

## Expression and Purification of Recombinant Mayaro Virus Envelope Glycoproteins E1 and E2 to Develop a Mayaro Virus Detection System

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Mayaro virus (MAYV) is a mosquito-transmitted *alphavirus* that produces an acute, usually non-fatal, febrile illness including Mayaro fever. Like other alphaviruses, the MAYV E1 and E2 envelope glycoproteins are major viral surface antigens that play a key role in host recognition and infection. Here, we report expression and purification methods for recombinant MAYV E1 (rE1) and rE2 using a baculovirus system. Enzyme-linked immunosorbent assays (ELISA) revealed that rE1 and rE2 were antigenic and reacted with human anti-MAYV IgG and IgM. Cross-reactivity was also confirmed with human anti-Chikungunya virus (CHIKV) IgG and IgM. Furthermore, we developed an immunochromatographic strip test (IST) with rE2 to diagnose MAYV infection. Thus, purified rE2 may be valuable tool for rapidly diagnosing MAYV infection.

**Key Words:** Baculovirus/insect cell system, recombinant envelope glycoprotein 1 (rE1), recombinant envelope glycoprotein 2 (rE2), immunochromatographic strip test (IST), Mayaro virus (MAYV)

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## INTRODUCTION

Mayaro virus (MAYV) was found on the island of Trinidad and has caused small outbreaks in several parts of tropical South America. MAYV is a single strand positive RNA virus of the *alphavirus* genus in the family *Togaviridae* and is similar to chikungunya virus (CHIKV) (1-3). Like other alphaviruses, MAYV is an arbovirus (arthropod-borne virus) which is mostly transmitted by *Haemagogus* mosquitoes. Recent studies have shown that *Aedes aegypti* mosquitos can spread MAYV worldwide (4-6).

MAYV infection causes a CHIKV-like, acute, usually non-fatal, febrile illness with a 3-5 day duration that includes fever (known as Mayaro fever), joint pain, headache, rashes, and muscle ache. Some patients develop persistent, terrible joint pain that lasts up to a year after infection (7, 8). MAYV infection can be confirmed by viral isolation, RT-PCR, and serological diagnosis using hemagglutination

inhibition tests and enzyme-linked immunosorbent assays (ELISA); however, these methods are unresponsive for a great number of infectious samples and live virus analysis may require biosafety laboratories. Furthermore, due to the antigenic similarity of MAYV and CHIKV, there is an acute need to develop more specific and precise methods for diagnosing MAYV infection (9).

The MAYV genome is a 12kbp long single-stranded RNA containing two open reading frames (ORFs). It encodes four non-structural proteins (nsP1, nsP2, nsP3, and nsP4) and five structural proteins (C, E3, E2, 6K, and E1). The structural envelope glycoproteins E1 and E2 are imbedded in the envelope on the viral surface. E1 mediates the fusion of the virus with the host cell, while E2 is mainly involved in attaching viruses to host cells (10). As for alphaviruses, E1 and E2 are targets of the anti-MAYV antibody response; therefore, E1 and E2 would be useful targets for immunodiagnostic analysis.

In this study, we explain the generation of soluble MAYV E1 and E2 using a baculovirus/insect expression system. Recombinant envelope proteins E1 (rE1) and rE2 were successfully expressed and purified in soluble forms, with rE2 better expressed and more stable than rE1. ELISA revealed that rE1 and rE2 were antigenic and reactive for specifically detecting human anti-MAYV IgG or IgM. Cross-reactivity was also evaluated using human anti-CHIKV IgG or IgM. In particular, rE2 displayed a higher ELISA value than rE1. Furthermore, we developed an immunochromatographic strip test (IST) using rE2 to detect human anti-MAYV IgG or IgM, demonstrating the potential applicability of rE2 in a diagnosis system to detect MAYV infection.

## MATERIALS AND METHODS

### Construction of recombinant expression vector containing MAYV envelope glycoproteins E1 and E2

Synthetic MAYV DNA encoding E1 (GenBank # KT818520, residues 1~442) or E2 (GenBank # KT818520, residues 1~421) and a C-terminal six histidine tag (His-tag) were obtained from Bioneer. Two enzyme sites, SmaI and XbaI, were created at the 5' and 3' ends of the *E1* and *E2* genes, respectively. *E1* and *E2* were amplified by PCR using the following primers: E1F, 5'-CCCGGGGGATCCTACGAGCACACG-3'; E1R, 5'-TCTAGATTGCG CCAAGTCATTGCAGTGCTGGATA-3'; and E2F, 5'-CCCGGGGGATCCAGCACTGC AAAT-3'; E2R, 5'-TCTAGACGTAGGATGCAGTCCGTAGTAGTATTC-3' (restriction sites underlined). The PCR products were cloned into corresponding sites in pAcGP67A (Pharmingen). The *E1* and *E2* genes inserted downstream of the robust *polh* promoter to create pAcGP67A-E1 and pAcGP67A-E2.

### Insect cell culture and transfection

The insect cell line *Spodoptera frugiperda* (Sf9) was cultured in Insect Sf-900 SFM serum-free media (Invitrogen, Waltham, Massachusetts, USA) at 28°C. Sf9 cells were co-transfected with Baculogold™ DNA (Pharmingen, San Diego, California, USA) and the recombinant vector pAcGP67A-E1 or pAcGP67A-E2, as demonstrated by the manufacturer. Several rounds of culture were performed to amplify the recombinant virus and a high viral titer stock solution was collected. To express the protein on a bigger scale, *Trichoplusia ni* BTI-TN-5B1-4 (High Five™) insect cells (Invitrogen, Waltham, Massachusetts, USA) were grown in Insect Hi-Express serum-free media previous infection with the high titer virus. High Five™ insect cells were used to express MAYV rEP1 and rEP2 after infection with the recombinant baculovirus.

### Purification of recombinant envelope protein (rE1) and rE2

Typically, 1 L of recombinant baculovirus-infected insect cell cultures was harvested approximately 72 h post-infection and the cells were centrifugated at 5000×g for 15 min to recover only the supernatant. The supernatant was dialyzed against binding buffer (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl), loaded onto a Ni<sup>2+</sup>-nitrilotriacetic acid (Ni-NTA) agarose column

(Qiagen, Hilden, Germany) equilibrated with the binding buffer, and washed with washing buffer (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 50 mM imidazole). rE1 and rE2 were eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 500 mM imidazole) and the presence of purified protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The eluted glycoproteins were dialyzed with Slide-A-Lyzer (3.5 K MWCO; Thermo Scientific, Waltham, Massachusetts, USA) in phosphate buffered saline (PBS, pH 7.4) overnight at 4°C, and stored in PBS (pH 7.4) or 5% (v/v) glycerol at -70°C.

## Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses

The supernatants were separated on NuPAGE Novex 4-12% Bis-Tris SDS-PAGE gels in MES-SDS running buffer (Invitrogen, Waltham, Massachusetts, USA) and visualized by staining with Coomassie brilliant blue. Gel images were obtained using a gel documentation system (AE-9000 E-graph, ATTO Corp, Tokyo, Japan).

For western blot analysis, proteins were separated on SDS-PAGE gels and transferred to polyvinylidenedifluoride (PVDF) membranes using a Trans-Bolt system (Bio-Rad, Hercules, California, USA). The membranes were blocked with 5% skim milk in 1× PBS containing 0.05% Tween-20 (PBST) for one hour at 25°C, probed with mouse anti-His-tag antibodies (Bethyl Laboratories, Inc., Montgomery, Texas, USA) at a 1:3000 dilution for one hour at 25°C, washed six times with PBST, and incubated with HRP-conjugated goat-anti mouse IgG antibodies at a 1:10,000 dilution for 1 h at 25°C. To visualize antibodies bound to the proteins, ECL luminal kits were used as enzyme substrates and the blots were visualized on a chemiluminescence imaging system (WSE-6200H LuminoGraph II, ATTO Corp, Tokyo, Japan).

## Mayaro antigen-specific ELISA

rE1 and rE2 reactivity against human anti-MAYV IgG and IgM was tested (11). A 96-well microtiter plate (Costar 3690, Corning Inc., New York, USA) was coated with 100 ng/well of purified rE1 or rE2 diluted to 1 µg/mL in 0.1 M carbonate-bicarbonate buffer (pH 9.0) overnight at 4°C. Unbound antigens were discarded and the wells were blocked with 2% BSA in PBS for 60 min at 25°C. After washing, the wells were incubated at 37°C for 2 h with MAYV positive controls (human anti-MAYV IgG or IgM) from an anti-MAYV ELISA kit (Euroimmun, Luebeck, Germany). CHIKV positive controls (human anti-CHIKV IgG or IgM) from an anti-CHIKV ELISA kit (Euroimmun, Luebeck, Germany) were used to test cross-reactivity. The plates were washed six times with PBST and incubated at 37°C for 1 h with HRP-conjugated anti-human IgG or IgM diluted at 1:10,000. Bound antibodies were detected by adding 3, 3', 5, 5'-tetramethylbenzidine (TMB, Sigma, St. Louis, Missouri, USA) and optical density was measured at 450 nm using an ELISA microplate reader (Thermo Scientific, Waltham, Massachusetts, USA). Bars represent the average absorbance of three measurements and the error bars indicate standard deviations (SD) of triplicate measurements.

## Immunochromatographic Strip Test (IST) method

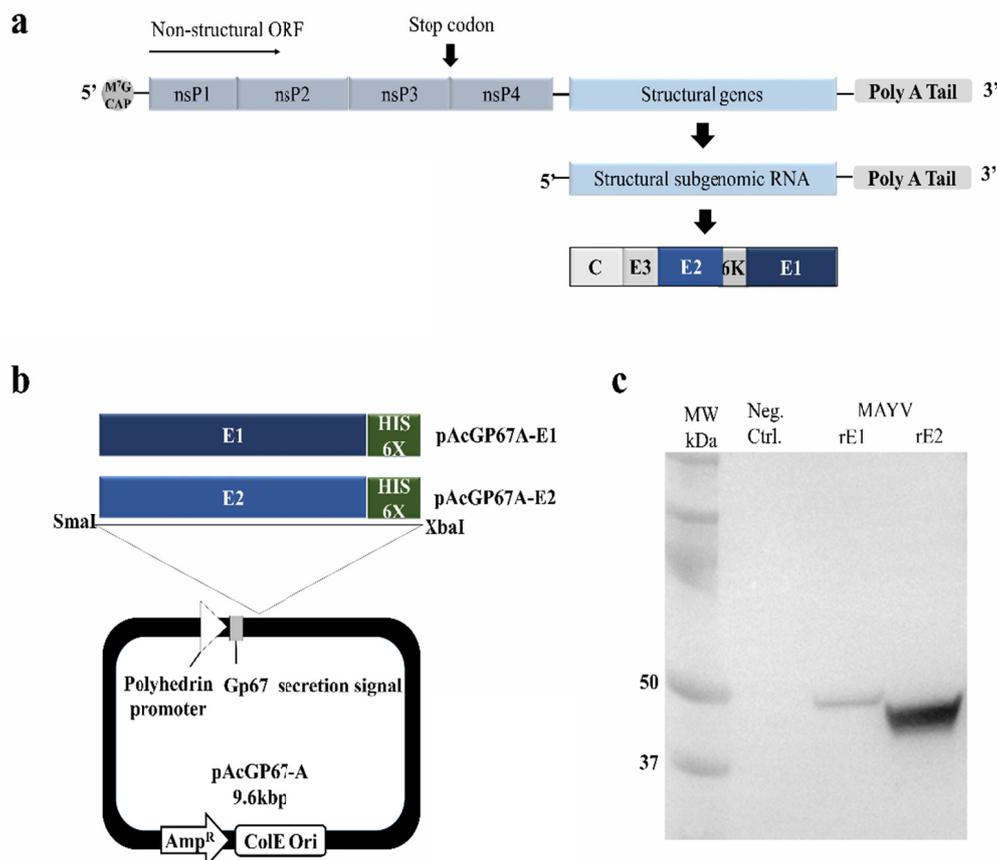
The IST system in this study used a working solution (PBST) and test strips. Nitrocellulose (NC) membranes, laminated cards, absorbent pads, and sample pads were obtained from Millipore. Red latex beads were supplied by Expedeon. Goat anti-mouse IgG antibodies and other chemicals were purchased from Sigma-Aldrich. The strip sensor was composed of a sample pad, NC membrane, absorption pad, and a backing card. The latex beads were covalently conjugated to rE2 lysine residues, soaked onto the conjugate pad, and dried at 37°C for 2 h. Anti-human IgG (0.1 mg/mL) or IgM (0.1 mg/mL) antibodies were immobilized on the test line and anti-His-tag antibodies (0.1 mg/mL) were deposited on the control line on a NC membrane using a dispenser (DCI100; Zeta Corporation, Gunpo-si, Gyeonggi-do, Korea). It was assembled on a backing card with a 2 mm overlap between each component of the strip sensor. To verify IST performance, we used MAYV positive controls (human anti-MAYV IgG and IgM) from an anti-MAYV ELISA kit (Euroimmun, Luebeck, Germany) and CHIKV positive controls (human anti-CHIKV IgG or IgM) from an anti-CHIKV ELISA kit (Euroimmun, Luebeck, Germany).

Samples (50  $\mu$ L of each) were pipetted onto the sample pad and migrated along the strip membrane by capillary action. Buffer-only (PBST with human serum albumin) was also tested as a negative control (Neg. Ctrl.). For data analysis, the color signal intensity of the test lines was quantified by calculating the average pixel intensity inside the test line using a custom script in the Fujifilm multi-gauge program (*Fuji photo film Co, Ltd., Tokyo, Japan*). Data sets were analyzed statistically using Origin software.

## RESULTS

### Construction of recombinant baculovirus to express MAYV rE1 and rE2 in insect cells

To express secreted versions of MAYV rE1 and rE2, we generated two recombinant expression constructs incorporating an N-terminal gp67 secretion signal peptide for recombinant protein secretion and a C-terminal His 6X tag for purification. The MAYV *E1* and *E2* genes, 442 bp at positions 1~442 of *E1* and 421 bp at positions 1~421 of *E2*, were inserted into the pAcGP67A vector at *Sma*I and *Xba*I restriction sites, respectively (Fig. 1a and b). Sf9 insect cells were transfected with selected cloned vectors (pAcGP67A-E1 or pAcGP67A-E2 plasmid) and wild type viral DNA. Each gene was inserted into the viral genome via homologous recombination to produce recombinant baculovirus (12, 13).



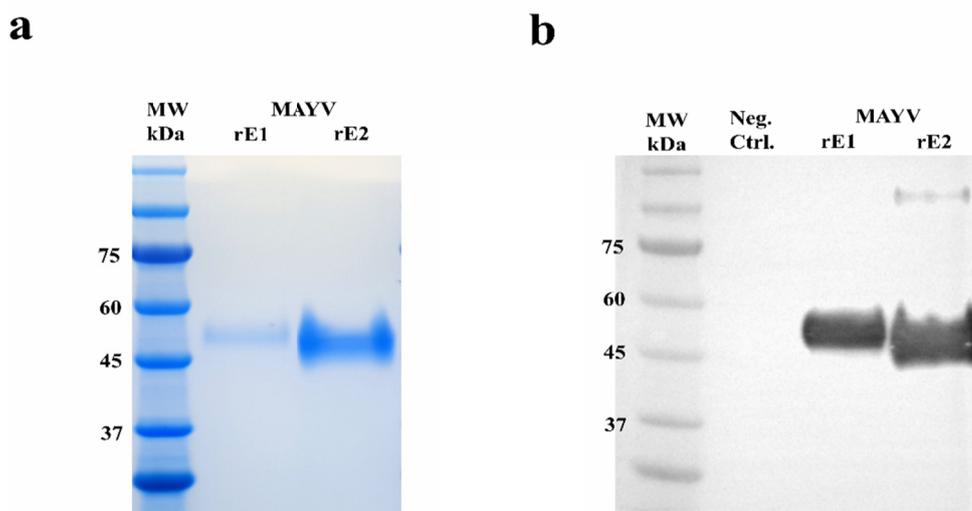
**Fig. 1.** MAYV rE1 and rE2 expression using a recombinant baculovirus/insect system. (a) Schematic representation of MAYV genome organization. (b) Schematic of the pAcGP67A-E1 and pAcGP67A-E2 constructs generated. C-terminal His-tags were used to purify the MAYV E1 and E2 proteins. (c) Western blot analysis of media from pAcGP67A-E1- and pAcGP67A-E2-transfected Sf9 cells using anti-His-tag antibodies conjugated to horseradish peroxidase. Uninfected Sf9 cell cultivation media was used as a negative control (Neg. Ctrl.).

Sf9 cells were directly infected with cell culture medium containing the recombinant baculovirus to produce a high titer baculovirus stock. rE1 and rE2 expression by the infected Sf9 cells was studied on a small scale ( $1 \times 10^6$  cells in a 25 cm<sup>2</sup> flask). Western blot analyses revealed protein bands at 50 kDa (Fig. 1c) and 48 kDa, corresponding well with the calculated molecular masses of rE1 and rE2.

### Overexpression and purification of MAYV rE1 and rE2 in High-Five™ insect cells by recombinant baculoviruses

Soluble rE1 and rE2 were overexpressed by infecting a new High-Five insect cell batches with high titer recombinant baculovirus (14) and then secreted into the extracellular medium. The highest rE1 and rE2 yields were obtained 72 h post infection.

rE1 and rE2 secreted into the medium were purified using a Ni-NTA affinity column, which can especially bind with the C-terminal His-tag in rE1 and rE2. The proteins were eluted with binding buffer containing 500 mM imidazole, analyzed by SDS-PAGE (Fig. 2a), and their concentrations were established using Bradford assays. The total yields of MAYV rE1 and rE2 were 0.2 mg/L and 2 mg/L at a concentration of  $1 \times 10^7$  High-Five™ cells/mL. The Ni<sup>2+</sup> affinity purification of secreted rE1 and rE2 resulted in a protein recovery of ~60% and purity of 95%. The purified proteins were recognized by immunoblot analysis using the anti-His6X antibodies (Fig. 2b).



**Fig. 2.** Analysis of MAYV rE1 and rE2 overexpression and purification by High Five™ cells. (a) SDS-PAGE of purified MAYV rE1 and rE2. The gel was stained with Coomassie brilliant blue R-250. (b) Western blot analysis of purified MAYV rE1 and rE2. After being transferred to a PVDF membrane, the proteins were detected by anti-His-tag antibodies conjugated to horseradish peroxidase. Uninfected High-Five™ insect cell cultivation media was used as the Neg. Ctrl.

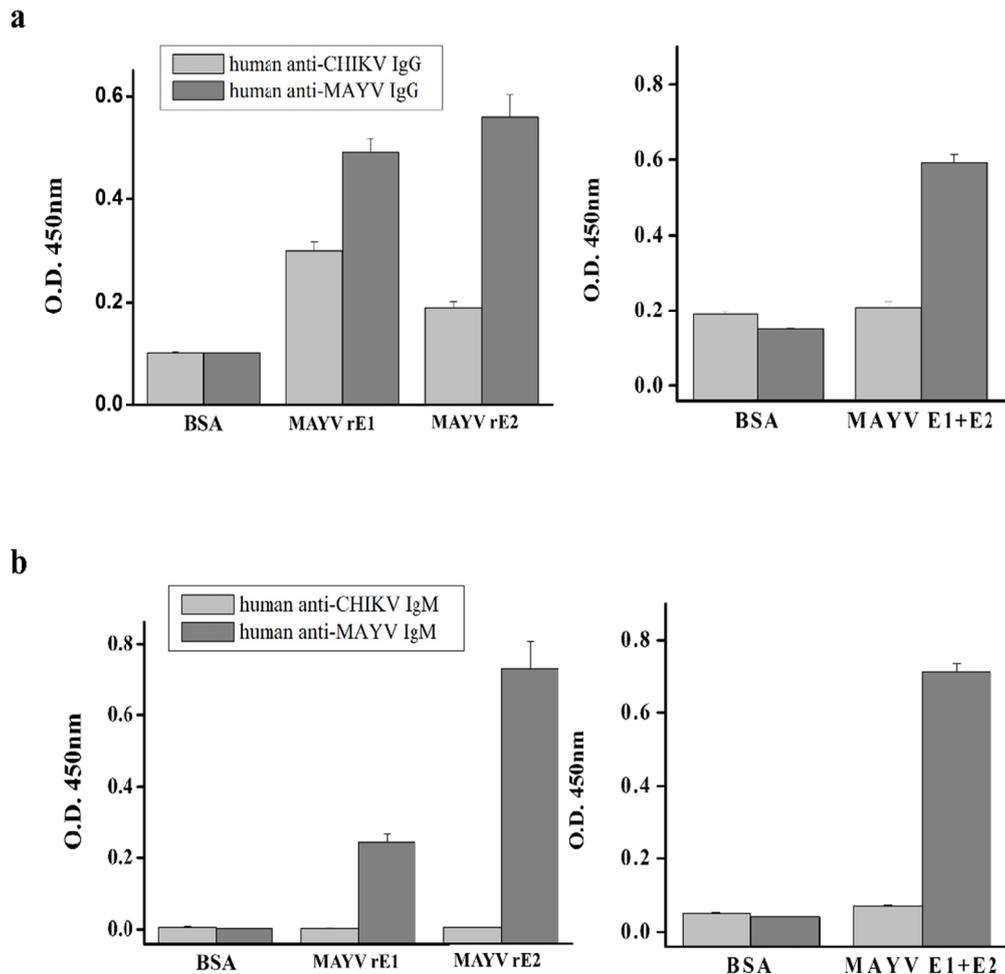
### Antigenic rE1 and rE2 characterization

To assess the antigenicity of purified rE1 and rE2, we measured their antigenic reactivity using a modified ELISA (Euroimmun, Luebeck, Germany) with positive (human anti-MAYV IgG or IgM) and negative controls from the anti-MAYV ELISA kit. Human anti-CHIKV IgG or IgM ELISAs (Euroimmun, Luebeck, Germany) were used to evaluate cross-reactivity with the rE1 and rE2 MAYV ELISAs.

As shown in Fig. 3, rE1 and rE2 exhibited significantly higher reactivity with human anti-MAYV IgG and IgM than the Neg. Ctrl. (BSA). IgG and IgM ELISAs indicated that rE1 and rE2 reacted with anti-MAYV IgG and IgM; rE1 showed a specific response but a very low signal value, whilst rE2 showed higher reactivity than rE1. Additionally, rE1 displayed low reactivity with anti-CHIKV IgG (Fig. 3a), but rE2 showed no cross-reactivity in the anti-CHIKV IgG or IgM ELISAs. These results indicate that both proteins could react with anti-MAYV IgG and IgM in infected sera; however, rE2 was more reactive than rE1 with anti-MAYV IgG and IgM and showed no cross-reactivity for anti-CHIKV IgG and IgM. Therefore, we concluded that rE2 would be more suitable for MAYV diagnosis than rE1.

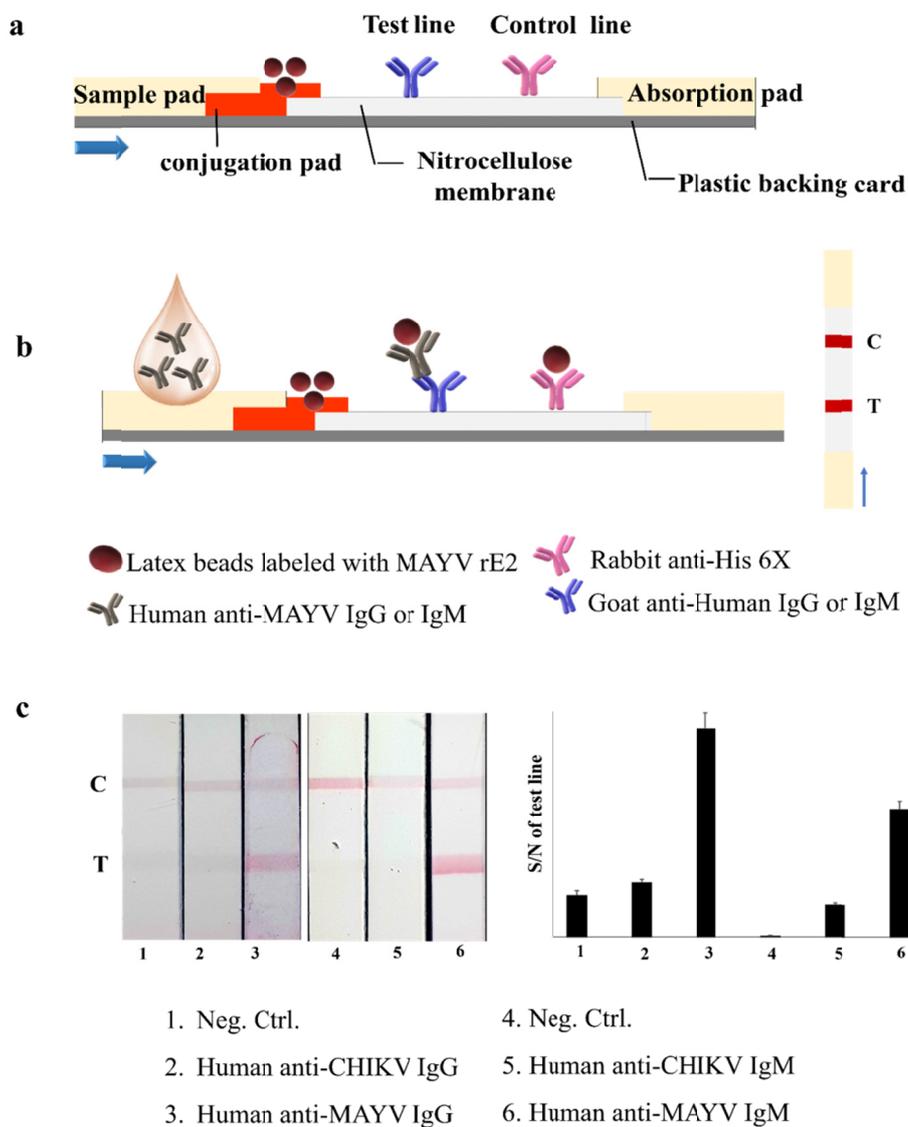
### Immunochromatographic Strip Test (IST) using human MAYV IgG and IgM

To estimate the ability of the IST to detect human anti-MAYV IgG and IgM, we developed a MAYV rE2 antigen strip for MAYV detection. As described in Fig. 4a, the IST consisted of a backing plate, absorption pad, NC membrane, conjugate pad, and sample pad. Red latex beads were labeled with purified rE2 and loaded onto the conjugate pad. Anti-human IgG or IgM and anti-His-tag antibodies were immobilized on the test and control lines, respectively.



**Fig. 3.** rE1 and rE2 reactivity against human anti-MAYV IgG (a) and IgM (b). rE1 and rE2 cross-reactivity were determined by ELISA using anti-MAYV IgG and IgM or anti-CHIKV IgG and IgM. Each microplate well was coated with 100 ng of purified recombinant proteins. Bars indicate the mean absorbance at 450 nm. Error bars indicate standard deviations (SD) of triplicate measurements.

First, samples including human anti-MAYV IgG or IgM were dropped onto the sample pad. After the sample had moved to the conjugate pad, human anti-MAYV IgG or IgM in the sample captured red latex beads labeled with rE2 to produce target complexes. When the target complexes flowed across the test line, they were captured by anti-human IgG or IgM. Complex accumulation produced a visible color on the test line. Free red latex beads labeled with rE2 flowed across the control line and were captured by anti-His-tag antibodies (Fig. 4b). To verify IST performance, we used MAYV (human anti-MAYV IgG and IgM) and CHIKV (human anti-CHIKV IgG or IgM) positive controls from the anti-MAYV and anti-CHIKV ELISA kits (Euroimmun, Luebeck, Germany), respectively. Human anti-MAYV IgG and IgM caused a band to appear on the test line of the IST within 15 min (Fig. 4c). No nonspecific bands appeared on the test line and human anti-CHIKV IgG and IgM were tested simultaneously. Positive bands were only observed in the MAYV positive sample.



**Fig. 4.** Schematic illustration of the MAYV IST assay (a, b) and an image of MAYV IST using rE2 to detect human anti-MAYV IgG and IgM (c). BSA was used as a Neg. Ctrl. Quantification of band intensity at the strip test line. Normalized pixel intensity was quantified using Image software. Intensity values were calculated relative to the control line intensity. The graph presents the ratio of signal to noise (S/N) on the test line.

## DISCUSSION

Mayaro virus is an emerging epidemic prevalent in tropical regions of South America; its range has recently spread into Central America and Caribbean island nations (15). The virus causes acute febrile illness and can cause long term joint pain in some patients after infection. Currently, there are no permitted vaccines or treatments for MAYV infection and transmission (4). MAYV is diagnosed by virus genome-wide detection analysis using viral isolation or a viral standard; however, this method is limited to viremic phase plasma which usually lasts no longer than 5 days (16).

Recently, the envelope proteins of alphaviruses have been studied to develop a method of MAYV diagnosis (17). MAYV encodes envelope glycoproteins; the E1 protein is primarily required in cell fusion, E2 binds to receptors of cell for entry. E1 and E2, surface protein, are significant targets for neutralizing antibodies in the host immune response against most alphaviruses (2). Until now, serological assays for commercial MAYV diagnosis have not been available.

In this study, we expressed and purified MAYV rE1 and rE2 in a baculovirus expression system as efficient antigens for serological diagnosis. The *E1* and *E2* genes of MAYV were cloned into a pAcGP67A vector that used the gp67 secretory signal sequence of the baculovirus protein to secrete the recombinant proteins. The six coding codons of histidine residues downstream of the *E1* and *E2* genes were translated into polyhistidine peptides at the C-terminals of E1 and E2 for protein purification. Insect cell expression systems are often used for mass production of transformatively modified biologically active proteins. 48 hours after infection, viral infected cells produced a small amount of proteins recognized by anti-His 6X antibodies; the major one had a molecular weight of 50 kDa. When High Five™ cells were infected, these proteins were nearly entirely secreted into the extracellular medium, with the highest levels obtained 3 days post-infection and decreasing thereafter due to proteases from lysed cells (18). Thus, we obtained rE1 and rE2 secreted into the extracellular medium for 72 h using High Five™ insect cells. Recombinant His-tagged rE1 and rE2 proteins were successfully eluted from the column. rE1 and rE2 purity were found to exceed 95 % in the reducing condition. The yield was 0.2 mg and 2 mg of rE1 and rE2 per liter of media, respectively, with rE1 yield much lower than rE2 yield. Smith JL, et. al reported that MAYV rE1 and rE2 are expressed as inclusion bodies in bacterial expression system (2). However, to our knowledge, no studies have reported the expression and purification of MAYV rE1 and rE2 in insect cells.

Because infected patient samples were difficult to obtain, purified rE1 and rE2 were tested with MAYV positive controls from human anti-MAYV IgG and IgM ELISA kits (Euroimmun) to confirm their reactivities. The rE1 and rE2 ELISAs were able to detect human anti-MAYV IgG and IgM; in particular, rE2 was more reactive than rE1. Cross-reactivity between related alphaviruses is a major concern in serological diagnostic analysis (19). To solve this common problem, we measured cross-reactivity against human anti-CHIKV antibodies. As shown in Fig. 3, rE2 showed no cross-reactivity with anti-CHIKV IgG/IgM, whilst rE1 exhibited low reactivity with human anti-CHIKV IgG. Therefore, rE2 MAYV ELISA is a suitable tool for diagnosing MAYV infection which can be performed on acute serum samples and can be used for serological investigations.

The IST analysis is a recognized POCT (point-of-care testing) technique. The method enables inexpensive and rapid analysis by users (20). As shown in Fig. 4, the principle of the strip sensor was based on the immune system (antigen-antibody reaction) between anti-human IgM and IgG (in the test line) and red latex beads labeled with rE2 complex human anti-MAYV IgG and IgM (in samples). When the sample was loaded on the strip, human anti-MAYV IgG or IgM in the samples captured red latex beads labeled with rE2 to form a target complex. During the sample moved to the end of the strip, the target complex is combined with capture antibodies on the test line to form a red signal on the test line. Free red latex beads labeled with rE2 were captured by anti-His-tag antibodies on the control line. We concluded that the strip sensor had no interaction with CHIKV. All results from the IST were checked by ELISA, and the results of the two analysis methods were relatively well matched.

In conclusion, we expressed purified MAYV rE1 and rE2 in insect cells and showed that MAYV ELISA and an IST assay

based on rE1 or rE2 could be useful tools for detecting human anti-MAYV IgG without cross-reactivity with CHIKV, which may be useful for serological screening during possible MAYV outbreaks. Thus, we believe that MAYV rE1 and rE2 have great potential as diagnostic reagents for detecting MAYV infection.

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