Epidemiology and spatio-temporal analysis of West Nile virus in horses in Spain between 2010 and 2016

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Summary

During the last decade, West Nile virus (WNV) outbreaks have increased sharply in both horses and human in Europe. The aims of this study were to evaluate characteristics and spatio-temporal distribution of WNV outbreaks in horses in Spain between 2010 and 2016 in order to identify the environmental variables most associated with WNV occurrence and to generate high-resolution WNV suitability maps to inform risk-based surveillance strategies in this country. Between August 2010 and November 2016, a total of 403 WNV suspected cases were investigated, of which 177 (43.9%) were laboratory confirmed. Mean values of morbidity, mortality and case fatality rates were respectively 7.5%, 1.6% and 21.2%, respectively. The most common clinical symptoms were: tiredness/apathy, recumbency, muscular tremor, ataxia, incoordination and hyperesthesia. The outbreaks confirmed during the last seven years, with detection of WNV RNA lineage 1 in 2010, 2012, 2013, 2015, 2016, suggest an endemic circulation of the virus in Spain. The spatio-temporal distribution of WNV outbreaks in Spain was not homogeneous, as most of them (92.7%) were concentrated in western part of Andalusia (southern Spain) and significant clusters were detected in this region in two non-consecutive years. These findings were supported by the results of the space-time scan statistics permutation model. A presence-only MaxEnt ecological niche model was used to generate a suitability map for WNV occurrence in Andalusia. The most important predictors selected by the ENM were: mean annual temperature (49.5% contribution), presence of *Culex pipiens* (19.5% contribution), mean annual precipitation (16.1% contribution) and distance to Ramsar wetlands (14.9% contribution). Our results constitute an important step for understanding WNV emergence and spread in Spain and will provide valuable information for the development of more cost-effective surveillance and control programs and improve the

protection of horse and human populations in WNV endemic areas.

Keywords: West Nile Disease, Emerging disease, Maximum Enthropy, MaxEnt, SaTScan; Risk-based surveillance

Introduction

West Nile disease (WND) is a re-emerging zoonotic disease in Europe and neighboring countries. The causative agent, West Nile Virus (WNV), is a positive, single-stranded, enveloped RNA virus classified within the Japanese encephalitis virus serogroup, in the genus *Flavivirus* (family Flaviviridae). To date, seven (or nine according to some authors) different WNV lineages have been identified, but only lineage 1 and 2 have been associated with human and horse cases (Rizzoli et al., 2015).

The virus is mainly transmitted by ornithophilic mosquitoes of the *Culex pipiens* complex and their hybrids in an enzootic life-cycle in which certain birds act as natural reservoir hosts, amplifying the virus. Although mammals are susceptible to infection, most are considered dead-end or incidental hosts, as the viraemia is too low to infect competent vectors. Humans can also become infected by blood transfusion, organ transplantation, intrauterine transmission, handling of infected carcasses and breast feeding (CDC, 2017). In general, WNV infection in humans and horses is asymptomatic or associated with influenza-like illness; however, in some cases (< 1%) the infection can lead to severe neurological symptoms and mortality (Hayes et al., 2005).

Compared with the rapid and widespread distribution of WNV in the United States after its introduction in 1999 (CDC, 2017), in Europe, WNV was initially considered to have minor health effects, with sporadic cases in human and horses. However, the number of notified WNV outbreaks caused by both lineage 1 and lineage 2 **has** significantly increased in Mediterranean Basin during the last decade, which has raised concerns in relation to both public and animal health (Hernández-Triana et al., 2014; Benjelloun et al., 2016).

Before the 2010 WNV epidemic in Spain, antibodies had already been detected in humans, horses and wild bird species, but only sporadic clinical cases were reported in humans and raptors in Spain (Kaptoul et al., 2007; Jiménez-Clavero et al., 2008; García-Bocanegra et al., 2011a). In late summer 2010, the first WNV horse outbreak was reported in southern Spain (Andalusia). Throughout that year, 35 further cases were reported in horses, and also 2 human cases were confirmed in the region (García-Bocanegra et al., 2011b). Since then, WNV outbreaks in horses have been reported every year. The goals of this study were: (1) to describe the main epidemiological and clinical findings of the WNV outbreaks in horses during the period 2010-2016 in Spain, (2) to assess the spatio-temporal distribution of WNV, and (3) to identifying the drivers of WNV occurrence in the endemic areas in Spain using a presence only maximum entropy ecological niche model.

Materials and methods

Descriptive analysis

After the confirmation of the first WNV outbreak in a horse herd on the 31st of August 2010, a passive surveillance system which included horses, humans, wild birds, and mosquitoes was launched by the veterinary and health authorities in Spain. All the herds in which horses with clinical symptoms compatible with WND were observed, were investigated by veterinary officers. Blood samples were obtained from suspected horses by puncture of the jugular vein. Brain and cerebrospinal fluid samples were also collected from dead or euthanized animals.

Serum samples from all investigated horses were tested to detect IgM antibodies against WNV using a commercial competitive ELISA (cELISA; IDEXX IgM WNV Ab, IDEXX Lab). Furthermore, **248 of the 403 (61.5%) investigated herds (including 115**

IgM-positive herds and 133 IgM-negative herds) were randomly selected and analyzed by a commercial blocking ELISA (bELISA; Ingezim West Nile compac R.10.WNV.K3, Ingenasa Lab), which detects IgG antibodies against one epitope of the prM-E protein of the flaviviruses of the Japanese Encephalitis antigenic group. Both ELISAs were performed according to the manufacturer's recommendations. **Ninety-four of 177 (53.1%) of the IgM-positive herds, as well as 31 of 133 (23.3%) of the IgM-negative herds, were selected, using a convenience sampling, and analyzed also by** virus serum-neutralization test (VNT) against WNV (strain Eg101) according to the OIE guidelines **(Table 1).** Blood and cerebrospinal fluid samples (CFS) were analyzed for detection of WNV lineage 1 and 2 by real time RT-PCR as previously described (Del Amo et al., 2013). All laboratory tests were performed at the National WNV Reference Laboratory in Algete (Madrid, Spain).

An outbreak was defined as a herd with at least one confirmed case. A case was defined as a horse with clinical symptoms that were compatible with WND and confirmed by the National Reference Laboratory as positive by detection of IgM antibodies to WNV or RT-PCR positivity. The WNV-specific morbidity was expressed as the number of WND cases in the investigated horse herds divided by the total number of horses in these affected herds. The WNV-specific mortality was calculated as the number of deaths due to WNV infection divided by the total number of horses in the confirmed herds. The WNV-specific case fatality was expressed as the proportion cases that died of WNV infection by the total number of WND cases.

Epidemiological data were collected during clinical inspections using a standardized questionnaire in Andalusia, the region where the majority of outbreaks (92.7%) were

reported. Information on the characteristics of the herds and affected animals was also recorded in this region for the descriptive analysis.

Spatio-temporal cluster analysis

A spatio-temporal analysis was carried out in Andalusia. Geolocations (UTM coordinates) of all investigated horse herds were provided by the Regional Government of Andalusia and the Spanish Ministry of Agriculture Food and Environment. Data from August 2010 to November 2016 were analyzed using a space-time scan statistic, with a space-time permutation model (Kulldorff et al., 2005), to detect the presence of areas and time periods with significant aggregation of WNV outbreaks in horse herds. Space-time permutation model, similarly to the other more commonly used Bernoulli- and Poisson-based models, creates thousands of overlapping cylinders over the study area and compare the observed number of cases within the cylinder (i.e., at particular space -based of the cylinder- and particular time -height of the cylinder-) to the "expected" number of cases in that cylinder. The main difference of the permutation model is that the expected is calculated using only the cases as described by Kulldorff et al, 2005. The maximum spatial and temporal window were set up to be 50% of the study region surface and 15% of the study period (one year), respectively. The number of Monte Carlo simulations was set to 999 for the cluster scan statistic. Analyses were run using SaTScan™ v9.4.4. Clusters were considered to be significant at $P < 0.05$.

Ecological Niche Modeling

Risk areas for WNV outbreaks occurrence in Andalusia were detected using the presence-only maximum entropy ecological niche model (MaxEnt) (Phillips et al., 2006). The model was performed with the MaxEnt program version 3.3.3 via the “dismo” package

in R Studio version 1.0.44 (Hijmans et al., 2016). Briefly, the maximum entropy ecological niche model looks at the association between the presence data and several environmental predictors known to be related to the disease in order to characterize the most important environmental requirements for the disease agent to be present and estimate a suitability probability in sampled and non-sampled geographic areas.

In order to determine the suitability area for WNV occurrence in Andalusia, the MaxEnt model used the WNV outbreaks locations in the region between 2010 and 2016 as presence data and 10.000 randomly chosen background points from Andalucía as "Pseudo-Absence" data. Potential predictors for the MaxEnt model consisted of a set of 14 climatic, environmental and demographic factors that were previously described as important for WNV presence. The model was calibrated with a default convergence threshold, a regularization of 1 and a number of iterations of 1,000. In addition, a logistic model was used to ensure that predictions gave estimates between 0 and 1 for the spatial suitability per map cell. Climatic predictors were provided by the Regional Government of Andalusia (CMAOT, 2016). They included mean annual temperature (ºC), mean maximum annual temperature (ºC), mean minimum annual temperature (ºC), mean annual rainfall (mm) and average number of rainy days per year (days). Environmental/demographic variables included altitude (m), evotranspiration defined as theoretical water requirements by the vegetation cover (mm), type of soil, distance to wetlands of national importance for water birds (Ramsar wetlands) (Km) (ICWII, 2017), land cover, presence of *Culex pipiens*, presence of *Culex theilery*, horse herd density and human population density. Human and horse densities (at municipality level), altitude, evotranspiration, type of soil, distance to Ramsar wetlands and land cover were provided by the Regional Government of Andalusia

(CMAOT, 2016). The land cover was obtained from the Land Cover Change 2006-2012 (http://land.copernicus.eu/pan-european/corine-land-cover/lcc-2006-2012/view). Raster maps of presence/absence of *C. pipiens* and *C. theileri* were generated based on the criteria defined by Tran et al. (2013). In brief, they evaluated whether different mosquito species were present in each CORINE (Coordination of Information on the Environment, http://www.eea.europa.eu) land cover class based on a literature review and the opinion of expert entomologists. Therefore, presence or absence of *C. pipiens* and *C. theileri* (dichotomous variable) throughout Andalusia was defined as based on the corresponding CORINE land cover class at each location. The 2006 CORINE land cover map was obtained from the European Environment Agency website (http://www.eea.europa.eu). All predictors were rescaled in rasters format with 100m × 100m spatial resolution, the same extent and the common UTM 30N projection. Correlation between predictors was assessed using pairwise Spearman's rank correlation coefficient (*rho*). When the correlation between two variables was 0.5 or higher, only the variable more biologically linked to WNV occurrence was included in the model. A first "full" MaxEnt model was fit with the **14** potential predictors. Predictors that contributed to 5% or more to the first model were selected to be run in a final "reduced" model. The final model was evaluated after partitioning the presence data into a training and a testing using the A k-fold method (Jung and Hu, 2015). A total of 80% of the WNV outbreaks locations were randomly selected for model building, whereas the remaining 20% locations were set aside for external validation. Model performance was assessed using the area under the curve (AUC) of the receiver operating characteristics curve (ROC) using the "dismo" package in R. Since AUC has been shown to be influenced by spatial sorting bias, the calibrated AUC (AUCc) was also used as suggested by Hijmans et al. (2012). The AUCc provides a more accurate estimate

of the real performance of a model.

The Jackknife training gain test and percent contribution were used to estimate the contribution of each predictor in the final model. Predictors with the highest training gains or those that reduced the training gain the most when left out of the model, were considered the most valuable variables to the model. The final model was then used to generate the corresponding suitability map for WNV occurrence in Andalusia and a partial plot for the contribution of each predictors in the model were generated.

Non-confirmed herds were also overlaid over the WNV suitability map in order to assess how many of them where located in high risk areas. Specifically, WNV suspected cases were classified into two categories ("high risk" or "low risk") based on the median value of the outbreaks. Map was created using ArcMap version 10.3 (ESRI, Environmental Systems Resource Institute, www.esri.com).

Results

Descriptive analysis

Between August 2010 and November 2016, 403 suspected horse herds were investigated, 177 (43.9%) were confirmed as WNV outbreaks (presence of both clinical symptoms and IgM antibodies or RT-PCR positivity to WNV in at least on horse) (Table 1, Fig. 1). Within the WNV positive herds, 236 (8.5%) of the 2,779 horses were considered clinically suspected, of which 215 were confirmed as cases, resulting in a mean WNV-specific morbidity of 7.7%. The mean age of the cases was 7 years (ranging between one and 22 years), and the census of the infected herds varied from 1 to 325 horses (median = 4). Vaccination programs were not implemented in any of the confirmed or non-confirmed

209 herds and movement of animals were not performed in these herds one month before the
210 outbreak was reported. In those WNV positive herds, mean WNV-specific mortality and
211 WNV-specific case fatality rates were 1.6% and 21.2%, respectively. The most common
212 clinical symptoms detected in the positive herds were: tiredness/apathy (74.2%),
213 recumbency (54.8%), muscular tremor (51.6%), ataxia (48.4%), incoordination (48.4%)
214 and hyperesthesia (45.2%). Fever (32%), anorexia (23%) and convulsion (23%) were also
215 frequently observed (Fig. 2).

216 Presence of IgG antibodies was confirmed in 107 (93.0%) of the 115 WNV
217 confirmed **(IgM-positive)** herds tested (Table 1). WNV neutralizing antibodies were
218 observed in 50 (53.2%) of the 94 confirmed herds that could be tested using VNT, with
219 titers of 1:10 in 20% of them, 1:20 in 30%, 1:40 in 18%, 1:80 in 22% and 1:160 in 10%.
220 Ten samples (10.6%) could not be analyzed due to cytopathic effects. Besides, presence of
221 IgG antibodies was detected in 41 of the 133 (30.8%) non-confirmed herds analyzed. WNV
222 neutralizing antibodies were detected in 14 out of 31 (45.2%) non-confirmed **(IgM-**
223 **negative)** herds analyzed by VNT, with titers of 1:20, 1:40, 1:80, 1:240 and 1:640 in two,
224 five, four, two and one horse herds, respectively.

225 WNV RNA was found in 14 (4.2%) out of 332 horse herds. Within the positive
226 herds, WNV RNA was detected in 5 (2.2%) of the 229 blood samples of the analyzed
227 horses, and in 11 (61.1%) of the 18 CFS from dead or euthanized animals. Two horses were
228 WNV RNA positive in both blood and CFS. WNV RNA was detected in 2010, 2012, 2013,
229 2015 and 2016. All RT-PCR positive samples were confirmed as WNV lineage 1.

230 The first outbreak was reported on the 31th of August 2010 in a horse herd in Cádiz
231 province (Andalusia). Since then, outbreaks have been reported every year during the

studied period (Fig. 3). The total number of outbreaks per year was: 36 (20.3%) in 2010, 6 (3.4%) in 2011, 3 (1.7%) in 2012, 35 (19.8%) in 2013, 8 (4.5%) in 2014, 17 (9.6%) in 2015 and 72 (40.7%) in 2016. There was a clear seasonal pattern in the outbreak temporal distribution. Outbreaks were concentrated between the months of July and January, and peaked in September (Fig. 3). The last three outbreaks were reported on the 11th of November 2016, two in Badajoz province (Extremadura) and one in Avila province (Castile and Leon). A total of 82 municipalities located in four different regions have been affected by the WNV outbreaks that occurred in Spain between 2010 and 2016. Most of the outbreaks were located in Andalusia (92.7%), followed by Extremadura (3.9%), Castile and Leon (2.8%) and Castile La Mancha (0.7%). One herd reported outbreaks in three consecutive years (2014-2016), and there were three other herds that reported outbreaks two different years (2010-2012, 2014-2015 and 2015-2016). The remaining 173 horse herds affected reported a single outbreak.

Spatio-temporal analysis

The space-time permutation model identified two statistically significant clusters ($P < 0.001$) centered in the west part of Andalusia (Fig. 1 and Table 2). The most likely cluster included 36 outbreaks and was located in south-western Andalusia (Cádiz province) in September 2010. Another cluster, with 34 outbreaks, emerged in August 2016 in central western Andalusia (Seville province).

MaxEnt modeling

Mean annual temperature (49.5% contribution), presence of *Culex pipiens* (19.5% contribution), mean annual rainfall (16.1% contribution) and distance to Ramsar wetlands (14.9% contribution) were identified as the most important predictors for WNV occurrence

255 in Andalusia (Fig. 4). The AUC of the final MaxEnt model was 0.918 and the AUCc values
256 0.914. Spearman correlation showed very low correlation between the selected predictors
257 (see Supplementary Fig. 1). Results of Jackknife and partial plots of the variables in the
258 final model are shown in Supplementary Fig. 2 and Supplementary Fig. 3. A total of 85 of
259 the 226 (37.6%) non-confirmed herds were identified in "high risk" areas (Fig. 4).

260 **Discussion**

261 *Descriptive analysis*

262 Even though antibodies to WNV had been previously detected in different species
263 in Spain, clinical cases in horses or humans were not reported until 2010 (García-
264 Bocanegra et al., 2011b). Since 2010, a total of 403 suspected WNV outbreaks were
265 investigated, and almost half of them (43.9%) were confirmed as outbreaks. The occurrence
266 of WNV **outbreaks** over Spain was not homogeneous, as it was higher in the western part
267 of Andalusia (Fig. 1). However, the temporal evolution confirms important changes in the
268 spread of WNV in the last decade in Spain, in agreement with those previously reported in
269 other European countries (ECDC, 2016).

270 Clinical signs observed in WNV infected horses included both general and nervous
271 signs, being similar to those previously reported (Murgue et al., 2001; Kutasi et al., 2011;
272 Porter et al., 2011; van Galen et al., 2013; Bouzalas et al., 2016). The WNV-specific case
273 fatality found in Spain (21%) was in accordance the values reported in Italy in 2011 (25%)
274 (Cantile et al., 2000; Autorino et al., 2002), but was lower than that reported in other
275 countries such as Hungary in 2008 (29%), Greece in 2010 (30%), France in 2000 (45%)
276 and Morocco in 2003 (56%) (Murgue et al., 2001; Schuffenecker et al., 2005; Kutasi et al.,

2011; van Galen et al., 2013; Bouzalas et al., 2016). Discrepancies in fatality rate among countries may be explained by differences in the viral strains involved or individual factors (Rios et al., 2010; Porter et al., 2011). In this respect, cross-immunity associated to previous exposure to other flaviviruses may also influence the clinical presentation of the disease (Tesh et al., 2002; Rodríguez et al., 2010). Several flaviviruses, including Usutu virus, Bagaza virus and Meaban virus have circulated in the study area in the last decade (García-Bocanegra et al., 2012a, Jurado-Tarifa et al., 2016). Consequently, laboratory analyses are required to confirm or exclude WNV infection.

Given the low viral load and short viremia in horses and humans, the voluntary vaccination in horses as well as the late appearance of clinical symptoms, frequently when the viremia phase is over, confirmation of WNV outbreaks is based on both presence of clinical symptoms and the detection of early IgM antibodies against WNV. Based on that criteria, 177 (43.9%) of the 403 suspected herds were confirmed as WNV outbreaks. VNT positivity was only confirmed in 53.2% of the total confirmed herds. Additionally, two cases were negative by VNT but positive using RT-PCR. Although VNT is recommended by OIE as the gold standard method for WNV diagnosis particularly in areas with circulation of other flaviviruses, our results indicate that the combination of clinical symptoms and detection of IgM antibodies against WNV, may be a good criterion to confirm a case in specific epidemiological scenarios.

A high percentage of WND cases (93.0%; 107 of the 115 WNV positive herds) presented both IgM and IgG antibodies. Experimental WNV infections indicate that IgM antibodies can be found in serum around 7-10 days post-infection (dpi) until 1-3 months, while IgG-specific antibodies can be detected for several years after infection (Ostlund et

al., 2001; Durand et al., 2002; Castillo-Olivares and Wood, 2004; Bouzalas et al., 2016). WNV RNA was only detected in blood in 2.2% of the total analyzed animals from positive herds, which is consistent with the short viremia reported in this species (Bunning et al., 2002). The presence of WNV RNA in 11 of the 18 (61.1%) CFS confirms the higher persistence of the virus in central nervous system as well as the usefulness of using this fluid for RNA WNV detection (Kleiboeker et al., 2004). Although both WNV lineages 1 and 2 have been reported in Europe and the Mediterranean Basin (Calistri et al., 2010; Papa et al., 2011), all RT-PCR positive samples were confirmed as WNV lineage 1. This coincides with previous studies that reported only WNV lineage 1 in birds, horses and mosquitoes in Spain (Jiménez-Clavero et al., 2008; García-Bocanegra et al., 2011b; Vázquez et al., 2011). However, given the active circulation of WNV lineage 2 in other Mediterranean countries during the last few years (Hernández-Triana et al., 2014), its introduction and spread in Spain cannot be ruled out (Fros et al., 2015).

The highest risk period for WNV outbreaks occurrence in Spain ranges between mid-August and mid-November, concentrating 94.4% of the total horse outbreaks. This temporal distribution of WNV outbreaks is consistent with the findings in other Mediterranean countries (Murgue et al., 2001; Autorino et al., 2002; Porter et al., 2011; Kutasi et al., 2011) and USA states with Mediterranean-like climate (CDC, 2017). The outbreaks confirmed consecutively during the last seven years as well as the detection of WNV RNA lineage 1 in 2010, 2012, 2013, 2015 and 2016, suggest an endemic circulation of WNV in Spain. Annual reintroduction of the virus through transportation of migratory infected birds, infected vectors putatively from Africa or overwintering, may explain pathways for the endemic circulation observed in Spain in the last years. Further

phylogenetic analyses are needed to elucidate the origin and evolution of the viruses circulating in Spain.

Spatio-temporal distribution

Andalusia is the region of Spain with the largest populations of horses with about 219,198 animals, and the density ranges between 3.7 and 1.2 horses/km^2 in the western and eastern regions, respectively (MAPAMA, 2016). The provinces of Seville and Cadiz, where the two clusters were detected, were also consistently associated with the detection of the three human cases reported in Spain to date (Fig. 3). The strategic location within important wild birds migratory flyways, the high number of wetlands, environmental conditions, higher density of competent vectors and the high density of horses, are possible factors implicated in the higher spread of WNV in this area.

The immunity of the horse population to WNV is directly related with the natural exposure and the vaccination. Seroprevalence against WNV in horses in Andalusia after the first WNV epidemic was 7.1% (36/510), being significantly higher in areas where the outbreaks were reported (García-Bocanegra et al., 2012). Further studies are required to assess the evolution of the immunity in the horse populations after seven years of WNV circulation in this region. Even though vaccination is known to be an effective measure for WNV prevention and control, because of the cost of vaccines, its application is voluntary and commonly restricted to regions where outbreaks were previously detected. The secondary cluster was identified in the region of Andalusia with highest horses and human population density, which highlights the high risk of WNV potential infection in humans. In fact, confirmed WNV outbreaks in horses is considered an early indicator of the risk of exposure to humans (Saegerman et al., 2016).

MaxEnt modeling

We have shown that MaxEnt modeling can be successfully applied to emerging diseases, in which climatic and environmental characteristics play an essential role. The presented presence-only model showed high accuracy capturing risk areas for WNV outbreaks (AUCc=0.914). The WNV risk map accurately identifies areas at high risk for WNV outbreaks in both humans and animals and thus provides a useful tool to design more accurate and cost-effective surveillance and control programs. Our results were in general consistence with those previously obtained using Mahalanobis distance analysis (Conte et al., 2015). Similar risk areas were also identified by Sánchez-Gómez et al. (2017) using a logistic regression-based spatial model with a limited number of predictors. The suitability areas obtained in our study provide a much higher spatio-temporal resolution than previous studies, allowing to refine the implementation of target interventions. Additionally, the MaxEnt model also highlighted new high risk areas for WNV occurrence (Fig. 4), which provides valuable information to guide surveillance strategies for early detection of new cases in currently free areas. Interestingly, the MaxEnt model identified WNV risk areas in central regions in Andalusia where, although outbreaks have not been reported yet, seropositivity to WNV has been detected in different species including horses, birds and wild ruminants (García-Bocanegra et al., 2012b, García-Bocanegra et al., 2016; Jurado-Tarifa et al., 2016). Moreover, a high percentage (37.6%; 85/226) of non-confirmed herds were located in high suitability areas. This finding, together with the high percentage of non-confirmed herds positive to IgG antibodies (30.8%; 41/133), most of them (45.2%; 14/31) also confirmed by VNT, indicate that the number of WNV outbreaks confirmed in horses in Spain may be underestimated. The notification of WND clinically suspected horses to the official veterinary services, which usually occurs at late stages of the disease,

370 as well the relatively short duration of WNV-specific IgM antibodies, are possible factors
371 that can hamper the confirmation of clinically suspected cases.

372 The final presence-only model mostly relied on climatic and environmental factors
373 to determine the WNV suitability in Spain between 2010 and 2016. Specifically, mean
374 annual temperature and mean annual rainfall which were the most important climatic
375 predictors for WNV occurrence. Areas with mean annual temperature higher than 18º C
376 were found most risk for WNV (Supplementary Fig. 2). Different studies have reported the
377 influence of temperature on the risk of WNV transmission (Ben Hassine et al., 2014; Paz,
378 2015). Increased temperature accelerates both WNV amplification and transmission
379 through the acceleration of the development of competent vectors, increasing the biting
380 behavior, increasing the reproductive rate, increasing the duration of the breeding season,
381 and by reducing the extrinsic incubation period (Chevalier et al., 2014; Paz, 2015).
382 Moreover, areas with annual precipitation between 550 and 1100 mm were found most risk
383 for the presence of WNV (Supplementary Fig. 2). These are consistent with the notion that
384 regions with wet and warm climatic conditions are risk habitats for WNV transmission
385 (Ozdenerol et al., 2013; Mughini-Gras et al., 2014; Paz, 2015). As the impact of rainfall in
386 WNV transmission is not as straightforward as temperature, the influence of rainfall on
387 WNV transmission remains controversial. Even though previous studies demonstrated that
388 the rainfall is positively correlated with both the presence of mosquitoes and WNV
389 outbreaks in humans (Papa et al., 2010; Bisanzio et al., 2011; Hartley et al., 2012;
390 Chevalier et al., 2014), heavy precipitation may have a negative effect on their abundance
391 through the dilution of nutrients and the flushing of the breeding sites (Koenraadt et al.,
392 2008). Furthermore, drought may concentrate resources for both avian reservoirs and

393 mosquitoes, leading to more likely contact between both groups of species.

394 Areas close to Ramsar wetland (< 0.5 km) were also found to be suitable for WNV
395 occurrence (Supplementary Fig. 2). This finding is consistent with previous observations
396 (Rodríguez-Prieto et al., 2012; Valiakos et al., 2014; Bargaoui et al., 2015; Sánchez-Gómez
397 et al., 2017). WNV transmission depends on the co-occurrence in space and time of both
398 virus, competent vectors and susceptible bird hosts. Wetlands provide suitability habitats
399 for mosquito larva presence (Bian et al., 2006). Moreover, Ramsar wetlands present a
400 particularly high diversity and abundance of wild bird species, increasing the risk of WNV
401 transmission to both natural reservoirs and dead-end hosts close to these areas (Valiakos et
402 al., 2014).

403 The final model included the presence of *C. pipiens* as the second most relevant
404 predictor of WNV occurrence. This result is consistent with other studies that identified this
405 mosquito species as the primary vector of WNV in Europe (Hubálek and Halouzka, 1999;
406 Calistri et al., 2010; Chevalier et al., 2014). Although data on the distribution of *Culex*
407 species are still limited in Spain, previous studies have shown that *C. pipiens, C.*
408 *perexiguus, C. theileri* and *C. modestus* are the main *Culex* species detected in western
409 Andalusia (Aranda et al., 2009, Vázquez et al., 2011, García-Bocanegra et al., 2012b). The
410 absence of *C. theileri* in the final model could indicate a less relevant role of this vector
411 species in WNV transmission in the study area. Future studies should be conducted
412 considering other competent vector species (eg. *C. perexiguus* and *C. modestus*) as well as
413 other environmental and climatic variables for which data was not available in this study
414 (e.g., relative humidity, seasonality) to assess the implication of these variables in the WNV
415 occurrence in Spain.

Conclusions

The results obtained in this study contribute to a better understanding of WNV transmission in Spain. The outbreaks confirmed consecutively during the last seven years, as well as the detection of WNV RNA lineage 1 in five different periods, suggest an endemic circulation of the virus in the south part of the country. The spatio-temporal distribution of WNV outbreaks in Spain was not homogeneous, as significant clusters were detected in western Andalusia in two non-consecutive years. Additionally, the highest number of outbreaks reported in 2016 compared to the previous years and the expansion to northern areas outside Andalusia province, suggests an active circulation and expansive trend. Therefore, there is a potential risk of WNV spread to previously unaffected areas within the Iberian Peninsula in the following years. The identification of WNV risk areas and spatio-temporal clusters in this study can serve to inform risk-based, more cost-effective strategies towards better prevention and control of WND in Spain. In order to better prevent future WNV cases in the horses and humans, specific measures, including vaccination programs, risk-based surveillance and outreach and communication of horse owners and the general public should be implemented in the identified areas.

Conflict of interest statement

None of the authors of this study has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Figure legends

Fig. 1. Spatial distribution of the 177 confirmed WNV outbreaks in Spain between 2010 and 2016. Black dots indicate outbreaks in horse herds.

Fig. 2. Frequency in which different clinical symptoms associated to WNV infection were observed in herds affected by WND in Spain (2010-2016). Black and grated bars indicate general and nervous symptoms, respectively.

Fig. 3. Temporal evolution (in weeks) of WNV outbreaks in Spain during the period 2010-2016.

Fig. 4. Map of Andalusia (southern Spain) showing high risk areas for WNV occurrence. Color gradient represents the WNV occurrence risk. Black and green dots indicate the confirmed and non-confirmed WNV outbreaks in horses, respectively. Yellow crosses indicate WNV outbreaks in human.

Table legends

Table 1. Results of the laboratory analyses for WNV in horse herds between August 2010 and November 2016, in southern Spain.

Table 2. Results of the space-time permutation model for WNV outbreaks in horses between August 2010 and November 2016, in Andalusia, southern Spain.

TABLE 1 Results of the laboratory analyses for West Nile virus (WNV) in horse herds between August 2010 and November 2016, in southern Spain

Laboratory diagnostics	% Confirmed herds (positive/tested)	% Non-confirmed herds (positive/tested)	% Investigated herds (positive/tested)
IgM antibodies	100 (177/177)	0.0 (0/226)	43.9 (177/403)
IgG antibodies	93.0 (107/115)	30.8 (41/133)	59.7 (148/248)
Neutralizing antibodies	53.2 (50/94)	45.2 (14/31)	51.2 (64/125)
WNV RNA	7.9 (14/177)	0.0 (0/155)	4.2 (14/332)

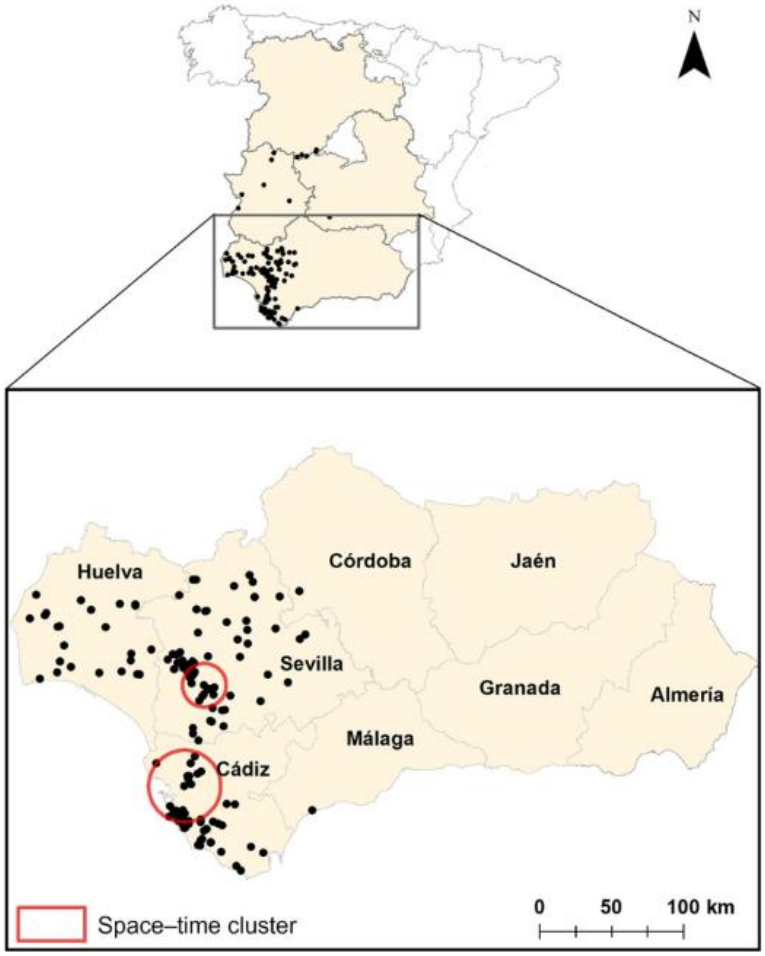

FIGURE 1 Spatial distribution of the 177 confirmed West Nile virus outbreaks in Spain between 2010 and 2016. Black dots indicate outbreaks in horse herds [Colour figure can be viewed at wileyonlinelibrary.com]

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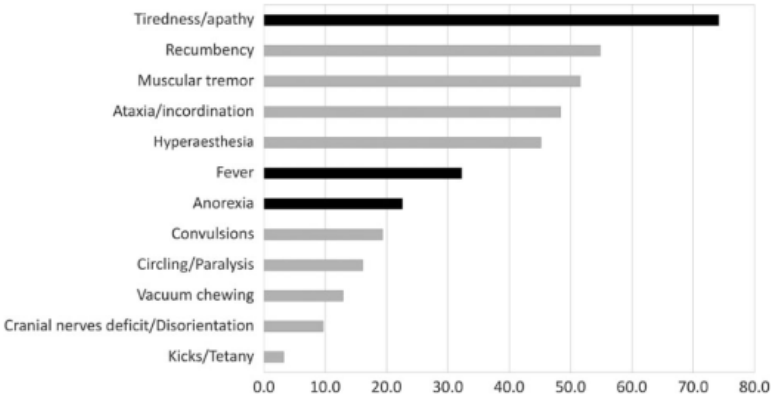

FIGURE 2 Frequency in which different clinical symptoms associated to West Nile virus infection was observed in herds affected by West Nile disease in Spain (2010–2016). Black and grated bars indicate general and nervous symptoms, respectively

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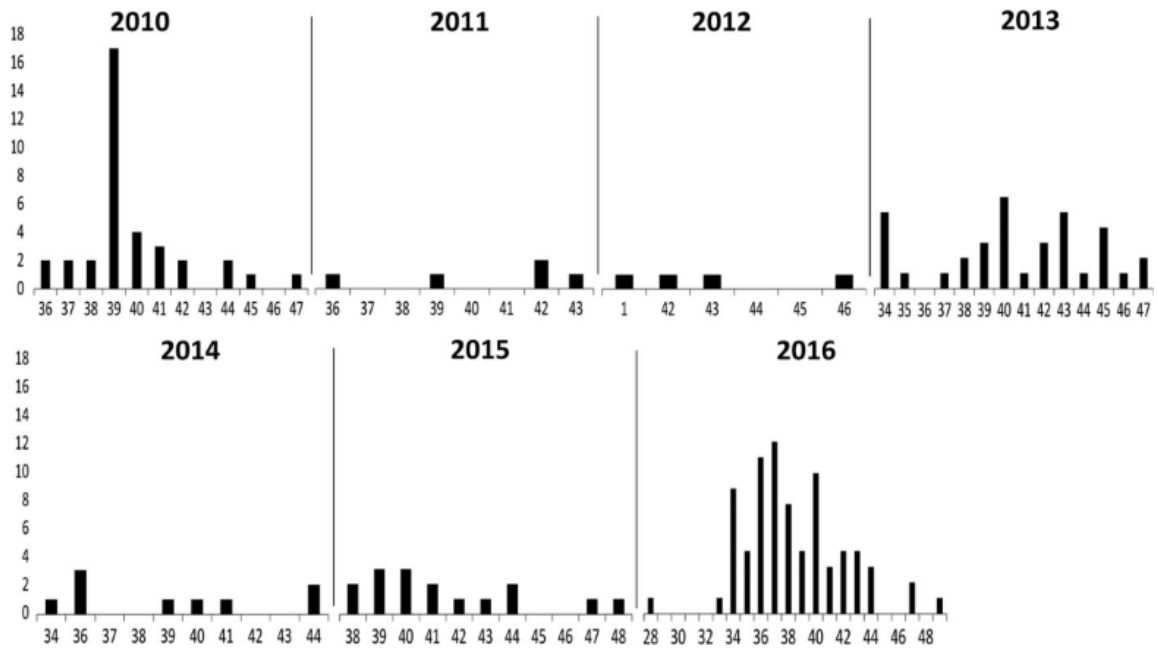

FIGURE 3 Temporal evolution (in weeks) of West Nile virus outbreaks in Spain during the period 2010–2016

TABLE 2 Results of the space–time permutation model for West Nile virus outbreaks in horses between August 2010 and November 2016, in Andalusia, southern Spain

Cluster	Radius (km)	Cluster time frame	No. of observed cases	No. of expected cases	Observed/expected	*p*-Value
1	25.00	31 August to 29 September 2010	36	8.45	4.26	<.001
2	15.17	4 August to 7 September 2016	34	7.62	4.46	<.001

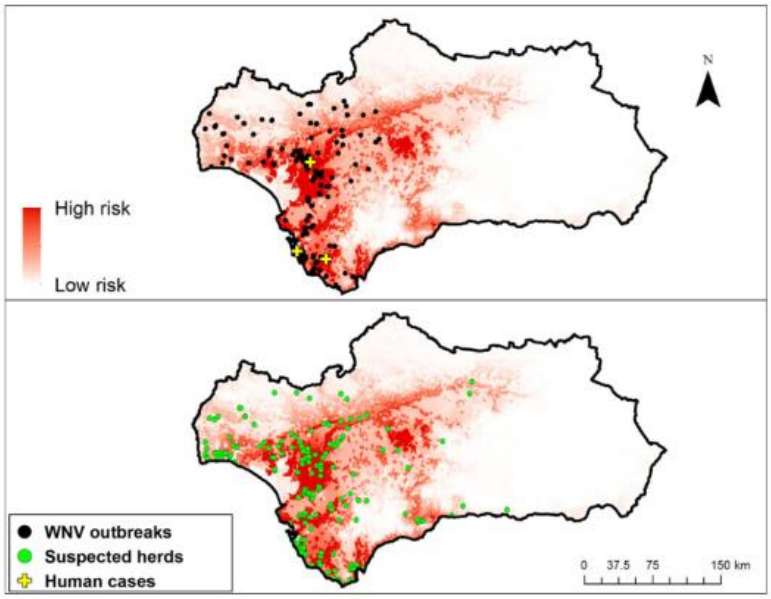

FIGURE 4 Map of Andalusia (southern Spain) showing high-risk areas for West Nile virus (WNV) occurrence. Colour gradient represents the WNV occurrence risk. Black and green dots indicate the confirmed and non-confirmed WNV outbreaks in horses, respectively. Yellow crosses indicate WNV outbreaks in human [Colour figure can be viewed at wileyonlinelibrary.com]