

Wild Birds in Romania Are More Exposed to West Nile Virus Than to Newcastle Disease Virus

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Abstract

The aim of this study was to evaluate the seroprevalence of West Nile virus (WNV) and Newcastle disease virus (NDV) in wild and domestic birds from Romania. During 2011–2014, 159 plasma samples from wild birds assigned to 11 orders, 27 families, and 61 species and from 21 domestic birds (*Gallus gallus domesticus*, *Anas platyrhynchos domesticus*) were collected. The sera were assayed by two commercial competitive enzyme-linked immunosorbent assay (cELISA) kits for antibodies against WNV and NDV. We found a high prevalence of WNV antibodies in both domestic (19.1%) and wild (32.1%) birds captured after the human epidemic in 2010. Moreover, the presence of anti-NDV antibodies among wild birds from Romania (5.4%) was confirmed serologically for the first time, as far as we are aware. Our findings provide evidence that wild birds, especially resident ones are involved in local West Nile and Newcastle disease enzootic and epizootic cycles. These may allow virus maintenance and spread and also enhance the chance of new outbreaks.

Key Words: West Nile virus—Newcastle disease virus—Wild birds—Romania.

Introduction

WEST NILE VIRUS (WNV) is recognized as an emerging and re-emerging pathogen, which is classified within the genus *Flavivirus*, family *Flaviviridae*, and is phylogenetically and antigenically related to other encephalitis viruses. WNV is transmitted by mosquitoes and causes fever and encephalitis in humans, equines, and occasionally wild birds (Hernández-Triana et al. 2014). Two lineages of WNV have been described, lineage 1 being responsible for repeated disease outbreaks in countries of the Mediterranean basin, whereas lineage 2 was thought to be restricted to sub-Saharan Africa, but it is currently emerging in Europe (Hernández-Triana et al. 2014).

Two important West Nile epidemic episodes were reported in Romania, the first in 1996 by Campbell et al. (2001) and a second in 2010 by Neghină (2011), but there are other studies that have confirmed the presence of WNV in Romania (Ceianu et al. 2001). In addition, recent serological surveys carried out on susceptible species, known as sentinels or reservoir hosts (*e.g.*, horses, wild and domestic

birds) reported that WNV, known to be endemic in the Danube Delta, has emerged in the eastern part of Romania as well (Ludu Oslobanu et al. 2014).

All strains of the type 1 avian paramyxovirus (APMV-1) belong to the order *Mononegavirales*, family *Paramyxoviridae*, genus *Avulavirus* (Mayo 2002), and are commonly referred to as Newcastle Disease virus (NDV). NDV is cosmopolitan and is responsible for one of the most ubiquitous viral diseases of birds (Alexander and Senne 2008). NDV is the causative agent of Newcastle disease, a severe disease in birds associated with substantial economic losses to the poultry industry worldwide. Genetic analysis identified two major NDV classes, I and II (Czeglédi et al. 2006). Class I isolates are generally of low virulence and have been recovered primarily from waterfowl of the family *Anatidae*. Class II viruses comprise the vast majority of isolates of diverse virulence, infesting poultry (gallinaceous birds) and pet and wild birds alike. Both classes have nine genotypes, identified as 1–9 in class I and I–IX in class II (Kim et al. 2007).

On the basis of serological and molecular studies, Newcastle disease has been confirmed in wild birds in many

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countries, e.g., France, Germany, and Serbia (Artois et al. 2002, Schettler et al. 2003, Vidanović et al. 2011). In Romania, the information on the presence of NDV in wild birds is scarce (Spînu et al. 2009). The last outbreaks of Newcastle disease in Romania were mentioned in 4-week-old broilers at a farm in Ialomița County, and, most recently, a new outbreak was reported in backyard domestic birds in Constanța County, both areas being located in southern Romania (World Organisation for Animal Health [OIE] 2014).

Materials and Methods

Bird capture and sampling

Between April 1 and July 15, in both 2011 and 2012, adult wild birds ($n=159$ individuals, 61 species) were captured across Romania by use of mist nets and various traps recommended for birds of different sizes (Fig. 1). The captured species consisted of temperate European birds (Table S1; Supplementary Data are available at www.liebertpub.com/vbz) that vary greatly in body size and cover a large taxonomic range. All birds were captured during their breeding season. Depending on body size, 50–250 μL of blood was collected in heparinized capillary tubes by venipuncture of the brachial vein. Blood samples were kept on ice in a cooling box for up to 10 h and subsequently centrifuged at $\sim 6200 \times g$ for 5 min. The plasma was separated from red blood cells and was stored at -20°C until screening for WNV and NDV. After bleeding, birds were marked individually with a numbered aluminum ring, and standard biometry measures were taken. All birds were released in good condition after sampling and measurements.

In 2014, backyard domestic birds (*Gallus gallus domesticus*, $n=16$, and *Anas platyrhynchos domesticus*, $n=5$) originating from private households in the Sfântu Gheorghe and Pădurea Letea, Danube Delta were also sampled. Approximately 500 μL of blood was collected from each bird, and the sera were removed, transferred to Eppendorf tubes after clotting, and stored at -20°C until laboratory testing.

Enzyme-linked immunosorbent assay

A competitive enzyme-linked immunosorbent assay (cELISA) for detection of immunoglobulin G (IgG)-type anti-WNV antibodies was used following the instructions of the manufacturers (commercial kit, ID Screen West Nile Competition Multi-species, ID.vet; Innovative Diagnostics, Grabels, France). The positive cutoff value was assigned using a positive/negative (P/N) ratio of <0.3 . The results were expressed as sample/negative control (S/N) percentages according to the formula following the manufacturers' indications: $S/N\% = \text{optical density (OD) sample} / (\text{OD negative control} \times 100)$. Samples were deemed as positive with $S/N \leq 40\%$, doubtful between 40% and 50%, and negative when $\geq 50\%$.

Anti-NDV IgG-type antibodies were detected by a cELISA with sera diluted to 1:25 following the manufacturers' instructions (commercial kit, ID Screen Newcastle Diseases Competition, ID.vet; Innovative Diagnostics, Grabels, France). The positive cutoff value was assigned using a P/N ratio of <0.6 . The results were expressed as percent inhibition (PI) percentages according to the formula: $PI\% = (\text{OD negative control} - \text{OD sample}) / (\text{OD negative control} \times 100)$. Sera with a PI $>40\%$ were deemed as positive, between 30% and 40% doubtful, and $<30\%$ as negative.

Statistical analyses

Point estimates and 95% confidence intervals (CI) for the prevalence of anti-WNV and anti-NDV antibodies were established. These parameters were calculated overall for temporal variation (year of sampling) and also for host-dependent factors (taxonomic order, migratory behavior). The differences in prevalence among avian orders and sampling years, as well as between migratory behavior types (resident vs. migratory) were statistically analyzed by a chi-squared independence test. These grouping factors were tested sequentially. A p value of ≤ 0.05 was considered to be statistically significant. The statistical analysis was performed using the EpiInfo 2000

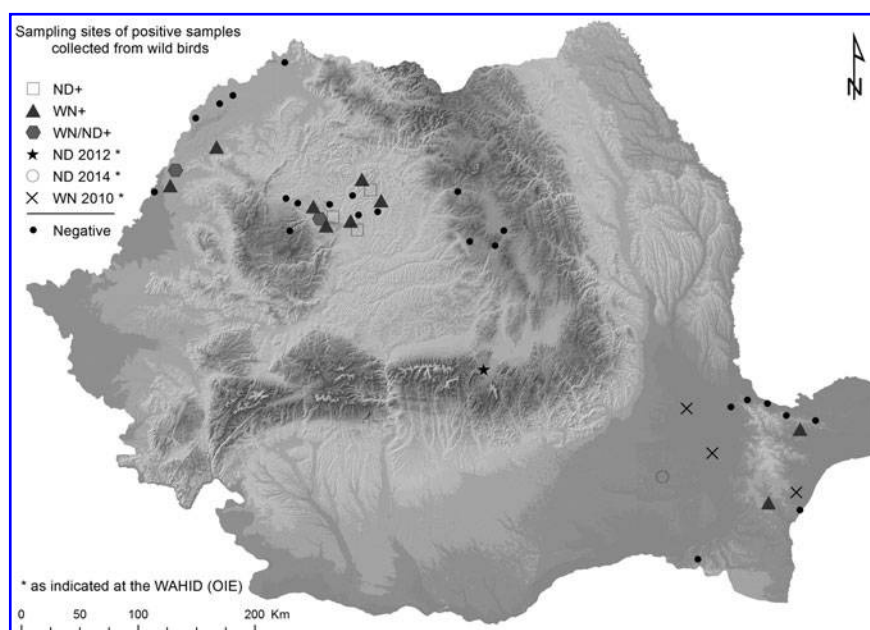


FIG. 1. Sampling sites of positive samples collected from wild birds.

software version 3.5.2 (Centers for Disease Control and Prevention in Atlanta, GA)

Results

The overall seroprevalence of WNV infection in wild birds captured in Romania was 32.14% (27/84; 95% CI 22.36–43.22), whereas that of NDV infection was 5.41% (8/148; 95% CI 2.36–10.37; Table 1). See Figure 1 for the sampling sites of positive samples.

There was no significant difference in the rate of seroprevalence when comparing WNV and NDV ($p=0.21$) or sampling years 2011 and 2012 ($p=0.15$), respectively. The simultaneous presence of anti-WNV and anti-NDV antibodies was found in three wild birds (3/159; 1.89%), two *Falco tinnunculus* and one *Columba livia*. Antibodies against one of the two viruses, either WNV or NDV, were found in 29 wild birds (18.24%; 29/159) belonging to 16 species. There were 14 wild bird species (eight orders) and four species (three orders), with at least one individual being seropositive for WNV or NDV, respectively. The prevalence of anti-NDV IgG antibodies was significantly higher in resident birds than in migratory ones ($p=0.005$). The overall seroprevalence of WNV infection in domestic birds was 19.05% (4/21; 95% CI 5.45–41.91).

Seroconversion induced by WNV was observed in both *G. gallus domesticus* and *A. platyrhynchos domesticus*. No anti-Newcastle disease antibodies were obtained in domestic birds.

Discussion

The presence of WNV and NDV among wild birds in Romania was serologically confirmed by cELISA both from birds sampled in the Danube Delta and outside this area. Bird migration has been considered one of the major drivers for translocation of WNV or NDV (Rappole et al. 2000, 2003, Brault 2009, Klaassen et al. 2012, Gale et al. 2013). The Danube Delta is a remarkable wetland area, designated as a

Ramsar Wetlands of International Importance and a World Heritage Site, where numerous migratory bird species breed and/or stop over during their migratory journey.

Several surveys conducted in Romania showed the presence of anti-WNV antibodies in wild and domestic birds using plaque reduction neutralization tests or a virus neutralization assay (Savage et al. 1999, Ludu Oslobanu et al. 2014). Although the virus neutralization test is regarded as the standard in flavivirus serology and is generally more specific than other serological techniques (Weingartl et al. 2003), the cELISA has been shown to be appropriate for WNV serological surveys (Ebel et al. 2002, Blitvich et al. 2003). The prevalence of anti-WNV antibodies in wild birds found in the present survey is higher compared with that obtained in eastern and southeastern Romania (Ludu Oslobanu et al. 2014). It should be considered that the samples from the wild birds were collected 1–3 years after the 2010 Romanian WNV outbreak. Variations in the species analyzed, serological methods, migratory behavior, climate conditions, epidemic time, sample size, or origin of the samples may also explain the differences encountered (García Bocanegra et al. 2011).

WNV lineage 1 and WNV lineage 2 were responsible for the outbreaks of 1996 and 2010 in Romania, respectively (Savage et al. 1999, Sîrbu et al. 2011). However, WNV lineage 2 was detected in a *Hyalomma marginatum marginatum* tick collected from a juvenile song thrush (*Turdus philomelos*) in the Romanian Danube Delta (Kolodziejek et al. 2014). Further molecular studies are timely for identifying to which lineage the strains currently circulating in wild birds from Romania belong.

WNV is a zoonotic pathogen, which is transmitted in natural cycles between mosquitoes, particularly *Culex* spp., and birds (Savage et al. 1999). It was shown that mosquito species are capable of transmitting WNV to humans and other mammals (Reiter et al. 2010). More than 19% of the examined domestic birds (*G. gallus domesticus*, *A. platyrhynchos*) raised in backyards in the Danube Delta area

TABLE 1. SEROCONVERSION INDUCED BY WEST NILE VIRUS AND NEWCASTLE DISEASE VIRUS IN WILD BIRDS

Category	West Nile virus		Newcastle Disease virus	
	No. of examined	No. of positive (%)	No. of examined	No. of positive (%)
Order				
Charadriiformes	3	0 (0)	4	0 (0)
Coraciiformes	6	1 (16.67)	9	0 (0)
Columbiformes	4	3 (75)	7	5 (71.43)
Cuculiformes	2	0 (0)	2	0 (0)
Falconiformes	8	8 (100)	9	2 (22.22)
Galliformes	4	2 (50)	4	0 (0)
Gruiformes	1	1 (100)	1	0 (0)
Passeriformes	46	10 (21.74)	97	1 (1.03)
Pelecaniformes	1	1 (100)	2	0 (0)
Piciformes	5	0 (0)	9	0 (0)
Strigiformes	4	1 (25)	4	0 (0)
Year				
2011	53	20 (37.74)	91	4 (4.40)
2012	31	7 (22.58)	57	4 (7.02)
Migratory behavior				
Resident	53	18 (33.96)	77	8 (10.39)
Migratory	31	9 (29.03)	71	0 (0)

were WNV seropositive. The presence of *Culex* mosquitoes in Romania and especially in the Danube Delta (Savage et al. 1999) and the presence of seropositive wild and domestic birds represent risk factors for WNV transmission to humans.

Although its zoonotic importance is limited, NDV remains a constant threat to the poultry industry (Cattoli et al. 2011). NDV is frequently isolated from wild birds in Europe (Artois et al. 2002, Vidanović et al. 2011). In a previous study, Spînu et al. (2009) reported the presence of anti-NDV antibodies in wild birds captured in Romania—Strigiformes: Eurasian eagle-owl (*Bubo bubo*), tawny owl (*Strix aluco*), scops owl (*Otus scops*); Falconiformes: common kestrel (*Falco tinnunculus*); and Ciconiiformes: white stork (*Ciconia ciconia*). In the present survey, seroconversion induced by NDV was observed in: Columbiformes: rock dove (*C. livia*), collared dove (*Streptopelia decaocto*); Falconiformes: common kestrel (*F. tinnunculus*) and Passeriformes: Eurasian magpie (*Pica pica*). Opposite to our expectations, NDV seroprevalence was significantly higher in resident birds than in migratory birds. Although migratory wild birds are involved in NDV distribution (Alexander et al. 2011), resident wild birds are apparently responsible for perpetuating NDV and promoting local outbreaks of the disease.

Potential future outbreaks caused by WNV and NDV can directly affect the health of birds and humans and may impact on biodiversity as well. Moreover, the impact of NDV on intensively raised birds could strongly influence the economic and social future of those involved in poultry farming.

Conclusions

Involvement of wild birds in West Nile disease and Newcastle disease outbreaks from Europe was previously demonstrated. Considering the present serological surveys conducted on wild and domestic birds from Romania, the possibility of new outbreaks cannot be excluded.

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Author Disclosure Statement

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