OTKA 120118

Novel detection and identification methods for the surveillance of emerging and endemic arboviruses in the Pannonian Basin

Final report

1. Introduction and scientific background of the project

Arthropod-borne viruses (arboviruses) are an ecological group of viruses "which are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts and haematophagous arthropods" (Cumakov et al., 1967). Arboviruses are mainly classified into the *Reo-*, *Toga-*, *Flavi-* and *Bunyaviridae* families (Hubálek et al., 2014); however, arthropod vectors may contribute to the transmission of several other viruses (e.g. members of the *Herpesviridae, Poxviridae, Asfarviridae, Orthomyxovridae, Rhabdoviridae* and *Retroviridae* families). The most frequent arthropod vectors of these viruses are mosquitoes and ticks, which are usually biological vectors of the agents, as the viruses are actively propagating in the cells of these invertebrates. Additionally, certain insects (e.g. horse-flies, *Tabanidae*) may transfer viruses between vertebrate hosts as mechanical vectors. In these cases, the mouthparts are contaminated with viruses during biting, and they are directly transmitted to another host (Verwoerd, 2015).

The number of known arbovirus species exceeds 500. Several of them are important human pathogens (e.g. Yellow fever virus, Dengue virus, Chikungunya virus, Japanese encephalitis virus, Tick-borne encephalitis virus) or animal pathogens (e.g. Bluetongue virus, African horse sickness virus, Akabane virus, Nairobi sheep fever virus). In contrast, others can cause disease in animals and humans (e.g. West Nile virus [WNV, *Flaviviridae, Flavivirus*], Rift Valley virus) (Viglietta et al., 2021). Most of these viruses are distributed in tropical and subtropical regions because their competent vectors are sensitive to lower temperatures (e.g. winter frosts). However, competent vectors of many exotic arboviruses are also present in regions with a moderate climate, providing the opportunity for local transmission of these viruses in case of introduction. Global changes have a significant impact on the spread of arboviruses. On the one hand, due to the changing (warming) climate, the geographical ranges of important vectors are slowly expanding towards the poles; on the other hand, the increasing intensity of international and intercontinental trade of goods and travel of people increases the risks for the introduction of exotic viruses (Laycock et al., 2022). Within the last two decades, several exotic arboviruses

emerged in Europe or the United States of America (e.g. WNV, Chikungunya virus, Dengue virus, Bluetongue virus, Schmallenberg virus, Usutu virus [USUV, *Flaviviridae*, *Flavivirus*], Zika virus) and caused considerable veterinary and public health problems. Additionally, exotic mosquito vectors (e.g. *Aedes albopictus, Aedes koreicus, Aedes japonicus*) also emerged recently in Europe (ECDC 2022a).

Due to the complexity of arbovirus ecology and transmission cycles, the available tools for controlling these agents are limited; they are mainly based on vector control approaches and immunisation of susceptible hosts. If arboviruses are introduced with amplifying hosts and competent vectors available, they often become endemic. Depending on the availability and abundance of hosts and vectors, the intensity of virus activity may vary over time (e.g. annual seasonality or outbreaks in certain years). Assessment of virus activity is an essential prerequisite of control measures because continuous control activities are usually not feasible, while specific control actions must be implemented at the very beginning of the increase of virus activity to avoid significant outbreaks (EFSA and ECDC 2020).

The most significant human arbovirus in the Pannonian Basin is the Tick-borne encephalitis virus (TBEV, *Flaviviridae, Flavivirus*). The presence of this virus in the region has been known for decades. Due to the vaccination available to the public since the 1990s, the annual number of diagnosed human cases in Hungary is usually below 100 (Csohán et al., 2015). Disease in animals is rarely detected (Varga et al., 2018).

Clinical cases of mosquito-borne flaviviral diseases were first diagnosed in Hungary in 2003 (Glávits et al., 2005). A genetic lineage 1 WNV caused encephalitis in geese and humans. Next year another WNV strain emerged in Hungary and caused central nervous disease and mortality in wild birds (Bakonyi et al., 2006). This virus, however, belonged to the genetic lineage 2 of WNV and was considered an exotic genotype in Europe. In subsequent years, this WNV strain became endemic in the region and caused several encephalitic cases in horses, humans, wild birds and other vertebrate species. Since its emergence, our research groups have been intensively investigating the activity and spread of the strain in Central and Sothern Europe, documenting numerous details of the genetic diversity, pathogenicity and vector ecology of the virus (Erdélyi et al., 2007; Kecskeméti et al., 2007; Kutasi et al., 2011; Papa et al., 2011; Bakonyi et al., 2013 and 2016; Rudolf et al., 2014 and 2015; Szentpáli-Gavallér et al., 2014 and 2016). We have also documented the emergence of another flavivirus, the USUV, in Hungary and other European countries, genetically characterised the virus and determined its pathogenicity in different vertebrate hosts (Bakonyi et al., 2004, 2005a, 2007 and 2014;

Weissenböck et al., 2004 and 2013; Chvala et al., 2005, 2006 and 2007; Meister et al., 2008; Rubel et al., 2008; Manarolla et al., 2010; Steinmetz et al., 2011; Hubálek et al., 2014; Rudolf et al., 2015.). We have contributed to the genetic characterisation of further WNV genetic lineages in Central Europe (Bakonyi et al., 2005b; Hubálek et al., 2010; Kemenesi et al., 2014; Ergunay et al., 2015) and genetically characterised other arboviruses (Nemes et al., 2004; Bakonyi et al., 2013). Within our previous research projects on this topic (OTKA K67900, OTKA D48647, FP7 261466 "Vectorie", FP7 261504 "EDENext"), we could successfully describe several details of WNV and USUV ecology, and we developed a model for an integrated surveillance system comprising active and passive monitoring elements to assess virus activity (Szentpáli-Gavallér et al., 2016). However, the system has not yet been applied at a large scale, and as project supports expired, surveillance activities were also interrupted. In addition to its original function, this system would be suitable for simultaneously monitoring a wider range of mosquito-borne viruses; therefore, developing detection systems for other viruses and their integration into the monitoring system could provide significant added value.

Another important equine virus, the Equine infectious anaemia virus (EIAV, Retroviridae, Lentivirus), emerged a few years ago in Hungary (Bolfa et al., 2015). Between 2008 and 2015, more than 60 infected horses were diagnosed and culled. Our research group also contributed to the partial genetic characterisation of the detected virus strains. These studies, however, revealed some weaknesses in EIAV diagnostic methods; therefore, improvements in this field could increase the sensitivity and specificity of future assays and hence facilitate the control measures against the spread of the virus. Horse flies are considered to play a role in the transmission of EIAV, although no data is available on whether this way of transmission is significant in Hungary. Therefore, we planned studies that would focus on detecting the virus in horse flies collected from the surroundings of EIAV-infected horses.

2. Hypotheses, key questions, and aims of the project

1. The previously developed and currently available combined surveillance method (with active and passive components) had been appropriate for the targeted monitoring and surveillance of the activity of certain flaviviruses. However, the collection of a representative number of samples requires considerable effort and is rather expensive, which limits the widespread and routine application of this method. The project aimed to develop and validate novel sample collection and processing methods for the cost-effective surveillance and monitoring of selected

arboviruses in mosquitoes and other flying arthropods. The sensitivity, efficacy and reliability of these novel methods were planned to be compared with the currently available conventional surveillance system. The new system should be suitable for the cost-effective provision of fine-scale spatial and temporal data adequate for epidemiological risk assessment and potential early warning.

2. Besides the mosquito-borne arboviruses we had discovered and characterised between 2004 and 2015 in Hungary, the presence of other viruses (e.g. WNV lineage 9) was also recognised (Kemenesi et al., 2014). Moreover, no recent data was available on the presence and occurrence of other arboviruses endemic in Central Europe (e.g. certain orthobunyaviruses and togaviruses). The samples collected within the project were also planned to be tested for the presence of these viruses.

3. The available scientific data on the occurrence of the aforementioned viruses in the neighbouring countries of the Pannonian Basin are diverse. By providing the methodology and scientific support for the uniform application of the novel surveillance system, we planned to obtain comparable data on the occurrence and spread of arboviruses in the region.

4. Between 2008 and 2015, Equine Infectious Anaemia re-emerged in Hungary, and a significant number of indigenous cases were diagnosed. Besides changes in the international transportation and trade of horses, differences in the sensitivity of the applied diagnostic tests may also play a role in the increase of the detected infections. Another aim of the project was to evaluate the currently available diagnostic methods for EIAV and develop further ones for the rapid and reliable identification of infected horses.

During our earlier studies, we successfully detected and characterised the presence of West Nile virus (WNV) and Usutu virus (USUV) in Hungary in both vertebrate hosts and mosquito vectors. We have developed and operated active and passive surveillance systems to investigate the spread of these viruses. The aim of our currently planned project was to develop novel sample collection and processing methods to increase the cost-effectiveness and detection efficiency of the monitoring of flavivirus seasonal activity. The specificity and sensitivity of these methods will be validated through comparison with the conventional methods. Samples collected during the project will also be tested for the presence of further arboviruses (e.g. flavi-, bunya-, and togaviruses), and the surveillance will be extended to selected collection sites in the neighbouring countries within the Pannonian Basin. Phylogenetic and epidemiological analyses of the detected viruses will be carried out. Additionally, we would like to compare the

currently available methods for the detection of Equine infectious anaemia virus (EIAV) on samples collected from Hungarian EIA cases, determine the most reliable diagnostic methods and develop further ones with increased specificity and sensitivity. The presence of EIAV RNA would be tested in horse-fly samples collected from the environment of infected horses. Our project was planned to provide a basis for establishing sustainable, long-term vector-borne pathogen surveillance and monitoring systems to provide reliable data on the presence or emergence of arboviruses in the Pannonian Basin.

3. Results

3.1. Development of flavivirus surveillance methods

The reliability of arbovirus surveillance and monitoring systems depends on the number of samples and the sensitivity of detection methods. Collection and identification of invertebrate vectors and virus detection are the crucial components of arbovirus surveillance. Large-scale insect vector collection and identification are expensive, limiting the widespread application of surveillance and monitoring systems. The currently available, conventional, combined WNV and USUV surveillance system contains passive and active components. While virological and serological investigations of horses, humans and wild birds showing clinical signs of encephalitis or dying due to it are the most important passive elements of the surveillance, collection, identification and virological testing of mosquitoes and serological screening of horses, humans and wild birds are the main active components.

3.2. Sugar-based baits

The main principle of the novel sample collection method planned in the project is based on the application of sugar-based baits for collecting mosquito saliva and detecting arbovirus nucleic acid in the samples (Hall-Mendelin et al., 2010). Mosquito vectors feed on sugar syrups attractant baits and contaminate them with saliva, so arboviruses shed via the saliva can be detected by molecular diagnostic assays. The presence of arboviruses at a particular sampling site can be screened by this method. Where positive screening results are obtained, targeted vector surveillance (with mosquito collection and identification) may ensue. Screening may therefore complement and increase the surveillance efficacy. This method was successfully used in Australia and California for mosquito-borne virus surveillance (Lothrop et al., 2012; van den Hurk et al., 2014) but has not been tested in Europe yet. Based on the results of these studies, it would provide a rapid and inexpensive screening method for the rapid detection of

arbovirus activity at the collection sites. Therefore, the conventional methods could be applied subsequently at the identified positive sites, which would significantly increase the efficacy of the sample collection efforts and lower their costs.

In the first two years of the project development of sugar baits for mosquito sampling, deposition of the baits at the mosquito collection sites and operation control, as well as testing of mosquito pools and sugar bait samples for the presence of flavivirus (WNV and USUV) nucleic acid with real-time RT-PCR assays were conducted. To develop a sugar-bait-based system for mosquito saliva sampling, we have investigated the stability of WNV in sugar solution and the infection efficacy in *Culex pipiens* mosquitoes under laboratory conditions. Although we could observe that mosquitoes were drinking the bait solution, and virus RNA was detected in the pre-treated sugar solution during the subsequent days, the qRT-PCR could not reveal the presence of the virus in the mosquitoes.

During the 2016-2019 mosquito seasons, we included the sugar-bait sample collection during the mosquito trapping and tested the baits for the presence of flaviviruses by qRT-PCR. The results of the tests were all negative, and as the classical entomological methods provided more and more samples in the following years that needed laboratory efforts, this, altogether with the difficulties caused by the change of the principal researcher and the Covid-19 pandemic, we could not continue the investigation on the application of the sugar-bait method on virus detection in mosquitoes. In 2019 we started negotiations with our international partners in Slovakia, Serbia and Austria to extend the sugar bait-based sampling methods to selected sites in these countries. However, the implementation of the sample collections failed due to the technical problems described above.

3.3. RT-LAMP

Currently, the detection of viruses in surveillance samples is mainly based on real-time RT-PCR assays demonstrating virus-specific RNA. This method is sensitive and specific but requires special and expensive equipment. We planned to develop real-time reverse transcription loop-mediated isothermal amplification (RT-LAMP) assays for the detection of WNV and USUV RNA in the samples. This method is more specific and sensitive than real-time RT-PCR, does not require expensive equipment and results are provided within a short time (Parida et al., 2004; Li et al., 2011). Therefore, screening results could be obtained even during fieldwork, and positively screened sites could be further sampled. Within the project, the conventional and novel surveillance methods were planned to be applied simultaneously to

compare their specificity and sensitivity and validate the reliability of the novel methods. The development of an RT-LAMP assay for detecting WNV RNA in samples was conducted in the first 3 years of the project. Two promising primer sets were selected in two suitable genomic regions of Lineage 2 WNV and tested on WNV reference strains. Unfortunately, neither primer set amplified the target reliably under previously developed conditions leading to a detailed investigation of the problem. The main hindrance in the development effort arises from the high genetic variation of WNV strains which makes it very difficult to find suitable multiple primer sites (minimum of 6 needed for one assay) that would reliably allow the detection of at least one lineage of WNV. However, further efforts could be made to find and test additional oligonucleotide candidates for this assay. Parallelly, we searched for a pan-flavi real-time RT-PCR system to screen the mosquito samples collected in the study. The aim of the pan-flavi method was to reduce the costs and the time needed to detect all the flaviviruses already present in Hungary and those currently "exotic" ones that are – although with a relatively low-risk rate – expected to be introduced to European countries.

3.4. Pan-flavi qRT-PCR

There are several pan-flavi RT-PCR detection methods described in the literature. Scaramozzino et al. (2001) published a heminested method targeting the NS5 region that detected DENV, JEV, USUV, WNV, WSLV, YFV and ZIKV and was found to be satisfying in serum samples of human patients. However, the original paper suggests conventional PCR be used in both rounds of the nested reaction, we tested the method by applying a SYBR Green real-time PCR in the second round to reduce the need for agarose gel electrophoresis by sorting the suspected samples based on the fluorescence emission curves. Vina-Rodriguez et al. (2017) suggest an assay combining a SYBR Green-based RT-qPCR with a low-density DNA microarray to identify the viruses detected in mosquitoes. Based on the description of the method, this would detect DENV, JEV, USUV, WNV, SLEV, YFV, ZIKV, MVV, LIV and TBEV. As we had no equipment to conduct the microarray part of the method, we tested whether the melting curve analysis could provide useful information to determine positivity and sequence differences for basic identification, followed by more specific RT-PCR reactions to confirm the results. Moreover, Elizalde et al. (2020) described a Taqman-based method that detects and differentiates viruses belonging to the Japanese encephalitis (JE) serocomplex (JEV, WNV, USUV. MVEV and SLEV) and the Ntaya serocomplex (TMUV and BAGV/ITV) being present in bird samples. We tested all these methods as a basis of our quest to find a pan-flavi qRT-PCR method, and we found not only were the mosquito samples collected in Hungary

between 2018 and 2021 all negative, but we also had to realise that even if the methods worked well on some WNV and USUV isolates we used as references, some of our field samples collected from birds and mammals that were previously tested positive on the presence of WNV and were sequenced as well, turned out to be negative by the methods mentioned above. This was a shocking revelation

In the final year of the project, we decided to focus on WNV and USUV, as they are already present in Hungary. The European Union Reference Laboratory for Equine Diseases lists the method described by Del Amo et al. (2013) among the reliable tests for screening samples. However, we found the described USUV-specific probe with locked nucleotides very expensive; therefore, we tested the combination of the Del Amo WNV-specific primers and probes with other USUV-specific primers and probes described by Nikolay et al. (2014) and Cavrini et al. (2011). The problem was the same as before: the reaction worked well on the reference isolates but not on our field samples that were known to be positive for WNV. We realised that the WNV strains present in Hungary are genetically different from the reference isolates; therefore, the WNV-specific primers needed to be modified. Following the repeated assessment of the specificity of the original Del Amo primers on an alignment of target region sequences retrieved from 480 WNV strains deposited in GenBank, we have designed a couple of new reverse primers. One of these was found to work successfully in the modified reaction with the original forward primer and probes, allowing the safe detection of all our WNV strains, including those undetectable by the original system. Following further optimisation steps, we performed a dilution series-based calibration of assay sensitivity and determined that its detection threshold is 10.7 virus particles in 1 microlitre of sample. We also tested the multiplex combination of the WNV-specific modified Del Amo system and the USUV-specific qRT-PCRs described by Nikolay et al. (2014) and Cavrini et al. (2011), and we found that the Cavrini method works more reliably and seems to be more sensitive compared to the Nicolay method. The combined modified Del Amo/Cavrini method was successfully tested on mosquito samples collected in Hungary during the 2022 mosquito season.

3.5.Surveillance of flaviviruses in vectors and hosts

3.5.1. Mosquito sampling and screening

In the 2017 mosquito season, we collected 150 mosquitoes belonging to 7 species. They were organised in 17 sample pools. The mosquito pools were tested for the presence of WNV nucleic acid by real-time RT-PCR assays described by Kolodziejek et al. (2014). All samples tested negative. We used oil-impregnated cards to collect and sample mosquitoes in artificial nests of

predatory birds. We detected WNV in *Culex pipiens* mosquitoes collected by this method. We also contributed to a study by Czech researchers on the overwintering of WNV in mosquitoes. WNV lineage 2 RNA was detected in three pools of *Culex pipiens* mosquitoes collected in 2017 at two study sites (Rudolf et al., 2017)

At the start of the 2018 mosquito season, we identified seven sampling sites for mosquito monitoring studies in Hungary. Between June and October 2018, we collected mosquitoes on 15 days/nights at these sites. Mosquitoes were captured using light traps with a combination of CO_2 attractants. Sugar baits were also included in the traps or were disseminated in the surroundings of the traps. In the study period, 3106 mosquitoes belonging to 10 species were trapped, identified and ordered into 396 pools. The mosquito samples and the sugar baits were tested for flaviviruses using quantitative, real-time RT-PCR assay. All samples tested negative.

In cooperation with the Zoonotic and Vector-borne Viruses Research Group at the University of Veterinary Medicine, Vienna, we developed and tested sound traps to collect *Uranotaenia unguiculata* mosquitoes. The traps collected equivalent numbers of female *Ur. unguiculata* as low-hanging light-baited downdraft traps. Vertebrate hosts of *Ur. unguiculata* were identified as *Pelophylax lessonae* and *P. ridibunda* (Anura: *Ranidae*) species group frogs from the blood found in engorged females. In addition to confirming infection by WNV lineage 4c, a potentially integrated flavivirus sequence was detected in male mosquitoes. A novel *Alphamesonivirus 1* (Nidovirales: *Mesoniviridae*) was found to be widespread in the *Ur. unguiculata* population (Camp et al., 2018a).

Between July and October 2019, we collected mosquitoes on 48 days/nights at the following sampling sites: Budapest (several locations), Siklós, Sződliget, Gyál, Szentendre, Kecskemét and Miskolc. Mosquitoes were captured using light traps with a combination of CO2 attractants. Sugar baits were also included in the traps or were disseminated in the surroundings of the traps. In the study period, 448 mosquitoes belonging to 11 species were trapped, identified and ordered into 105 pools. About half (56) of the pools contained *Aedes albopictus* mosquitoes, most of them trapped in Budapest, indicating the massive presence of the species in the area. RNA extracts from the mosquito pools were tested for WNV- and USUV-specific nucleic acid by RT-PCR described by Scaramozzino et al. (2001). However, none of the pools was found positive for the tested viruses. The results of the study conducted in the 2018 and 2019 season were summarised in a thesis work at the University of Veterinary Medicine.

After purchasing 5 BG-Sentinel mosquito traps, mosquito sample collection continued between the end of June and the end of September 2020. Altogether 499 mosquito samples of 5750 individuals were collected at 10 sites, including nature conservation areas (Izsák-Kolon tó, Kunszentmiklós, Soroksár Botanical Garden), rural (farm) environment (Inárcs, Hőgyész, Szokolya) and city environment (Budapest 7th district, 19th district, Gyál). The collected mosquito species were identified; the samples belong to 13 species, namely *Aedes japonicus, Aedes koreicus, Aedes vexans, Anopheles hyrcanus, Anopheles maculipennis, Anopheles plumbeus, Coquillettidia richiardii, Culex modestus, Culex pipiens, Culiseta annulata, Ochlerotatus caspius, Ochlerotatus leucomelas* and *Uranotaenia unguiculata*. Moreover, *Aedes albopictus* samples were collected in Budapest, Nagykanizsa (1 sample) and Barcs (1 sample), including citizen contributions resulting in 66 single individuals. The samples were tested applying the qRT-PCR method described by Vina-Rodriguez et al. (2017), and all of them were found to be negative for the presence of flaviviruses. The results were summarised in a thesis work at the University of Veterinary Medicine.

The mosquito sample collection continued between March and October 2021 using the BG-Sentinel mosquito trap in 16 sampling sites, including nature conservation areas (Kardoskút red-footed falcon colonies, Vácrátót Botanic Garden) and the city environment (Budapest 14th district, Dunakeszi, Gyál, Göd, Kardoskút village, Budapest Óbuda Island). At the Campus of the Hungarian University of Agricultural and Life Sciences, Faculty of Horticulture, a permanent trap was established, collecting samples 24/7 during the whole mosquito season. Altogether 548 mosquito samples of 7547 individuals were collected and identified; the samples belong to 14 species, namely *Aedes albopictus, Aedes koreicus, Aedes vexans, Anopheles atropalpus, Anopheles claviger, Anopheles maculipennis, Anopheles plumbeus, Coquilettidia richiardii, Culex modestus, Culex pipiens, Culiseta annulata, Ochlerotatus caspius, Ochlerotatus flavescens* and *Ochlerotatus geniculatus*. Testing the samples applying a qRT-PCR method described by Elizalde et al. (2020), none of the samples was positive for the presence of WNV and USUV.

In cooperation with the University of Veterinary Medicine Vienna, between July and October 2021, we collected mosquito samples at 3 horse farms (Üllő, Pusztazámor and Nyergesújfalu) to investigate the virus transmission. The horse farms were chosen as there were WNV cases in the 2018 mosquito season. Altogether 251 mosquitoes classified into 61 pools were collected and were identified as *Aedes vexans, Anopheles maculipennis, Anopheles plumbeus, Culex modestus, Culex pipiens, Culiseta annulata* and *Ochlerotatus caspius*. The samples were tested

by 3 methods; a conventional nested RT-PCR suggested by the Vienna researchers, the qRT-PCR applied this year in our laboratory that was published by Elizalde et al. (2020), and later on, in 2022, we re-checked the samples by the modified Del Amo/Cavrini method as well. All samples were negative for the presence of WNV and USUV. The study was summarised in a students' scientific circle thesis and presentation at the University of Veterinary Medicine, Budapest.

In the sixth year of the project, the collection of mosquito samples continued between May and November 2022 using the BG-Sentinel mosquito traps. The sampling sites included zoos (Győr, Szeged, Nyíregyháza, Debrecen) and nature conservation areas (Lajta-Hanság National Park and Körös-Maros National Park) as well as human settlements. Altogether 25,317 mosquitoes were trapped and sorted into 2,224 pools based on the species, the place and the time of collection. Most trapped mosquitoes were *Culex pipiens* (21,925 animals in 1,666 pools) and the invasive *Aedes albopictus* (2,489 animals in 212 pools). Apart from the *Aedes albopictus*, two other invasive species were also detected, *Aedes koreicus* in 55 pools (181 animals) and less frequently the *Aedes japonicus* (6 animals in 5 pools). *Aedes albopictus* was detected in Budapest, Debrecen, Eger and Dunakeszi; *Aedes koreicus* was found in Budapest, Pest county, Tatabánya, Szeged, Eger and Marcali, while *Aedes japonicus* was trapped in Budapest, Vác and Lábatlan. Moreover, the following mosquito species were identified among the samples: *Aedes vexans, Anopheles plumbeus, Aedes cinereus/geminus, Anopheles maculipennis, Coquellitidia richiardii, Culex hortensis, Culex modestus, Culiseta annulata, Ochlerotatus dorsalis, Ochlerotatus annulipes, Ochlerotatus geniculatus, Ochlerotatus pulcritarsis, Ochlerotatus caspius, Uranotaenia uguiculata, Anopheles hyrcanus, Culiseta longioreolata* and 11 individuals that were identified as members of the *Phlebotomus* genus. This year the newly developed, modified De Amo-Nicolay/Cavrini multiplex qRT-PCR method was applied to screen the mosquitoes on the presence of WNV lineage 1 and lineage 2 strains as well as USUV. Until the submission of the final report, 6 samples (5 pools of *Culex pipiens* and 1 pool of *Ochlerotatus dorsalis*) were found to be positive for WNV Lineage 2, and 7 pools of mosquitoes (5 *Culex pipiens*, 1 *Aedes albopictus* and 1 *Aedes vexans*) were found to be infected by USUV.

In 2022 we followed different sampling methods. We applied permanent traps on the campus of the Hungarian University of Agricultural and Life Sciences, Faculty of Horticulture in Budapest, in the zoo of Debrecen, in the Körös-Maros National Park (Kardoskút), as well as in Vácrátót and Vác. We received samples from citizens participating in the mosquito monitor program organised by the Centre for Ecological Research. Moreover, in cooperation with

colleagues of the National Public Health Center and the Veterinary Diagnostic Directorate of the National Food Chain Safety Office, we conducted mosquito trapping at locations where human, horse or bird WNV cases were reported. The cases were reported between week 34 (22 August) and week 42 (18 October), and the findings are summarised in Table 1. Of all samples, 1 WNV and 2 USUV positive samples were detected before the first reported cases in Debrecen and Vác, respectively. Following the first reported human and bird cases from Mosonmagyaróvár and Győr, we found 1 WNV-positive *Aedes vexans* pool and 2 USUV-positive *Culex pipiens* pools in Jánossomorja and 1 USUV-positive *Culex pipiens* pool in Győr. Following the human cases reported in Csongrád-Csanád County, we trapped WNV-positive *Culex pipiens* pools in Szeged (1 pool) and Mórahalom (2 pools). The USUV-positive *Aedes albopictus* pool was collected in Eger. The qRT-PCR products were sequenced and found to be specific for either WNV Lineage 2 or USUV, but due to the end of the project on 30 September 2022, the detailed genetic investigation could not be performed. These results indicate virus activity and also that the multiplex qRT-PCR method we developed is specific enough to detect the WNV and USUV strains present in Hungary and sensitive enough to detect even a few virus copies in mosquito pools containing low numbers (1-20) of individuals, therefore, should be used for surveillance in the future.

Table 1.: Mosquito surveillance linked to human West Nile fever cases between W34 (22 August) – W42 (18 October) 2022

Human WNV infection	**Mosquito collection**	**Virus found in mosquito**
Mosonmagyaróvár	Győr, Jánossomorja	USUV, WNV-L2
Szeged	Szeged Zoo	WNV-L2
Mórahalom	Mórahalom	WNV-L2
Szentes	Szentes	negative
Dunakeszi	Dunakeszi	negative (or not tested)
Vác	Vác (permanent trap)	USUV (or not tested)
Erdőkertes	no mosquito collection	-
Kőkút	no mosquito collection	-
Nyíregyháza	Nyíregyháza Zoo	negative
Tiszabercel	Tiszabercel	negative
Eger	Eger	USUV
Szerencs	Szerencs	negative

3.5.2. Virus activity in birds and mammals

From September 2016 to October 2017, we tested 187 wild birds for flavivirus infections (97 in 2016 and 90 in 2017). We detected the Usutu virus (USUV) in 12 wild birds (predominantly

blackbirds) in Hungary in 2016. We have determined the complete genome nucleotide sequences of two USUVs detected in 2016 in Hungary and Austria. Genome comparisons with other European and African strains revealed a novel virus lineage that emerged in the countries (Bakonyi et al., 2017).

In 2017 we detected 5 cases of the Usutu virus and 1 case of West Nile virus infection. We have also collected serum samples from horses in the surroundings of areas with known flavivirus activity and tested them for the presence of anti-flavivirus antibodies using the competitive IgG ELISA method. Our investigations revealed >50% seropositivity in non-vaccinated animals. In cooperation with research groups of the University of Veterinary Medicine and Pharmacy in Kosice, Slovakia, we have studied the prevalence of flavivirus infections in horses (n = 145) and birds (n = 109) by ELISA and confirmation by neutralisation test (VNT). WNV antibodies have been detected in 11.7% of tested horses and 11.9% of tested birds and confirmed in 6.9% of horse and 9.2% of bird samples. None of the WNV seropositive or dubious horses had WNV IgM (ELISA), and none of the tested horses had USUV neutralising antibodies. Autochthonous WNV infections have been confirmed in 16.7% of horses. Antibodies against Tick-borne encephalitis virus were detected in 6.2% of horses and in 3.4% have been confirmed by VNT. Confirmed WNV seropositive were eight raptors, one white stork, and one house sparrow. We have also determined the complete genome sequence of a WNV detected in an avian sample collected in Slovakia and identified unique nucleotide substitutions in the viral genome (Csank et al., 2018; Dorko et al., 2018; Drzewnioková et al., 2018).

In 2018 we continued the passive surveillance activities testing samples from dead wild birds and horses with central nervous signs for the presence of flaviviruses using immunohistochemistry and nucleic acid detection methods. The West Nile virus (WNV) activity in 2018 was the most intensive ever recorded in the country. From January to October 2018, we tested 136 wild birds for flavivirus infections. We detected 15 WNV and 3 Usutu virus (USUV) infections in wild birds. Additionally, we have diagnosed West Nile neuroinvasive disease in 104 horses and one Bactrian camel in Hungary using laboratory methods (RT-PCR and ELISA). The results of the virus detection and the genome sequencing in cooperation with colleagues in the public health field were published in 2 scientific papers (Nagy et al., 2018; Zana et al., 2020).

Considering the high numbers of cases and losses caused by WNV infections amongst birds of prey, we have initiated a study to investigate the immune response of birds to WNV antigens. In cooperation with a research group at the Oregon Health & Science University (OHSU),

Portland, USA, we obtained inactivated WNV antigens to test avian immune reactivity in the goose model. This compound was a vaccine candidate, and we hoped it could provide a prophylactic solution against West Nile neuroinvasive disease in birds of prey. However, based on the virus neutralisation (VN) test results, the vaccine did not provide convincing antibody titers in the goose model even after 3 doses; therefore, the testing in protected or highly protected species of birds of prey was not permitted by the relevant authorities.

Between October 2018 and September 2019, we tested 279 dissected dead wild birds and 11 swab samples collected from European roller (*Coracias garrulus*), and a blood sample collected from a penguin kept at Budapest Zoo for the presence of flavivirus infections. We have detected 3 WNV (house sparrow, goshawk and ostrich), and the penguin was diagnosed positive for the presence of anti-WNV IgG, while none of the birds was found positive for the presence of USUV. Additionally, we tested 267 horses on the presence of anti-WNV IgG by ELISA method: 188 were found positive, indicating a previous (asymptomatic) infection and, consequently, seroconversion. Acute West Nile neuroinvasive disease was diagnosed in 31 out of 341 horses in Hungary using laboratory methods (RT-PCR and IgM ELISA). Apart from the penguin discussed among the wild birds, samples of mammalian animals, i.e. a camel and a fallow deer kept at Budapest Zoo, were investigated upon clinical signs – the camel was diagnosed positive on the presence of WNV, while the fallow deer was found negative.

In 2020 a total of 486 wild birds belonging to 56 species were subjected to pathological investigation and sampling for virological testing. Among them, 1 blackbird (*Turdus merula*) was positive for the Usutu virus, and one Northern goshawk (*Accipiter gentilis*) was positive for WNV. Moreover, blood samples from 2 ferruginous ducks (*Aythya nyroca*) showing CNS signs were collected at the Budapest Zoo and tested for the presence of viral nucleic acid and antibodies, with a negative result. Blood samples were collected from 15 magpies (*Pica pica*) and 14 hooded crows (*Corvus cornix*) in the Kiskunsági Nemzeti Park. Among them, 3 magpies and 3 hooded crows were found to be positive for anti-WNV IgG by both ELISA and VN tests. These animals were also tested by RT-PCR and found negative for WNV, USUV and TBE.

Before and during the 2020 mosquito season, 195 horse blood samples from all over Hungary were tested for antibodies against WNV, USUV and TBEV with ELISA and VN tests as well. Among them, 167 were found to be positive for anti-WNV IgG. Moreover, 2 horses sampled in October and December 2019 and 1 horse sampled in September 2020 tested positive in the anti-WNV IgM ELISA by the National Food Chain Safety Office Veterinary Diagnostic

Directorate. The blood samples were handed over to our research group for viral nucleic acid detection, but the RT-PCR investigation ended with negative results.

In 2021, blood samples from WNV susceptible birds (western jackdaw, red-footed falcon and common kestrel) were collected. Apart from the serological investigations described below, we planned to investigate the samples on the presence of viral nucleic acid by qRT-PCR. We realised that separating the buffy coat is difficult due to the small volume of the bird-origin blood samples and results in the loss of viral nucleic acid in the sample. However, nucleic acid extraction from whole blood samples is hindered by fibrin coagulation due to reactions with reagents. In the 6th year of the project, we were planning to find a solution to this problem.

In the 5th year of the project, a total of 120 blood samples of wild birds belonging to 3 species /western jackdaw (*Coloeus monedula*), red-footed falcon (*Falco vespertinus*) and common kestrel (*Falco tinnunculus*)/ were collected in Kardoskút. The samples were taken from adults and chicks, and the samples were tested on the presence of antibodies by ELISA that detects anti-WNV, anti-USUV and anti-TBE antibodies. All the jackdaw samples were found negative; among the red-footed falcon samples, 29 were positive, while among the common kestrel samples, 58 were detected positive in the ELISA. As the method detects antibodies against three viruses, an immune fluorescence test is needed to clarify the antibody specificity, which was planned to be performed during the 6th year of the project, but due to the problems at the customs, the quality upon arrival of the ordered fluorescent-labelled antibodies needed was not suitable for the test.

In 2022 upon agreement with Sopron University, University of Debrecen, and the Körös-Maros National Park, we collected blood samples from birds. From the Lajta-Hanság National Park until the final report submission, we investigated 44 blood samples from crows to survey the seropositivity in the population. Among the samples, IgG antibodies were detected by ELISA in 1 adult and 2 juvenile (hatched either in 2021 or 2022) birds. From Debrecen, we received 50 blood samples and carcasses of 120 hooded crows until the end of September 2022. The serological investigation performed by ELISA test revealed that until the sampling, 4 birds got infected by either WNV, USUV or TBEV. Unfortunately, the blood samples are not linked to the carcasses; therefore, we have no data on the age of the birds sampled or any pathological lesions that would indicate an approximate time of the infection. PCR testing of organ samples detected 9 WNV lineage 2 infections in the examined sample set, establishing that the WNV circulation period in Debrecen lasted from 16 July to 15 September 2022. From Kardoskút, we received 287 blood samples from red-footed falcon, kestrel and jackdaw nestlings. We found

65 birds positive for the presence of anti-WNV/USU/TBEV IgG. We also investigated 8 serum samples from Eastern imperial eagle nestlings sampled during their ringing at the lowland habitats East and South East of Budapest, and none of them were found to be positive for WNV/USUV/TBEV antibodies.

As in the ELISA test, there is cross-reaction between the antigen and the antibodies produced against WNV, USUV and TBEV, virus neutralisation test, or, considering the small volume of the serum available, immunofluorescence test is needed to determine the specificity of the antibodies. However, due to financial reasons, this investigation has not been performed until the submission of the final report.

We also worked on finding a method to perform nucleic acid investigation and subsequent qRT-PCR on the coagulated blood after separating the serum from the birds' blood samples. Due to the presence of fibrin and the enormous amount of DNA originating from the nucleated red blood cells, nucleic acid purification is difficult: the lysis step designed for mammal samples results in a gel-like mass that is almost impossible to press through the filter catching the nucleic acid. Therefore we have tested several commercial kits designed to extract total nucleic acids from biological samples and successfully adapted a kit utilising a proteinase K digestion step to solve this problem. This kind of testing and optimisation exercise is of great importance for the wise and efficient utilisation of the limited number and often very small quantities of biological samples obtainable from endangered bird species.

This year, we received serum samples of 2 anti-WNV IgM-positive horses from the Veterinary Diagnostic Directorate of the National Food Chain Safety Office. The qRT-PCR performed on these samples turned out negative. One of the horses was kept in Tatabánya, and the other in Domonyvölgy. Mosquito trapping was conducted at these locations right after the serological diagnosis of the horses, but all mosquito pools collected were found to be negative for flaviviruses. The horse kept at the horse farm in Domonyvölgy was imported from Italy a week before the onset of the clinical signs, and therefore we did the qRT-PCR investigations with the hope of detecting a virus that is different from the ones circulating in Hungary. However, this data explains the mosquito negativity at this location.

Moreover, in 2022 we performed an IgG-detecting ELISA test on 76 serum samples of horses. The samples arrived at our laboratory with the question of the need for anti-WNV vaccination before or during the mosquito season. The majority of the samples were positive; in case of negative results, we recommended the vaccination of the horses.

Regarding anti-WNV immunity, we also performed a 4-year-long study on the time length of seropositivity after natural infection by WNV. The horses participating in the investigation were asymptomatically infected in 2018; anti-WNV IgM-detecting ELISA confirmed the infection. In 2019-2022, 36, 38, 38 and 30 horses were tested, respectively. Most horses were positive in the IgG ELISA test throughout the 4 years. One horse was negative in 2019 but seroconverted in 2020, while another was positive in the 2019-2020 sampling and became negative in 2021. The ELISA results were also confirmed by the VN test, determining that the antibodies were specific to WNV; thus, we excluded the serological cross-reaction with USUV and TBEV. These results show that the seropositivity flowing natural infection lasts for at least 4 years even after asymptomatic infection; however, it depends on the individual and most probably the age of the horse, as the one becoming negative was over 17 years old and therefore, the capability of its immune system might be weaker compared to younger animals. Altogether, the conclusion is that before the mosquito season, anti-IgG detecting serological test is needed to determine the need for vaccination. The study was summarised in a thesis work at the University of Veterinary Medicine, Budapest.

3.6.Surveillance of the presence of other arboviruses

3.6.1. Chikungunya virus

Chikungunya is a viral disease caused by a virus (Chikungunya virus, CHIKV) classified into the *Togaviridae* family *Alphavirus* genus. The virus was first identified in Tanzania in 1953 and is transmitted by *Aedes* mosquitoes from human to human. The word “chikungunya” means “what bends up”, indicating the severe joint pain the patients suffer from, besides fever and rash, as well as chronic arthritis associated with the infection. Diagnostic tests are available, but no antiviral treatment or licensed vaccine exists. The disease is notifiable at the EU level.

Humans are the major source of the chikungunya virus (urban cycle); moreover, primates serve as reservoirs in Africa, maintaining a sylvatic cycle of infection. Chikungunya is endemic in all (sub) tropical regions of America, Africa and Asia. In 2005-2006, the major outbreak in the Indian Ocean resulted in imported cases found in Asia, Australia, the USA, Canada and continental Europe. In 2007, following the introduction of the virus by an infected traveller, an outbreak of autochthonous chikungunya virus infections took place for the first time in Europe (Italy), with over 300 cases. In 2010, 2014 and 2017, a few autochthonous cases were reported in France, while in Italy, the 2017 outbreak resulted in over 400 cases, about half of them confirmed by laboratory investigations. As there are three major genetic lineages recognised,

identifying the origin of the infection is possible by a detailed genetic investigation of the virus detected in the patients. Based on these epidemiological events, the risk of the chikungunya virus spreading in the EU is high due to importation through infected travellers, the presence of competent vectors in many countries (particularly around the Mediterranean coast) and population susceptibility.

The main vectors of the chikungunya virus are *Ae. aegypti* and *Ae. albopictus* mosquito species. Up to now, the presence of *Ae. aegypti* has been reported only in Madeira; however, *Ae. albopictus* is established in the southern and central countries of mainland EU and is spreading. When the environmental conditions are favourable, in areas where *Ae. albopictus* is established, viraemic travel-related cases may generate a local virus transmission as demonstrated by the sporadic events of chikungunya virus transmission since 2007.

The presence of *Ae. albopictus* in Hungary was first reported in 2014 near Baja, and since then, it has been trapped every year in increasing numbers, not only near the southwestern border but, indicating its range to expand, also in other parts of the country, including Budapest. This finding resulted in the inclusion of a CHIKV-detecting qRT-PCR into the testing of the mosquitoes collected in this project. For the surveillance, we applied two qRT-PCR systems as suggested by Thirion et al. (2019), with the primers and probe set published by Panning et al. (2008) for screening and another primer-probe set described by Pastorino et al. (2005) for confirmation. In 2019, 56 Ae. albopictus mosquitoes were trapped, most in Budapest and some in Miskolc, Gyál and Siklós. In 2020 not only the number increased to 157, but also the sites of catching: the majority of the mosquitoes were trapped in Budapest, and a few of them was found in Marcali, Dunakeszi, Nagyszalonta, Nagykanizsa and Barcs.In these two years, the animals were investigated individually. In 2021 a record number, 2920 *Ae. albopictus* mosquitoes sorted into 173 pools based on the time and place of trapping were investigated; they were caught mostly in Budapest (in the permanent trap located at the campus of the Hungarian University of Agricultural and Life Sciences, Faculty of Horticulture) and Pest county (Dunakeszi, Göd, Gyál), and a few of them were found in Balatinalmádi, Székesfehérvár. In the 2022 season, 1232 Asian tiger mosquitoes were collected and sorted into 138 pools; as in the previous year, the permanent trap established at the campus of the Hungarian University of Agricultural and Life Sciences, Faculty of Horticulture in Budapest was the main source of the animals, but some were found in other settlements in Pest county too (Dunakeszi, Vác, Budaörs), as well as in Battonya and Eger. Between 2014 and 2019, each year, 1-5 imported chikungunya cases were reported by the National Public Health Center; in 2020 and 2021, due to the travel restrictions

resulting from the Covid-19 pandemic, the number of cases was 0. The investigated mosquito samples were found negative for the presence of CHIKV, which agrees with the ECDC statement that no events of autochthonous transmission have been reported in the EU/EEA since 2017.

3.6.2. Bunyaviruses

In cooperation with researchers of the Institute of Virology, University of Veterinary Medicine, Vienna, Austria and the Departamentul Sanatate Publica, Facultatea de Medicina Veterinara, USAMV, Iasi, Romania, serum samples were collected from wild boars and, in Austria, deers and roe deers, and were investigated by virus microneutralisation assay for the presence of antibodies specific for Tahyna virus (TAHV, *Peribunyaviridae*, *Orthobunyavirus* genus). Altogether 15% of the wild boar samples were found positive; the seropositivity rate in Hungary varied between 0% and 50% (Csongrád county), indicating the activity of the virus in the wild animal populations (Camp et al., 2018b). Based on this finding, we planned the development of RT-PCR and RT-LAMP-based methods for detecting the nucleic acids of orthobunyaviruses (Tahyna virus, Sedlec virus, Schmallenberg virus) and the determination of the specificity and sensitivity of the assays using reference virus strains. In 2019 we tested mosquito samples for the presence of orthobunyavirus RNA with RT-PCR and RT-LAMP methods, but all mosquito samples collected were found negative for orthobunyavirus RNA. As the Covid-19 pandemic caused severe technical problems in purchasing reagents and laboratory supplies, between 2020 and 2022, the testing was suspended; however, we are planning to resume the screening of the collected samples in the future.

3.6.3. Equine infectious anaemia virus

Based on the Equine infectious anaemia (EIA) outbreak in 2015, among the plans of the project, we wanted to compare the currently available methods for detecting Equine infectious anaemia virus (EIAV) on samples collected from Hungarian EIA cases, determine the most reliable diagnostic methods and develop further ones with increased specificity and sensitivity. The presence of EIAV RNA was planned to be tested in horse-fly samples collected from the environment of infected horses. We hope that our studies provide a basis for establishing sustainable, long-term pathogen surveillance and monitoring systems to provide reliable data on the presence or emergence of the virus in the Pannonian Basin.

Efficient control measures of Equine infectious anaemia require reliable, sensitive and rapid diagnostic methods. A comparison of the available assays on samples collected from EIAV-

infected horses in Hungary was planned to help identify the best diagnostic methodology, and the development of further ones may increase the efficacy of sanitary prophylaxis.

The re-emergence of EIA in Hungary raised serious concerns among horse keepers. Control of the spread of EIAV is necessary for horse health management and the maintenance of the animal health situation of the country. Improved diagnostic methods and assays could facilitate the early recognition of EIAV-infected horses, improving the efficacy of virus control measures.

Between 2016 and 2018, we compared the specificity and sensitivity of the currently available assays for the diagnostics of Equine Infectious Anaemia (AGID, ELISA, PCR) on samples collected from EIAV-infected horses identified in the previous years in Hungary. The so-far published RT-PCR assays for EIAV have been tested for specificity and sensitivity. Two assays were able to detect the virus in the majority of samples from seropositive horses. In cooperation with the Medical University in Vienna, we have demonstrated that certain molecular diagnostic methods (e.g. metagenomic analysis) may provide a false positive result for EIAV. The next-generation sequencing approach on Hungarian EIAVs failed, most probably due to RNA degradation in the samples.

In the following years, the EIAV investigation was not continued. One of the reasons was the leave of the PhD student assigned to the topic, who resigned from the Veterinary Diagnostic Directorate of the National Food Chain Safety Office and decided to give up her PhD studies. Equine infectious anaemia is a notifiable disease that, by national regulation, can be investigated only in the Veterinary Diagnostic Directorate of the National Food Chain Safety Office; therefore, the lack of a PhD student working there seriously hindered the work on the topic. This, combined with the change in the principal investigator, resulted in a lack of capacity to deal with the study. Moreover, and this is also related to the resignation of the colleague, we have not received information about the EIAV outbreaks in Hungary from the Veterinary Diagnostic Directorate of the National Food Chain Safety Office, and therefore we did not have a motivation for efforts among the other technical problems caused by the Covid-19 pandemic and the capacity needed to deal with the increasing number of mosquito samples.

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The impact of climate change on the epidemiology of some mosquito-borne viruses [Thesis, University of Veterinary Medicine, Budapest, supervisor: Petra Forgách] / Kaia Seim Jakobsen (2021)

Surveillance on the presence of flaviviruses and Chikungunya virus in mosquito vectors [Thesis, University of Veterinary Medicine, Budapest, supervisor: Petra Forgách] / Se-eun Choi (2021)

A nyugat-nílusi vírus szúnyogvektorokban való jelenlétének vizsgálata korábban fertőzött lóállományok közvetlen közelében [TDK, University of Veterinary Medicine, Budapest, supervisors: Orsolya Korbacska-Kutasi and Petra Forgách] / János Kiss (2022)

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