Antibodies to West Nile Virus and related Flaviviruses in wild boar, red foxes and others mesomammals from Spain

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Abstract

Red foxes (*Vulpes vulpes*), wild boar (*Sus scrofa*) and Iberian pigs (*Sus scrofa domestica*) that are raised extensively outdoors, as well as other wild mesomammals from south central Spain and wild boar from Doñana National Park (DNP), were tested for antibodies against West Nile Virus (WNV) and related flaviviruses by ELISA and against WNV by VNT. Mean flavivirus seroprevalence according to ELISA was 20.4 ±7.8% (21 out of 103) in red foxes, 12.6 ± 2.8% (69 out of 545) in wild boars, and 3.3 ± 2.7% (6 out of 177) in Iberian pigs. A stone marten (*Martes foina*) also tested positive. Flavivirus seroprevalence in wild boar was significantly higher in DNP, and increased with age. Haemolysis of the serum samples limited interpretation of VNT to 28 samples, confirming WNV seroprevalence in one red fox, four Iberian pigs and nine wild boars. ELISA positive, microVNT negative samples suggest presence of non-neutralizing antibodies against WNV or antibodies to other antigenically related flaviviruses. Despite the importance of wetlands

for flavivirus maintenance and amplification, WNV/flavivirus seroprevalence in wild boar and red foxes was not associated to wetland habitats. This is the first report of exposure of red foxes to WNV. With view to use of the tested species as sentinels for flavivirus activity, limited exposure of Iberian pigs that would be available for regular sampling, low numbers of foxes collected and concentration of wild boar harvest in the winter season are mayor drawbacks.

Keywords: Flavivirus, West Nile virus, Red fox, Wild boar, Iberian pig, Seroprevalence

1. Introduction

Flaviviruses have a worldwide distribution, and a number of them such as West Nile Virus (WNV) are the cause of zoonoses of considerable importance for public health. The flavivirus (family *Flaviviridae*) members of the Japanese encephalitis antigenic complex (JEV) are maintained in a mosquito vector and bird reservoir cycle where humans, wild and domestic mammals can be implicated as incidental hosts (Weissenböck et al., 2010). Flavivirus activity has been rather limited in Spain until the last decade in which evidence of circulation of different mosquito-borne flaviviruses has been found with increasing frequency. In Spain, flavivirus activity main comprises three mosquito-borne flaviviruses (WNV, USUV and BAGV; Figuerola et al., 2007; Busquets et al., 2008; Vazquez et al., 2010, 2011; Agüero et al., 2011).

WNV is a re-emerging zoonotic virus responsible of outbreaks in humans, domestic animals (horses) and wildlife. In Spain, evidence of exposure to WNV has been found in humans (Kaptoul et al., 2007; Anonymous, 2010), birds (Figuerola et al., 2007; Höfle et al., 2008; López et al., 2008; Jiménez-Clavero et al., 2008), mosquitos (Vázquez et al., 2010, Sotelo et al, 2011a), and horses (Jiménez-Clavero et al., 2010; OIE, 2010).

55 USUV has caused disease and mortality in birds in Europe and recent human cases in Italy
56 (Pecorari et al., 2009), but as yet in Spain it has only been detected in mosquitoes
57 (Busquets et al., 2008; Vázquez et al., 2011) and no related disease has been reported in
58 birds or humans. In contrast, Bagaza virus (BAGV), a Flavivirus of the Ntaya serocomplex
59 that had never been detected before in Europe, was recently isolated from an outbreak of
60 lethal disease in free-living game birds in Southern Spain (Agüero et al., 2011).
61 Mammals can be naturally exposed to flaviviruses, either by bite from infected vectors, or
62 by ingestion of infected carrion or diseased prey (Austgen et al., 2004; Marra et al., 2004).
63 In fact, serological evidence of exposure to flaviviruses, mainly WNV, has been reported
64 in wild and domestic mammals from America, Africa, Asia and Eastern Europe (Root et
65 al., 2005; Bentler et al., 2007; Halouzka et al., 2008; Ohno et al., 2009, El-Harrak et al.,
66 2011). In Spain, a report from 1980 described the presence of antibodies to flaviviruses in
67 rodents (Chastel et al., 1980). WNV seropositivity has been evidenced in horses (Jiménez-
68 Clavero et al., 2010) and also clinical disease and mortality have been reported in this
69 species (OIE, 2010). Recently, we reported on exposure of wild juvenile ungulates, namely
70 wild boar and Iberian red deer (*Cervus elaphus*) to flaviviruses (Boadella et al., 2011).
71 Flavivirus exposure of free-living mammals with a broad geographic range and high
72 population density, such as wild boar (Acevedo et al., 2007), red fox (*Vulves vulpes*), and
73 other species could be a useful indicator of viral circulation and expand the knowledge on
74 virus ecology in Mediterranean ecosystems (Platt et al., 2008). Likewise, the extensively
75 reared Iberian pig could come in contact with vectors and thus be a useful source of
76 information on flavivirus activity in the region.
77 The objective of this work is to determine, through antibody detection, the degree of
78 exposure to flaviviruses (especially WNV) of wild boar, red foxes, and other medium sized
79 wild and domestic mammals in South-Central Spain and to further assess whether this

information can be useful to monitor flavivirus activity in a given area. Also, the data collected in this study from wild boar will be analyzed to test whether it is correct to assume that the risk of exposure to flaviviruses is higher in wetland habitats, where a high mosquito abundance (Dale and Knight, 2008) and a high concentration of a wide range of wild bird species, either resident or migratory (López et al., 2008) is present, and during specific seasons, in which mosquito activity is more likely. Thus, in this work we compare antibody prevalence data among different geographical regions, habitats and seasons. The effect of host factors such as age, sex and body condition on seroprevalence is also assessed.

2. Material and methods

2.1 Study area

The study includes 23 sampling sites in south-western Spain (41º15 N and 38º04 N, 5º20 O and 0º59 W, minimum altitude=244m, maximum altitude=2274m, relative humidity=64%, mean temperature=14ºC), and one in Doñana National Park (DNP; 36º56 N, 6º21 W, mean altitude=12m, relative humidity=80%, mean temperature=19ºC), in the Guadalquivir river marshes in southern Spain (Figure 1). Of the 23 sampling sites in south-western Spain, 2 correspond to wetlands with abundance of migratory and resident birds; 9 are devoted to agriculture and 12 are made up of typical Mediterranean forest in small mountain chains. DNP is one of the main wetlands in Western Europe with high density and diversity of resident and migratory birds.

2.2 Sampling

Blood samples of wild boar (n=545), red fox (n=103) and other wild mammals (European wild cat *Felis silvestris silvestris* n=1, stone marten *Martes foina* n=6, European otter *Lutra*

lutra n=2, European badger *Meles meles* n=7, common genet *Genetta genetta* n=2) were obtained from hunting drives, trapping programs for National Park management or from animals found dead, mostly road-kills. Blood was collected from the thoracic cavity of freshly dead animals. Blood of Iberian pigs (n=177) was collected from the infraorbital sinus during the annual official active sanitary surveillance procedures (Real Decreto (RD) 1186/2006, published October 13th, 2006, now replaced by RD 599/2011, published April 29th 2011).Wild boars were sampled in 2007-2010, Iberian pigs in 2009-2010, red foxes in 2006-2008 and other small mammals in 2003-2007. Wild boar and red fox samples were grouped by season (spring, summer, autumn, winter) and habitat type (wetland, Mediterranean forest, and agricultural crops) for statistical analysis.

The age of wild boars was determined using dentition patterns, classifying them into piglets (< 7 months), weaners (7 to 12 months), juveniles (12 to 24 months) and adults (>2years; Matschke, 1967). When kidney data was available, the kidney fat index (KFI) was obtained as indicator of body condition (Batcheler and Clarke, 1970).

Red fox samples were classified according to sex and age (juveniles and adults), and the mean KFI was obtained when possible.

All Iberian pigs sampled were adults and body condition information was not available.

Upon arrival at the laboratory, blood samples were centrifuged for at least 10 min at 2,000*g* for serum separation and the serum was stored at -20º C until testing.

2.3 Serological tests

Presence of antibodies against WNV and closely related flaviviruses was analysed using a commercial competitive enzyme–linked–immunosorbent assay (cELISA; ID Screen©® West Nile Competition, ID Vet, Montpellier, France) based on purified whole WNV antigen for detection of antibodies directed against the PrM-E envelope protein common to flaviviruses. The test was performed according to manufacturer's instructions.

To confirm ELISA positive samples, neutralizing antibody titers to WNV were determined by a micro virus-neutralization test (micro VNT) previously described by Figuerola et al. (2007), using Vero cells and WNV strain E101.

2.4 Molecular tests

To determine WNV and flavivirus genome presence, nucleic acids of available tissue (spleen) of antibody-positive wild boar (n=69) were extracted (*High Pure RNA Tissue Kit*, Roche Diagnostics, Barcelona, Spain), and analysed by real time reverse transcription-polymerase chain reaction (RRT- PCR) for WNV (TaqMan MGB PCR, QuantiTEC Probe® RT-PCR, Qiagen, Madrid, Spain; Jiménez-Clavero et al., 2006), and Flavivirus (QuantiTEC® SYBR®Green RT-PCR, Qiagen, Madrid, Spain) detection (Moureau et al., 2007).

2.5 Statistical analysis

A Chi square ($\chi 2$) test for homogeneity was used to compare the mean flavivirus seroprevalence in wild boar between sampling sites and between DNP and the mean seroprevalence from the combined sampling sites from south-western Spain.

To study factors that affect exposure to flaviviruses in wild boar we performed a generalized mixed model (GzMM) where flavivirus seropositivity was the response variable and “sex”, “age class”, “season”, “sampling year” and “habitat” were the categorical explanatory variables. Sample origin was included a random variable. For this analysis we used a binomial error and a logit link. Also a generalized mixed model (GzMM) was performed in order to study the relationship between body condition (as log10-transformed KFI, continuous response variable) and WNV antibody presence in wild boar. We included “WNV seropositivity” (as categorical 0=absence, 1=presence),

"sex", "age class", "season" and "origin" (all of them as categorical) as explanatory variables. "WNV seropositivity" interactions with "sex" and "age" were added to the models. Sampling year was included as random variable. Identity error and a normal link were applied. Finally the Chi square ($\chi 2$) test for homogeneity was used to compare flavivirus seroprevalence in red foxes among age groups and according to sex. All analyses were performed with the SPSS software package, version 19.0 (IBM SPSS Statistics, New York, NY, USA).

3. Results

Antibodies against flaviviruses were detected by ELISA in 20.4 ±7.8% (21 out of 103) of the red foxes, 12.6 ± 2.8% (69 out of 545) of the wild boars, in 3.3 ± 2.7% (6 out of 177) of the Iberian pigs and in one stone marten (Table 1, Figure 1). In wild boar, a significantly higher mean seroprevalence was found in DNP (27±7.1%) as compared to sampling sites in south-western Spain (Figure 1, $\chi^2 = 45.764$, d.f. 6, $p< 0.001$) and the general mean prevalence in south-western Spain (6.9±2.5%) respectively $\chi^2=31.7$, d.f. 1, $p < 0.05$). Habitat, season and sampling year did not affect flavivirus seroprevalence in wild boar. Also, flavivirus seroprevalence in wild boars and red foxes was apparently not affected by sex and body condition. However, adult wild boar had a significantly higher flavivirus antibody prevalence (19.7± 5.8%, 35 out of 178), than juveniles (7.8± 5.2%, 8 out of 103), weaners (6.5± 4.7%, 7 out of 107), and piglets (5± 9.6%, 1 out of 20) (GLzMM, F=4.136, d.f. 4, $p<0,05$, Figure 2).

Due to the strong haemolysis in sera from wild boars and red foxes, only 32% (21 wild boar, 6 Iberian pig and 1 red fox samples) of the total samples tested by microVNT gave a readable result. In wild boar, WNV neutralizing antibodies were found in 9 of the 69 ELISA positive samples (13%). More precisely 5 of 41 ELISA positive samples from DNP

185 (12.2%), 2 of 7 ELISA positive samples from wetland habitats in south-western Spain
186 (28.6%), and 2 of 7 samples (28.6%) from Mediterranean forest habitat had WNV
187 neutralizing antibodies (Table 1, Figure 1). In the case of the Iberian pig, none of the
188 samples was haemolytic, and WNV neutralizing antibodies were detected in four (66.6%)
189 of the six ELISA positive samples (Figure 1). The stone marten ELISA positive sample
190 was negative by VNT. One sample of a red fox from a Mediterranean forest habitat
191 presented a high titre of WNV neutralizing antibodies, while neutralization titres for WNV
192 in wild boar and Iberian pigs were relatively low (Table 2).
193 All spleen samples analyzed by RRT-PCR were negative for the presence of WNV and
194 flavivirus genome.
195
196 **4. Discussion**
197 The results of the present study newly confirm the exposure of red foxes, Iberian pigs and
198 a stone marten from south-central Spain to flaviviruses. Mesomammals have previously
199 been confirmed to be exposed to WNV and other flaviviruses. However, to our knowledge,
200 this is the first report of flavivirus exposure in red fox and stone marten. Antibodies to
201 flaviviruses, mainly WNV, have been found in other members of the *Canidae* family, such
202 as gray foxes (*Urocyon cinereoargenteus*) or coyotes (*Canis latrans*; Bischof and Rogers,
203 2005; Bentler et al., 2007), and other mesomammals from North America (Dietrich et al.,
204 2005; Root et al., 2005; Bentler et al., 2007; Gómez et al., 2008; Blitvich et al., 2009), but
205 had not been reported from Europe. Disease due to WNV has been documented in both
206 wolves (*Canis lupus*) and domestic dogs in connection with WN fever outbreaks in horses
207 and humans (Lanthier et al., 2004) and dogs have actually been proposed as potential
208 sentinels for WNV surveillance (Resnick et al., 2008).

209 The high flavivirus antibody prevalence rate evidenced in red foxes could be due to higher
210 exposure to frequent infected mosquito bites or to consumption of infected prey, although
211 this route of infection has only been reported in experimentally infected domestic cats and
212 naturally infected raptorial birds (Garmendia et al., 2000; Austgen et al., 2004).
213 Iberian pigs that could be more easily accessible than wild boar and red foxes for sampling
214 as sentinels were apparently less exposed to flavivirus. In a study including juvenile
215 individuals we showed that wild boar could be an interesting sentinel species for
216 flaviviruses surveillance (Boadella et al., 2011). As the frequently flavivirus positive red
217 foxes are generally available only in low numbers and as wild boar is mostly harvested in
218 winter, Iberian pigs that are farmed extensively and thus exposed to mosquitoes could be a
219 valuable alternative, not the least because they are accessible for sampling around the year,
220 and as blood samples are of better quality, than samples obtained from wild boar carcasses.
221 Our study shows however that exposure to flaviviruses in this species is much lower than
222 in wild boar in the same period. Previously, antibodies against WNV have been found in
223 feral swine in North America by ELISA with a mean prevalence in 2001-2004 (22.5%)
224 similar to the one encountered in DNP, and by VNT in 6.5% wild boar in the Czech
225 Republic (Gibbs et al., 2006; Halouzka et al., 2008).
226 In this study results for flavivirus seroprevalence in mesomammals by ELISA and WNV
227 seroprevalence by VNT differed. Currently, VNT is considered the gold standard to
228 confirm exposure to WNV (Dauphin and Zientara, 2006). Here, the high degree of
229 haemolysis present in the serum samples of wild boar and red foxes has only allowed
230 correct interpretation in a reduced number of samples (n=28). ELISA, on the other hand, is
231 less prone to haemolysis interference, but detects antibodies of cross related flaviviruses,
232 particularly viruses of the Japanese encephalitis serocomplex. Thus, in this study we
233 discuss flavivirus seroprevalence in general, as ELISA positive, microVNT negative

234 samples could contain either non-neutralizing antibodies against WNV or antibodies to
235 other antigenically related flaviviruses. However, to date only the mosquito borne USUV
236 and BAGV, and the tick borne Spanish sheep encephalomyelitis virus (SSEV) have been
237 reported in the country (Marin et al., 1995; Busquets et al., 2008; Agüero et al., 2011).
238 The substantially higher flavivirus seroprevalence found in DNP as compared to south-
239 central Spain (CLM) suggests a potential link to a habitat that favours vector abundance
240 and reservoir host (wild bird) presence. Nevertheless, no association of flavivirus
241 seroprevalence to wetland habitats could be established in wild boar in south-central Spain.
242 Flavivirus, namely WNV activity has been shown to vary with time due to climatic factors
243 that affect vector abundance (e.g. Platonov et al., 2008). In this study, flavivirus exposure
244 was detected between 2005 and 2010. For the years 2003 and 2004 only data for three
245 badgers, two genets, a wild cat and a stone marten were available, which is insufficient to
246 conclude about flavivirus activity in the study area. We were unable to detect a relation of
247 seroprevalence to year or season, however we also do not know about the duration of
248 antibody persistence in our test species, and most of our samples were from the winter (low
249 mosquito density) season which is when hunting drives take place.
250 The significantly higher seroprevalence found in adult wild boars in comparison to
251 juveniles, weaners and piglets (Figure 2), coincides with previous results in North
252 American feral swine (Gibbs et al., 2006) and in camels (El Harrak et al., 2011), and could
253 be explained by a longer time span of possible exposure or due to antibody persistence. In
254 adult pigs, antibodies to JEV have been found to persist for more than three years possibly
255 due to frequent re-inoculation by mosquito bites (Geevarghese et al., 1994), while other
256 experimental studies detected persistent antibodies in absence of re-infections only until 28
257 days post infection (Blitvich et al., 2003; Teehee et al., 2005).

Finally, in this work, we found antibodies against WNV and/or other flaviviruses but viral genome was not detected, suggesting absence of active infection or low possibilities of viral genome detection due to transitory viraemia or other host characteristics. Material other than spleen that might have been more suitable for flavivirus genome detection was not available for this study.

The results obtained in this study document the exposure of widely distributed wild and domestic mammals in Spain to flaviviruses, specifically in areas where flavivirus activity has been previously reported, but do not reveal Iberian pigs as good sentinel species for flavivirus surveillance. With the samples available for this study we could neither demonstrate increased exposure to flavivirus in wetlands nor a relation of flavivirus exposure to season and thus mosquito abundance. The association of Flavivirus prevalence with age suggests that juvenile individuals may be of more interest for surveillance. Additional studies aimed at evaluating flavivirus circulation in other regions and the degree of exposure of other widely distributed mammals would be of interest.

Conflict of interest statement

The authors have no conflict of interest.

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Table 1. Sampling effort by year and habitat type, and flavivirus and WNV seroprevalence by blocking ELISA and WNV neutralization test in wild boar, red fox, and Iberian pigs.

Species	Habitat	Wetland location	2006			2007			2008			2009			2010		
			n	ELISA pos.	VNT rd/+	n	ELISA pos.	VNT rd/+	n	ELISA pos.	VNT rd/+	n	ELISA pos.	VNT rd/+	n	ELISA pos.	VNT rd/+
Wild boar	Mediterranean forest					30	3	0/0	34	3	0/0	48	1	1/1	19	0	0/0
	Wetland	DNP SW		------		46	13	3/3	40	12	4/0	40	6	3/1	26	10	4/1
	Agriculture					30	1	1/1	55	2	2/2	76	2	2/0	2	0	0/0
Total wild boar				-----		18	0	0/0	62	10	1/0	15	1	0/0	4	0	0/0
						124	17	4/4	191	32	7/2	179	10	6/2	51	10	4/1
Red fox	Mediterranean forest		11	0	0/0	17	7	1/1	45	10	0/0						
	Wetland		11	1	0/0	--	--	--	--	--	--		----			----	
	Agricultre		1	0	0/0	16	3	0/0	2	0	0/0						
Total red fox			23	1	0/0	33	10	1/1	47	10	0/0		---			---	
Iberian pig	Mediterranean forest											--	--	--	20	0	--
	Wetland			---			----			---		--	--	--	--	--	--
	Agriculture											86	3	3/3	71	3	3/1
Total Iberian pig				---			---			---		86	3	3/3	91	3	3/1
Total samples			23	1	0/0	157	27	5/5	238	42	7/2	265	13	9/5	142	13	7/2

Table 2. Summary of antibody titres against WNV detected in free-living wild boars, red foxes and Iberian pigs

Species	5	10	20	40	80	160	240	480
Wild boar	1	3	2	1	0	2	--	--
Red fox	--	--	--	--	1	--	--	1
Iberian pig	--	--	1	1	1	1	--	--

514 Figure 1. Flaviviruses seroprevalence in wild boar, red fox and Iberian pig from south-

515 central, Spain. Stars indicate locations with VNT positive samples.

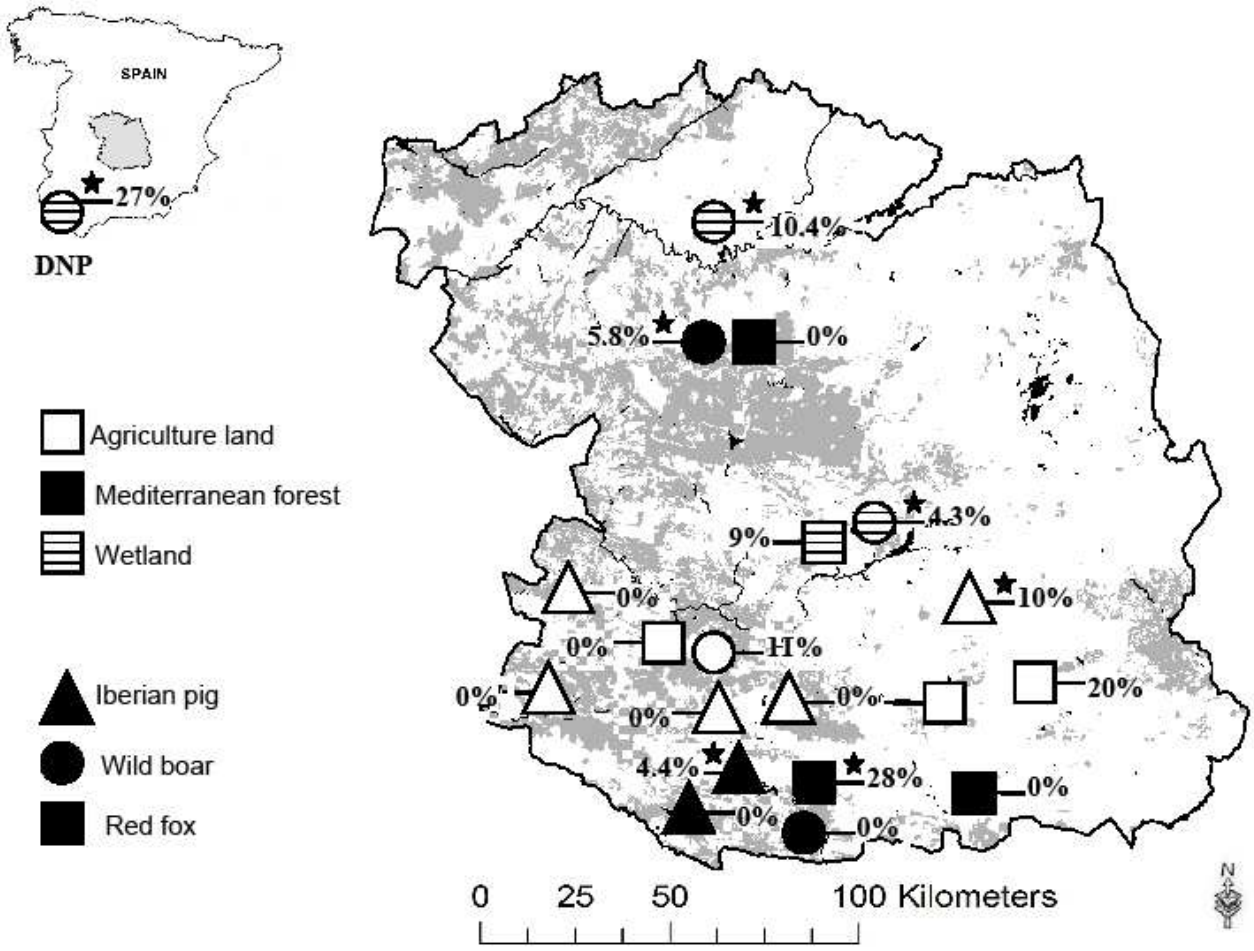

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Figure 2. WNV/Flavivirus seroprevalence in wild boars of south central Spain increases with age.