Brief Summary of Ph.D. Thesis

Microbiological diagnostic investigation of endemic zoonotic flaviviruses in Hungary

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Introduction

Examination of the emerging and re-emerging viruses is the most challenging field in virological research. An emerging disease can be defined, as a disease which has appeared in a population for the first time, or that might have existed previously, but is rapidly increasing in incidence or geographical range. Viral zoonoses are typically emerging agents and can be characterized as infectious diseases caused by a virus that has spilled over to humans from an animal (usually a vertebrate). The term 'arbovirus' (**ar**thropod-**bo**rne virus) is not part of the current viral taxonomy, it applies to any virus that is transmitted to humans and/or other vertebrates by certain species of blood-feeding arthropods, such as mosquitos or ticks. In Hungary, the most important arboviruses, which pose a significant risk to public or animal health are the members of the *genus Flavivirus, family Flaviviridae*. Therefore, our study is mainly focusing on endemic flaviviruses, such as *tick-borne encephalitis virus* (TBEV) and *West Nile virus* (WNV).

Detection of virus-specific antibodies in serum and cerebrospinal fluid (CSF) is the primary and most widely

used method for the diagnosis of flavivirus infections, whereas previous experience has shown that the virus nucleic acid can no longer be detected in blood or CSF at the onset of clinical symptoms. A major problem of the currently used methods for flavivirus serology is the sequence similarity of structural proteins of flaviviruses. Consequently, antibodies against a flavivirus can cross-react with the structural proteins of another one, significantly complicating the specific diagnosis. This is of importance in areas, where different flaviviruses co-circulate, for example, WNV and TBEV in Hungary. Due to the cross-reactive antibodies, differential diagnosis of secondary flavivirus infections or co-infections is particularly difficult. As there is a reasonable need for case confirmation and highly sensitive methods, the characterization of viral strains and isolation of new variants are also on-demand. Therefore, in the last few years, the development and introduction of molecular assays have become particularly relevant in the laboratory diagnosis of human flavivirus infections. Surveillance studies that enable to assess virus circulation in the given geographical area are also required for public health implications and response actions. According to the Hungarian law, the laboratory

diagnosis of human viral zoonotic infections across the entire territory of Hungary is centralized and performed only at the National Reference Laboratory (NRL) for Viral Zoonoses of the National Public Health Center; Hungary (NPHC). Therefore, it is necessary to follow recent trends in laboratory diagnosis and continuously improve the laboratory capacities, as well as to implement surveillance studies.

Objectives

1. **Introduction of molecular biological assays to the diagnostic laboratory routine, especially for the detection of TBEV and WNV RNA.**
   Sera, cerebrospinal fluid (CSF) and urine samples of serologically confirmed, acutely ill TBEV and WNV patients were tested by PCR method. Our aim was to determine which sample type was most suitable for viral nucleic acid detection. PCR positive samples were sequenced, and phylogenetic analysis has also been performed. Virus isolation was carried out using PCR positive clinical specimens.

2. **Ascertain the length of virus excretion in PCR positive cases by follow-up studies.** Serial urine samples of long-term hospitalized West Nile neuroinvasive disease (WNND) patients were tested by PCR and PCR positive urine samples were also used for virus isolation.

3. **Besides urine, we started collecting anticoagulant treated whole blood samples to improve the WNV diagnostic sensitivity.** Our further aim was to compare the two sample types.

4. **Introduction of Usutu virus (USUV) specific molecular biological and serological assays.** For differential diagnostic purposes, WNND and West Nile fever (WNF) patients have been tested retrospectively, at the end of the 2018 WNV transmission season.

5. **Assess the WNV prevalence of WNV-specific antibodies in the samples of blood donors collected in 2016.** Although, the number of diagnosed WNND cases have gradually increased since 2004, when the first cases were reported, a comprehensive serological survey to gain information about the seroprevalence of WNV in the Hungarian human population has not been conducted since 1999/2000. Anti-WNV antibody determination from plasma samples of 2112 donors was performed. The study was conducted by a collaboration between the Confirmatory Laboratory of the Hungarian Blood Transfusion Service and the National Reference Laboratory for Viral Zoonoses (NPHC).

Materials and Methods

Molecular biological assays:

1. TBEV, WNV, and USUV-specific PCR tests were performed on serum, whole blood, CSF, and urine samples of serologically confirmed or probable cases of TBEV and WNV. Aliquots of each clinical specimen were frozen and stored at -80°C until further use.

2. We optimized reverse-transcription real-time and nested PCR methods based on previously published protocols and we also designed primer sets for nested PCR and sequencing.

3. PCR positive samples were sequenced by Sanger-method and the complete genome sequencing of one selected strain was also performed by next generation sequencing. Phylogenetic analysis was also carried out.

4. For virus isolation PCR positive samples were inoculated into *in vitro* cell cultures, followed by intracranially injection of the sample into suckling mice. Virus replication was confirmed by real-time PCR.

Serological methods:

1. Acute or recent TBEV and WNV infections were diagnosed by the detection of virus-specific IgM, IgA, and IgG response. For antibody determination, commercially available ELISAs and *in house* developed indirect immunofluorescent assay (IFA) were used.

2. IFA and/or ELISA results were confirmed by hemagglutination inhibition (HI) and virus neutralization assays. As a result of the PhD study, HI tests were replaced by serum microneutralization.

3. For determination of WNV seroprevalence among blood donors, IFA was used as a primary method. Cross-reactivity was excluded by end-point titration. HI tests and WNV microneutralization assay were used for the confirmation of anti-WNV IgG reactive results. The presence of anti-WNV IgM antibodies was also determined by IFA.

Results

The laboratory diagnosis of flavivirus infections – which previously based only on serological methods – has been developed by the introduction of molecular biological assays, at the National Reference Laboratory for Viral Zoonoses (NPHC).

1. As a result of the introduction of newly advanced diagnostic tools, WNV RNA was detected in human clinical samples, for the first time in Hungary, during the 2014 transmission season. In three patients with neurological symptoms, viral RNA was detected from both urine (n=3 patients) and serum specimens (n=2 patients), albeit for a longer period and in higher copy numbers with urine. Phylogenetic analysis of the NS3 genomic region of three strains and the complete genome of one selected strain demonstrated that all three patients had lineage 2 WNV infections. Our finding affirms the utility of viral RNA detection in urine as a molecular diagnostic procedure for the diagnosis of WNV infections.

Altogether, 93 acute WNV infections were investigated between 2015 and 2017. Sera, CSF, and urine samples

were tested by PCR. WNV was generally detectable in urine at a higher load and for a longer time. Altogether, 45.45% of patients (n=77) and 57.26% of tested urine samples (n=117) proved to be PCR positive, while in serum samples only 16.86% of tested patients (n=83) and 18.39% of tested samples (n=83) had positive results. WNV RNA was least likely to be detected in CSF samples, as only two patients (n=49) had weak positive or inconclusive (Ct>40.00) PCR results. WNV RNA kinetics in the urine and blood was also investigated. WNV RNA was detectable in 75% of the tested urine samples between days 6 and 10 after symptom onset. 63% and 78% of tested samples were PCR positive between days 11-15 and days 16-20, respectively.

WNV RNA persisted in the urine for a longer time than in serum, as it was still detectable at 30 days after onset in over 38% of cases. Serum was PCR positive in less than 30% of cases, during the first 16 days after symptom onset.

Acutely ill TBEV infected patients were also involved in the study. Between 2016 and 2017, serum (n=33), whole blood (n=6) CSF (n=22), and urine (n=23) samples of 30 serologically diagnosed patients were investigated by RT-PCR method. Two sera, one urine, and one CSF sample

of four patients tested positive and the European subtype of TBEV could be identified. This was the first time that TBEV RNA could be detected from human clinical samples in Hungary.

2. In 2015, a follow-up study was carried out in case of 5 WNND patients. WNV nucleic acid was detectable in urine even for several weeks (on days 23; 24; 27; 36; and 40) after symptom onset and viral RNA was present at higher concentration compared with other samples. Virus isolation from urine of two patients was also successful.

3. During the 2018 WNV transmission season, similarly to other endemic areas in Europe, a large number of human WNV infections was reported in Hungary. The cumulative number of cases in 2018 (n=225) exceeded the cumulative sum of cases in the previous 14 years. The increased number of cases allowed the collection and comparison of additional sample types, such as anticoagulated whole blood and urine. The total number of WNV PCR positive patients was n=53. In 45.3% of cases, both sample types (whole blood and urine) were PCR positive. Viral RNA could be detected in whole blood, while urine was negative in 18.9%. Urine sample was not

available, but whole blood PCR positivity confirmed the acute WNV infection in 9.4% of patients. Urine proved to be PCR positive and whole blood was not sent to the laboratory (15.1%) or it was PCR negative (5.7%) in approximately 20% of cases. The use of either urine or whole blood samples for molecular diagnostics instead of serum improved the efficiency of viral RNA detection; however, the appropriate sample types were available for only half of the patients. In addition, the current case definition criteria in the EU specify 'Detection of WNV nucleic acid in blood or CSF' as laboratory test for case confirmation of a WNV infection. Therefore, rapid case confirmation was highly facilitated by whole blood PCR results. Based on our sequence data, lineage 2 WNV strains were identified as etiological agents of human infections in 2018.

4. For differential diagnostic purposes USUV diagnostic tests were also implemented. All whole blood and urine samples that gave negative results in the WNV real-time PCR assay were further tested for the presence of USUV. In one whole blood sample from 2018, USUV RNA was detected. Therefore, this patient was classified as a confirmed case of USUV infection, after having first been

interpreted as a probable WNV infection based only on WNV serological results. Based on the partial sequence of the viral genome, obtained in the nested PCR from the blood sample, USUV lineage Europe 2 was identified. The current report emphasizes the need for USUV diagnostic assays. Targeted investigations of cases with the clinical suspicion of aseptic meningitis or viral encephalitis might reveal USUV in the etiology of further human cases.

5. Among the 2112 plasmas evaluated for anti-WNV IgG antibodies in 2016, the overall estimated unweighted prevalence of anti-WNV antibodies was 2.19%. The highest seroprevalence was calculated in Southern Great Plain region, followed by Southern Transdanubia. Northern Great Plain and Central Hungary showed also high seroprevalence.

Conclusions

During the five-year period of the study, we detected WNV and TBEV in human clinical specimens, for the first time in Hungary. We also successfully isolated WNV from urine samples. WNV is detectable in urine at a higher load and for a longer time than in plasma or CSF. WNV excreted in urine is also infectious since it could be isolated in 11 patients. Testing the urine samples may improve the sensitivity of molecular methods for the diagnosis of WNV infection because the viral load is generally higher than in other specimens, especially during the later phase of the infection. The presence of high viral load allows virus isolation supporting reference laboratory capacity. In five long-term hospitalized WNND patients, viral nucleic acid was detectable and could be isolated in urine even for several weeks after symptom onset and viral RNA was present at higher concentration compared with other samples. Nevertheless, the duration of virus excretion may be longer in most severe WNND cases. In our study serial sample collection was limited to patients who developed a more severe course of the disease and needed intensive hospital care for a longer period. According to the European Union's case definition criteria,

a PCR positive result in urine sample alone does not allow laboratory case confirmation of WNV. Testing of blood and CSF samples is therefore required for rapid case confirmation, however, in fact, most of the patients are no longer viremic at the time of symptom onset due to the short duration of the viremic phase and the low viral load in serum. From the beginning of the 2018 transmission season, we started collecting anticoagulated whole blood samples for WNV PCR analysis. EDTA-treated whole blood and urine samples, the most appropriate specimens for viral RNA detection, were available only from 48% and 64% of patients, respectively. Testing of both whole blood and urine samples supported the sensitivity of the diagnosis of WNV infections, because the effectivity of viral nucleic acid detection has been highly improved. In 74% (n=39) of the PCR positive patients (n=53) the acute WNV infection could be confirmed by whole blood PCR positivity. By comparing the whole blood and serum sample of the same patient, in 78% (n=32) cases WNV RNA was detected, while serum remained negative.

Despite of WNV, in human TBEV cases, the molecular biological methods seemed to be less effective, due to the low level of PCR positive patients (n=4) and randomly

received results in different clinical specimens. Therefore, the application of serological methods remains essential.

The introduction of USUV-specific assays in the differential diagnosis of flaviviruses has become topical, recently. Compared with WNV, USUV is less likely to be associated with severe infections in humans, except in immunocompromised individuals. Only a few clinically manifested cases have been described so far. In 2018 we report the first human USUV infection in Hungary; it had a neurological manifestation, characterized as aseptic meningitis. The enzootic cycle of WNV and USUV is similar, their geographic distribution overlaps, and the clinical manifestations of USUV infection are rather like WNV. As USUV and WNV show considerable similarities and that an accurate laboratory diagnosis presents some difficulties due to the serological cross-reactivity, the USUV-related human infections might be underestimated. Our findings underline the importance of thoroughly performed serological tests combined with molecular assays to differentiate WNV and USUV infections and to avoid unrecognized human USUV cases.

The use of molecular diagnostic tools for viral RNA detection directly from clinical specimens allows amplification of viral genomic regions suitable for

sequencing and, hence, the genetic characterization of the viral strains by phylogenetic analysis. One potential hypothesis in explaining the dramatic increase in WNV cases in 2018 is that a genetic change in the virus resulted in enhanced virulence and/or replication. Our recent sequence data from 2018 suggest that there is no evidence for the emergence of a novel viral variant. Lineage 2 WNV strains were similar to those were circulating in Hungary in previous years. However, the lack of full genome sequences limited this conclusion. Additionally, several studies have shown that certain environmental factors, such as temperature and precipitation anomalies, can be predictors for WNV transmission. Increased precipitation in the spring can facilitate the early expansion of vector populations, while the activity of WNV transmission cycle is associated with drought conditions and elevated temperature.

Although, the annual number of diagnosed WNV cases has gradually increased in the last decade, a comprehensive serological survey to gain information about the seroprevalence of WNV in the Hungarian human population has not been conducted since 2004, when the first human WNV infections were reported. Finally, a total of 2112 randomly selected voluntary whole

blood donors were enrolled in the WNV seroprevalence study. The total weighted seroprevalence [2.19% (95% CI: 1.64% - 2.90%)] estimated in our recent study was significantly higher ($p = 0.001$) than that calculated in samples collected in 1999/2000 (0.61%, 95% CI 0.00–1.2). (Unpublished data of the National Reference Laboratory for Viral Zoonoses, NPHC.) The former survey was conducted and verified by the same laboratory methods described in our present study, but samples were collected from the general population with a more extended age distribution. After categorization of donors by residence regions, a clear-cut ranking could be observed among the regions in the WNV seroprevalence. Southern Great Plain region indicated the highest WNV seroprevalence as well as WNV cumulative incidence. Although, a pretty good correlation could be observed between the estimated seroprevalence and the cumulative incidence in six regions, a higher cumulative incidence could have been expected in the case of Southern Transdanubia.

New scientific results

1. In Hungary we first detected WNV RNA in human clinical specimens (serum, whole blood, CSF, and urine) and we successfully isolated the virus from urine.
2. WNV whole genome sequencing from a human clinical sample was performed for the first time in Hungary. WNV lineage 2 circulation has been established in the country.
3. Based on the results of follow-up studies, we found that WNV RNA could be detected in the urine samples of WNND patients even for weeks after the onset of symptoms.
4. Serological screening of healthy blood donors revealed that between 1999/2000 and 2016, the WNV seroprevalence increased significantly, and the most affected regions were Southern Transdanubia, followed by Southern Great Plain, Northern Great Plain and Central Hungary.
5. We confirmed a human USUV infection for the first time in Hungary and we first detected TBEV in human clinical specimens (serum, urine, and CSF).

Publications based on the results of the PhD dissertation

Research papers in peer-reviewed journals

1. **Anna Nagy,** Enikő Bán, Orsolya Nagy, Ferenczi Emőke, Ágnes Farkas, Krisztián Bányai, Szilvia Farkas, Mária Takács. (2016). **Detection and sequencing of West Nile virus RNA from human urine and serum samples during the 2014 seasonal period.** Arch Virol., 161, 1797−1806. https://doi.org/10.1007/s00705-016-2844-5

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3. **Anna Nagy**, Orsolya Nagy, Katalin Tarcsai, Ágnes Farkas, Mária Takács. (2018). **First detection of tick-borne encephalitis virus RNA in clinical specimens of acutely ill patients in Hungary**. Ticks Tick Borne Dis., (9)3, 485−489. https://doi.org/10.1016/j.ttbdis.2017.12.017

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1. **Nagy, Anna**, Marina Varga, Mária Takács, Katalin N Szomor. (2013). **"Retrospective study for screening Usutu virus antibodies in two different groups of human population in Hungary."** In Acta Microbiologica et Immunologica Hungarica, (60)1, 191–192.

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