

Recombinant Polypeptide Antigen-Based Immunoglobulin G Enzyme-Linked Immunosorbent Assay for Serodiagnosis of Dengue[∇]

Flavia B. Dos Santos,^{1*} Rita Maria R. Nogueira,¹ Monique R. Q. Lima,¹ Thatiane S. De Simone,¹ Hermann G. Schatzmayr,¹ Elezer M. B. Lemes,² Eva Harris,³ and Marize P. Miagostovich¹

Department of Virology, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro 21045-360, Brazil¹; Department of Technological Development, Bio-Manguinhos, Rio de Janeiro 21045-360, Brazil²; and Division of Infectious Diseases, School of Public Health, University of California, Berkeley, California 94720-7360³

Received 18 December 2006/Returned for modification 8 February 2007/Accepted 8 March 2007

We have developed an indirect enzyme-linked immunosorbent assay for detection of anti-dengue virus (DENV) immunoglobulin G antibodies using four recombinant DENV envelope polypeptides as antigens, which demonstrated a sensitivity of 89.4% and a specificity of 93.3%. These easily produced antigens are a feasible, cost-effective alternative for generating reagents for dengue serological tests.

The accurate and efficient diagnosis of dengue is important for clinical care, surveillance, pathogenesis studies, and vaccine research. Dengue viruses consist of four serotypes (DENV1 to -4) (17) and cause a spectrum of disease ranging from self-limited illness (dengue fever) to more severe forms of disease (dengue hemorrhagic fever/dengue shock syndrome) (25). Dengue is one of the most important mosquito-borne viral diseases in the world (10), with 2.5 to 3 billion people at risk for infection and tens of millions of dengue cases annually. Nonetheless, the disease remains significantly underreported (15). Although numerous commercial kits for serological diagnosis of dengue are available, some of which have shown good sensitivity and specificity (3, 8), their cost poses a financial burden to many countries where dengue is endemic.

Enzyme-linked immunosorbent assay (ELISA)-formatted tests to detect anti-DENV immunoglobulin G (IgG) antibodies have been developed as an alternative method to the hemagglutination inhibition test, which has served as the “gold standard” for the diagnosis and classification of DENV infections (4, 5, 12, 18, 25). Moreover, an IgG-ELISA was reported to be an accurate and reliable assay for characterization of human immune responses in primary and secondary DENV infections (18). However, these ELISAs require viral antigen produced, in limited quantities, by growing DENV in cell culture or in suckling mouse brain, which is insufficient for the large-scale serological screening made necessary by the spread of the disease. Furthermore, the use of whole viruses or crude extracts represents potential health hazards through exposure to infectious virus particles. To overcome this problem, we developed and evaluated an in-house ELISA for detecting anti-DENV IgG antibodies using as the antigen a mixture of DENV1 to -4 recombinant polypeptides, as its use for IgM capture has been previously demonstrated (23). Fragments of

approximately 500 bp from the 5′ end (nucleotides 1093 through 1585) of the DENV1 to -4 E genes were amplified by reverse transcription-PCR (RT-PCR) and cloned into an expression vector, and the immunogenic E polypeptides (~25 kDa) were easily expressed in *Escherichia coli* (7, 23).

The 271 sera used in this study were from the Flavivirus Laboratory, Oswaldo Cruz Institute/FIOCRUZ, Brazil (1998 to 2005). Laboratory-positive DENV infection was defined in patients experiencing a febrile illness consistent with dengue according to WHO criteria (25), in accordance with which infection was confirmed by DENV isolation (9), detection of DENV RNA by RT-PCR (16), detection of anti-DENV IgM antibodies by IgM antibody capture ELISA (19), and/or a fourfold or greater rise in anti-DENV IgG-ELISA titer in paired acute- and convalescent-phase sera (18). Individuals were classified as negative for DENV infection if results were negative by all the methods described above. A panel of 113 paired sera from laboratory-confirmed dengue cases and 45 single samples from nondengue cases was divided into six groups: groups A to C, paired sera from patients infected with DENV1 ($n = 21$), DENV2 ($n = 15$), and DENV3 ($n = 25$), respectively; group D, paired sera from serologically confirmed DENV-infected patients whose serotype was not identified ($n = 52$); group E, single sera from healthy individuals confirmed as dengue negative ($n = 15$); group F, unpaired sera from individuals positive for anti-yellow fever virus antibodies and negative for anti-DENV antibodies ($n = 10$); group G, single sera from measles patients ($n = 10$); group H, unpaired sera from rubella patients ($n = 10$). When paired sera were analyzed, the convalescent-phase sera were collected approximately 14 days (range, 13 to 18) after the acute-phase serum sample.

The standard IgG-ELISA was performed as previously described (18). For the recombinant polypeptide-based IgG-ELISA (REC IgG-ELISA), 96-well plates (Immulon Dynatech Industries, Inc., Chantilly, VA) were coated with 75 μ l of a mixture of DENV1 to -4 recombinant polypeptides (6 μ g/ml of each per well) and incubated overnight at 4°C. Plates were blocked in phosphate-buffered saline (PBS), pH 7.4, 3.5% nor-

* Corresponding author. Mailing address: Flavivirus Laboratory, Department of Virology, Oswaldo Cruz Institute/FIOCRUZ, Av Brasil, 4365, Manguinhos, Rio de Janeiro 21045-360, Brazil. Phone and fax: 55(21) 2598-4373. E-mail: flaviab@ioc.fiocruz.br.

[∇] Published ahead of print on 28 March 2007.

TABLE 1. Sensitivity and specificity of REC IgG-ELISA in confirming DENV infection in paired dengue sera and controls

Group ^a	No. of sera with indicated REC IgG-ELISA result/total no. tested ^b	
	Negative	Positive
A (DENV1 cases; 21 pairs)	5/21 (23.8)	15/21 (71.4)
B (DENV2 cases; 15 pairs)	2/15 (13.3)	13/15 (86.7)
C (DENV3 cases; 25 pairs)	1/25 (4.0)	24/25 (96.0)
D (DENV cases, serotype not identified; 52 pairs)	3/52 (5.7)	49/52 (94.3)
Total for groups A-D	12/113 (10.6)	101/113 (89.4)
E (healthy individuals; <i>n</i> = 15)	14/15 (93.3)	1/15 (6.7)
F (yellow fever cases; <i>n</i> = 10)	7/10 (70.0)	3/10 (30.0)
G (measles cases; <i>n</i> = 10)	9/10 (90.0)	1/10 (10.0)
H (rubella cases; <i>n</i> = 10)	9/10 (90.0)	1/10 (10.0)
Total for groups E-H	39/45 (86.7)	6/45 (13.3)

^a Subjects in groups A to D had confirmed DENV infection; subjects in groups E to H had no DENV infection.

^b Values in parentheses are percentages.

mal goat serum, and 0.05% Tween 20 (PNT) for 1 h at 37°C. One hundred microliters of serum diluted 1:40 in PBS with 1.5% nonfat dry milk was added, plates were incubated at 37°C for 1 h, and then 40 μ l of horseradish peroxidase-conjugated anti-human IgG (Sigma Chemical Co.) diluted 1:2,000 in PNT was added. After 1 h at 37°C, 100 μ l/well of 2,2-azino-di-3-ethyl-benzthiazoline sulfonate substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added. Plates were incubated for 30 min at room temperature, and the optical density (OD) was measured at 405 nm. Each serum sample was tested in duplicate wells uncoated or coated with the recombinant polypeptides, and negative and positive controls were included in each plate. The cutoff OD value for seropositivity was set at ≥ 0.20 since this value was consistently above the mean adjusted OD plus 3 standard deviations for the negative control sera. Recombinant polypeptides from DENV1 to -4 were obtained as previously described (7, 23).

In this study, REC IgG-ELISA yielded 89.4% (101/113) sensitivity in well-characterized paired dengue sera (groups A to D) and 93.3% specificity with one false-positive case out of 15 sera from healthy individuals (group E). Sensitivities ranged from 71.4% to 96% depending on the DENV serotype (Table 1). Sensitivity was determined based on the results obtained from the analysis of both acute- and convalescent-phase sera from dengue cases, while specificity was determined by the analysis of unpaired dengue-negative serum samples. Potential cross-reactivity with other related diseases was investigated; low reactivity was observed in individuals positive for yellow fever virus (group F), and one serum specimen from groups positive for measles and rubella (groups G to H) cross-reacted, with ODs close to the cutoff value for the DENV recombinant peptides (Fig. 1).

A total of 52 primary and 61 secondary cases were characterized by the standard IgG-ELISA using mouse brain antigen. The REC IgG-ELISA showed sensitivities of 78.9% (41/52) and 98.4% (60/61) in primary and secondary cases, respectively (Fig. 2). Even though IgM antibody is the marker of choice in dengue diagnosis, some patients with secondary DENV infections produce low or undetectable levels of IgM, and the IgM

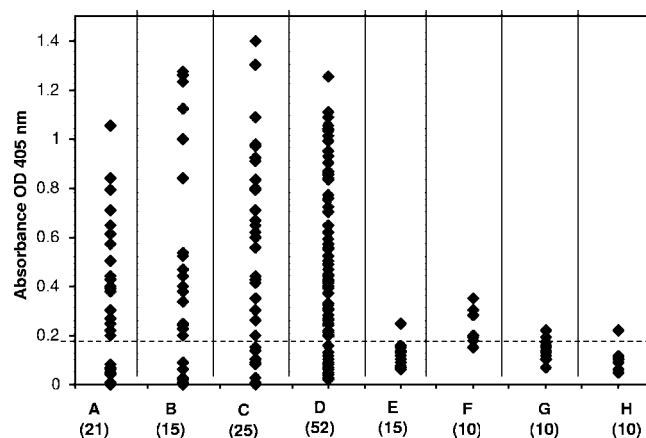


FIG. 1. IgG antibody response in dengue patients and control groups to the recombinant polypeptides as determined by REC IgG-ELISA. Absorbance (OD_{405}) values are shown for reactions with paired sera from DENV-infected patients (DENV1 to -3) and unpaired sera from healthy individuals and from yellow fever-, measles-, and rubella-positive cases. The cutoff value is represented by the dashed line.

response may be slow to appear and may be short-lived (13, 20, 22, 24). Previous studies have shown that IgG is a more sensitive marker than IgM in secondary DENV infections (14). In fact, REC IgG-ELISA was more sensitive in confirming secondary dengue cases (98.4%) than the IgM antibody capture ELISA (85.2%). The vast majority of symptomatic DENV infections in endemic regions are secondary infections, and the REC IgG-ELISA is highly reliable for diagnosing these cases. Furthermore, the use of paired acute- and convalescent-phase samples is recommended to avoid false-positive results in areas

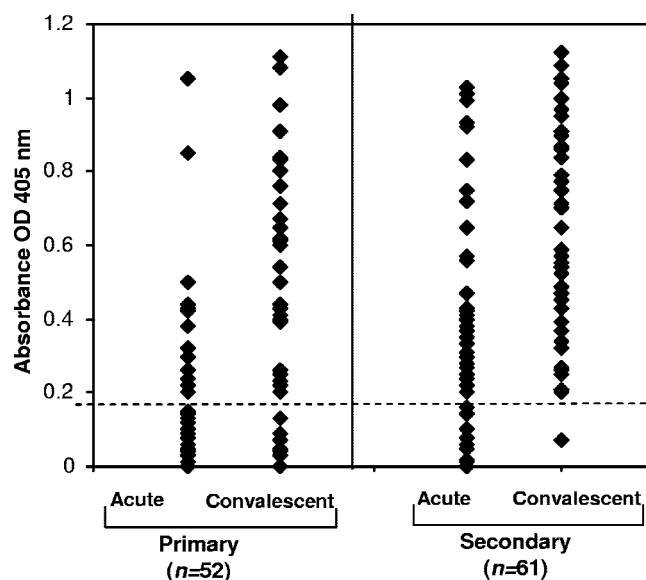


FIG. 2. IgG antibody response to DENV recombinant polypeptides as determined by REC IgG-ELISA in acute- and convalescent-phase sera from dengue patients presenting primary and secondary infections.

where dengue is endemic due to the long persistence of DENV IgG antibodies in many patients with secondary infection (21).

Our recombinant polypeptides, representing the N-terminal third of the DENV envelope, are highly expressed, easy to purify, and have already shown a high sensitivity and specificity in diagnosing DENV infections (7, 23). Another assay for dengue diagnosis using recombinant proteins consisting of the N-terminal 80% of the envelopes of the four serotypes yielded 90% sensitivity and 86% specificity (6). Recently, results obtained using a protein composed of DENV E, NS1, and NS3 epitopes expressed in *E. coli* in ELISAs were consistent with those obtained with a commercial kit (1).

Recombinant DENV antigens produced in *E. coli*-based expression systems effectively address issues related to biohazard risks, cost, and specificity associated with the use of virus antigen-based diagnostic assays. The DENV E polypeptides presented here are useful not only for diagnosis of dengue based on IgM antibodies (23) but for detection of anti-DENV IgG responses as well.

We are grateful to Lee W. Riley and to José Paulo Gagliardi Leite for assistance in standardizing the REC IgG-ELISA and to Eliane S. M. de Araújo, José Farias Filho, Jaqueline Bastos Santos, and Natália Plínio de Souza for technical support. We thank Marilda Siqueira from the Laboratory of Respiratory Viruses at Oswaldo Cruz Institute/FIOCRUZ for providing the measles and rubella samples.

This research was supported by Fogarty International Center grant TW 00905, NIH, and by PDTIS/FIOCRUZ grant RDI06/IM07, Brazil. M.P.M., R.M.R.N., and H.G.S. were fellows of Conselho Nacional de Desenvolvimento Científico e Tecnológico (grant 40.0164/98-1).

REFERENCES

- Anandarao, R., S. Swaminathan, S. Fernando, A. M. Jana, and N. Khanna. 2006. Recombinant multiepitope protein for early detection of dengue infections. *Clin. Vaccine Immunol.* **13**:59–67.
- Reference deleted.
- Blacksell, S. D., P. N. Newton, D. Bell, J. Kelley, M. P. Mammen, Jr., D. W. Vaughn, V. Wuthiekanun, A. Sungkakum, A. Nisalak, and N. P. Day. 2006. The comparative accuracy of 8 commercial rapid immunochromatographic assays for the diagnosis of acute dengue virus infection. *Clin. Infect. Dis.* **42**:1127–1134.
- Chungue, E., G. Marche, R. Plichart, J. P. Boutin, and J. Roux. 1989. Comparison of immunoglobulin G enzyme-linked immunosorbent assay (IgG-ELISA) and haemagglutination inhibition (HI) test for the detection of dengue antibodies. Prevalence of dengue IgG-ELISA antibodies in Tahiti. *Trans. R. Soc. Trop. Med. Hyg.* **83**:708–711.
- Clarke, D. H., and J. Casals. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am. J. Trop. Med. Hyg.* **7**:561–573.
- Cuzzubbo, A. J., T. P. Endy, A. Nisalak, S. Kalayanarooj, D. W. Vaughn, S. A. Ogata, D. E. Clements, and P. L. Devine. 2001. Use of recombinant envelope proteins for serological diagnosis of dengue virus infection in an immunochromatographic assay. *Clin. Diagn. Lab. Immunol.* **8**:1150–1155.
- dos Santos, F. B., M. P. Miagostovich, R. M. Nogueira, H. G. Schatzmayr, L. W. Riley, and E. Harris. 2004. Analysis of recombinant dengue virus polypeptides for dengue diagnosis and evaluation of the humoral immune response. *Am. J. Trop. Med. Hyg.* **71**:144–152.
- Groen, J., P. Koraka, J. Velzing, C. Copra, and A. D. Osterhaus. 2000. Evaluation of six immunoassays for detection of dengue virus-specific immunoglobulin M and G antibodies. *Clin. Diagn. Lab. Immunol.* **7**:867–871.
- Gubler, D. J., G. Kuno, G. E. Sather, M. Velez, and A. Oliver. 1984. Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. *Am. J. Trop. Med. Hyg.* **33**:158–165.
- Gubler, D. J., and M. Meltzer. 1999. Impact of dengue/dengue hemorrhagic fever on the developing world. *Adv. Virus Res.* **53**:35–70.
- Reference deleted.
- Innis, B. L., A. Nisalak, S. Nimmannitya, S. Kusalerdchariya, V. Chongswadi, S. Suntayakorn, P. Puttisri, and C. H. Hoke. 1989. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *Am. J. Trop. Med. Hyg.* **40**:418–427.
- Innis, B. L. 1997. Antibody response to dengue virus infection, p. 221–243. *In* D. J. Gubler and G. Kuno (ed.), *Dengue and dengue hemorrhagic fever*. CAB International, New York, NY.
- Lam, S. K. 1993. Rapid dengue diagnosis and interpretation. *Malays. J. Pathol.* **15**:9–12.
- Lam, S. K. 1995. Application of rapid laboratory diagnosis in dengue control. *Asian Pac. J. Mol. Biol. Biotechnol.* **3**:351–355.
- Lanciotti, R. S., C. H. Calisher, D. J. Gubler, G. J. Chang, and A. V. Vorndam. 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J. Clin. Microbiol.* **30**:545–551.
- Lindenbach, B. D., and C. M. Rice. 2001. *Flaviviridae*: the viruses and their replication, p. 991–1041. *In* D. M. Knipe and P. M. Howley (ed.), *Fields virology*. Lippincott Williams & Wilkins, Philadelphia, PA.
- Miagostovich, M. P., R. M. Nogueira, F. B. dos Santos, H. G. Schatzmayr, E. S. Araujo, and V. Vorndam. 1999. Evaluation of an IgG enzyme-linked immunosorbent assay for dengue diagnosis. *J. Clin. Virol.* **14**:183–189.
- Nogueira, R. M., M. P. Miagostovich, E. Lampe, R. W. Souza, S. M. Zagne, and H. G. Schatzmayr. 1993. Dengue epidemic in the stage of Rio de Janeiro, Brazil, 1990-1: co-circulation of dengue 1 and dengue 2 serotypes. *Epidemiol. Infect.* **111**:163–170.
- Ruechusatawat, K., K. Morita, M. Tanaka, S. Vongcheree, S. Rojanasuphot, P. Warachit, K. Kanai, P. Thongtradol, P. Nimnakorn, S. Kanungkid, and A. Igarashi. 1994. Daily observation of antibody levels among dengue patients detected by enzyme-linked immunosorbent assay (ELISA). *Jpn. J. Trop. Med. Hyg.* **22**:9–12.
- Shu, P. Y., and J. H. Huang. 2004. Current advances in dengue diagnosis. *Clin. Diagn. Lab. Immunol.* **11**:642–650.
- Vaughn, D. W., S. Green, S. Kalayanarooj, B. L. Innis, S. Nimmannitya, S. Suntayakorn, A. L. Rothman, F. A. Ennis, and A. Nisalak. 1997. Dengue in the early febrile phase: viremia and antibody responses. *J. Infect. Dis.* **176**:322–330.
- Videa, E., M. J. Coloma, F. B. Dos Santos, A. Balmaseda, and E. Harris. 2005. Immunoglobulin M enzyme-linked immunosorbent assay using recombinant polypeptides for diagnosis of dengue. *Clin. Diagn. Lab. Immunol.* **12**:882–884.
- Vorndam, A. V., and G. Kuno. 1997. Laboratory diagnosis of dengue virus infection, p. 313–333. *In* D. J. Gubler and G. Kuno (ed.), *Dengue and dengue hemorrhagic fever*. CAB International, New York, NY.
- World Health Organization. 1997. *Dengue haemorrhagic fever: diagnosis, treatment, prevention and control*. World Health Organization, Geneva, Switzerland.