West Nile virus past infections in the general population of Southern Spain

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OBJECTIVE. To analyze the prevalence of past and recent infections by West Nile virus (WNV) and the risk factors associated with WNV exposure in a representative population from southern Spain.

METHODS. Sample size was established for an estimated prevalence of past WNV infections of 5 ± 2.5% in 504 subjects. A pre-stratification was performed according to age distribution and place of residence. After random telephone solicitation and acquisition of informed consent, a serum sample was collected and an epidemiologic survey performed on all participating subjects. Samples were tested with ELISA-IgG and MAC-ELISA to detect specific IgG and IgM antibodies; results were confirmed by the plaque reduction neutralization test (PRNT).

Multivariate analysis using a forward stepwise logistic regression model was performed to assess potential risk factors associated with WNV exposure.

RESULTS. Prevalence of past WNV infections confirmed by PRNT in the 504 participants was 0.6%, affecting mainly older persons (mean age 65 ± 23 vs. 34 ± 22 years; p = 0.018), those living in rural areas (5.4% vs. 0% in urban areas; p = 0.01), and individuals with risk professions (prevalence 2.8% vs. 0%; p = 0.048). None of the five recent infections detected by MAC-ELISA was confirmed by PRNT.

CONCLUSIONS. These results strongly suggest past circulation and exposure of the human population to WNV in Southern Spain.

Key words: Flavivirus. West Nile Virus. Seroprevalence studies. Spain.

Seroprevalencia de infecciones por el virus del Nilo Occidental en la población general del sur de España

OBJETIVO. Analizar la prevalencia de infecciones pasadas y recientes por el virus del Nilo Occidental (VNO), así como los factores de riesgo asociados con la exposición al mismo, en una muestra representativa de la población del sur de España.

MÉTODOS. El tamaño de la muestra se estableció para una seroprevalencia de infección pasada del 5 ± 2.5% en 504 sujetos, preestratificándose ésta por edad y lugar de residencia. Los voluntarios se incluyeron tras solicitud telefónica y consentimiento, y a todos ellos se les realizó una extracción de suero y una encuesta epidemiológica. Las muestras se analizaron para detectar IgG e IgM específicas, mediante enzimoinmunoensayo (ELISA-IgG) y enzimoinmunoensayo por captura (MAC-ELISA), confirmando ulteriormente los resultados positivos mediante reducción-neutralización en placa (PRNT).

Finalmente se realizó un análisis de regresión logística multivariante paso a paso hacia delante para determinar los posibles factores asociados con la exposición al VNO.

RESULTADOS. La prevalencia de infecciones por VNO confirmadas por PRNT en los 504 sujetos incluidos fue del 0.6%, y se vieron principalmente afectadas las personas de mayor edad (edad media 65 ± 23 frente a 34 ± 22 años; p = 0.018), las que vivían en áreas rurales (prevalencia del 5.4% frente al 0% en áreas urbanas; p = 0.01), y las personas con profesiones de riesgo (prevalencia del 2.8% frente al 0%; p = 0.048). Por el contrario, ninguna de las infecciones recientes detectadas por MAC-ELISA fue confirmada ulteriormente por PRNT.

CONCLUSIONES. Estos resultados apoyan firmemente la circulación en el pasado del VNO, así como la exposición humana del mismo, en áreas del sur de España.


Introduction

The global ecology of West Nile virus (WNV) has experienced noteworthy changes in recent years. From an endemic pattern of infection in specific areas dotted with sporadic local outbreaks every 25-30 years1-3, this flavivirus has spread to non-endemic areas over the past 10 years, causing a significant burden of human and animal disease4,5. This expansion, the causes of which are not yet totally understood, has been featured by the forthcoming...
of human outbreaks preceded by severe and highly mortal epizootia affecting mainly indigenous birds11,12. An increase in WNV virulence has also been noted in these outbreaks, confirmed by human mortality ranging between 4.3% in Romania in 1996 and 16.7% in Israel in 20004,6. Because of these issues, WNV is now considered a paradigm of emerging pathogens13,14.

The presence of circulating WNV strains has also increased in the Mediterranean basin in the past years, in countries geographically and climatologically closely related to Spain, such as Italy, France, Tunisia, Algeria, and Morocco15. Many factors occur in our environment to enable the introduction and expansion of WNV in Spain: a) the ubiquitous presence of wetlands (particularly the Doñana National Park in southwestern Spain, one of the most extensive in Europe), and stable populations of various ornithophilic Culex spp. mosquitoes; b) the strategic geographical location for numerous migratory bird co-ining from endemic areas of Africa (e.g., storks and flamingos), which annually share these natural areas and wetlands with indigenous birds, and c) recent animal and human outbreaks occurring in many bordering countries, configuring a virtual "ring" of WNV circulation around Spain11,12.

Despite these issues, there is little data assessing the ecology and possible circulation of WNV in sylvatic cycles occurring in our geography. With respect to humans, no cases of confirmed acute infection have been declared, and the few existing seroepidemiologic studies were performed with techniques having poor specificity (indirect hemagglutination)15,16. Thus, the current impact of human exposure to WNV in Spain remains uncertain. The aim of the present study was to analyze the prevalence of past and recent WNV infections and the possible risk factors associated with exposure to WNV in a representative population from southern Spain.

Material and methods

Geographical area and study population

Inclusion criteria and sample collection

Serological techniques

Sample size/stratification

Seroprevalence was determined in the overall sample, and by place of residence and age group. Continuous variables are expressed as

Inclusion criteria and sample collection

Subjects of all age groups were selected by random telephone solicitation, with the exception of the youngest age group (0-14 years). Subjects from this last group were pre-selected in our two central laboratories, which receive blood samples from the entire area (primary care, outpatient clinic, and hospital). Based on the pre-selection, we ultimately selected samples originating from primary care centers and pre-anesthetic assessment in outpatient clinic (to avoid selection of hospitalized, chronically ill, or immunosuppressed children). Subsequently, legal guardians were asked by phone for informed consent to use the remaining serum for our study, and were questioned to detect possible exclusion criteria. Once informed consent had been granted, the serum sample was stored at –80 °C and an epidemiologic survey was performed on all participating subjects. The data collected included gender, age, profession, place of residence, outdoor activities, travel over the past 12 months to other parts of Spain and Europe, contact with animals, and insect bites in the past month. Risk professions were defined as those involving close contact with animals (farmers, stockkeepers, veterinarians), nature and wetlands (rangers, forest officers), or mosquitoes (entomologists and pest control workers). The following persons were excluded: those unable to answer the epidemiologic survey, those who had traveled to endemic areas in the previous 12 months, and persons previously vaccinated against yellow fever, hospitalized in the last 30 days, with febrile diseases in the last 30 days, or with any type of immunosuppression.

Serological techniques

Samples were processed and tested for WNV IgG by enzyme-linked immunosorbent assay (ELISA) and for WNV IgM by antibody-capture ELISA (MAC-ELISA). For other flaviviruses (dengue, yellow fever, and tick-borne encephalitis), the assays for dengue IgG (Panbio Diagnostics, Queensland, Australia) and for TBE IgG and IgM were assayed by commercial diagnostic methods. Dengue IgM was assayed by a plaque reduction microneutralization test against WNV (PRNT). For this purpose, samples were tested in duplicate and assayed twice. Two-fold dilutions (25, 50, 125, 250) of the samples ranging from 1:16 to 1:256 were placed in a 96-well tissue culture microplate (Nunc Serological assay for WNV IgM by antibody-capture ELISA (MAC-ELISA) was performed at 37 °C, 50 μL of a Vero R5 cell suspension containing 4x103 cells/well, was added to each well. Cultures were maintained for 7 days at 37 °C and 5% CO2, and then fixed with a solution containing 10% formaldehyde and 1% naphthol blue-black dye. The endpoint titer was defined as the highest serum dilution that showed no cytotoxic effect in 50% of cell cultures. All those procedures were performed in a level 3 biosafety facility.

Serology showing an IgG/IgM titer against WNV underwent further commercial serological analysis against other flaviviruses (dengue and tick-borne encephalitis (TBE)). The assays for dengue IgG (Panbio Diagnostics, Queensland, Australia) and for TBE IgG and IgM (Dade Behring, Deerfield, IL, USA) were based on indirect ELISA. The method for dengue IgM was a p-chain capture ELISA (Panbio Diagnostics).

Definition of WNV infection

Subjects with any ELISA IgM titer were considered as having possible past WNV infection, subjects with any MAC-ELISA IgM titer were considered as having possible recent WNV infection. Those with any ELISA IgG or MAC-ELISA IgM titer, subsequently confirmed by PRNT assay, were considered as having definitive past or recent WNV infection, respectively.

Statistical analysis

Seroepidemiologic survey, those who had traveled to endemic areas in the previous 12 months, and persons previously vaccinated against yellow fever, hospitalized in the last 30 days, with febrile diseases in the last 30 days, or with any type of immunosuppression.
TABLE 1. Epidemiological features of subjects with past West Nile virus infection in the population of southern Spain

<table>
<thead>
<tr>
<th>Subject (gender, age, place of residence, risk-profession, outdoor activities)</th>
<th>ELISA-IgG*</th>
<th>MAC-ELISA-IgM*</th>
<th>PRNT</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, 77 years, rural, yes, no 217VM</td>
<td>3.75</td>
<td>Negative</td>
<td>&gt; 256</td>
<td>WNV past infection</td>
</tr>
<tr>
<td>Male, 80 years, suburban, yes, no 7VM</td>
<td>3.33</td>
<td>Negative</td>
<td>128</td>
<td>WNV past infection</td>
</tr>
<tr>
<td>Male, 38 years, rural, yes, no 112VR</td>
<td>3.18</td>
<td>Negative</td>
<td>64</td>
<td>WNV past infection</td>
</tr>
<tr>
<td>Female, 30 years, urban, no, no 234VR</td>
<td>1.79</td>
<td>Negative</td>
<td>&lt; 16</td>
<td>Not confirmed</td>
</tr>
<tr>
<td>Female, 30 years, urban, yes, no 117VM</td>
<td>1.53</td>
<td>Negative</td>
<td>&lt; 16</td>
<td>Not confirmed</td>
</tr>
<tr>
<td>Cases with WNV IgG positive result</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, 38 years, urban, yes, yes 1199YR</td>
<td>Negative</td>
<td>4.39/negative**</td>
<td>&lt; 16</td>
<td>Not confirmed</td>
</tr>
<tr>
<td>Female, 5 years, urban, no, no 219VM</td>
<td>Negative</td>
<td>2.23/negative**</td>
<td>&lt; 16</td>
<td>Not confirmed</td>
</tr>
<tr>
<td>Male, 20 years, suburban, no, no 76VM</td>
<td>Negative</td>
<td>1.86/na***</td>
<td>&lt; 16</td>
<td>Not confirmed</td>
</tr>
<tr>
<td>Female, 11 years, urban, no, no 228VR</td>
<td>Negative</td>
<td>1.66/negative**</td>
<td>&lt; 16</td>
<td>Not confirmed</td>
</tr>
<tr>
<td>Male, 19, years, urban, yes, no 284VR</td>
<td>Negative</td>
<td>1.31/negative**</td>
<td>&lt; 16</td>
<td>Not confirmed</td>
</tr>
</tbody>
</table>

Results

Five subjects (nearly 1% of the 504 included) presented anti-WNV IgG titers with a mean ELISA absorbance ratio of 2.72 ± 1 (table 1); none of the remaining sera had indeterminate absorbance ratios. Prevalence of definitive past WNV infection confirmed by PRNT was 0.6% (3 positive samples, with PRNT titers of 64, 128, and > 256, respectively (table 1), and was significantly higher in rural areas (P = 0.01). Comparisons of demographic and epidemiological features between subjects with and without past WNV infection are shown in table 2. No differences were detected with the exception of activity in any risk profession (P = 0.04), which was associated with prior WNV exposure. Multivariate analysis detected no independent risk factors associated with past WNV infection.

Five subjects (nearly 1% of the 504 included) showed IgM titers against WNV with a mean MAC-ELISA absorbance ratio of 2.03 ± 1.15 (table 1); WNV IgG antibodies were not detected in any of these subjects, and none of the remaining sera had indeterminate absorbance ratios. None of these individuals presented specific anti-WNV neutralizing antibodies when PRNT assay was performed (table 1). Application of a background subtract procedure to four of the remaining sera also yielded negative results.

TABLE 2. Epidemiological features of subjects with definitive past WNV infection in the population of Southern Spain, compared with those without previous exposure

<table>
<thead>
<tr>
<th>Epidemiologic feature</th>
<th>Prevalence of past WNV infection</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>1.2/0%</td>
<td>NS</td>
</tr>
<tr>
<td>Risk profession (yes/no)</td>
<td>2.8/0%</td>
<td>0.04</td>
</tr>
<tr>
<td>Travel to rural areas (yes/no)</td>
<td>1.2/0.3%</td>
<td>NS</td>
</tr>
<tr>
<td>Outdoor activities (yes/no)</td>
<td>0/6%</td>
<td>NS</td>
</tr>
<tr>
<td>Contact with animals (yes/no)</td>
<td>0.5/0.7%</td>
<td>NS</td>
</tr>
<tr>
<td>Insect bites in past month (yes/no)</td>
<td>0/6%</td>
<td>NS</td>
</tr>
</tbody>
</table>

Results

Analysis of the IgG immune response against dengue and TBE viruses in the 5 subjects with anti-WNV IgG titers by ELISA showed IgG titers against dengue in sera from 5 subjects (subjects 1, 2, and 3 in table 1); no IgG immune response was detected against TBE virus. Similarly, analysis of IgM immune response against dengue and TBE viruses in the 5 subjects with anti-WNV IgG titers by ELISA detected IgM titers against dengue in the serum of one subject (subject 6 in table 1). Application of a subtract background assay to this serum sample (in the presence and absence of specific dengue antigen), the specific IgM immune response was not confirmed, and therefore ruled out. No IgM immune response against TBE virus was detected in any of the 5 subjects.

Discussion

The data from the present study show the presence of definite past WNV infection in the general population of southern Spain, confirming exposure of the population to this pathogen. These data complete previous preliminary studies carried out in Spain with less specific techniques that reported past infection rates of 8% in the human population of the northwestern part of the country and 3.1%...
in small mammals collected from several parts. In addition, the findings are in accordance with the results of a similar recent study performed in northeastern areas of the country, which detected about 0.2% of confirmed WNV past infection in the general population.

Definitive past WNV infection was characterized by affecting older persons, subjects living in rural areas, and those having risk professions. This epidemiological pattern probably reflects the means of acquisition, which may have been in the former past (older people are exposed during longer periods of time), and in subjects with closer contact to the wild cycle of WNV, since rural areas are located closer to wetlands, and therefore to reservoirs and potential vectors; the same is true for persons with risk professions.

As other authors have pointed out, despite documented circulation of the virus, population exposure to WNV, and even declaration of sporadic outbreaks affecting equine cattle in surrounding countries, epidemiologic feature contrasts with the dynamics of WNV disease in North America, in which WNV has expanded considerably, causing more than 7,000 cases of human disease since 1999. Several factors may be related to this difference. Natural immunization of the majority of the population due to early exposure to WNV is hardly admissible, because the prevalence rates found in the present study are far from those reported in endemic areas, which range from 8% to 15%. Partial immunization conferring some kind of protection against WNV due to population exposure to antigenically-related flaviviruses is also unlikely, because no indigenous cases or evidence of circulation of the Japanese encephalitis virus group or dengue virus have been reported in Spain, and yellow fever was eradicated in the final years of the 19th century. Exposure to other flaviviruses (mainly TBE virus, which is endemic in vast territories of Europe) could highlight the plausibility of the cycle to “jump” to bridging vectors (mosquitoes that bite both birds and humans), and to indigenous biologic vectors, making the ELISA techniques with respect to closely related flavivirus unrecognized in our area. The same is true for persons with risk professions. This epidemiological pattern probably reflects the means of acquisition, which may have been in the former past (older people are exposed during longer periods of time), and in subjects with closer contact to the wild cycle of WNV, since rural areas are located closer to wetlands, and therefore to reservoirs and potential vectors; the same is true for persons with risk professions.

In conclusion, our data have documented the presence of definite past WNV infection in the general population of southern Spain. These infections were associated with older age, rural areas, and risk professions.

### Acknowledgements

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### References


