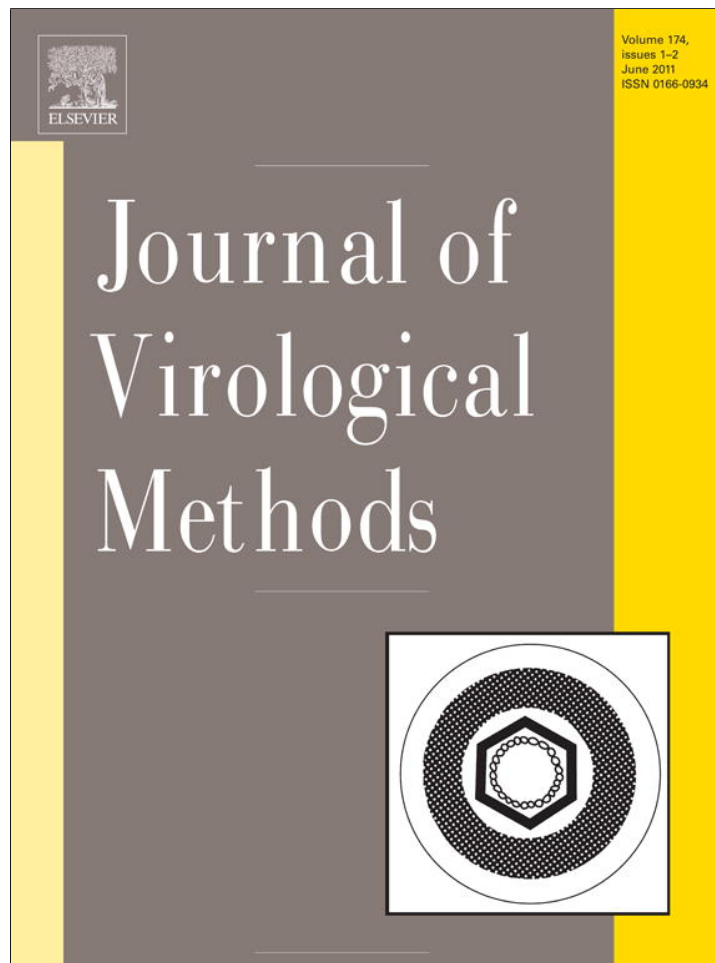


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Development and evaluation of a new epitope-blocking ELISA for universal detection of antibodies to West Nile virus

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West Nile virus (WNV) is an emerging zoonotic pathogen with a wide range of hosts, including birds, horses and humans. The development and evaluation of the performance of a new enzyme-linked immunosorbent assay (ELISA) are described for rapid detection of WNV-specific antibodies in samples originating from an extensive range of vertebrates susceptible to WNV infection. The assay uses a monoclonal antibody (MAb) which binds whole virus particles and neutralizes infection *in vitro* by recognizing a neutralizing epitope within the envelope (E) glycoprotein of the virus. This MAb, labelled with horseradish peroxidase, was used to compete with WNV-specific serum antibodies for virus-binding *in vitro*. The epitope-blocking ELISA was optimized in a manner that enabled its validation with a number of experimental and field sera, from a wide range of wild bird species, and susceptible mammals. The new ELISA exhibited high specificity (79.5–96.5%) and sensitivity (100%), using the virus-neutralization test as reference standard. It also required a much lower volume of sample (10 µl per analysis) compared to other ELISAs available commercially. This new method may be helpful for diagnosis and disease surveillance, particularly when testing samples from small birds, which are available in limited amounts.

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1. Introduction

West Nile virus (WNV, family *Flaviviridae*, genus *Flavivirus*) is an arthropod-borne zoonotic, epizootic and epornitic pathogen. In recent years WNV has spread in many parts of the world, constituting a remarkable example of an emerging pathogen (Brault, 2009; Gould et al., 2001; Kramer et al., 2008). WNV is maintained in nature through a transmission cycle involving mosquitoes as vectors and wild birds as reservoir hosts. Epidemiologically, horses and humans are dead-end hosts since they acquire infection by infected mosquito bites and develop clinical illness, but are unable to transmit the virus. The most severe clinical outcome in these species includes neurological signs such as meningitis and encephalitis, leading to death. Case fatality in humans is approximately 3–4%, whereas in horses it ranges from 23 to 43%. Many bird species are susceptible to WNV infection, which can be fatal in some species (McLean et al., 2002). Individuals surviving the infection

develop long-lasting immunity provided by specific antibodies in serum.

Serological tests for WNV-specific antibodies rely mainly on ELISA, hemagglutination inhibition or IFA techniques for screening, and virus-neutralization tests for confirmation (Shi and Wong, 2003). A critical issue regarding the specificity of these serological tests is cross-reactions with other flaviviruses. This is of particular importance in those areas where co-circulation of several flaviviruses occurs (Kuno, 2003). Among the above methods, virus-neutralization test provides the highest specificity, and for this reason it is considered as the “gold standard” for WNV-antibody detection. However, the virus-neutralization test is a complex, cumbersome and time-consuming technique, which is not suitable for large-scale testing in surveillance schemes. This method also requires live virus for testing samples, which involves the risks associated with manipulation of hazardous zoonotic pathogens. A high-containment facility (BSL-3) is required for sample processing, and is often not available. Of the above serological tests appropriate for the screening of antibodies to WNV in sera, ELISA is the format used most widely, as it is versatile, reproducible, and easy to standardize. Due to the clinical relevance of this disease

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for humans and horses, a number of different ELISA methods for WNV-specific antibody detection in humans and horses have been developed (Feinstein et al., 1985; Kleiboeker et al., 2004; Long et al., 2006; Lorono-Pino et al., 2009; Prince et al., 2004) and are available commercially. Nonetheless, for serological surveillance in wild bird hosts, a different approach is needed for the screening of samples, given the wide range of susceptible avian species with highly diverse immunoglobulins and associated antigenic differences. As a consequence, indirect ELISA methods rely on secondary antibodies which are specific for the avian species under test. There are two alternative strategies to circumvent this problem: firstly, the use of a “wide range” of secondary antibodies obtained from mixtures of antisera raised in bird species representative of the WNV host range, which will cross-react with immunoglobulins of as many birds as possible (Ebel et al., 2002), and secondly, the use of competitive or blocking ELISAs which are based on the ability of the host immunoglobulins to compete for antigenic binding sites, or block the binding of specific antibodies to WNV (Blitvich et al., 2003a,b). Currently, to the best of our knowledge, the availability of commercial multi-species ELISAs for WNV antibody detection is limited to only one assay kit, which is a monoclonal antibody-based competition ELISA (IdVet ID Screen[®] West Nile Competition, IdVet, Montpellier, France). One important drawback of this ELISA is the 50 μ l sample volume required for each test. This amount cannot be obtained from some, since many host species are small-size birds such as passerines, from which only limited amounts of blood can be obtained due to health and animal welfare concerns. The objective of the present study was to develop a new ELISA fulfilling the needs for an optimal serological screening test, suitable for disease surveillance viz: a rapid test amenable for high throughput screening and automation; a unique test valid for the range of WNV host species, including many species of wild birds and susceptible mammals; a small volume of serum. With this aim, a new epitope-blocking ELISA technique was developed and evaluated as a screening test for detection of WNV antibodies in a range of vertebrate species susceptible to WNV infection.

2. Materials and methods

2.1. Viruses and cells

The NY'99 034EDV WNV strain, belonging to WNV lineage 1, isolated from an infected crow, was obtained from the National Veterinary Services Laboratories, United States Department of Agriculture (USDA), Ames, IA, and was propagated and titrated in Vero cells. The Eg101 WNV strain (lineage 1) and the E6 clone of Vero cells used for virus propagation and titration were obtained from Dr. Herve Zeller (Institut Pasteur de Lyon, France). Other WNV strains used in experimental inoculations of animals, also propagated in Vero cells, were: Spain/2007 (strain GE1b/B), isolated in the laboratory (Jiménez-Clavero et al., 2008); Morocco/2003 (strain 04.05), kindly provided by Dr. M. El Harrak, Biopharma, Rabat, Morocco (Schuffenecker et al., 2005); Kunjin (strain KJ539), and Kun MP502-66; kindly provided by Dr. A. Tenorio, CNM-ISCIII, Majadahonda, Spain, and B956 (type strain, Uganda, 1937, lineage 2) from the American Type Culture Collection (ATCC, cat. no: VR1510). The SAAR-1776 strain of Usutu virus (USUV), obtained from A. Buckley (Centre for Ecology and Hydrology, Oxford, UK) was propagated and titrated in Vero cells.

2.2. Antigen production

Culture fluids from Vero cells infected with NY'99 034EDV WNV strain were harvested when a cytopathic effect (cpe) was observed (generally 3–4 days after inoculation). Virus inactivation was car-

ried out by the addition of 0.05% (v/v) β -propiolactone (Sigma, St Louis, MO) to the culture fluids after a freeze–thaw step, followed by overnight incubation at 4 °C, plus 2 h at 37 °C to eliminate residual toxicity. Virus inactivation in the culture fluid was verified following three blind passages in Vero cell monolayers without the development of cpe. Viral extracts for rabbit immunization were obtained as follows: the inactivated cell-cultured virus fluid was clarified by centrifugation at 10,000 \times g for 30 min. The inactivated virus was purified from the supernatant by ultracentrifugation at 140,000 \times g for 2 h through a 25% (w/v) sucrose cushion. The pellet was resuspended in phosphate-buffered saline solution (PBS). This concentrated antigen extract was kept at –20 °C until use. Viral antigen for ELISA was obtained as follows: infected cells were collected from the inactivated culture medium by centrifugation at 2000 \times g for 30 min. Cell pellets were resuspended in PBS containing protease inhibitors and disrupted by one freeze–thaw cycle at –70 °C. Finally cells were lysed in PBS containing 0.5% Triton X-100, 0.1% Sarkosyl and 10 mM DTT, pH 7.2 and soluble extract was clarified by centrifugation at 15,000 \times g for 15 min at 4 °C and stored at –70 °C.

2.3. Polyclonal antisera and monoclonal antibodies

Polyclonal antisera to WNV (NY'99 034EDV WNV strain) were raised in two New Zealand White rabbits by three consecutive subcutaneous injections (at 0, 15 and 39 days) with 50 μ g WNV antigen extract, prepared as described above, and resuspended in complete Freund's adjuvant (first dose), incomplete Freund's adjuvant (second dose) and PBS (third dose). The WNV antiserum was obtained from blood taken from the ear marginal vein of the rabbits at 49 days after the first antigen dose. Polyclonal antiserum raised to USUV in rabbits was a generous donation from Dr. Tamas Bakonyi, Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent István University, Budapest, Hungary.

Production of hybridoma cell lines secreting MAbs specific for WNV was performed essentially as described previously (Sanz et al., 1985). Briefly, cells of the mouse myeloma X63/Ag 8.653 were fused with spleen cells of Balb/c mice immunized with purified virus (at least 3 times with 50 μ g/doses). Screening for the presence of WNV specific antibodies in the cell supernatant was performed by indirect ELISA using the recombinant ectodomain (domain III), derived from the 3' end of the E glycoprotein gene of WNV NY'99 034EDV strain, and cloned into an expression plasmid. This recombinant ectodomain (rdIIIIE1) was expressed in Sf9 insect cells infected with baculovirus. Specific antibody-secreting hybridomas were cloned by limiting dilution in presence of hybridoma cloning factor. MAb 1D11 (IgG2b) was selected due to its optimal reactivity both to the E glycoprotein and to the virus, in epitope-blocking and virus-neutralization assays *in vitro*.

2.4. ELISA

ELISA plates (Maxisorp, NUNC, Roskilde, Denmark) were coated with WNV antigen extract diluted to 5 μ g protein/ml in PBS and incubated overnight at 4 °C. After washings, wells were blocked with 1% bovine serum albumin in PBS (blocking solution) for 1 h at room temperature. Following the removal of the blocking solution, serum samples were added to the wells at 1:5 dilution in blocking solution (final volume: 50 μ l/well) and incubated overnight at 4 °C. Duplicate wells were used for negative and positive control sera, which were made up, respectively, with rabbit non-immune serum and rabbit anti-WNV antiserum (1:1600 dilution) prepared as described above. After the washings, MAb 1D11 conjugated to horseradish peroxidase was added to the plates (50 μ l/well) at 1:10,000 dilution in PBS with 1% BSA (bovine serum albumin) and 0.05% Tween 20, and incubated for 1 h at room tempera-

ture. Subsequent to the final washing step, 50 μ l/well of substrate solution (TMB) was added to each well and the chromogenic reaction allowed to develop at room temperature for 15 min in the dark. Finally, 50 μ l/well of stop solution (0.5 M H₂SO₄) was added and the optical density (OD) in each well read at 450 nm. Results were valid when the OD in negative control wells was over 0.8 OD units, and the OD of the positive control wells was below 0.35 OD units. Cut-off value was set at 30% percentage of inhibition, $PI = 100 - [(OD_{\text{sample}}/OD_{\text{negative control}}) \times 100]$. However, the ELISA sets a range of “doubtful” results between 30% and 40% of inhibition, and such results will be considered as positive in this study since in common diagnostic practices sera yielding doubtful results have to be confirmed by virus-neutralization.

2.5. Virus-neutralization and haemagglutination-inhibition tests

The detection and titration of neutralizing antibodies to West Nile or Usutu viruses were performed by a micro-virus neutralization test as described previously (Figuerola et al., 2007a). Briefly, serum samples were inactivated at 56 °C for 30 min prior to testing. Two-fold dilutions of test sera (25 μ l) in Eagle's Minimal Essential Medium (EMEM) supplemented with L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin, were incubated with the same volume (25 μ l) of 100 TCID₅₀ of either WNV strain Eg101 or USUV strain SAAR-1776, for 1 h at 37 °C and 5% CO₂. This was followed by the addition of 50 μ l of a suspension (2×10^5 cells/ml) of Vero E6 (for WNV) or Vero (for USUV) cells in the same medium, plus fetal calf serum to reach a final concentration of 5%. The plates were incubated further for 6–7 days at 37 °C and 5% CO₂ atmosphere until cytopathic effects (cpe) were observed in control wells containing 10 TCID₅₀ of virus. Virus-neutralization positive samples were those inhibiting cpe at 1:5 or higher dilutions. Samples were titrated by testing serial serum dilutions from 1:5 to 1:320. Neutralizing serum titre was regarded as the highest value of the reciprocal serum dilution giving a complete absence of cpe.

For the haemagglutination inhibition tests, sera were suspended 1:5 in a mixture of borate saline (pH 9.0) and 25% kaolin, allowed to stand at room temperature with periodic shaking, and centrifuged at 950 \times g for 30 min. A 40% goose red blood cell (RBC) suspension in saline was added (50 μ l) to each serum and incubation at room temperature for 20 min was allowed, following which the sera were centrifuged at 340 \times g for 10 min. Two fold dilutions of sera in borate saline (25 μ l) ranging from 1:10 to 1:5120 were prepared in 96 well micro titre plates. Wesselsbron virus, a *Flavivirus* group specific antigen propagated in suckling mice brains and purified by sucrose density gradient centrifugation, was added per well at a concentration of 4 haemagglutinating units (4HAU) (25 μ l). The plates were incubated at 4 °C overnight, following which 40% goose RBCs (50 μ l) were added and allowed to stand at room temperature for 60 min. A serum sample was regarded as positive when haemagglutination was inhibited at a dilution of 1:80, suspect or doubtful at 1:40, and negative at a dilution below 1:40.

2.6. Samples from experimental infections

Bird sera. Sera from red-legged partridges (*Alectoris rufa*) inoculated experimentally with live West Nile viruses were obtained as described elsewhere (Sotelo et al., 2011). Briefly, groups of red-legged partridges ($n = 10$) were inoculated with WNV Spain/2007 (strain GE1b/B) and WNV Morocco/2003 (strain 04.05) respectively. A third group of 10 birds was injected with diluent (DMEM) and served as uninfected controls. Blood was collected from a subset of animals from all the groups, including the controls, on different days post-inoculation (dpi) from the ulnar vein of the wing and allowed to clot for 1 h at 37 °C, followed by incubation at 4 °C overnight. Following incubation, the coagulated blood was cen-

trifuged at approximately 600 \times g for 10 min to separate the serum. Sera from the control group was obtained also at the same time points.

Mice sera. Sera from mice (*Mus musculus*) were obtained during experimental inoculations with live WNV and USUV for a different study whose details will be presented elsewhere (Sotelo et al., 2011). Essentially, groups of $n = 6$ –10 Swiss mice were inoculated with 1–10,000 pfu of the following viral strains: WNV NY'99 034EDV (lineage 1a); WNV Kunjin KJ539 (lineage 1b); WNV B956 (lineage 2); WNV Kun MP502-66 (putative lineage 6); USUV strain (SAAR-1776). Blood was drawn from the tail or maxillary veins prior to inoculation and at 27 dpi in those mice surviving the infection. These samples were analyzed by a number of techniques prior to this study, thus, only a limited amount remained available of some, which were prevented from being further tested.

2.7. Field samples

Sera from mammals. A panel of 91 sera was obtained from feral horses (*Equus caballus*) inhabiting the Guadalquivir marshes, Southern Spain. These horses were essentially asymptomatic, and were subjected previously to serosurveillance in the search for WNV-specific neutralizing antibodies (Jiménez-Clavero et al., 2007, 2010). Sera from South African horses presenting with neurological signs suggestive of WNV infection ($n = 69$) in the first quarter of 2010 were tested for diagnostic purposes, while giraffes (*Giraffa camelopardialis*) ($n = 80$) were tested for export certification to various countries. South African ruminant sera ($n = 87$), including 85 bovines (*Bos taurus* and *Bos indicus*), 1 buffalo (*Syncerus caffer*) and 1 caprine (*Capra hircus*) were obtained from a serological survey for several enzootic diseases causing production losses and neurological disorders, including WNV. All the South African samples were tested at the ARC Onderstepoort Veterinary Institute (OVI) – molecular epidemiology and diagnostics programme (MEDP).

Sera from wild birds. A panel of 146 sera was obtained from wild birds belonging to 12 different species (five families, three orders), captured in the Guadalquivir marshes, Southern Spain. These sera were collected and tested by a commercial assay (IdVet ID Screen[®] West Nile Competition, IdVet, Montpellier, France), following manufacturer's instructions. Additionally, two panels of sera from jackdaws (*Corvus monedula*) from Guadalquivir marshes ($n = 244$) and flamingo chicks (*Phoenicopterus ruber*) from Fuente de Piedra and other locations in Southern Spain ($n = 310$) were tested.

Sera from wild birds infected with Usutu virus. Sera from five wild birds (1 common kestrel, *Falco tinnunculus*; 3 common buzzards, *Buteo buteo* and 1 Western marsh harrier, *Circus aeruginosus*) were kindly supplied by Herbert Weissenböck, Institute of Pathology and Forensic Veterinary Medicine, University of Veterinary Medicine, Vienna, Austria.

2.8. Animal welfare and ethical issues

All animal work performed in this study was supervised and approved by the INIA's Ethical Committee for Animal Experimentation to meet animal welfare and ethical rules in the European Union, which are in agreement with the American Physiological Society's Guiding Principles on the Care and Use of Animals.

3. Results

3.1. Testing of sera from wild birds

A panel of sera from wild birds was selected on the basis of previous results of a commercial competitive ELISA (c-ELISA) for the detection of WNV antibodies in a wide range of hosts (IdVet ID

Table 1
Analysis of a panel of wild bird sera using three different serological techniques: the new epitope-blocking ELISA, a commercially available ELISA (IdVet ID Screen® West Nile Competition) and the gold-standard virus-neutralization test (VNT).

Bird species (common name)	Bird species (scientific name)	ELISA IDVet® positive/total	New epitope-blocking ELISA positive/total	VNT positive/total
Mallard	<i>Anas platyrhynchos</i>	1/13	2/13	0/13
Gadwall	<i>Anas strepera</i>	0/1	0/1	0/1
Pochard	<i>Aythya ferina</i>	0/1	1/1	0/1
Cattle egret	<i>Bubulcus ibis</i>	0/3	0/3	0/3
Stork	<i>Ciconia ciconia</i>	6/23	2/23	1/23
Common coot	<i>Fulica atra</i>	28/37	24/37	13/37
Crested coot	<i>Fulica cristata</i>	13/24	13/24	4/24
Moorhen	<i>Gallinula chloropus</i>	0/4	1/4	0/4
Marbled teal	<i>Marmaronetta angustirostris</i>	0/1	0/1	0/1
Red-crested pochard	<i>Netta rufina</i>	0/3	0/3	0/3
Spoonbill	<i>Platalea leucorodia</i>	1/30	2/30	1/30
Glossy ibis	<i>Plegadis falcinellus</i>	0/6	0/6	0/6
Total		49/146	45/146	19/146
Diagnostic sensitivity (%)		100	100	–
Diagnostic specificity (%)		76.4	79.5	–

Table 2
Analysis of serum samples obtained from red-legged partridges inoculated experimentally with two different strains of WNV: Spain/2007 and Morocco/2003. ($n = 10$ per group). VNT: virus-neutralization test. GMT: geometric mean titre (antilogarithm of the arithmetic mean titre expressed in log units).

Days post-inoculation	WNV strain Spain/2007			WNV strain Morocco/2003		
	eb-ELISA (positives/analyzed)	VNT (positives/analyzed)	VNT (GMT)	eb-ELISA (positives/analyzed)	VNT (positives/analyzed)	VNT (GMT)
–1	0/10	0/10	–	0/10	0/10	–
3	2/7	0/7	–	0/7	0/7	–
5	–	–	–	1/2	0/2	–
6	6/7	0/7	–	1/5	0/5	–
7	–	–	–	0/1	0/1	–
10	5/5	5/5	17.4	1/1	1/1	10
12	2/2	2/2	40	2/2	2/2	40
14	1/1	1/1	40	1/1	1/1	40
20	3/3	3/3	50.1	2/2	2/2	20

Screen® West Nile Competition). The panel comprised 49 c-ELISA-positive and 97 c-ELISA-negative samples belonging to 12 different species, representing five different taxonomic families. The birds shared the Guadalquivir marshes ecological habitat, where recent WNV circulation was shown to occur (Figuerola et al., 2007b). Samples were tested in parallel using the new epitope-blocking ELISA (eb-ELISA) and the “gold standard” virus-neutralization test (Table 1). Virus-neutralization yielded 19 positive results, the new eb-ELISA detected 46, while the c-ELISA resulted in 49 positive samples. The diagnostic specificity for this panel of wild bird samples when utilizing virus-neutralization as the reference standard, was 79.5% and 76.4% for the new eb-ELISA and c-ELISA, respectively. Diagnostic sensitivity was 100% for both assays.

In order to determine the earliest time post-infection the eb-ELISA could detect WNV specific antibodies, red-legged partridges were infected experimentally with two lineage 1 viruses, Spain/2007 and Morocco/2003, and bled at different days post inoculation (dpi) until the end of the experiment at dpi 20. The sera were tested in parallel using virus-neutralization test and the new eb-ELISA. As shown in Table 2, the new eb-ELISA detected WNV-specific antibodies as early as 3 dpi, before the demonstration of neutralizing antibodies by the virus-neutralization test, which only occurred at 10 dpi. Both virus-neutralization test and the new eb-ELISA detected antibodies to WNV until the end of the experiment. Sera from the control (non-inoculated) group of partridges were all negative in the eb-ELISA.

In order to test the performance of the new eb-ELISA in wild bird surveillance programs, a panel of sera was examined from wild birds such as corvids (jackdaws, *C. monedula*) and flamingos (*P. ruber*), which presumably play a role in the epidemiology of WNV infections. Among the 244 jackdaw samples tested, three were found positive. Of them, two were subjected to the virus-

neutralization for confirmation, since the third serum sample was exhausted before this test could be carried out. One out of two eb-ELISA positive jackdaw samples was confirmed as WNV antibody positive by virus-neutralization. With respect to the flamingo samples, only three (out of 310) of the sera tested gave positive results by the eb-ELISA, and none were positive by virus-neutralization.

3.2. Tests of sera from mammals

Ninety-one serum samples from feral horses free-ranging in the Doñana National Park (Guadalquivir marshes), Spain, were tested in parallel by the new epitope-blocking ELISA and by the “gold-standard” virus-neutralization. When tested by eb-ELISA, eight serum samples were positive, the rest were negative. By virus-neutralization, five serum samples were positive while the rest were negative. Using virus-neutralization as the “gold standard”, the diagnostic specificity and sensitivity of the new eb-ELISA in this panel of horse samples was of 96.5% and 100%, respectively (Table 3).

A total of 236 sera from South African mammals were subjected to serological testing. As a serological test for routine testing for

Table 3
Analysis on a panel of sera from feral horses in Doñana National Park, Spain. VNT: virus-neutralization test.

	eb-ELISA	VNT
Positive	8	5
Negative	83	86
Total	91	91
Diagnostic sensitivity (%)	100	–
Diagnostic specificity (%)	96.5	–

Table 4

Summary of the serological analyses performed in South African mammals.

Species	No sera	WNV positive (HAI) (pos/analyzed)	WNV positive (eb-ELISA) (pos/analyzed)	Specificity ELISA (vs. HAI)	Sensitivity eb-ELISA (vs. HAI)
Horse	69	14/47 (NA: 22)	26/69 (21/47) ^a	79% (n = 47) ^a	100% (n = 47) ^a
Giraffe	80	6 (+1 dbt ^b)/80	4/80	100%(n = 80)	67% (n = 80) ^a
Bovine	85	NA	17/85	NA	NA
Buffalo	1	NA	1/1	NA	NA
Caprine	1	NA	0/1	NA	NA
Total	236	20/127	25/127	95.3% (n = 127)	83.3% (n = 127)

HAI: haemagglutination-inhibition assay. NA: not analyzed (in HAI).

^a In parentheses, results within the group of samples analyzed by HAI.^b dbt: doubtful.

WNV antibodies, the ARC-OVI used haemagglutination inhibition (HAI), and not virus-neutralization, thus the results obtained with the new eb-ELISA were compared with those of the HAI test, and the comparison was carried out only in giraffes and a proportion of equines. A summary of these results is shown in Table 4. Among the equine sera, 47 were tested by HAI, and 14 HAI positive results were obtained, while the eb-ELISA yielded 21 positive results within this group (n = 47), and 26 in the whole batch of equine samples (n = 69). None of the equine samples found positive by HAI were negative by the eb-ELISA, hence the eb-ELISA resulted in 79% specificity and 100% sensitivity when measured against the HAI test for horse samples. With regard to giraffes, 6 out of 80 were positive by the HAI test, and one gave a doubtful result. However, within this group of samples only four yielded positive results by the eb-ELISA, the rest remaining negative. This resulted in 100% specificity, while the sensitivity was of 67% (the HAI indeterminate results were not regarded as positive). Of the 87 samples from ruminants examined by the eb-ELISA, 18 were positive, where 17 were of bovine origin and one was from a buffalo. A single caprine serum examined gave negative results.

3.3. Analytical specificity

Sera from mice inoculated with different genetic lineages of WNV (lineages 1a, 1b, 2 and putative lineage 6) as well as with the closely related Usutu virus, were tested. Using WNV as antigen, most sera from WNV-inoculated mice were positive by virus-neutralization (26 out of 34). All virus-neutralization-positive sera were positive by the eb-ELISA, which detected antibodies against all the WNV lineages used for the inoculations. Two out of eight sera from USUV-inoculated mice were also able to neutralize homologous USUV *in vitro*, and none of these were positive by the eb-ELISA Table 5. Remarkably, most of the mice infected with USUV, and some infected with WNV strains B956 (lineage 2) (particularly at low virus doses) and Kun MP502-66 (putative lineage 6) did not seroconvert, suggesting that either the infectious dose used in some of the inoculation experiments was low or that mice are barely susceptible to infection with certain viruses employed, particularly USUV.

In order to determine specificity and cross-reactivity with other flaviviruses, five USUV-positive serum samples from wild birds obtained from an USUV epizootic outbreak affecting wild birds in Austria, subsequent to the introduction of this virus in 2001, were subjected to analysis by the new eb-ELISA. All were positive for USUV-neutralizing antibodies by virus-neutralization (titre of 1:10 in all cases). Only one serum (corresponding to a Western marsh harrier) was positive by the eb-ELISA. The sample was found negative for WNV neutralizing antibodies by virus-neutralization.

Finally, the specificity of the new eb-ELISA was assessed for antibodies raised to WNV and USUV in rabbits. While the eb-ELISA detected anti-WNV Abs with a titre over 1:160, it detected hardly anti-USUV at 1:5 dilution. For comparison, both antisera were

titrated by the virus-neutralization test against their homologous viruses and each had a titre of 1:160.

4. Discussion

A new ELISA was developed which fulfils the needs for an optimal serological screening suitable for WNV surveillance programs. The ELISA was valid for a wide range of species. In the present study, it was shown to be able to detect specific antibodies to WNV in 16 different species of wild birds, encompassing a wide range of avian families with potential involvement in WNV epidemiology, including corvids (often considered important hosts for WNV), such as jackdaws (*C. monedula*) which are abundant in Southern Europe, and flamingos (*P. ruber*), which are found frequently seropositive to WNV in Southern Europe (Figuerola et al., 2007a), and, due to their migratory behaviour, are regarded as putative WNV carriers in Europe and Africa (Jourdain et al., 2007). The ELISA could also detect WNV-specific antibodies in storks, coots, spoonbills, partridges and mallards; that is, in all bird species from which WNV-neutralizing antibodies were demonstrated in the present study. The ELISA was also able to detect WNV-specific antibodies in different species of mammals, including equines, giraffes, bovines, buffaloes and mice. Since the ELISA is based on epitope blocking, it would presumably detect all types of WNV antibodies from any host species, which can bind the epitope recognized by MAbs 1D11, preventing its binding. In this regard, the performance of the new eb-ELISA is comparable to an epitope-blocking ELISA described previously (Blitvich et al., 2003a,b). An advantage of the new eb-ELISA is the need for only 10 µl of serum sample to perform the test, which is important particularly considering the low serum volume usually available from some small birds like passerines, which are also important hosts for WNV (Kilpatrick et al., 2006). Previous ELISAs available commercially require five times this volume for testing serum.

The diagnostic sensitivity of the new eb-ELISA in wild bird sera was 100% when compared to that of virus-neutralization which is regarded as the "gold standard" for WNV antibody detection. The implication is that no positive sample by virus-neutralization gave negative results by the eb-ELISA. However, 27 eb-ELISA-positive sera were negative by the virus-neutralization, leading to a diagnostic specificity of 79.5%. Although this specificity is slightly higher than that of the commercial c-ELISA to which it was compared (76.4%), a considerable number of ELISA positive samples were not confirmed by virus-neutralization. These positive results could have resulted from cross reactions and warrant further investigations. Nonetheless, results from birds infected experimentally (red-legged partridges) showed that some early infection sera (between 3 and 10 days post-inoculation) could be positive by eb-ELISA while remaining negative by virus-neutralization. This strongly supports a notion of insufficient sensitivity of the "gold standard" virus-neutralization, rather than low specificity of the eb-ELISA, as one of the likely causes of the discrepancy observed between the two techniques. In addition, humoral immune responses to infectious pathogens comprise both neutral-

Table 5
Summary of the results obtained with sera from mice inoculated with different WNV and USUV strains. Note that some sera analyzed by virus-neutralization test were not analyzed by eb-ELISA. This was due to the limited volume available of some of the samples.

Lineage/virus inoculated in mice	Strain	Inoculation dose (pfu/mice)	eb-ELISA (no. positive/analyzed)	Virus-neutralization test ^a (no. positive/analyzed)
WNV L1a	NY99	1000	2/2	3/3
WNV L1b	Kunjin KJ359	1000–10,000	5/5	7/7
WNV L2	B956	10–1000	5/7	5/8
WNV L6	Kun MP502-66	1000–10,000	6/6	11/16
Usutu	SAAR-1776	10,000	0/8	2/8

^a Virus-neutralization test was performed using the same virus species as used for the inoculations (WNV E101 strain or USUV SAAR 1776 strain).

izing and non-neutralizing antibodies, and WNV is not an exception in this respect (Diamond et al., 2008). WNV infection could elicit non-neutralizing antibodies which bind the epitope recognized by the MAb 1D11, thus blocking the attachment of the MAb, and yield positive ELISA results.

The eb-ELISA results were also compared to another serological technique used widely for screening purposes, the HAI test. Similar to the comparison of the ELISA with virus-neutralization, none of the positive samples by HAI test were found negative by the eb-ELISA, exception for two serum samples from giraffes (Table 4). The HAI test was performed with a Wesselsbron antigen, a *Flavivirus* group-specific antigen that cross-reacts with antibodies of a wide range of flaviviruses, especially in this type of assay (Blackburn and Swanepoel, 1980). Wesselsbron is endemic in South Africa, where it affects domestic and wild ruminants. A likely explanation for the HAI-positive/eb-ELISA-negative results could be the exposure of the giraffes to Wesselsbron, not the WN virus; hence the low sensitivity observed (67%). This would be more consistent with the fact that the sensitivity of the ELISA in horses was 100%.

Field sera from birds and mammals showed that the test was able to detect antibodies to WNV circulating in different geographical areas, representing distinct epidemiological situations: firstly, a Western Mediterranean country (Spain) characterized by low WNV activity, and where sporadic cases of WN disease have been recorded only recently (Bofill et al., 2006; Jiménez-Clavero et al., 2008) and overall seroprevalence for WNV neutralizing antibodies in wild birds was measured recently (Figueroa et al., 2008); secondly, South Africa, where WNV is endemic in the country, and fatal, or severe to mild forms of disease have been recorded in humans, horses, ostrich (*Struthio camelus*) and a dog (*Canis lupus familiaris*) (Venter et al., 2009). The viruses circulating in the Western Mediterranean region, belonging to lineage 1 (Sotelo et al., 2009), differ also from those found in South Africa, generally belonging to lineage 2 (Botha et al., 2008). The results obtained in this study showed that the new eb-ELISA can be useful for surveillance programs both in epidemic and endemic situations, irrespective of the WNV lineage which is circulating. Antibodies from mice infected experimentally with different WNV lineages, including 1a, 1b, 2 and a putative new lineage (lineage 6) were detected in the eb-ELISA, which also showed low cross-reactivity with USUV antibodies, not only in experimental sera from mice, but also in sera from wild birds infected naturally. However, a low level seroreactivity to USUV antibodies was ascertained, as one serum (out of five) from USUV-infected wild birds was reactive by the WNV eb-ELISA, and a weak (but positive) reaction was also seen when a USUV-specific rabbit antiserum was tested. Serological cross-reactions between members of the Japanese encephalitis group of flaviviruses have been documented (Banerjee and Deshmane, 1987). For this reason, the new eb-ELISA should be used as a screening assay and all positive sera should be confirmed by virus-neutralization. Ideally, the neutralization test should not only consist of WNV as antigen, but include other flaviviruses circulating in the area of study. The virus eliciting the seroconversion will be identified by compar-

ing the virus-neutralization titres obtained against each *Flavivirus* (Table 5).

In conclusion, a new epitope-blocking ELISA for the detection of WNV-specific antibodies in a wide range of host species has been developed. The performance of this test in both experimental and field serum samples was adequate for use for diagnostic and disease surveillance programs. This ELISA has been made available commercially (Ingezym West Nile Compac, INGENASA, Madrid, Spain).

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