

Comparison of Complete Genome Sequences of Usutu Virus Strains Detected in Spain, Central Europe, and Africa

Tamás Bakonyi,^{1,2} Núria Busquets,³ and Norbert Nowotny^{1,4}

Abstract

The complete genomic sequence of Usutu virus (USUV, genus *Flavivirus*, family Flaviviridae) strain MB119/06, detected in a pool of *Culex pipiens* mosquitoes in northeastern Spain (Viladecans, Catalonia) in 2006, was determined and analyzed. The phylogenetic relationship with all other available complete USUV genome sequences was established. The Spanish sequence investigated showed the closest relationship to the USUV prototype strain SA AR 1776 isolated in South Africa in 1959 (96.9% nucleotide and 98.8% amino acid identities). Conserved structural elements and enzyme motifs of the putative polyprotein precursor were identified. Unique amino acid substitutions were recognized; however, their potential roles as virulence markers could not be verified. Comparisons of the polyprotein precursor sequences of USUV strains detected in mosquitoes, birds, and humans could not confirm the predicted role of unique amino acid substitutions in relation to virulence in humans. Phylogenetic analysis of a partial coding section of the NS5 protein gene region indicated that USUV strains circulating in Europe form three different genetic clusters. Broad and targeted surveys for USUV in mosquitoes could reveal further details of the geographic distribution and genetic diversity of the virus in Europe and in Africa.

Key Words: Usutu virus—Flavivirus—Spain—Phylogenetic analysis—Virulence marker.

Introduction

USUTU VIRUS (USUV), A MEMBER OF THE mosquito-borne group of the genus *Flavivirus*, family Flaviviridae, was originally isolated from a pool of *Culex univittatus* mosquitoes in Ndumu, Natal, South Africa in 1959 (Woodall et al. 1964, McIntosh 1985). Subsequently, the virus was isolated from different species of mosquitoes and birds in Africa, and sporadic human infections were reported in central Africa (for review, see Nikolay et al. 2011). However, USUV in Africa received only scant attention, and it had never been associated with bird mortality. In 2001, a strain of USUV emerged in Austria and caused a significant die-off of wild birds, especially Eurasian blackbirds (*Turdus merula*) (Weissenböck et al. 2002). The virus managed to overwinter, became a resident pathogen, spread locally, and continued to

cause wild bird mortality in the eastern part of Austria (Weissenböck et al. 2003, Chvala et al. 2007). Since 2004, however, USUV-associated bird mortality decreased and the antibody prevalence against USUV increased significantly, suggestive of the development of herd immunity (Meister et al. 2008, Buchebner et al. 2013). Although no USUV-related bird deaths were observed in Austria since 2006, the same virus strain was identified as the cause of sporadic or epizootic wild bird mortality in Hungary (Bakonyi et al. 2007), Italy (Mannarola et al. 2009), Switzerland (Steimetz et al. 2011), the Czech Republic (Hubálek et al. 2012), and Germany (Jöst et al. 2011, Becker et al. 2012).

Recent retrospective studies indicated that USUV was already present in Italy in 1996 and may have been the etiological agent of episodes of wild bird mortality since then (Weissenböck et al. 2013). Neuroinvasive human USUV

¹Viral Zoonoses, Emerging and Vector-Borne Infections Group, Institute of Virology, University of Veterinary Medicine Vienna, Vienna, Austria.

²Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest, Hungary.

³Centre de Recerca en Sanitat Animal (CRESA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain.

⁴Department of Microbiology and Immunology, College of Medicine and Health Sciences, Sultan Qaboos University, Muscat, Oman.

infections were reported from immunocompromised patients in Italy (Cavrini et al. 2009, Pecorari et al. 2009). Genetic comparisons demonstrated very close relatedness between the USUV strains circulating in central Europe (Chvala et al. 2007, Hubálek et al. 2012, Gaibani et al. 2013, Nikolay et al. 2013, Weissenböck et al. 2013). The presence of USUV was also reported from the United Kingdom, on the basis of serological examinations of different species of birds (Buckley et al. 2003, Buckley et al. 2006); however, no associated bird mortality has been reported from the United Kingdom.

In the northeast region of Spain (Catalonia), USUV was detected in 2006 in a pool of *Culex pipiens* mosquitoes without associated bird deaths (Busquets et al. 2008). In Spain, the virus was subsequently detected in *Cx. perexiguus* in 2009 (Vázquez et al. 2011), and in two sick song thrushes (*Turdus philomelos*) in 2012 in southern Spain (Höfle et al. 2013). Phylogenetic comparisons of the Spanish strains, however, indicated significant differences between USUVs of mosquito and bird origin (Höfle et al. 2013).

Recently, the complete genome sequences of five USUV strains from central and western Africa were determined and analyzed (Nikolay et al. 2013). The study revealed that USUV strains isolated in Africa between 1969 and 2007 exhibited greater genetic diversity than USUVs detected in central Europe. One African strain (ArB1803, isolated from in the Central African Republic in 1969) showed considerable genetic differences compared to the other known USUVs; therefore, it was suggested to classify this divergent strain as a subtype of USUV (Nikolay et al. 2013).

The complete genome sequence of an USUV strain isolated from a human patient with neurological symptoms in Italy in 2009 has been determined recently (Gaibani et al. 2013). The authors identified amino acid substitutions in the envelope (E) protein and nonstructural protein 5 (NS5), as well as substitutions at the 3'-untranslated region (UTR), which, according to the authors, might be genetic markers for a human neuroinvasive phenotype. The complete genome sequence of another USUV strain (HB81P08), isolated from a human febrile patient in the Central African Republic in 1981, has been determined by Nikolay et al. (2013). Unique amino acid substitutions were identified in the NS2a, NS3, and NS5 protein regions, as well as nucleotide deletions in the 3'-UTR.

The aim of this study was to determine the complete genome sequence of the USUV strain detected in *Cx. pipiens* in 2006 in Spain (Busquets et al. 2008), and to compare it with other USUV sequences, particularly at the predicted putative virulence marker loci.

Materials and Methods

USUV RNA was demonstrated in a pool of three female *Cx. pipiens* mosquitoes, which were captured in mid-August 2006 in the center of the village of Viladecans, from the Delta del Llobregat, Catalonia, Spain (Busquets et al. 2008). The mosquitoes were stored in AVL buffer (Qiagen, Hilden, Germany) and were used for the determination of the complete genome sequence of this USUV strain designated MB119/06. Viral RNA was extracted from 140 µL of mosquito homogenate using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The viral genome was amplified in continuous

TABLE 1. ADDITIONAL SPECIFIC OLIGONUCLEOTIDE PRIMERS USED FOR AMPLIFICATION AND SEQUENCING OF THE COMPLETE GENOME OF USUV MB119/06

Name	Sequence 5' to 3'	Product length (bp)
Usu 42f	TGGAGGATCGTGAGATTAAC	1161
UsuSp 1202r	GTTGGACAATTGGAGACAGT	
UsuSp 1326f	GACTCATGGCAACTATTC	567
UsuSp 1892r	TCCGTACACATGCTATAGGT	
UsuSp 3651f	TCTACTCGTGCTGATTCTTG	414
UsuSp 4064r	CCGATGATGATGAGAGTGAT	
UsuSp 7249f	CTGATTACTGCGGCAGCTCT	138
UsuSp 7387r	TAGCCACCAAGCCATCTACC	
UsuSp 8499f	ACATGCCAATCAGGAGAAGA	714
UsuSp 9213r	TTCAACACCTCCTCCAGAAT	

Numbers in primer names indicate the 5' end annealing positions. f, forward (genomic) primer; r, reverse (complementary) primer.

RT-PCR systems using overlapping oligonucleotide primer pairs and the QIAGEN OneStep RT-PCR Kit (Qiagen, Hilden, Germany). Amplification products were sequenced using the Sanger dideoxy method, as described earlier (Bakonyi et al. 2004). For a few gene regions, in which the originally designed primers did not result in specific PCR products, further primer pairs were designed based on adjacent specific sequences and used for amplification and sequencing of the missing regions. The additionally designed primers are listed in Table 1. After excluding primer regions, the sequences were compiled and aligned with all complete USUV genome sequences deposited in the GenBank database, using the Align Plus 4 (Scientific and Educational Software) and ClustalX (Thompson et al. 1997) programs. To specifically determine the genetic relationship of USUV strains circulating in Spain, an alignment of a partial NS5 protein coding region was also generated, in which the nucleotide sequences of USUV strains detected in *Cx. perexiguus* in Spain in 2009 (Vázquez et al. 2011) and in two song thrushes (*T. philomelos*) in Spain in 2012 (Höfle et al. 2013) were included. Accession numbers are listed in Table 2. Putative amino acid sequences of the polyprotein precursors were also aligned and analyzed.

Phylogenetic reconstruction analyses were performed on the alignments employing the maximum likelihood (ML) method based on the Tamura-Nei model, neighbor-joining method, minimum evolution method, unweighted pair group method with arithmetic mean (UPGMA), and maximum parsimony method of the MEGA5 software package (Tamura et al. 2011). The stability of the trees was tested by bootstrap resampling analysis of 1000 replicates. The probable relationships were displayed in phylograms. Murray Valley encephalitis virus (MVEV; GenBank accession number NC_000943) was used as outgroup to root the trees.

Results

The complete genome sequence of USUV MB119/06 is composed of 11,064 nucleotides and contains an open reading frame between nucleotide positions 97 and 10,401, coding for a 3434-amino-acid-long putative polyprotein precursor. Conserved structural elements and putative enzyme motifs of

TABLE 2. USUTU VIRUS NUCLEOTIDE SEQUENCES INCLUDED IN THE PHYLOGENETIC ANALYSES

Code	Strain	Isolation origin			GenBank accession number	Reference
		Country	Year	Host		
SA 1959	SA Ar 1776	South Africa	1959	<i>Cx. neavei</i>	AY453412	Bakonyi et al. 2004
CAR 1969	ArB1803	Central African Republic	1969	<i>Cx. perfuscus</i>	KC754958	Nikolay et al. 2013
SE 1974	ArD19848	Senegal	1974	<i>Cx. perfuscus</i>	KC754954	Nikolay et al. 2013
CAR 1981	HB81P08	Central African Republic	1981	Human	KC754955	Nikolay et al. 2013
SE 1993	ArD101291	Senegal	1993	<i>Cx. univittatus</i>	KC754956	Nikolay et al. 2013
AT 2001	Vienna 2001	Austria	2001	Blackbird	AY453411	Bakonyi et al. 2004
AT 2002	Meise H	Austria	2002	Blue tit	JQ219843	Becker et al. 2012
HU 2005	Budapest	Hungary	2005	Blackbird	EF206350	Bakonyi et al. 2007
SP 2006	MB119/06	Spain	2006	<i>Cx. pipiens</i>	KF573410	This paper
SE 2007	ArD192495	Senegal	2007	<i>Cx. neavei</i>	KC754957	Nikolay et al. 2013
IT 2009A	Italia 2009	Italy	2009	Blackbird	JF266698	Savini et al. 2011
IT 2009B	Bologna/09	Italy	2009	Human	HM569263	Gaibani et al. 2013
SP 2009	HU10279 09	Spain	2009	<i>Cx. perexiguus</i>	HQ833022*	Vázquez et al. 2011
GE 2011	BH65/11-02-03	Germany	2011	Blackbird	HE599647	Becker et al. 2012
SP 2012	Flavi1	Spain	2012	Song thrush	KC437386*	Höfle et al. 2013

*NS5 gene region, partial coding section.

the polyprotein precursor, described in Bakonyi et al. (2004) (12 Cys residues in the E and NS1 protein regions; putative integrin-binding domain, and receptor-binding domain for virus attachment to sulfated proteoglycans at the E protein region; catalytic triad, and substrate binding pocket of trypsin-like serine protease motif at the NS3 protein region; and RNA-dependent RNA polymerase motif at the NS5 protein region) were identified. Within the RNA helicase motif DEAH/DEAD at the carboxyl terminal of the NS3 protein region, an amino acid substitution was observed at H₁₇₉₁D. The putative *N*-glycosylation sites of the E protein, predicted by Nikolay et al. (2013), were also present in this Spanish USUV strain.

The genome sequence of USUV MB119/06 was aligned with 12 complete USUV genome sequences deposited in GenBank database. The nucleotide identity rates varied between 96.9% (strain SA Ar 1774, South Africa, 1959) and 78.3% (strain ArB1803, Central African Republic, 1969). Amino acid identity rates of the polypeptide precursor varied between 98.8% (strain Vienna_2001, Austria, 2001) and 94.4% (strain ArB1803, Central African Republic, 1969). The phylogenetic relationships between the investigated USUV genome sequences were inferred by five different statistical algorithms. Principally, similar topology trees were obtained; however, the bootstrap support values varied at particular junctions, depending on the applied method. One of the best resolution and bootstrap supports was obtained with the ML algorithm (Fig. 1). The USUV MB119/06 complete genome sequence (marked in red with a black dot in Fig. 1) showed the closest phylogenetic relatedness to the SA Ar 1776 strain (isolated from *Cx. neavei* in South Africa in 1959). Another African isolate (ArD19848, isolated from *Cx. perfuscus* in Senegal in 1974) forms a monophyletic cluster with the above-mentioned two sequences. All other USUV sequences from Europe (marked with grey background, Fig. 1) and Africa cluster in distinct groups.

Partial nucleotide sequences of the NS5 protein-coding region of two further Spanish USUVs are also available in

GenBank database (Vázquez et al. 2011, Höfle et al. 2013). Nucleotide sequences of the complete USUV genomes between nucleotide positions 8377 and 9305 were aligned with the above-mentioned partial sequences. The phylogram obtained with the ML method indicated the closest relationship of the MB119/06 USUV sequence with USUV HU10279 09, detected in *Cx. perexiguus* in Spain in 2009 (Vázquez et al. 2011) (Fig. 2). The other Spanish USUV (Flavi1, detected in

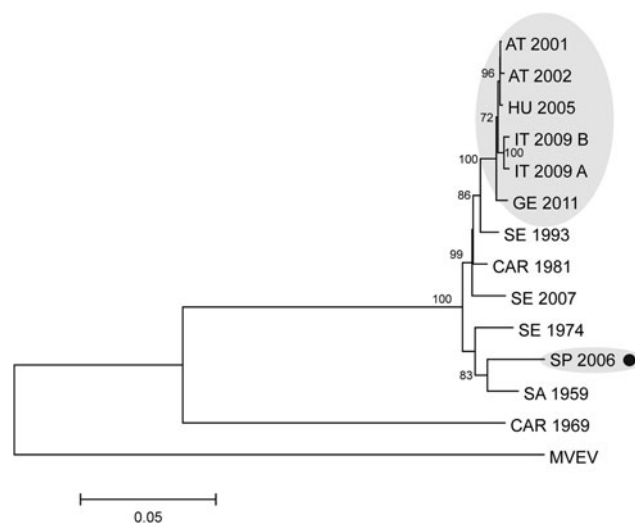


FIG. 1. Phylogram demonstrating the genetic relationships of Usutu virus (USUV) complete genome nucleotide sequences. Details on the viruses are provided in Table 2. Murray Valley encephalitis virus (MVEV; GenBank accession number NC_000943) was used as outgroup to root the tree. The USUV MB119/06 sequence described in this article is marked with a black dot. European clusters are highlighted with grey background. Bootstrap values >70 are displayed at nodes. The horizontal bar on the left represents the genetic distance.

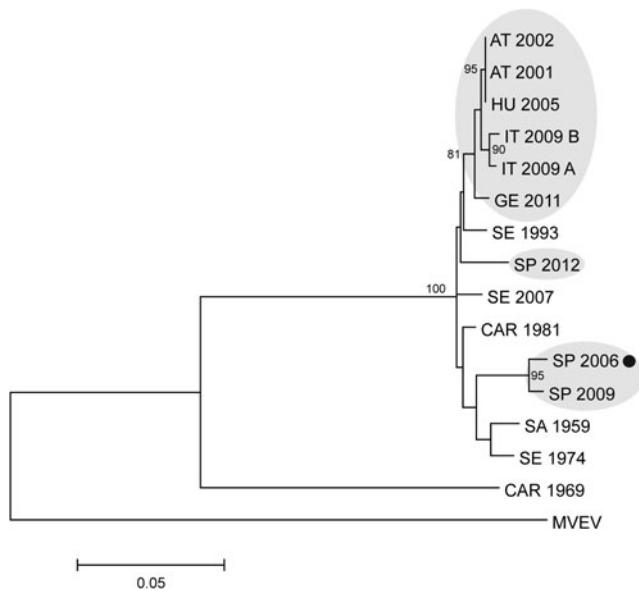


FIG. 2. Phylogram demonstrating the genetic relationships of Usutu virus (USUV) nucleotide sequences in a partial NS5 (RNA-dependent RNA polymerase) gene region. Details on the viruses are provided in Table 2. Murray Valley encephalitis virus (MVEV; GenBank accession number NC_000943) was used as outgroup to root the tree. The USUV MB119/06 sequence described in this article is marked with a black dot. European clusters are highlighted with grey background. Bootstrap values > 70 are displayed at nodes. The horizontal bar on the left represents the genetic distance.

two song thrushes in 2012; Höfle et al. 2013) formed a separated branch (Fig. 2, marked in pink), related to a sequence from Senegal, and sequences of central European USUVs (the latter marked in blue, Fig. 2). The ML phylogenetic reconstructions of the putative polypeptide precursor amino acid sequences indicated similar clustering of the viruses; however, generally lower bootstrap support values were obtained (tree not shown).

In their analyses of sequences of African USUVs (Nikolay et al. 2013) and of a human isolate from Italy (Bologna/09, Gaibani et al. 2013), the authors predicted potential virulence markers and unique substitutions. Nikolay et al. (2013) identified amino acid substitutions A₁₂₀V and M₂₂₈I, specific to USUVs from Africa/Europe. The MB119/06 sequence is identical to the African viruses at these loci. The authors also characterized a human USUV isolate from Africa (strain HB81P08 isolated in the Central African Republic in 1981). Unique amino acid substitutions L₁₂₉₉S, H₁₉₇₇Y, and Q₂₇₀₂H were identified, as well as a deletion of the 3'-UTR between nucleotide positions 10494 and 10510. These mutations were found neither in the MB119/06 virus nor in the human isolate from Italy (Bologna/09). Gaibani et al. (2013) described two unique amino acid substitutions in the Bologna/09 strain. The S₅₉₅G substitution in the E protein region is also present in the MB119/06 virus, but not in the African and in the central European strains. The D₃₄₂₅E substitution in the NS5 protein region is present only in the Italian sequence. Finally, the putative amino acid sequence of MB119/06 contains 24 unique amino acid substitutions compared to the other USUV

sequences analysed (K₁₀₁R, V/A₁₁₂L, T₁₅₃M, D/E₁₇₂G, S₂₇₃G, A₄₅₄V, V₅₆₃I, A₆₃₆V, H₉₆₇Y, V/T₁₀₆₇I, A₁₂₂₇V, A₁₂₃₆T, L₁₂₇₀F, I₁₄₆₀V, K₁₆₄₅R, H₁₇₇₁Y, H₁₇₉₁D, G₁₉₈₁S, V₂₀₀₉I, P₂₃₀₁H, N₂₃₅₅T, D₂₅₅₂E, H₃₀₅₅Y, and V₃₃₂₂I).

Discussion

The emergence and spread of USUV in central Europe has been investigated intensively and is well documented. The virus strain most likely emerged in Italy in 1996 or earlier (Weissenböck et al. 2013). The virus established itself in the region and circulated in avian hosts and mosquito vector species. The strain spread northward to Austria, Hungary, Switzerland, Germany, and the Czech Republic. Phylogenetic analyses indicate that presumably the same strain has been circulating and dispersing in the region (Chvala et al. 2007, Jöst et al. 2011, Becker et al. 2012, Hubálek et al. 2012, Gaibani et al. 2013, Nikolay et al. 2013, Weissenböck et al. 2013). It has obviously been pathogenic to particular species of birds (especially blackbirds, owls, and sparrows). Contrary to this, bird mortality was not or only exceptionally associated with USUV strains from Africa and Spain; and in the United Kingdom, only serological evidence indicated the presence of USUV both in migratory and sedentary bird populations (Buckley et al. 2003, Buckley et al. 2006). These observations point toward differences in the virulence of USUV strains.

The phylogenetic analysis of the complete genome sequence of the Spanish USUV strain MB119/06 confirmed the previous inferences on the genetic relatedness of the virus, on the basis of partial nucleotide sequence data (Busquets et al. 2008, Höfle et al. 2013). This virus is more closely related to the prototype USUV strain from South Africa than to the central European strain and to strains isolated in central and western Africa. A closely related USUV was detected in 2009 in southern Spain in *Cx. perexiguus*, indicating that this virus strain is circulating in the country, although it does not cause significant/detectable wild bird mortality. Because the principal vertebrate hosts of USUV are birds, migratory species of birds may play a role in the geographic spread of the virus. Because the bird migratory routes between Africa and western Europe are typically via Gibraltar and Spain, it is a reasonable scenario that USUVs from Spain (or from western Africa) were introduced to the United Kingdom. If these strains are not virulent to birds, they might covertly circulate in the populations. Targeted surveillance studies for USUV in mosquitoes in Western Europe could support this hypothesis.

Cases of USUV-associated avian neurological illness were recently reported in Spain (Höfle et al. 2013). On the basis of the phylogenetic analysis of NS5 partial sequences, the authors revealed that the USUV detected in song thrushes in 2012 is different from the USUVs detected in mosquitoes in Spain before. The authors hypothesize that this USUV was introduced to Spain by migrating birds from northern Europe. Our phylogenetic analysis involved novel USUV sequences from Africa described by Nikolay et al. (2013). The phylogram indicates that the USUV detected in 2012 in song thrushes in Spain is not monophyletic with the USUVs circulating in central Europe. Therefore, it is unlikely that the central European strain reached Spain. It seems more likely that a strain from central or western Africa was introduced. However, the thrush infections were detected in November, 2012, when southward bird migration is dominant in Spain.

Identification of virulence markers could play a key role for understanding USUV epidemiology. The analysis of increasing numbers of complete sequences might reveal candidate marker loci. Our study also identified unique amino acid substitutions in USUV strain MB119/06. The effects of these mutations on the virulence of USUV should further be investigated using, *e.g.*, site-specific mutagenesis (Brault et al. 2007). Particularly important is the aspect of a possible human pathogenicity of USUV.

Within this study, we compared the complete genome sequences of the so-far available two human USUV isolates (HB81P08 from the Central African Republic, 1981, and Bologna/09 from Italy, 2009). Unique nucleotide or amino acid changes, present only in the USUVs of human origin, but not in the other analyzed sequences, were not found. Therefore, it is possible that the cumulative effect of multiple genetic markers influence the virulence of USUV in humans. However, the described human cases differ significantly in their clinical presentations. The patient in the Central African Republic suffered from a febrile illness with rash. These are frequent symptoms of flavivirus infections (*e.g.*, in mild cases of West Nile, dengue, and yellow fever); therefore, it is quite possible that USUV may cause such a disease manifestation, although the exclusive role of USUV in the etiology of this case could not be confirmed. The human neuroinvasive USUV cases in Italy were diagnosed in immunocompromised patients (Cavrini et al. 2009, Pecorari et al. 2009). Impaired immune defense may allow the invasion of viruses, which are otherwise nonpathogenic for healthy people. Therefore, in these cases, the reactions of the host organisms might have played a greater role in the pathogenesis of the USUV infection, rather than an increased virulence of the virus involved.

Conclusions

The phylogenetic analysis of all currently available complete USUV sequences from Africa, Spain, and central Europe revealed a significant genetic diversity of the virus. The viruses circulating in Europe belong to (at least) three different genetic clusters. The USUVs detected in mosquitoes in Spain are genetically more closely related to African isolates than to central European ones. The viruses differ apparently in their virulence for avian hosts. Therefore, the geographical distribution of USUV is probably wider than so far identified because of covert cycles in bird hosts and mosquito vectors. Comparative genomic analyses revealed several differences among USUV strains. They might—or might not—act as virulence marker loci. The effects of these potential markers should be investigated further in controlled experiments. Considering the potential public health impact of USUV (conditional human pathogen), its interference in the serological diagnosis of pathogenic human flaviviruses due to cross-reactions, and its pathogenicity for several species of birds, a broader, targeted surveillance activity would be necessary to assess the geographical distribution and abundance of USUV in Europe and in Africa.

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Author Disclosure Statement

No competing financial interest exists.

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Address correspondence to:

Tamas Bakonyi
Viral Zoonoses

Emerging and Vector-Borne Infections Group
Institute of Virology
University of Veterinary Medicine Vienna
Veterinaerplatz 1
1210 Vienna, Austria

E-mail: Tamas.Bakonyi@vetmeduni.ac.at