

## Correspondence

Alan Buckley

abuck@ceh.ac.uk

## Serological evidence of *West Nile virus*, *Usutu virus* and *Sindbis virus* infection of birds in the UK

Alan Buckley,<sup>1</sup> Alistair Dawson,<sup>2</sup> Stephen R. Moss,<sup>1</sup> Shelley A. Hinsley,<sup>2</sup> Paul E. Bellamy<sup>2</sup> and Ernest A. Gould<sup>1</sup>

<sup>1</sup>Centre for Ecology and Hydrology Oxford, Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford OX1 3SR, UK

<sup>2</sup>Centre for Ecology and Hydrology, Monkswood, Abbots Ripton, Cambridge PE28 2LS, UK

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The introduction and rapid dispersal of the African flavivirus *West Nile virus* (WNV) throughout North America, and the high fatality rate due to encephalitis in birds, horses, other wildlife species and humans, has attracted major attention worldwide. *Usutu virus*, another flavivirus, came to prominence in 2001, when it was identified as the agent responsible for a drop in the bird population in Austria; previously this encephalitic virus was found only in birds and mosquitoes in Africa. *Sindbis virus*, a pathogenic alphavirus that causes arthritis, is widespread throughout Africa, Europe, Asia and Australia, infecting a range of arthropods and vertebrates and is genetically related to encephalitic viruses in North America. Currently there is no evidence that any of these viruses cause disease in the UK. Here the presence of virus-specific neutralizing antibodies is reported in the sera of resident and migrant birds in the UK, implying that each of these viruses is being introduced to UK birds, possibly by mosquitoes. This is supported by nucleotide sequencing that identified three slightly different sequences of WNV RNA in tissues of magpies and a blackbird. The detection of specific neutralizing antibodies to WNV in birds provides a plausible explanation for the lack of evidence of a decrease in the bird population in the UK compared with North America. The potential health risk posed to humans and animals by these viruses circulating in the UK is discussed.

## INTRODUCTION

*West Nile virus* (WNV) is an **arbo**virus (**ar**thropod-**bo**rne virus) in the family *Flaviviridae*, genus *Flavivirus*. Before its inadvertent introduction into North America in 1999, probably from Israel or Egypt (Lanciotti *et al.*, 1999), it was known to be widespread throughout Eurasia (Hubalek & Halouzka, 1999). The virus was first isolated in the West Nile District of Uganda in 1937 (Smithburn *et al.*, 1940).

Molecular epidemiological evidence indicates that WNV radiated from Africa northwards to the Mediterranean and southern European regions, eastwards to India, central and southern Asia and finally to Australia as Kunjin virus (Gould, 2002). The natural life cycle of this virus involves ornithophilic mosquitoes, particularly but not exclusively, *Culex* species, and birds that migrate between Africa, Europe and Asia (Work *et al.*, 1955). Non-migratory birds become infected when WNV-infected mosquitoes feed on them. Evidence that the virus is transmitted between resident and migratory birds by infected mosquitoes can be obtained by detecting WNV-specific antibody, infectious virus or RNA in the non-migratory birds or in nearby animals. The risk of human epidemics is greatest in countries where ornithophilic mosquitoes occur at high densities in urban areas near to slow moving rivers or large areas of water that attract migratory birds. The mosquitoes feed on the infected birds, become infected and amplify the virus, which is then transmitted to the next host on which the mosquito feeds. In 1996, an epidemic in Romania involved hundreds of human cases with 17 fatalities (Savage *et al.*, 1999) and there are no obvious reasons at the moment why, in the future, similar epidemics should not spread throughout Europe. Recently, France (Murgue *et al.*, 2000) and Italy (Cantile *et al.*, 2000) have also experienced outbreaks of WN fever in horses and WNV-seropositive birds have been identified in Poland (Juricova *et al.*, 1998) and the Czech Republic (Juricova *et al.*, 1993). Serological studies on humans in the Ebro Delta, Spain, for antibodies to alphaviruses, flaviviruses and a bunyavirus showed an infection rate of 12.5 % for at least one of the viruses tested. In Ampolla, San Jaime and Montells, 30 % of human sera tested were positive for WNV antibodies (Lozano & Filipe, 1998). However, the most publicized emergence of WNV occurred in New York during the late summer of 1999. Within 2 months, 62 human cases of West Nile encephalitis and six fatalities were recorded (Roehrig *et al.*, 2002). Subsequently, the virus overwintered successfully and dispersed throughout the USA, causing more than 3600 confirmed cases and more than 270 deaths by the end of 2002.

*Usutu virus* (USUV), which is closely related to WNV, was first isolated from mosquitoes in South Africa in 1959 (Woodhall, 1964). Subsequent isolations have been made from birds, mosquitoes and mammals, one from *Praomys* species and one from a man with fever and rash (Karabatsos, 1985). In Africa, the virus circulates between birds and mosquitoes with mammals being inadvertent hosts if bitten by infected mosquitoes. There are no reports of severe disease in man and USUV was known only in Africa prior to 2001 when it was isolated unexpectedly from birds dying of encephalitis in Vienna, Austria (Weissenböck *et al.*, 2002).

*Sindbis virus* (SINV), the type species of the genus *Alphavirus*, circulates in the same ecological habitat as WNV in Africa (Olson & Trent, 1985) and is transmitted between birds by ornithophilic *Culex* species of mosquito. Migratory birds transport the alphaviruses great distances and many migratory birds have

antibodies to SINV. Moreover, SINV has been isolated from the blood and tissues of these birds (Ernek *et al.*, 1977; Jupp *et al.*, 1986), but there is no evidence that it causes mortality or sickness in these birds. A strain of SINV isolated in Finland in 1982 during an outbreak of polyarthritis was identified as being closely related to a South African virus isolated in 1963 (Shirako *et al.*, 1991). From studies on the inland plateau and Natal lowlands in South Africa (Jupp *et al.*, 1986), WNV and SINV have been shown to be transmitted to birds by the same mosquito species, particularly *Culex univittatus*.

There are no published studies concerned with the presence of WNV, USUV or SINV in the UK and there is no scientific evidence to suggest that any of these viruses are endemic in the UK. However, many birds migrate annually from regions in Africa where WNV, USUV and SINV co-circulate and are actively transmitted between birds and mosquitoes. It is, therefore, possible that they are carried by birds to the UK and transmitted via indigenous mosquitoes to non-migratory birds and to other wildlife species. As part of a systematic programme of research to see whether or not such viruses are present and active in the UK, we initiated a survey for the presence of virus-specific neutralizing antibodies in the sera of healthy migratory and non-migratory birds. Specific neutralizing antibodies against WNV, USUV and SINV were detected in the bird sera, implying that the viruses are being introduced into the UK by migrant birds and then transmitted to and within resident bird populations by local mosquitoes.

## METHODS

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**Collection of samples from birds.** Sera were collected from 30 different species of wild or farm birds: *Turdus merula* (blackbird); *Sylvia attricapilla* (blackcap); *Buteo buteo* (buzzard); *Corvus corone* (carrion crow); *Fringilla coelebs* (chaffinch); *Gallus domesticus* (free-range chicken); *Anas platyrhynchos* (domestic duck); *Prunella modularis* (dunnock); *Sylvia borin* (garden warbler); *Parus major* (great tit); *Picus viridis* (green woodpecker); *Delichon urbica* (house martin); *Corvus monedula* (jackdaw); *Garrulus garrulus* (jay); *Falco tinnunculus* (kestrel); *Sylvia curruca* (lesser whitethroat); *Carduelis cannabina* (linnet); *Pica pica* (magpie); *Anthus pratensis* (meadow pipit); *Phoenicurus phoenicurus* (redstart); *Emberiza schoeniculus* (reed bunting); *Acrocephalus scirpaceus* (reed warbler); *Erithacus rubecula* (robin); *Acrocephalus schoenobaenus* (sedge warbler); *Turdus philomelos* (song thrush); *Accipiter nisus* (sparrowhawk); *Sturnus vulgaris* (starling); *Hirundo rustica* (swallow); *Meleagris gallopavo* (domestic turkey); *Sylvia communis* (whitethroat).

Most birds were sampled in Cambridgeshire, UK ( $n=296$ ) between October 2001 and September 2002, others, from Dorset ( $n=14$ ), were obtained in May 2002 (mainly migrants returning to the UK) and south Wales ( $n=43$ , house martin and swallow) in August 2002 (migrants about to leave the UK). Carrion crows

( $n=26$ ) and magpies ( $n=45$ ) were trapped during pest control programmes and brains and sera were collected. Other species were caught in mist nets, bled and released. Amongst these were 69 juveniles, i.e. hatched during 2002 (15 species). Three species of free-range poultry (chicken, turkey and duck) were also sampled in Cambridgeshire in May 2002.

**Virus strains.** WNV strain 99-34940-31A, designated WN-NY, isolated in New York State, USA in 1999, and WNV strain DAK Ar B 310, passage 13, designated WN-DAK, isolated from a mosquito collected in Central African Republic in 1967, were supplied by R. E. Shope and R. B. Tesh, University of Texas, USA. WNV isolated from Israel in the early 1980s, designated WN-Is, was supplied by M. Halevy, Israel Institute for Biological Research. USUV strain SAAR 1776, passage 9, isolated from a mosquito and designated USUV, was supplied by J. S. Porterfield, formerly of Oxford University, UK. SINV strain SAAR 86, passage 5, isolated from a mosquito pool and designated SINV, was supplied by J. Casals, formerly of Yale Arbovirus Research Unit, USA. *Louping ill virus*, strain 91/330 Preston, from H. W. Reid, Moredun Research Institute, Edinburgh, UK, was isolated from a sheep and designated LIV.

**Plaque reduction neutralization test (PRNT).** Sera, heat-inactivated at 56 °C for 30 min, were coded and tested with no knowledge of their identity. They were diluted twofold from 1:10 to 1:320 in MEM with Earle's salts (Invitrogen). Aliquots of diluted virus (125 µl) containing 50 p.f.u. were added to 125 µl of diluted serum and incubated at 37 °C for 1 h. Vero cell monolayers in 24-well tissue culture plates were washed with serum-free medium and drained before the virus/serum mixture was added and incubated at 37 °C for 1 h. The inoculum was removed from the cells and 1 ml 1.5 % low-gelling-temperature agarose in MEM containing 2 % FBS was added to each well. Plates were incubated for 5–7 days at 37 °C. Monolayers were fixed with 10 % formaldehyde in saline, stained with 0.1 % naphthalene black, washed, dried and the plaques were counted. Negative serum controls were included in each experiment.

As the sera were obtained from healthy birds, it was assumed that antibody, if present, might be of low avidity and of low titre. Therefore, PRNT data were analysed by scoring the neutralization results at high and lower stringency as follows: firstly, the highest dilution of antibody that neutralized 90 % of the plaques was recorded as the PRNT<sub>90</sub> endpoint; secondly, using the same titrations, the highest dilution of antibody that neutralized 50 % of the plaques was recorded as the PRNT<sub>50</sub> endpoint.

**Indirect immunofluorescence (IF) tests.** WN-NY virus was used to infect Vero cells grown on glass coverslips for 40 h, which were then fixed in ice-cold acetone for 5 min. These cells were treated with 100 µl of diluted chicken serum and incubated at 37 °C for 45 min before washing in PBS at 37 °C

for 10 min. Rabbit anti-chicken FITC-conjugated antibody (Sigma) was added at the recommended dilution before incubation at 37 °C for 45 min. After washing in PBS, WNV-specific monoclonal antibody (mAb 546) was added. Coverslips were incubated at 37 °C for 45 min, washed and sheep anti-mouse biotinylated antibody (Amersham) was added at the recommended dilution. After incubation for 45 min at 37 °C, the coverslips were washed and Texas red conjugated with Streptavidin (Amersham) was added for 10 min at room temperature. Coverslips were washed for 10 min in warm PBS and then transferred to warm water before drying and mounting in glycerol/DABCO on glass slides. An Olympus UV light microscope fitted with exclusion filters to observe fluorescein or Texas red fluorescence was used to examine the dual-labelled infected cells.

**Western blot analysis.** Aliquots (50 µl) of normal and WN-NY virus-infected 20 % (w/v) mouse brain suspension in PBS were diluted 1:1 with SDS-PAGE denaturing loading buffer and boiled for 3 min. Replicate 10 µl loadings were applied to a 10 % polyacrylamide gel, together with Seebule-2 protein molecular mass markers (Novex). The gel was run at 200 V and then immersed in Western blotting buffer (20 % MeOH, 20 mM Tris and 15 mM glycine) for 10 min before blotting onto an Immobilon-P transfer membrane (Millipore, IVPH00010) at 12 V for 2 h. The membrane was cut into strips containing one track of each uninfected and WNV-infected brain sample. The strips were then incubated in blocking buffer (5 %, w/v, milk powder and 0.01 % Tween 20 in PBS) for 1 h at room temperature. The test chicken or positive control mouse anti-WNV, antisera were diluted at 1:50, 1:250 and 1:500 in blocking buffer and added to individual strips of membrane that were incubated overnight at 4 °C. Strips were washed four times with blocking buffer and were then incubated with rabbit anti-chicken alkaline phosphatase (Sigma, F8888) or rabbit anti-mouse alkaline phosphatase (Sigma, A4312), as appropriate. All strips were then washed four times with blocking buffer and developed using Sigmafast NBT/BCIP (Sigma, B5655).

**RT-PCR sequencing.** Viral RNA was extracted using the RNAagents kit (Promega) following the manufacturer's instructions. Primers used for nested RT-PCR were based on conserved regions of the known sequences of WNV (AF260967) and Kunjin (D00246) virus envelope protein. The nucleotide positions of the primers are as follows; first forward primer 1092–1114 (F1) and second forward primer 1181–1201 (F2); first reverse primer 2014–1995 (R1) and second reverse primer 1960–1941 (R2). First-strand cDNA synthesis was performed using Superscript II reverse transcriptase (Life Technologies) with primer R1. A nested PCR was used to amplify the DNA. The first reaction utilized primers F1 and R1 and the second F2 and R2 to produce a product of 779 bp. PCR products were gel-purified and both strands sequenced using a PE Biosystems cycle sequencing kit with primers F2 and R2.

## RESULTS

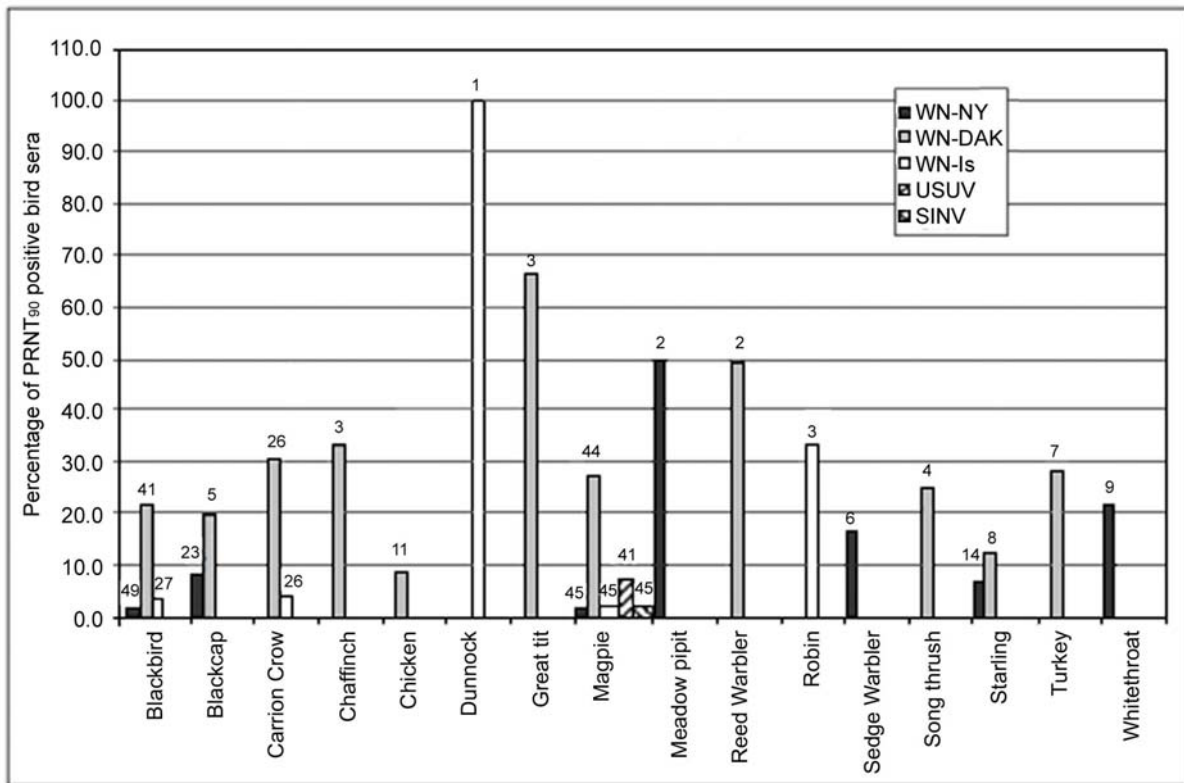
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### **Bird sera contain WNV- and/or USUV- and/or SINV-specific PRNT antibodies**

A total of 353 serum samples were obtained from either UK-resident or migrant birds and poultry showing no signs of disease. Since only small volumes were available for PRNT analysis for many of the sera, they were tested initially for WNV-specific antibodies using only the WN-NY strain of virus. Those for which the volumes were larger were also tested for antibodies against two other strains of WNV, one strain of SINV (113 samples) and one strain of USUV (91 samples). The only known endemic flavivirus in the UK is LIV. This tick-borne virus is antigenically distinguishable from WNV by PRNT analysis. A strain of LIV was, therefore, included in all tests as a control reagent. The first set of results presented below was carried out using the high stringency PRNT<sub>90</sub> method of analysis.

Of the 353 sera tested, 52 (14.7 %) were PRNT<sub>90</sub> positive for WNV, with antibody titres ranging between 1/10 and 1/40. The results of these positive neutralization tests are shown in Fig. 1; they also include the species of bird from which sera were obtained and tested against each of the six viruses. Of the 52 WNV-positive sera, 36 neutralized only WN-DAK, nine neutralized only WN-NY, four neutralized only WN-Is, one neutralized WN-DAK and WN-Is, and the other two of the 52 WNV-positive sera were also positive for USUV. In addition, one serum sample was positive only for USUV and another was positive only for SINV. No sera were positive for LIV at the PRNT<sub>90</sub> level. We conclude from these results that WNV is being introduced into a significant proportion of UK-resident birds, presumably by mosquitoes feeding on migrant birds.

The PRNT<sub>90</sub> endpoint is the standard method that has been used to identify WNV-neutralizing antibodies in dead and dying birds in the USA. However, this high stringency endpoint may not be appropriate for the UK since there is no evidence of disease in birds, and antibodies may be of much lower titres compared with birds in the USA and may also have low avidity. Therefore, since the control results were satisfactory, the neutralization data were re-scored using the lower stringency PRNT<sub>50</sub> end point. The PRNT<sub>50</sub> results (Table 1) using three strains of WNV, and USUV, SINV and LIV, also define the bird species from which each serum sample was collected. Of 353 bird sera, 66 % were positive for WN-NY with neutralizing antibody titres ranging from 1/10 to >1/320. The two other strains of WNV (WN-DAK and WN-Is) produced higher [157 positive out of 172 tested (91.3 %)] and slightly lower [84 positive out of 141 tested (59.6 %)] proportions of positive sera. Two sera were positive against LIV; one of these, from a robin, was negative for WNV and the other, from a turkey, was positive for WNV.



**Fig. 1.** Histogram depicting the percentage of antibody-positive bird sera, as determined by the PRNT<sub>90</sub> method. The results are presented for each bird species tested against up to five viruses, i.e. WN-NY, WN-DAK, WN-Is, USUV and SINV, respectively. LIV (the sixth virus), which was the negative control, is not shown in the histogram. Each serum sample was titrated from 1/10 to 1/320. All sera showing at least 50 % plaque reduction at a 1:10 dilution (or more) were considered positive. The numbers of bird sera tested against each virus, for each bird species, are shown above the columns.

Of the 353 sera, 113 were analysed by PRNT<sub>50</sub> using SINV: 57 (50.4 %) contained detectable SINV-neutralizing antibodies and, of the 103 WNV-positive sera (i.e. from the 113 sera), 48 were negative for SINV. Therefore, more than 50 % of the resident birds sampled during the early spring months had been exposed to both WNV and SINV.

**Table 1.** Summary of analysis of bird sera tested by the PRNT<sub>50</sub> method against six viruses

Positive sera are defined as those that reduce the plaque count by at least 50 % at a 1:10, or higher, dilution. ND, Tests that were not carried out due to availability of serum.

Bird species	Virus (%)					
	WN-NY (n)	WN-DAK (n)	WN-Is (n)	USUV (n)	SINV (n)	LIV (n)
Blackbird	78 (49)	98 (41)	74 (27)	100 (3)	56 (9)	0 (43)
Blackcap	87 (23)	100 (5)	100 (1)	ND	50 (2)	0 (5)
Buzzard	100 (1)	100 (1)	100 (1)	ND	ND	0 (1)
Carrion crow	85 (26)	100 (26)	58 (26)	54 (26)	62 (26)	0 (26)
Chaffinch	56 (9)	100 (3)	100 (4)	ND	ND	0 (4)
Chicken	71 (14)	82 (11)	60 (10)	0 (7)	13 (8)	0 (14)
Duck	83 (6)	100 (2)	0 (1)	0 (1)	67 (3)	0 (6)
Dunnock	64 (14)	50 (2)	100 (1)	ND	67 (3)	0 (9)
Garden warbler	75 (4)	ND	ND	ND	ND	ND
Great tit	61 (36)	100 (3)	ND	ND	ND	0 (13)
Green woodpecker	0 (1)	100 (1)	0 (1)	0 (1)	0 (1)	0 (1)
House martin	50 (18)	100 (1)	ND	ND	100 (1)	ND
Jackdaw	0 (6)	0 (6)	0 (6)	0 (6)	0 (6)	0 (6)
Jay	100 (2)	ND	ND	ND	ND	ND
Kestrel	0 (1)	ND	ND	ND	ND	ND
Lesser whitethroat	100 (1)	ND	ND	ND	ND	ND
Linnet	100 (1)	ND	ND	ND	ND	ND
Magpie	67 (45)	93 (44)	62 (45)	68 (41)	51 (45)	0 (45)
Meadow pipit	100 (2)	ND	ND	ND	ND	ND
Redstart	100 (1)	ND	ND	ND	ND	ND
Reed bunting	100 (1)	100 (1)	ND	ND	ND	ND
Reed warbler	71 (7)	100 (2)	100 (2)	ND	ND	ND
Robin	83 (18)	67 (3)	100 (3)	ND	50 (2)	25 (4)
Sedge warbler	50 (6)	ND	100 (2)	ND	ND	ND
Song thrush	80 (5)	100 (4)	0 (1)	ND	0 (1)	0 (4)
Sparrowhawk	0 (1)	ND	ND	ND	ND	0 (1)
Starling	93 (14)	100 (8)	0 (2)	ND	ND	0 (14)
Swallow	8 (25)	ND	ND	ND	ND	ND
Turkey	29 (7)	86 (7)	0 (7)	67 (6)	83 (6)	14 (7)
Whitethroat	100 (9)	100 (1)	100 (1)	ND	ND	ND
Total	353	172	141	91	113	203

Also, 91 of the original bird sera representing eight species were tested for USUV-neutralizing antibodies and 49 of these (53.8 %) were positive by the PRNT<sub>50</sub> method. One serum sample from a magpie was positive for USUV and SINV but negative for WNV, and 35 of the 42 USUV-negative sera were positive for WNV, demonstrating that the PRNT<sub>50</sub> method can discriminate between USUV- and WNV-specific neutralizing antibodies. It is also worth noting that in six of the sera, the titres against USUV were equal to or higher than those against WNV, implying that these birds had been exposed to USUV and WNV. It is important to emphasize that even though a lower stringency threshold is being used, these results demonstrate specificity for each virus tested.

Table 2 presents details of the PRNT<sub>50</sub> results for which there was sufficient serum for all of the viruses to be tested. A wide range of results was obtained, including (a) WNV positive, (b) WNV and USUV positive, (c) WNV, USUV and SINV positive, (d) WNV and SINV positive, (e) USUV and SINV positive and (f) SINV positive. These results demonstrate (a) the high sensitivity and specificity of the test, (b) that individual UK-resident birds are being exposed to at least three different mosquito-transmitted arboviruses, and (c) that WNV, USUV and SINV are widespread throughout all of the sites investigated.

**Table 2.** Antibody titres (PRNT<sub>50</sub>) of 87 bird sera tested against six viruses.

Bird sera arranged by collection site and time of collection starting at the earliest date.

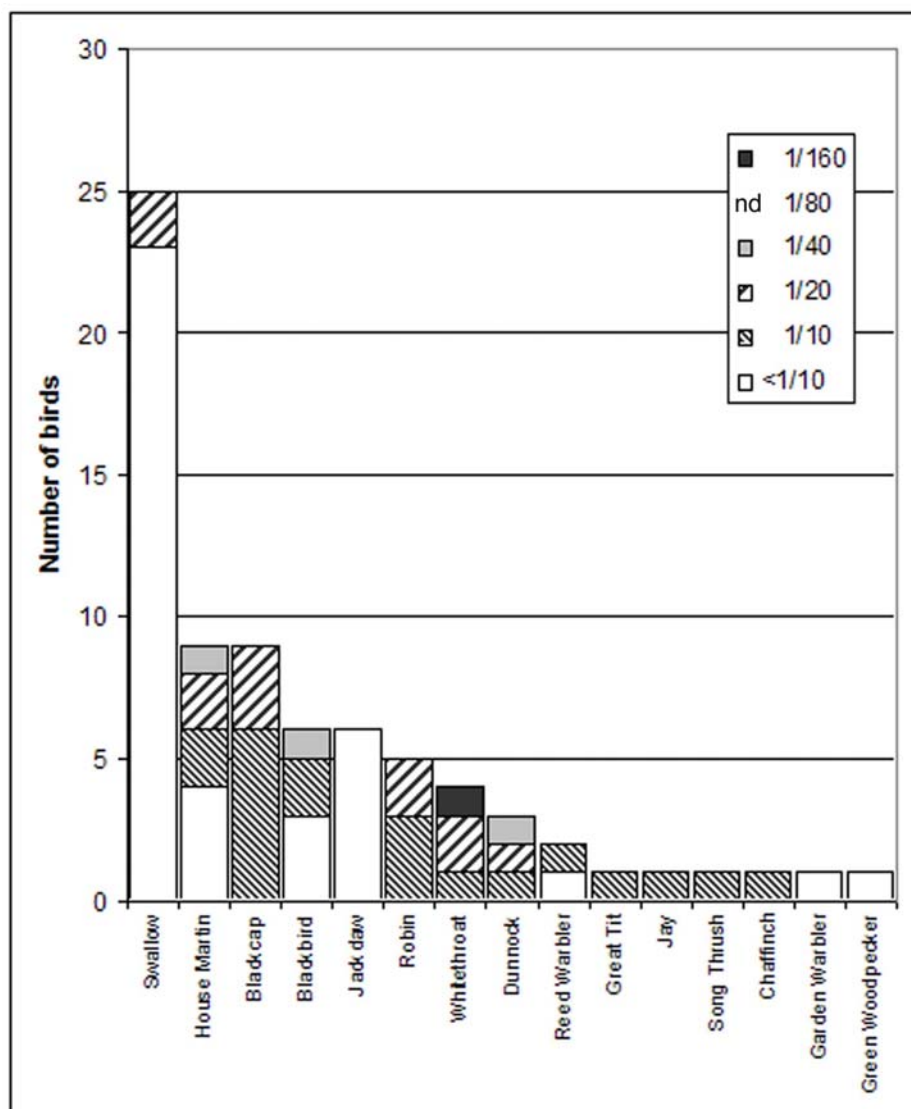
Site 1, Abbots Ripton; Site 2, Croxton; Site 3, Monks Wood; Site 4, Stamford; Site 5, Whittlesey.

NT, Not tested.

Site	Species	Sample	PRNT <sub>50</sub> antibody titre							
			WN-NY	WN-DAK	WN-Is	LIV	USUV	SINV		
1	Carrion crow	WNV 202	1/20	1/10	1/80	<1/10	1/160	1/40		
		WNV 203	1/40	1/40	1/40	<1/10	1/20	<1/10		
		WNV 204	1/40	1/40	1/40	<1/10	1/10	<1/10		
		WNV 209	1/20	1/40	1/20	<1/10	1/10	<1/10		
		WNV 210	1/40	1/40	<1/10	<1/10	1/20	1/10		
		WNV 228	1/20	1/20	<1/10	<1/10	<1/10	1/20		
		WNV 229	1/20	1/40	<1/10	<1/10	<1/10	1/20		
		WNV 230	1/40	1/20	1/20	<1/10	1/40	1/10		
		WNV 231	1/20	1/10	1/40	<1/10	<1/10	1/40		
		WNV 241	<1/10	1/40	<1/10	<1/10	1/10	<1/10		
	Magpie	WNV 191	1/10	1/20	1/10	<1/10	1/20	<1/10		
2	Carrion crow	WNV 188	1/40	1/40	1/20	<1/10	1/20	1/20		
		WNV 189	1/20	1/10	<1/10	<1/10	<1/10	1/10		
		WNV 196	1/20	1/20	1/10	<1/10	1/40	1/10		
		WNV 197	1/40	1/40	<1/10	<1/10	1/40	1/20		
		WNV 198	1/20	1/40	1/80	<1/10	<1/10	1/10		
		WNV 199	1/10	1/20	1/20	<1/10	1/40	1/10		
		WNV 208	1/10	1/20	1/10	<1/10	1/10	<1/10		
		WNV 226	1/10	1/20	<1/10	<1/10	<1/10	<1/10		
		WNV 227	1/20	1/20	1/20	<1/10	<1/10	1/20		
		WNV 238	1/10	1/80	1/10	<1/10	<1/10	1/10		
		WNV 239	1/10	1/40	<1/10	<1/10	1/10	<1/10		
		WNV 240	1/20	1/40	<1/10	<1/10	<1/10	<1/10		
		WNV 242	1/40	1/80	1/40	<1/10	1/20	1/10		
		WNV 246	<1/10	1/80	<1/10	<1/10	<1/10	<1/10		
		WNV 247	<1/10	1/80	<1/10	<1/10	<1/10	<1/10		
		WNV 248	<1/10	1/20	1/40	<1/10	<1/10	1/10		
			Magpie	WNV 109	1/20	1/20	<1/10	<1/10	NT	1/10
				WNV 111	1/20	1/20	<1/10	<1/10	1/20	<1/10
				WNV 128	<1/10	1/20	<1/10	<1/10	1/20	<1/10
				WNV 129	<1/10	<1/10	<1/10	<1/10	1/20	1/20
		WNV 131	<1/10	<1/10	1/40	<1/10	NT	1/20		
		WNV 132	<1/10	1/20	<1/10	<1/10	1/20	1/10		
		WNV 133	<1/10	1/20	1/10	<1/10	1/40	1/20		
		WNV 135	<1/10	1/10	<1/10	<1/10	1/10	<1/10		

		WNV 142	<1/10	1/10	<1/10	<1/10	1/20	1/10
		WNV 143	<1/10	1/10	<1/10	<1/10	NT	1/40
		WNV 144	<1/10	1/80	1/10	<1/10	1/10	1/10
		WNV 145	1/10	1/20	1/10	<1/10	1/40	<1/10
		WNV 146	1/20	1/20	<1/10	<1/10	1/20	<1/10
		WNV 147	<1/10	1/10	<1/10	<1/10	<1/10	1/10
		WNV 168	1/20	1/20	1/20	<1/10	1/10	1/40
		WNV 169	1/10	1/20	1/40	<1/10	1/10	<1/10
		WNV 170	1/10	1/20	1/40	<1/10	1/40	1/10
		WNV 171	1/20	1/20	1/40	<1/10	1/10	1/10
		WNV 172	1/10	1/20	1/40	<1/10	1/10	1/10
		WNV 173	1/40	1/20	1/10	<1/10	<1/10	<1/10
		WNV 174	1/40	<1/10	1/20	<1/10	1/20	<1/10
		WNV 175	1/10	1/20	1/20	<1/10	1/40	1/10
		WNV 176	1/40	1/40	<1/10	<1/10	1/20	<1/10
		WNV 177	1/20	1/20	<1/10	<1/10	1/10	1/10
		WNV 178	1/10	1/20	<1/10	<1/10	1/20	<1/10
		WNV 186	1/40	1/40	1/40	<1/10	1/40	<1/10
		WNV 187	1/10	1/40	1/10	<1/10	1/20	<1/10
		WNV 190	1/20	1/40	1/40	<1/10	1/20	1/10
		WNV 192	1/20	1/20	1/20	<1/10	1/20	1/20
		WNV 193	1/80	1/40	1/20	<1/10	1/20	<1/10
		WNV 200	1/40	1/80	1/40	<1/10	<1/10	1/20
		WNV 201	1/40	1/80	1/10	<1/10	<1/10	1/20
		WNV 205	1/20	1/10	1/10	<1/10	<1/10	1/20
		WNV 206	1/40	1/40	1/80	<1/10	1/10	1/10
		WNV 207	1/40	1/40	1/80	<1/10	<1/10	<1/10
		WNV 225	1/20	1/40	<1/10	<1/10	<1/10	<1/10
		WNV 243	<1/10	1/20	<1/10	<1/10	<1/10	<1/10
		WNV 244	<1/10	1/20	<1/10	<1/10	<1/10	<1/10
		WNV 245	<1/10	1/20	<1/10	<1/10	1/20	<1/10
		WNV 262	1/20	1/40	1/40	<1/10	NT	<1/10
3	Blackbird	WNV 121	1/20	1/20	<1/10	<1/10	1/10	<1/10
		WNV 126	1/20	1/20	<1/10	<1/10	1/40	<1/10
	Magpie	WNV 371	<1/10	1/40	1/10	<1/10	<1/10	<1/10
		WNV 372	1/20	1/40	1/20	<1/10	<1/10	1/10
		WNV 373	<1/10	1/40	1/10	<1/10	<1/10	<1/10
		WNV 374	1/20	1/20	1/10	<1/10	<1/10	
4	Green woodpecker	WNV 281	<1/10	1/20	<1/10	<1/10	<1/10	
5	Chicken	WNV 232	<1/10	<1/10	1/10	<1/10	<1/10	<1/10
		WNV 234	1/40	1/40	1/20	<1/10	<1/10	<1/10
		WNV 235	1/20	1/20	<1/10	<1/10	<1/10	<1/10
		WNV 236	1/40	1/40	1/40	<1/10	<1/10	<1/10
		WNV 237	1/20	1/20	1/20	<1/10	<1/10	<1/10
			WNV 272	<1/10	1/20	<1/10	<1/10	<1/10
	Duck	WNV 152	<1/10	1/10	<1/10	<1/10	<1/10	<1/10
		WNV 161	<1/10	1/10	<1/10	<1/10	1/10	1/20
	Turkey	WNV 162	<1/10	1/20	<1/10	<1/10	1/10	1/40
		WNV 163	<1/10	<1/10	<1/10	<1/10	<1/10	1/10
WNV 164		1/10	1/10	<1/10	<1/10	1/10	1/20	
WNV 165		<1/10	1/20	<1/10	<1/10	1/20	<1/10	
		WNV 166	<1/10	1/20	<1/10	<1/10	1/10	

Amongst the 353 sera, 75 were collected from juveniles, i.e. birds that had hatched during 2002 and were, therefore, unlikely to have left the UK. Of these 75 sera (Fig. 2), 35 produced WNV-positive PRNT<sub>50</sub>, implying that they had been exposed to infectious WNV in the UK during the first year of their lives. In contrast, six jackdaws that were unlikely to have been exposed to mosquitoes because they had been hand-reared indoors were negative in all PRNT assays.

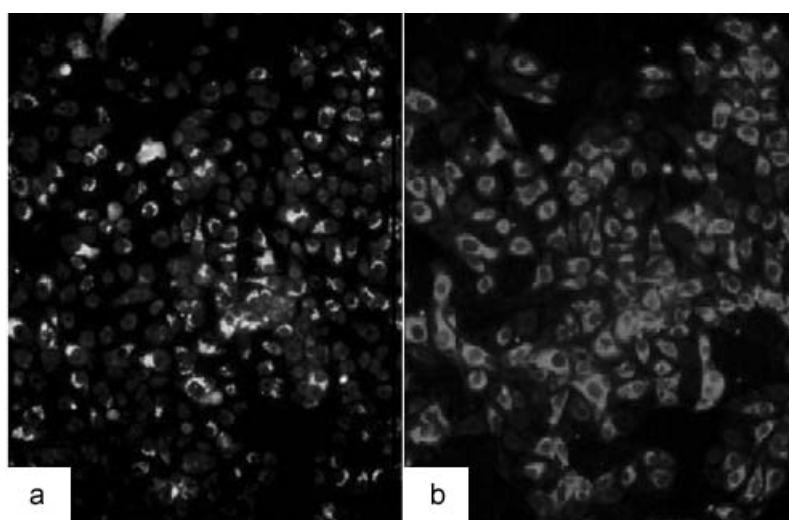


**Fig. 2.** Histogram depicting the antibody titre obtained when 75 samples of sera from 15 species of juvenile birds (hatched in 2002) were tested by the PRNT<sub>50</sub> method against WNV (WN-NY strain). The shading identifies the titre and the height depicts the number of sera producing that titre.

### Detection of WNV antibodies by IF tests

Whilst the PRNT is a robust and widely accepted method of demonstrating the presence of flavivirus-specific antibodies in sera, it was felt necessary to confirm by independent serological methods that the bird sera contained antibodies that react with WNV. Therefore, indirect IF tests were performed on four PRNT-positive and two PRNT-negative free-range chicken sera, using Vero cells infected with WN-NY. The cells were double-treated with WNV-positive chicken serum and a WNV-specific mAb (mAb 546). They were then labelled with fluorescein-conjugated anti-chicken serum (Sigma) and Texas red-0001-9341 © 2003 SGM

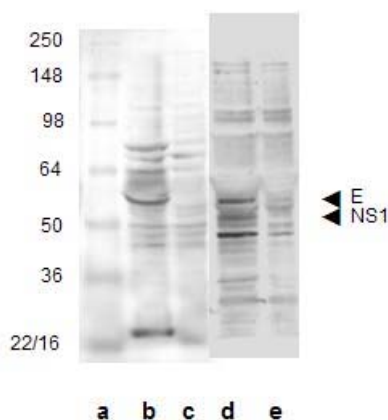
conjugated anti-mouse serum (Sigma). Three of the four PRNT-positive chicken sera were positive in IF tests at a 1:1000 dilution and the fourth at a 1:100 dilution of serum (i.e. significantly higher titres than were observed by PRNT), producing WNV-specific cytoplasmic fluorescence in the virus-infected cells (Fig. 3). In Fig. 3(a), the chicken serum identified infected cells, as indicated by the brilliant white aggregates distributed around the nucleus of infected cells (illustrated in black and white). This pattern of fluorescence is identical to that produced by non-structural NS1 proteins (Gould *et al.*, 1985), implying that the virus has replicated in the bird and stimulated NS1-specific antibodies. In Fig. 3(b), the cytoplasmic fluorescence obtained using a WNV E protein-specific mAb (Gould *et al.*, 1990), in the same cells, shows a different distribution of the viral E protein, i.e. diffuse distribution (illustrated as a greyish white colour).



**Fig. 3.** Indirect immunofluorescence double-labelling of WNV-infected Vero cells with PRNT-positive chicken serum and WNV-specific mAb 546. (a) Chicken serum produced a limited distribution of fluorescence, primarily around the nucleus of infected cells (brilliant white appearance). This is typical of NS1 protein distribution in the cytoplasm (Gould *et al.*, 1985). (b) The WNV-specific mAb 546 produced typical diffuse cytoplasmic fluorescence representative of E protein in infected cells (greyish-white appearance).

### Confirmation of the presence of WNV-specific antibodies by Western blot analysis

The presence of both E- and NS1-specific antibodies in chicken serum was also confirmed by Western blot analysis with optimized concentrations of WNV antigen and PRNT-positive chicken serum that labelled both E and NS1 proteins (Fig. 4). The control mouse anti-WNV serum (Fig. 4, track b), which was prepared using non-infectious WNV, labelled the E protein but not the NS1 protein.



**Fig. 4.** Western blot of WNV-infected mouse brain labelled with chicken serum. (a) Molecular mass markers in kDa; (b, d) WNV-infected brain; (c, e) uninfected brain. Tracks 'a-c' were treated with mouse anti-WNV hyperimmune serum, followed by alkaline phosphatase-conjugated anti-mouse serum (Sigma) and tracks 'd' and 'e' were labelled with WNV-specific chicken serum diluted 1:250 and alkaline phosphatase labelled anti-chicken serum. Arrow heads indicate the WNV E (53 kDa) and NS1 (50 kDa) proteins.

## Detection of WNV-specific RNA in the sera and brains of birds

A nested RT-PCR was performed to test for the presence of WNV RNA using primers based on WNV sequence in 60 bird sera and 32 brains of carrion crows and magpies. Five magpies (three sera and two brains) produced identical WNV-specific sequence (655 bp from the 779 bp PCR product) that differed by two nucleotides from the published New York strain of WNV. A sixth magpie produced a product with four nucleotide changes and one blackbird serum sample yielded a product with three nucleotide changes. The precise nucleotide substitutions, compared with the published New York strain, are shown in Table 3. Control positive and negative tests were included. All experiments were repeated and precautions were taken to eliminate contamination of the tests. These results confirm the presence of WNV in UK birds. At the present time, the same brain samples have yielded no RT-PCR products when appropriate SINV primers have been used.

**Table 3.** Sequence variation of WNV RT-PCR products from UK birds compared with the WN-NY virus sequence  
A nucleotide change that resulted in an amino acid change is indicated with an asterisk.

Sample	Source	Nucleotide position							Sequence accession no.
		1285	1508	1539	1726	1762	1795	1898	
127	Serum	T	G	A	T	T	G*	T	AY321578
144	Serum	T	G	A	G	T	A	C*	AY321579
145	Serum	T	G	A	G	T	A	C*	AY321579
146	Serum	T	G	A	G	T	A	C*	AY321579
171	Brain	T	G	A	G	T	A	C*	AY321579
175	Brain	T	A	G	G	C	A	T	AY321780
193	Brain	T	G	A	G	T	A	C*	AY321579
WN-NY	New York, 1999	C	G	A	G	T	A	T	AF260967

## Failure to isolate infectious virus from healthy birds

At the time of this work, it was not possible to attempt virus isolation using newborn mice. However, attempts were made to isolate infectious virus from 53 antibody-positive bird sera and 32 brains of carrion crows or magpies by placing 50 µl of serum or brain suspension diluted in 1 ml MEM onto Vero cell monolayers in 24-well tissue culture plates and incubating the monolayers at 37 °C for up to 7 days. Immunofluorescence microscopy using the WNV-specific mAb 546 or a SINV-specific mAb 30.11 (Chanas *et al.*, 1982) and a fluorescein-conjugated anti-mouse serum were used to test each culture for the presence of virus. None of 53 sera or 32 bird brains yielded infectious WNV or SINV. Serial passage of the supernatant medium onto fresh Vero cells also failed to isolate infectious virus.

## DISCUSSION

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Although the scope of this investigation is limited by the number and volumes of bird sera available for analysis, we conclude that at least three arboviruses, WNV, USUV and SINV, have been introduced into UK-resident birds from migrant birds, as identified in sera collected in 2001 and 2002. The observation that sera collected from resident birds contain neutralizing antibodies against WNV, USUV and SINV, and the antibody-positive results with the juvenile birds, imply that these viruses are being actively transmitted to the indigenous UK bird population. Moreover, a relatively high proportion of resident birds were positive, implying efficient transmission from the migrant bird population. Further support for the view that WNV is present in resident birds in the UK was also obtained by RT-PCR sequencing of RNA extracted from sera and brain tissue of magpies and a blackbird, i.e. resident birds.

Because mosquito densities in the UK are relatively low compared with warmer countries, such as North America, southern Europe, Africa and Asia, the likelihood of successful transfer of the virus to local birds from migrants might be expected to be low. However, using high stringency analysis, i.e. PRNT<sub>90</sub>, 22.7 % (36 positives from 172 tested using WN-DAK virus) of the UK bird sera tested were shown to have neutralizing antibodies against WN-DAK and 3.3 and 0.9 % had neutralizing antibodies against USUV or SINV, respectively. This suggests that WNV, USUV and SINV are being introduced into UK-resident birds and share overlapping habitats, apparently without causing an obvious reduction in the bird population. Therefore, these viruses are either avirulent strains or local bird species have been exposed to them for many years and have developed herd immunity. It is known that in the USA, neutralizing antibodies to the closely related *St Louis encephalitis virus* (SLEV) persist for at least 2 years in house sparrows, leaving them resistant to re-infection with SLEV (McLean *et al.*, 1983). In contrast, North American avian and equine species frequently develop fatal infections to WNV, presumably because it was introduced to the United States for the first time in, or just before, 1999, i.e. there was virtually no pre-existing herd immunity. Alternatively, North American WNV may be more virulent for birds than the WNV found in the Old World. An equivalent observation was reported recently for birds in Austria that were shown to be fatally infected by USUV, an African virus that quite possibly has only arrived in Austria recently from Africa (Weissenbock *et al.*, 2002).

In the PRNT analyses, some sera produced neutralization that was specific for WNV, some were specific for USUV and others showed reactivity with both viruses, in some cases being higher for WNV and, in others, higher for USUV. This is entirely consistent with the known specificity of the PRNT (Porterfield, 1980). The results reflect the history of virus exposure of each bird.

Prior to these analyses, we had compared 10 strains of WNV (including lineages I and II) for sensitivity to neutralization by WNV using an immune mouse serum. We observed variation in neutralization sensitivity that did not define the difference between lineage I and II viruses. Variation in neutralization sensitivity is consistent with previous observations for WNV and *Yellow fever virus* (Hammam *et al.*, 1965; Peiris *et al.*, 1982; Buckley & Gould, 1985; Blackburn *et al.*, 1987). In PRNT<sub>90</sub> tests, WN-DAK (22.7 %) was significantly more sensitive to neutralization by bird sera than either WN-NY (2.6 %) or WN-Is (5.0 %). The choice of WN-DAK together with the use of the high sensitivity PRNT<sub>50</sub>, rather than the high stringency PRNT<sub>90</sub>, at least partly explains the relatively higher proportion of positive birds observed in our survey compared with reports in Europe. In the USA, the neutralization test for WNV antibody detection in sera uses a 90 % threshold and the New York strain of virus. Whilst this is clearly acceptable in situations where the animals become sick and develop high avidity antibodies, the animals under test in Europe are healthy and have not shown overt clinical symptoms. The neutralizing potency of their sera is likely to be lower. By presenting the results using both PRNT<sub>50</sub> and PRNT<sub>90</sub> endpoints we showed that whilst there was still a significant neutralization-positive bird population, the proportion of positives was lower using the PRNT<sub>90</sub> method. Nevertheless, taking into account the fact that these sera contained high antibody titres by IF analysis, we believe that they reflect more accurately the presence of virus-specific antibodies in birds in the UK. Moreover, a non-neutralizable virus fraction of at least 10 % has been demonstrated in tests that employ sera from the early stage of primary infections (Della-Porta & Westaway, 1978), and this could account, in part, for the lower sensitivity of the PRNT<sub>90</sub> data. Because all tests were performed using coded sera and because the hand-reared jackdaw fledglings (also supplied as coded sera), which had no exposure to mosquitoes or other arthropods, were PRNT<sub>50</sub> negative in all tests, we conclude that the PRNT<sub>50</sub> results are robust.

USUV- or SINV-neutralizing antibodies were not detected as frequently as WNV antibodies in the same sera, suggesting that USUV and SINV may be introduced by migrant birds but may not circulate efficiently amongst the birds and mosquitoes in the areas where the sera were collected. Clearly more bird samples will need to be analysed before this can be confirmed.

Juvenile birds do not migrate until the autumn of their first year. Of 69 wild juveniles (i.e. excluding the six hand-reared jackdaws), 35 caught during the summer months were WNV positive by PRNT<sub>50</sub>, suggesting that they have been exposed to WNV within the UK. Since overall antibody levels in adults were relatively low, it is unlikely that the detected antibody in the juveniles represents residual, maternally transferred antibody. Therefore, extensive mosquito trapping and analysis for the presence of WNV and possibly other arboviruses seems justified.

Other possible sources of transmission of these viruses between indigenous birds include mites and ticks (Shah *et al.*, 1960; Calisher & Karabatsos, 1988; Niklasson, 1988; Sixl *et al.*, 1988), especially where large numbers of birds roost together at the same site or in their nests. Therefore, in addition to mosquitoes, birds and mammals, including bats and humans, samplings of mites and ticks in areas where the virus circulates may yield evidence of these additional routes of transmission. Two of the birds that had either low titre or no antibody against WNV produced positive tests in PRNT against LIV; this virus is associated most often in the UK with encephalitis in moorland sheep and grouse, which become infected by *Ixodes ricinus* when they feed on animals and birds (Reid, 1984), although encephalomyelitis due to LIV has also been detected in farm animals on lowland grazing areas in Ireland, where the grasses have a high moisture content.

Determination of the nucleotide and deduced amino acid sequence of a region of the WNV envelope gene demonstrated very close similarity of one isolate with the WN-NY strain of WNV and slightly lower similarity with two others. At this stage, it is too early to interpret the significance of these data.

It is significant that WNV, USUV and SINV appear to be carried by the birds as persistent infections, i.e. healthy birds carrying very low levels of virus in serum and possibly other tissues without showing any clinical signs of infection. It is known that WNV can be transmitted to mosquitoes feeding on apparently healthy birds (Theiler & Downs, 1973). This is an extremely efficient method of virus dispersal and possibly explains the success of these viruses in becoming dispersed throughout many parts of the Old World and now the New World (Mackenzie *et al.*, 2002).

What are the implications of these observations for humans in the UK? Currently, there is no evidence that British citizens suffer from febrile illness, fatal encephalitis or polyarthritis arising from the bite of mosquitoes infected with WNV, USUV or SINV. On balance, it seems unlikely that these viruses do present significant health problems to humans, birds or horses in the UK, since the likely risk of exposure to WNV-, USUV- or SINV-infected mosquitoes for humans living in urban or peri-urban areas at the present time should be reasonably low. Nevertheless, as the impact of climate change takes effect and as more people spend increasing periods of time in the countryside, where mosquitoes are likely to occur in the highest densities, the risk of human exposure to encephalitic infection by WNV will almost certainly increase.

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## REFERENCES

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- Blackburn, N. K., Thompson, D. L. & Jupp, P. G. (1987).** Antigenic relationship of West Nile strains by titre ratios calculated from cross-neutralization test results. *Epidemiol Infect* **99**, 551–557.
- Buckley, A. & Gould, E. A. (1985).** Neutralization of yellow fever virus studied using monoclonal and polyclonal antibodies. *J Gen Virol* **66**, 2523–2531.
- Calisher, C. H. & Karabatsos, N. (1988).** Arbovirus serogroups: definition and geographic distribution. In *The Arboviruses: Epidemiology and Ecology*, pp. 19–57. Edited by T. P. Monath. Boca Raton, FL: CRC Press.
- Cantile, C., Di Guardo, G., Eleni, C. & Arispici, M. (2000).** Clinical and neuropathological features of West Nile virus equine encephalomyelitis in Italy. *Equine Vet J* **32**, 31–35.
- Chanas, A. C., Gould, E. A., Clegg, J. C. & Varma, M. G. (1982).** Monoclonal antibodies to Sindbis virus glycoprotein E1 can neutralize, enhance infectivity, and independently inhibit haemagglutination or haemolysis. *J Gen Virol* **58**, 37–46.
- Della-Porta, A. J. & Westaway, E. G. (1978).** A multi-hit model for the neutralization of animal viruses. *J Gen Virol* **38**, 1–19.
- Ernek, E., Kozuch, O., Nosek, J., Teplan, J. & Folk, C. (1977).** Arboviruses in birds captured in Slovakia. *J Hyg Epidemiol Microbiol Immunol* **21**, 353–359.
- Gould, E. A. (2002).** Evolution of the Japanese encephalitis serocomplex viruses. In *Japanese Encephalitis and West Nile Viruses*, pp. 391–404. Edited by J. M. Mackenzie, A. D. Barrett & V. Deubel. Berlin: Springer-Verlag.
- Gould, E. A., Buckley, A., Cammack, N., Barrett, A. D. T., Clegg, J. C. S., Ishak, R. & Varma, M. G. R. (1985).** Examination of the immunological relationships between flaviviruses using yellow fever virus monoclonal antibodies. *J Gen Virol* **66**, 1369–1382.
- Gould, E. A., Buckley, A., Higgs, S. & Gaidamovich, S. (1990).** Antigenicity of flaviviruses. *Arch Virol* (Suppl. 1), S137–S152.
- Hammam, H. M., Clarke, D. H. & Price, J. L. (1965).** Antigenic variation of West Nile virus in relation to geography. *Am J Epidemiol* **82**, 40–55.
- Hubalek, Z. & Halouzka, J. (1999).** West Nile fever: a reemerging mosquito-borne viral disease in Europe. *Emerg Infect Dis* **5**, 643–650.
- Jupp, P. G., McIntosh, B. M. & Blackburn, N. K. (1986).** Experimental assessment of the vector competence of *Culex* (*Culex*) *neavei* Theobald with West Nile and Sindbis viruses in South Africa. *Trans R Soc Trop Med Hyg* **80**, 226–230.
- Juricova, Z., Hubalek, Z., Halouzka, J. & Machacek, P. (1993).** Virologic detection of arboviruses in greater cormorants. *Vet Med Praha* **38**, 375–379.
- Juricova, Z., Pinowski, J., Literak, I., Hahm, K. H. & Romanowski, J. (1998).** Antibodies to alphavirus, flavivirus, and bunyavirus arboviruses in house sparrows (*Passer domesticus*) and tree sparrows (*P. montanus*) in Poland. *Avian Dis* **42**, 182–185.
- Karabatsos, N. (1985).** *International Catalogue of Arthropod-Borne Viruses*, 3rd edn. San Antonio, TX: Am Soc Trop Med

Hyg.

- Lanciotti, R. S., Roehrig, J. T., Deubel, V. & 21 other authors (1999).** Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science* **286**, 2333–2337.
- Lozano, A. & Filipe, A. R. (1998).** Antibodies against the West Nile virus and other arthropod-transmitted viruses in the Ebro Delta region. *Rev Esp Salud Publica* **72**, 245–250.
- Mackenzie, J. M., Barrett, A. D. & Deubel, V. (2002).** The Japanese encephalitis serological group of flaviviruses: a brief introduction to the group. In *Japanese Encephalitis and West Nile Viruses*, pp. 1–10. Edited by J. M. Mackenzie, A. D. Barrett & V. Deubel. Berlin: Springer-Verlag.
- McLean, R. G., Mullenix, J., Kerschner, J. & Hamm, J. (1983).** The house sparrow (*Passer domesticus*) as a sentinel for St. Louis encephalitis virus. *Am J Trop Med Hyg* **32**, 1120–1129.
- Murgue, B., Murri, S., Zientara, S., Durand, B., Durand, J. P. & Zeller, H. (2000).** West Nile outbreak in horses in southern France, 2000: the return after 35 years. *Emerg Infect Dis* **7**, 792–796.
- Niklasson, B. (1988).** Sindbis and Sindbis-like viruses. In *The Arboviruses: Epidemiology and Ecology*, pp. 167–176. Edited by T. P. Monath. Boca Raton, FL: CRC Press.
- Olson, K. & Trent, D. W. (1985).** Genetic and antigenic variations among geographical isolates of Sindbis virus. *J Gen Virol* **66**, 797–810.
- Peiris, J. S., Porterfield, J. S. & Roehrig, J. T. (1982).** Monoclonal antibodies against the flavivirus West Nile. *J Gen Virol* **58**, 283–289.
- Porterfield, J. S. (1980).** Antigenic characteristics and classification of *Togaviridae*. In *The Togaviruses*, pp. 13–46. Edited by R. W. Schlesinger. New York: Academic Press.
- Reid, H. W. (1984).** Epidemiology of louping ill. In *Vectors in Virus Biology*, pp. 161–178. Edited by M. A. Mayo & K. A. Harrap. London: Academic Press.
- Roehrig, J. T., Layton, M., Smith, P., Campbell, G. L., Nasci, R. & Lanciotti, R. (2002).** The emergence of West Nile virus in North America: ecology, epidemiology and surveillance. In *Japanese Encephalitis and West Nile Viruses*, pp. 223–240. Edited by J. S. Mackenzie, A. D. Barrett & V. Deubel. Berlin: Springer-Verlag.
- Savage, H. M., Ceianu, C., Nicolescu, G. & 7 other authors (1999).** 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* **61**, 600–611; erratum **62**, 162.
- Shah, K. V., Johnson, H. N., Rao, T. R., Rajagopalan, P. K. & Lamba, B. S. (1960).** Isolation of five strains of Sindbis virus in India. *Indian J Med Res* **48**, 300–308.
- Shirako, Y., Niklasson, B., Dalrymple, J. M., Strauss, E. G. & Strauss, J. H. (1991).** Structure of the Ockelbo virus genome and its relationship to other Sindbis viruses. *Virology* **182**, 753–764.
- Sixl, W., Stunzner, E. & Withalm, H. (1988).** Serological examination for antibodies against West Nile virus, Semliki virus and chikungunya virus in laboratory mice, parasitized by nidicolle fauna from swallow's nests. *Geogr Med Suppl* **1**, 51–55.
- Smithburn, K. C., Hughes, T. P., Burke, A. W. & Paul, J. H. (1940).** A neurotropic virus isolated from the blood of a native of Uganda. *Am J Trop Med Hyg* **20**, 471–492.
- Theiler, M. & Downs, W. G. (1973).** *The Arthropod-borne Viruses of Vertebrates: an Account of the Rockefeller Foundation Virus Program (1951–1970)*. London: Yale University Press.
- Weissenböck, H., Kolodziejek, J., Url, A., Lussy, H., Rebel-Bauder, B. & Nowotny, N. (2002).** Emergence of Usutu virus, an African mosquito-borne flavivirus of the Japanese encephalitis virus group, central Europe. *Emerg Infect Dis* **8**, 652–656.
- Woodhall, J. P. (1964).** The viruses isolated from arthropods at the East African Virus Research Institute in the 26 years ending December 1963. *Proc E African Acad* **2**, 141–146.

**Work, T. H., Hurlbut, H. S. & Taylor, R. M. (1955).** Indigenous wild birds of the Nile Delta as potential West Nile virus circulating reservoirs. *Am J Trop Med Hyg* **4**, 872–888.

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