

# Evidence of a humoral response to a novel protein WARF4 embedded in the West Nile virus NS4B gene encoded by an alternative open reading frame

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**Abstract.** West Nile virus (WNV) is a flavivirus that is maintained in a bird-mosquito transmission cycle. Humans, horses and other non-avian vertebrates are usually incidental hosts. However, WNV is a neurotropic virus, which requires an efficient humoral response for the control of a neuroinvasive infection. The WNV genome encodes three structural (capsid, premembrane/membrane and envelope) and seven non-structural proteins. Bioinformatic analysis performed on the WNV genomes detected a conserved alternative open reading frame restricted to the lineage I virus. To quickly verify the existence of this putative protein, entitled West Nile Alternative Reading Frame 4 (WARF4), we produced a prokaryotic recombinant source of WARF4 and verified its immunogenicity *in vivo* by analyzing 43 horse serum samples, of which 15 were positive for antibodies to WNV premembrane and envelope (prM-E) proteins. Specific antibodies to WARF4 were significantly detected in 5 out of the 15 serum samples testing positive for antibodies to prM-E WNV proteins. Our findings provide evidence of a significant antibody response to the WARF4 protein in the serum of the horse testing positive for antibodies to prM-E proteins, thus indicating that this antigen might be a potential tool for further characterization of the immune response of WNV infections in humans as well.

## Introduction

Following its original isolation, West Nile virus (WNV) was implicated in sporadic outbreaks of mild viral illness in Africa, the Middle East, western Asia and Europe (1,2). Since 1999, the virus has emerged as a new threat to humans and mammals in the western hemisphere (3,4). Phylogenetic analyses reveal two distinct lineages (5): lineage I, which has a worldwide distribution and is involved in human and equine outbreaks, and lineage II, which is present in Africa's sub-Saharan region and is not associated with clinical manifestations in humans (6,7). Although several studies are in progress for a better understanding of WNV pathogenesis, the mechanisms of immunological escape of West Nile remain unclear (8). Moreover, it is suggested that unidentified factors may be involved in the West Nile disease (9). WNV can be transmitted to humans and can cause encephalitis, depending on age and the immunity status of the exposed individual (3). Antibody-positive blood components are less efficient transmitters of the virus than antibody-negative components, thus indicating that neutralizing antibodies are essential for the control of WNV infection *in vivo* and that an efficient humoral response is critical for the control of a neuroinvasive WNV infection (10,11).

To obtain a major number of proteins without modifying the genome size, viruses often make use of an alternative reading frame gene embedded in the mean coding strand. The existence of superimposed genes, first noted in bacteriophage  $\phi$ X174 (12), is a phenomenon well characterized in viruses as well as in other organisms (13-15). Among the flaviviridae, the use of an alternative open reading frame protein (ARFP) has been reported for hepatitis C viruses (HCV) (16). After humans, horses constitute the majority of mammals infected with WNV (17). The close phylogenetic relationship between HCV and West Nile led us to analyze the WNV genome, searching for alternative gene products. Our bioinformatic analysis detected six ARFPs, one of them restricted to lineage I of WNV. To quickly verify the existence of this putative

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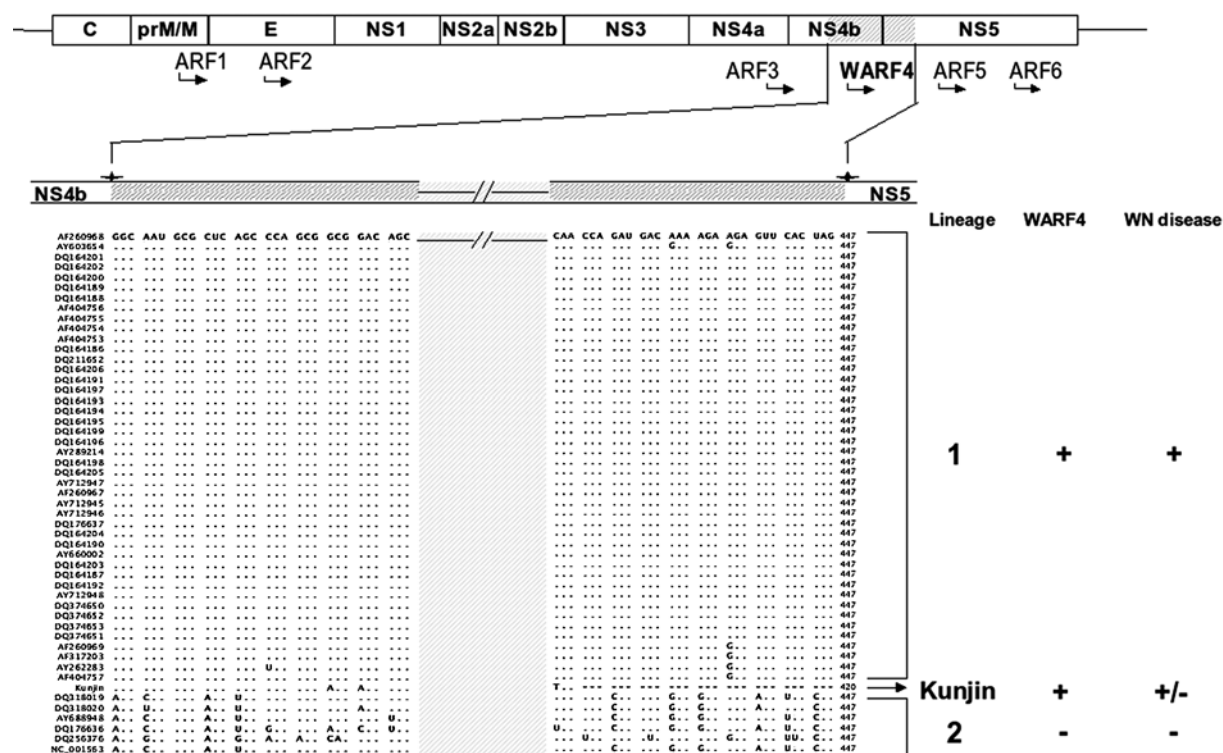


Figure 1. Schematic organization of the West Nile genome and positions of the alternative reading frames. Illustrated in detail are the partial alignment of WARF4 and its association with viral lineage and disease.

protein, entitled West Nile Alternative Reading Frame 4 (WARF4), we produced a prokaryotic recombinant source of WARF4 and tested its immunogenicity *in vivo* by analyzing 43 horse serum samples, of which 15 were positive for antibodies to WNV premembrane and envelope (prM-E) proteins. Our findings provide evidence of a significant antibody response to the WARF4 protein in the prM-E protein positive horse, thus indicating that this antigen might be a potential tool for further characterization of the immune response to WNV infections in humans as well.

Materials and methods

**Bioinformatic analysis.** Fifty West Nile viral genomes (Fig. 1) were retrieved from GenBank. ARFs were detected by CLC Sequence Viewer ([www.clcbio.com](http://www.clcbio.com)). The search parameters included any codon used as start codon and an ARF protein length of at least 100 amino acids including a stop codon. Further analyses were carried out using ClustalX (<http://www.clustal.org/>).

**Serum samples and genomic RNA.** Forty-three horse serum samples were collected by Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana during the serological surveillance activities conducted in Toscana (Italy) between 1998 and 2007. The sera were tested according to the manufacturer's instructions for anti-West Nile IgG using the commercial Kit ELISA ID Screen® West Nile Competition (ID.VET, Montpellier-France). West Nile genome RNA was obtained from the European Network for Diagnostics of Imported Viral Disease (ENIVD) during the External Quality Assurance (EQA) for molecular detection of

West Nile virus. The nucleic acids were extracted using the NucleoSpin RNA Virus Kit according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany).

**Cloning and expression of WARF4 protein.** The fragment of 394 bp spanning the WARF4 position 7327-7720 (accession number AF260967) was amplified in a single step RT-PCR using 5' forward oligonucleotide (gttgaggaattcttccaaacctctcccaag) and 3' reverse oligonucleotide (atgcgcggatcccagcggcgacagcggctgg). The fragment was cloned into the expression vector pRSETC and then expressed in the BL21 Star (DE3)pLysS competent cells (Invitrogen, CA). The his-tagged recombinant protein (WARF4) was purified under denaturing condition with Ni-NTA Agarose resin (Qiagen, Germany). Proteins were visualized by Coomassie Blue staining.

**Western blot analysis.** Approximately 200 ng of the WARF4 protein or the bacterial cell extract transformed with the plasmid vector alone was separated by SDS/PAGE and then transferred to a nitrocellulose membrane. After blocking, membranes were incubated with horse serum (1:1000) overnight at 4°C. The 1:1000 dilution was chosen for testing as the highest serum concentration that lacked substantial background reactivity. After washing, the filters were incubated with anti-horse IgG whole-molecule peroxidase conjugate (Sigma) and developed by a chemiluminescent kit (Sigma) as previously described (18-20). Criteria of positivity was the appearance of an immunoreactive band in the WARF4 sample, co-migrating with the one visualized by the anti-his antibody and not in the bacterial cell extract used as negative control.

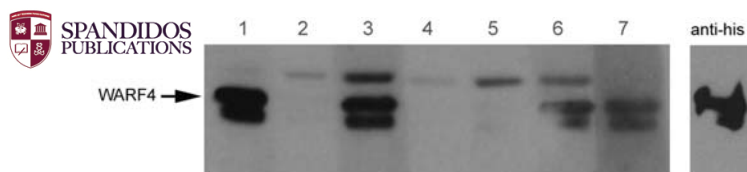


Figure 2. Immunoreactivity of horse sera to the WARF4 recombinant protein. Seven prM-E protein positive horse serum samples assayed for humoral response to the recombinant protein WARF4 are shown. Four serum samples (1, 3, 6, 7) specifically detected the WARF4 protein by Western blotting. Reactivity with the anti-his antibody to WARF4 recombinant protein is shown as the positive control.

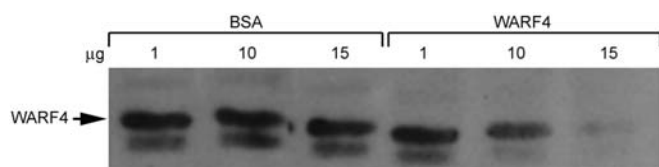


Figure 3. Specificity of the anti-WARF4 antibodies. The analysis was performed to confirm the specificity of the serum antibodies to WARF4 protein by a competition assay. One WARF4 positive horse serum sample preincubated with increasing amounts of BSA as negative control (lanes 1, 2, 3) and WARF4-free antigen (lanes 4, 5, 6) is shown. The samples were then analyzed by Western blotting.

**Competition assay.** Competition assay was performed to confirm the specificity of the serum IgG for the WARF4 protein. Increasing concentrations of the WARF4 purified protein or bovine serum albumin (BSA) (1-15 µg) were incubated for 30 min at room temperature with horse serum testing positive for antibodies to WARF4 protein. The serum was then tested for its reactivity with WARF4 by Western blotting as described above.

**Statistical analysis.** Associations were considered significant at p-values  $\leq 0.05$  by a 2x2 contingency table.

## Results

*In silico* analysis detected six ARFs (Fig. 1). Among the others, the ARF4 appears to be the longest (447 bp) and most conserved from the genomes, present in 88% (44/50). This novel alternative reading frame, entitled WARF4, begins from a +2 position when compared to the coding strand. This novel potential gene is embedded between the NS4b C-terminal residue and the NS5 N-terminal residue (7311-7757 bp, accession number AF260967), starting from a GGC triplet and terminating with a TAG stop codon. The WARF4 amino acid sequence does not show an apparent homology with other known protein sequences. Assuming that the initiation codon is the first in the alternative frame, the protein would contain 148 AA and shows an isoelectric point of 9.4. Bioinformatic analysis predicts a myristoylation signal in position 1 to 10 AA with a low probability of false-positive prediction. In order to prove the actual existence of the WARF4 protein, we produced a prokaryotic recombinant source of WARF4 and verified its immunogenicity *in vivo* by

Table I. Evidence of antibodies to WARF4 protein in horse sera testing positive for prM-E WNV proteins.

	IgG anti-prM-E		Total horse sera
	(+)	(-)	
IgG anti-WARF4			
(+)	5 <sup>a</sup>	1	6
(-)	10	27	37
Total horse sera	15	28	43

<sup>a</sup>For statistical analysis, results were analyzed by a 2x2 contingency table. A significant association was found between antibodies to prM-E proteins and WARF4 protein (p<0.015).

analyzing its reactivity with 43 horse serum samples by Western blotting. Among the others, 15 horse sera showed antibodies against prM-E WNV proteins, while 28 lacked reactivity to the same proteins. Criteria of positivity to WARF4 recombinant protein was the appearance of an immunoreactive band in the WARF4 sample, co-migrating with the one visualized by the anti-his antibody and not in the bacterial cell extract used as negative control. Two proteins in the WARF4 sample were detected in several horse serum samples as well by the anti-his antibody (Fig. 2). The lower molecular weight protein might represent a degradation product of the full length WARF4 recombinant protein. Overall, we detected serum antibodies to WARF4 recombinant purified protein in 5 out of 15 horses which simultaneously displayed antibodies against the prM-E proteins. Serum antibodies were also detected in one of 28 horses testing negative for antibodies to WNV prM-E proteins. To demonstrate the specificity of the humoral response to WARF4, a competition assay was performed (Fig. 3). When using the highest WARF4 protein concentration as competitor, the serum reactivity disappeared thus confirming the presence of specific IgG for the WARF4 protein. A significant association was found between the presence of antibodies to prM-E and WARF4 proteins in horse serum (p<0.015) (Table I).

## Discussion

West Nile virus, which has become endemic in North America, is a positive-sense RNA neurotropic virus (10,11,17). The WNV genome encodes 3 structural (capsid, premembrane/ membrane and envelope) and 7 nonstructural proteins (10,11,17). Neutralizing antibodies are essential for the control of the WNV infection (10,11,17). It was demonstrated that MHC molecules can also present peptides encoded in alternate translational reading frames (21). The general importance of out of frame proteins was highlighted for their ability to enhance immunity responses (21). Furthermore, it was demonstrated that these alternative products may have a critical role in the pathogenesis of viral infections (22,23). The WNV genome has been completely sequenced, and our computer-assisted analysis indicates the presence of 6 ARFs. Among the others, alignment analysis shows that the ARF4 (entitled West Nile Alternative Reading



Frame 4, WARF4), represents a unique feature of lineage I WNV genomes, that are often associated with clinical symptoms. In order to prove the actual existence of the WARF4 protein, we produced a prokaryotic recombinant source of WARF4 and verified its immunogenicity *in vivo* by analyzing its reactivity with 43 horse sera by Western blotting. The horse sera were also characterized for their reactivity to well known premembrane and envelope (prM-E) WNV proteins by a commercial ELISA kit.

Our results demonstrated a differential reactivity of the horse sera. Five serum samples displayed antibodies for both WARF4 and prM-E WNV proteins; 10 sera, testing positive for prM-E antibodies were WARF4 negative. Conversely, one serum sample lacking immunoreactivity to prM-E appeared, instead, reactive to the WARF4 recombinant protein. Our results indicate a statistically significant association between the presence of antibodies to prM-E proteins and WARF4 in the horse sera ( $p < 0.015$ ) (Table I). The reactivity of horse sera to prM-E protein but not to WARF4 might be related to the ARF4 expression level. Indeed, ARF proteins are reported to be usually less expressed compared to ORF proteins (24). The fact that a single horse had specific antibodies to the WARF4 protein but not to the prM-E proteins might suggest cross-reactivity of antibodies with co-circulating flavivirus protein (25) or a different sensitivity of the assay used for testing the presence of antibodies.

We have not yet investigated the translation mechanisms of this novel encoded protein. A cap-dependent ribosomal scanning process is involved in WN virus translation, although a different strategy was reported for other flaviridae (26). Since the WARF4 gene lacks an AUG codon, a potential alternative translation mechanism could take into account the ribosomal frame shift; nevertheless other non-canonical translation mechanisms cannot be excluded. At this stage it is not possible to speculate on the function of this novel protein. Furthermore, it must be highlighted that the presence of antibodies to the WARF4 protein needs to be observed in humans infected by WNV. Overall, our findings provide the evidence of an antibody response to the WARF4 protein in the serum of horse testing positive for antibodies to prM-E proteins, thus indicating that this antigen might be a potential new tool for further characterization of the immune response of WNV infections in humans as well. However, further studies are necessary to show that the WARF4 protein is produced in the context of WNV infection. On the other hand, it will be intriguing to investigate whether a potential association exists, between the evidence of a WARF4 specific immune response and the outcome of the human WNV disease. In this context, it is also suggestive that lineage II WNV genomes lacking WARF4 are not associated to human clinical disease.

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