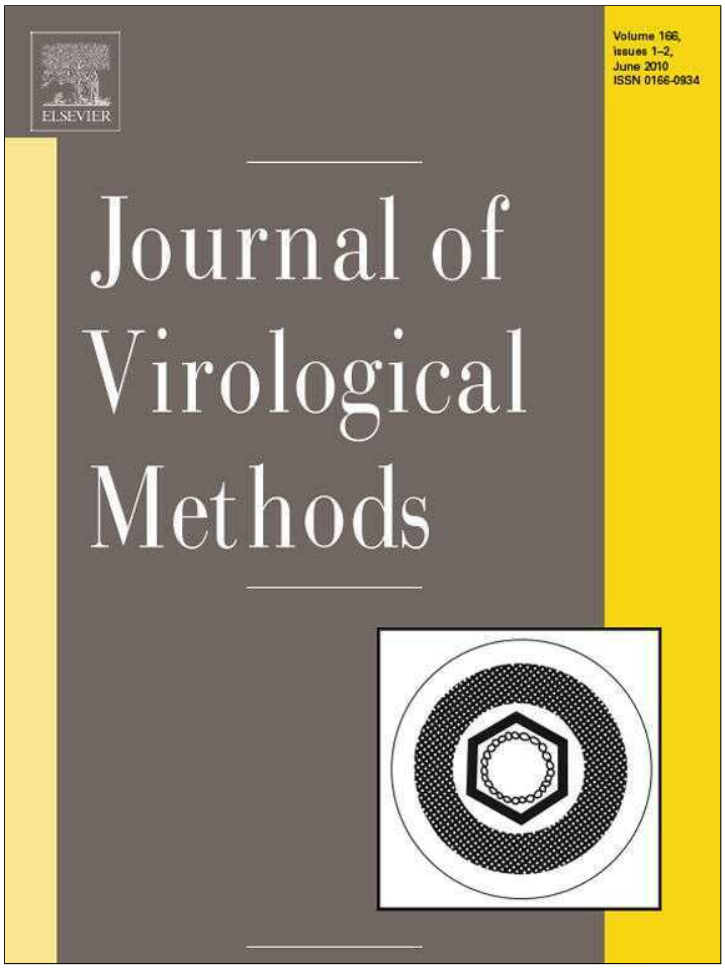

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Evaluation of an enzyme-linked immunosorbent assay for detection of West Nile virus infection based on a recombinant envelope protein produced in *Trichoplusia ni* larvae

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A B S T R A C T

West Nile virus (WNV), a Flavivirus distributed most widely, is presenting lately variable epidemiological and ecological patterns, including an increasing virulence that has already caused over 1000 human deaths in USA. Currently, diagnosis of WNV is achieved mainly by enzyme-linked immunoassays (ELISAs) based on the use of inactivated whole WNV (iWNV) as antigen, although results have to be confirmed by plaque reduction neutralization tests (PRNTs). Expression of WNV envelope recombinant E (rE) protein and its usefulness as ELISA antigen are described. Production of rE was achieved upon infection of *Trichoplusia ni* insect larvae with a recombinant baculovirus. Once optimized, the rE-based ELISA was validated with a battery of mouse and equine sera characterized previously. Concordance with the iWNV-based ELISA used routinely was good (95%), as it was with the reference PRNT (90%), with specificity of 94.4% and sensitivity of 88.1%. Production of rE protein in insect larvae allows for an easy, low cost and quite large-scale yield of partially purified antigen which is suitable for serological diagnosis of WNV, without the need for manipulation of large quantities of infective virus.

1. Introduction

West Nile virus (WNV) is an arbovirus (genus *Flavivirus*; family *Flaviviridae*) that was first isolated in Uganda in 1937 (Smithburn et al., 1940). Maintained in an enzootic cycle between mosquito-vectors and birds, other vertebrates, such as horses and humans, can be also infected (Blitvich, 2008). Although WNV infection is asymptomatic in the majority of cases, viral neural tropism can lead to death due to encephalitis and meningitis (Blitvich, 2008). Of special relevance is the high virulence of the infection in North America where, since it was first reported in 1999 (Anderson et al., 1999; Lanciotti et al., 1999), it has caused over 1000 human deaths (www.cdc.gov/ncidod/dvbid/westnile). WNV is endemic in regions of Africa, Western Asia, Oceania, Europe, and nowadays in America, where it spread very rapidly from north to south (Blitvich, 2008; Zeller and Schuffenecker, 2004).

WNV diagnosis is achieved routinely by serological assays (Dauphin and Zientara, 2007) but, due to the cross-reactivity of flaviviruses, plaque reduction neutralization tests (PRNTs) must be used as the reference assay for specific diagnosis. PRNT is a laborious and time-consuming technique that has to be carried out in bio-safety level 3 (BSL-3) facilities with the consequent risks for the personnel involved in live virus manipulation; therefore, several enzyme-linked immunosorbent assays (ELISAs) have been developed for serological detection of anti-WNV antibodies in humans (Hogrefe et al., 2004; Loroño-Pino et al., 2009; Malan et al., 2004; Martin et al., 2000; Tardei et al., 2000) and animal samples (Blitvich et al., 2003; Davies et al., 2001; Ebel et al., 2002). ELISAs are easier to perform, allow the screening of a large number of samples in a considerably reduced time, and may help to exclude negative samples. The antigen used in ELISA for diagnosis of WNV in most reference laboratories consists of inactivated whole virus (iWNV), either obtained from infected cell cultures or from inoculated suckling mouse brains. This type of antigen is also used in most commercial ELISAs for detection of anti-WNV immunoglobulin G (IgG) in human and veterinarian samples.

To produce WNV recombinant antigens the structural prM/E proteins have been the preferred targets (Davies et al., 2001; Hogrefe et al., 2004; Muerhoff et al., 2004; Wang et al., 2001), although other approaches have also been applied (Kitai et al., 2007; Wong et al., 2003). This study describes the production, in *Trichoplusia ni* (cabbage looper) insect larvae, of WNV-E protein soluble fragment using a baculovirus expression system. This method

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represents an efficient way of protein production which maintain the glycosylation capabilities offered by the insect cell system without the requirement of sterile conditions or growth media, it is easy to scale-up and yields a highly concentrated protein extract at a relatively low cost (Kost et al., 2005).

Partially purified soluble recombinant WNV-E protein (rE) was evaluated for use as an antigen in an indirect ELISA for detection of anti-WNV IgG antibodies using a battery of equine sera characterized previously (Alonso-Padilla et al., 2009). Comparison of the ELISA based on rE protein with the ELISA based on the use of iWNV as antigen shows a good equivalence in sera reactivity, with good specificity and sensitivity when compared with the "gold-standard" PRNT technique.

2. Materials and methods

2.1. Virus and cells

West Nile virus NY99 flamingo 385-99 strain was propagated and titrated on Vero cells (African green monkey kidney cells) as described by Tardei et al. (2000). Plaque reduction neutralization tests (PRNTs) were conducted on Vero cells with WNV using twofold serial sera dilutions, as described previously (Beaty et al., 1989). Titers were calculated as the reciprocal of the serum dilution, diluted at least 1:40, which reduced plaque formation ≥90% ($PRNT_{90}$). Heat-inactivated antigen (iWNV) was produced from Vero cells infected with WNV and processed as described elsewhere (Blitvich et al., 2003). All experiments which involved the use of live virus were performed in bio-safety level 3 (BSL-3) containment facilities.

Recombinant baculovirus growth and titrations were carried out in Sf21 (*Spodoptera frugiperda*) cells as described previously by Jiménez de Oya et al. (2009a).

2.2. Cloning and expression of WNV recombinant E protein (rE)

Viral genomic RNA was extracted from 140 μl of WNV infected Vero cell culture supernatant using QIAamp®Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A cDNA fragment encoding the soluble fragment of the E protein (nucleotide positions 967-2322, GenBank accesison no. AF196835) was generated by RT-PCR (SuperScript™ One-Step RT-PCR, Invitrogen, Carlsbad, CA, USA) using primers forward 5′-CCAGCGGATCCCTTCAACTGTTTAGGAATG-3′ and reverse 5′-GCCTCCGGTACCGAGCGGAATGCTCCTCCGAA-3′ (BamHI and KpnI restriction sites included in the primers are underlined). The purified PCR product (QIAquick® PCR Purification Kit, Qiagen) was digested with BamHI and KpnI, purified again, and inserted into BamHI and KpnI digested pFastBacMel-B2 vector under the control of the polyhedrin constitutive promoter as described previously (Jiménez de Oya et al., 2009a). pFastBacMel-B2 includes the melitin signal peptide after the polyhedrin constitutive promoter, which is known to head translation in insect cells through the Golgi apparatus and the cell secreting pathway (Mroczkowski et al., 1994), allowing glycosylation of the insert and its posterior secretion. Recombinant baculovirus expressing the E protein (rE-Bac) was produced using the Bac-to-Bac system (Invitrogen) following the manufacturerĩs instructions.

To obtain recombinant E (rE) protein on a large scale in an easy and economical way, groups of 10 specimens of *Trichoplusia ni* (*T. ni*) larvae were inoculated near the proleg with 10^5 plaque forming units (PFU)/larva of rE-Bac, or with a similar dose of an irrelevant control baculovirus (Ir-Bac), as described previously (Jiménez de Oya et al., 2009b; Pérez-Filgueira et al., 2006).

2.3. Partial purification of WNV recombinant rE protein

WNV rE protein and an irrelevant control product (Ir-BacNi) were obtained partially purified from larvae extracts after rough homogenization (BagMixer, Interscience, Saint Nom la Bretèche, France) of 10 larvae in 20 ml volume of solubilising solution containing: 10 mM imidazole (Merck, Mollet del Vallés, Spain), 500 mM NaCl (Merck), 1% Triton X-100 (Sigma–Aldrich GmbH, Steinheim, Germany), 3 mM dithiothreitol (Sigma–Aldrich) and a protease inhibitor cocktail (Complete, Roche Diagnostics, Mannheim, Germany). Homogenates were cleared after two centrifugation steps at $8000 \times g$ and $14{,}500 \times g$ in a JA-20 rotor (Avanti™ J-25 I Centrifuge, Beckman Coulter, Brea, CA, USA), along with a filtration step through Miracloth (Merck), and three ultrasonication cycles (UP200S, Hielscher, Teltow, Germany) in between. The final supernatant was then loaded onto His-GraviTrap Ni-rich resin columns (GE Healthcare, Alcobendas, Spain). For the partial purification process, concentrations of 10, 20 and 500 mM imidazole were used for the binding, washing and elution buffers, respectively. Protein concentration was determined by Bradford reaction (Bio-Rad Protein Assay, München, Germany), as well as by gel densitometry using known amounts of purified bovine serum albumin. After the partial purification ten inoculated larvae processed yielded up to 5 mg of soluble protein when inoculated with rE-Bac.

2.4. Protein expression

Western blot analysis was carried out as described by Jiménez de Oya et al. (2009b). The following reagents were used as primary antibodies: an anti-His mouse monoclonal antibody (Clonetech Inc. Laboratories, Mountain View, CA, USA), an anti-WNV hyperimmune mouse ascitic fluid, (anti-WNV-HMAF; Desprès et al., 2005; kindly gifted by Dr. P. Desprès, Institut Pasteur, Paris, France), the available commercially 3.67G mouse monoclonal antibody (Millipore Ibérica SA, Madrid, Spain) and a negative control mouse sera (Cordoba et al., 2007). All primary antibodies, but 3.67G (1:500) and negative control sera (1:100), were used at a 1:1000 dilution.

2.5. rE-based ELISA

Partially purified rE protein was evaluated as an enzyme-linked immunosorbent assay (ELISA) antigen. Ninety-six wells microplates (NUNC, Roskilde, Denmark) were coated overnight at 4 °C with 50 μl/well of rE protein diluted 1:100 in coating buffer (0.015 M Na_2CO_3, 0.030 M $NaHCO_3$; pH 9.6). All following incubations were carried out for 1 h at 37 °C. Plates were blocked with 100 μl/well of blocking solution (3% skimmed milk in phosphate buffered solution with 0.05% Tween 20, PBST) and then washed twice with PBST before adding 50 μl/well of anti-WNV antibodies dilutions of: either 3.67G mouse monoclonal, a positive sera pool from mice infected experimentally (MPS; Cordoba et al., 2007), anti-WNV-HMAF, a positive serum from a rabbit inoculated with live WNV (RIS; kindly provided by Dr. M.A. Jiménez-Clavero, CISA-INIA, Valdeolmos, Spain). A mice negative sera pool (MNS) was included as control. After primary antibody incubation, plates were washed three times with PBST and 50 μl/well of protein G horseradish peroxidase conjugated (Invitrogen), diluted 1:5000 in blocking solution, was added as secondary antibody. Four additional washing steps were performed before adding 50 μl/well of substrate solution (*O*-phenylene diamine dihydrochloride–0.056% H_2O_2). Substrate reaction was allowed to develop in the dark at room temperature for 10 min, then stopped by the addition of H_2SO_4 3N (50 μl/well) and read at 492 nm in an ELISA microplate reader (TECAN Genios Ag, Vienna, VA, USA).

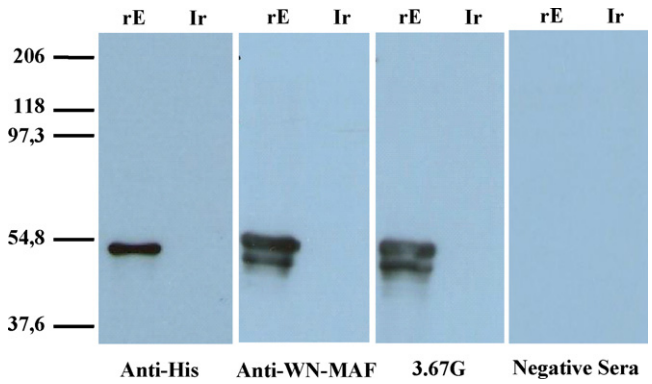

Fig. 1. Western blot analysis of WNV recombinant enveloped (rE) protein. Western blot analysis of rE and an irrelevant recombinant protein (Ir-BacNi) were stained with an anti-His monoclonal antibody (anti-His), anti-WNV hyperimmune mouse ascitic fluid (anti-WNV-HMAF), 3.67G mouse monoclonal antibody, and a mice negative sera pool as described in Section 2. Molecular size markers are shown on the left.

3. Results

3.1. Cloning and expression of WNV rE recombinant protein

Partially purified rE protein expressed in *T. ni* larvae was detected by Western blot at the expected size of approximately 50 kDa by a series of primary antibodies (Fig. 1); however, with the exception of the anti-His antibody, all anti-WNV specific antibodies tested recognized a double band pattern, probably being the lower species a degradation product lacking the His tag. None of the antibodies tested recognized similarly processed extracts from larvae inoculated with an irrelevant baculovirus (Ir-BacNi) and uninfected mice serum used as negative control did not react with the antigens.

3.2. Optimization of the rE-based ELISA

The antigenicity of the partially purified rE protein as ELISA antigen was confirmed with serial dilutions of a battery of positive and negative antibodies of different origin (mouse monoclonal and polyclonal sera, mouse hyperimmune ascitic fluid and rabbit immune serum) as described above (see Section 2.5). All antibodies tested, except the negative control sera, reacted with rE and their reactivity correlated with neutralization capability (Fig. 2).

For ELISA optimization, microplates were coated overnight at 4 °C with serial dilutions of rE antigen (62.5 ng to 2.0 μg per well); then, characterized previously as positive ($n = 8$) and negative ($n = 8$) murine (Cordoba et al., 2007) and positive ($n = 8$) and negative ($n = 8$) equine sera (Estrada-Franco et al., 2003; a kind gift from Dr. J.G. Estrada-Franco, UTMB, Galveston, USA), were used as primary antibodies as described in Section 2.5. Mouse and equine positive sera reactivity on rE containing wells was proportional to antigen concentration (Fig. 3), remaining the reactivity plateau with A_{492} values above 1.0 at a 0.5 μg/well concentration of antigen (average $A_{492} = 1.1$; sd = 0.35; and $A_{492} = 1.2$; sd = 0.16, respectively). Average reactivity of murine and equine negative sera were $A_{492} = 0.08$ (sd = 0.01) and $A_{492} = 0.16$ (sd = 0.06). These values were similar to the background of the assay that was tested by including in every third column of the plates a negative antigen (Ir-BacNi) as suggested by Ebel et al. (2002), confirming the specificity of the antigen–antibody reaction.

3.3. Criteria for determining positive reactivity by anti-WNV ELISA

To establish the cut-off of the assay, the above-mentioned eight characterized previously negative horse sera, which gave an average A_{492} value of 0.16 (range = 0.09–0.26, sd = 0.06) on positive antigen containing wells, were pooled and used as negative control. These values were similar (average $A_{492} = 0.15$; sd = 0.03) to those obtained on negative antigen containing wells. As described previously (Martin et al., 2000), a sample was considered positive when the mean A_{492} values on positive antigen containing wells was at least twice that of the mean A_{492} values on negative antigen containing wells, and the positive/negative (P/N) ratio was ≥2.0. P/N ratio was calculated by dividing the absorbance of the sample reacted on viral antigen by the absorbance of the negative control serum on the same antigen.

3.4. Comparison of rE-based with iWNV-based ELISA and $PRNT_{90}$

Once the rE antigen concentration (0.5 μg/well) and serum dilution (1:100) were set up, the same procedure was performed over a battery of 60 equine serum samples characterized previously from Mexico, 42 of which specifically reacted against WNV based on their $PRNT_{90}$ and hemagglutination inhibition (HI) reactivity and 18 were negative (Alonso-Padilla et al., 2009). The results were compared with those obtained in parallel on plates coated with heat-inactivated antigen (iWNV). Specificity of the antigens and background reactivity were determined by including in every third column of the coated plates their corresponding negative antigens, either Ir-BacNi or processed uninfected cell lysates (Mock-Vero).

The concordance between both ELISAs was 95% (kappa = 0.89) and the specificity and sensitivity showed by the rE-based ELISA when compared with the iWNV-based ELISA was 100% and 92.5%,

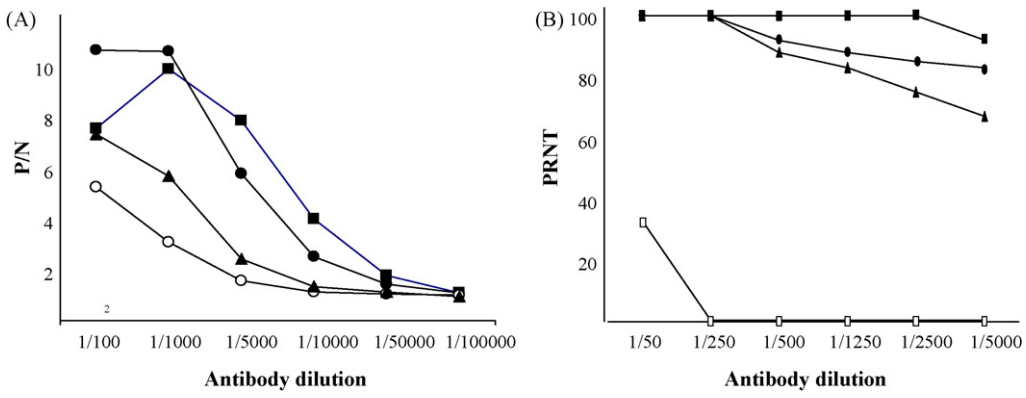

Fig. 2. ELISA reactivity (A) and neutralization capability (B) of antibodies. rE-based ELISA and plaque reduction neutralization tests were conducted as described in Sections 2.5 and 2.1, respectively. Rabbit immune serum (solid squares), hyperimmune mouse ascitic fluid (solid circles), 3.67 mouse monoclonal antibody (solid triangles), and mouse positive (open circles) and negative (open squares) polyclonal sera pools were tested. P/N, positive/negative ratio. PRNT, plaque reduction neutralization tests.

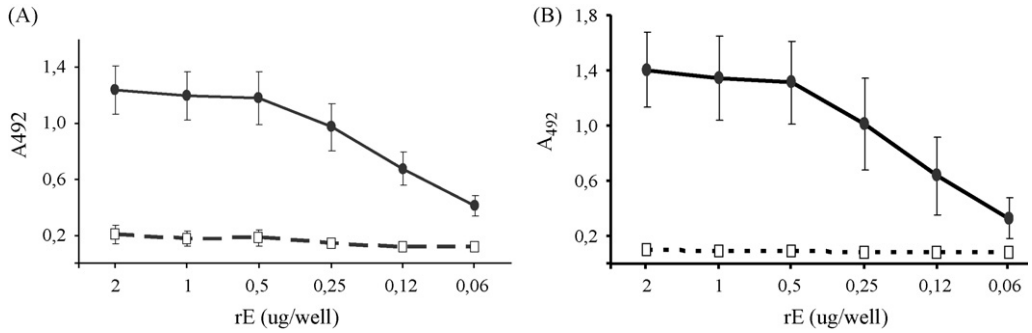

Fig. 3. Optimization of rE antigen concentration. Equine (A) and murine (B) positive (filled circles) and negative (open squares) sera characterized previously were tested by ELISA against twofold dilutions (2.0–0.06 μg/well) of partially purified rE protein. Data are shown as the average absorbance (A_{492}) of eight negative and eight positive samples of each animal species. Standard deviations for each point are shown.

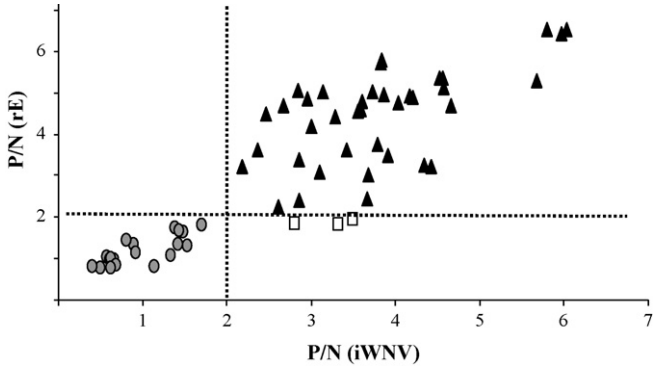

Fig. 4. Comparison of the reactivity of rE antigen and iWNV-based ELISAs. ELISAs were performed as described in Section 2.5. Data are expressed as P/N ratios calculated as described in Section 3.3. Vertical and horizontal dotted lines represent the cut-off of the assays. Filled triangles and circles represent sera that reacted positively and negatively, respectively, in both assays. Open squares represent sera that were positive only by the iWNV-based tests.

Table 1
Concordance of equine sera reactivity between the rE-based ELISA and the PRNT.

	Number of sera with the indicated reactivity by the $PRNT_{90}$		ELISA total
	Positive	Negative	
Results by the rE-based ELISA			
Positive	37	1	38
Negative	5	17	22
$PRNT_{90}$ total	42	18	60

Concordance, 90% (kappa = 0.78).

respectively. In the only three discordant cases, the reactivity on rE antigen was close to the cut-off of the assay (Fig. 4). Next, the rE-based ELISA results were compared with that of $PRNT_{90}$, as the gold-standard (Table 1). The concordance found was 90% (kappa = 0.78), with a 94.4% specificity, since only one ELISA positive sera was negative by PRNT. On the other hand, the sensitivity was 88.1%, although all five discordant samples showed P/N values (average P/N = 1.9; sd = 0.12) very close to the cut-off of the assay (P/N $\geq$2.0), and three of them presented low neutralizing titers ($PRNT_{90}$ = 40).

4. Discussion

Unless a non-infectious recombinant antigen is available, ELISA diagnosis of WNV involves working with infectious virus to produce inactivated antigens from infected cell cultures or suckling mouse brains (Blitvich et al., 2003; Ebel et al., 2002; Loroño-Pino et al., 2009; Malan et al., 2004; Martin et al., 2000; Tardei et al., 2000), with the risks that the procedure implies for lab personnel and the highly sophisticated BSL-3 containment facilities needed to grow this zoonotic agent. In search for an accurate non-infectious WNV recombinant antigens for diagnosis and based on previous investigations with other flavivirus models, such as Dengue virus (DENV) or Tick-borne encephalitis virus (TBEV), initial studies focused on viral structural (prM-E) recombinant proteins (Davies et al., 2001; Hogrefe et al., 2004; Muerhoff et al., 2004), since they are highly exposed to the host immune system and bear most of the neutralizing epitopes described (Roehrig, 2003; Wang et al., 2001). Lately, it has been reported that the soluble fragment of the WNV-E protein obtained with baculovirus vectors is folded properly in the absence of prM and is capable of detecting anti-WNV IgG antibodies in a variety of animals infected with virus (Bonafé et al., 2009). Even its crystal structure was determined expressing the protein in insect cells inoculated with a recombinant baculovirus (Nybakken et al., 2006). Hence, baculovirus expressed recombinant E protein likely acquires conformations similar to that present on the native virion and, therefore, would be recognized by sera from WNV animals infected naturally, making it a suitable antigen for WNV diagnosis by ELISA.

Although WNV antigens production using baculovirus expression vectors had been described previously (Bonafé et al., 2009; Qiao et al., 2004), to our knowledge, this is the first time that live insect larvae have been used for it. These recombinant protein factories allow the production of high yields of glycosylated recombinant proteins in an easy to scale-up and low cost procedure. The conditions used here for the production of WNV antigen yield up to 0.5 mg of partially purified rE protein per larva in 72 h. Since 0.5 μg/well was used in the developed ELISA, this indicates that an estimated 1000 individual determinations can be obtained from a single larva.

The accuracy of rE protein as a diagnostic antigen was confirmed by comparison of the *in house* rE-based ELISA with the inactivated whole virus antigen (iWNV) based assay used widely, and validated with the reference test (PRNT). The results showed a quite good concordance between both ELISAs (95%). Likewise, concordance of the rE-based ELISA with the PRNT was also good (90%). The specificity when compared with the reference technique was 94.4%. As suggested previously (Alonso-Padilla et al., 2009), the single non-neutralizing sample that was positive in both ELISAs could reflect reactivity to other related Flavivirus circulating in the geographical area examined and not assayed in the referred work. The sensitivity observed was 88.1%, but all five neutralizing samples that were rE ELISA negative showed P/N values just below the cut-off of the test, and three of them presented also low neutralization titers ($PRNT_{90}$ = 40). As three of the five were positive by iWNV ELISA, the explanation for the other two neutralizing samples might be due to

the presence of neutralizing IgM specific antibodies that were not tested in the present study.

WNV is showing currently variable epidemiological and ecological patterns, with the common feature of a continuous spread. All these, and the possibility that ongoing climatic warming would enlarge mosquito susceptible habitats and breeding areas, makes the availability of a reliable, easy, economic and safe diagnostic procedure of great importance for investigation of infections with WNV.

This study describes the successful production of WNV recombinant envelope E (rE) protein upon inoculation of *T. ni* larvae with a recombinant baculovirus. This procedure allows for an easy, economical and relative large-scale production of rE that proved to be a suitable antigen for detection of anti-WNV antibodies in horse serum samples collected in field.

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