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Short Communication

West Nile virus serosurveillance in pigs, wild boars, and roe deer in Serbia



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ABSTRACT

West Nile virus (WNV) is maintained in nature in an enzootic transmission cycle between birds and mosquitoes, but it also infects many other vertebrates, including humans and horses, in which it can induce severe neurological diseases; however, data about virus circulation in other mammals is scarce. WNV has a history of recent outbreaks in Europe, including Serbia, where it was identified for the first time in 2010 in mosquitoes and in 2012 in birds and humans, being responsible for over 300 confirmed human cases and 35 deaths there along 2013. To assess WNV circulation among mammals in the country, 688 samples obtained from 279 farm pigs, 318 wild boars, and 91 roe deer were investigated for the presence of antibodies to WNV by enzyme-linked immunosorbent assay (ELISA) and viral neutralization test (VNT), and the specificity of their reactivity was assayed against Usutu virus (USUV). ELISA-reactive sera were identified in 43 (15.4%) pigs, 56 (17.6%) wild boars, and 17 (18.7%) roe deer. Of these, 6 (14%), 33 (59%), and 4 (23.5%) respectively, neutralized WNV. One out of the 45 ELISA negative sera tested, from a roe deer, neutralized WNV. Cross-reactivity neutralization test indicated that all deer and pigs neutralizing sera were WNV specific, while in 5 (15.2%) of the wild boar samples the specificity could not be established. Four wild boar sera showed USUV specificity. All these data confirm the circulation of both flaviviruses in Serbia, and highlight the need for the implementation of global coordinated surveillance programs in the region.

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1. Introduction

West Nile virus (WNV) is the most worldwide distributed flavivirus. Its transmission cycle involves ornithophilic

mosquito-vectors and birds, although the virus occasionally infects other vertebrates, including humans and horses, in which it may cause sporadic disease outbreaks that may result in fatalities (Jeffrey Root, 2013; Martin-Acebes and Saiz, 2012).

Based on phylogenetic analyses up to nine WNV lineages have been proposed (Pachler et al., 2014), although lineages 1 and 2 are by far the most worldwide widespread (Martin-Acebes and Saiz, 2012). In Europe, until 2004, when a lineage 2 strain was firstly isolated in Hungary, only lineage 1 strains were circulating but, since

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then lineage 2 strains have been isolated in humans and animals in several countries (Bagnarelli et al., 2011; Bakonyi et al., 2006; Erdelyi et al., 2007; Hernandez-Triana et al., 2014; Kurolt et al., 2014; Papa, 2012; Petrović et al., 2013; Savini et al., 2012, 2013; Wodak et al., 2011), being already responsible for dozens of human deaths.

In Europe, the presence of anti-WNV antibodies has been demonstrated in several bird species, but few data are available from mammals other than human or horses, as serological evidence of WNV infection has been reported only in a few domestic and wild mammal species (Jeffrey Root, 2013). Free-living mammals are naturally exposed to flaviviral infections and, thus, they may be involved in the virus transmission cycle.

In 2012, the first outbreak of WNV clinical infection in humans was reported in Serbia with a total of nine fatalities (Popovic et al., 2013), and 1 year later the largest human WNV outbreak in Europe was described there (<http://ecdc.europa.eu/>). Before that anti-WNV IgG had been detected in humans (Petrić et al., 2012), birds (Petrović et al., 2013), and horses (Lupulovic et al., 2011), and WNV-RNA had been amplified from mosquito (Petrić et al., 2012) and bird tissue (Petrović et al., 2013) pools. In the light of all these data, the aim of the present study was the serological surveillance of WNV activity in farm pigs, and, as free ranging wild mammals, in wild boar and roe deer, to have a better knowledge of WNV activity in the country that would help to implement state-of-the art surveillance systems.

2. Methods

2.1. Samples

Blood samples from swine (*Sus scrofa domestica*), wild boars (*Sus scrofa*), and roe deer (*Capreolus capreolus*) were collected at different locations around the country (Fig. 1). Swine samples ($n = 279$) from growing (2- to 3-month-old, $n = 60$), pre-fattening (4-month-old, $n = 39$), fatteners (8-month-old, $n = 60$), gilts (8- to 10-month-old, $n = 60$), and sows (1–4 years, $n = 60$) were collected in three big industrial farms with closed and isolated buildings and artificial ventilation (1000–3000 sows/farm) in three municipalities from the northern part of Serbia during

the end of 2010 and the beginning of 2011. Wild boar samples ($n = 318$) were from animals of age less than 6 months ($n = 70$), 6–18 months ($n = 90$), 1.5–2.5 years ($n = 72$) and more than 2.5 years of age ($n = 86$) that were hunted from October 2011 to April 2012 on the territory of 37 municipalities, of which 20, 7 and 10 were at northern, north-western and south-eastern part of Serbia, respectively. Three to seven years-old roe deer ($n = 91$) were hunted from April to August 2011 and during spring 2012 on the territory of 10 municipalities from northern part of Serbia. All samples were kept at -20°C until use.

2.2. Enzyme-linked immunosorbent assay (ELISA)

Anti-WNV IgG was detected using an adapted inactivated WNV-based enzyme linked immune-sorbent assay, ELISA, as described (Cordoba et al., 2007; Escribano-Romero et al., 2013). The positive cut-off value was assigned using a positive/negative (P/N) ratio of ≥ 2 , calculated by dividing the mean absorbance of the test serum reacted on viral antigen by the absorbance of the negative control serum on viral antigen.

2.3. Virus neutralization test (VNT)

Presence of anti-WNV specific neutralizing antibodies was assayed in all ELISA positive sera, as well as in 45 randomly selected ELISA negative samples. VNT was performed as described (Alonso-Padilla et al., 2009) in susceptible Vero cells using two-fold serial dilutions of serum (starting from 1:20) and a fixed amount (100 PFU) of a WNV-NY99 strain (Gen Bank acc. no. KC407666) (Cordoba et al., 2007; Martin-Acebes and Saiz, 2011). Titers were calculated as the reciprocal of the serum dilution that completely inhibited cytopathic effect. In all 44 WNV-VNT positive samples, and in 107 randomly selected negative ones, as control of flaviviral specific reactivity, VNT was similarly performed with Usutu virus (USUV), the only other flavivirus of the Japanese encephalitis serocomplex described so far to be circulating in Europe (Vazquez et al., 2011). As commonly accepted, VNT titers 4-fold higher to either virus were taken as a proof of the specificity of the infection. Finally, VNT was also performed with lineage 2 WNV (SRB-Novi Sad/12), the

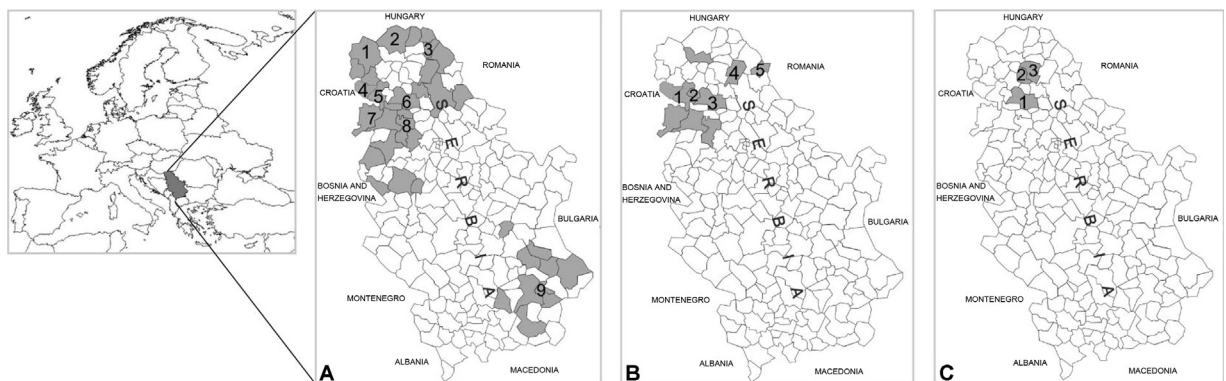


Fig. 1. Sampling areas of wild boars (A), roe deer (B) and farm pigs (C). Number illustrates the locations where anti-WNV and/or anti-USUV VNT antibody positive samples were found (details in Table 1).

only strain recently isolated in Serbia (Petrović et al., 2013), in 64 sera corresponding to 6 and 20 lineage 1 neutralizing pig and wild boar sera, and to 18 and 20 non-neutralizing sera, respectively.

3. Results

A total of 688 sera from farm pigs, wild boars, and roe deer collected in Serbia (Fig. 1) were analyzed to assess WNV circulation in the country. WNV ELISA-reactive sera were detected in 43/279 (15.4%), 56/318 (17.6%), and 17/91 (18.7%) of the pigs, wild boars, and roe deer sera tested, respectively.

Among the ELISA positive samples, WNV neutralizing antibodies were detected in 6 (14%), 33 (59%), and 4 (23.5%) of the sera, respectively, with neutralizing antibodies titers ranging between 1/80 and 1/640 in pigs, and 1/40 and 1/320 in wild boars. All VNT positive roe deer samples had a 1/40 neutralizing titer. None of the ELISA negative sera tested (13 from pigs, 18 from wild boars, and 14 from roe deer) were WNV-VNT positive, except for one roe deer sample that was repeatedly ELISA negative, but VNT positive with a titer of 1/80. Unfortunately, no serum remained to test the possible presence of IgM. To confirm the results described above with WNV lineage 1 strain (NY-99), VNT was performed in a representative set of 24 swine and 40 wild boar sera with the lineage 2 strain (SRB-Novi Sad/12) that was circulating in Serbia at the time of sampling (Petrović et al., 2013). All 26 sera tested that neutralized the WNV lineage 1 strain also neutralized the lineage 2 strain with similar titers, 1/40 to 1/640 for swine and 1/40 to 1/320 for wild boar. Likewise, sera that did not neutralize lineage 1, neither neutralized lineage 2 virus.

To assess flaviviral antigenic cross-reactivity (Lobigs and Diamond, 2012), VNT were similarly performed with Usutu virus (USUV), the only other flavivirus of the Japanese encephalitis serocomplex described so far to be circulating across Europe (Vazquez et al., 2011). A total of the 14 out of 151 sera tested (all 116 from ELISA positive and 35 from ELISA negative animals) neutralized USUV in cell culture. Among WNV-ELISA positive samples, USUV neutralizing antibodies were found in 2 (4.7%) pigs (with titers of 1/40 and 1/160, respectively), and 11 (19.6%) wild boar (titers range 1/40 to 1/160), but in none of the 17 roe deer sera. Following worldwide accepted specificity criteria, all pig and roe deer sera were WNV specific, while in 5 of the 33 (15.2%) wild boar samples the specificity could not be established. On the other hand, three of the wild boar sera resulted USUV specific, as they did not neutralize WNV. In addition, one WNV ELISA and VNT negative sera also neutralized USUV, resulting in a total of four USUV neutralizing specific wild boar sera.

All farm pig and roe deer sera tested were sampled on the northern and north-western part of the country, and positive samples were found scattered around the sampling locations. Wild boar sera were sampled in the same regions and also in the south-eastern part of Serbia and, except one, all ELISA and VNT anti-WNV antibody positive sera were also found at the northern part of Serbia. No differences in seroprevalence were recorded among wild boars and roe deer in relation to the age of the

animals. In pigs seroprevalence was higher among older animals, as 38 (88.4%) of all WNV-ELISA positive samples correspond to sows (1–4 year-olds), which represented only 21.5% (60/279) of the total pigs tested, that is, WNV prevalence among sow population was as high as 63.3% (38/60).

4. Discussion

Exposure of wild and domestic mammals to WNV infection has been documented in some species (Jeffrey Root, 2013); however, nowadays it is difficult to evaluate the role of these animals in the life cycle of the virus in nature. Several species, such as squirrels, deer, pigs, and wild boars, have been proposed as useful for surveillance programs (Boadella et al., 2012; Gibbs et al., 2006; Jeffrey Root, 2013; Padgett et al., 2007; Santaella et al., 2005). Thus, assessment of the activity of WNV among mammals would help in the implementation of better surveillance and control programs.

In the present study, the prevalence of WNV reactive antibodies among farm swine, wild boars, and roe deer from Serbia was assayed in the light of the recently WNV activity described in the country, where in 2012 the first outbreak of WNV clinical infection in humans was reported with a total of 70 West Nile fever cases and 9 fatalities (Popovic et al., 2013), and where along 2013 over 300 new human cases were diagnosed in what was the largest human WNV outbreak in Europe during that year (<http://ecdc.europa.eu/>). Likewise, seropositivity among birds (Petrović et al., 2013) and horses (Lupulovic et al., 2011; Petrović et al., 2013, 2014), including the seroconversion of previously negative animals (Petrović et al., 2014), and viral detection from mosquitoes (Petrić et al., 2012) and birds (Petrović et al., 2013), from which lineage 2 isolates were isolated and characterized, has been recently described in the country.

Most of these data were originated from the northern part of country, and from capital city Belgrade surroundings, and little information, especially for animal populations, about other regions of Serbia is available. Here, despite the fact that all tested pigs and roe deer were collected in the North, data from wild boars indicate that WNV/flavivirus circulation is certainly more intensive in this part of the country than in the South, as only 1 ELISA and VNT anti-WNV antibody positive wild boar sample was found among those originated from southern Serbia (Fig. 1 and Table 1). The northern part of the country, the Vojvodina Province and the capital city Belgrade, is an agricultural region with 90% flat field bordered with big rivers (Danube, Sava and Tisza) and a few wetlands. This region is a well-known resting and nesting area of many migratory and resident wild birds, which could be one of the main reasons of the higher flavivirus circulation and incidence of infection among humans and animals reported there.

Prevalence of anti-WNV IgG (15.4%, 17.6%, and 18.7%) was comparable among the three species tested (pigs, wild boars, and roe deer). These numbers are in the range of the few reports carried out before in other regions of the world in feral swine (22.5%) (Gibbs et al., 2006), red foxes (20.4%),

Table 1

WNV and/or USUV VNT positive samples per location (hunting ground/pig farm) found in the species tested referred to Fig. 1.: wild boars (A), roe deer (B), and pigs (C).

Specie	Locations	Number of VNT positives samples	
		WNV	USUV
Wild Boars (A)	1	1	1
	2	2	3
	3	1	0
	4	5	2
	5	1	0
	6	1	0
	7	14	5
	8	7	1
	9	1	0
Roe Deer (B)	1	1	0
	2	1	0
	3	1	0
	4	1	0
	5	1	0
Pigs (C)	1	1	1
	2	4	0
	3	1	1

wild boars (12.6%) (Gutierrez-Guzman et al., 2012), and deer (12.7%) (Santaella et al., 2005).

Neutralizing antibodies against WNV were detected in 14%, 59%, and 23.5% of WNV-ELISA positive samples from pigs, wild boars and deer samples, respectively. These figures are lower than that reported in Spain in a few pigs (66.6%, 4/6), and slightly higher than in wild boars (42.8%, 9/21) (Gutierrez-Guzman et al., 2012). The neutralizing capability of these sera against the WNV lineage 2 strains that has recently colonized Europe was assayed using the only strain isolated so far in Serbia (SRB-Novi Sad/12) concomitantly to the first outbreak reported in the country (Petrović et al., 2013). All sera tested neutralized both WNV lineages strains to a similar extent.

As flaviviral antigenic cross-reactivity is a well-known phenomenon (Lobigs and Diamond, 2012), to further assess flaviviral specificity of the sera tested, their neutralization capability was assayed against the other only mosquito-born flavivirus (USUV) currently circulating in Europe (Vazquez et al., 2011), including Serbia (Lupulovic et al., 2011; Petrović et al., 2013). Following worldwide criteria, all 6 and 5 neutralizing pig and roe deer sera, and 28 (84.8%) of wild boar sera were WNV specific, while in the remaining 5 (15.2%) no flaviviral specificity could be determined. Four wild boars (33.3%) resulted USUV specific, as they did not neutralize WNV. These results confirm the co-circulation of both viruses in Serbia and the need for an accurate diagnosis of circulating flaviviruses (Lupulovic et al., 2011; Petrović et al., 2013).

Several mammals have been proposed as sentinel models for WNV activity, mainly horses (Angelini et al., 2010; Barbic et al., 2012; Barzon et al., 2013; Calzolari et al., 2010; Mattar et al., 2011), but vaccination campaigns hampered its usefulness, as no DIVA vaccine is available. Beside horses, it has been proposed that deer may be useful indicators of recent WNV activity, as they rarely travel long

distances, typically have a relatively long life, and are accessible to mosquito bites (Santaella et al., 2005). Likewise, wild boars have been also recommended as accurate sentinel for flavivirus surveillance (Boadella et al., 2012), but, as they are mostly hunted in winter, a less WNV active season, its usefulness is questionable. Our data suggest that domestic pigs could probably be more feasible for this purpose. It should be noted that 88.37% (38/43) of the ELISA anti-WNV antibody positive pig sera were from older animals, 1–4 year old sows, which represent only 21.5% of the 279 total pig sera tested. However, pigs were from big industrial farms with closed and isolated buildings and artificial ventilation, where infection and seroconversion should be less likely than in free ranging or backyard pigs, which are much more exposed to the vector bites and could probably be better sentinel animals. In any case, further studies are needed to confirm it.

In brief, our results corroborate the circulation of both WNV and USUV among wild and domestic mammals in Serbia, mainly in the North, and point to a need to implement state-of-the art wide-scale multinational surveillance systems in the region.

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