

## EMERGENCE OF MOSQUITO-BORNE BUNYA-, TOGA-, AND REOVIRUSES IN CENTRAL EUROPE

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**Abstract** In this paper the most important ecological characteristics of arthropod-borne (arbo) viruses and the recent emergence of some pathogens in Europe are reviewed. In early July 2007 the first autochthonous Chikungunya virus outbreak occurred in Europe between July and September 2007. A local transmission cycle involving *Aedes albopictus* mosquitoes was demonstrated. In 2006, Bluetongue virus emerged for the first time in north-western Europe. *Culicoides imicola*, which is not present in north-western Europe, is the principal vector; in north-western Europe, however, other *Culicoides* species served as vectors. Tahyna and Sedlec bunyaviruses were first isolated in central Europe; Tahyna virus is known to be a human pathogen, Sedlec virus was isolated from a wild bird, and it is unknown whether this virus may also affect humans, farm, or pet animals. Phylogenetic analysis of Tahyna virus indicates that the central European strains of the virus are closely related to each other. Genetic investigations of Sedlec virus shows that it is a member of the Simbu group of orthobunyaviruses, and related to Oropouche virus and Akabane virus. Integrated research of entomologists, virologists and other experts is necessary to develop of early warning networks and control systems for the protection of the European human and animal populations.

**Key Words** Arbovirus, Tahyna virus, Sedlec virus, RT-PCR, phylogenetic analysis

### INTRODUCTION

The World Health Organization defined arthropod-borne (arbo) viruses as “viruses, which are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by hematophagous arthropods; they multiply and produce viraemia in the vertebrates, multiply in the tissues of arthropods, and are passed on to new vertebrates by the bites of arthropods after a period of extrinsic incubation” (Griffin, 2007). The initial investigations on arboviruses employed antigenic characteristics for their classification. Based on the cross-reactivity in hemagglutination inhibition tests, arboviruses were divided into three groups, named A, B and C (Porterfield, 1986). Further studies revealed that these viruses are belonging to different virus families and genera. Arbovirus group A viruses are classified into the *Alphavirus* genus of the family *Togaviridae* (*alpha* reflects on group A). Group B viruses belong to the *Flavivirus* genus of the *Flaviviridae* family, while group C viruses are members of the *Orthobunyavirus*, *Phlebovirus* and *Nairovirus* genera of the *Bunyaviridae* family (Casals, 1963). Three virus genera of the *Reoviridae* family, *Orbivirus*, *Coltivirus* and *Seadornavirus* also comprise viruses, which are predominantly transmitted by arthropod vectors.

In this study the main characteristics of selected arboviruses of *Bunya*-, *Toga*- and *Reoviridae* are reviewed, with a particular focus on their vectors, and their emergence in the continental climate zones of Europe.

The genus *Alphavirus* includes 28 virus species, which are antigenically classified into at least 7 serocomplexes. Alphaviruses are disseminated world-wide. Although vertebrate hosts (i.e. migratory birds, small mammals) may be involved in the transmission cycle in certain virus species, persistent and congenital infections in humans have also been described; the most important factors in the spread of alphaviruses are mosquito vectors. *Aedes*, *Culex* and *Culiseta* mosquitoes are the main vectors of alphaviruses, and the

incubation period in them is relatively short (2 to 7 days) compared to other arboviruses. Several alphavirus infections remain inapparent, but encephalomyelitis, reticuloendothelial infections, arthritis and rash are the most frequent and most typical symptoms of alphavirus-induced diseases. The different alphaviruses show high diversity in geographical distribution. In America the Eastern-, Western- and Venezuelan equine encephalitis viruses are the most important pathogens, which are able to cause severe diseases in humans and domesticated animals (predominantly in horses). Sindbis virus is the most widely distributed alphavirus, which causes arthritis, rash and fever in humans. In Europe, disease outbreaks have been observed mainly in Scandinavia (Ockelbo, Pogosta fever) and in Russia (Karelian fever); however, seropositive hosts were detected in other regions as well (Griffin 2007).

Chikungunya virus (CHIKV) is another member of the genus *Alphavirus*. This virus causes severe arthritic symptoms in humans, hence the name in Swahili, which means “that which bends up”. CHIKV has been causing epidemics in India, Southeast Asia, Indonesia, the Philippines and most of sub-Saharan Africa. Since 2005, large outbreaks have been reported in several islands in the Indian Ocean and in India (Das et al., 2007). In Reunion Island (French Territory) almost 266,000 people (about 35% of the population) had a clinical form of chikungunya (Renault et al., 2007). In 2006, the regional health bureau processed 254 death certificates that mentioned chikungunya as a cause of death, compared with none in 2005. Travellers from areas affected by CHIKV have been diagnosed with chikungunya fever in several European countries (Beltrame et al., 2007), but local transmission involving mosquitoes has not occurred until 2007. In the tropics, the rural cycle of CHIKV involves *Ae. africanus* and *Ae. furcifer*, and wild primates are the main vertebrate hosts, while the urban cycle occurs in humans and involves *Ae. (Stegomyia) aegypti*. Within the last few years, CHIKV has adapted to a new vector, *Ae. albopictus (Stegomyia albopicta)*, the “Asian Tiger Mosquito”, and spread to new geographic areas (de Lamballerie et al., 2008). *Ae. albopictus* was introduced to Italy in 1990 through the import of used vehicle tires, and the mosquito could adapt to the Mediterranean climate and spread in several regions of Italy (Romi, 1995). In August 2007 a high number of febrile illnesses was diagnosed in Castiglione di Cervia and in Castiglione di Ravenna, two small villages in the province of Ravenna, region Emilia-Romagna, Italy. Serological testing and RT-PCR confirmed the diagnosis of chikungunya fever. In addition, CHIKV was detected by RT-PCR in *Ae. albopictus* mosquitoes. Within one month approximately 200 cases have been diagnosed in the region. The index case is supposed to be a traveller coming from an affected area in the Indian subcontinent. The patient arrived in Italy in the middle of June and developed symptoms soon after when he was in Castiglione di Cervia. The peak of the epidemic curve occurred during the third week of August. In the great majority of the patients, the disease was mild and self-limiting. Usually fever lasted for a few days in most patients and a macular rash appeared in more than 50% of cases; however, arthralgia was intense and often persistent even after the abatement of fever. One death occurred, in an 83-year-old man with severe underlying conditions (Angelini et al., 2007).

Several other alphaviruses (i.e. O’nyong-nyong virus, Igbo Ora virus, Ross river virus, and Barmah Forest virus) cause local outbreaks in the human population in tropical regions, but these viruses were not introduced into Europe yet.

*Bunyaviridae* is one of the most populated virus families. The *Orthobunyavirus* genus alone contains more than 150 viruses, which are found all around the world. These viruses are mainly vectored by mosquitoes, but in some instances culicoid flies (midges) are the principal vectors. The California encephalitis serogroup viruses are the most significant human pathogens, especially in North America, where La Crosse virus (LACV) causes epidemics of encephalitis mainly in children, while the Jamestown Canyon virus usually causes more severe disease in adults. The principle vector of LACV is *Aedes triseriatus*, while Jamestown Canyon virus is vectored by *Culex inornata* (Schmaljon and Nichol, 2007).

In Europe two California encephalitis serogroup orthobunyaviruses, Tahyna virus (TAHV) and Inkoo virus (INKV) have been detected so far. Tahyna virus was first isolated in eastern Slovakia in 1958 from a pool of *Ae. caspius* mosquitoes (Bárdos and Danielová, 1959), and is transmitted transovarially in *Ae. vexans* and *Culiseta annulata* mosquitoes. Small mammals are the main vertebrate hosts of TAHV (Vapalathi et al., 1996). Human infection results in a febrile illness with respiratory and gastrointestinal symptoms, and

occasionally meningitis, but probably most infections remain subclinical (Grimstad, 1988). The virus has also been isolated from serum of sick children in central Europe (Bárdos et al., 1975), and from mosquito pools in the European and Asian regions of Russia, even in the Arctic (Lvov et al., 1985; Lvov et al., 1997). An antigenically related virus, Lumbo virus, was isolated from mosquitoes in Africa and Asia. In Africa, Bunyamwera and Ngari viruses are responsible for several acute human febrile illnesses, while in South America Oropouche virus caused similar epidemics. In the Far East and in Australia, Akabane virus is an important cause of foetal malformations in cattle (Schmaljon and Nichol, 2007).

Phleboviruses are mainly transmitted by sandflies (Phlebotomidae) and by mosquitoes. The Rift Valley fever virus (RVFV) is the agriculturally and medically most important member of this genus. It is vectored by mosquitoes, and causes extensive outbreaks of abortions, and generalized febrile and hemorrhagic disease in ruminants. RVFV also causes acute febrile illness in humans, and less frequently, encephalitis. Other phleboviruses, such as Toscana virus (Charell et al., 2005), the Sandfly virus Naples, and the Sandfly virus Sicily were isolated from human febrile illnesses, and from phlebotomus flies in Europe. Uukuniemi virus was isolated from *Ixodes ricinus* ticks in Europe. Nairoviruses are principally transmitted by ticks, but the two most important representatives, Crimean - Congo hemorrhagic fever virus and Nairobi sheep fever virus, are also vectored by culicoid flies and mosquitoes (Schmaljohn and Nichol, 2007).

Within the *Reoviridae* family orbiviruses are the most significant arboviruses. In veterinary aspects bluetongue virus (BTV) and African horse sickness virus (AHSV) are the most important members of the genus. Both of them are transmitted by culicoid flies (midges), and damage endothelial cells of the vertebrate hosts, which usually manifests in oedema, serosal haemorrhages, respiratory symptoms, hypotension and shock (Roy, 2007). BTV causes serious outbreaks in ruminants, mainly in sheep and cattle. The disease was first described in Africa, but later it emerged in Cyprus, the Middle East, Asia, the Mediterranean regions of Europe, the United States, South America, and Australia. In Europe certain BTV serotypes (1, 2, 4, 9 and 16) are endemic in some Mediterranean countries. In the northern parts of Europe BTV was not present until August 2006, when the virus unexpectedly emerged in the Netherlands and in Belgium. Although the principal vector of BTV, the *Culicoides (Avaritia) imicola*, is not resident in these areas, the virus was successfully vectored by other native culicoides species, i.e. *C. (A.) obsoletus*, *C. (C.) pulicaris*, and caused an explosive outbreak reaching most western European countries, and causing more than 50,000 cases in more than 20,000 livestock until autumn 2007 (AFSSA, 2008). Surprisingly the emergent BTV strain belongs to the serotype 8, hence not a southern European strain spread to the north, but an exotic strain was introduced, most probably from Africa.

Serious African horse sickness outbreaks were recorded in central and southern Africa since 1569 (Theal, 1899). This virus can cause mortality levels >95% in susceptible horse populations. Equids are the main vertebrate hosts of the virus, and it is vectored by midges, including *C. imicola*. AHSV was repeatedly introduced into Spain in 1966 and in 1987. In the latter case, a subclinically infected zebra from Namibia was the source of the outbreak, the virus was maintained in the country until 1990, and in 1989 it spread to Portugal as well (Mellor and Hamblin, 2004).

Coltiviruses, i.e. the Colorado tick fever virus in North America, and the Eyach virus in Europe, are tick-borne human pathogens, while viruses of the *Seadornavirus* genus (i.e. Banna virus) are mosquito-borne human pathogens in South-East Asia (Attoui et al., 2005).

The occurrence, ecology (in vertebrate hosts and in invertebrate vectors), epidemiology, and clinical impact of the previously-mentioned viruses greatly vary. The global changes of the recent decades, including the intensive increase of the Earth's human population, the industrial animal-keeping technologies, the intensifying international travel and trade, and the climate changes significantly influence the spreading potential of arboviruses and their arthropod vectors. Our investigations focus on the early and sensitive detection and molecular characterization of previously unknown, emerging, or less investigated arboviruses. Our flavivirus studies are subjects of another talk (Bakonyi et al., in this issue). This paper gives examples for the molecular detection and characterization of mosquito-borne orthobunyaviruses in central Europe.

## MATERIALS AND METHODS

### Samples

The Tahyna virus prototype strain “92” G was isolated from a pool of *Ochlerotatus (Ae.) caspius* mosquitoes, which were collected in the Tahyna village, East Slovakia, on 19. 07. 1958 (Bárdos and Danielová, 1959). A fourth suckling mouse brain passage virus was used for our investigations. The TAHV strain T16 was isolated from the heparinized blood of a 4-year-old boy with Valtice fever, in Drnholec near Mikulov, South Moravia, Czech Republic, 25. 08. 1974 (Bárdos et al., 1975). The TAHV strain T19 was isolated from a pool of *Culiseta (Theobaldia) annulata* mosquitoes collected in Murakeresztrúr, southern Hungary, in 1967 (Molnár, 1982).

Two further TAHV infections were detected by molecular methods from human patients suffering from fever and rash (Baden, Austria, 30. 08. 2004, and Vienna, Austria, 15. 09. 2004). A pathogenic agent designated AV 172 was isolated from the blood of a Reed Warbler (*Acrocephalus scirpaceus*) in southern Moravia, Czechoslovakia, in 1985 (Hubálek et al., 1990). The virus was identified as a probably new species within the family *Bunyaviridae*, designated Sedlec virus (SEDV). Suckling mouse brain passage 4 (from 18. 12. 1985) was used in the molecular investigations.

### Detection of TAHV and SEDV

Development of molecular methods for the detection and investigation of TAHV and SEDV nucleic acid: The genome of bunyaviruses is negative-sense, single-stranded RNA, forming three genomic segments, the small (S), medium (M), and large (L) segment. The complete sequences of TAHV S and M segments are deposited in the GenBank database under accession numbers Z86497 and AF229129, respectively (NCBI, 2008). Oligonucleotide primer pairs were designed for the amplification of overlapping nucleotide sequences of the genome segments. These specific primers were employed in reverse-transcription polymerase chain reactions (RT-PCRs) on the purified RNA of the virus strains. The amplification products were sequenced, and the overlapping regions were compiled to continuous sequences of the strains. Thereafter the sequences were subjected to phylogenetic analyses using the Neighbour-Joining statistical method by the ClustalX (Thompson et al., 1997) and PHYLIP softwares (Felsenstein, 2004).

The probable genetic relatedness of the strains was demonstrated in phylogenetic trees with the help of the TreeView 1.6.6. software. Because no sequence information for the L segment of TAHV was available in the GenBank, sequences of other bunyaviruses were aligned and the most conserved genomic regions were determined. Consensus primers were designed that anneal these conserved regions, and if nucleotide diversity was observed within the primer-annealing sites, degenerated primers (containing a mixture of nucleotide alternatives at the variable loci) were constructed. The primers were applied in RT-PCR assays, and the amplification products were identified by direct sequencings and BLAST search against gene bank databases. The nucleotide sequence of SEDV has not been investigated before. Therefore the previously described consensus primers were applied in RT-PCR assays for the amplification and subsequent determination of partial regions of the virus genome.

## RESULTS AND DISCUSSION

Partial sequences of the S, M and L genome segments of five central European TAHV genotypes were determined. The sequences shared 98 to 100 % nucleotide identity with each other, and with the prototype sequences deposited in the GenBank database. The phylogenetic analysis of the sequences revealed very close genetic relatedness between the strains. Although the different orthobunyaviruses significantly differ in nucleotide sequences, the results of our study indicate that the central European TAHV strains, which were isolated within ~ 400 km apart from each other, and within ~ 40 years, are very similar to each other. The Czech and Austrian genotypes were detected from cases of human febrile illness, and the sequence comparisons did not show significant differences between these strains; the same was true for the Slovakian and Hungarian strains, which were isolated from mosquitoes. The data indicate that TAHV is a widespread arbovirus in central Europe and might play an important role in not further specified human febrile diseases. Probably due to the aspecific symptoms (flu-like disease) TAHV infections of humans are likely to be underdiagnosed, and the real incidence of the disease is probably much higher. RT-PCR might be a suitable method for the rapid detection of TAHV from the peripheral blood mononuclear cells of viraemic patients, and hence could improve the diagnostics of acute TAHV infections.

Using consensus primers, overlapping parts of the SEDV L segment were amplified, and a 1793 nucleotide long continuous sequence of the segment was determined. It shares the highest (66%) identity with Oropouche virus of the Simbu group of *Orthobunyaviridae*. Because the Simbu group viruses are transmitted by culicoid flies and mosquitoes, further investigations are necessary to reveal the exact arthropod vectors of this virus. Due to its sensitivity and specificity, RT-PCR could be a suitable tool for the screening of midge and mosquito samples for the presence of SEDV. Because other members of the group, such as Oropouche virus and Akabane virus, are significant human and animal pathogens, the probable medical impact of the virus shall be clarified in subsequent investigations.

## CONCLUSIONS

Within the last twenty years several new mosquito-borne viruses emerged in Europe. Some of them, like Chikungunya virus, or Dengue virus (*Flaviviridae*) are typically introduced by travellers. Other viruses, like African horse sickness virus, were imported with infected animals. In most cases, however, the exact origin and route of the introduction remains unknown. Migratory birds are the suspected carriers of West Nile- and Usutu flaviviruses, while in the case of Bluetongue virus the spread of serotype 8 to Western Europe remains still unclear. Besides the local transmission, certain insects may also be involved in the long distance spread of arboviruses, either by travelling on vehicles, ships and airplanes, or carried by the wind (e.g. midges).

Due to the more frequent introduction of exotic invertebrates to Europe, and because of climatic changes, new arthropod species may adapt to the moderate climate, overwinter and become residents in Europe (i.e. *Ae. albopictus*, *C. imicola*). These arthropods may be competent vectors for such viruses, which were not present in the continent before, therefore the vertebrate host populations (including humans and animals) are highly susceptible for such infections, and the diagnostic and control measures (available tests, vaccines, treatment) are to a much lesser extent available yet, compared to those for pathogens which are endemic to Europe. The European CHIKV, BTV and Usutu virus outbreaks forebode that so-far exotic viruses with similar ecology, e.g. dengue virus, AHSV, RVFV, Japanese encephalitis virus and yellow fever virus, may also emerge in Europe and could cause serious human and animal disease outbreaks.

On the other hand, the ecology and medical impact of several resident arboviruses, such as Tahyna virus, Sedlec virus, Toscana virus, Sindbis virus, and others are not completely acknowledged yet. Due to the advances in molecular biology, robust diagnostic tools became available for the rapid and sensitive detection of viruses. Comprehensive surveillance and monitoring investigations are necessary for the early detection of the introduction and spread of exotic arboviruses in Europe. Besides the accurate identification of the vector and host species, and the pathogens, — according to their ecological characteristics —, early detection networks and effective control measures shall be developed. This activity requires intensive collaboration of entomologists, virologists, and several other experts in the affected areas (e.g. medical, nature conservation, legislation, and agriculture).

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