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Prevalence of West Nile Virus Neutralizing Antibodies in Wild Birds from the Camargue Area, Southern France

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ABSTRACT: The Camargue area of southern France experienced the re-emergence of West Nile Virus (WNV) in the late summer of 2000 and 2004. Immediately preceding the 2004 outbreak, samples were collected from 432 birds of 32 different species captured in mist nets and from 201 Cattle Egret (*Bubulcus ibis*) nestlings sampled in their nests between 1 April and 12 June 2004. West Nile virus neutralizing titers of ≥40 were detected in 4.8% (95% confidence limit, 2.9–7.5%) of the adult birds and in 1.6% (0.3–4.6%) of the egret nestlings. Migratory passerines had a higher prevalence of WNV neutralizing antibodies (7.0%) than did resident and short-distance migratory passerines (0.8%), suggesting exposure to WNV or a related flavivirus during overwintering in Africa.

Key words: Antibodies, Camargue, serosurvey, West Nile virus, wild birds.

West Nile virus (WNV, *Flaviviridae*, *flavivirus*) is maintained and amplified by natural transmission between birds and ornithophilic mosquitoes, with occasional spillover infections that cause fever or neurologic symptoms in mammals, including horses and humans. West Nile virus only emerged recently in the New World, but has been present in the Old World since 1937 (Zeller and Schuffenecker, 2004). The first WNV outbreak in horses in France occurred during the summer of 1962 in the Camargue region, a wetland area characterized by very diverse bird communities (Jourdain et al., 2007a). A second epizootic in the Camargue occurred in 2000, with 76 equine cases (Murgue et al., 2001). Monitoring of sentinel ducks and chickens revealed minimal transmission of WNV during 2001 and 2002, and no indications of transmission were detected in 2003; however, in late July 2004, a sentinel chicken from Saintes-Marie-de-la-Mer seroconverted, indicating that local WNV transmission had occurred. The first equine case was reported in late August 2004, and 31 others were confirmed by mid-October (Zeller et al., 2004).

These equine cases and seroconversions in sentinel birds indicate an otherwise covert WNV cycle involving wild birds and mosquito vectors. Two previous studies have investigated WNV infections of wild avifauna in the Camargue. The first, conducted between 1978 and 1979, failed to detect any WNV hemagglutinating antibodies in 80 wild birds, mostly Yellow-legged Gulls (*Larus cachinnans*; Rollin et al., 1982). The second study, which was done shortly after the 2000 epizootic in horses, reported the presence of WNV neutralizing antibodies in Mallards (*Anas platyrhynchos*), Yellow-legged Gulls, and Common Magpies (*Pica pica*; Hars et al., 2004). We here report the results of a serologic survey of wild birds during the spring and early summer of 2004, shortly before WNV was detected in sentinel birds and in horses.

We concentrated on species likely to be involved in WNV introduction or amplification in the Camargue, as described previously (Jourdain et al., 2007c). These species included many small passerine birds, because species of the order Passer-

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iformes are believed to be among the most competent for WNV transmission (Work et al., 1955; Komar et al., 2003). We also sampled Cattle Egrets (*Bubulcus ibis*) because they are often in close contact with horses, breed in colonies where *Culex* mosquito vectors are abundant (Mouchet et al., 1970), and have been postulated as amplifying hosts for WNV (Rodrigues et al., 1981; Hubalek, 2004). A recent study reported that experimentally infected Cattle Egret chicks do not develop a high level of viremia (Reisen et al. 2005) but, in the Camargue wetlands, even a low viremia level in birds may contribute to virus amplification. In this area, the major WNV vector is the mosquito *Culex modestus*, which does not occur in the USA but is an extremely efficient laboratory vector (Balenghien et al., 2007; Balenghien et al., in press) that can probably become infected after feeding on a bird with a low level of viremia. West Nile virus neutralizing antibody was measured to determine seroprevalence in these two groups of birds (small passerines and Cattle Egrets). If WNV is introduced by migratory birds and then amplified in local bird populations, we hypothesized that long-distance migratory passerines should have higher antibody prevalence than other passerine species, and that antibodies should also be present in local Cattle Egrets.

Passerine birds were captured daily from 1 April to 12 May 2004, about 30 km southeast of Saintes-Maries-de-la-Mer (site one), using eight mist nets placed in bushes located a few hundred meters behind the beach in an attempt to preferentially capture migratory birds immediately after their crossing of the Mediterranean Sea (Fig. 1). The species, sex, and age were recorded. Cattle Egret nestlings (7–25 days old) were caught manually in a major colony located approximately 20 km northwest of Saintes-Maries-de-la-Mer (site two) between 23 June and 12 July 2004. Each bird was ringed and bled from the brachial vein into heparinized capillary tubes before being released. Blood samples were centrifuged and plasma kept frozen at −20 C prior to testing. Plasma samples were tested for neutralizing antibodies to WNV in a BLS3 laboratory using a microneutralization protocol, as described (Malkinson et al., 2002; Figuerola et al., 2007). Briefly, plasma samples were inactivated at 56 C for 30 min and serially diluted twofold from 1:20 to 1:640 in 96-well plates. Each sample was tested in duplicate, and cellular toxicity was evaluated on a well containing uninfected cells. All other wells were primed with 100 median tissue culture infective doses of the France 05.21/00 WNV equine strain (accession No. AY268132) in 50 µL of Eagle's medium supplemented with antibiotics and fetal calf serum. After incubation for 1 hr at 37 C in a 5% CO_2 atmosphere, 5×10^4 Vero E6 cells in 100 µL were added to each well. The final plasma dilutions ranged from 1:40 to 1:1280. Plates were incubated for 6 days at 37 C and then stained with crystal violet. The neutralizing titer was calculated as the highest dilution completely preventing cytopathic effect. Confidence intervals were calculated using the binomial law (Sokal and Rohlf, 1995).

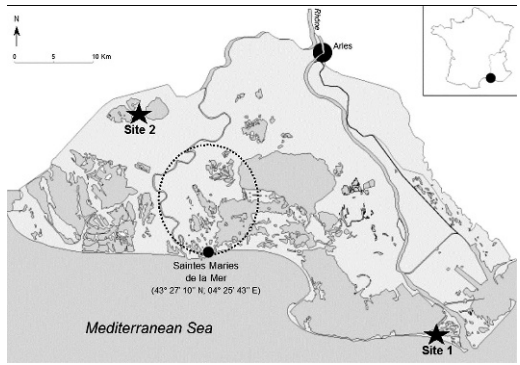


FIGURE 1. Map of the Camargue area showing the study sites. The epicenter of the 2004 West Nile fever outbreak in horses was located in the circled area.

Overall, 432 samples from 32 species (including three nonpasserine species) from site one were tested. Thirty-nine samples were toxic and were excluded from the study. The remaining 393 plasma

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TABLE 1. West Nile virus neutralizing antibody titers measured in plasma samples from birds captured in site 1 (upper section: passerine birds, lower section: non passerine birds) and site 2. Species for which only 1 or 2 individuals were available for testing are grouped as "other species".

Study site	Bird species	Long distance migrant	Number tested	Titer 40	Titer 80	Titer 160	Titer 320	Titer 640	Cell toxicity
Site 1	*Phylloscopus trochilus*	X	96	3	3				7
	Sylvia atricapilla		85	2		1			12
	Phoenicurus phoenicurus	X	50	1	1				5
	Erithacus rubecula		38						5
	Luscinia megarhynchos	X	27	2					1
	Ficedula hypoleuca	X	27		1				3
	S. borin	X	24						1
	S. communis	X	16		2			1	
	Muscicapa striata	X	11						
	Phylloscopus collybita		8						
	Lanius senator	X	7			1			1
	Phoenicurus ochruros		6						1
	S. cantillans	X	5						
	Hippolais polyglotta	X	4						1
	Passer domesticus		4						1
	Other species (n=14)	(X)	19						3
	Upupa epops	X	3		1				
	Other species (n=2)	(X)	2						
Site 2	*Bubulcus ibis* (nestlings)		201	1		1	1		14
Both sites	All species		633	9	8	3	1	1	53

samples included 19 (from eight species) showing titers ≥40 (Table 1), corresponding to an overall antibody prevalence of 4.8% (95% confidence limit, 2.9–7.5%). Interestingly, all positive samples, except three from Blackcaps (*Sylvia atricapilla*) which may winter in the Mediterranean basin, came from long-distance migrants that winter in Africa south of the Sahara. Within the passerines, the prevalence of antibody-positive birds was significantly higher for long-distance migrants (7.0%) than for residents and short-distance migrants (0.8%) (χ^2=7.36, P<0.01). These data support other studies in England (Buckley et al., 2003), Spain (Lopez et al., 2008), and other European countries that suggest migrants frequently have antibodies to flaviviruses. Conversely, only three of the 201 Cattle Egret nestlings sampled from site two had WNV neutralizing antibodies; titers ranged from 40 to 320 (Table 1). The corresponding antibody prevalence was 1.6% (95% confidence limit, 0.3–4.6%), which is much lower than that observed in previous studies from Russia, India, and Israel (Berezin, 1971; Rodrigues et al., 1981; Jamgaonkar et al., 2003; Mumcuoglu et al., 2005). Because all nestlings were less than 25 days old, antibodies were likely of maternal origin (Rodrigues et al., 1981; Reisen et al., 2005) and thus indicate exposure of adult females to flaviviruses either in the Camargue or elsewhere in the Mediterranean Basin. Because maternal antibody titers decline after hatching, it is likely that some samples with low neutralizing antibody titers (10 or 20) were missed.

The present study was conducted shortly before the emergence of WNV cases in horses in late August 2004 and the isolation of a WNV strain from two resident birds, a House Sparrow (*Passer domesticus*) and a Common Magpie in October 2004 (Jourdain et al., 2007b). Phylogenetic analysis of these two isolates showed that they were closely related to other strains previously isolated from horses in the western Mediterranean Basin (Jourdain et al., 2007b). However,

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we cannot preclude the possibility that WNV was re-introduced in the Camargue by migratory birds because only few strains from a limited geographic range were available for phylogenetic analyses. Results from our serologic survey, performed during the spring of 2004, indicated a higher prevalence of WNV antibodies in long-distance migratory passerines than in other passerines, which suggests that a large proportion of migrants are exposed to WNV or closely related flaviviruses on their wintering grounds in Africa. However, the long persistence of WNV antibodies in birds (McIntosh et al., 1969; Gibbs et al., 2005; Wilcox et al., 2007), and the fact that no parallel testing was done to rule out cross-reactive flaviviruses as the causative infection (blood sample volumes were kept to a minimum to avoid survival risks to the studied birds), both prevent a formal identification of the virus responsible for the detected antibodies. Other possible viruses include those from the Japanese encephalitis (e.g., Yaounde virus, Usutu virus) or the Yellow fever (e.g., Uganda S virus) serologic groups, viruses which have occasionally been isolated from birds in sub-Saharan Africa (Mackenzie et al., 2002; Simpson, 1993). Usutu virus is considered to be established in Central Europe (Chvala et al., 2007; Meister et al., 2008). Formal evidence for introduction of WNV into the Camargue from Africa, or elsewhere, will require a search for acute infection by WNV during migration. This has been shown in Israel for White Storks (*Ciconia ciconia*) migrating south (Malkinson et al., 2002) and in Cyprus for a Barred Warbler (*Sylvia nisoria*) migrating north (Watson et al., 1972), but requires considerable time and effort. The present serologic study should help to identify species that are frequently exposed to flaviviruses and that, therefore, might be considered as privileged target species for viral isolation studies.

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