Journal of Virological Methods 187 (2013) 114–120

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Recombinant envelope protein-based enzyme immunoassay for IgG antibodies is comparable to neutralization tests for epidemiological studies of dengue infection

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Article history:
Received 18 January 2012
Received in revised form
12 September 2012
Accepted 13 September 2012
Available online 24 September 2012

Keywords:
Dengue virus
Serology
Diagnosis
ELISA
Recombinant envelope proteins

A B S T R A C T

Dengue virus (DENV) is the most prevalent arbovirus in the world, found mainly in tropical regions. As clinical manifestations present frequently as nonspecific febrile illness, laboratory diagnosis is essential to confirm DENV infections and for epidemiological studies. Recombinant envelope (E) antigens of four serotypes of DENV were used to develop an immunoglobulin G enzyme-linked immunosorbent assay (IgG-ELISA). To evaluate the IgG-ELISA, a panel of serum samples that had been tested previously by a plaque reduction neutralization test (PRNT) was investigated for the presence of anti-E antibodies against the four DENV serotypes. IgG-ELISA was found to have a sensitivity (91%) and specificity (98%) at a receiver-operating characteristic (ROC) optimized cutoff and demonstrated high performance as well as good indexes. A concordance of 97% was achieved between both assays, and only 21/704 (3%) samples were not concordant. The results of the present study demonstrate a moderate correlation between neutralizing antibody titers and IgG-ELISA values. These findings indicate that the recombinant protein-based IgG-ELISA is a suitable method for routine serodiagnosis, monitoring and seroepidemiological studies of DENV infections.

1. Introduction

Dengue virus (DENV), a member of the family *Flaviviridae*, is the most prevalent mosquito-borne viral disease in tropical and subtropical regions of the world. Nearly 2.5 billion people in more than 100 countries live in dengue-endemic regions, which include the Caribbean, South and Central America, Africa, and Southeast Asia (Gubler, 2002). Approximately 100 million cases of dengue infections, including an estimated 250,000–500,000 severe cases of dengue hemorrhagic fever or dengue shock syndrome and approximately 25,000 deaths occur annually in the world (Halstead, 2007). According to the Pan American Health Organization (PAHO), dengue killed 1167 individuals in Latin America in 2010, and 1.8 million cases were confirmed.

There are four serotypes of *Dengue virus*: DENV-1, DENV-2, DENV-3, and DENV-4. Infection with one DENV induces lifelong protective immunity to the serotype of the virus that produced the infection. To date, no effective commercial vaccine or treatment exists for dengue infections, so early detection, epidemiological studies, and the management of severe disease are essential to prevent death (Deen et al., 2006). Therefore, health care resources, such as clinical and laboratory diagnosis and hospital admissions, represent a financial burden to many countries where dengue is endemic, including Brazil.

The detection of antibodies by serological methods is very useful for dengue diagnosis. There are a variety of serological tests that can measure anti-DENV antibodies including the hemagglutination inhibition test, complement fixation test, fluorescent antibody test, enzyme-linked immunosorbent assay (ELISA), and plaque reduction neutralization test (PRNT). However, only PRNT measures the

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0166-0934
http://dx.doi.org/10.1016/j.jviromet.2012.09.012

biological parameter of in vitro virus neutralization, and for this reason, it is the most virus specific serologic test among the flaviviruses (WHO, 2007). PRNT is a serotype-specific test for DENV, correlating well with serum levels of protection from viral infection (Guzmán and Kourí, 2004). Indeed, PRNT is regarded as the "gold standard" serological method for the diagnosis of DENV (Thomas et al., 2009). Quantitative PRNT values demonstrate a good correlation with the patient's immune state prior to DENV serotype infection and cross-reaction to other serotypes (Morens et al., 1985). However, PRNT has some practical disadvantages because it is technically demanding, labor intensive and time consuming. In addition, as a variable bioassay, the test is difficult to standardize between different laboratories. ELISA, however, is rapid, simple to perform, relatively inexpensive, easier to standardize and, importantly, can be automated. However, there are a lack of data describing the sensitivity and specificity of IgG-ELISA compared to PRNT for dengue antibody detection. To date, there are no published technical efficacy trials correlating the presence of neutralizing antibodies and IgG detected by immunoenzymatic assays.

Although PRNT may be the most useful serological method for detecting serotype-specific antibodies, it employs a DENV-infected cell culture supernatant that increases the risk of potential health hazards via exposure to infectious virus particles. For this reason, the simplified production of recombinant proteins or synthetic peptides in bacteria has been proposed as an antigen source (dos Santos et al., 2004), which is often the limiting reagent in DENV diagnostic assays. Indeed, the use of recombinant proteins as antigens avoids the problems associated with whole-virus DENV antigen preparations (Videa et al., 2005).

The serological method developed in this study utilizes recombinant E proteins of DENV as antigens. These proteins have already been proven to elicit protective antibodies in mice and monkeys (Clements et al., 2010; Men et al., 2000, 1991; Putnak et al., 2005; Putnak, 1994; Raviprakash et al., 2000) and have been used as an antigen in the rapid diagnosis of DENV infection (Makino et al., 1991). Most recombinant DNA-based strategies focus on the E protein because it is the major DENV structural protein that is known to contain the principal neutralizing epitopes, and it is considered to be the viral immunodominant protein (Lai et al., 2008; Lin et al., 1994).

The present work describes the development and evaluation of an indirect ELISA based on DENV E recombinant proteins of all four serotypes as a tool for diagnosis and seroepidemiological studies.

2. Materials and methods

2.1. Serum samples

In this study, an IgG-ELISA was evaluated using 704 serum specimens obtained from the Secretaria Municipal de Saúde of Belo Horizonte. From June 2006 until March 2007, samples were collected randomly from healthy volunteers in Belo Horizonte (Minas Gerais State, Brazil), an endemic area for DENV 1–3. The samples were stored at −20 °C for later use. All of the 704 samples were screened previously for neutralizing antibodies by PRNT in a blind panel, and a detailed clinical–epidemiological description of the cohort is described elsewhere (Pessanha et al., 2010).

Written informed consent to participate in the study was obtained from each person (or their guardian). All data were handled confidentially and anonymously. This study was reviewed and approved by the Comitê de Ética em Pesquisa of Secretaria Municipal de Saúde of Belo Horizonte (protocol 037.2005).

2.2. Recombinant protein expression

For the cloning and expression of the DENV 1, 2, 3 and 4 recombinant E proteins, all four DENV serotypes were propagated in *Aedes albopictus* C6/36 cells and used as a source of viral RNA for reverse transcription PCR. DENV-1 was isolated from a dengue fever (DF) patient infected during an outbreak in 2002 in Rio de Janeiro (kindly provided by Ricardo Galler, Instituto Oswaldo Cruz, Brazil). DENV-2 and DENV-3 were isolated by our laboratory from a DF patient and a dengue hemorrhagic fever (DHF) patient, respectively. The DENV-4 used was the isolate H402276 (Osanai et al., 1983). Viral RNA extraction was performed using the QIAmp Viral RNA kit (Qiagen, Inc., Valencia, CA, USA). The cDNA fragments were amplified by PCR using specific primers for each DENV serotype: Den1E-F (ATAGGATCCCGGTGCGTGGGAATAG) and D1E-R (TATAGTCGACTTA AGTTGCTTCAAACATTTTTCCTATG), Den2E-F (TATAGGATCCCGTTGCATAGGAATATCAAATAGAG) and Den2E-R (TATAGTCGACTTATGTTGTCTCAATCATTTGGCC), Den3E-F (TATAGGATCCAGATGTGTGGGAGTAGGAAAC) and Den3E-R (TATACTGCAGTTATCTGGCAGTGGCCTCG), and Den4E-F (TATAGGATCCCGATGCGTAGGAGTAGGAAAC) and Den4E-R (AGCAAGCTTTTATGTGGACTCAAACATCTTGCC). The PCR products were cloned into a pGEM-T vector (Promega Corp., Madison, WI, USA) and subcloned into a pQE-9 vector (Qiagen, Inc., Valencia, CA, USA). To confirm the integrity of the ORFs, all four constructions were sequenced at least 3 times in both orientations (MegaBACE 1000 Sequencer, GE Healthcare, Buckinghamshire, UK) using pQE universal primers.

The recombinant proteins represent 80% of the N-terminus of the viral E proteins (E_{80}) of DENV 1, 2, 3 and 4. We chose the N-terminal 80%-length recombinant E protein (encoding amino acids 1–404) of DENV 1–4 as antigens because together they may be recognized by specific antibodies to each DENV serotype. The plasmid was transformed into an *Escherichia coli* host strain M15 containing the pREP4 plasmid, which encodes the *lac* repressor and the kanamycin marker. The resulting recombinant proteins were analyzed by expression screening, wherein the IPTG-induced bacteria were lysed directly in a sample buffer and analyzed by SDS-PAGE (data not shown).

The four recombinant proteins were purified by nickel affinity chromatography under denaturing conditions. Based on SDS-PAGE, the proteins were more than 90% pure and had a molecular mass of approximately 50 kDa (data not shown). We obtained yields of up to 10.3, 4.8, 3.3, and 9.0 mg of Den1E, Den2E, Den3E and Den4E, respectively, per liter of bacterial culture.

2.3. Enzyme-linked immunosorbent assay

For the IgG-ELISA, Nunc Maxisorp™ 96-well ELISA plates were filled with 100 μl/well of an equal mixture of the DENV 1–4 recombinant proteins (40 ng of each antigen/well) diluted in 0.01 M carbonate buffer at pH 9.6. After overnight incubation at 4 °C, the plates were washed three times with 300 μl/well of phosphate buffer solution containing 0.05% Tween 20 (PBS-T) and blocked with 1% bovine serum albumin (BSA) (Sigma–Aldrich, USA) in PBS-T for 2 h at room temperature (RT). After the washing procedures, the serum sample was diluted at 1:50 in PBS-T with 0.1% BSA, 100 μl/well of serum dilution was added in duplicate, and the plates were incubated at RT for 60 min. After washing again, the reactions were developed using 100 μl/well of a rabbit-anti-human IgG antibody conjugated to horseradish peroxidase (Sigma–Aldrich, USA), diluted at 1:5000 in PBS-T with 0.1% BSA. After incubation at RT for 60 min, the plates were washed four times, and the substrate o-phenylenediamine dihydrochloride (Sigma–Aldrich, USA) and 0.03% hydrogen peroxide were added. After incubation at RT for 10 min, the reaction was terminated by the addition of 40 μl of 2 M sulfuric acid to each well and read at 492 nm using a Biochrom

Asys Expert Plus microplate reader. Positive control (serum shown by PRNT to have DENV 1–4 neutralizing antibody titers ≥ 1:30) and negative control (PRNT < 1:30) were included in each run.

2.4. Plaque reduction neutralization test

The serum samples were screened for specific antibodies against DENV-1, 2 and 3 in LLC-MK_2 cells using a 50% plaque reduction criteria as described previously (Russell et al., 1967). The serum was inactivated (56 °C for 30 min) and diluted to a 1:30 screening dilution in duplicate. The PRNT results were registered in qualitative (positive and negative) terms. Neutralizing antibody titers ≥1:30 were interpreted as positive and lower titers were interpreted as negative. Positive and negative controls sera were 2-fold diluted beginning with a 1:30 dilution until 1:240 to determine the titer of neutralizing antibodies against DENV 1–3. Each serum was tested in duplicate, and the plaque-forming unit (PFU) was recorded as the average of the number observed in each of the two cultures. The samples (Pessanha et al., 2010) which show discordant results with the IgG-ELISA were retested.

2.5. Data analysis

Statistics were performed using GraphPad Instat Software, version 5.0, and the receiver-operator characteristic (ROC) curve analysis was carried out using MedCalc Software, version 7.3.0.0. This curve optimizes the interpretation of the ELISA results when there are well-defined positive and negative populations available for analysis. The ROC curve is a graphical plot of the sensitivity versus "100 – specificity" for a binary classifier system, using various cutoff values. This allows the cutoff value that gives the best balance of sensitivity and specificity to be selected for the test being considered (Zweig and Campbell, 1993). To determine which cutoff value provided the most accurate result, the area under the curve (AUC) was calculated.

3. Results

3.1. Performance of IgG ELISA

To evaluate the IgG-ELISA, 704 serum samples, tested previously by PRNT, were used to investigate the presence of specific antibodies against DENV. A total of 100 PRNT-positive samples and 604 PRNT-negative samples were used. The results associated with the mean optical density obtained with an IgG-ELISA of each of the 704 serum samples were analyzed to establish the cutoff value by the ROC curve method (Fig. 1). The results were considered positive when the optical density at 492 nm was higher than 0.524.

The results summarized in Table 1 indicate that 97% of the samples tested by IgG-ELISA and PRNT were concordant between both assays. Positive samples with both assays were assigned as true positives (TP), and those samples whose results were negative for both assays were assigned as true negatives (TN). Using PRNT results as true values, the false-negative rate of the IgG-ELISA test was 9% (9/100), and the false-positive rate was 1.9% (12/604).

Table 1
Comparison between PRNT and IgG-ELISA tests (positive and negative results).

ELISA/PRNT results	No. of samples	% of total
$ELISA^{+}/PRNT^{+}$ (TP)	91	12.9
$ELISA^{-}/PRNT^{-}$ (TN)	592	84.1
$ELISA^{+}/PRNT^{-}$ (FP)	12	1.7
$ELISA^{-}/PRNT^{+}$ (FN)	9	1.3
Total	704	100

TP, true positive; TN, true negative; FP, false positive; FN, false-negative.

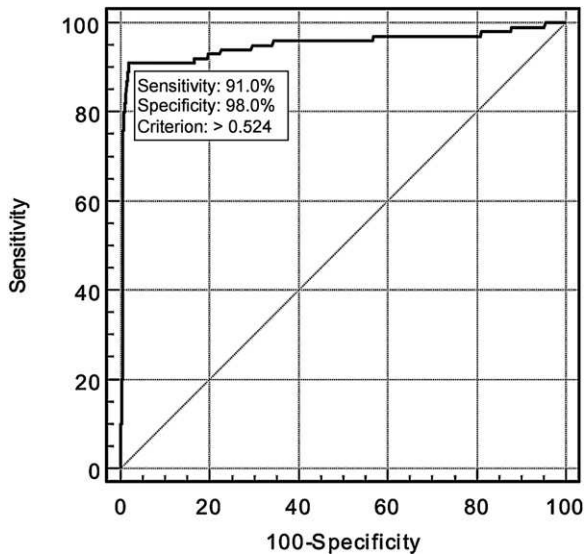

Fig. 1. The ROC curve for 704 serum samples tested by PRNT and IgG-ELISA. The AUC, which indicates accuracy, was determined for the mean of the OD duplicates and was equal to 0.950, resulting in 91% sensitivity and 98% specificity, for a cutoff value of 0.524.

The AUC indicated that the IgG-ELISA was, on average, 95% accurate, and the 95% confidence interval of the AUC for IgG-ELISA ranged from 93.2 to 96.5% (Table 2). The optimal cutoff point of 0.524 resulted in a sensitivity of 91% and a specificity of 98% as shown in Table 2 and Fig. 1. This optimal cutoff point helped to classify tentatively the samples as TP, TN, false positive (FP) or false negative (FN). Of the 100 PRNT-positive serum samples, 91 were considered to be TP because the samples presented IgG-ELISA readings above the optimized cutoff point, while 9 PRNT-positive sera were diagnosed as FN because the IgG-ELISA results were below this cutoff point. Among the 604 PRNT-negative sera, 592 serum samples presented values below the ROC-optimized cutoff when tested by IgG-ELISA and were hence considered to be TN (Fig. 2). The optimal IgG-ELISA cutoff was associated with a positive likelihood ratio (LR) of 45.80 and a negative LR of 0.09 (Table 2). The probability of TP serum samples with positive ELISA test results (positive predictive value) was 88.3%, while the probability of TN serum samples with negative IgG-ELISA test results (negative predictive value) was 98.5% (Table 2).

Additionally, all 9 FN samples were negative in the Panbio Dengue IgM capture ELISA, except one. In contrast, all 12 FP samples were positive by the PanBio Dengue IgG Capture ELISA (data not shown).

Table 2
ROC analysis of the IgG-ELISA performance in identification of prior DENV infections (n = 704 serum samples).

Indice	IgG-ELISA
AUC	0.950
AUC at 95% confidence interval	0.932–0.965
Sensitivity (%)	91.0
Specificity (%)	98.0
Test positive predictive value (%)	88.3
Test negative predictive value (%)	98.5
Optimal test cutoff	0.524
Positive likelihood ratio	45.80
Negative likelihood ratio	0.09
Prevalence	14.2%

AUC, area under the curve.

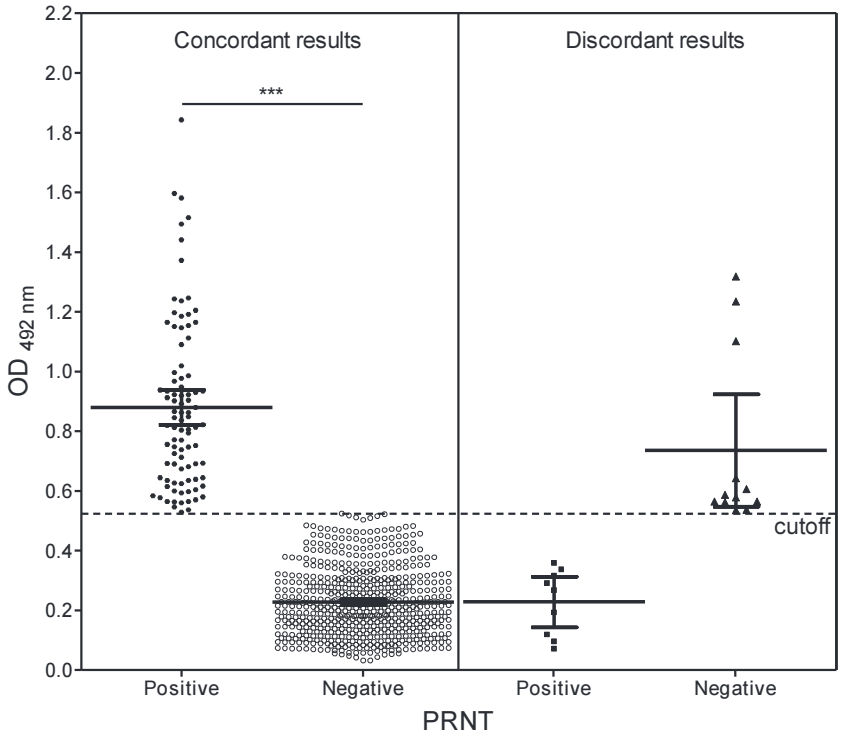

Fig. 2. Scatter plot of the levels of serum IgG against DENV ($OD_{492\,nm}$) compared to the PRNT results. Concordant results are defined as positive or negative for both methods and discordant results as false-positive or false-negative (***$P < 0.0001$, Mann–Whitney statistical test). The horizontal dotted line indicates the cutoff value (0.524).

3.2. Correlation of IgG-ELISA with antibody titer and serotype distribution measured by PRNT

The use of the IgG-ELISA as a quantitative assay compared to PRNT was assessed. Fig. 3 presents the results of the antibody determination in individual serum specimens of the 704 samples by the use of PRNT and IgG-ELISA in a two-dimensional distribution. The correlation between the anti-dengue IgG-ELISA and PRNT values results in $\rho = 0.537$ after Spearman's correlation ($P < 0.0001$, 95% confidence interval for ρ between 0.48 and 0.59).

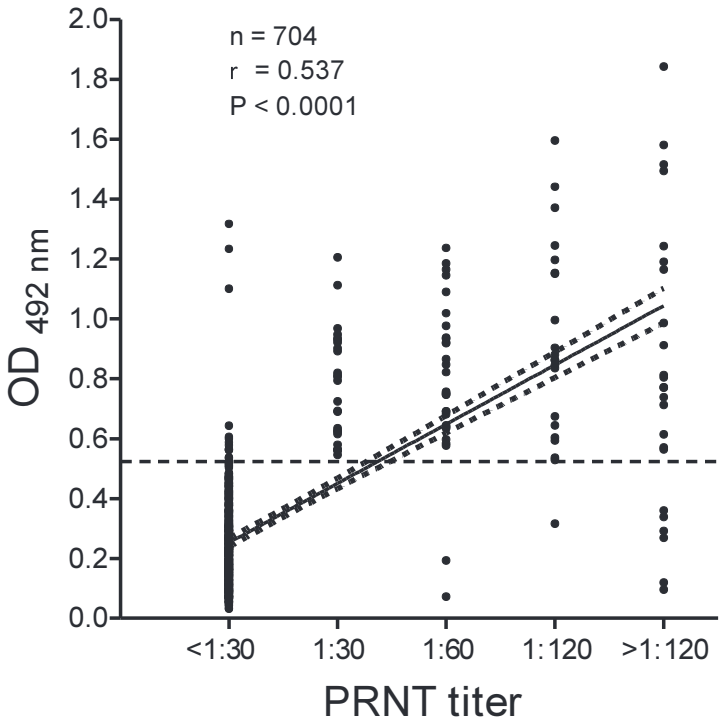

Fig. 3. Regression analysis (95% correlation coefficient and regression line) of 704 samples tested by dengue PRNT and IgG-ELISA. The horizontal dotted line indicates the cutoff value (0.524).

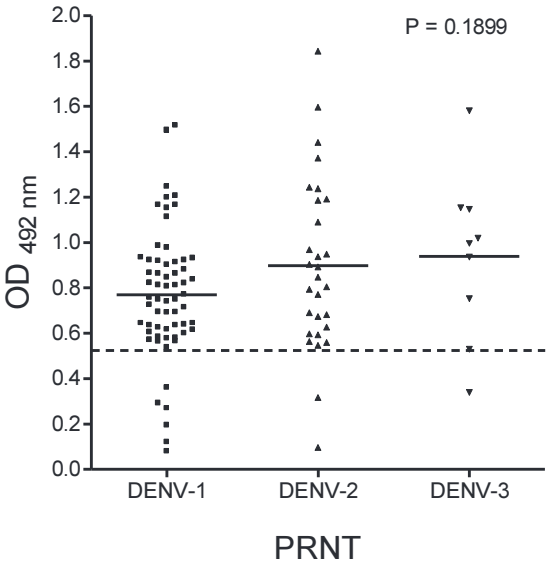

Fig. 4. Relationship between serotype-specific distribution and IgG-ELISA values. PRNT was used to distinguish the immune state to different DENV serotypes. The distribution of PRNT positive sera for DENV serotypes 1, 2 and 3 was 62, 29, and 9 samples, respectively. The three medians (short horizontal lines) are not statistically significant ($P = 0.1899$, Kruskal–Wallis statistical test). The horizontal dotted line indicates the cutoff value (0.524).

The samples that were negative by IgG-ELISA and positive by PRNT presented neutralizing antibody titers ranging from 1:60 to >1:120 (representative data are shown in Fig. 3). As shown in Fig. 3, the IgG-ELISA was able to detect low titers (1:30) as well as high titers of neutralizing antibodies (>1:120).

The graph in Fig. 4 presents the results of specific neutralizing antibody determination in 100 individual serum specimens by the use of PRNT and IgG-ELISA in a two-dimensional distribution. An analysis using a Kruskal–Wallis test revealed that the apparent difference between the three medians are not significant statistically ($P = 0.1899$).

Regarding the specific serotype, the IgG-ELISA is able to detect, with similar sensitivity, neutralizing antibodies to DENV-1, 2 and 3, as the sensitivity for each serotype was 90.3, 93.1 and 88.9%, respectively. These data were determined by calculating the number of TP results divided by the sum of the TP results plus the number of FP results.

Neutralizing antibodies to DENV-4 were investigated additionally in all nine FN sera samples, but none had detectable titers by PRNT (data not shown).

4. Discussion

Although PRNT has been used for over 30 years as a quantitative method to detect anti-DENV neutralizing antibodies, the assay is laborious to perform, and a period of at least 7 days is required to obtain accurate diagnostic results; however, it is still considered the gold standard for determining immunity against DENV. Several dengue immunoenzymatic assays have been developed with different formats as alternatives to PRNT because they are simple, rapid, and easy to perform (de Paula and Fonseca, 2004). Furthermore, the whole virus production required for neutralizing tests has potential biohazards associated with the handling of live virus during the preparation of diagnostic reagents. Thus, the replacement of neutralizing tests is an attractive possibility.

In recent years, numerous ELISA kits have been developed and used to diagnose dengue infections (Buerano et al., 2000; dos Santos et al., 2007; Nunes et al., 2011; Palmer et al., 1999; Parida et al., 2001; Sathish et al., 2002; Vaughn et al., 1999), but there have been studies examining the detection of anti-DENV antibodies using the four serotype E recombinant proteins (Cuzzubbo et al., 2001; dos Santos et al., 2007; Ludolfs et al., 2002; Videa et al., 2005).

Here, we have exploited a bacterial expression system for the production of E proteins. The E gene was truncated at its carboxy terminus to remove the hydrophobic membrane anchor segment to maximize the expression of the protein, as recommended by others in different systems (Delenda et al., 1994; Men et al., 2000, 1991; Raviprakash et al., 2000). Bacterial expression is perhaps the most employed commonly expression system for the production of non-glycosylated recombinant proteins. The organism is relatively simple to manipulate, and expressed proteins are purified easily under denaturing conditions. Additionally, refolding of the protein is not necessary for diagnostic purposes (Tripathi et al., 2008).

Anti-DENV IgG antibodies in human sera cross-react with all four dengue virus serotypes (Nawa et al., 2000) and even with other flaviviruses (Cuzzubbo et al., 1999; Rivetz et al., 2009; Vaughn et al., 1999; Wu et al., 1997). Hence, we proposed the use of a mixture of the four serotype recombinant DENV antigens as reagents. Although an indirect IgM-ELISA with a mixture of recombinant antigens bound to a polystyrene plate has already been evaluated (Videa et al., 2005), this IgG-ELISA is the first immunoenzymatic assay based on recombinant DENV E proteins to have comparable results to PRNT. Moreover, IgG antibodies against DENV 1–3 are detected simultaneously by our test using a single serum dilution rather than additional dilutions for each serotype neutralization, as required by PRNT.

The comparative evaluation performed on the MedCalc program resulted in a cutoff that represents the distinction limit between positive and negative samples. An ELISA result equal to or lower than the cutoff is negative, and a higher result is positive. Although 683 (97%) samples evaluated by PRNT and IgG-ELISA showed concordant results, 21 samples (3%) tested by IgG-ELISA were discordant to the PRNT results. Two hypothesis may explain the differences in the results: (i) Positive samples verified only by PRNT have undetectable IgG levels by IgG-ELISA, whereas specific IgM is the first class of neutralizing antibodies that result from a recent DENV infection; or (ii) the recombinant antigens used in this study have epitopes that are recognized by specific antibodies (a phenomenon known as cross-reaction) of other viruses of the *Flavivirus* genus, such as *Yellow fever virus* (YF). However, the second hypothesis is unlikely because 83% of the individuals tested by our assay were vaccinated previously against YF (Pessanha et al., 2010). This number represents more than five times the positive IgG anti-dengue samples. Moreover, no *West Nile virus*, *Japanese encephalitis virus* (JE) or *Saint Louis encephalitis virus* infections were reported in Brazil until the end of the sampling collection. In addition, there were no cases of DENV-4 reported in the sample collection period.

The sensitivity and the specificity results obtained for the IgG-ELISA are comparable to other serological methods that are available commercially for the detection of antibodies to DENV in Brazil, such as Panbio ELISA Dengue IgG, Panbio Dengue Duo Cassette and Pathozyme Dengue IgG (ANVISA, 2003). Two commercial ELISAs, the PanBio Dengue IgM capture ELISA and the PanBio Dengue IgG capture ELISA (PanBio Pty Ltd., Brisbane, Australia), also detect cross-reactive antibodies against JE and YF virus (Rivetz et al., 2009; Yew et al., 2009).

The results reported herein indicate that our ELISA, based on pooled recombinant E antigens, provided a reliable diagnosis of DENV infections comparable to PRNT because high sensitivity and specificity were obtained by the IgG-ELISA and were associated with high accuracy. In addition, IgG-ELISA OD values also presented a good correlation to neutralizing antibody titers as detected by PRNT (Fig. 3), allowing inferences about the antibody levels in a similar manner to PRNT. Furthermore, recombinant antigen-based

assays, compared to virus-infected cell culture antigen assays, can provide a valuable resource for serodiagnosis because they are potentially cheaper, and the cost of most commercial kits for dengue diagnosis is a limitation in many dengue-endemic countries.

Epidemiological studies may play major role in evaluating the prevalence of dengue fever and in providing indicative values that reflect the effectiveness of vector control strategies, especially in endemic areas where DENV infections are frequent (Teixeira et al., 2005). According to the present study conducted in 2006 and 2007, a considerable proportion of the population in Belo Horizonte had anti-DENV IgG antibodies (14.2%), which increases the risk of DHF/DSS cases once dengue becomes endemic in this city. According to the Secretaria de Vigilância em Saúde (SVS), DENV-3 was introduced recently into Minas Gerais State. This introduction correlates well with our findings of serotype-specific dengue cases, as types 1 and 2 represented 91% of dengue infections determined by PRNT in our study.

Several serological survey studies have been conducted in Brazil in the last few decades involving randomly collected samples tested by PRNT, hemagglutination inhibition, or immunoenzymatic assays (Figueiredo et al., 2004; Lima et al., 2007, 1999; Siqueira et al., 2004; Vasconcelos et al., 2000, 1999). However, this work is the first serological survey effort to compare PRNT to an IgG-ELISA based on DENV recombinant antigens.

DENV possesses epitopes that are shared by other flaviviruses, such as JEV, and as a result, antibodies generated against cross-reactive epitopes during infection may give rise to false-positive tests (Koraka et al., 2002; Vaughn et al., 1999). This cross-reactivity occurs more often with IgG antibodies and less often with IgM antibodies (Cuzzubbo et al., 1999). The IgG-ELISA described in this study has proven to be very reliable and presented no cross-reaction to anti-YF virus antibodies.

5. Conclusions

In summary, our IgG-ELISA has proven to be rapid and easy to perform, overcomes some of the limitations associated with PRNT, and has great potential for use in the serodiagnosis and seroepidemiological study of DENV infections. In addition, the assay has the potential for use in other applications, such as vaccine studies in clinical trials, as the system is able to detect effectively dengue neutralizing antibodies. Additionally, the high specificity of IgG-ELISA in detecting DENV antibodies enables its use in areas where exposure to *Flavivirus* antigens of YFV occurs through vaccination.

Conflicts of interest statement

The authors have no conflicts of interest concerning the work reported in this paper.

Acknowledgements

The authors are grateful to Angela Sana Lopes, Ilda V. Gama, Andresa A. Carvalho, Edel F.B. Stancioli, Mauricio L. Nogueira and Jordana G.A. Coelho-dos-Reis, Glaura C. Franco and colleagues from Laboratório de Vírus (ICB-UFMG) for their excellent technical support. The authors are also thankful to all of the volunteers, without whom this work would not have been possible, and for the financial support provided by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), INCT and PRONEX-Dengue, Departamento de Ciência e Tecnologia do Ministério da Saúde (DECIT/MS). FGF, CAB, PCP and EGK are fellows from CNPq.

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