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West Nile Virus Monitoring of Migratory and Resident Birds in Germany

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Abstract

West Nile virus (WNV) is a mosquito-borne flavivirus naturally circulating in wild bird populations. The virus is also capable to infect a broad range of vertebrate species. Humans and equines are highly susceptible and can develop mild flu-like illnesses as well as severe encephalitis leading to fatalities. Most recently, WNV was found to circulate in countries close to Germany, such as France, Czech Republic, Italy, Austria, and Hungary. Given this epidemiological situation its spread to Germany cannot be ruled out. As no data on the WNV situation were available for Germany for the most recent past, we have conducted a serological survey to reveal WNV antibodies in wild birds. More than 2700 blood samples from migratory and resident birds representing 72 species that were collected during 2005–2009 were tested using an immunofluorescence assay and partly by micro-virus neutralization test. By immunofluorescence assay WNV-reactive antibodies could be demonstrated in 11 wild bird species. Similarly, WNV-neutralizing antibodies were revealed in migratory birds belonging to 10 species, but not in resident birds. According to the absence of WNV-reactive antibodies in resident birds and the absence of WNV-specific RNA in all investigated bird samples, there is currently no evidence for a WNV circulation in Germany.

Key Words: Arbovirus(es)—Birds—Germany—Transmission—West Nile Virus.

Introduction

West Nile virus (WNV) is an arthropod-borne *Flavivirus* (family *Flaviviridae*) belonging to the Japanese encephalitis virus group (Lindenbach et al. 2007). From the two major genetic lineages described (Berthet et al. 1997, Lanciotti et al. 1999, 2002, Beasley et al. 2004), WNV lineage 1 strains are distributed worldwide. Virus strains of lineage 2 were found exclusively in sub-Saharan Africa and in Madagascar in the past, but recently infections were also found in Europe (Bakonyi et al. 2006, Erdélyi et al. 2007, Weissenböck et al. 2010). According to Bondre et al. (2007) WNV can be grouped into five distinct lineages. Recently, it was suggested that WNV can be classified into seven distinct lineages (Mackenzie and Williams 2009). WNV is transmitted by hematophagous arthropod vectors, predominantly mosquitoes of the *Culex* species (Hurlbut 1956, Hubálek 2008), to wild birds, which serve as natural reservoir hosts. Infection of most wild bird species is asymptomatic. However, some species, primarily corvids (e.g., crows and jays) and birds of prey, can succumb to deadly disease. WNV can also be transmitted to a broad range of other nonvertebrate (e.g., ticks) and vertebrate hosts [for review see van der Meulen et al. (2005) and Blitvich (2008)]. Humans and equines are highly susceptible and develop diseases spanning from moderate flu-like symptoms to fatal encephalitis (Castillo-Olivares and Wood 2004, Hayes et al. 2005b, Davis et al. 2006, Sejvar and Marfin 2006). Since its first isolation in 1937 (Smithburn et al. 1940), WNV circulates

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in many countries in sub-Saharan Africa (incl. Madagascar), Southern and Eastern Europe, Eurasia, Asia, and Australia (Hubálek and Halouzka 1999, Hall 2000, Petersen and Roehrig 2001, Zeller and Schuffenecker 2004, Hayes et al. 2005a, Kramer et al. 2007). When WNV was imported into New York City in 1999 (Jia et al. 1999, Lanciotti et al. 1999, Nash et al. 2001), this infection spread rapidly over the Americas causing morbidity and high mortality in the naive bird populations (LaDeau et al. 2007), in horses (Castillo-Olivares and Wood 2004, van der Meulen et al. 2005), and also in humans (Hayes and Gubler 2006, CDC 1999–2009). In Europe, indigenous human WNV cases have been reported in France, Romania, Hungary, Russia, Ukraine, and Italy (Hannoun et al. 1964, Panthier et al. 1968, Tsai et al. 1998, Hubálek and Halouzka 1999, Krisztalovics et al. 2008, Barzon et al. 2009). Important equine and/or avian outbreaks have been reported recently from France (Durand et al. 2002, Jourdain et al. 2007), Italy (Autorino et al. 2002, Monaco et al. 2009), and Hungary (Blitvich 2008). In Hungary WNV lineage 1 was found in birds in 2003 (Bakonyi et al. 2006) but disappeared thereafter. Instead, WNV lineage 2 cases were detected in 2004 and 2005 (Bakonyi et al. 2006, Erdélyi et al. 2007) and 2007. In summer 2008, a widespread outbreak of WNV lineages 2 infection was observed all over Hungary and in the eastern part of Austria affecting birds, equines, and human beings (Bakonyi and Nowotny, pers. comm.) (Kristalovics et al. 2008, ProMED-mail 2009). This indicates that there is an ongoing WNV epidemic that may also spread on to Germany. Rabensburg virus representing WNV lineage 3 (Bakonyi et al. 2005) has been isolated from Culex mosquitoes in the Czech Republic in 1997, 1999, and 2006 (Hubálek et al. 2010). However, there was no human or animal disease associated with this virus and only low virulence was observed in experimental transmission studies. Few data are available on the current WNV situation in Germany. In an earlier study, antibodies to WNV were found in several migratory and resident bird species (Linke et al. 2007), but WNV-specific RNA has so far never been detected in any of the previous studies (Schirrmeier et al. 2004, Hlinak et al. 2006, Linke et al. 2007, Ziegler et al. 2010). Therefore, there was no evidence in the past indicating the presence of indigenous WNV infections, even though potential vector mosquitoes are present in Germany (Mohrig 1969, Weitzel et al. 2006).

However, in the light of global trade and travel and also possible climate changes and because of the first appearance of WNV in Austria, Hungary, and Italy, we decided to carry out a large-scale study on the WNV antibody status of migratory and resident birds belonging to a broad variety of species in Germany. The demonstration of antibodies in resident birds would ultimately prove that WNV is already circulating locally.

Materials and Methods

Sera/plasma

Birds were bled by puncturing their wing veins, and after blood separation sera were stored at −20°C and cruors at −70°C. Reference sera from vaccinated and naturally WNV-infected chicken with a known titer (kindly provided by the U.S. Department of Agriculture) were used to calibrate the test system. Prevaccination and postvaccination (West Nile-Innovator®; Fort Dodge Animal Health) sera from chicken and ducks served as negative and positive controls.

Indirect immunofluorescence assay

A modified protocol of a commercial WNV immunofluorescence assay (IFA) slide test kit (Euroimmun) was used generally following the manufacturer's instructions. Bird sera were used at 1/30 dilutions and goat anti-bird fluoresceine-isothiocyanate-labeled antibodies (Bethyl Laboratories) diluted 1/100 for the detection of binding antibodies. As these labeled antibodies were only low or nonreactive to immunoglobulins (Ig) of some wild bird species as determined in a dot blot format (Table 1), IgY of these species was observed by using another conjugate, that is, rabbit anti-bird antibodies (ICL) diluted 1/200 followed by goat anti-rabbit fluoresceine-isothiocyanate-labeled antibodies (Invitrogen) at 1/200 dilution. Endpoint titrations were carried out for reactive and inconclusive samples.

Micro-virus neutralization test

Micro-virus neutralization tests (micro-VNTs) were carried out using a 96-well plate format using a published

Table 1. Detection of Avian Immunoglobulin Y by Commercial Anti-Bird Conjugates as Determined by Dot-Blot

Common name/species	*Goat α bird IgY (Bethyl)*	*Rabbit α bird IgY (ICL)*
Chicken/*Gallus gallus domesticus*	+++	++
Wood pigeon/*Columba palumbus*	+	+
Duck/*Anas platyrhynchos*	+++	++
Greylag goose/*Anser anser*	+	+
Mute swan/*Cygnus olor*	+++	++
Peregrine/*Falco peregrino*	+++	+++
Osprey/*Pandion haliaetus*	++	+++
Red kite/*Milvus milvus*	++	++
Black kite/*Milvus migrans*	+	++
Lesser spotted eagle/*Aquila pomarina*	+++	++
Goshawk/*Accipiter gentilis*	++	++
Honey buzzard/*Pernis apivorus*	−	+
White stork/*Ciconia ciconia*	+	++
Common tern/*Sterna hirundo*	++	+++
Willow warbler/*Phylloscopus trochilus*	+	+++
Pied flycatcher/*Ficedula hypoleuca*	+	+++
Garden warbler/*Sylvia borin*	+	+++
House martin/*Delichon urbicum*	+++	+++
Barn swallow/*Hirundo rustica*	++	+++
Rook/*Corvus frugilegus*	+	+++
Horse/*Equus ferus caballus*	−	−
Human	−	−

IgY, immunoglobulin Y.

method with minor modifications (Linke et al. 2007). Dilutions of heat-inactivated sera in the tissue culture medium were incubated in duplicate for 1 h at 37°C with equal volumes of 100 tissue culture infectious doses ($TCID_{50}$) of a WNV lineage 1 strain (NY99) and then added to 1-day-old Vero E6 cell monolayers. After 1 h incubation at 37°C, a fresh cell culture medium was added and cytopathic effects were recorded by crystal violet staining after 6–7 days. Titers were calculated following the Behrens–Kaerber method. Neutralization effects at 1/10 or higher dilutions were qualified positive. Positive samples were retested using dilutions ranging from 1/10 to 1/2560 to determine end point titers. Reference sera from vaccinated and naturally infected chicken were used for calibration.

One-step quantitative real-time–polymerase chain reaction assay

Viral RNA was extracted from whole blood or cruor by using the QIAmp Viral RNA kit or RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Viral RNA was detected by a one-step quantitative real-time–polymerase chain reaction (qRT-PCR) assay (Eiden et al. 2010). Primers and probe were targeted to the region coding for the nonstructural protein NS2A, which enables the detection of both WNV lineages 1 and 2. The qRT-PCR assay was performed employing the Mx3000P QPCR system (Agilent/Stratagene) using the Quantitect Probe RT-PCR Kit (Qiagen) in a total volume of 25 μL.

Results

In this study 2736 avian blood samples originating from 72 species of migratory and resident birds were tested for the presence of WNV antibodies (Table 2). Samples were collected in the period 2005–2009 at multiple rural and urban locations in several German states (Fig. 1). The birds were trapped with mist nets and funnel traps (small passerines) or cannon nets (greylag goose), individually marked with numbered metal or plastic rings, bled, and released. Small migratory passerine birds were captured and bled at the time of return from their wintering sites. Nestlings were ringed and bled at nest sites (white stork, osprey, and hooded crow). Several injured wild birds were bled in animal clinics during veterinary treatment.

Using an indirect IFA, 23 of the 2736 tested samples representing 11 wild bird species showed antibodies to WNV with titers ranging from 1/15 up to 1/1920 (Table 3). Five of these avian species were long-distance (trans-Saharan) migrants, four species were short-distance or partial migrants, and two species were residents. The majority of 463 tested white storks were nestlings, whereas 2 of the 3 IFA-reactive samples originated from adult birds. Another three samples of white stork nestlings remained inconclusive. All 22 ospreys tested by IFA—of which 2 were reactive—originated from nestlings or juveniles. Two samples of an adult barn swallow that was accidentally sampled twice in June and August 2008 were IFA reactive with a considerable drop in titer from 1/1920 down to 1/480 a few weeks later. Three IFA-reactive sera from resident birds originated from a juvenile common magpie (*Pica pica*) and two adult tawny owls (*Strix aluco*). Sera from two wood pigeons (*Columba palumbus*), one garden warbler (*Sylvia borin*), one tawny owl (*S. aluco*), one long-eared owl (*Asio otus*), and one lesser spotted eagle (*Aquila pomarina*) gave inconclusive IFA results.

Results for 1086 samples that were also analyzed by micro-VNT are shown in Table 4. Two of the 28 white stork sera that exhibited WNV-neutralizing antibodies originated from adult individuals, whereas all the others were taken from nestlings. Because of the very small sample volumes, it was not possible to investigate most of the Passeriformes by micro-VNT. Therefore, the findings in some IFA-reactive small Passerine plasmas (common redstart, blackcaps, etc.) could not be reconfirmed by micro-VNT. However, none of the resident bird species sera with IFA reactive (common magpie and two tawny owls) or inconclusive results contained WNV-neutralizing antibodies, whereas a few IFA negative sera showed virus-neutralizing activity (gray heron, red kite, herring gull, and Eurasian woodcock). In summary, of 23 samples found reactive by IFA, 6 samples could not be tested by micro-VNT because of insufficient sample volumes. From the 17 IFA-reactive samples that were also tested by micro-VNT, 9 showed also neutralizing antibodies, but 8 IFA-reactive samples could not be confirmed by micro-VNT. On the other hand, 30 IFA-negative samples from migratory birds showed WNV-neutralizing antibodies. Compared to the micro-VNT as gold standard, the IFA used here was found to be highly specific (99%), although not as sensitive (25%) due to the false-negative results.

Detection of viral RNA was also attempted by a one-step qRT-PCR on 237 blood samples (94 birds of prey, 69 Ciconiiformes [mainly white storks], 43 Anseriformes, 19 Columbiformes, 4 Gruiformes [Eurasian coots], 3 Charadriiformes [2 herring gulls and 1 black-headed gull], 3 Passeriformes [2 common redstarts and 1 rook], 1 great spotted woodpecker, and 1 little grebe). In none of these blood samples WNV-specific RNA was revealed.

Discussion

WNV outbreaks or virus activity have been observed in Central European countries most recently (Bakonyi et al. 2006, Figuerola et al. 2007, Höfle et al. 2008, Hubálek et al. 2008a, 2008b, Jiménez-Clavero et al. 2008, López et al. 2008, Weissenböck et al. 2010). Migratory birds can act as carriers for this zoonotic pathogen and carry it over long distances (Hubálek 2000, Malkinson et al. 2001, 2002, Malkinson and Banet 2002). The detection of WNV antibodies in resident bird populations is indicative for a local virus circulation. Germany is located not far away from the most recent outbreaks in Austria and Hungary; therefore, it is necessary to closely monitor the WNV status of birds as indicators for a potential virus introduction. Thus, we carried out a serological study on migratory and resident birds captured in Germany. In most cases, where only spurious amounts of sera were available, these were initially screened by indirect IFA. Reactive sera as well as sera from species from which larger amounts of blood samples were available were also analyzed by micro-VNT. Two different IFA conjugates were used that detect IgY of a broad variety of avian species. Most reactive samples with high antibody titers gave corresponding results in both methods, and 96% of the micro-VNT-positive sera were detected by IFA too. As the micro-VNT displayed a higher analytical sensitivity, IFA missed some low reactive samples. However, the small serum volumes, for

Table 2. Bird Species and Total Number (*n*) of Wild Bird Individuals Sampled in Germany During 2005–2009 That Were Analyzed by Immunofluorescence Assay and Micro-Virus Neutralization Test, Respectively, and Migration Status of Birds

Order (-formes)	*Common name/species*	*IFA,* n = *2736*	*micro-VNT,* n = *1086*	*Migratory status*
Ciconii-/	White stork/*Ciconia ciconia*	463	461	LD
Ardei-	Gray heron/*Ardea cinerea*	16	16	PM, LD, SD, R
	Bittern/*Botaurus stellaris*	1	1	PM, LD, SD, R
Accipitri-	Common buzzard/*Buteo buteo*	56	55	PM, SD
	Goshawk/*Accipiter gentilis*	44	44	R
	European sea eagle/*Haliaeetus albicilla*	43	42	R
	Osprey/*Pandion haliaetus*	22	22	LD
	Sparrow hawk/*Accipiter nisus*	21	21	PM, SD
	Red kite/*Milvus milvus*	18	18	SD
	Lesser spotted eagle/*Aquila pomarina*	17	17	LD
	Black kite/*Milvus migrans*	12	12	LD
	Honey buzzard/*Pernis apivorus*	3	3	LD
	Griffon vulture/*Gyps fulvus*	1	1	SD[a]
	Marsh harrier/*Circus aeruginosus*	1	1	LD
Falconi-	Kestrel/*Falco tinnunculus*	18	18	PM, SD
	Peregrine/*Falco peregrino*	6	4	R
	Hobby/*Falco subbuteo*	1	1	LD
Strigi-	Long-eared owl/*Asio otus*	8	8	PM, SD
	Tawny owl/*Strix aluco*	7	7	R
	Barn owl/*Tyto alba*	3	3	R
	Tengmalm's owl/*Aegolius funereus*	1	1	PM, SD
Anseri-	Mute swan/*Cygnus olor*	79	69	SD
	Greylag goose/*Anser anser*	43	42[b]	PM, SD
	Swan goose/*Anser cygnoides*	26	0	R[c]
	Mallard/*Anas platyrhynchos*	21	16	SD
	Common merganser/*Mergus merganser*	3	0	SD
	Canada goose/*Branta canadensis*	4	4	PM, SD[c]
	Gadwall/*Anas strepera*	4	0	LD
	Whooper swan/*Cygnus cygnus*	2	1	LD, SD
	Red-breasted merganser/*Mergus serrator*	2	0	SD
	Egyptian goose/*Alopochen aegyptiacus*	1	0	PM, SD[c]
	Lesser white-fronted goose/*Anser erythropus*	1	1	SD
	Greater scaup/*Aythya marila*	1	1	SD, LD[a]
	Greater white-fronted goose/*Anser albifrons*	1	1	SD
	Mandarin duck/*Aix galericulata*	1	1	R[c]
Charadrii-	Herring gull/*Larus argentatus*	207	3	SD
	Common tern/*Sterna hirundo*	25	2	LD
	Eurasian woodcock/*Scolopax rusticola*	8	8	SD
	Black-headed gull/*Larus ridibundus*	4	1	SD
	Great black-backed gull/*Larus marinus*	3	0	SD
Passeri-	Barn swallow/*Hirundo rustica*	469	3	LD
	Pied flycatcher/*Ficedula hypoleuca*	367	0	LD
	Garden warbler/*Sylvia borin*	134	1	LD
	Willow warbler/*Phylloscopus trochilus*	97	0	LD
	Hooded crow/*Corvus cornix*	94	88	R
	Blackcap/*Sylvia atricapilla*	75	0	SD, LD
	Common redstart/*Phoenicurus phoenicurus*	75	0	LD
	House martin/*Delichon urbicum*	47	0	LD
	Spotted flycatcher/*Muscicapa striata*	45	0	LD
	Reed warbler/*Acrocephalus scirpaceus*	9	0	LD
	Common magpie/*Pica pica*	9	9	R
	Marsh warbler/*Acrocephalus palustris*	8	0	LD
	Common blackbird/*Turdus merula*	7	6	PM, SD, R
	Eurasian jay/*Garrulus glandarius*	7	7	R
	Lesser whitethroat/*Sylvia curruca*	5	0	LD
	Rook/*Corvus frugilegus*	2	1	SD
	Greenfinch/*Carduelis chloris*	2	1	SD
	Common raven/*Corvus corax*	2	2	R
	Starling/*Sturnus vulgaris*	2	2	SD
	Hawfinch/*Coccothraustes coccothraustes*	1	1	SD
	Chaffinch/*Fringilla coelebs*	1	1	SD

(*continued*)

TABLE 2. (CONTINUED)

Order (-formes)	*Common name/species*	*IFA, n = 2736*	*micro-VNT, n = 1086*	*Migratory status*
Columbi-	Wood pigeon/*Columba palumbus*	32	31	SD
	Domestic pigeon/*Columba livia f. dom.*	4	4	R
Phalacro-coraci-	Great cormorant/*Phalacrocorax carbo*	8	1	PM, SD
Pici-	Great spotted woodpecker/*Dendrocopos major*	9	8	R
	Green woodpecker/*Picus viridis*	1	1	R
Podicipedi-	Little grebe/*Tachybaptus ruficollis*	1	1	PM, SD
Grui-	Eurasian coot/*Fulica atra*	6	6	PM, SD
	Great bustard/*Otis tarda*	3	2	R
	Common crane/*Grus grus*	1	1	SD
	Common moorhen/*Gallinula chloropus*	1	1	PM, SD
Apodi-	Common swift/*Apus apus*	1	1	LD
Other	Other	13	0	

[a]Zoo/park bird.
[b]Including 15 cytotoxic samples not analyzable.
[c]Neozoon bird species.
IFA, immunofluorescence assay; VNT, virus neutralization test; R, resident species; PM, partial migrant; SD, short-distance migrant; LD, long-distance migrant.

example, from passerines, make the IFA indispensable and the method of choice. No indirect WNV antibody enzyme-linked immunosorbent assay for detection of avian IgY antibodies from a wider range of species is available to date. A commercially available competitive enzyme-linked immunosorbent assay (ID Screen®; West Nile Competition, ID Vet) is suitable for the multi-species detection of antibodies, but it requires sample volumes that are larger than those that can be obtained from small birds (e.g., passerines).

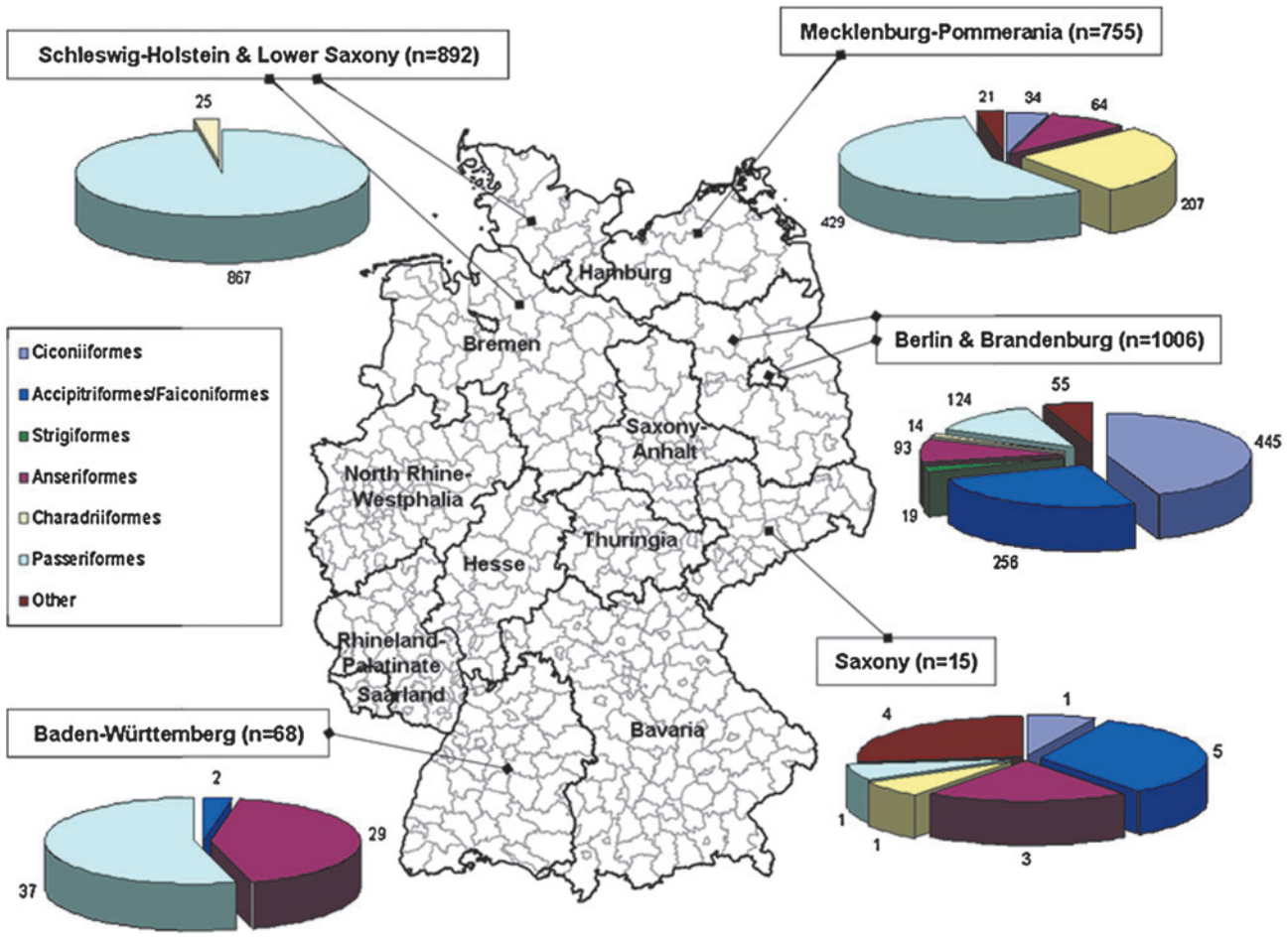

FIG. 1. Location of sampling sites of wild bird species from Germany.

Table 3. Serum Titers of Immunofluorescence-Assay-Reactive Samples

Common name/species	n	Titer of IFA-reactive samples							
		15	30	60	120	240	480	960	1920
White stork/*Ciconia ciconia*	463		1	2					
Osprey/*Pandion haliaetus*	22		1			1			
Tawny owl/*Strix aluco*	7			2					
Barn swallow/*Hirundo rustica*	469					1	2[a]		1[a]
Willow warbler/*Phylloscopus trochilus*	97	1							
Common redstart/*Phoenicurus phoenicurus*	75						1		
Blackcap/*Sylvia atricapilla*	75	4							
Common magpie/*Pica pica*	9				1				
Wood pigeon/*Columba palumbus*	32			2					
Great cormorant/*Phalacrocorax carbo*	8				1				
Eurasian coot/*Fulica atra*	6	1	1						

n = tested individuals of species.
[a]Including two samples of one barn swallow accidentally captured and bled twice in summer 2008.

Antibodies to WNV were found in 23 of 2736 (0.8%) tested samples by IFA and in 41 of 1086 (3.7%) sera by micro-VNT. These results compared generally to those reported from earlier studies (Linke et al. 2007). IFA reactions can be caused by cross-reacting antibodies due to closely related Flaviviruses. Cross-reactions with closely related viruses from the Japanese encephalitis virus group can also occur in the highly specific virus-neutralization test. Usutu virus (USUV) is such a close relative of WNV that is also circulating in Central Europe; it emerged in Austria in 2001 (Weissenböck et al. 2002, Bakonyi et al. 2004), and spread recently to Hungary (Bakonyi et al. 2007), Switzerland, and Italy (Manarolla et al. 2010). The endemic in Austria has been well characterized. Initially, USUV caused major fatalities primarily among resident birds like blackbirds, owls, and birds of prey (Weissenböck et al. 2003, Chvala et al. 2007), peaking in 2003, whereas from 2004 onward, USUV-associated bird mortality decreased in Austria significantly. Serological studies indicated in 2003 and 2004 a rather small proportion of seropositive wild birds (<10%), whereas the percentage of seroreactors raised to >50% in 2005 and 2006, suggesting the establishment of herd immunity (Meister et al. 2008). For Germany, only few data on the USUV situation are available. In samples collected in the period 2002–2005, Linke et al. (2007) found that 3 out of 25 WNV-IFA-reactive samples (originating from migratory birds, i.e., white stork, common redstart, and osprey) contained USUV-neutralizing but not WNV-neutralizing antibodies. Hence, although unlikely, IFA-WNV-reactive samples in the present study could also indicate USUV infections in these birds. Forty-five sera that were reactive in the WNV IFA or micro-VNT were therefore also assayed in a USUV-micro-NT (using USUV strain Vienna_2001) with negative results (titers <1:10) in all cases (data not shown).

Further, there is no evidence for wild bird infections with tick-borne encephalitis virus, another closely related flavivirus that is endemic in some areas of Germany, although birds can be infested by tick-borne encephalitis virus–infected ticks (Ernek et al. 1968, Waldenström et al. 2007).

In the study presented here three sera from resident birds (one common magpie and two tawny owls) were reactive in the IFA screen. These IFA reactions were not confirmed by micro-VNT, which indicates nonspecific or cross-reactive antibodies. Vice versa, WNV-neutralizing antibodies were found exclusively in migratory birds (i.e., long-distance, short-distance, and intermediate migrating birds) and were most likely the consequence of WNV infections at the resting sites in Africa or Southern Europe or during their journey to

Table 4. Neutralizing Serum Titers by Micro-Virus Neutralization Test

Common name/species	n	Neutralizing serum titers by micro-VNT									
		10	15	20	30	60	80	120	160	240	640
White stork/*Ciconia ciconia*	461	14	10	3		1					
Grey heron/*Ardea cinerea*	16				1						
Osprey/*Pandion haliaetus*	22						2			1	
Red kite/*Milvus milvus*	18				1						
Eurasian woodcock/*Scolopax rusticola*	8			1							
Black-headed gull/*Larus ridibundus*	1	1									
Barn swallow/*Hirundo rustica*	3								1[a]		1[a]
Wood pigeon/*Columba palumbus*	32					1					
Great cormorant/*Phalacrocorax carbo*	1							1			
Eurasian coot/*Fulica atra*	6	1			1						

Titers >1:10 are considered to be positive; n = tested individuals of species.
[a]Including two samples of one Barn Swallow accidentally captured and bled twice in summer 2008.

Central Europe. Low or borderline micro-VNT titers were also found in nestlings or juveniles of such migratory birds (e.g., white storks). These antibody levels are much lower than the titers that are found in experimentally (Komar et al. 2003) or naturally WNV-infected birds (Figuerola et al. 2007). We therefore assume that these antibodies represent maternal antibodies that have been passed on via egg yolk from WNV-competent adults. This assumption is also supported by the absence of WNV-neutralizing antibodies in all resident bird species investigated. Assuming cross-reacting antibodies to other non-WNV Flaviviruses would be another, although less likely, explanation.

For a successful long-distance spread and the establishment of a self-sustained transmission cycle in a new environment, several factors are critical: suitable population densities of relevant host and vector species, congruent population ecologies, and also environmental and climatic conditions that allow the productive amplification in these hosts. Essentially, infected birds from WNV enzootic areas must endure the long and exhaustive flight to Central Europe and then be blood-sucked by susceptible mosquitoes. Alternatively, a sustained infection cycle within groups of migratory birds is also conceivable, but less likely. WNV infections are characterized by only a short viraemia in most, if not all, affected birds (Komar et al. 2003) and a minimum blood virus titer is required for a successful vector infection (Goddard et al. 2002, Turell et al. 2002). The WNV amplification in mosquitoes strongly depends on ambient temperatures and in new vertebrate species on their innate and adaptive immune responses (Reisen and Brault 2007).

On the other hand, the multiple WNV cases in the United States and Canada as well as the emergence and recent spread of a lineage 2 WNV in Hungary and Austria (Bakonyi et al. 2006, ProMED-mail 2009, Weissenböck et al. 2010) strongly emphasize that this virus–bird–mosquito infection cycle can also operate in more temperate regions. Therefore, a WNV introduction into Germany by migratory birds or other routes followed by a self-sustained perpetuation is also conceivable. Resting sites for wild birds where a high population density exists and where susceptible vectors live are high-risk areas for such an introduction. The use of sentinel birds that are detained at such sites could be a suitable WNV-monitoring approach (Buckley et al. 2006, Chevalier et al. 2008, Globig et al. 2009, Ziegler et al. 2010). Likewise, monitoring programmes for horses could be useful for predicting the risk of human WNV infections (Ward and Scheurmann 2008).

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