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Development of monoclonal antibodies against prM of Dengue virus 4

Desenvolvimento de anticorpos monoclonais para prM de Dengue vírus 4

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Abstract
***Introduction**: dengue is a most common mosquito-borne viral disease in the Americas and tropical countries. **Objective**: in this work, mice were hyperimmunized with DENV 4 antigen to produce monoclonal antibodies (mAbs). **Methodology**: DENV 4 (GenBank KC806069) was inoculated in C6/36 cell monolayers cultivated in Leibovitz's 15 medium supplemented with 5% fetal bovine serum and incubated at 28 °C. The virus stock was submitted to concentration and ultracentrifugation and stored at -80 °C until use (VC DENV 4). Balb/c mice were injected intraperitoneally with 50µg of DENV-4 and successive intraperitoneal injections of 25 µg of VC DENV 4 with Freund's incomplete adjuvant were performed. The spleen cells were fused to SP2/0 myeloma cells with PEG 1540 and distributed in 96-well microplates with Iscove's modified medium with Hipoxantina–Aminopterina–Timidina. Hybridoma screening by indirect ELISA showed positive results for six mAbs, and their characterization was performed by Western blotting and Indirect Immunofluorescence (IFI) techniques. **Results**: the six mAbs showed strong recognition of prM (24/29 kDa), and minor reaction to E protein (66 kDa), E/E protein dimer (105 kDa), and NS1 (49 kDa) protein in two mAbs. The use of mAbs anti-prM as a diagnostic tool using IFI has been demonstrated to detect DENV-4 antigen in infected cells or tissues. **Conclusion**: DENV 4 generate mAbs with strong reactivity to prM with potential use to confirm the presence of DENV 4 antigen in tissues or infected cells.*
Keywords: *Monoclonal antibodies. DENV 4. prM.*

Resumo
Introdução: a dengue é uma doença viral transmitida por mosquitos comumente das Américas e países tropicais. **Objetivo**: neste trabalho, camundongos foram hiperimunizados com antígeno DENV 4 para produzir anticorpos monoclonais (mAbs). **Metodologia**: DENV 4 (GenBank KC806069) foi inoculado em monocamadas de células C6 / 36 cultivadas em meio Leibovitz 15 suplementado com 5% de soro fetal bovino e incubadas a 28°C. O estoque viral foi submetido à concentração, ultracentrifugação e armazenado a -80 °C (VC DENV 4). Camundongos Balb / c foram injetados intraperitonealmente com 50 µg de VC DENV-4 e injeções intraperitoneais sucessivas de 25 µg de antígeno com adjuvante incompleto de Freund. As células do baço foram misturadas a células SP2/0 com PEG 1540 e distribuídas em microplacas de 96 poços com meio Iscove Modificado em presença de Hipoxantina – Aminopterina – Timidina. A triagem de hibridomas por ELISA indireto apresentou resultados positivos para seis mAbs, e sua caracterização foi realizada por técnicas de Western blotting e Imunofluorescência Indireta (IFI). **Resultados**: os seis mAbs mostraram forte reconhecimento de prM (24/29 kDa) e reação menor à proteína E (66 kDa), dímero de proteína E / E (105 kDa) e proteína NS1 (49 kDa) em dois mAbs. O uso de mAbs anti-prM como uma ferramenta de diagnóstico utilizando IFI demonstrou eficacia em detectar o antígeno DENV-4 em células ou tecidos infectados. **Conclusão**: o mAbs produzidos para DENV 4 demonstraram uma forte reatividade contra prM, e poderiam ser uma ferramenta de uso potencial no diagnóstico de DENV 4 .
Palavras chaves: Anticorpos monoclonais. DENV 4. prM.

INTRODUCTION

The development of monoclonal antibodies (mAbs) by Kohler and Milstein (1975)[1] led to the production of excellent analytic tools for the characterization of biomolecules. In this context, mAbs have been useful for studying the intracellular localization, early expression, or polyprotein cleavage of viral proteins. mAbs could also provide insight

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on how to inhibit the viral replication cycle as well as on identifying potential viral proteins that could be targeted for the laboratory diagnostic tests or antiviral development.

This work presents mAbs toward the M precursor protein (prM) of the Dengue virus 4 (DENV 4). Dengue is the most common mosquito-borne viral disease in the tropical and subtropical regions that affects humans, causing 50–100 million infections annually[2].

DENV is a member of the Flaviviridae family, Flavivirus genus, with four antigenically distinct serotypes (DENV1–4). In Brazil, the DENV-4 reemerged in 2010 after 31 years, rapidly spreading through the population. Since then, DENV-4 has become the dominant serotype, although in some regions of the country, all four serotypes are still in circulation[3,4].

The DENV genome is a positive-sense RNA strand encoding a large precursor viral polyprotein that is cleaved by cellular and viral proteases to generate three structural proteins C (capsid, 11 kDa), prM/M (membrane, 27/31 kDa), and E (envelope, ~55 kDa) and seven non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5[5]. The processing and cleavage of the large viral polyprotein in flavivirus-infected cells to obtain structural and non-structural viral proteins are still under investigation. However, it was demonstrated that E, prM and NS1 proteins are all transported together to the surface of the cell. Moreover, the E, prM and C proteins co – precipitate with NS1 protein and some studies have revealed that prM, is intimately associated with E glycoprotein to prevent premature viral fusion inside the host cell[6].

*In this work, we obtained mAbs against DENV 4 with strong immune response to prM as well as some reactivity against E and NS1 proteins. We discuss whether the observation of multiple bands could reflect shared antigenic determinants as a consequence of the cleavage of the polyprotein during viral maturation***.**

METHODOLOGY

Virus and cells

Aedes albopictus clone C6/36 *(ATCC® CRL-1660) cell line was grown in Leibovitz's 15 (L-15) medium (Gibco®,USA) supplemented with 5% fetal bovine serum (FBS), 1% non-essential amino acids (Invitrogen USA), 10% of tryptose phosphate broth (Sigma Aldrich, St. Louis, USA) and incubated at 28 °C.*

DENV 4 (GenBank Accession Number KC806069) was inoculated in C6/36 cell monolayers (MOI 0.1). After a viral cytopathic was evident in 90 % of the monolayer, the cultures were frozen and thawed and clarified at 10.000 g 20 minutes 4 °C. DEN-4 viral stock was subjected to standard assay of virus titer measurement using Vero cells monolayers. The virus stock containing 10 $^{6.5}$ $DICT_{50}$/mL was submitted to PEG 8000 concentration and ultracentrifugation as described in Fontes et al.[7]. The viral stock (VC DENV 4) was stored at -80 ◦C until use.

Production of mAbs

Balb/c mice at 6 e 8 weeks of age were injected intraperitoneally with 50µg of VC DENV-4 emulsified with Freund's complete adjuvant (Sigma–Aldrich, St. Louis, USA) and successive three intraperitoneal injections of 25 µg of VC DENV 4 with Freund's incomplete adjuvant were performed. The mouse experiments were submitted and approved by the Ethics Committee of Animals -Universidade Federal da Bahia, protocol number 044/2013. After mice were sacrificed, their spleen cells (5 × 10^6) were fused to SP2/0 myeloma cells (1 × 10^6) with PEG 1540 (Sigma–Aldrich, St. Louis, USA). The mixed cells were centrifuged and the pellet was suspended in Iscove's modified medium with Hypoxanthine–Aminopterin–Thimidine (HAT, Gibco Co.USA) and distributed in 96-well microplates. Further details of the procedure can be found elsewhere[7,8].

The screening of hybridomas was performed by indirect ELISA, in a polystyrene microplate coated with C6/36 DENV-4 infected pellet cells (30µg) and non-infected pellet cells. Goat Anti-Mouse IgG Antibody, HRP conjugate against Mouse IgG was used as secondary antibodies. The positive hybridomas were selected and cloned twice by limiting dilution. After expanded in a culture flask, the hybridoma cells were collected and injected into Pristane-treated BALB/c mice (10^6 cells/mouse) to obtain ascitic fluid.

Characterization of mAbs

Viral proteins identification: Polyacrylamide gel electrophoresis (PAGE) andWestern-blot assay

VC DENV 4 was resuspended in Laemmli sample buffer (30mMTris [pH 6.8], 2-mercaptoethanol, 2% sodium dodecyl-sulfate (SDS), 10% glycerol and 0.01% bromophenol) and heated at 100 ◦C for 3 min.

Analysis of viral proteins was undertaken using gradient (5-15 %) polyacrylamide slab gel electrophoresis (SDS-PAGE). Protein molecular weight markers (SigmaMarker™ wide range 6,500-200,000 Da, USA) were included in each run. After electrophoresis, the gels were either stained with Coomassie brilliant blue (Coomassie Brilliant Blue R-250, Bio-Rad, USA) or the viral proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane by eletroblotting (100V, 1 h). The membrane was cut into strips and blocked in 5% skim powder milk in PBS (pH 7.4) for 1 h at 37 ◦C. After that, the membrane was incubated with mAbs (ascitic fluid 1:5) at 4 °C overnight. After the membrane strips were washed with PBS-Tween 20 (PBS-T 0.05%) 3 times for 10 min, secondary anti-mouse HRPO-conjugate antibody (Sigma Aldrich Chemicals Co., USA) (1:1000) was applied and incubated for 1 h at 37 ◦C. Then the strips were washed 3 times for 10 min each in PBS-T and the substrate 3,3 diamino benzidine (DAB) 10 mg/ml and H_2O_2 were added for colour development.

Detection of viral antigen by Indirect immunofluorescence (IFI)

IFI in C6/36 DENV 4 infected cells

IFI was performed following the protocol described before[9]. The C6/36 cells were infected with DENV 4 (GenBank Accession Number KC806069) at a multiplicity of infection (MOI) of 0.1. Mock-infected cells were run in parallel to the viral infected cells to serve as negative controls. After a viral cytopathic effect was evident, a mixture of infected cells and negative control cells (1 infected: 10 uninfected cells, respectively) were deposited (25 ul) in each spot of immunofluorescence slides and after drying, the cells were fixed in a cold acetone (-20 °C). Each mAb (ascitic fluid 1:8) were incubated overnight and then, secondary antibody anti-mouse IgG FITC-conjugated (1:40) (Sigma–Aldrich, St. Louis, USA) was added. After washing, a last rinse with Evans blue 0,01% was applied. The positive cells were visualized as a green apple color in the cytoplasm (Zeiss AxioLab, 400X).

Diagnosis of Flavivirus: DENV 1, DENV 2, DENV 3 and ZIKV

Monolayers of C6/36 cells were infected with viral strains of DENV 1, DENV 2 and DENV 3 (DENV viral reference strains obtained from Evandro Chagas Institute, Brazil) and ZIKV (GenBank KU940224.1). Mock-infected cells were run in parallel to the viral infected cells to serve as negative controls. Fresh L15 medium was added and incubated at 28°C until a a viral cytopathic was evident. C6/36 infected cells from each viral strain were mixed with C6/36 uninfected cells (proportion of 1:10) and deposited (25 ul) in each spot of immunofluorescence slides. The IFI test was performed following the protocol described cited above (Material and Methods 2.1).

Viral Isolation

DENV-4 positive sera sample by RT-PCR [10] was inoculated (25 ul) in monolayers of C6/36 cell lines. Fresh L15 medium supplemented with 5% fetal bovine serum (FBS), 1% non-essential amino acids (Invitrogen USA), 10% of Tryptose Phosphate Broth (Sigma Aldrich, Brazil) was added and incubated at 28 °C. After seven days, the cells and supernatant were frozen and unfrozen, and after centrifugation (10.000 g 20 minutes, 10 °C) the supernatant was submitted to a new culture cell. After three blind passages, the virus isolated was confirmed by RT-PCR[10] and IFI using mAb F10 as cited before (Material and Methods 2.1)

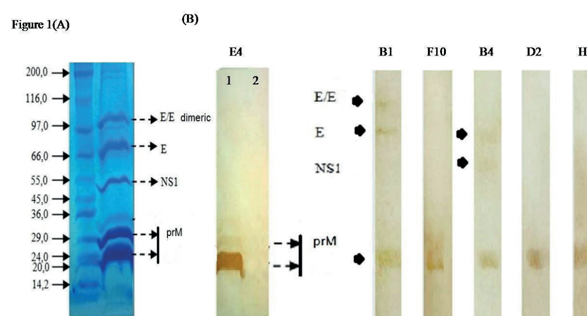
Detection of DENV 4 in midguts after oral experimental infection in mosquitoes *Aedes aegypti*

Seven-day-old female mosquitoes (n=10) were deprived of meal (sucrose) for 24 h and then were infected by feeding with caprine blood (1 ml) infected with 300 ul of DENV 4 viral suspension ($10^{6.5}$ DICT50/mL). The control group (n=10) was submitted to the same procedure except infected blood. After 5 days post-oral infection the mosquitoes (infected and control group) were dissected and the midguts were placed on a slide to fix them in cold acetone (-20 °C) 10 minutes, and submitted to IFI as cited above. All preparations were examined by fluorescent microscopy (Zeiss AxioLab, 400x).

RESULTS

The protein pattern of DENV-4 obtained to produce mAbs can be visualized in the Figure 1A. Five bands with molecular weights between 24 to 105 kDa were observed on a SDS-PAGE (gradient gel 5–15%). The two lowest band of 24 kDa and 29 kDa suggested the presence of protein prM. Other protein bands of 49 and 66 kDa correspond to NS1 and E, respectively and the 105 kDa band is the E/E dimeric form of E protein.

Figure 1 – *Identification of DENV-4 proteins.*



(A) Gradient 5-15% SDS-PAGE viral fractionation profile of PEG-DENV-4 under reducing conditions stained with Coomassie Blue; (B) Western-blot: Each mAb (ascitic fluid) was diluted 1:5 and after overnight incubation was added HRP – conjugated goat anti-mouse IgG. Arrows indicate viral protein according to molecular weight (SigmaMarker™ wide range 6,500-200,000 Da) Lane 1: VC-DENV-4. Lane 2: Uninfected C6/36 cells (Negative control).

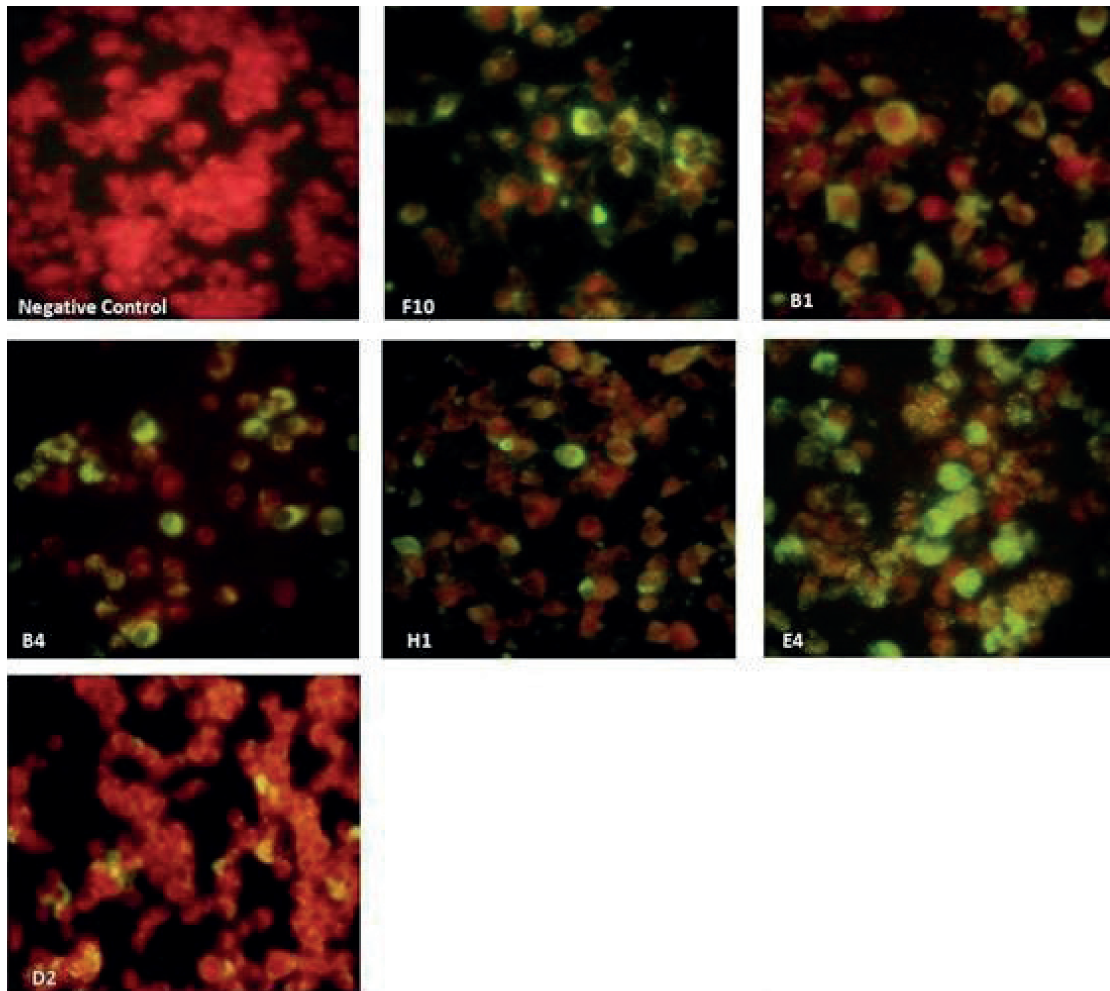
Fonte: Authors, 2022

The DENV-4 antigen generated a good immune response in mice, allowing for the generation of six MAbs, named B1, D2, E4, F10, H1, B4, with different characteristics as shown through subsequent characterization.

Western blotting analysis revealed differences in the reactivities of the mAbs (Figure 1B). All the mAbs were able to recognize the epitopes under denaturing conditions with strong immunoreactivity to prM protein. It was also observed mAbs with the simultaneous reactivity to prM, NS1 and E proteins (mAbs B1 and B4).

The IFI technique demonstrated that mAbs recognize the presence of DENV-4 antigen in C6/36 infected cells. As shown in Figure 2, the mAbs F10, B1, H1, E4, and B4 were clearly positive in the IFI reaction. However, the D2 showed weak reactivity.

Figure 2 – *Indirect immunofluorescence using mAbs against DENV-4*



The images represent each of the six mAbs incubated with C6/36 culture cells infected with DENV-4. Negative control: uninfected C6/36 with mAb E4. The presence of apple green fluorescence indicates detection of DENV-4 in infected cells. Fluorescence microscopy magnification, 400X.

Fonte: Authors, 2022

The IFI technique was also applied to differential diagnosis between close related flavivirus DENV and ZIKV. All mAbs recognize the four immunologically related serotypes DENV 1-DENV 4 in infected cells. The comparative results of the characteristic each mAb is showed in Table 1. It is observed that mAbs F10, H1 and E4 can differentiate between DENV 4 and ZIKV. Moreover, mAbs B1 and B4 that reacted at the same time with prM, NS1 and E proteins cross-reacted with ZIKV (Table 1)

Table 1 – Reactivity of the Anti-Dengue virus prM monoclonal antibodies

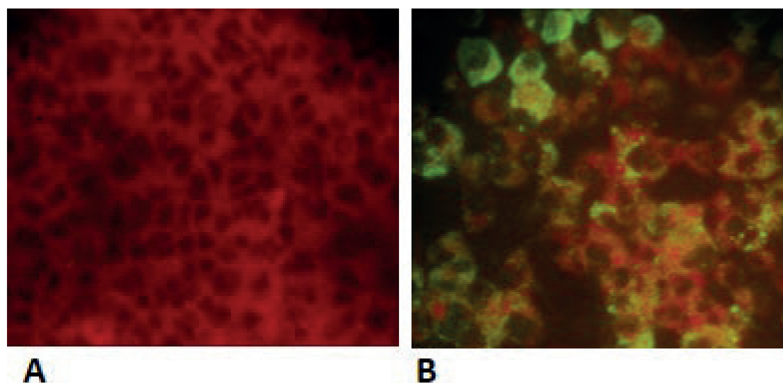
mAb	**Western – Blot Assay**	**Immunofluorescence Assay (IFA) (*)**	**IFA DENV 1/2 /3**	**IFA Cross-reactivity ZIKV**
B1	E Dimer; E; prM	++	+	+ (*)
B4	NS1; E; prM	++	+	+
D2	prM	+	+	ND
F10	prM	+++	+	-
H1	prM	++	+	-
E 4	prM	+	+	-

* *Immunofluorescence assay: (+) positive reaction; (-) negative reaction*
Fonte: Authors, 2022

It was also confirmed the use of mAbs as a potential tool to confirm the virus isolate (Figure 3) or to detect

viral antigen in tissues (Figure 4). The midguts of Aedes Aegypti mosquitoes orally infected with DENV 4 were assessed by fluorescence microscopy and it was confirmed the presence of the virus in the intestinal epithelium cells.

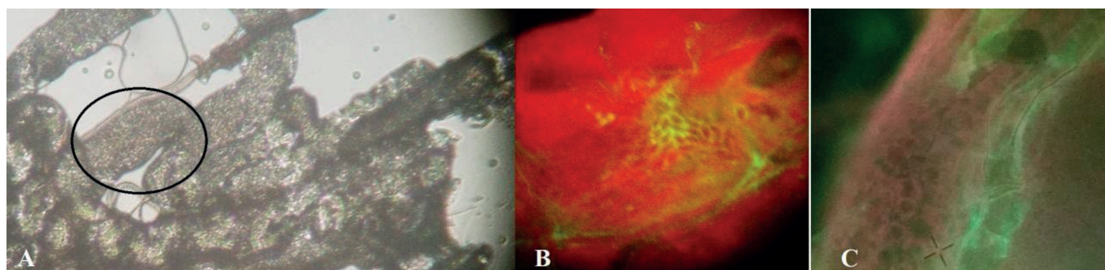
Figure 3 – *Indirect immunofluorescence technique using anti-Dengue 4 prM monoclonal antibody to confirm DENV 4 virus isolate*



(A) Uninfected C6/36 cells, negative control; (B) Positive viral isolate in C6/36 cells using mAb F10.

Fonte: Authors, 2022

Figure 4 – *Detection of viral antigen in DENV 4 infected midguts from Aedes aegypti mosquitoes.*



(A) Aedes aegypti midgut section exposed to IFI reaction (circle; 100x). (B) Midgut section of Aedes aegypti infected with DENV-4 (5 days after infection) and incubated with mAb F10. Small foci of DENV-4 antigen were detected in the midgut epithelium. (C) Uninfected Aedes aegypti midgut section (negative control) The presence of apple green fluorescence cells indicates the detection of DENV 4 antigen (Fluorescence microscopy magnification 400X, Zeiss Axiolab).

Fonte: Authors, 2022

DISCUSSION

Monoclonal antibodies have become key components and essential tools for laboratory diagnostic tests of various pathogens. In this work, the DENV 4 antigen inoculated in mice allowed the obtention of mAbs with different biochemical characteristics and protein reactivities. The protein pattern of DENV 4 that was used as antigen showed the provable presence of prM, E, NS1 and dimeric form E viral proteins with significant amount of prM. Mosquito culture cells infected with DENV are mentioned to contain large amounts of prM compared to other cultured VERO, BHK and KB cells[11]. Possibly the low efficiency of cellular peptidases in mosquito cells allows the detection of higher amounts of this protein[12]. Furthermore, prM in the analysis by SDS-PAGE was detected as a double band. Several reports mention that prM is a glycosylated protein with different glycosylation profiles that are reflected in multiple bands [11]. The first studies with cells infected with DENV show that the use of drugs that inhibit glycosylation or endoglycosis enzymes, multiple prM bands disappear or only one is shown, results consistent with the concept that these multiple bands represent a single protein with different glycosylation pattern[12].

The present work shows that all mAbs reacted strongly to prM. Some authors have demonstrated mAbs with high immunoreactivity against prM protein for DENV[13]. This is in agreement with the natural DENV infections in humans, as the prM protein is highly antigenic and the highest rates of anti-M antibodies occur during dengue infections[14,15]

The reactivity of DENV 4 mAbs were not exclusive against prM. The mAbs were also shown to react against the E, NS1 proteins and dimeric form E protein. Multiband reactivity could reflect shared antigenic determinants in viral proteins during viral maturation (precursors). Reinforcing this concept, it is known that during viral replication, prM and E are closely associated to prevent premature viral fusion within the cell and can also co-precipitate with NS1. NS1 is secreted and resides in

the plasma membrane and recently, NS1 was shown to interact with prM / E glycoproteins to help fold and envelop the cytoplasmic membrane for the production of new infectious DENV particles [16-18].

The use of these mAbs as diagnostic tools to detect the DENV 4 antigen in cell cultures (viral isolation) or in tissues was successful, some of them were even useful to differentiate DENV and ZIKV antigens. In this regard, mAbs with reactivity directed solely at prM did not cross-react with ZIKV. Some authors report that the E and NS1 proteins contain a high level of amino acid sequence homology that elicit cross-reactive antibodies in the flavivirus group[19,20]. However, prM is one of the proteins that has a low level of amino acid sequence homology (48-52%) in flaviviruses, favoring its use in differential diagnostic tests [20,21].

CONCLUSION

DENV 4 generate mAbs with strong reactivity to prM with potential use to confirm the presence of DENV 4 antigen in tissues or infected cells. Nevertheless, more studies are in progress to evaluate their use in other diagnosis tests for DENV or other flaviviruses.

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