JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2007, p. 3870–3874 Vol. 45, No. 12
0095-1137/07/$08.00+0 doi:10.1128/JCM.01390-07
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Emergence of Usutu Virus in Hungary▽

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Received 11 July 2007/Returned for modification 16 August 2007/Accepted 26 September 2007

In 2001, Usutu virus (USUV), a mosquito-borne flavivirus of the Japanese encephalitis virus serogroup related to West Nile virus and previously restricted to sub-Saharan Africa, emerged in wild and zoo birds in and around Vienna, Austria. In order to monitor the spread of the infection, a dead bird surveillance program was established in Austria and in neighboring Hungary. In Hungary, 332 dead birds belonging to 52 species were tested for USUV infection between 2003 and 2006. In the first 2 years, all birds investigated were negative. In August 2005, however, USUV was detected in organ samples of a blackbird (*Turdus merula*), which was found dead in Budapest, Hungary, by reverse transcription-PCR, immunohistochemistry, and in situ hybridization. In July and August 2006, a further six dead blackbirds tested positive for USUV, and the virus was isolated from organ samples of one bird. These birds were also found in urban areas of Budapest. The nearly complete genomic sequence of one Hungarian USUV strain was determined; it was found to share 99.9% identity with the strain that has been circulating in Austria since 2001. This result indicates that the USUV strain responsible for the blackbird die-off in Budapest most likely spread from Austria to Hungary instead of being independently introduced from Africa.

In 2001, an episode of increased wild bird mortality was observed in and around Vienna, Austria. Unexpectedly, Usutu virus (USUV) was isolated from the affected birds; for the first time this African flavivirus emerged in central Europe (14). An extended outbreak was observed in the subsequent year in the same area and predominantly involved blackbirds (*Turdus merula*) (15), which indicated that USUV survived the Austrian winter and adapted to the local mosquito populations. Detailed investigations were carried out, including the genetic characterization of the emerging USUV strain (1) and host susceptibility studies (2, 6, 7), in order to assess the ecological and epidemiological impacts of the virus. A dead bird surveillance program was introduced in Austria to monitor the occurrence and spread of the infection (8). Seasonal USUV-induced encephalitis outbreaks were observed during five consecutive years in the east Austrian wild bird populations. While a limited geographic spread of the virus was noticed, the number of cases peaked in 2003 and since then has markedly decreased. At the same time the number of USUV antibody-positive birds increased significantly, indicating a rather rapid development of herd immunity (12).

Prompted by the geographic proximity and the preliminary data on the spread of the virus, a dead bird surveillance program was also established in neighboring Hungary. The observations from the 4 years of surveillance are presented in this study.

MATERIALS AND METHODS

Dead bird samples. Since 2003, continuous monitoring activity was performed by ornithologists of the Ócsa Bird Ringing Station Society at a bird ringing camp near Budapest, Hungary. Encephalitic wild birds obtained during the avian influenza monitoring programs of the Central Veterinary Institute were also included in the investigations. Additional wild bird carcasses were submitted by the conservation officers of BirdLife Hungary, bird watchers, veterinarians, and townspeople. Within 4 years, 332 dead birds belonging to 52 species were collected, identified, necropsied, and investigated for the presence of USUV (Table 1).

RT-PCR, sequencing, and sequence analysis. Viral nucleic acids were extracted from homogenates of the brains of the birds by standard methods (9, 14). When brains were not available, spleens and livers were used as sample materials. RNA extracts were subjected to one-step reverse transcription-PCR (RT-PCR) assays with Japanese encephalitis virus (JEV) complex-specific primers (primers WNV10090f [GARTGGATGACVACRGAAGACATGCT] and WNV10807r [GGGGTCTCCTCTAACCTCTAGTCCTT]) (1, 14) and USUV-specific primers (primers Usu9170f [AGGACCATTGGTTAGGAAGA] and Usu9704r [GGCTTGACAACACAATCATC]) (16). The specific amplification products were directly sequenced (1), and the sequences were identified by using the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/BLAST/). The nearly complete genomic sequence of one Hungarian USUV strain was determined by direct sequencing of overlapping amplification products (1); the sequence was aligned with the available USUV sequences deposited in the GenBank database by using the Align Plus (Scientific and Educational Software) and ClustalX (13) programs.

Histopathology, IHC, and ISH. Brain tissue samples of selected birds and tissues of other organs (e.g., liver, spleen, kidney, heart, and lung) were fixed in 7% neutral buffered formalin for subsequent histological and immunohistological examinations. Paraffin-embedded tissue sections (4 μm) were stained with

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▽ Published ahead of print on 3 October 2007.

TABLE 1. Dead birds collected in Hungary between 2003 and 2006 and investigated for USUV infection

Species		No. of birds
English name	Latin name	
Barn swallow	*Hirundo rustica*	14
Black redstart	*Phoenicurus ochruros*	3
Blackbird	*Turdus merula*	33
Blackcap	*Sylvia atricapilla*	22
Blue tit	*Parus caeruleus*	33
Brambling	*Fringilla montifringilla*	6
Bullfinch	*Pyrrhula pyrrhula*	2
Chaffinch	*Fringilla coelebs*	4
Chiffchaff	*Phylloscopus collybita*	10
Common buzzard	*Buteo buteo*	2
Common kestrel	*Falco tinnunculus*	1
Common redstart	*Phoenicurus phoenicurus*	1
Common treecreeper	*Certhia familiaris*	1
Common whitethroat	*Sylvia communis*	1
Dunnock	*Prunella modularis*	5
Garden warbler	*Sylvia borin*	2
Goldcrest	*Regulus regulus*	1
Golden oriole	*Oriolus oriolus*	2
Goldfinch	*Carduelis carduelis*	1
Grasshopper warbler	*Locustella naevia*	1
Great spotted woodpecker	*Dendrocopos major*	1
Great tit	*Parus major*	24
Greenfinch	*Carduelis/Chloris chloris*	23
Gray heron	*Ardea cinerea*	1
House sparrow	*Passer domesticus*	2
Kingfisher	*Alcedo atthis*	1
Lesser whitethroat	*Sylvia curruca*	5
Little bittern	*Ixobrychus minutus*	2
Long-eared owl	*Asio otus*	1
Long-tailed tit	*Aegithalos caudatus*	1
Marsh warbler	*Acrocephalus palustris*	3
Middle spotted woodpecker	*Dendrocopos medius*	1
Moustached warbler	*Acrocephalus melanopogon*	8
Penduline tit	*Remiz pendulinus*	2
Pheasant	*Phasianus colchicus*	1
Pied flycatcher	*Ficedula hypoleuca*	1
Pigeon	*Columba livia*	1
Red-backed shrike	*Lanius collurio*	5
Reed bunting	*Emberiza schoeniclus*	7
Reed warbler	*Acrocephalus scirpaceus*	12
Robin	*Erithacus rubecula*	51
Savi's warbler	*Locustella luscinioides*	7
Sedge warbler	*Acrocephalus schoenobaenus*	4
Short-toed treecreeper	*Certhia brachydactyla*	1
Song thrush	*Turdus philomelos*	5
Starling	*Sturnus vulgaris*	2
Stonechat	*Saxicola torquata*	1
White stork	*Ciconia ciconia*	6
Tree sparrow	*Passer montanus*	3
Winter wren	*Troglodytes troglodytes*	3
Wood warbler	*Phylloscopus sibilatrix*	1
Yellow-legged gull	*Larus cachinnans*	1
Total		332

hematoxylin-eosin and examined by light microscope. Immunohistochemistry (IHC) by the avidin-biotin complex technique and with rabbit USUV antiserum as the primary antibody was performed with the tissue sections (16). Selected tissue sections were also subjected to an in situ hybridization (ISH) assay with a digoxigenin-labeled USUV-specific oligonucleotide probe (16).

Virus isolation. Virus isolation attempts were carried out with the organ samples (brain, liver, kidney, lung, and spleen) of selected USUV RNA-positive blackbirds. The tissue samples were homogenized and suspended in phosphate-buffered saline. After centrifugation (4,000 × *g*, 10 min), the supernatants were inoculated onto 1-day-old, confluent primary goose embryo fibroblast cell cultures (2). The cell cultures were incubated at 37°C for 3 to 5 days and were checked daily for the occurrence of cytopathic effects (CPEs). Three subsequent passages were made, and cell cultures showing specific CPEs were further investigated by RT-PCR and sequencing of the amplification products.

Nucleotide sequence accession number. The nearly complete genome sequence of the USUV Budapest-2005 strain was submitted to the GenBank database under accession number EF206350.

RESULTS

Dead bird surveillance. All birds collected in 2003 and 2004 tested negative for USUV nucleic acids and antigens. In August 2005, however, the Central Veterinary Institute received a dead blackbird found in the residential area of centrally located District XI of Budapest. Both the JEV complex- and the USUV-specific RT-PCR assays gave positive results with an homogenate of the brain of the bird; sequencing of the amplification products revealed that USUV was detected in the sample. The case history included observations of several (up to eight) sick and dead blackbirds within the same area during the preceding week. The observed mortality during August and September 2005 was reported to reach 18 birds in total.

In July and August 2006, nine blackbirds were received from the territory of Budapest for avian influenza monitoring. Six of these samples were suitable for further testing, and all six proved positive for USUV by RT-PCR. These birds were found in Districts XVI and XVIII of the city (three birds in each district), which are distinct from the location of the outbreak in the previous year (Fig. 1). All further birds tested in 2005 and 2006 were found to be negative for USUV.

Histopathology, IHC, and ISH. Gross lesions observed in all cases were rather unspecific, such as general congestion of internal organs, as well as splenomegaly and hepatomegaly. Histopathological investigations revealed acute hepatitis with multiple inflammatory and necrotic foci (Fig. 2A), various degrees of focal necrosis in the spleen, acute mucous enteritis, mild and focal perivascular lymphohistiocytic infiltrations in the kidney and heart, focal myocardial degeneration (Fig. 2B), vacuolar degeneration of tubular epithelial cells in the kidney, and perivascular and perineuronal edema with very few lymphohistiocytic perivascular cuffs in the brain. USUV antigen was detected by IHC in selected bird samples. In the brains, single accumulations of immunoreactive glial cells in the cerebellum and focal perivascular signals in the entire brain were present; in other cases, multiple IHC-positive foci were

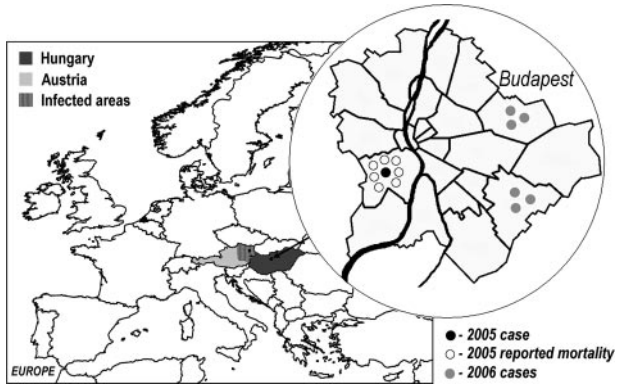

FIG. 1. Blackbird mortality due to USUV infection in Budapest, Hungary.

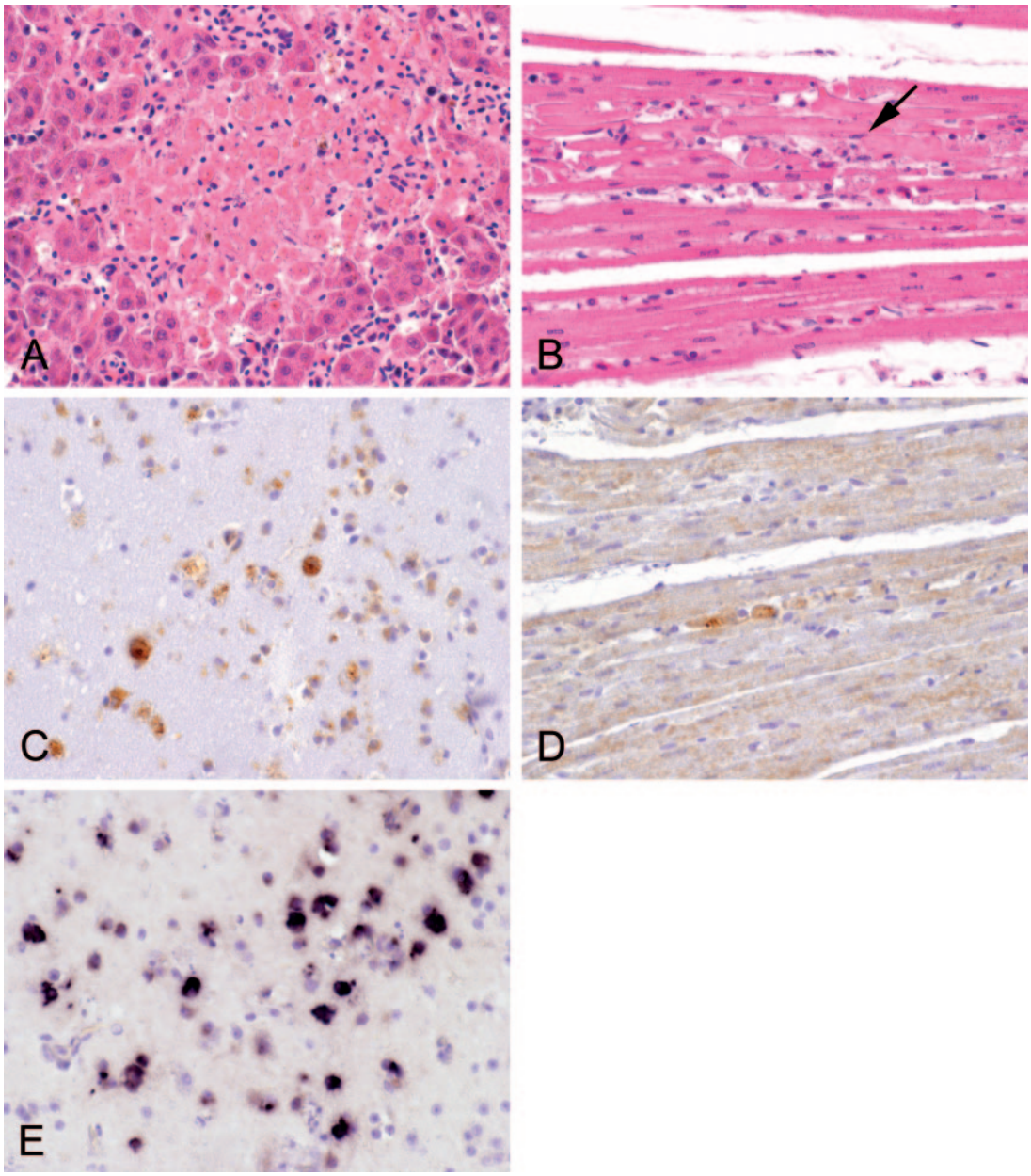

FIG. 2. Histopathological lesions and detection of viral signals by IHC and ISH in USUV-infected blackbird organs. (A) Liver; acute necrotizing hepatitis, hematoxylin-eosin staining. (B) Heart; focal myocardial degeneration (arrow), hematoxylin-eosin staining. (C) USUV antigen in the brain; multiple IHC-positive foci. (D) USUV antigen in the heart; IHC-positive myocardial cells. (E) USUV nucleic acid in the brain; multiple ISH-positive neurons. Magnifications, ×396.

observed predominantly in the cerebral cortex (Fig. 2C). In the hearts, a large number of myocardial cells and sometimes interstitial cells contained USUV antigens (Fig. 2D). Many positive signals in the capsule and red pulp of the spleen were seen, and lung sections also contained single positive cells. A positive reaction was also observed in several areas of the pancreas, as well as in crypt epithelia, connective tissue cells, and the intramural ganglia of the intestines. Due to the strong background staining of hepatocytes in the liver and tubular epithelial cells in the kidneys, the results of the IHC tests were inconclusive for these tissue samples. USUV-specific nucleic acid was demonstrated in brain sections by ISH (Fig. 2E).

Virus isolation. Tissue homogenates of the brain and the visceral organs of the blackbirds were inoculated onto primary goose embryo fibroblast cell cultures. After 3 days of incubation, focal cell rounding and shrinkage were observed in the cell cultures inoculated with brain and liver homogenates of a blackbird found dead in Budapest in August 2006. Within 2 days, 80 to 90% of the cells showed CPEs; the cells lost their adherence to the surface of the culturing flask and floated in the supernatant. The CPE was more pronounced in the sub-

TABLE 2. Nucleotide and amino acid differences between the Vienna-2001 and Budapest-2005 USUV strains

Coding region	Nucleotide			Amino acid		
	Position	USUV Vienna-2001	USUV Budapest-2005	Position	USUV Vienna-2001	USUV Budapest-2005[a]
Pre-M	623	C	T	176	T	I
E	2181	C	T	695	S	.
NS2a	3686	C	T	1197	T	I
NS2a	4067	T	C	1324	I	T
NS2b	4366	C	T	1424	L	.
NS3	5432	C	T	1779	A	V
NS4a	6700	C	T	2203	L	.
NS4b	7197	T	G	2367	F	L
NS5	7689	A	G	2530	R	.
NS5	7992	T	C	2631	Y	.
NS5	9321	A	G	3075	R	.
NS5	9556	C	T	3154	L	.
NS5	10311	A	G	3405	R	.
3′ Untranslated region	10948	A				

[a] Periods indicate that the amino acid is the same as that in USUV Vienna-2001.

sequent two passages. No CPE or cell degeneration was observed in the control cells. An USUV-specific RT-PCR assay with the cell culture isolate gave a positive result, and sequencing of the amplification product confirmed the identity of the isolate.

Sequence analysis. To reveal the origin of the infection and the genetic relatedness between the USUV strains which emerged in Vienna and Budapest, the nearly complete genome sequence of the Budapest-2005 strain was identified by direct sequencing of overlapping amplification products (14). The sequence (GenBank accession number EF206350) was aligned to the genome records of the USUV Vienna-2001 (GenBank accession number AY453411) strain and the South African USUV reference strain, SAAR-1776 (GenBank accession number AY453412). The Budapest-2005 strain shared 99.9% nucleotide identity with the Vienna-2001 strain and 97% nucleotide identity with the SAAR-1776 strain. Thirteen nucleotide substitutions and 1 nucleotide insertion/deletion were found by comparison of the Vienna and the Budapest strains. The nucleotide substitutions were equally distributed over the genome and resulted in five changes of the deduced amino acid sequence of the putative polyprotein precursor. The insertion/deletion was found in the 3′ untranslated region within a six-/seven-mer A signal (at nucleotide position 10948). The nucleotide and amino acid changes are presented in Table 2.

Partial nucleotide sequences of the E protein-coding region (between nucleotide positions 1175 and 1529) of the viruses detected in 2006 were determined, in order to identify mutations in the main surface protein gene, which is supposed to act as neutralizing antigen. Single C-to-T transitions were found in the USUVs from 2006 at nucleotide positions 1231 (three viruses), 1317 (one virus), and 1431 (one virus), compared to the sequence of 2005 strain. None of these substitutions caused changes in the putative amino acid sequence of the E protein.

DISCUSSION

The results of this study indicate that, following its emergence in Austria, the USUV strain circulating in Austria most likely spread to Hungarian wild bird populations instead of being independently introduced from Africa. While in the last 2 years USUV-induced mortality decreased in the blackbird populations of the eastern federal states of Austria, an increased spread of the pathogen was observed, with USUV cases observed in all four eastern Austrian federal states (Vienna, Lower Austria, Burgenland, and Styria) (8). Although the emergence of the virus in Hungary was not unexpected, it is surprising that the first positive case was diagnosed about 200 km away from the closest known Austrian focus of USUV infections (the federal state of Burgenland). Already in 2003, however, information was received on reduced numbers of blackbirds in the parks of some Hungarian cities close to the Austrian border (personal communications with local ornithologists and veterinarians); the few blackbird samples submitted from this region for RT-PCR investigations, however, were found to be negative for USUV. Presumably, the infection had spread through the country earlier, but it remained unrecognized, because it did not cause mass mortality among the wild birds. Nonetheless, the fact remains that USUV emerged in 2001 in Vienna, i.e., in the largest city of Austria, and in 2005 in Budapest, i.e., in the largest city of Hungary, and not in remote areas.

Recent studies reported on seroconversions to USUV in wild birds and poultry in the United Kingdom (3, 4) and in wild birds in Germany (10). Interestingly, USUV-induced bird mortality was not observed in those countries, and the etiologic virus was not detected in those cases either by molecular methods or by isolation. The results of those studies indicate a more widespread distribution of USUV in Europe. Molecular and biological characterization of these so far putative USUV strains could provide valuable information on differences in the virulence of the European strains.

According to the observations in Austria and Hungary, blackbirds are probably one of the most susceptible hosts of the central European strain of USUV, and the infections are often lethal in this species, at least at the beginning of an epidemic. Blackbirds, however, show significant differences in ecology and population density, depending on their habitats. In the forests their population density is much lower than that in city parks (11). Therefore, on the one hand, the chances of

virus spread are probably lower in forests, because the mosquito vectors have a wider choice of target species, while on the other hand, blackbird mortality in the forests is less conspicuous, since the chances that dead birds will be found are very low. On the contrary, blackbird population densities are much higher in urban habitats; hence, the chances for USUV transmission by mosquitoes are higher, dead birds are easily found, and there is an increased public awareness and sensitivity to bird mortality events. All these factors contribute to the higher probability of detection of emerging infections in urban wildlife in general. During our investigations, 33 blackbirds were tested, and 15 of them were found in city parks or residential areas, including all USUV-positive birds.

The pathological findings for the USUV-positive blackbirds are largely identical to those reported earlier for birds from Austria (5). In both the Hungarian and the Austrian birds, the lesions and morphological evidence of viral replication in a large variety of organs and tissues point toward multiorgan failure as the cause of death.

The genome sequence of the Hungarian USUV strain shares a high level of similarity with that of the Austrian strain, which was isolated 4 years earlier. The putative amino acid sequence of the USUV precursor polyprotein contains conserved elements, which are in common in the JEV group of flaviviruses (such as Cys residues of the E and NS1 proteins, which are involved in intramolecular disulfide bonds; a putative integrin-binding domain and a receptor-binding domain for binding to sulfated proteoglycans of the E protein; a catalytic triad and a substrate binding pocket of a trypsin-like serine protease at the N terminus and an RNA helicase motif at the C terminus of the NS3 protein; as well as an RdRp motif at the C terminus of the NS5 protein) (1). The amino acid substitutions in the Hungarian USUV strain did not affect these conserved loci.

If the virus exhibits spreading kinetics and an epizootiology in Hungary similar to those seen in Austria, recurrent blackbird mortality will be expected in the summer and fall of 2007. Very recently, at the beginning of July 2007, two dead blackbirds were found in Budapest. USUV nucleic acid was detected in them by RT-PCRs. Detailed investigations of the genotype involved as well as the testing of further bird samples from the 2007 epidemic season are being carried out.

It is interesting to note that while the mortality from USUV infection was declining significantly in Austria, the virus emerged at locations 200 km apart; it would not be surprising if this virulent, central European strain of USUV also emerges in the near future in other European cities and countries.

ACKNOWLEDGMENTS

This study was funded by grants OTKA D48647 and OTKA K67900 and the Bolyai János Research Grant.

We thank Gergő Halmos and Pál Morandini (BirdLife Hungary) for providing wild bird samples. The technical assistance of Nora Nedorost and Klaus Bitterman is gratefully acknowledged.

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