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EMERGENCE OF MOSQUITO-BORNE FLAVIVIRUSES IN CENTRAL EUROPE

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Abstract Although the majority of flaviviruses are endemic in tropical or sub-tropical region, certain species are often detected in Europe as well. In central Europe, the Tick Borne Encephalitis Virus is endemic in several geographic regions. Amongst the mosquito-borne flaviviruses the West Nile virus (WNV) emerged in several European countries within the last 50 years. Outbreaks of WNV encephalitis in humans and horses were reported, and strains were isolated form wild bird and mosquito species too. Recently, new WNV strains were isolated in Central Europe from mosquito vectors and from encephalitic cases of vertebrate host. The aim of this study was to reveal the origin and genetic relatedness of these strains.

Another mosquito borne flavivirus, the Usutu virus (USUV) emerged in 2001 in Austria. Previously the virus was detected only in Africa. In Austria the USUV exhibited high pathogenicity to wild birds, especially to the Eurasian Blackbird (*Turdus merula*). Within the last seven years USUV became resident pathogen in Austria and the infection spreads to the neighbouring countries. The main epidemiological observations of the Austrian USUV outbreak are discussed in this study. The complete genome sequences of Central-European flavivirus strains were determined and submitted to phylogenetic analyses. A WNV strain detected in encephalitic geese in Hungary in 2003 exhibited the closest genetic relationship (~98% nucleotide identity) to WNV strains isolated in Israel and in the United States. Another Hungarian WNV strain was isolated from a goshawk (*Accipiter gentilis*) in 2004. This virus showed the highest identity rates (~96%) to WNV strains of lineage 2 isolated in Africa. The same strain re-emerged in 2005 in *Accipiter* species and in a sheep, as well as in 2007 in birds and in a horse. A third WNV strain was isolated from *Culex pipens* mosquitoes in 1997 in the Czech Republic. This isolate shared only 75% to 77% nt identities with strains of WNV lineages 1 and 2. The USUV strains, which were detected in Austria and Hungary, share >99% identity with each other, and 97% identity with the South-African strain. The virus strains that emerged in Switzerland and Italy in 2006 were nearly identical with the Austrian and Hungarian USUV isolates. The results of the investigations revealed a diversity of flaviviruses in Central Europe and the role of migrating birds as possible carriers of exotic flaviviruses.

Key Words West Nile virus, Usutu virus, encephalitis

INTRODUCTION

Arthropods are carriers and vectors of several parasites and microbial agents (Hill et al., 2005). Certain arthropod-borne diseases are major threats for public and veterinary health. Besides ticks and flies, mosquitoes are the most important vectors of human and animal pathogens (*Plasmodium spp*.) (Tuteja, 2007). Several viruses are transmitted by arthropods (so-called arthropod-borne viruses or arboviruses.). The term “arbovirus” is an ecological classification; the arthropod-transmitted viruses are belonging to several

different virus families (Rehle, 1989). Although several DNA viruses (i.e. the myxoma virus [*Poxviridae*] or the African swine fever virus [*Asfarviridae*]) are vectored by arthropods, most of the arboviruses are RNA viruses, and belonging to the *Reoviridae, Togaviridae, Flaviviridae* and *Bunyaviridae* virus families (Calisher, 1994, Lundström, 1999). These viruses are usually rather sensitive for the environmental conditions; therefore the arthropod vectors protect them from the inactivating effects. Moreover, several arthropods are so-called biological vectors of the viruses. These viruses infect and multiplicate in the arthropods' cells and often infect the offspring, which maintain the virus infections in the population.

Table 1. Mosquito-borne flaviviruses in Central Europe.

Code	Name	Lineage, clade	Isolation		
			Year	Host	Origin
NY99a	WNV HNY1999	1a	1999	Human	New York, USA
NY99b	WNV NY99flamingo38299	1a	1999	Flamingo	New York, USA
Is98	WNV IS98STD	1a	1998	Stork	Israel
Hu03	WNV goose-Hungary/03	1a	2003	Goose	Hungary
It98	WNV Italy1998Equine	1a	1998	Horse	Italy
Ro96	WNV RO9750	1a	1996	*Culex pipiens*	Romania
Rus99a	WNV VLG4	1a	1999	Human	Volgograd, Russia
Rus99b	WNV LEIV-Vlg99-27889	1a	1999	Human	Volgograd, Russia
Tu97	WNV PaH001	1a	1997	Human	Tunisia
Fr00	WNV PaAn001	1a	2000	Horse	France
Eg51	WNV Eg 101	1a	1951	Human	Egypt
Chin01	WNV Chin-01	1a	1950s	?	Russia
Kunjin	WNV Kunjin MRM61C	1b	1960	*Culex annulirostris*	Australia
Sarafend	WNV Sarafend	2	Laboratory strain		
Ug37	WNV B956 (WNFCG)	2	1937	Human	Uganda
Hu04	WNV goshawk-Hungary/04	2	2004	Goshawk	Hungary
RabV	Rabensburg virus (97-103)	3	1997	*Culex pipiens*	Czech R.
Rus98	WNV LEIV-Krnd88-190	4	1998	*Dermacentor marginatus*	Caucasus

The majority of arbovirus infections occur in the tropical and subtropical regions due to the warm climate as well as the abundance and diversity of vector and host species. Several arboviruses are, however, present in the temperate zones too. Within the *Flaviviridae* family the genus *Flavivirus* comprises more than 50 virus species (Gould et al., 2001, ICTVdb, 2006). These viruses are grouped as tick-borne viruses, mosquito-borne viruses and viruses with no known arthropod vector. Some of these viruses usually cause diseases associated primarily with fever, arthralgia and rash, i.e. the Dengue virus (DV) and the West Nile virus (WNV). Others usually cause encephalitis syndrome, i.e. the Tick-Borne Encephalitis viruses (TBEV), the Japanese Encephalitis virus (JEV), the St. Louis Encephalitis virus (SLEV), and the Murray Valley Encephalitis virus (MVEV). The third main clinical manifestation of flavivirus infection is haemorrhagic fever, i.e. Yellow fever virus (YFV) and Omsk haemorrhagic fever virus (OHFV). However, some flaviviruses may cause diverse diseases, i.e. WNV encephalitis, or dengue haemorrhagic fever (Davis et al., 2006, Halstead, 2007).

In central Europe TBEV is endemic in several regions and causes hundreds of human infections each year (Charrel et al., 2004). The presence of WNV in Europe is also known for decades; however clinical manifestation of the infections were previously recorded only in the Mediterranean and eastern regions (i.e.

France, Italy, Romania, Ukraine, Belarus) (Hubálek and Halouzka, 1999). Within the last ten years new WNV strains were isolated in The Czech Republic from mosquito vectors and in Hungary from encephalitic cases of vertebrate host. This study discusses the genetic relatedness and possible origin of these strains.

A mosquito-borne flavivirus, the Usutu virus emerged in 2001 in Austria (Weissenböck et al., 2002). Previously this virus was found only in the sub-Saharan region of Africa, and was considered as a minor pathogen. In Austria the virus caused severe mortality in urban wild bird populations, especially in blackbirds (Weissenböck et al., 2003). The main genetic characteristics of the virus and the epidemiological observations of the USUV outbreak are presented in this study.

MATERIALS AND METHODS

Samples

A flavivirus strain (97-103) was isolated by intracranial inoculation of suckling mice with homogenates of female *Culex pipiens pipiens* mosquitoes, which were collected in the Czech Republic, following a flood of the river Morava in 1997 (Hubálek et al., 1998, 1999). The collection site was very close to the Austrian town of Rabensburg, consequently the isolate 97-103 was later tentatively called Rabensburg virus (RabV). Another antigenically identical strain (99-222) was isolated from *Cx. pipiens* mosquitoes in the same location two years later (Hubálek et al., 2000).

An outbreak of encephalitis was observed in a goose farm in Hungary, close to the river Danube in 2003 (Glávits et al., 2005). Brain sample of a six-week old goose (*Anser anser domestica*) was used for the molecular detection and characterization of the causative agent. In 2004 a goshawk (*Accipiter gentilis*) showed central nervous system (CNS) symptoms and died in a raptor rehabilitation centre in the south-eastern region of Hungary (Erdélyi et al., 2007). Organ samples of the bird were submitted to virological and molecular investigations for the identification of the causative agent. Further goshawk and sparrowhawk (*Accipiter nisus*) samples were submitted for investigations in 2005 from the same raptor keeping facilities (Erdélyi et al., 2007). A four-year-old ewe showed CNS symptoms and died on a sheep farm in 2005. A flavivirus was isolated from the brain samples of the animal and was identified with molecular methods (Kecskeméti et al., 2007). A horse with CNS symptoms was hospitalized at the Üllő Large Animal Clinic of the Veterinary Faculty, Budapest in September 2007. Due to the worsening symptoms, the horse was euthanized a few days after. The animal was necropsied and the cause of the disease was investigated with histopathological and molecular methods.

Increased wild bird mortality was observed in the late summer 2001 in a city park in Vienna, Austria. Predominantly blackbirds (*Turdus merula* L.) were involved, however five great grey owls (*Strix nebulosa*) also died in the Vienna Zoo Schönbrunn (Weissenböck et al., 2002). A virus was isolated from the brain sample of a blackbird and was characterized by serological and molecular methods. Within 2002 and 2007 dear bird surveillance programmes were established in Austria (Chvala et al., 2007) and in Hungary (Bakonyi et al., 2007). Dead wild birds were collected in urban parks, wild bird rehabilitation centres, and in ringing camps. Birds were identified, necropsied and were tested for flavivirus infections with histopathological and molecular methods (Chvala et al., 2004). Similar to the Vienna outbreak in 2001, increased wild bird mortality was observed in Zurich, Switzerland and in an owl farm close to Milan, Italy in 2006. Dead birds and bird brain samples were submitted for pathological and virological investigations.

Detection and Identification of Flaviviruses

The samples were processed using standard histopathological and virological methods. Tissue samples were fixed in formalin, embedded in paraffin; histological sections were made, stained with hematoxylin and eosin, and investigated under light microscope. Immunohistocemistry (IHC) was used for the specific detection of virus antigens in the cells using WNV and USUV specific sera (Weissenböck et al., 2002; Erdélyi et al., 2007). Viruses were isolated by the intracerebral infection of suckling mice, inoculation into the allantoic cavity of embryonated eggs, or the infection of primary cell cultures and permanent cell lines (Hubálek et al., 1998; Bakonyi et al., 2005b). Viruses were antigenically characterized with plaque reduction microneutralization tests, with indirect immunofluorescence, and with haemagglutination inhibition tests (Hubálek et al., 1999; Meister et al., 2008). The virus nucleic acid was detected in the samples with WNV,

USUV and JEV-group specific primers in reverse transcription - polymerase chain reaction (RT-PCR) assays as well as with *in situ* hybridization (ISH) tests using specific oligonucleotide probe molecules (Weissenböck et al., 2002; Bakonyi et al., 2006).

Determination of complete nucleotide sequences and phylogenetic analyses: Oligonucleotide primer pairs were designed to amplify overlapping cDNA sequences on the complete genome of WNV, RabV and USUV in RT-PCR assays (Bakonyi et al., 2004, 2005a, 2006). The amplification products were directly sequenced in both directions; the partial sequences were aligned and compiled. The nucleotide sequences of the strains were submitted to phylogenetic analysis using the Neighbour-Joining statistical method by the ClustalX (Thompson et al., 1997) and PHYLIP softwares (Felsenstein, 2004). Sequences were compared to other flavivirus sequences deposited in GenBank databases (NCBI). The probable genetic relatedness of the strains was demonstrated in phylogenetic trees with the help of the TreeView 1.6.6. software.

RESULTS AND DISCUSSION

The complete genome sequence of the Rabensburg strain was determined and the coding sections for the putative virus proteins were identified (Bakonyi et al., 2005a). The 10972 nucleotide (nt) long sequence was aligned with other WNV sequences in the GenBank. The RabV showed 73-77% nt identity with other WNV strains. Previous studies revealed two distinct genetic lineages of WNV (Lanciotti et al., 2002; Charrel et al, 2003). Lineage 1 contains strains from all around the world, and sub-divided into at least three clusters (1a: worldwide strains, 1b: Indian strains, 1c: Australian strains [Kunjin virus]). Lineage 2 contains strains, which were detected only in the sub-Saharan Africa, and in Madagascar before. The phylogenetic tree indicates that RabV is genetically separated from the previously known WNV strains, and represents a new, third lineage of the virus (Figure 1). Another WNV strain was found in the GenBank database, which is also secluded from the known WNV strains, and from the RabV too; therefore this strain presumably represents a fourth lineage of WNV. This particular virus (Krnd88-190) was isolated from *Dermacentor marginatus* tick in the Caucasus in 1988 (Lvov et al., 2004). Virulence studies revealed that the RabV strain is less virulent to mice than the WNV lineage 1 topotype strain Eg-101 (Bakonyi et al., 2005a).

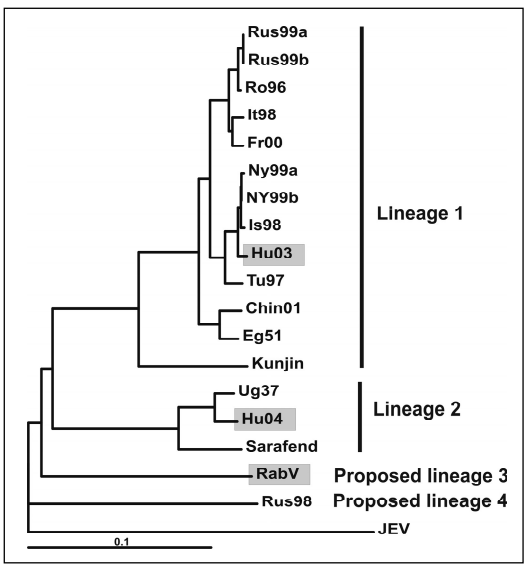

Figure 1. Phylogram based on the complete nucleotide sequences of selected WNV strains demonstrating the genetic relatedness of these strains. Abbreviations are listed in Table 1. Bars indicate different lineages of WNV. The strains which were characterized in this study are marked with grey background.

WNV was detected in the brain of the goose which died in an encephalitic outbreak in Hungary in 2003 (Glávits et al., 2005). The complete genome sequence of the virus was amplified by overlapping RT-PCR reactions and was determined by direct sequencings (Bakonyi et al., 2006). Phylogenetic analysis of the strain revealed that this virus belongs to the lineage 1 of WNV. It has shown the closest genetic relationship (98% nt identity) with WNV strains isolated in Israel in 1998, and in the New York in 1999 (Figure 1). WNV emerged in the America first in 1999 in New York (Anderson et al., 1999). The virus caused serious outbreaks in the cities, in wild bird populations, and several human and horse encephalitic cases were

reported. Within five years WNV spread around the North American continent (Hayes and Gubler, 2006). The origin of the virus is unknown, but - according to the phylogenetic studies - the closest relatives are isolates from Israel (Lanciotti et al., 1999). This virus strain was first isolated from migrating white stork (*Ciconia ciconia*) fledglings, which have hatched in the summer of 1998 in central Europe (Malkinson et al., 2002.). The WNV strain, which was isolated in 2003 in Hungary, is probably a descendant of the virus, which was introduced into Israel and into the USA five years before.

The goshawk brain sample, which was collected in 2004 in southeast Hungary, gave positive reaction in WNV-specific RT-PCRs, and in IHC investigations (Bakonyi et al., 2006, Erdélyi et al., 2007). The complete genome sequence of the virus was determined and phylogenetic analysis was performed. The WNV strain showed the highest (96%) nt similarity to the WNV lineage 2 topotype strain B 956 (Figure 1). This is the first case when a neuroinvasive WNV lineage 2 strain was detected outside of Africa. The same virus re-emerged in 2005 in the same raptor rehabilitation centre, and caused mortality in goshawks and in a sparrowhawk. In 2005 a virus was isolated from the brain sample of a ewe, which was kept about 20 km from the previously-mentioned raptor rehabilitation centre, and died in CNS symptoms (Kecskeméti et al., 2007). RT-PCR investigations and partial sequencings revealed that the virus strain is practically identical with the lineage 2 WNV strain, which emerged in Hungary one year before. A horse was hospitalized with CNS symptoms and died in September, 2007. RT-PCRs and IHC investigations detected WNV in the brain and spinal chord samples of the animal. The partial nucteotide sequence of the virus was determined, and it has showed >99 % identity with the goshawk, sparrowhawk, and sheep strains. The horse was kept 30 km from the sheep farm, where the ewe case emerged in 2005. The results of these studies indicate, that a previously exotic (presumably African), neuroinvasive, lineage 2 WNV strain developed efficient host-vector cycle in the Hungarian continental climate, over-wintered, circulates for at least three years, and causes sporadic CNS diseases in birds and in mammals.

A virus was isolated from a blackbird sample, which was received during an episode of increased wild bird mortality in Vienna in 2001. The virus was identified by IHC, ISH, and RT-PCT, using JEV-group specific primers (Weissenböck et al., 2002). The partial nucleotide sequence of the virus indicated that the Usutu virus emerged in Austria. The complete genome sequence of the Vienna USUV strain and the reference, South African USUV strain (Sa Ar 1776) has been determined; they share 97% nt identity (Bakonyi et al., 2004). The USUV has been detected in further blackbirds in and around Vienna, and in great grey owls in the Vienna Zoo. The mortality of urban wild birds was monitored in the eastern federal states of Austria between 2002 and 2005 (Weissenböck et al, 2003; Chvala et al., 2007). The number of the recorded cases (mainly blackbirds) increased until 2003, but a sudden decrease was observed in 2004 (Figure 2.), and in 2007 no USUV positive cases were found in Austria. On the other hand, the number of USUV seropositive wild birds significantly increased in the last three years of the survey (Figure 3.) (Meister et al., 2008). Mosquitoes were collected at Austrian regions with USUV activity. The virus was detected in *Culex pipiens, Culex hortensis, Culex territans, Culiseta annulata, Aedes vexans* and *Aedes rossicus* pools, using RT-PCR and real-time RT-PCR (unpublished data). Limited dead wild bird monitoring was also preformed in the central region of Hungary since 2003. The USUV first emerged in Hungary in 2005 in urban blackbirds in Budapest (Bakonyi et al., 2007). The complete genome sequence of the virus was determined. It shows 99.9% nt identity with the Vienna 2001 USUV strain. USUV activity was also observed in 2006 and in 2007 in the blackbird population in Budapest.

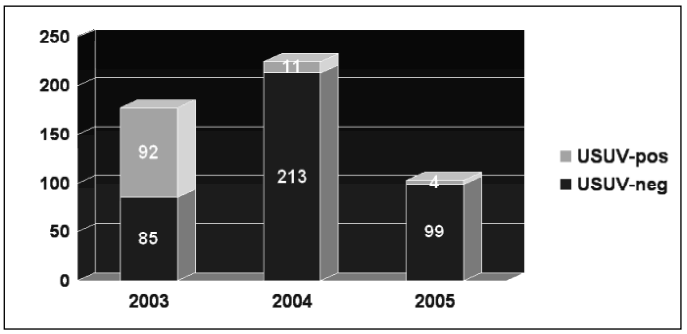

Figure 2. Number of wild birds submitted for USUV investigations within the Austrian monitoring programme.

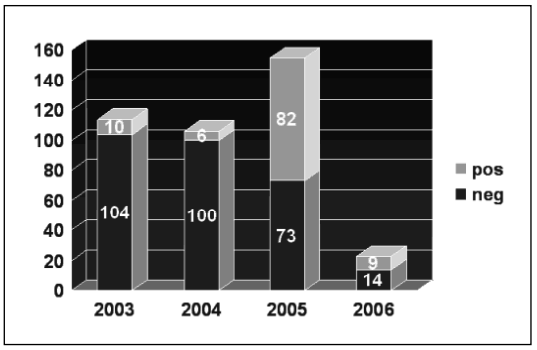

Figure 3. Number of wild bird sera submitted for USUV-specific antibody detection in the Austrian monitoring programme.

Increased wild bird mortality (predominantly blackbirds and house sparrows [*Passer domesticus*]) was recorded in Zurich, Switzerland in July and August, 2006. The USUV was detected in the brain samples of the several birds by RT-PCR and IHC. The partial genome sequence of the virus exhibits >99% identity to the Vienna-2001 and Budapest-2005 blackbird strains. Great grey owls and hawk owls (*Surnia ulula*) showed CNS symptoms and died in an owl farm close to Milan, Italy in August, 2006. The USUV was detected in the brain samples of the birds by RT-PCR and IHC. The partial genome sequence of the virus was practically identical with the strains emerged in Austria, Hungary and Switzerland. The virus re-emerged in 2007 in a little owl (*Athene noctua*) at the same owl farm, and also in blackbirds and sparrows in the city of Milan.

Our observations indicate that USUV established a successful ecological cycle in the central European wild bird and mosquito populations. The virus shows considerable neurovirulence in blackbirds, in sparrows, and in different owl species (*Strigiformes*). The incidence of the virus in Austria decreased, probably due to the establishment of herd immunity in the host populations (Meister et al., 2008). Meanwhile the virus spread to neighbouring European countries and caused local enzootics in urban wild bird populations.

CONCLUSIONS

Our investigations revealed that at least four different mosquito-borne flavivirus strains, WNV lineage 1, WNV lineage 2, RabV, and USUV, were present within the last ten years in central Europe, and periodic or consecutive activity was recorded in different years' epidemic seasons. Some of these viruses caused symptomatic CNS diseases and mortalities in bird and mammal species. Besides the outbreaks in animals, WNV-induced meningitis and encephalitis cases were diagnosed in human patients in Hungary between 2003 and 2006, using serological methods (Bakonyi et al., 2006).

The previously mentioned flaviviruses share common surface antigens; therefore cross-reactions might complicate the accurate serological diagnosis of the infections (Heinz, 1986). The direct diagnosis of newly emerging strains (i.e. WNV lineage 2) requires improved molecular diagnostic methods, because the assays, which are routinely used in some European diagnostic laboratories, might not detect these strains (Niedrig et al., 2006).

Although the exact route of the introduction of exotic flaviviruses to central Europe is unknown, the role of migrating birds is presumed. These viruses established successful mosquito vector — vertebrate host cycles and some of them became resident endemic pathogens in some areas. The geographic spread of some viruses (WNV lineage 2, USUV) was also observed. Therefore continuous monitoring activity would be necessary to follow the spread of these viruses in the region.

Currently specific treatment and vaccines are not available for the protection of birds or humans from the previously mentioned flaviviruses. Only mosquito control measures could reduce the risk of the infection of vertebrates and the development of serious diseases (CDC). Therefore the use of improved mosquito control measures is strongly emphasized.

The climatic changes and the increased international travelling, trade, and transport raise the risk of introduction of new pests (i.e. exotic mosquito species) and pathogens (i.e. exotic viruses) into Europe. Therefore integrated monitoring activity and emergency preparedness is necessary to protect the human communities and animal populations from newly emerging pests, pathogens and diseases.

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