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Detection of Specific Antibodies in Saliva during Dengue Infection

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Saliva was collected prospectively from patients presenting with suspected dengue infection 4 to 8 days after the onset of symptoms and assayed by a commercial dengue immunoglobulin M (IgM) and IgG capture enzyme-linked immunosorbent assay (ELISA) (PanBio Dengue Duo ELISA). Laboratory diagnosis was based on virus isolation and on hemagglutination inhibition (HAI) assay and an in-house IgM and IgG capture ELISA. With a positive result defined as either salivary IgM or IgG levels above the cutoff value, an overall sensitivity of 92% was obtained for both primary- and secondary-dengue patients (22 of 24), while no patients with non-flavivirus infections ($n = 11$) and no healthy laboratory donors ($n = 17$) showed elevation of salivary antidengue antibody (100% specificity). Salivary IgG levels correlated well with serum HAI titer ($r = 0.78$), and salivary IgG levels could be used to distinguish between primary- and secondary-dengue virus infections.

In terms of morbidity, mortality, and economic costs, dengue is the most important mosquito-borne disease in the world, with an estimated 100 million cases annually (13). Initial infection with one of the four serotypes of dengue virus (primary-dengue virus infection) may lead to dengue fever, which is a self-limiting, febrile disease with a low mortality rate, while reinfection with a different dengue serotype (anamnestic or secondary-dengue virus infection) may lead to more-serious forms of the disease (e.g., dengue hemorrhagic fever or dengue shock syndrome) (1, 9, 14). Recently, commercial tests have been described for the detection of anti-dengue immunoglobulin M (IgM) and IgG antibodies in serum (2, 11, 12, 21, 23). Potential problems with the use of serum include the requirement of consent and cooperation of the patient, which is often unavailable due to social or religious reasons, the need for a trained venipuncturist and the need to separate serum before testing, and the difficulty and added risk of venipuncture in children, the group most commonly affected by dengue in areas where infection is endemic.

Most body fluids contain antibodies, although at much lower levels than those in blood. Thus, these sources of antibody are unsuitable as diagnostic specimens, in spite of the obvious advantages and convenience of samples such as saliva. Salivary antibodies have been reported to be useful for the diagnosis of a number of infections, including AIDS, leptospirosis, measles, mumps, hepatitis A and B, and rubella (3–6, 15–17). In this study we examined the ability of the PanBio Dengue Duo enzyme-linked immunosorbent assay (ELISA) to detect both IgM and IgG antibodies to dengue with saliva samples.

Sera and saliva samples were collected prospectively from patients presenting at the Kamphaeng Phet Provincial Hospital in northern Thailand. Saliva was collected by using a commercially available collection device (Omni-Sal; Salivary Diagnostic Systems, Singapore). This device dilutes saliva twofold in the buffer provided. After collection, saliva was stored at −80°C until assayed blindly by the Dengue Duo ELISA. Diagnosis was based on assay of blood or sera by using in-house ELISA, hemagglutination inhibition assay (HAI), or viral isolation performed at the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand (8, 21). Of the 35 patients from Thailand enrolled in the study, 2 had primary dengue, 22 had secondary dengue, and 11 had no laboratory evidence of dengue infection despite the presence of clinical symptoms compatible with dengue fever. Saliva was also collected from 17 healthy Australian laboratory staff members.

The Dengue Duo ELISA has been shown to be useful in the diagnosis of dengue infection with sera (2, 12). It detects IgM and IgG separately by a capture assay format and was performed by the procedure recommended by the manufacturer (2), except that saliva was diluted 1:2 in the assay diluent provided before the addition of 100 μl to each well of the assay plate (final dilution, 1:4). Positive, negative, and calibrator control sera used in the kit were also run alongside the saliva samples, though these were diluted 1:100 in the diluent provided. Results were expressed as the ratio of the absorbance in test samples divided by the absorbance of the calibrator sera. A ratio of 0.6 was found to give the best distinction between dengue infection and other conditions. A positive sample was defined as having a sample/calibrator absorbance ratio of ≥0.6, and a negative sample was defined as having a sample/calibrator absorbance ratio of <0.6. Dengue virus infection was characterized by the elevation of either IgM or IgG, with a negative sample defined as having both IgM and IgG ratios of <0.6.

High sensitivity and specificity were obtained when saliva was used for the detection of anti-dengue virus antibodies, with 22 of 24 (92%) of dengue virus infections showing elevation of either IgM or IgG (Table 1). Of the patients with dengue virus infection, 8 showed elevation of both salivary IgM and IgG (all secondary infections); 3 showed elevation of salivary IgM only (two primary infections and one secondary infection); 11 showed elevation of salivary IgG only (all secondary infec-

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TABLE 1. Elevation of salivary anit-dengue antibodies in dengue and nondengue patients

Diagnostic group	No. of patients with elevated salivary antibody/total no. of patients (%) with:		
	IgM ratio of ≥0.6	IgG ratio of ≥0.6	IgM ratio of ≥0.6 or IgG ratio of ≥0.6
Primary dengue	2/2 (100%)	0/2 (0%)	2/2 (100%)
Secondary infection	9/22 (41%)	19/22 (86%)	20/22 (91%)
No evidence of active dengue	0/11 (0%)	0/11 (0%)	0/11 (0%)
Laboratory staff	0/17 (0%)	0/17 (0%)	0/17 (0%)

tions); and 2 with secondary infections were negative for both IgM and IgG. The date of the onset of symptoms was also available for 24 patients. Salivary antibodies were elevated in 2 of 2 patients by day 4, in 4 of 6 patients at day 5, and in all 16 patients tested between days 6 and 8. None of the 11 patients with clinically suspected dengue and no laboratory evidence of infection produced a positive result in the saliva assay (100% specificity). Furthermore, none of the saliva samples from 17 healthy laboratory donors showed elevation of anti-dengue IgM or IgG by this assay (Table 1). Specificity needs to be evaluated among patients with non-dengue flavivirus infections such as Japanese encephalitis, yellow fever, West Nile fever, and tick-borne encephalitis. Some false-positive results should be expected due to antibody cross-reactivity. However, these diseases can often be distinguished on the basis of clinical and epidemiological information.

Salivary IgM levels in primary-dengue virus infections were higher than those found in secondary-dengue virus infection, while salivary IgG levels were generally higher in secondary infections (Fig. 1). Similar results have been reported with sera from dengue patients (2, 8, 11, 18, 20). Consequently, the comparison of salivary IgM and salivary IgG levels could be used to distinguish between primary- and secondary-dengue infection, as observed in other studies with sera (2, 8, 11, 18). In this study, patients with primary-dengue infections had elevated levels of IgM without detectable IgG, while the majority of patients with secondary dengue (86%) showed elevated levels of IgG with or without detectable IgM. This finding indicates that the cutoff selected for IgG detects high levels of IgG characteristic of secondary- but not of primary- or past dengue virus infections (7, 10, 22). This result is also reflected in the excellent correlation between salivary IgG levels and HAI titer (Fig. 2).

Salivary IgG and IgM levels determined by the Dengue Duo ELISA were compared to serum levels of IgM and IgG found by the AFRIMS in-house ELISA (8) (Fig. 3). Serum and salivary IgG levels showed excellent correlation (Pearson's $r = 0.93$; $P < 0.0001$), though the correlation between IgM levels in serum and saliva was not significant (Pearson's $r = 0.25$, $P = 0.2014$). The serum and salivary ELISAs gave the same result (positive or negative) for IgM in 20 of 30 serum samples, while the same result was obtained for IgG in 25 of 30 serum samples. The majority (8 of 10) of discrepant sera in the IgM assays were positive in the serum test and negative in the saliva test, while most (4 of 5) of the discrepant serum samples in the IgG assays were positive by the saliva test and negative by the serum test.

Saliva has been reported to be an alternative to sera as a source of antibodies for the diagnosis of a number of diseases, including AIDS, leptospirosis, measles, and mumps, and for hepatitis A and B and rubella infections (3–6, 15–17). This

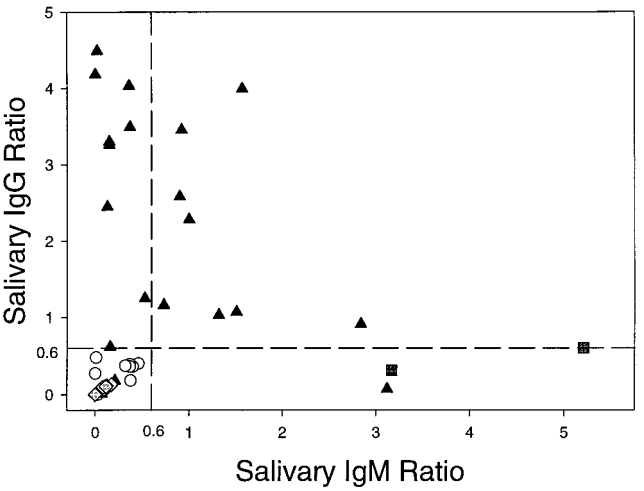

FIG. 1. Comparison of anti-dengue IgG and IgM antibody levels in saliva. Based on viral isolation, HAI, and an in-house ELISA, 2 patients were diagnosed with primary dengue (squares), 22 were diagnosed with secondary dengue (triangles), and 11 had no evidence of dengue infection (circles). Seventeen laboratory donors are also represented (diamonds). Cutoff ELISA ratios of 0.6 for IgM and IgG are shown by broken lines.

study suggests that saliva is a useful alternative to sera for the diagnosis of dengue. The use of the antibody capture assay format has been shown to be superior to indirect ELISA for the detection of salivary antibodies, and this superiority is likely to be due to the removal of competing antibody in the capture step (15). Antibody concentrations in saliva are 19.9 mg/100 ml (IgA), 1.4 mg/100 ml (IgG), and 0.2 mg/100 ml (IgM), and these levels are approximately 1/10, 1/800, and 1/400 of those in serum (15). An IgA capture ELISA has been reported to have utility in dengue diagnosis, though IgA appears more slowly than IgM and is shorter-lived (19). In this study, investigation of IgA levels in saliva by an in-house antibody capture ELISA gave results similar to those of the IgM

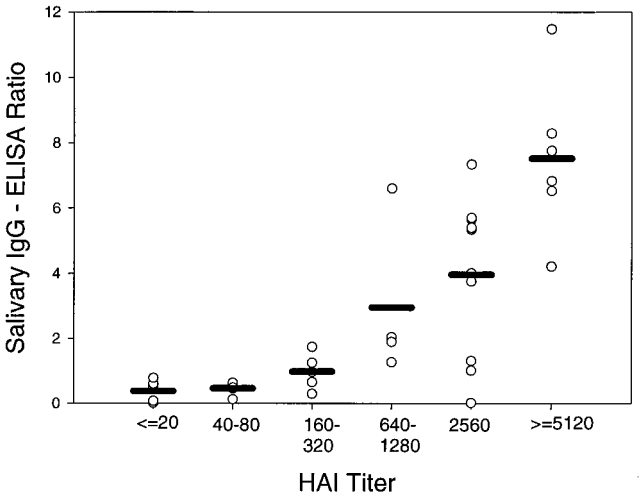

FIG. 2. Correlation between salivary IgG levels determined by the PanBio Dengue Duo ELISA and serum antibody levels determined by HAI. Individual assay values are shown by open circles, while the mean ELISA IgG ratio for different groupings is shown by a horizontal bar. There was a significant association between mean ELISA IgG ratio and HAI titer (analysis of variance, $F = 14.2$; $P < 0.0001$) as well as a significant correlation between individual IgG levels and HAI titer (Pearson's $r = 0.78$, $P < 0.0001$).

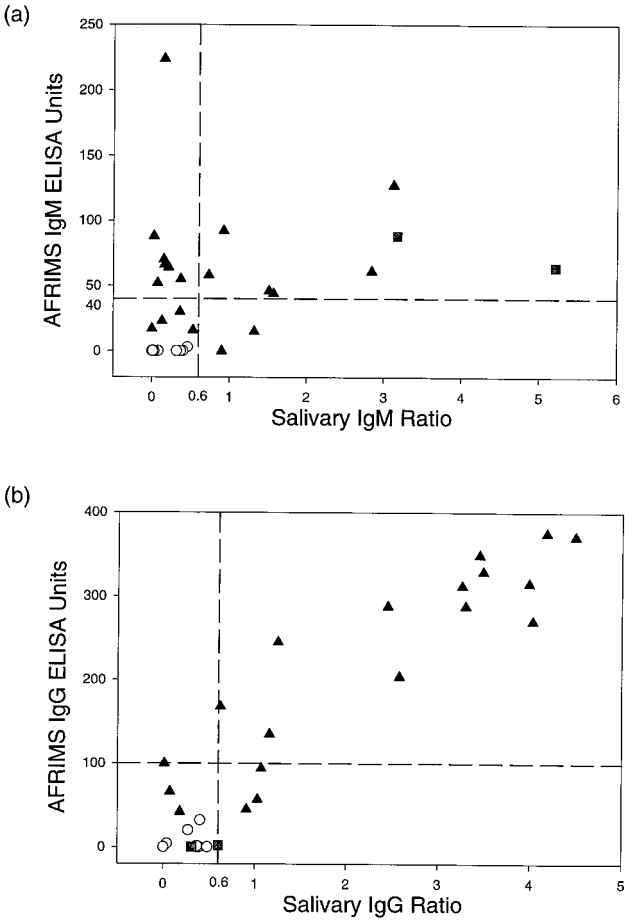

FIG. 3. Correlation between salivary antibody levels determined by the PanBio Dengue Duo ELISA and serum antibody levels determined by the AFRIMS Capture ELISA for Ig. Two patients diagnosed with primary dengue (squares), 21 patients diagnosed with secondary dengue (triangles), and 7 patients with no evidence of dengue infection (circles) are represented. Cutoff values of 0.6 (PanBio IgM and IgG), 40 (AFRIMS IgM), and 100 (AFRIMS IgG) are shown by broken lines.

capture ELISA, with fewer than half the patients with secondary-dengue infection having elevated IgA (data not shown).

Saliva-based assays should be particularly useful for epidemiological studies (to document recent dengue virus infections in a healthy population or among those experiencing mild disease) and for the diagnosis of acute disease when blood collection is difficult due to cultural factors or difficult venous access, especially in very young children.

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REFERENCES

1. **Burke, D. S., A. Nisalak, D. E. Johnson, and R. M. Scott.** 1988. A prospective study of dengue infections in Bangkok. Am. J. Trop. Med. Hyg. **38:**172–180.
2. **Chew, T. S., A. Cuzzubbo, and P. L. Devine.** 1998. Evaluation of commercial capture enzyme-linked immunosorbent assay for the detection of immunoglobulin M (IgM) and IgG antibodies produced during dengue infection. Clin. Diagn. Lab. Immunol. **5:**7–10.
3. **Da Silva, M. V., E. D. Camargo, A. J. Vaz, and L. Batista.** 1992. Immunodiagnosis of human leptospirosis using saliva. Trans. R. Soc. Trop. Med. Hyg. **86:**560–561.
4. **De Azevedo Neto, R. S., A. Richards, D. J. Nokes, A. S. B. Silveira, B. J. Cohen, S. D. Passos, V. A. U. F. de Souza, D. W. G. Brown, C. S. Pannuti, and E. Massad.** 1995. Salivary antibody detection in epidemiological surveys: a pilot study after a mass vaccination campaign against rubella in Sao Paulo, Brasil. Trans. R. Soc. Trop. Med. Hyg. **89:**115–118.
5. **Frerichs, R. R.** 1994. Saliva assays for HIV antibody diagnosis. Labmed. Int. **5–6:**16–19.
6. **Friedman, M. G., M. Phillip, and R. Dagan.** 1989. Virus-specific IgA in serum, saliva and tears of children with measles. Clin. Exp. Immunol. **75:** 58–63.
7. **Gubler, D. J.** 1996. Serological diagnosis of dengue/dengue haemorrhagic fever. Dengue Bull. **20:**20–23.
8. **Innis, B. L., A. Nisalak, S. Nimmannitya, S. Kusalerdchariya, V. Chongswasdi, 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.
9. **Innis, B. L.** 1995. Dengue and dengue hemorrhagic fever, p. 103–146. *In* J. S. Porterfield (ed.), Kass handbook of infectious diseases. Exotic viral infections. Chapman & Hall Medical, London, United Kingdom.
10. **Innis, B. L.** 1997. Antibody responses to dengue virus, p. 221–243. *In* D. J. Gubler and G. Kuno (ed.), Dengue and dengue haemorrhagic fever. CAB International, New York, N.Y.
11. **Lam, S. K., M. Y. Fong, E. Chungue, S. Doraisingham, A. Igarashi, M. A. Khin, Z. T. Kyaw, A. Nisalak, C. Roche, D. W. Vaughn, and V. Vorndam.** 1996. Multicentre evaluation of dengue IgM dot enzyme immunoassay. Clin. Diagn. Virol. **7:**93–98.
12. **Lam, S. K., and P. L. Devine.** 1998. Evaluation of capture ELISA and rapid immunochromatographic test for the determination of IgM and IgG antibodies produced during dengue infection. Clin. Diagn. Virol. **10:**75–81.
13. **Monath, T. P.** 1994. Dengue: the risk to developed and developing countries. Proc. Natl. Acad. Sci. USA **91:**2395–2400.
14. **Nimmannitya, S.** 1993. Management of dengue and dengue haemorrhagic fever, p. 55–61. *In* P. Thongcharoen (ed.), Monograph on dengue/dengue haemorrhagic fever. World Health Organization Regional Office for South-East Asia, New Delhi, India.
15. **Parry, J. V., K. R. Perry, and P. P. Mortimer.** 1987. Sensitive assays for viral antibodies in saliva: alternative to tests on serum. Lancet **11:**72–75.
16. **Parry, J. V., K. R. Perry, S. Panday, and P. Mortimer.** 1989. Diagnosis of hepatitis A and B by testing saliva. J. Med. Virol. **28:**255–260.
17. **Perry, K. R., D. W. G. Brown, J. V. Parry, S. Panday, C. Pipkin, and A. Richards.** 1993. Detection of measles, mumps and rubella antibodies in saliva using antibody capture radioimmunoassay. J. Med. Virol. **40:**235–240.
18. **Ruechusatsawat, 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.
19. **Talarmin, A., B. Labeau, J. Lelarge, and J. L. Sarthou.** 1998. Immunoglobulin A-specific capture enzyme-linked immunosorbent assay for diagnosis of dengue fever. J. Clin. Microbiol. **36:**1189–1192.
20. **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.
21. **Vaughn, D. W., A. Nisalak, S. Kalayanarooj, T. Solomon, N. M. Dung, A. Cuzzubbo, and P. L. Devine.** 1998. Evaluation of rapid immunochromatographic test for diagnosis of dengue virus infection. J. Clin. Microbiol. **36:** 234–238.
22. **Vorndam, V., and G. Kuno.** 1997. Laboratory diagnosis of dengue virus infections, p. 313–333. *In* D. J. Gubler and G. Kuno (ed.), Dengue and dengue haemorrhagic fever. CAB International, New York, N.Y.
23. **Wu, S. J. L., B. Hanson, H. Paxton, A. Nisalak, D. W. Vaughn, C. Rossi, E. A. Henchal, K. R. Porter, D. M. Watts, and C. G. Hayes.** 1997. Evaluation of dipstick enzyme-linked immunosorbent assay for detection of antibodies to dengue virus. Clin. Diagn. Lab. Immunol. **4:**452–457.