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Production of recombinant NS1 protein and its possible use in encephalitic flavivirus differential diagnosis

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ABSTRACT

Saint Louis encephalitis virus (SLEV) and West Nile virus (WNV) are two of the major causes of arboviral encephalitis in the Americas. The co-circulation of related flaviviruses in the Americas and prior vaccination against flaviviruses pose problems to the diagnostic specificity of serological assays due to the development of cross-reactive antibodies. An accurate diagnosis method capable of differentiating these related viruses is needed. NS1 is a glycosylated, nonstructural protein, of about 46 kDa which has a highly conserved structure. Anti-NS1 antibodies can be detected within 4–8 days after the initial exposure and NS1 is the least cross-reactive of the flaviviral antigens. This study was aimed to generate SLEV and WNV NS1 recombinants proteins for the development of a flavivirus diagnostic test. Local Argentinian isolates were used as the source of NS1 gene cloning, expression, and purification. The protein was expressed in *Escherichia coli* as inclusion bodies and further purified by metal-chelating affinity chromatography (IMAC) under denaturing conditions. Human sera from SLEV and WNV positive cases showed reactivity to the recombinant NS1 proteins by western blot. The unfolded NS1 proteins were also used as immunogens. The polyclonal antibodies elicited in immunized mice recognized the two recombinant proteins with differential reactivity.

1. Introduction

Saint Louis encephalitis virus (SLEV) and West Nile virus (WNV) (genus *Flavivirus,* family *Flaviviridae*), are positive-sense RNA viruses with a 30- to 35-nm icosahedral core surrounded by a lipid bilayer envelope. The genome is composed of ten genes coding for three structural proteins (C, M, and E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) [1,2].

SLEV is the major representative of the Japanese encephalitis serocomplex in the Americas. Since the first identification of SLEV in St. Louis, Missouri, in 1933, it has caused more than 50 outbreaks and numerous epidemics in the United States, and was one of the major causes of arboviral encephalitis until the introduction of West Nile virus in 1999 [3,4]. Although SLEV has been known to occur in Argentina and Brazil since the 1960s, human cases of SLEV infection have only been reported sporadically. The first confirmed outbreak of SLEV outside North America was reported in 2005 in Argentina, where 47 confirmed clinical cases of SLEV infection, including nine fatalities, were reported in Córdoba province [5]. Since 2005, small outbreaks and sporadic cases have been reported in this country [6,7].

WNV is currently the most widely distributed of the encephalitic flaviviruses. First isolated in Uganda in 1937, WNV circulation has been described in Africa, Europe, Asia, and America. Since its introduction in 1999 in New York, it has rapidly dispersed throughout the Americas, affecting people from the central region of Canada to South America [1]. The first report of the introduction of WNV in Argentina was in January 2005, when the virus was detected in birds [8]. After this, evidence of the circulation of the virus in horses was reported in 2006 and 2010 [9].

Diagnosis of human Flavivirus cases is rarely accomplished by isolating the virus or antigen detection, since viremia levels may be very low or absent by the time the clinical manifestations appear [10]. For

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this reason, it depends almost exclusively on indirect methods. Thus, serological tests are established as the primary method for flaviviral diagnosis, making it possible to detect IgM or IgG from sera or cerebrospinal fluid (CSF) 4–8 days after the onset of symptoms [11,12].

The gold-standard serological method for detecting immune responses is the Plaque Reduction Neutralization Test (PRNT), which can detect specific viral neutralizing antibodies (NTAb) and differentiate between closely related members of the genus Flavivirus. The PRNT is a very laborious technique that takes at least a week and requires appropriately timed sampling of acute and convalescent sera. Also, the handling of the live virus requires skilled personnel, and Biosafety level (BSL)-3 facilities that are not commonly available in most clinical settings. The procedure for the antigen preparation is time-consuming, and the biological risk limits the use of viruses that do not circulate in the region [12,13].

Many commercial and in-house serological tests have been developed to diagnose flavivirus infections [14–18], but the co-circulation of related flaviviruses in the Americas and prior vaccination against flaviviruses, like Yellow Fever, pose problems to the diagnostic specificity of serological assays due to the development of cross-reactive antibodies [12]. In South America, in addition to WNV and SLEV, flaviviruses that should also be considered when testing patients' sera include Dengue 1–4, Zika, Yellow Fever, Ilheus, Bussuquara and Rocio [19–22]. In many cases, this complexity leads to an indeterminate detection of the infecting etiology, concluding the diagnosis as probable SLEV/WNV.

NS1 protein is a highly conserved nonstructural protein that contains nearly 352 amino acid residues (depending on viral species) and shows a molecular weight that depends on post-traductional glycosylations (46 kDa approximately) [23]. NS1 also appears to have a role in immune evasion, as it has been shown to attenuate complement activation [24]. While a large amount of NS1 remains in the infected cell, it is also actively secreted as a hexameric form at relatively high levels. During the acute phase of infection, both forms are demonstrated to be immunogenic and stimulate a non-neutralizing antibody response [12]. NS1 is an early diagnostic marker, as a high NS1 level circulates in the acute phase and can be detected in the sera of patients with primary and secondary infections, up to the 9 t h day after the onset of symptoms [25,26]. Anti-NS1 IgM antibodies can be detected within 4–7 days after the initial exposure and may persist more than one year. In comparison, anti-NS1 IgG is reliably detected eight days after the onset of symptoms [13]. It has also been reported that NS1 is the least cross-reactive of the flaviviral antigens and has the most specific reactivity when attempts are made to differentiate WNV from SLEV immune sera [27].

We describe the production of recombinant NS1 proteins from SLEV and WNV using local Argentinian isolates as the source of NS1 gene sequences. The method used in this study might be suitable for producing NS1 proteins from strains of other geographical regions or other related viruses with no or minimal modification. The recombinant NS1 proteins retain their immunological properties and can potentially be used in the development of an NS1 antigen-based flavivirus diagnostic method, and this would be pan-specific or Japanese Encephalitis Virus serocomplex specific diagnostic method.

2. Materials and methods

2.1. Viral strains, RNA extraction, and reverse transcription

The SLEV epidemic strain (CbaAr-4005) was isolated from *Cx. Quinquefasciatus* mosquitoes collected during the human encephalitis outbreak in 2005 [28]. Viral stocks were obtained by inoculating monolayers of VERO cells in 75 cm^2 tissue culture flasks with virus diluted 1:10 in Eagle's minimum essential medium (MEM) containing 2% fetal bovine serum (FBS). Cells and supernatant were then harvested when 75% cytopathic effects were observed. Cell debris were removed by centrifugation and the viral supernatant was aliquoted and stored at −80 °C, according to Diaz et al., (2006) [28].

Vero cell supernatant was used for viral RNA extraction using the commercial QIAamp viral RNA MiniSpin Kit (Qiagen) following the manufacturer's instructions. Extracted RNA was reverse-transcribed into cDNA using SuperScript III (Invitrogen) and random hexamers.

The WNV strain ArEq-001, whose sequence was used for the synthesis of NS1, was isolated from horses and sequenced by Morales et al. in 2006 [9].

2.2. Primers design and generation of PCR products

The SLEV strain CbaAr-4005 (GeneBank accession number FJ753286) was used as the source for the gene-specific sequence. The SLEV NS1 primers were designed to incorporate the restriction sites *Nde*I and *Xho*I followed by the specific gene sequence according to the corresponding SLEV strain. The PCR product was generated using the virus cDNA as template and the corresponding pair of primers. PCR amplification was performed using Pfu DNA Polymerase (Promega) using typical PCR conditions as follows: after an initial denaturation step at 94 °C for 2 min, the reaction was amplified for 35 cycles. Each cycle consisted of a denaturation step at 94 °C for 20 s, annealing at 50 °C for 20 s, extension at 72 °C for 1 min and a final extension step at 72 °C for 6 min, followed by storage at 4 °C. PCR products were then purified from 0.8% (w/v) agarose gel using QIAquick gel extraction kit (Qiagen).

2.3. Construction of the recombinant expression plasmid

The expression vector pET-22 b (+), a His-Tag (HT) fusion vector, was used to generate the expression clones of NS1-HT fusion proteins of SLEV and WNV. In the first place, the generated PCR product was subcloned in the pGEM-T (Promega) vector following the manufacturer's instructions. The pGEM-T vector with the NS1 full-length nucleotide sequence of SLEV and the expression vector pET-22 b (+) (Novagen) were double digested by *Nde*I and *Xho*I restriction endonucleases (Thermo Fisher Scientific), gel purified and ligated. The ligation mixture was transformed into electrocompetent *E. coli* Top 10 cells, and bacterial colonies were analyzed by plasmid DNA isolation and restriction digestion fragment mapping and sequenced using T7 promoter and T7 terminator universal primers.

The NS1 full-length nucleotide sequence of WNV strain ArEq-001 (GeneBank accession number GQ379160) was codon optimized for *E. coli* expression and chemically synthesized by Epoch Life Science Inc., USA. The restriction sites *Nde*I and *Xho*I were incorporated, and the sequence was cloned in pET-22 b (+).

2.4. Bacterial strains, culture, and DNA transformation

Electrocompetent TOP10 E. *coli* cells were used to generate the recombinant plasmids. The expression of recombinant proteins was conducted using *E. coli* Rosetta (DE3) strain cells. Transformation of DNA into *E. coli* competent cells was performed using the electroporation method (Gene Pulser, Bio-Rad) followed by 1 h recovery at 37 °C in Luria Bertani (LB) medium. The transformants were spread onto LB agar plates containing the corresponding antibiotic.

Recombinant bacterial colonies were analyzed by plasmid DNA isolation and restriction digestion fragment mapping, and sequencing using the plasmid corresponding universal primers.

2.5. Expression of the NS1-HT proteins

A single bacterial colony was inoculated in 10 ml LB medium with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and allowed to grow overnight with shaking at 37 °C. The overnight culture was then diluted (1:50) in fresh 500 ml LB medium containing the antibiotics and