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Serological evidence of co-circulation of West Nile and Usutu viruses in equids from western Spain

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Abstract

West Nile virus (WNV) is a mosquito-borne emerging virus in Europe with capacity to cause neurological complications such as encephalitis or meningoencephalitis in humans, birds or equids. In Spain, WNV is actively circulating in mosquitoes, birds and horses in different regions, but never has been deeply studied in Extremadura. Therefore, the aim of this study was to evaluate the seroprevalence of WNV in equids of those areas and to analyse the risk factors associated with exposure to the virus. A total of 199 out of 725 equids presented antibodies against WNV by competition ELISA (27.45%), while 22 were doubtful (3.03%). Anti-WNV IgM antibodies were detected in 16 equids (2.21%), and 3 animals were doubtful (0.41%). All ELISA-reactive positive/doubtful sera ($N = 226$) were further tested by micro-virus neutralization test (VNT), and a total of 143 horses were confirmed as positive for WNV, obtaining a seroprevalence of 19.72% in equids of western Spain. In addition, specific antibodies against USUV were confirmed in 11 equids. In 24 equids, a specific flavivirus species (detected by ELISA test) could not be determined. The generalized linear mixed-effects models showed that the significant risk factors associated with individual WNV infection in equids were the age (adults) and hair coat colour (light), whereas in USUV infections, it was the breed (pure). Data demonstrated that WNV and USUV are circulating in regions of western Spain. Given the high WNV seroprevalence found in equids from the studied areas, it is important to improve the surveillance programmes of public health to detect undiagnosed human cases and to establish a vaccination programme in equid herds in these regions.

KEYWORDS
Flavivirus, horses, risk factors, seroprevalence, vector-borne pathogens

1 | INTRODUCTION

West Nile virus (WNV) is a mosquito-borne zoonotic agent belonging to the genus *Flavivirus*, family Flaviviridae (Angenvoort, Brault, Bowen, & Groschup, 2013). Mosquitoes are the vectors and birds are the main reservoir. Mammals, such as humans and equids, are generally considered dead-end hosts, as viraemia resulting from WNV infection is insufficient to contribute to the amplification cycle (Bunning et al., 2002; Sotelo, Fernández-Pinero, & Jiménez-Clavero, 2012). WNV infections rarely result in clinical disease

but can occasionally cause outbreaks that seriously affect animals and human health (Beck et al., 2013). Although the incidence of WNV has been estimated to be below 10% in equids, mortality rates can approach 50% among those that present clinical disease (Angenvoort et al., 2013).

Antibodies against WNV have been detected in equids from different Spanish regions. In Majorca Island, a study of seroprevalence showed a seropositivity rate of 6.4% to WNV (Vanhomwegen et al., 2017). In Doñana National Park (Huelva), south-west Spain, WNV seropositivities of 8.3% and 2.6% were detected in 2005 and 2007, respectively, in feral horses (Jiménez-Clavero et al., 2010; Jiménez-Clavero, Tejedor, Rojo, Soriguer, & Figuerola, 2007). García-Bocanegra et al. (2012) found that 7.1% of the horses of different Andalusian provinces (southern Spain) presented serological evidence of infection by WNV. In central Spain, 1.35% of horses were WNV-seropositive (Abad-Cobo et al., 2017).

Phylogenetic analysis revealed that WNV strains are grouped into nine genetic lineages, with lineage 1 and lineage 2 currently associated with disease outbreaks (Christova et al., 2020; Petersen, Brault, & Nasci, 2013). Prior to 2004, only lineage 1 strains were detected in animals and humans in Europe (Zeller & Schuffenecker, 2004). Lineage 2 was first isolated in 2004 in Hungary and Russia; since then, this lineage has dispersed to eastern, central/southern European countries, causing hundreds of cases in humans, birds and horses (Hernández-Triana et al., 2014; de Heus et al., 2019; Ravagnan et al., 2015; Rizzoli et al., 2015; Savini et al., 2012). Currently, lineage 2 predominates in Europe in different species (Vilibic-Cavlek et al., 2019). In Spain, WNV outbreaks in horses have been detected since 2010 until 2019 (European Centre for Disease Prevention & Control, 2019; García-Bocanegra et al., 2012, 2018), produced mainly by lineage 1 (García-Bocanegra et al., 2011; Ministerio de Agricultura, 2019). However, WNV lineage 2 has also been detected in Catalonia (northern Spain) in northern goshawks (*Accipiter gentilis*) (Busquets et al., 2019) and in mosquitoes (Busquets, Alba, Allepuz, Aranda, & Núñez, 2008; Vázquez et al., 2011).

Usutu virus (USUV) was firstly identified in Europe in 2001, when it was determined to be the causative agent of a mass mortality event in birds (Weissenböck et al., 2002). According to several studies, USUV seems to be actively circulating in mosquitoes, birds and horses from different regions of Spain (Ferraguti et al., 2016; Höfle et al., 2013; Jurado-Tarifa et al., 2016; Llorente et al., 2013; Vázquez et al., 2011; Roiz et al., 2019) but there is no information about it in the Extremadura region and other bordering areas.

The objective of this study was to assess the presence and distribution of WNV seropositivity in equids from western Spain, including the provinces of Badajoz and Cáceres and bordering areas of Huelva, Salamanca and Toledo. The study also analyses the possible presence of USUV in those areas and the different risk factors associated with WNV and USUV infections in horses.

2 | MATERIAL AND METHODS

2.1 | Study design and data collection

A cross-sectional survey was carried out in 2018 and 2019 to analyse the seroprevalence of WNV in equid herds from western Spain, including mainly the provinces of Badajoz and Cáceres and some herds of Huelva, Salamanca and Toledo. In addition, few samples ($N = 2$) from 2017 were also added to the analysis. This area is characterized by a continental Mediterranean climate, with hot and dry summers and mild winters.

The sample size was estimated using Win Episcope 2.0 (www.winepi.net) with an expected prevalence of 8% (according to previous studies in Spain) and an expected error of 2.5%. In 2018, the equine census in studied area was approximately 40.569 animals (Ministerio de Agricultura, 2019). The calculated sample size was 453. We increased up to 766 serum samples to have enough samples representing the different areas.

Blood samples were collected from equids (743 horses, 6 mules and 17 donkeys), of which 41 had been previously vaccinated against WNV. Blood samples were centrifuged at 1,006 *g* for 10 min at 4°C within the next 24 hr after collection, to separate the serum, which was stored at −20°C until further analysis. Vaccinated animals were excluded from the study.

For each sampled animal, an accompanying form with information of the farm and the location, as well as on the animal (age, vaccination status, movements, among others), was filled. One animal died one week after taking the blood sample, and the animal was necropsied. Different tissues were analysed for the presence of WNV and USUV by multiplex real-time RT-PCR as detailed in 'Viral genome detection assays' section.

2.2 | Antibody detection assays

2.2.1 | ELISA tests

Serum samples were tested by two commercial ELISA kits for the presence of WNV total antibodies (INgezim West Nile Compac, Ingenasa, Spain) and WNV-specific IgM (INgezim West Nile IgM, Ingenasa, Spain) following the manufacturer's instructions. Positive and doubtful sera in ELISA tests were then confirmed for WNV-specific antibodies by using a micro-virus neutralization test (VNT), performed as described below.

2.2.2 | Virus-neutralization test (VNT)

West Nile virus-neutralizing antibodies were titrated by VNT, the OIE (World Organization for Animal Health)-prescribed gold standard method for serological diagnosis of WNV, using 96-well microtitre plates as previously described (Llorente et al., 2019). VNTs were performed in parallel against WNV strain Eg-101 (GenBank accession no.

AF260968) and USUV SAAR-1776 (GenBank accession no. AY453412), in order to differentiate specific neutralizing antibody responses against these two different flaviviruses that are known to cross-react in the ELISA tests, and probably co-circulate in the studied area. Neutralizing immune response was assigned as specific for the virus giving VNT titre at least fourfold higher than the titre obtained for the other virus (Calisher et al., 1989). When titre differences did not reach this threshold, the result was considered inconclusive and the virus which caused the infection was considered an undetermined flavivirus.

2.3 | Viral genome detection assays

2.3.1 | Nucleic acid extraction

Viral RNA was extracted from tissue homogenates (blood, hair, serum, spleen, lung, brain, cerebellum, heart, liver and kidney), using a previously described method (Pérez-Ramírez et al., 2017).

2.3.2 | Real-time RT-PCR

Viral genome load was measured by real-time RT-PCR, using a previously described method that detects and differentiates lineage 1 WNV, lineage 2 WNV and USUV in a single reaction (del Amo et al., 2013). Lineage 1 WNV (NY99 strain), lineage 2 WNV (B956 strain) and USUV (SAAR strain) RNAs were included as positive controls in RT-PCR assay obtaining an expected Ct value of 30 ± 2 for each target. Negative controls, without detectable Ct value, were also included. Samples with Ct value >40.00 were considered negative.

2.4 | Statistical analysis

Descriptive statistics with frequencies and percentages were generated for each of the pathogen identified. The prevalence of antibodies against WNV, USUV and other flaviviruses was estimated with the exact binomial confidence intervals of 95%. Generalized linear mixed-effects models (GLMM) with a 'logit' link function and binomial distribution were used to investigate which risk factors were associated with WNV and USUV seroprevalence in equids from the studied areas. Separate models were fitted for each virus. The infection status of each individual (infected or uninfected) for each virus was included as the dependent variable, while gender (male and female), age (young: <5 years; adult: 5–20 years; and geriatric: >20 years), breed (crossbreed, purebred and other equids), body score (according to Henneke scale: thin: <4; moderate: 4–7; and fat: >7), colour (light and dark), type of housing (stall, pasture and mix), aptitude (leisure sport and reproduction), competition (yes and not), water (presence or absence of at least one pond within a 100 m radius from the herd), hypersensitivity to insect bites (yes and not), size of herd (small: <5; medium: 6–19; and big: >20) and seasons (spring, summer, autumn and winter) were included as categorical independent explanatory variables. The province, the equid exploitation nested in province and the year of sampling were included as random factors to account for the temporal and geographical stratification of the sampling design. The chi-square tests were calculated using an ANOVA type III, and post hoc analyses were performed using Tukey contrasts test when necessary. For each GLMM, we tested the collinearity between independent variables by using the variance inflation factor (VIF) (Zuur, Ieno, & Elphick, 2010). The GLMM overdispersion was checked using the Pearson statistic (ratio of the Pearson chi-square to its degrees of freedom), a common method used for assessing the deviance of goodness-of-fit statistics (Rodríguez, 2010). We found no evidence of collinearity between the variables included in the models or of overdispersion, as the Pearson dispersion statistics were always close to 1. All statistical analyses were conducted in R (v. 3.6.3; The R Foundation for Statistical Computing Platform 2020) using the packages: arm, car, lme4, MuMIn, multcomp, MASS, Matrix, Rcpp and stats.

3 | RESULTS

The cross-sectional study included 725 equids. Overall, 199 (27.45%, 95% CI: 24.26%–30.88%) presented antibodies to WNV by competition ELISA, while 22 (3.03%, 95% CI: 2.01%–4.55%) were doubtful. Anti-WNV IgM antibodies were detected in 16 equids (2.21%, 95% CI: 1.36%–3.55%), and 3 animals yielded doubtful results in this IgM assay (0.41%, 95% CI: 0.14%–1.21%) (Table 1).

Serologically positive and doubtful sera (including IgM) ($N = 226$) were subjected to VNT, confirming the presence of anti-WNV-specific antibodies in 143 equids (63.27%). Therefore, the actual WNV seroprevalence in equids from our study was 19.72% (95% CI: 16.66%–22.78%). The VNT also showed that 11 equids, positive or doubtful by ELISA tests, were positive for USUV (1.52%, 95% CI: 0.85%–2.70%) and other 24 positive/doubtful animals gave VNT titres that did not allow to differentiate between WNV and USUV antibody responses, thus being considered as infections by undetermined flavivirus (3.31%, 95% CI: 2.23%–4.88%) (Table 1).

In horses showing WNV-specific antibodies, neutralizing titres against WNV ranged from 1:20 to ≥1:1,280. The number and percentage of animals with NtAb against WNV according to each titre are shown in Table 1. The 40.5% of WNV-seropositive sera showed equal or higher than 1:320. Only one USUV-seropositive animal (out 11) showed a titre greater than 1:80 against USUV (Table 1).

At the herd level, 44 out of the 69 equine herds (63.77%; 95% CI: 51.98%–74.10%) had at least one WNV-seropositive animal, confirmed by VNT.

The distribution of positive herds to WNV, USUV or undetermined flaviviruses is represented in Figure 1. Herds with seropositive

TABLE 1 Total of positive and doubtful individuals according to ELISA test (competition and IgM; $N = 725$) and VNT ($N = 226$) techniques. Titres obtained by VNT in samples with WNV- and USUV-specific antibodies are shown

	ELISA		VNT		
	No of WNV-positive (%)	No of WNV doubtful (%)	No of WNV[a] (%)	No of USUV[a] (%)	No of undetermined flaviviruses[a] (%)
Competition	199 (27.45)	22 (3.03)	143 (19.72)	11 (1.52)	24 (3.31)
IgM	16 (2.21)	3 (0.41)			

No of individuals with specific neutralizing Abs according to VNT titres (%)		
Titres	**WNV**	**USUV**
1:10	0 (0)	2 (18.18)
1:20	12 (8.39)	5 (45.45)
1:40	13 (9.09)	1 (9.09)
1:80	31 (21.67)	2 (18.18)
1:160	29 (20.28)	1 (9.09)
1:320	26 (18.18)	0 (0)
1:640	22 (15.28)	0 (0)
≥1:1,280	10 (6.99)	0 (0)

[a]The percentage represents the individuals with specific WNV and USUV antibodies or with undetermined flaviviruses detected by VNT.

animals to WNV and USUV were distributed throughout the areas of study, including the provinces of Cáceres and Badajoz and close areas in Toledo and Huelva.

There were 13 horses with neurological signs compatible with WNV within 3 months prior to sampling, among which 5 were WNV-seropositive by IgM and competition ELISA, 4 were only seropositive by competition ELISA, and 4 were negative by both ELISA kits. Eight out of 13 horses with signs had neutralizing antibodies against VNT. The analysis of the sera obtained in these horses by RT-PCR was negative. One of the symptomatic horses died one week after blood sampling, and different tissues (spleen, lung, liver, heart, kidney, hair, brain and cerebellum) were collected at necropsy and analysed by RT-PCR. WNV lineage 1 RNA was detected in brain and cerebellum.

Prevalence of antibodies against WNV, USUV or undetermined flaviviruses depending upon different conditions (age, hypersensitivity to insect bites, aptitude, breed, body score, hair coat colour, competition, gender, presence of water, season, size of herd and type of housing) is shown in Table 2.

A total of 12 explanatory variables were tested in the statistical analysis. The GLMM showed that the significant risk factors associated with the individual risk of infection by WNV were as follows: age and hair coat colour (Table 3). Briefly, young equids showed less WNV seroprevalence than adults (Tukey post hoc test: estimate (±SE) = −0.76 ± 0.27, $z = -2.83$, $p < .001$). Also, dark hair coat animals presented less WNV antibodies than light individuals (−0.41 ± 0.20, $z = -2.04$, $p = .04$). No significant association was found with other risk factors (Table 3).

The analysis of risk factors for USUV infection is showed in Table 4. Thereafter, purebred horses showed higher levels of infection than crossbreed (Tukey post hoc test: estimate (±SE) = 1.56 ± 0.77, $z = 2.02$, $p = .04$).

4 | DISCUSSION

In this study, the seroprevalence observed for WNV in equids from western Spain was 19.7%, higher than observed in previous reports of the same kind in Spain, for example 8.3% WNV seropositivity in feral horses tested in Huelva between 2005 and 2008 (Jiménez-Clavero et al., 2010), and some years later, a 7.1% overall seroprevalence in equids from different provinces of Andalusia (south of Spain) (García-Bocanegra et al., 2012). Similar results were obtained in the Balearic Islands by Vanhomwegen et al. (2017), where 6.6% of horses showed antibodies against WNV. Between 2011 and 2013, a much lower seroprevalence was found in horses from central Spain (1.35%) (Abad-Cobo et al., 2017). In Portugal, the bordering country of the studied area, an analysis in 1,313 horses' serum samples showed a prevalence of 3% (Barros et al., 2011). In other Mediterranean countries, the prevalence was 31.1% in Morocco (Benjelloun et al., 2017), 17.4% in Algeria (Lafri et al., 2017), 8.5% in southern France (Durand et al., 2002) or 3.43% in Croatia (Barbić et al., 2012).

Although some of these different studies used different sampling or testing methodologies that could partially explain the differences observed in seroprevalence results, this cannot explain the great differences observed in previous studies from neighbouring areas of Spain, with comparable methodologies employed. Therefore, other factors may be implicated in the high WNV presence in the western areas of Spain, especially in the Extremadura region. In 2004, the first human case of WNV in Spain was reported from a resident of Barcelona, who was infected in Badajoz, one of the provinces included in this study (Kaptoul et al., 2007). Besides, WNV outbreaks in equids have been reported in this area since 2015 (Ministerio de Agricultura, 2019). It might occur that WNV circulation in this region

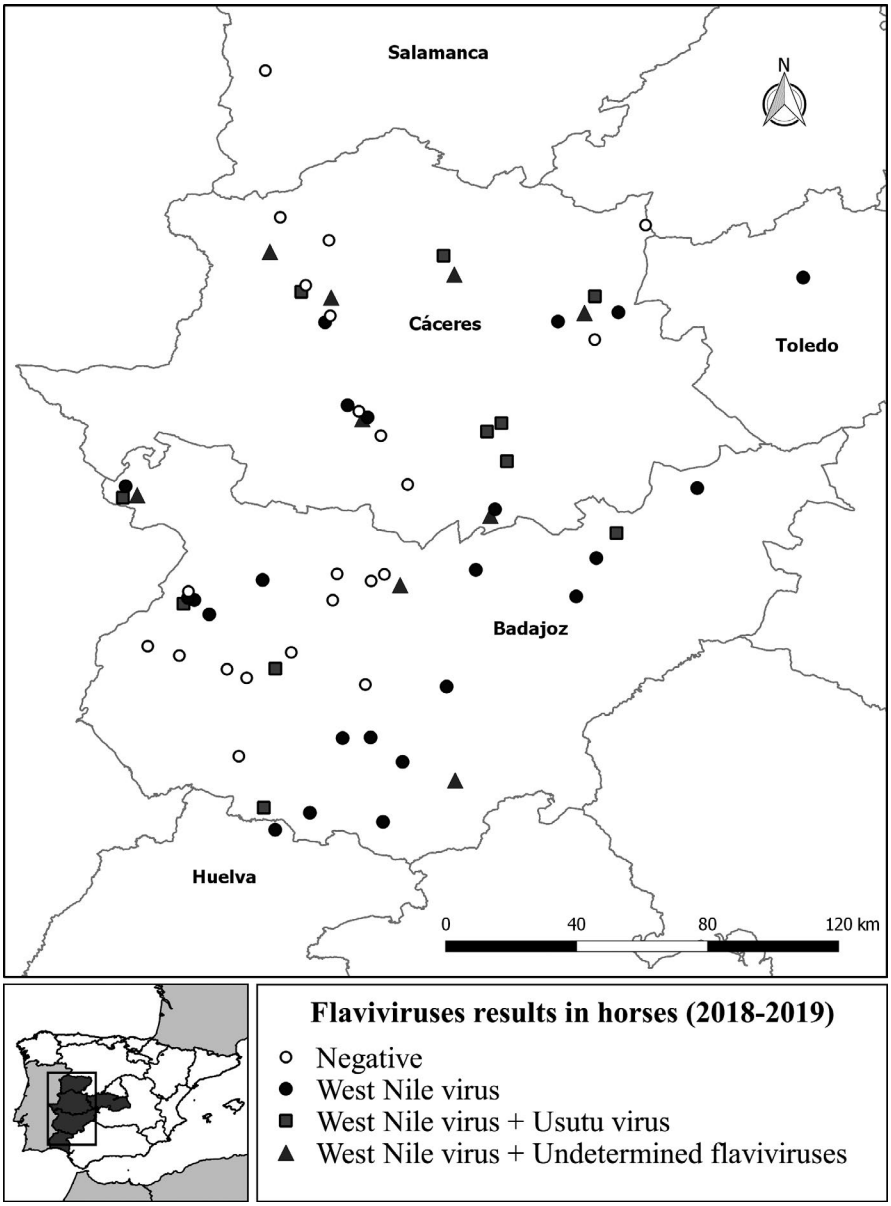

FIGURE 1 Distribution of flaviviruses in western Spain based on the geographical origin of the equine herds analysed. Map created by QGIS. 3.12.0 (2020). QGIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.org

has remained overlooked for years. This area presents favourable conditions for the maintenance and circulation of WNV. There are several natural areas included in the RAMSAR convention wetland and numerous natural protected areas with high density of birds and mosquitoes. On the other hand, the studied area is the Spanish region with the largest freshwater reserve of the country, which would facilitate the presence of competent vectors. The *Culex pipiens* s.l. mosquito species has been shown to be the main vector of WNV to equids in Europe (Martinet, Ferté, Failloux, Schaffner, & Depaquit, 2019). Previous studies have described *Cx. pipiens* as the most frequent and widespread mosquito species in Extremadura, followed by *Culex theileri* and *Culex univittatus* (Gangoso et al., 2020; Mixão et al., 2016), all of them highly competent vectors of WNV.

Finally, this study was carried out between 2017 and 2019. It is also important to point out that in Europe, the 2018 season was characterized by an unusual increase in the number of human and equid cases compared with records from previous years (see picture for human cases at https://www.ecdc.europa.eu/en/news-events/epidemiological-update-west-nile-virus-transmission-season-europe-2018). The most likely explanation for this trend is that weather conditions, elevated temperatures and precipitation anomalies, and potentially other environmental factors, were favourable for the earlier and bigger expansion of the vector population (Aberle et al., 2018; Bakonyi et al., 2017). Apparently, this was not the case for Spain, and particularly for Extremadura and adjacent regions, where reported WNV cases did not differ significantly from previous seasons. Our data, however, support a more intense transmission season in 2018 and early 2019 in the surveyed area, pointing out a possible underestimation of cases in the official reports (see map at https://www.ecdc.europa.eu/en/publications-data/west-nile-virus-outbreaks-among-equids-european-union-2018-transmission-season). This study remarks the need for reinforcement of monitoring and surveillance programmes for WNV in this area implemented by public administrations, together with other support actions such as raising awareness, motivating and

TABLE 2 Frequency of antibodies against WNV, USUV and undetermined flaviviruses in equids from western Spain using ELISA and VNT

Variable	Categories	No of samples	No of WNV-positive (%)	No of USUV-positive (%)	No of undetermined flavivirus positive (%)
Age	Young	202	26 (12.87)	1 (0.49)	6 (2.98)
	Adult	419	95 (22.67)	9 (2.15)	14 (3.34)
	Geriatric	104	22 (21.15)	1 (0.96)	4 (3.85)
Hypersensitivity to insect bites	Yes	89	18 (20.22)	1 (1.12)	5 (5.62)
	No	636	125 (19.65)	10 (1.57)	19 (2.99)
Aptitude	Leisure and sport	529	103 (19.47)	8 (1.51)	17 (3.21)
	Reproduction	196	40 (20.41)	3 (1.53)	7 (3.57)
Breed	Crossed	332	67 (20.18)	3 (0.90)	11 (3.31)
	Other equidae	22	4 (18.18)	0 (0)	1 (4.55)
	Pure breed	371	72 (19.41)	8 (2.16)	12 (3.23)
Body score	Thin	49	12 (24.49)	2 (4.08)	1 (2.04)
	Moderate	572	111 (19.41)	6 (1.05)	19 (3.32)
	Fat	104	20 (19.23)	3 (2.88)	4 (3.85)
Hair coat colour	Light	366	84 (22.95)	5 (1.37)	11 (3.01)
	Dark	359	59 (16.43)	6 (1.67)	13 (3.62)
Competition	Yes	114	22 (19.30)	1 (0.88)	6 (5.26)
	No	611	121 (19.80)	10 (1.64)	18 (2.95)
Gender	Female	373	76 (20.38)	7 (1.88)	15 (4.02)
	Male	352	67 (19.03)	4 (1.14)	9 (2.56)
Presence of water	Yes	535	117 (21.87)	11 (2.06)	19 (3.55)
	No	190	26 (13.68)	0 (0)	5 (2.63)
Season	Autumn	371	67 (18.06)	6 (1.62)	16 (4.31)
	Spring	71	16 (22.54)	1 (1.41)	1 (1.41)
	Summer	178	37 (20.79)	2 (1.12)	5 (2.81)
	Winter	105	23 (21.90)	2 (1.09)	2 (1.09)
Size of herd	Big	395	77 (19.49)	6 (1.52)	6 (1.52)
	Medium	283	58 (20.49)	4 (1.41)	18 (6.36)
	Small	47	8 (17.02)	1 (2.13)	0 (0)
Type of housing	Stall	262	49 (18.70)	3 (1.15)	9 (3.44)
	Pasture	385	78 (20.26)	7 (1.82)	11 (2.86)
	Mix	78	16 (20.51)	1 (1.28)	4 (5.13)
Total		725	143	11	24

Note: Age class (young <5 years, adult 5–20 years and geriatric >20 years); body score according to Henneke scale (thin <4, moderate 4–7 and fat >7); size of herd (small <5 horses, medium 6–19 horses and big >20 horses).

training clinical veterinarians to better prevent, detect and alert on this disease, which would likely improve not only to mitigate its effect on the equine populations, but also on public health.

Despite the high seroprevalence observed, only 5.35% of the equids were vaccinated against WNV in the studied area. In EEUU, the introduction of a vaccine in 2002 resulted in a remarkable reduction in the subsequent number of equine encephalitis cases in 2003, in contrast to the large increase in human cases (Ng et al., 2003). Therefore, equine vaccination should be encouraged and carried out to reduce the risk of WNV infection in horses in western Spain.

It is noteworthy that 19 animals (3 of them doubtful) presented WNV ELISA-IgM antibodies, of which 12 were confirmed positive by VNT to WNV or undetermined flaviviruses. These samples (positive IgM ELISA + VNT) were taken between September and November 2018 ($N = 8$) and between September and October 2019 ($N = 3$). One additional WNV IgM-positive horse was sampled at the beginning of March 2019. The presence of high WNV IgM antibodies is the best serological indication for a recent infection, as in horses persistence of these antibodies is less than three months (Angenvoort et al., 2013; Castillo-Olivares et al., 2011). Therefore, our results confirm that there has been an active circulation of the virus among

TABLE 3 Results of the GLMM analysing the relationships between the characteristics of individual equids (gender, age, body score, breed, hair coat colour, aptitude, competition, hypersensitivity to insect bites), herd characteristics (type of housing, size of herd, presence of water) and season, and the seroprevalence of WNV infection ($N = 725$)

Variable	Category	Estimate (±SE)	χ^2	*df*	*p*-value
Intercept	—	−0.98 (0.56)	3.06	1	.08
Gender	Females	0.00[a]	0.08	1	.78
	Males	−0.06 (0.23)			
Age	Adults	0.00[a]	8.13	2	.02
	Geriatrics	−0.05 (0.3)			
	Foals	−0.76 (0.27)			
Body score	Thin	0.00[a]	1.87	2	.39
	Normal	−0.34 (0.39)			
	Fat	−0.63 (0.47)			
Breed	Crossbreed	0.00[a]	0.19	2	.91
	Other equidae	−0.16 (0.63)			
	Purebred	−0.1 (0.26)			
Hair coat colour	Light	0.00[a]	4.07	1	.04
	Dark	−0.41 (0.2)			
Aptitude	Leisure and sport	0.00[a]	0.43	1	.51
	Reproduction	0.22 (0.33)			
Type of housing	Stall	0.00[a]	0.72	2	.69
	Pasture	0.01 (0.3)			
	Mix	−0.32 (0.42)			
Competition	No	0.00[a]	0.19	1	.66
	Yes	−0.15 (0.33)			
Presence of water	No	0.00[a]	1.42	1	.23
	Yes	0.4 (0.34)			
Hypersensitivity to insect bites	No	0.00[a]	0.03	1	.87
	Yes	−0.05 (0.32)			
Seize of herd	Big	0.00[a]	0.80	2	.67
	Medium	0.03 (0.32)			
	Small	−0.42 (0.52)			
Season	Autumn	0.00[a]	1.09	3	.78
	Spring	0.18 (0.47)			
	Summer	−0.26 (0.38)			
	Winter	0.17 (0.38)			

[a]Reference category.

horses in western Spain in the last 2 years (2018 and 2019). Possibly, the horse IgM-seropositive sampled in March 2019 had been probably infected in November–December 2018, confirming that year as one with the longest transmission season (European Centre for Disease Prevention & Control, 2019) or the WNV transmission could probably occur as early in the year, such as during the first three months of the year 2019.

The RT-PCR had identified WNV lineage 1 in the brain and cerebellum of a dead horse included in the study. Before the identification of WNV lineage 2 in Europe in 2004, all WNV outbreaks had been associated with lineage 1 (Bakonyi et al., 2006; Vilibic-Cavlek et al., 2019). In fact, WNV lineage 1 has been detected previously in birds, horses and mosquitoes from Spain (Vázquez et al., 2011; Sotelo et al., 2012) and it is still predominant in wide areas of Spain where WNV circulation is present (Sánchez-Gómez et al., 2017). According to our results, it seems that lineage 1 is currently widespread in western Spain and the finding of WNV lineage 1 in an outbreak affecting 2 owlets in the same area of study further corroborates this situation (unpublished data). However, in 2018, lineage 2 was detected for the first time in Catalonia (north-eastern Spain) in two northern goshawk individuals by passive surveillance (Busquets et al., 2019) and an additional WNV lineage with

TABLE 4 Results of GLMM analysing the relationships between the characteristics of individual equids (gender, age, body score, breed, hair coat colour, aptitude, competition, hypersensitivity to insect bites), herd characteristics (type of housing, size of herd, number of equids, presence of water) and season, and the seroprevalence of USUV ($N = 725$)

Variable	Category	Estimate (±SE)	χ^2	*df*	*p*-value
Intercept	—	−20.56 (162.93)	0.02	1	.89
Gender	Females	0.00[a]	1.03	1	.31
	Males	−0.75 (0.73)			
Age	Adults	0.00[a]	2.01	2	.36
	Geriatrics	−0.68 (1.12)			
	Foals	−1.48 (1.11)			
Body score	Thin	0.00[a]	3.69	2	.16
	Normal	−17.54 (0.93)			
	Fat	−1.01 (1.02)			
Breed	Crossbreed	0.00[a]	4.09	2	.13
	Other equidae	−17.55 (591.21)			
	Purebred	1.56 (0.77)			
Hair coat colour	Light	0.00[a]	0.51	1	.47
	Dark	0.47 (0.66)			
Aptitude	Leisure and sport	0.00[a]	0.76	1	.38
	Reproduction	−0.77 (0.89)			
Type of housing	Stall	0.00[a]	0.38	2	.83
	Pasture	−0.36 (0.86)			
	Mix	−0.78 (1.3)			
Competition	No	0.00[a]	0.46	1	.49
	Yes	−0.83 (1.22)			
Presence of water	No	0.00[a]	0.01	1	.91
	Yes	18.8 (162.93)			
Hypersensitivity to insect bites	No	0.00[a]	0.08	1	.77
	Yes	−0.32 (1.11)			
Seize of herd	Big	0.00[a]	0.14	2	.93
	Medium	−0.29 (0.77)			
	Small	−0.07 (1.19)			
Season	Autumn	0.00[a]	2.50	3	.47
	Spring	−0.63 (1.19)			
	Summer	−1.47 (0.94)			
	Winter	−0.23 (1.01)			

[a]Reference category.

a common evolutionary branch with lineage 4 was detected in mosquitoes in southern Spain (Vázquez et al., 2010). So, it will be important to keep WNV surveillance and to recognize the possible introduction of new lineages, such as lineage 2, associated with pathogenic potential in horses.

According to our results, there was a significant risk for WNV infection in horses associated with age, being adults (6–19 years) those with the highest probability of exposure compared to young animals (<5 years). Cardinale et al. (2017) found that the older horses (between 11 and 15 years old) had the higher percentage of WNV antibodies, and Hassine et al. (2014) also observed that risk of WNV infection increased with the age of animals. In other studies, no significant differences among age classes were observed in horses (García-Bocanegra et al., 2012; Vanhomwegen et al., 2017). Assuming that anti-WNV IgG usually persists several years after infection, our results would suggest the presence of an endemic and recurrent circulation of WNV over the time in the studied areas.

Our study also showed significant higher seroprevalence in the light hair coat colour ($N = 366$; 22.95%) as compared to the dark colour ($N = 359$; 16.43%). There are few studies that analyse this variable with seroprevalence in equids. Our results are consistent with those of Aharonson-Raz et al. (2014) in horses from Israel, who

observed a significantly lower seroprevalence in the dark colour group (black/dark, brown/dark, grey) as compared to the light colour group (grey/light appaloosa), indicating an association between colour and breed. Also, Azmi et al. (2017) found a significant lower seroprevalence in coloured horses (Paint horses and Appaloosas) than solid-coloured horses, but no significant differentiation was found between light and dark horses regarding seroprevalence. Other study conducted by Epp, Waldner, West, and Townsend in (2007) analysed the factors associated with WNV disease fatalities in horses. They found that light-coloured horses had greater odds of dying from WNV clinical disease than did dark-coloured horses, possibly due to some genetic link between immune response and colour. Mosquitoes use a combination of visual, thermal and chemical cues emitted by vertebrate hosts to locate blood meals (Hawkes & Gibson, 2016; Lehane, 2005). In general, darker colours are more attractive to host-seeking mosquitoes than light colours (Allan, Day, & Edman, 1987). However, contrary to this assumption but similar to our results (Yan, Gangoso, Martínez-de la Puente, Soriguer, & Figuerola, 2017) found that birds with a greater percentage of slightly attractive colours, that is, light brown, light green, yellow and white, were preferred by *Cx. pipiens*. These authors mention that it is the contrast against the dark background what can make light colour attractive to mosquitoes. Mosquitoes are attracted by reflected white light in a greater percentage than by black light due to greater brightness and sharper colour under conditions of poor visibility (night) than in daytime (Browne & Bennett, 1981). Because *Cx. pipiens* usually peaks at sunrise, sunset or at night (Becker et al., 2010) the lighter colours of horses can favour their attraction to feed.

In the current study, there was no effect for gender on seropositivity, and this finding is similar to that of Durand et al. (2002) in France, Hassine et al. (2014) in Jordania or Bażanów et al. (2018) in horses from Poland. Other studies have reported a higher incidence of WNV in male horses than females (Ostlund et al., 2001; Tber, 1996). In the same sense, our results indicate that WNV seropositivity among equids is not significantly associated with seasons, body score, type of housing, aptitude, competition, presence of water and size of herd.

Eleven equids were seropositive for USUV by VNT. Because only WNV ELISA-positive/doubtful samples were tested for USUV, it is likely that the actual seroprevalence for this pathogen in our study could be underestimated. Although the competition ELISA kit used in this work (INgezim West Nile Compac, Ingenasa, Spain) can cross-react with other flaviviruses as the manufacturer admit in the accompanying information, this cross-reaction may not occur in all USUV-positive antibody samples, since different sera from USUV-infected partridges ($N = 8$) analysed by INgezim ELISA test gave negative results (Llorente et al., 2019). In any case, the use of USUV-specific serological methods would be expected to increase the accuracy of the estimation of seroprevalence for this pathogen. However, these methods are still not widely available for use in horses.

It seems that USUV was first introduced to Europe, particularly in Spain, in two different moments: in the 1950s and then in the 1990s, along an eastern Atlantic migratory route (Engel et al., 2016). USUV has been detected previously in Spain in *Cx. pipiens* mosquitoes (Busquets et al., 2008) and in *Cx. perexiguus* (Vázquez et al., 2011). The USUV strains detected in *Cx. pipiens* mosquitoes in Spain were genetically more closely related to the African isolates than to central European ones (Bakonyi, Busquets, & Nowotny, 2014). This virus, or specific antibodies, had also been reported in birds from Spain (Ferraguti et al., 2016; Figuerola, Soriguer, Rojo, Tejedor, & Jimenez-Clavero, 2007; García-Bocanegra et al., 2011; Höfle et al., 2013; Jurado-Tarifa et al., 2016; Llorente et al., 2013). Phylogenetic comparisons of the Spanish strains indicated significant differences between USUVs of mosquito and bird origin (Höfle et al., 2013). USUV infection had also been confirmed in Spanish red deer (García-Bocanegra et al., 2016). Despite all this USUV information on mosquitoes, birds and mammals in Spain, there is very little information about USUV infections in horses. In Balearic Islands, Vanhomwegen et al. (2017) found a USUV seroprevalence of 1.2% in 172 Majorcan horses, similar to our results (1.52%). In Italy, studies in equids have shown higher percentages of USUV seroprevalences: between 89.2% in 2008 and 7.8% in 2009 (Savini et al., 2011). In the same way, a high seroprevalence of anti-USUV-neutralizing antibodies was found in horses in Poland (27.98%) (Bażanów et al., 2018). By contrast, other studies are coincident with our results, showing low seroprevalences for USUV in horses. In Slovakia, none of the tested horses were USUV-positive (Csank et al., 2018), and in Croatia, only 2 of 69 WNV ELISA-positive horses had USUV-neutralizing antibodies (Barbic et al., 2013). In the south of Tunisia, the analysis of 284 equids showed that 10 had USUV-neutralizing antibodies (Hassine et al., 2014). According to Savini et al. (2011), horses did not show a strong immune response against USUV. This species is not susceptible to the virus and does not induce high antibody titres (Hassine et al., 2014). In humans, USUV has been associated with cases of encephalitis indicating that it is also able to invade the central nervous system (Clé et al., 2019), such as other members of the Japanese encephalitis virus (JEV) complex. Our results show, for the first time, the presence of USUV in horses from western Spain, so it would be useful to implement early warning systems to detect USUV activity in order to assess the risk of its circulation for public health.

According to the statistical analysis, the pure breeds, specifically the warmblood breeds (Arabian and Thoroughbred), are more likely to present USUV antibodies. Few studies have addressed the influence of this factor in USUV horse infections. Some authors found differences between horse breeds and WNV infection (Ahmadnejad et al., 2011; Rios et al., 2009) but not with USUV infection (Bażanów et al., 2018). Ahmadnejad et al. (2011) also found that the Arab breed was a seropositive risk factor to WNV infection in horses of Iran and the quarter horses were four times more likely to be exposed to WNV than mixed breeds (Rios et al., 2009). In our case, the pure breeds, especially the Arabian breed and the Thoroughbred, seem to have a greater susceptibility to infection, possibly due to their training habits. These breeds are keeping outside, in pastures, where the concentration of vectors and birds is higher than in stalls. Also, these breeds

compete in endurance races consisting into run more than 40 kms in one day, which make them more exposed and more attractive (thermal and chemical cues) to possible vectors. The training is always outside with sessions of more than two hours of exercise in the vector feeding hours. This significant association was not observed in WNV infections, despite having a transmission cycle very similar to USUV. Therefore, considering this assumption and bearing in mind the low number of USUV-seropositive animals identified ($N = 11$), the final conclusions must be taken with caution. The differences between horse breeds and USUV infection could be also possible due to different genetic susceptibility to infection. In several mammalian species, like humans and mice, genes associated with the susceptibility to WNV infection have been reported (Bigham et al., 2011; Cahill, Conley, DeWan, & Montgomery, 2018; Mashimo et al., 2002). In horses, Rios et al. (2010) found associations concerned WNV-induced clinical disease. Concretely, they found six SNPs in the promoter region of the 2′–5′-oligoadenylate synthetase 1 isoform (OAS1) associated with lethal WN meningoencephalitis. However, other researchers (Stejskalova et al., 2019) found that none of 138 SNPs in OAS1 was associated with lethal WN disease in two different equid breeds. So, it could be possible an individual variability in antiviral defence mechanisms, but the process seems to be complex and remains largely unclear today.

Further studies on a larger group of animals are needed to identify and confirm the risk factors of infection to reach more evidence-supported and robust conclusions.

5 | CONCLUSIONS

Our findings reflect the high WNV seroprevalence in equids from Extremadura and adjacent regions, the highest data found until now in equids from Spain. The presence of WNV-positive anti-IgM horses confirmed an active circulation of the virus among horses of western Spain in 2018 and 2019. The co-circulation of WNV and USUV is widespread in western Spain, including all the Extremadura region. Equine vaccination programmes should be encouraged to reduce the risk of WNV. It is also important to improve the surveillance programmes of public health to detect undiagnosed human cases in western Spain.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

FG, DB-B and EF conceived the study. MÁJ-C and FL developed, designed and directed the analytical work at the laboratory; FG, DB-B, JMA, MM-C and EF collected samples and performed the experiments. FG, DB-B, PA-S and FL conducted the analyses. EF and MÁJ-C contributed to the reagents/materials/analysis tools. MF performed the statistical analyses. All authors read, contributed and approved the final version of the manuscript.

ETHICAL APPROVAL

The risk assessment was submitted to and approved by the ethics committee of the University of Extremadura (register no. 24/2017). To facilitate fieldwork, collaborations were established with veterinary clinicians and the samples were obtained during the routine clinical work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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