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Short communication

Circulation of zoonotic flaviviruses in wild passerine birds in Western Spain

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ABSTRACT

We explore the presence of zoonotic flaviviruses (West Nile virus (WNV) and Usutu virus (USUV)) neutralizing antibodies in rarely studied passerine bird species. We report, for the first time in Europe, WNV-specific antibodies in red avadavat and cetti's warbler, and USUV in yellow-crowned bishop. The evidence of WNV and USUV circulating in resident and migratory species has implications for both animal and public health. Future outbreaks in avian reservoir hosts may occur and passerines should be considered as priority target species in flavivirus surveillance programmes.

1. Introduction

Emerging and re-emerging infectious diseases represent a major threat to biodiversity and an important public health issue. West Nile virus (WNV) and Usutu virus (USUV) are closely related emerging vector-borne zoonotic arboviruses (Fam. Flaviviridae) co-circulating in many areas (Nikolay, 2015), and they are currently broadening their incidence and distribution in Europe (Bakonyi and Haussig, 2020; Vilibic-Cavlek et al., 2020). Indeed, West Nile fever is one of the most widespread viral diseases on the planet (Chancey et al., 2015) and a re-emerging viral disease in Spain (Sotelo et al., 2011), with a complex eco-epidemiology as it has been reported in more than 300 bird species (CDC C for DC and P., 2021). The recent unprecedented outbreaks accompanied with growing cases of human morbidity and mortality during last decade has prompted zoonotic flaviviruses as a major public health concern (Rodríguez-Alarcón et al., 2021; World Health Organization, 2022).

Both WNV and USUV are largely maintained in a mosquito-bird transmission cycle, with humans and other mammals considered as incidental ("dead-end") hosts. Therefore, avian species are key vertebrate reservoir hosts for zoonotic flavivirus studies. Although their re-emergence in Europe in early 2000's led to an intensified surveillance for infection in birds, some important gaps, such as the reservoir competence for many European avian species, remain unsolved (Rizzoli et al., 2015). Indeed, to minimise zoonotic outbreaks, it is essential to know the natural reservoirs that are involved in their emergence, amplification or dilution. In Europe, large bird species such as raptors are usually found affected in WNV outbreaks, representing a well-studied group in WNV seasonal circulation and introduction (Vidaña et al., 2020; Bravo-Barriga et al., 2021), although their role in maintaining an enzootic cycle is still unclear. This could be probably due to most of the WNV observations are by passive surveillance (e.g., carcass sampling) and from birds in recovery centres (Vidaña et al., 2020; Bravo-Barriga et al., 2021). However, the role of other bird species such as small passerines in local flaviviruses circulation has been ignored or underestimated in many WNV surveillance studies occurring

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in Europe. This is remarkable since passerines are the most diverse and abundant avian species in the world (Del Hoyo and Collar, 2016). Moreover, their presence in agricultural and urban environments may boost the risk and emergence of zoonotic diseases in humans (Gibb et al., 2020), thus constituting a research priority to detect possible introduction and spread of zoonotic viruses. Although the role of certain passerines species in WNV transmission is well known in other countries (particularly in USA), the role of small passerines as WNV and USUV reservoir and potential amplifying hosts of infection is less known in Europe. Many recent studies have explored WNV and USUV in poultry, dead wild birds or those admitted to wildlife rehabilitation centres, revealing a widespread circulation of flaviviruses among these birds (Folly et al., 2020; Bravo-Barriga et al., 2021; Napp et al., 2021). Nonetheless, asymptomatic individuals in wild populations are undersampled, and this could lead to missing important information to assess local circulation of flavivirus in avian communities, and to failing to detect new target species for wild bird disease surveillance. For example, the extensive capturing and sampling of wild birds in the Netherlands has allowed the recent report of the first locally acquired WNV detection in birds, thus revealing the potential role of this passerine species in enzootic transmission (Sikkema et al., 2020). Furthermore, because their ability to survive WNV infection and to develop detectable antibodies to WNV, exotic birds are considered as suitable species for conducting serosurveillance for this flavivirus (Hofmeister et al., 2015). Moreover, the role of migratory birds in the maintenance and worldwide dissemination of zoonotic pathogens such as flaviviruses would be also very valuable for public health risk assessment (Malik et al., 2021).

2. Methodology of study

We explore the prevalence of flavivirus (WNV and USUV) in 645 free-living individuals from 20 species and 14 families frequently found in human dominated landscapes. Birds were captured using mist nets in December 2018 and from January to October 2019 in four sampling areas in Extremadura, southwestern Spain (Azud de Badajoz: 38°51′0.5″ N, 7°1′19.8″ W; Casas Aisladas de Gevora: 38°55′55.9″ N, 6°57′44″ W; Sagrajas: 38°55′42.7″ N, 6°53′48.9″ W; Botoa: 38°53′10.3″ N, 6°55′32.5″ W. Fig. 1), a region with active flavivirus circulation (Guerrero-Carvajal et al., 2021). We classified as exotic species to invasive species that were introduced with human assistance outside their native range. Migratory species were categorized to those flying over long distances to spend the winter in different habitats, whereas resident species spend the whole year in their breeding grounds. Blood samples (c.a. 40 μl) were collected from the jugular vein of 45 native resident (10 species), 339 exotic resident (3 species) and 261 native migratory individuals (7 species) (Table 1). All native birds were released after blood sampling. According to Spanish laws, exotic species were delivered to authorities. Blood samples were kept at 4 °C and centrifuged within 24 h after sampling for 10 min at 11,000 rpm to separate the plasma, which was stored at − 80 °C until further analysis.

All serum samples were analysed by the blocking ELISA kit INGEZIM West Nile COMPAC (INgenasa, Spain). This test detects WNV antibodies, but it partially cross-reacts with other flaviviruses (Llorente et al., 2019), allowing the detection of USUV antibodies. The inhibition percentage (IP) of each sample was calculated following the manufacturer's protocol. Serum samples with IP equal to or higher than 40% were considered as positive; samples with IP equal to or lower than 30% were considered as negative. IP values between 30% and 40% were doubtful. Because flaviviruses within the same serocomplex (e.g., WNV and USUV) exhibit strong cross-reactions in serological tests, ELISA positive and doubtful samples were subsequently analysed by confirmatory micro virus–neutralization test (micro-VNT) against WNV (WNV E101, GenBank accession no. AF260968) and USUV (viral strain: USUV SAAR–1776, GenBank accession no. AY453412) for differential diagnosis, as described previously (Llorente et al., 2019). The detection of neutralizing antibody titres at least 4-fold higher for a given flavivirus

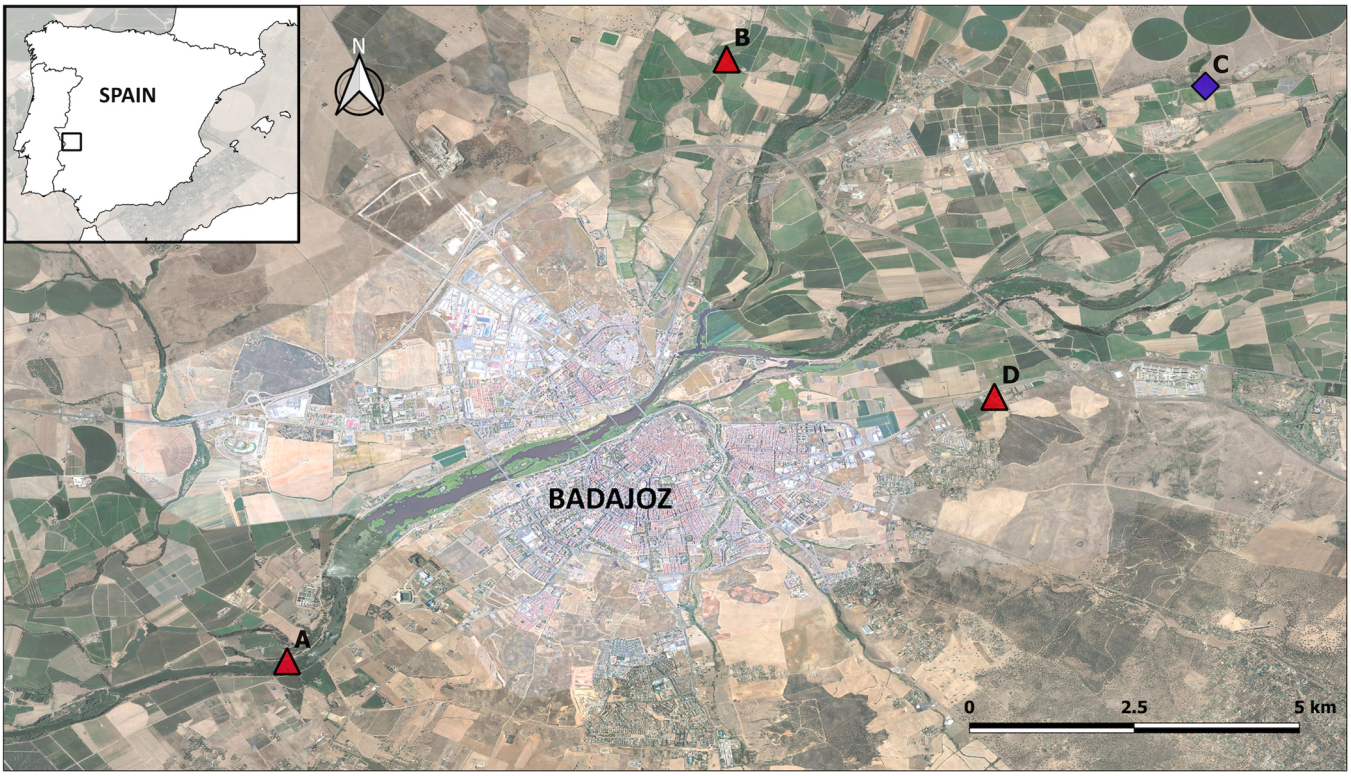

Fig. 1. Geographical distribution and positive VNT-results for WNV (red triangle), USUV (blue diamonds) in wild birds during 2018 and 2019 in Extremadura, southwestern Spain. Undetermined Flavivirus were found in all sampled localities (A: Azud de Badajoz; B: Casas Aisladas de Gévora; C: Sagrajas; D: Botoa). Map created by QGIS Geographic Information System, version 3.22.0 (2021). QGIS Association, http://www.qgis.org.

Table 1
Results obtained from ELISA and micro-VNT test for WNV and USUV in wild birds from Extremadura, southwestern Spain.

Family	Species name	Common name	Status	Sampling locality	N	Elisa positive (doubtful)	VNT analyzed	VNT positive WNV specific	VNT positive USUV specific	VNT positive Undetermined Flavivirus
Estrildidae	***Amandava amandava***	**Red avadavat**	**Exotic resident**	**B, C**	**120**	**3 (6)**	**4**	**1**	**0**	**3**
Estrildidae	*Estrilda astrild*	Common waxbill	Exotic resident	A, B, C	130	18 (22)	31	0	0	31
Ploceidae	***Euplectes afer***	**Yellow-crowned bishop**	**Exotic resident**	**B, C**	**89**	**13 (6)**	**15**	**0**	**1**	**12**
Hirundinidae	*Delichon urbicum*	House martin	Native migratory (S)	D	232	37 (13)	48	18	0	12
Motacillidae	*Anthus pratensis*	Meadow pipit	Native migratory (W)	A	1	0	0	0	0	0
Muscicapidae	*Erithacus rubecula*	European robin	Native migratory (W)	A	5	1 (1)	2	0	0	0
Muscicapidae	*Ficedula hypoleuca*	European pied flycatcher	Native migratory (P)	A	3	0	0	0	0	0
Muscicapidae	*Luscinia megarhynchos*	Common nightingale	Native migratory (S)	A	1	0	0	0	0	0
Phylloscopidae	*Phylloscopus collybita*	Common chiffchaff	Native migratory (W)	A, C	18	0 (1)	0	0	0	0
Turdidae	*Turdus philomelos*	Song thrush	Native migratory (W)	A	1	0	0	0	0	0
Alcedinidae	*Alcedo atthis*	Common kingfisher	Native resident	A	4	0	0	0	0	0
Aegithalidae	*Aegithatus caudatus*	Long-tailed tit	Native resident	A	4	0	0	0	0	0
Cettiidae	***Cettia cetti***	**Cetti's warbler**	**Native resident**	**A**	**9**	**1**	**1**	**1**	**0**	**0**
Fringillidae	*Serinus serinus*	European serin	Native resident	A	1	0	0	0	0	0
Paridae	*Cyanistes caeruleus*	Eurasian blue tit	Native resident	A	6	0	0	0	0	0
Paridae	*Parus major*	Great tit	Native resident	A	1	0	0	0	0	0
Passeridae	*Passer domesticus*	House sparrow	Native resident	C	1	1	1	0	0	1
Passeridae	*Passer hispaniolensis*	Spanish sparrow	Native resident	A	8	0	0	0	0	0
Sylviidae	*Sylvia atricapilla*	Eurasian blackcap	Native resident	A	2	0	0	0	0	0
Sylviidae	*Sylvia melanocephala*	Sardinian warbler	Native resident	A, C	9	1 (2)	2	0	0	2
					645	75 (51)	104 *	20	1	61

Note: In bold, species that have been detected with specific WNV and USUV antibodies for first time in Europe. * Because the limited amount of collected plasma, only 104 ELISA samples were analysed in parallel by micro-VNT against WNV and USUV. Sampling localities (A: Azud de Badajoz; B: Casas Aisladas de Gévora; C: Sagrajas; D: Botoa). Native migratory bird species were categorized as wintering (W), passage migrants (P) and bird species breeding in Spain during spring (S).

over the other was considered a proof of specificity (Calisher et al., 1989). When VNT titre differences did not reach this threshold, the result was considered inconclusive and the specific flavivirus causing the infection could not be determined.

We performed Chi-square test to determine if the proportion of individuals exposed to WNV or USUV varied with bird status (exotic resident, native migratory, native resident). All analyses were performed using PASW Statistics 18 statistical package for Windows.

3. Results

Overall, out of 645 samples, 75 (11.6%) specimens tested positive by ELISA, whereas 51 (7.9%) were doubtful. Due to the limitations of working with small birds and the limited amount of collected plasma, only 104 ELISA samples (71 positive and 33 doubtful) out of the 126 positive and doubtful samples were subsequently analysed in parallel by micro-VNT against WNV and USUV. Outcomes from VNT analyses revealed that 20 birds (19.23% of the samples analysed by VNT) showed specific antibodies for WNV, one for USUV (0.96%), and 61 (58.65%) were classified as undetermined flavivirus being not possible to determine the specificity of antibodies in these samples (Table 1). The remaining 22 samples (21.16%) tested by VNT were negative.

A total of 82 samples (48 exotic resident, 30 native migratory and 4 native resident) were confirmed by VNT to be exposed to WNV or USUV (Table1). There were no differences in the proportion of individuals exposed to WNV or USUV depending on their bird status (exotic resident = 14.95%, native migratory = 11.63%, native resident = 9.09%; Chi-square test: N = 623; $\chi^2 = 2.070$; g.l. = 2; p = 0.3515).

4. Discussion

This is the first WNV and USUV study carried out on free-range wild birds from Extremadura on their natural habitats. We confirmed flavivirus' exposure in 82 birds (12.7%), highlighting the importance of passerines for the correct assessment of circulation in the region. Notably, our findings in wild populations of passerines are similar to those recently reported by Napp et al. (2021) showing a 10% of seroprevalence of flaviviruses in poultry and wild birds from northeast Spain. In addition, a survey carried out mainly in larger birds (e.g., Pelecaniformes and Ciconiiformes) admitted to rehabilitation centres in Extremadura showed a higher flavivirus seroprevalence compared to our study (Bravo-Barriga et al., 2021), which could be explained because the expected higher number of cases of infectious diseases in birds arriving at rehabilitation centres (Camacho et al., 2016). However, we should be cautious with our records because the use of passive methods for bird capturing such as mist nets or traps may produce biased data about the number of infected birds in one population, given that the mortality and reduced activity of individuals infected with flavivirus (Hubálek et al., 2014) could reduce the capture probabilities among these birds. Moreover, working with passerines had important limitations; for instance, the volume of plasma is usually very scarce, thus, 22 positive and doubtful ELISA samples were excluded from the VNT analyses possibly underestimating the number of birds infected with flavivirus in the studied wild populations.

Overall, 48 exotic resident, 30 native migratory and 4 native resident individuals were confirmed as infected by flaviviruses by VNT. Specifically, all the exotic resident bird species showed antibodies for undetermined flavivirus. In addition, one juvenile red avadavat (*Amandava amandava*) captured in January 2019 showed specific antibodies for WNV and one adult yellow-crowned bishop (*Euplectes afer*) sampled in December 2018 for USUV. Because red avadavat normally reproduce during the last moths of the year in Spain (De Lope et al., 1985), the presence of antibodies against WNV in young individuals suggests an active circulation of flavivirus during autumn or early winter in our latitudes. Among the native migratory bird species, only house martins (*Delichon urbicum*) were confirmed as infected with undetermined flaviviruses (n = 12), and 18 birds captured in June 2019 showed antibodies for WNV. This high number of VNT-positive for WNV in house martins is particularly relevant, especially in the context that some WNV strains could have been introduced from Africa to Europe by migratory birds (García-Carrasco et al., 2021). Moreover, one out of the 18 VNT-positive for WNV house martins was a juvenile bird sampled prior to its first migration to Africa, confirming the circulation of the virus in the area in late winter – spring 2019 and supporting a potential role of this migratory species in the active circulation of WNV in Europe. Finally, three native resident bird species were infected by flaviviruses, with one adult cetti's warbler (*Cettia cetti)* sampled in October 2019 showing specific antibodies for WNV, and one house sparrow (*Passer domesticus*) and two sardinian warbler (*Sylvia melanocephala*) infected by undetermined flaviviruses.

The presence of antibodies to WNV and USUV in resident species and migratory individuals prior its first migration demonstrates the widespread circulation of these flaviviruses in passerine populations in south Europe across all the year. This outcome, together with the density, proximity to anthropogenic environments and contact frequency with humans of passerines, stress the importance to include these wild species in the routine surveillance programmes for zoonotic diseases. Remarkably, WNV-specific antibodies were found in red avadavat and cetti's warbler for the first time in Europe, while a seropositive house martin was already found in the studied area (Bravo-Barriga et al., 2021). In addition, USUV was reported for the first time in Europe in a yellow-crowned bishop (see Vojtíšek et al., 2021 for a recent review). Further studies would be needed to characterize the WNV and USUV strains present in these birds and to determine their potential origin, pathogenicity and the risk to public health.

Ethical issues

Bird trapping was carried out with all necessary permits with the current regional and national laws of Spain, and sampling on private land and residential areas were conducted with all the necessary permits and consent from the owners. Methods were evaluated and approved by the institutional Commission of Bioethics of University of Extremadura (CBUE 49/2019) and by Junta de Extremadura Local Government (87/2019).

Authors' contributions

AM and MF conceived the idea of the study; AM, MF, SM, JM, LGL and JAO collected the samples; DB-B, FG-C performed the ELISA assays; JM, PA-S and FL performed the VNT analyses. DB-B carried out the editing and visualization of geographical data. AM and MF wrote the first draft of the manuscript; AM, FdL, EF and MAJ-C contributed to the reagents/materials/analysis tools. All authors read, contributed to, and approved, the final version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Accessibility

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

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