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Dengue-1 envelope protein domain III produced in *Pichia pastoris*: Potential use for serological diagnosis

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ABSTRACT

Dengue is a major international public health concern. There is no drug to treat dengue virus infections and a vaccine is yet to be licensed. The laboratory diagnosis of dengue virus infection has been greatly improved during the last decade; therefore, the main limiting factor is the production of recombinant viral antigens on a large scale. Domain III of dengue virus envelope protein contains multiplex conformation-dependent neutralizing epitopes, making it an attractive diagnostic candidate. In this work, we have demonstrated the expression of dengue virus type 1 envelope domain III protein (EDIII-D1) in methylotrophic yeast, *Pichia pastoris* GS115. The recombinant secreted protein (sEDIII-D1) was purified by affinity chromatography and characterized by SDS–PAGE. Purified protein was recognized in immunoblot analysis and enzyme-linked immunosorbent assay (ELISA) with dengue-infected human serum samples. In conclusion, secreted expressions of domain III protein can be obtained in *P. pastoris* by methanol induction. This product has the potential to be used for the diagnosis of dengue infections.

Introduction

Dengue is one of the most serious mosquito-borne flavivirus diseases. Dengue fever (DF)[2] can be caused by any of four serotypes, dengue virus type 1, 2, 3, and 4 (DENV-1, DENV-2, DENV-3 and DENV-4) [1]. People living in tropical and subtropical areas are at risk of dengue virus infection. The World Health Organization estimates that there may be ~100 million cases of dengue infections worldwide every year, which result in 250,000–500,000 severe cases (dengue hemorrhagic fever [DHF]/dengue shock syndrome [DSS]) and approximately 25,000 fatalities [2,3]. There is no specific treatment for DF. Diagnosis of acute dengue virus infection on the basis of clinical syndromes is not reliable and, therefore, laboratory studies are normally relied upon to confirm diagnoses because more than half of infected individuals either are asymptomatic or have mild undifferentiated fevers [4].

DENV is a single-stranded RNA virus. The dengue virion has three structural proteins (capsid [C], envelope [E], and membrane [M]) and RNA codes for seven nonstructural proteins. The envelope of the DENV consists of a lipid bilayer containing two envelope-associated proteins: the E and M proteins; the glycoprotein E has three domains (I, II and III) [5]. Domain III (EDIII) contains approximately 100 amino acid (aa) residues, which are stabilized by a single S–S; it, therefore, lacks glycosylation sites. EDIII can fold independently into an immunoglobulin-like module which undergoes the most significant displacement in fusion transition [3,6,7]. Structural studies of intact virions have shown that EDIII is exposed and accessible on the virion surface [8]. This domain, which is responsible for receptor binding, contains multiple type and subtype specific neutralizing epitopes [3,9,10]. Furthermore, recombinant EDIII protein [11,12] and EDIII-specific mAbs [13] can block virus infectivity. EDIII is, therefore, an important candidate as a reagent for rapid viral diagnosis because nowadays the greatest current difficulty in conducting immunoenzymatic assays is obtaining suitable antigens. In Brazil, the antigens currently used for dengue diagnosis are structural viral proteins taken from the brains of artificially infected new-born mice, which is an arduous and expensive method. This is an important obstacle in the large-scale production of antigens used in diagnostic assays of infected patients. Commercially available kits using recombinant proteins or viruses have been used in routine dengue laboratory diagnosis, but are expensive. Worldwide, conducting rapid and inexpensive diagnostic tests is increasingly necessary to allow diagnoses on a larger scale, even in the remote outskirts of large cities. Due to this demand,

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[2] *Abbreviations used:* DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; AOX1, alcohol oxidase 1 gene; YPDS, yeast extract peptone dextrose sorbitol; ELISA, enzyme-linked immunosorbent assay; ROC, receiver operating characteristic.

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attempts to obtain better quality and higher quantities of antigens are being made using a heterologous system of protein expression in Eukaryotes.

In the last several years, the methylotrophic yeast *Pichia pastoris* has emerged as a powerful and inexpensive heterologous system for the production of high levels of functionally active recombinant proteins [14,15]. As a eukaryote, *P. pastoris* provides the potential for producing soluble correctly folded recombinant proteins that have undergone all of the post-translational modifications required for functionality [16]. Several S–S-linked proteins such as recombinant hepatitis B surface antigen and insulin have been successfully produced in *P. pastoris* [17–19].

In this study, we expressed the optimized gene encoding protein E domain III of DENV-1 in *P. pastoris* GS115. This system permits secretion of the recombinant protein (sEDIII-D1) into the culture supernatant, free of toxins/pyrogens (unlike *Escherichia coli* derived proteins). The purified protein has shown immunogenic potential.

Materials and methods

Microbial host strains and plasmid vector

The host strain used in this study was *P. pastoris* GS115. This strain is defective in the histidine dehydrogenasegene (his4) and has a functional copy of the alcohol oxidase 1 gene (AOX1). For secretory expression in this yeast, we used the integrative plasmid pPICZαA, which contains an *AOX1* promoter fused to the *Saccharomyces cerevisiae* pre-pro α factor secretory signal-encoding sequence and a zeocin-resistance marker for selection of yeast transformants. *P. pastoris* GS115 strain, plasmid pPICZαA, and *E. coli* DH5α used for cloning and replication of the construction were purchased from Invitrogen, Carlsbad, CA.

Creation of recombinant P. pastoris clone carrying EDIII-D1 gene

A 408 base-pair (bp) EDIII-dengue 1 gene (GenBank accession number NC_923865), codon optimized for expression in *P. pastoris*, was obtained by chemical synthesis (Genscript, NJ, USA). This gene was inserted into the *EcoR* I and *Not* I restriction sites of pPICZαA. The resultant construct, pPICZEDIII-D1, was linearized with *Sac* I and electroporated into *P. pastoris* GS115. In parallel, we also generated a blank control clone, harboring the empty parent vector. Zeocin-resistant high-copy transformants were selected on yeast extract peptone dextrose sorbitol (YPDS) plates containing zeocin (100, 200, and 500 μg/ml), as described in the Invitrogen Easy select TMPichia expression kit manual. (Invitrogen's Easy Select Pichia Expression kit manual). The presence of the EDIII-D1 gene insert was analyzed by direct colony PCR using a domain III-specific primer pair (Sense 5′-tcacaagaaggtgccatgcac-3′; Antisense 5′-agacaacttcaaagccttttc-3′).

Production of sEDIII-D1 recombinant protein

A verified transformant was inoculated in 5 mL YPD medium to a starter culture and maintained for 24 h at 30 °C in a shaker. This pre-culture was inoculated into 1L BMGY pH 6.0 (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.002% biotin, 100 mM sodium phosphate, 1% glycerol and 0.004% histidine). The culture was allowed to shake at 250 rpm at 30 °C for approximately 3 days, until it reached an OD600 of approximately 20. At this point, cells were pelleted by low-speed centrifugation, washed with sterile peptoned water (1% yeast extract, 2% peptone), and re-suspended in 100 mL BMMY pH 6.0 (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.002% biotin, 100 mM sodium phosphate, 1% methanol and 0,004% histidine) to induce protein expression. Induction was maintained for 72 h at 250 rpm/30 °C; methanol was added to a final concentration of 1% (v/v) at 12 h intervals. After the induction, the supernatants were collected by centrifugation and processed for the sEDIII-D1 purification.

Purification and characterization of recombinant protein sEDIII-D1

The culture supernatant containing sEDIII-D1 protein was freshly diluted with an equal volume of binding buffer pH 8.0 (20 mM sodium phosphate, 500 mM NaCl) and clarified by filtration through a 0.45 μm membrane, and then subjected to affinity chromatography. The clarified supernatant was loaded into a 1 ml HisTrap FF crude column, previously equilibrated with binding buffer, and attached to an AKTA purifier system (GE Healthcare Life Sciences, Uppsala, Sweden). A ten-column volume of binding buffer was passed through the column to remove non-specifically bound proteins and the specifically bound protein was retrieved from the column using an elution buffer (20 mM sodium phosphate, 500 mM NaCl, 300 mM Imidazole). Peak fractions were pooled together and dialyzed against PBS pH 8.0 using centrifugal filtration devices with a 10,000-molecular weight cut-off (Millipore); the protein estimation was determined with a BCA kit (Pierce Chemical Co., Rockford, USA). For characterization, the purified proteins were subjected to 15% SDS–PAGE. Separated proteins were electro-transferred onto a nitrocellulose membrane and processed for immunoblot analysis using anti-HisTag mAb (Sigma, USA) and dengue-infected human serum samples.

Serum samples

Sera from 171 patients were provided by the Central Public Healthcare Laboratory in the State of Rondônia (LACEN/RO) and the Central Blood Bank of Rondônia State, Brazil (FHEMERON/RO). All sera were tested in accordance with protocols approved by the Central Blood Bank of Rondônia State, Brazil Institutional Human/Animal Care and Use Committee, and all samples were anonymous.

The samples had previously been confirmed as IgM or IgG positive for dengue by MAC-ELISA IgM (Pan-Bio, Australia) and Dengue Duo IgM and IgG ELISA Capture Kits (Sanofi, NJ, USA). Of the 171 samples, 60 were IgG positive and 32 were IgG negative using Dengue Duo IgM and IgG ELISA Capture Kits; 50 were IgM positive and 29 were IgM negative using both kits MAC-ELISA IgM (Pan-Bio, Australia) and Dengue Duo IgM and IgG ELISA Capture Kits (Sanofi, NJ, USA).The totals were 110 positive and 61 negative samples.

Indirect enzyme-linked immunosorbent assay (ELISA) for detection of IgM and IgG

Purified sEDIII-D1 were used as a coating antigen for sensitization (1.0 μg/well) of 96-well polystyrene plates (Maxisorp; Nunc, Roskilde, Denmark) in a carbonate–bicarbonate buffer (pH 9.6) and incubated at 4 °C overnight. The plates were blocked for 30 min with block solution (PBS/5% fetal bovine serum). Serum samples were diluted 1/100 in block solution; 100 μL of each serum dilution was assayed in duplicate. The plates were incubated at 37 °C for 2 h and washed four times with PBS/1% Tween 20. Secondary anti-human IgM or IgG antibodies peroxidase-conjugated (Sigma, USA) was diluted 1/2500 in block solution added to the plates, which were incubated at 37 °C for 2 h, followed by five washes;100 μL of TMB (Sigma, USA) substrate solution (1 tablet TMB, 10 mL of 0.05 M phosphate–citrate buffer/pH 5.0, 2 μL of hydrogen peroxide) was then added. Plates were then incubated at room temperature for 20 min and the reaction was stopped with 50 μL of 2 M H_2SO_4. The plates were read with a multichannel

spectrophotometer (Multiskan, Thermo Scientific, USA) with a 450 nm filter.

Statistical analysis

Receiver operating characteristic (ROC) curves were analyzed to estimate the diagnostic cut off, sensitivity, and specificity (INSTAT software, GraphPad, San Diego, CA, USA).

Results

Creation of a recombinant P. pastoris clone expressing EDIII-D1

To create an EDIII-D1 secretory *P. pastoris* clone, we constructed an expression vector pPICZEDIII-D1 (Fig. 1B) by inserting a codon-optimized synthetic gene (Fig. 1A) into the plasmid pPICZαA. In this system, protein expression is under the control of the AOX1 promoter in response to methanol induction. The EDIII-D1 sequence was fused in frame to the *S. cerevisiae* pre-pro α-factor secretory signal and integrated into the genome of the *P. pastoris* host strain GS115 using the single cross-over strategy.

The recombinant protein EDIII of DENV-1 encoded in this system is composed of 138 amino acid residues; these domains possess the single S–S bond that is critical for structural and antigenic integrity [20]. The EDIII-D1 is predicted to be ~14,5 kDa in size and was expressed as fusions to a C-terminal polyhistidine (6xHis) tag to aid in detection and purification.

Transformants harboring the EDIII-D1 expression cassette were selected in the presence of 500 μg/mL zeocin, and then the presence of the integrated EDIII-D1 gene was further confirmed by direct colony PCR (Fig. 2A). A DNA fragment of about 400 bp was amplified from transformed GS115 cells, using specific an EDIII-D1 primer pair. The amplification with AOX1 primers confirmed that the domain gene had been integrated into the AOX1 locus on the genome of the *P. pastoris* GS115 host.

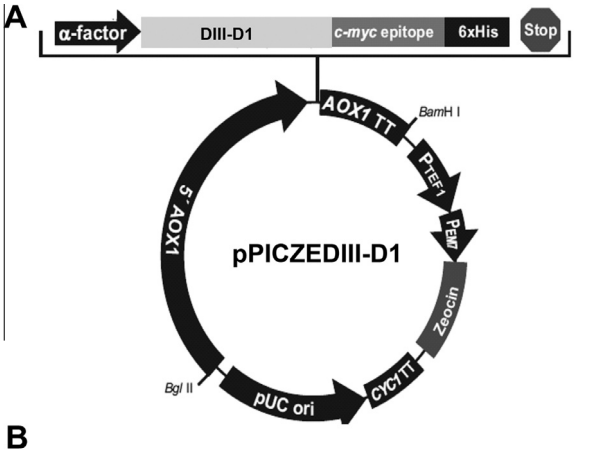

Fig. 1. The envelope domain III from dengue-1 antigen expression. (A) Codon-optimized synthetic gene of EDIII-D1. (B) Map of plasmid pPICZ-EDIII-D1. The EDIII-D1 gene was inserted into the *EcoR* I and *Not* I sites of the integrative vector pPICZαA under the control of the methanol-inducible AOX1 promoter (5′ AOX1). (C) Recombinant protein sequence.

A PCR-confirmed clone was assayed for the expression of EDIII-D1 gene product in small-scale testing with methanol induction for 3 days. Secreted expression of the recombinant protein is indicated by a 15 kDa protein band corresponding to the expected molecular weight of EDIII-D1 in the supernatant fraction (Fig. 2B). No similar band appeared in the blank control or in the supernatant without methanol induction. After confirmation of the secretion, this protein was called sEDIII-D1. Expression of the sEDIII-D1 protein was obtained during the first 24 h; therefore, induction with methanol for 3 days resulted in the maximal sEDIII-D1 expression (Fig. 2C). A nonspecific band of approximately 70 kDa, can also be observed in the material after methanol induction, but this band was not revealed in Western blot analysis (Fig. 3B and C).

Expression and purification of recombinant sEDIII-D1

The expression of recombinant sEDIII-D1 was done in conditions developed by Barr and collaborators that closely resembled the requirements of fermentor systems [21]. Therefore, the culture was conducted in two stages, first via the generation of biomass in a repressive buffered media, and then by the production phase, where the cells were re-suspended in small volumes of methanol induction-buffered media. The use of a buffered medium (pH 3.0–6.0) is known significantly reduce the proteolysis of the secreted recombinant protein [22,23].

Since the recombinant sEDIII-D1 protein contains a polyhistidine tag, it was recovered from the clarified methanol-induced supernatant by Ni-affinity chromatography. The entire process of purification was controlled and monitored by connecting the column to an AKTA system. Aliquots of fractions collected during the purification were analyzed by SDS–PAGE (Fig. 3A). It is evident that the bound fraction contains a major band of 15 kDa which is consistent with the predicted size of the sEDIII-D1 protein. An immunoblot analysis of the purified protein using anti-HisTag mAb and dengue positive serum appeared to confirm its identity (Fig. 3B and C). These data were confirmed by high sensitivity and specificity of IgM anti-dengue using sEDIII-D1 (Table 1).

Indirect ELISA for the detection of IgM and IgG anti-dengue

A panel of the 160 serum samples was evaluated with the purified and characterized sEDIII-D1 with an indirect enzyme-linked immunosorbent assay (ELISA); they were used to evaluate IgM and IgG anti-dengue. These samples had previously been characterized by commercially available serological tests (114 positive and 46 negative). For the IgM anti-dengue, the cut-off was defined at the point at which 0.204 nm, 99.12% specificity, and 86.96% sensitivity were observed. Therefore, the cut-off for IgG anti-dengue was 0.300 nm, 65.25% specificity, and 86.67% sensitivity (Fig. 4, Table 1).

Discussion

The global prevalence of dengue has grown dramatically in recent decades. This viral disease affects people in tropical and subtropical regions around the world, predominantly in urban and semi-urban areas, threatening more than 2.5 billion [24]. In endemic areas, secondary infections are most common and more highly associated with mortality [25,26]. Therefore, there is genuine demand for the rapid detection and differentiation of dengue virus infection diagnostic assays.

The dengue viral envelope protein is responsible for viral attachment by binding to cellular receptors [27,28]. Hence, the Ig-like envelope domain III protein, an independently folding domain that plays an important role in host receptor interactions,

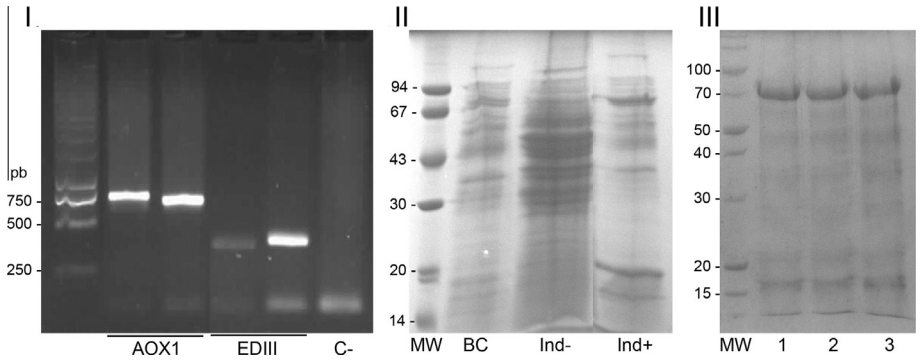

Fig. 2. Construction of *P. pastoris* expressing EDIII-D1 recombinant protein. (A) PCR amplification of AOX1 gene (AOX1) or dengue-1 domain III genes (EDIII) from two representative zeocin-resistant GS115transformants, C− negative control. (B) SDS–PAGE analysis of protein expression in the supernatant (BC) Blank control (GS115), (Ind−) supernatant without induction, and (Ind+) supernatant after methanol induction. (C) SDS–PAGE analysis of recombinant protein expression after 1, 2, and 3 days of methanol induction. (MW) Protein molecular weight markers (kDa).

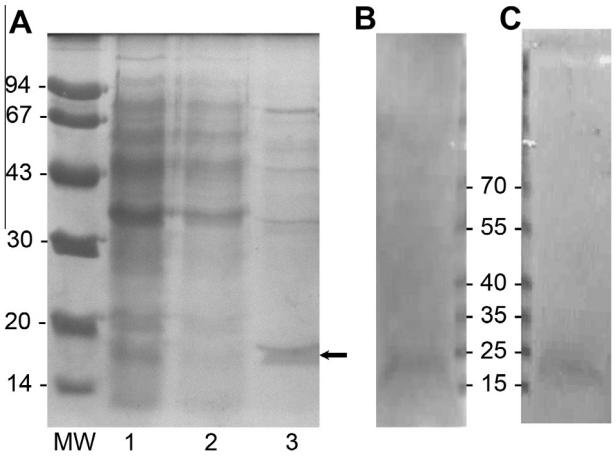

Fig. 3. Purification and characterization of the sEDIII-D1 protein. (A) Comassie blue-stained 15% reduced SDS–PAGE of samples obtained from each of the recombinant protein purification steps. (1) Total supernatant after methanol induction; (2) sample that did not interact with the affinity medium; (3) purified recombinant sEDIII-D1; (MW) Protein molecular weight markers (kDa). (B) Immunoblot of sEDIII-D1 after the purification. Aliquots of the purified material (3) were transferred to nitrocellulose and probed with anti-HisTag antibodies and dengue positive serum. (M) Pre-stained molecular weight markers (kDa). The arrows indicate the electrophoretic positions of the sEDIII-D1 protein.

Table 1
Sensitivity and specificity of IgM and IgG anti-dengue using sEDIII-D1. Receiver operating characteristic (ROC) curves were analyzed to estimate the diagnostic sensitivity and specificity.

	% Sensitivity	% Specificity
IgM	86.96	99.12
IgG	86.67	65.25

containing multiple serotype-specific conformation-dependent neutralizing epitopes [12,29] and exhibiting a very high degree of stability [30,31], has become an attractive candidate as a reagent for diagnosis of dengue infections.

During the last years, successful expressions of recombinant envelope protein dengue viruses have been demonstrated in different vector systems [32–34]. Although prokaryotic expression systems such as *E. coli* are simple to perform, they lack the modification mechanism of eukaryotic expression and it is difficult to purify recombinant proteins from inclusion bodies. The baculovirus expression system is usually used to produce recombinant proteins in eukaryotic cells, but they are not suitable for large-scale expression of foreign proteins. In the present study, the *P. pastoris* yeast expression system was used to produce dengue virus type 1 domain III proteins because of the simplicity of techniques needed for molecular genetic manipulation and its ability to produce foreign proteins extra cellularly. Furthermore, many post-translational modifications of eukaryotic systems, such as disulfide bond formation, can be performed in the yeast system. The recombinant vector pPICZEDIII-D1 described above was constructed with the EDIII-D1 gene in frame downstream from the sequence encoding the *S. cerevisiae* α signal peptide and upstream from the sequence encoding His-tag.

In accordance with the above objective, we demonstrated the secretory expression of the sEDIII-D1 protein in a two-phase cultivation, which included a glycerol batch phase, to accumulate biomass followed by an induction phase with methanol. The secretion of the recombinant protein into the growth medium is an important advantage because only low levels of endogenous protein are secreted to the media by the yeast itself, a process that facilitates following the protein purification steps [35,36]. The sEDIII-D1 recombinant protein was purified in Ni-affinity column chromatography and characterized by its electrophoretic mobility and its reactivity to 6xHis Tag mAbs and dengue-positive human serum.

Further purified and characterized sEDIII-D1 was successfully tested for the diagnosis of dengue infection in an indirect ELISA. From the 100 total human serum samples, the sensitivity was 86.67% and specificity 65.25% for IgG; sensitivity was 86.96% and specificity was 99.12% for IgM. The immunodominant epitopes preserved in the recombinant sEDIII-D1 protein certainly contributed to a high degree of sensitivity and specificity for the diagnosis of dengue infections. The common problem with dengue serological assays lies in detection of circulating cross-reactive antibodies against other Flaviviruses. Cross-reactivity was found to be minimum while identifying dengue virus infection, with in-house indirect ELISA employing pre-coated EDIII-D1 proteins.

Fry and collaborators reported that ideal results are obtained by combining antigen or antibody detection methods, thereby increasing the sensitivity and the specificity of serological tests [37]. Previous research indicates that variables such as infecting serotype, primary or secondary infection, time of sample collection, and the individual responses of patients are factors that can cause variations in the sensitivity and specificity of serological tests from 65% to 100% [3,37,38]. The sensitivity and specificity of sEDIII-D1 proteins in detecting antibodies from dengue-infected patients renders hope that it may be a promising candidate for the development of commercial and inexpensive dengue diagnostic kits. A similar strategy could lead to the production of recombinant domain III proteins of other serotypes. The level of purification facilitated by protein secretion has the potential to make this a highly cost-effective approach for dengue diagnosis, in a large scale production.

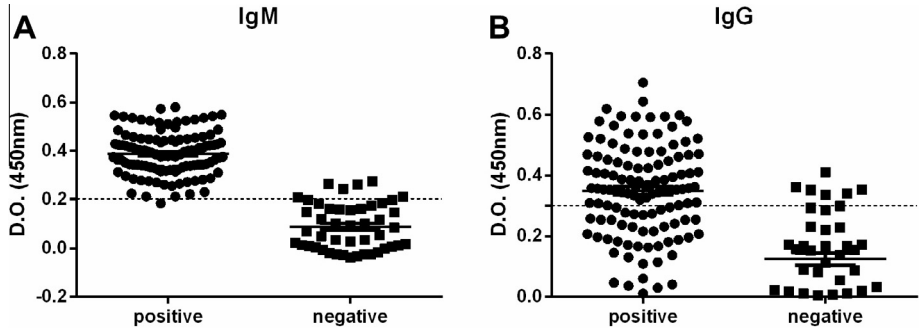

Fig. 4. Indirect ELISA using sEDIII-D1. Results of dengue-positive and negative (healthy individuals) samples in IgM (A) and IgG (B) detection. The horizontal line indicates the cutoff value of the assay.

Acknowledgments

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