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# Serosurvey of Blood Donors to Assess West Nile Virus Exposure, South-Central Spain

## Appendix

### Selection of Blood Donors and Sample Size Calculation

The blood donor samples comprised 10,232 serum samples, providing extensive coverage across the Ciudad Real province (42 municipalities) (Figure, https://wwwnc.cdc.gov/EID/article/30/7/24-0450-F1.htm). In a cross-sectional epidemiologic approach, we sought to determine the overall exposure rate of the human population in the study area by employing a representative subset. To achieve this, we calculated the minimum sample size necessary to estimate the prevalence of WNV exposure at the finest spatial resolution available for blood donor information, namely the municipality. Appendix Table 1 shows the 2020 human census and sample size per municipality. The required sample size for estimating the prevalence of exposure at the municipal level, considering an expected seroprevalence of 1.7% (based on findings a in Greek seroprevalence study [*1*]), was determined to be 26 donors per municipality, with a 5% of precision and 95% confidence interval. Sample selection was conducted in a balanced manner, according to the age (three classes: <30 years old, 30–50 years old, and >50 years old) and sex of donors per municipality.

### Serologic Methods

To detect IgG antibodies against WNV, the commercial ELISA West Nile Virus IgG DxSelect (Focus Diagnostics Inc., Cypress, California) were used. ELISA-positive samples were subjected to additional testing using a virus neutralization test (VNT) for WNV lineage 1 (WNV-1), Usutu (USUV) and Tick-borne encephalitis (TBEV). VNT is described in detail elsewhere

(*2*). We selected these viruses because are endemic in Europe, in fact, in recent years, the areas in Europe reporting flavivirus infections and specifically WNV, USUV, or TBEV have significantly increased (*3*). WNV and USUV are endemic in Spain and although no autochthonous TBEV human cases have been detected, recent animal seroprevalence studies indicate the possibility of TBEV circulation in horses and dogs from several areas in Spain (*4*,*5*). The titers of neutralizing antibodies were defined as the highest serum dilution that showed >90% neutralization of the virus challenge. Neutralizing antibody titers ≤1:8 were considered negative.

VNT used Vero E6 cells and the virus strains HU6365/08 (WNV-1, GenBank: JF707789.1), Neudorfl (TBE) and HU10279/09 (USUV, GenBank accession no. KX268472.1). A sample was considered WNV seropositive when it was reactive via ELISA in the VNT assay the antibody titer obtained against WNV was 4 times higher than the antibody titer obtained against another of the three tested flavivirus.

## Epidemiologic Survey

ELISA-positive donors underwent a telephone interview conducted by the blood bank staff, which an epidemiologic survey was performed to obtain information about their country of birth, WNV-endemic countries visited, vaccination history, and specific vaccinations against other flaviviruses, such as yellow fever virus (YFV) and Japanese encephalitis virus (JEV), due to the potential for cross-reaction in the ELISA results. Only the WNV-seropositive donor confirmed by VNT was informed of the results.

## Ethics Considerations

This study was designed and performed according to the Helsinki Declaration. All samples were integrated into 'Biobanco del Sistema Sanitario Pública de Andalucía (Nodo Hospital Universitario Reina Sofía-IMIBIC)'. All donors signed an informed consent form. The CEIC (Clinical Research Ethics Committee) of Hospital de Ciudad Real approved the collection of samples.

## Supplementary Results

Two donors with positive results in the IgG WNV ELISA assay and not confirmed by VNT against any of the three tested flaviviruses, were natives from countries where the virus is endemic (Colombia and Ecuador) and frequently traveled to their countries of origin. Therefore, the positive results from the WNV IgG ELISA are due to a previous flavivirus infection endemic to this area, such as dengue and/or Zika viruses. None of the ELISA-positive donors showed neutralizing antibodies against USUV or TBEV.

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**Appendix Table 1.** Demographic characteristics

| Municipality | Census persons (year 2020) | No. donations available | No. donors included |
|---|---|---|---|
| Agudo | 1,657 | 72 | 41 |
| Alcázar San Juan | 30,766 | 951 | 28 |
| Aldea del Rey | 1,631 | 30 | 29 |
| Almadén | 5,200 | 91 | 33 |
| Almagro | 8,905 | 448 | 31 |
| Almodóvar | 5,983 | 63 | 31 |
| Argamasilla de Alba | 6,955 | 216 | 31 |
| Bolaños de Calatrava | 12,019 | 273 | 27 |
| Calzada de Calatrava | 3,630 | 141 | 31 |
| Campo de Criptana | 13,312 | 122 | 30 |
| Carrión de Calatrava | 3,099 | 21 | 21 |
| Castellar de Santiago | 1,862 | 78 | 31 |
| Ciudad Real (Capital province) | 75,504 | 643 | 30 |
| Corral de Calatrava | 1,117 | 35 | 31 |
| Daimiel | 17,916 | 687 | 31 |
| Fuente El Fresno | 3,208 | 137 | 31 |
| Herencia | 8,456 | 230 | 31 |
| Malagón | 7,881 | 209 | 29 |
| Manzanares | 17,962 | 599 | 31 |
| Membrilla | 5,942 | 161 | 30 |
| Miguelturra | 15,498 | 189 | 28 |
| Montiel | 1,294 | 20 | 19 |
| Moral de Calatrava | 5,208 | 163 | 31 |
| Pedro Muñoz | 7,285 | 137 | 31 |
| Picón | 668 | 26 | 26 |
| Piedrabuena | 4,379 | 183 | 31 |
| Pozuelo de Calatrava | 3,586 | 105 | 28 |
| Puerto Lápice | 891 | 30 | 30 |
| Puertollano | 46,607 | 1154 | 26 |
| Retuerta del Bullaque | 927 | 22 | 22 |
| Robledo | 1,053 | 20 | 20 |
| San Carlos del Valle | 1,109 | 63 | 31 |
| Santa Cruz de Mudela | 4,085 | 92 | 26 |
| La Solana | 15,419 | 226 | 31 |
| Tomelloso | 36,168 | 1,019 | 30 |
| Torralba de Calatrava | 2,966 | 48 | 30 |
| Valdepeñas | 30,252 | 829 | 28 |
| Villahermosa | 1,790 | 39 | 31 |
| Villamanrique | 1,128 | 22 | 22 |
| Villanueva de los infantes | 4,869 | 128 | 31 |
| Villarrubia de los ojos | 9,762 | 459 | 31 |
| Villarta de San Juan | 2,739 | 51 | 31 |
| Total | 430,688 | 10,232 | 1,222 |

**Appendix Table 2.** Serology and epidemiology of ELISA-positive donors

| ID | Donation date | Age (years) | Sex | Country of origin | Travel in WNV-endemic area | Vaccines | IgG ELISA | VNT WNV1 | VNT USUV | VNT TBEV |
|---|---|---|---|---|---|---|---|---|---|---|
| WNV01 | Feb-18 | 30–50 | Male | Spain | No | No | Positive | Negative | Negative | Negative |
| WNV02 | Feb-18 | 30–50 | Female | Ecuador | Ecuador | NA | Positive | Negative | 8-Jan | Negative |
| WNV03 | Feb-18 | 30–50 | Male | Spain | No | No | Positive | Negative | Negative | Negative |
| WNV04 | Feb-18 | 30–50 | Male | Colombia | Colombia | No | Positive | Negative | Negative | Negative |
| WNV05 | Feb-18 | >50 | Male | Spain | No | No | Positive | Negative | Negative | Negative |
| WNV06 | Jan-18 | 30–50 | Female | Spain | No | No | Positive | Negative | Negative | Negative |
| WNV07 | Jan-18 | <30 | Male | Spain | No | No | Positive | 1/256 | 1/8_1/16 | Negative |
| WNV08 | Jan-18 | <30 | Male | Spain | NA | NA | Positive | Negative | Negative | Negative |
| WNV09 | Jan-18 | >50 | Female | Spain | No | No | Positive | Negative | Negative | Negative |
| WNV10 | Jan-18 | <30 | Male | Spain | No | No | Positive | Negative | Negative | Negative |
| WNV11 | Feb-18 | <30 | Male | Spain | NA | NA | Positive | Negative | Negative | Negative |
| WNV12 | Jan-18 | <30 | Male | Spain | No | No | Positive | Negative | Negative | Negative |
| WNV13 | Feb-18 | >50 | Male | Spain | No | No | Positive | Negative | Negative | Negative |
| WNV14 | Feb-18 | >50 | Female | Spain | No | No | Positive | Negative | Negative | Negative |
| WNV15 | Feb-18 | <30 | Female | Spain | No | No | Positive | Negative | Negative | Negative |
| WNV16 | Feb-18 | <30 | Male | Spain | No | No | Positive | Negative | Negative | Negative |
| WNV17 | Apr-18 | >50 | Male | Spain | No | No | Positive | Negative | Negative | Negative |
| WNV18 | Apr-18 | <30 | Female | Spain | No | No | Positive | Negative | Negative | Negative |
| WNV19 | Apr-18 | 30–50 | Female | Romania | Romania | No | Positive | Negative | Negative | Negative |

NA, unavailable information.