Epidemiol. Infect. (2003), **131**, 1023–1027. © 2003 Cambridge University Press
DOI: 10.1017/S0950268803001031 Printed in the United Kingdom

SHORT REPORT
Screening for West Nile virus infections of susceptible animal species in Austria

H. WEISSENBÖCK[1]*, Z. HUBÁLEK[2], J. HALOUZKA[2], A. PICHLMAIR[1], A. MADERNER[1], K. FRAGNER[1], J. KOLODZIEJEK[3], G. LOUPAL[1], S. KÖLBL[4] AND N. NOWOTNY[3,5]

[1] *Institute of Pathology and Forensic Veterinary Medicine, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria*
[2] *Medical Zoology Laboratory, Institute of Vertebrate Biology, Czech Academy of Sciences, Klásterní 2, CZ-69142 Valtice, Czech Republic*
[3] *Clinical Virology Group, Institute of Virology, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria*
[4] *Federal Ministry for Social Security and Generations, Franz Josefs Kai 51, A-1010 Vienna, Austria*
[5] *Department of Medical Microbiology, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates*

(*Accepted 19 August 2002*)

SUMMARY

Avian mortality and encephalomyelitis in equines are considered good indicators for West Nile virus (WNV) activity. We retrospectively tested 385 horse sera for WNV antibodies and looked for WNV nucleic acid and/or WNV antigen in paraffin embedded tissues from 12 horses with aetiologically unresolved encephalomyelitis and 102 free-living birds of different species which had been found dead. With the exception of four horses originating from eastern European countries investigated on the occasion of transit through Austria, all horse sera were negative. Nested RT-PCR of the horse tissues yielded no amplification of WNV-RNA. Also, all bird samples, examined by immunohistochemistry, *in situ* hybridization and nested RT-PCR were negative for WNV. These results indicate that currently WNV cannot be considered a significant pathogen in Austria.

West Nile virus (WNV) infections of humans and animals have been recognized for decades in Europe, Africa and Asia [1]. In 1999, the virus was introduced to North America, where it had spread from the state of New York to 26 eastern and southeastern US states and Canada by the end of 2001. WNV-infection in the US has been responsible for several human deaths, thousands of dead birds and several hundred fatal cases of severe neurological disease in horses. Lethal human infections together with avian mortality have also been recognized in WNV-outbreaks in Israel [2, 3], whereas recent outbreaks in Europe either presented as exclusively human (Romania [4–6]) or equine disease (Italy, France [7, 8]). Although not associated with significant disease outbreaks, WNV has been isolated in Central European countries such as the Czech Republic, Hungary and Slovakia [1]. WNV has not been isolated in Austria, although serological data from the 1970s indicated the presence of WNV or cross-reacting flaviviruses [9–13].

WNV surveillance is traditionally carried out by serology and virus isolation from mosquitoes or diseased vertebrates. These techniques are increasingly complemented by molecular biological methods such

* Author for correspondence.

as conventional reverse transcriptase (RT)-PCR or real time PCR [14–16]. WNV antigen or RNA can be detected in paraffin-embedded tissue samples by immunohistochemistry (IHC) or *in situ* hybridization (ISH) [17, 18]. In this study, we looked retrospectively for evidence of WNV infections in animals known to be highly susceptible, i.e. birds and horses. The study includes serological investigations of horses and immunohistochemical and molecular investigations of paraffin-embedded, archived tissue samples of horses and wild as well as captive birds. Selection criteria were unexplained deaths or encephalitis in birds, and encephalomyelitis in horses. In addition, during 2001, we encouraged the submission of dead wild birds and included these in the screening program. Sera collected from 350 horses from different regions in the eastern part of Austria, were tested for antibodies to WNV. Approximately one-quarter of the horses had a history of slight to moderate clinical signs suggesting CNS disease. In addition, 35 sera were tested from horses examined by a border veterinarian when the horses immigrated to, or were in transit through, Austria.

The sera were stored at $-20\,^{\circ}$C, and inactivated at 56 °C, for 30 min prior to testing. The presence of antibodies was analysed by plaque-reduction neutralization test [19].

The necropsy files of the Institute of Pathology and Forensic Veterinary Medicine were screened for birds fulfilling the following criteria: wild birds or captive birds held in outdoor aviary; cause of death unexplained or encephalitis. A total of 42 birds meeting these selection criteria was identified within a period of 14 years (1987–2000; Table 1). A total of 14 horses with records of meningitis, encephalitis and/or brain hemorrhage of undetermined aetiology were identified within the same time period.

In order to increase submission of bird samples in the year 2001 we published a request in a national ornithological journal for reports of obvious avian mortality and for carcasses or organs. In total, we received specimens from 55 native birds (Table 2), originating from the federal states of Upper Austria (24), Vienna (15), Lower Austria (6), Burgenland (4), Salzburg (2), Styria (1); for three samples the geographic origin could not be determined. Five great grey owls from the Zoo Schönbrunn (Vienna), which had died within a few days, were also obtained.

From the birds, paraffin embedded tissue blocks of kidney, liver, spleen, heart, intestine and, when available, brain were selected. From the horses, tissue blocks containing brain stem and, when available, spinal cord were selected.

Table 1. *42 birds retrospectively examined for WNV infection*

English name (number)	Scientific name
White stork (1)	*Ciconia ciconia*
Mallard (3)	*Anas platyrhynchus*
Moscovy duck (2)	*Cairina moschata*
Kestrel (2)	*Falco tinnunculus*
Peregrine falcon (2)	*Falco peregrinus*
Bearded vulture (1)	*Gypaetus barbatus*
Hen harrier (1)	*Circus cyaneus*
Chinese painted quail (2)	*Excalfactoria chinensis*
Plover (1)	*Charadrius* sp.
Street dove (6)	*Columba livia f. domestica*
Long-eared owl (2)	*Asio otus*
Blackbird (1)	*Turdus merula*
Song thrush (1)	*Turdus philomelos*
Bullfinch (1)	*Pyrrhula pyrrhula*
Starling (1)	*Sturnus vulgaris*
Magpie (1)	*Pica pica*
Rook (4)	*Corvus frugilegus*
Hooded crow (2)	*Corvus corone cornix*
African grey parrot (1)	*Psittacus erithacus*
Yellow crested cockatoo (1)	*Cacatua galerita*
Golden headed quetzal (1)	*Pharomachrus auriceps*
Zebra finch (4)	*Taeniopygia guttata*
Parrot finch (1)	*Erythrura psittacea*

The bird samples were subjected to immunohistochemical screening, using a previously described protocol [20], as birds with WNV infections have been shown to exhibit large quantities of antigen in various tissues [17]. Cases with positive or inconclusive signals were examined by ISH [21] and PCR.

In contrast, it has been reported [18] (and it is also our own experience) that WNV-IHC is relatively insensitive for the detection of WNV infection in horse tissues, and nested RT-PCR is much more sensitive. Thus we tested horse samples by nested RT-PCR only.

For RT-PCR amplification, RNA was extracted from 10 μm thick paraffin sections. After deparaffination in xylene, the pellets were washed in ethanol and dried. Subsequently, proteinase K digestion at 37 °C for 48 h was performed. After inactivation of proteinase K at 95 °C for 8 min, RNA was extracted with QIAamp RNA Blood Mini kit according to the manufacturers' instructions. Primers able to amplify WNV strains isolated in Africa (Eg 101, GenBank no. AF260968), Europe (Ro 97, GenBank no. AF260969) and America (NY 99 flamingo, GenBank no. AF196835) were designed using the

Table 2. *Birds included in the WNV screening program 2001*

English name (number)	Scientific name
Kestrel (1)	*Falco tinnunculus*
Street dove (1)	*Columba livia f. domestica*
Great grey owl (5)	*Strix nebulosa*
Black woodpecker (1)	*Dryocopus martius*
Great spotted woodpecker (2)	*Dendrocopos major*
Common swallow (1)	*Hirundo rustica*
Waxwing (1)	*Bombycilla garrulus*
Blackcap (3)	*Sylvia atricapilla*
Robin (1)	*Erithacus rubecula*
Black redstart (1)	*Phoenicurus ochruros*
Thrush (2)	*Turdus* sp.
Song thrush (1)	*Turdus philomelus*
Fieldfare (1)	*Turdus pilaris*
Blackbird (8)	*Turdus merula*
Great tit (2)	*Parus major*
Blue tit (1)	*Parus coeruleus*
Chaffinch (1)	*Fringilla coelebs*
Siskin (2)	*Carduelis* sp.
Greenfinch (2)	*Carduelis chloris*
Goldfinch (1)	*Carduelis carduelis*
Bullfinch (2)	*Pyrrhula pyrrhula*
Hawfinch (2)	*Coccothraustes coccothraustes*
House sparrow (7)	*Passer domesticus*
Starling (5)	*Sturnus vulgaris*
Eurasian jay (2)	*Garrulus glandarius*
Hooded crow (4)	*Corvus corone cornix*

SciED primer designer software. For WNV amplification the used primers were WNV-1 forward: 5′-TGGCAGAGCTTGACATTGAC-3′, WNV-1 reverse: 5′-GGTGCTCCTGGATCATTCAT-3′, amplifying a 220 bp product from the NS2b region of WNV. The nested primers for this amplification product were WNV-1N forward: 5′-GGCCATTCCAATGACTATCG-3′, WNV-1N reverse: 5′-ATCAAGCCGCACATCAACTC-3′, resulting in a 154 bp amplification product. An additional set of primers was: WNV-2 forward: 5′-CGCGTGTTGTCCTTGATTGG-3′, WNV-2 reverse: 5′-CTCCGCCGATTGATAGCACT-3′, amplifying a 230 bp product of the C region of WNV. Nested primers were: WNV-2N forward: 5′-TTGGCGTTCTTCAGGTTCAC-3′, WNV-2N reverse: 5′-GCCGATTGATAGCACTGGTC-3′, resulting in a 142 bp amplification product. As internal check for RNA integrity β-actin m-RNA was amplified by RT-PCR using the primers ACT-horse forward: 5′-TGGACTTCGAGCAGGAGATG-3′ and ACT-horse reverse: 5′-CAGGAAGGAGGGCTGGAAGA-3′ in horses and ACT forward: 5′-TGGAYTTCGAGCAGGAGATG-3′ and ACT reverse: 5′-CARGAARGADGGYTGGAAGA-3′ in all bird species. Both primer pairs generate a 140 bp amplification product. Accidential genomic DNA amplification was ruled out by the negative outcome of conventional PCR with omission of the RT step.

The PCR conditions were optimized using paraffin embedded organ material from a WNV-positive bird and a WNV-positive horse (kindly provided by M. Hall). Reverse transcription and amplifications were performed in a continuous RT-PCR method as previously described [22].

None of the sera of the horses originating from Austria showed detectable antibodies to WNV. In contrast, 4 of the horses imported from other countries were seropositive at titres of 1:32–1:256. All of these were exported from Hungary and were travelling through Austria with their final destination Germany. Two seropositive horses (PRNT-titres 1:256 and 1:128–256 respectively) were detected in a group of 18 animals imported in February 2000. They were 14 geldings and 4 mares between 3 and 7 years of age and their breed was unknown. They were collected within Hungary to be sold to Germany as a team of carriage horses. None of them showed clinical signs during their examination by the border veterinarian. The other two seropositive animals (PRNT-titres 1:32–64 and 1:32) were found among 10 horses which entered the EU-border in Nickelsdorf in June 2001. They were born in Hungary and had no obvious clinical signs when examined at the border. Further details about their sex, age and breed were not available. It proved impossible to obtain further blood samples or to get further clinical information on any of these animals after they had entered Germany.

Using IHC, WNV-antigen was easily detected within the kidney and brain of positive controls of birds. Of the 42 Austrian birds tested retrospectively (Table 1), 35 reacted clearly negative. In seven birds, reaction products were discovered, which did not match the distribution pattern of controls and published cases [17]. Tissue samples from the organs with reaction products were investigated by ISH and nested RT-PCR, using both primer sets. With both techniques, all organs were clearly negative.

Of the birds included in the 2001 study, 6 blackbirds were submitted from a mass die-off, and the five great grey owls had died within a few days of each other. All other birds were found singly. All the blackbirds and great grey owls showed positive

signals by IHC in many organs including brain. Further investigations [22] confirmed that the cause of death in the blackbirds and owls was not WNV but another flavivirus of the Japanese Encephalitis virus group, Usutu virus. WNV could not be demonstrated. Of the remaining birds, five showed inconclusive signals by IHC: in four birds we found granular reaction products in the spleen, and in one bird diffuse or granular staining of virtually all neurons in the brain. Using PCR and ISH, all these birds were WNV-negative.

Nested RT-PCR was positive in paraffin embedded tissue from the equine control case. None of the horses with aetiologically unclear nonsuppurative encephalitis showed amplification products.

RNA integrity was confirmed by RT amplification of β-actin mRNA in all of the avian samples and in all but one of the equine samples.

WNV infections can occur as epidemics with obvious morbidity and mortality in warmblooded hosts including human beings. Such epidemics are usually not overlooked and eventually diagnosed on the basis of laboratory tests [23, 24]. However, WNV infections may also occur in a more unspectacular way, with only a few human cases and relatively mild signs [25], and these outbreaks may be easily overlooked. In Austria, there have been no obvious epidemics of meningoencephalitis in human beings or horses and no significant bird die-offs within the last decade, except for the 2001 episode. However, single seropositive birds, mammals and humans in the seventies [9–13] and the more recent isolation of WNV close to the Austrian border [26] indicate the occasional presence of WNV in Austria and make it a possible cause of avian deaths and equine encephalomyelitis. It has been suggested that infected mosquitoes, birds and horses are good indicators for WNV activity [27].

We were able to exclude WNV as possible cause of death or encephalitis in the birds and horses examined. These results strongly suggest that WNV infections have not been missed as causes of meningoencephalitis and mortality in horses and birds during the last decade in Austria. Thus, it seems that both currently and in the past WNV has not been a significant pathogen in Austria.

Although no evidence of WNV infection was found in animals originating in Austria, several horses originating from Hungary were seropositive. These horses all appeared clinically healthy at the time of blood sampling and were most likely not vireamic. Definitive proof, however, is lacking, as we were not able to test for the presence of virus in the blood or monitor the antibody titres over time.

Although WNV was not detected in wild birds, during the course of this investigation, a flavivirus closely related to WNV, Usutu virus, was identified as cause of avian mortality, predominantly in blackbirds (*Turdus merula*), in Austria in 2001 [22]. These results clearly show the importance of animal disease and mortality surveillance in the context of emerging flavivirus diseases.

ACKNOWLEDGEMENTS

Many thanks to Bernadette Murgue, Thomas Briese, Tracey McNamara, and Mark Hall who provided WNV antiserum, plasmids containing WNV sequences, and positive reference material, respectively. We thank Ingrid Friedl for excellent technical assistance and Stephan Weigl and Wolfgang Zenker who provided the majority of the 2001 birds. This study received financial support from the Hochschuljubiläumsstiftung of the City of Vienna.

REFERENCES

1. Hubálek Z, Halouzka J. West Nile fever – a reemerging mosquito-borne viral disease in Europe. Emerg Infect Dis 1999; **5**: 643–650.
2. Giladi M, Metzkor-Cotter E, Martin DA, et al. West Nile encephalitis in Israel, 1999: The New York connection. Emerg Infect Dis 2001; **7**: 659–661.
3. Weinberger M, Pitlik SD, Gandacu D, et al. West Nile fever outbreak, Israel, 2000: epidemiologic aspects. Emerg Infect Dis 2001; **7**: 686.
4. Cernescu C, Nedelcu NI, Tardei G, Ruta S, Tsai TF. Continued transmission of West Nile virus to humans in southeastern Romania, 1997–1998. J Infect Dis 2000; **181**: 710–712.
5. Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI. West Nile encephalitis epidemic in southeastern Romania. Lancet 1998; **352**: 767–771.
6. Savage HM, Ceianu C, Nicolescu G, et al. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. Am J Trop Med Hyg 1999; **61**: 600–611.
7. Cantile C, Di Guardo G, Eleni C, Arispici M. Clinical and neuropathological features of West Nile virus equine encephalomyelitis in Italy. Equine Vet J 2000; **32**: 31–35.
8. Murgue B, Murri S, Zientara S, Durand B, Durand JP, Zeller H. West Nile outbreak in horses in southern France, 2000: the return after 35 years. Emerg Infect Dis 2001; **7**: 692–696.
9. Aspöck H, Graefe G, Kunz C, Radda A. Antikörper gegen Arboviren in Staren (*Sturnus vulgaris L.*) in

Österreich. Zentralbl Bakteriol [Orig A] 1972; **221**: 141–142.
10. Aspöck H, Kunz C, Picher O, Böck F. Virologische und serologische Untersuchungen über die Rolle von Vögeln als Wirte von Arboviren in Ost-Österreich. Zentralbl Bakteriol [Orig A] 1973; **224**: 156–167.
11. Gresiková M, Thiel W, Batiková M, Stünzner D, Sekeyová M, Sixl W. Haemagglutination-inhibiting antibodies against arboviruses in human sera from different regions in Steiermark (Austria). Zentralbl Bakteriol [Orig A] 1973; **224**: 298–302.
12. Sixl W, Batiková M, Stünzner D, Sekeyová M, Sixl-Voigt B, Gresiková M. Haemagglutination-inhibiting antibodies against arboviruses in animal sera, collected in some regions in Australia. II. Zentralbl Bakteriol [Orig A] 1973; **224**: 303–308.
13. Sixl W, Stünzner D, Withalm H. Untersuchungen auf Anthropozoonosen bei Landarbeitern in Ostösterreich. I. Mitteilung. Zentralbl Bakteriol [Orig A] 1976; **234**: 265–270.
14. Lanciotti RS, Kerst AJ, Nasci RS, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 2000; **38**: 4066–4071.
15. Eidson M, Kramer L, Stone W, Hagiwara Y, Schmit K. Dead bird surveillance as an early warning system for West Nile virus. Emerg Infect Dis 2001; **7**: 631–635.
16. Andreadis TG, Anderson JF, Vossbrinck CR. Mosquito Surveillance for West Nile virus in Connecticut, 2000: isolation from Culex pipiens, Cx. restuans, Cx. salinarius, and Culiseta melanura. Emerg Infect Dis 2001; **7**: 670–674.
17. Steele KE, Linn MJ, Schoepp RJ, et al. Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. Vet Pathol 2000; **37**: 208–224.
18. Cantile C, Del Piero F, Di Guardo G, Arispici M. Pathologic and immunohistochemical findings in naturally occuring West Nile virus infection in horses. Vet Pathol 2001; **38**: 414–421.
19. Hubálek Z, Savage HM, Halouzka J, Juricova Z, Sanogo YO, Lusk S. West Nile virus investigations in South Moravia, Czechland. Viral Immunol 2000; **13**: 427–433.
20. Weissenböck H, Suchy A, Holzmann H. Tick-borne encephalitis in dogs: neuropathological findings and distribution of antigen. Acta Neuropathol 1998; **95**: 361–366.
21. Graber HU, Muller CF, Vandevelde M, Zurbriggen A. Restricted infection with canine distemper virus leads to down-regulation of myelin gene transcription in cultured oligodendrocytes. Acta Neuropathol 1995; **90**: 312–318.
22. Weissenböck H, Kolodziejek J, Url A, Lussy H, Rebel-Bauder B, Nowotny N. Emergence of Usutu virus, an African mosquito-borne flavivirus of the Japanese encephalitis virus group, central Europe. Emerg Infect Dis 2002; **8**: 652–656.
23. Briese T, Jia XY, Huang C, Grady LJ, Lipkin WI. Identification of a Kunjin/West Nile-like flavivirus in brains of patients with New York encephalitis [letter]. Lancet 1999; **354**: 1261–1262.
24. Platonov AE. West Nile encephalitis in Russia 1999–2001: were we ready? Are we ready? Ann NY Acad Sci 2001; **951**: 102–116.
25. Hubálek Z, Halouzka J, Juricova Z. West Nile fever in Czechland [letter]. Emerg Infect Dis 1999; **5**: 594–595.
26. Hubálek Z, Halouzka J, Juricova Z, Sebesta O. First isolation of mosquito-borne West Nile virus in the Czech Republic [letter]. Acta Virol 1998; **42**: 119–120.
27. Marfin AA, Petersen LR, Eidson M, et al. Widespread West Nile virus activity, eastern United States, 2000. Emerg Infect Dis 2001; **7**: 730–735.