10.

5.

6. **List of Publications**

1. **Szentpáli-Gavallér K.**, Antal L., Tóth M., Kemenesi G., Soltész Z., Dán Á., Erdély K., Bányai K., Bálint Á., Jakab F., Bakony T.: Monitoring of West Nile virus in mosquitoes between 2011–2012 in Hungary. Vector Borne and Zoon. Dis., 14(9). 648–655. 2014.

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3. **Szentpáli-Gavallér Katalin**, Dán Ádám, Erdélyi Károly, Bálint Ádám, Somhegyiné

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2. **Szentpáli-Gavallér Katalin**, Bálint Ádám,

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3. Bakonyi Tamás, **Szentpáli-Gavallér Katalin**, Papp László, Soltész Zoltán, Kemenesi Gábor, Bernhard Seidel, Zdenek Hubálek, Norbert Nowotny: Detection of West Nile virus in culicid mosquitoes in Central Europe. 6th European Mosquito Control Association Workshop (EMCA 2011). Budapest, 2011. szept. 12–15.

4. **Szentpáli-Gavallér Katalin**, Antal László, Tóth Mihály, Kemenesi Gábor, Soltész Zoltán, Dán Ádám, Erdélyi Károly, Bányai Krisztián, Bálint Ádám, Jakab Ferenc, Bakonyi Tamás: Monitoring of West Nile virus in mosquitoes between 2011–2012, Hungary. Congress of Hungarian Society for Microbiology. Keszthely, 2013. okt. 16–18. (poster)

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