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Evaluation of a Dipstick Enzyme-Linked Immunosorbent Assay for Detection of Antibodies to Dengue Virus

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Accurate serological confirmation of dengue (DEN) infection is difficult, because simple reliable assays for the detection of DEN antibodies are not available. To address this problem, a dipstick enzyme-linked immunosorbent assay (ELISA) was evaluated. The dipstick contained dots of serially diluted DEN 2 antigen. To detect immunoglobulin G (IgG), the dipstick was processed through four reaction cuvettes containing test serum, enhancer, enzyme-conjugated anti-human IgG and IgM antibody, and substrate. Total assay time was 45 min. To detect IgM, the serum was passed through a protein G device to remove IgG. The dipstick was then processed as before, except that the incubation times were longer and enzyme-conjugated anti-human IgM was used. The total assay time was 3 h. The dipstick ELISA results were compared with results from microplate ELISA. The IgG dipstick ELISA showed a sensitivity of 95.2% and a specificity of 100% compared to an IgG microplate ELISA with serum samples from 125 individuals living in an area in which DEN is endemic. In tests with 75 serum samples from patients with clinically suspected acute DEN infections, the IgM dipstick ELISA showed a sensitivity of 97.9% and specificity of 100% compared to those of an IgM antibody capture microplate ELISA. These results showed that the dipstick ELISA was a sensitive and specific test for the detection of either DEN IgM or IgG in human serum. The dipstick ELISA was also shown to be useful for detecting seroconversions to DEN IgM or IgG in paired serum samples from 20 patients with virus isolation-confirmed acute DEN infections.

The four serotypes of dengue virus (DEN 1, 2, 3, and 4) are widely distributed throughout the tropical and subtropical regions of the world (11). These viruses, primarily transmitted by the mosquito *Aedes aegypti*, are estimated to infect over 100 million people each year, causing thousands of cases of DEN fever and the more severe DEN hemorrhagic fever (DHF) (8, 9). The latter disease has been strongly associated with sequential infections by two different serotypes of DEN virus (9).

The most common serological techniques for the diagnosis of DEN are the hemagglutination inhibition (HI) test (7), which detects total antibody, and the microplate enzyme-linked immunosorbent assay (ELISA) (6, 12, 16), which is commonly formatted to detect immunoglobulin G (IgG) or IgM antibody separately. The HI test and the IgG microplate ELISA usually require paired acute- and convalescent-phase serum samples collected a week or more apart for definitive diagnosis based on a fourfold rise or fall in DEN antibody titers (24). The IgM antibody capture ELISA frequently allows a presumptive diagnosis to be made during the acute phase of DEN illness with a single serum sample because of the shorter duration of IgM antibody following infection. These methods, while providing high sensitivity and specificity, are laborious, rely on trained personnel, and require the use of several reagents that are not commercially available.

The objective of this study was to evaluate a simple diagnostic assay for identifying DEN antibody in human serum or plasma specimens. The format used was a semiquantitative dipstick ELISA previously developed for detecting antibodies to rickettsiae (14, 15, 22). The dipstick ELISA results were compared with those of a standard IgG microplate ELISA and IgM antibody capture microplate ELISA for the detection of DEN IgG and IgM antibody, respectively.

MATERIALS AND METHODS

DEN antigens. For initial dipstick development, several formalin-inactivated crude and purified antigens of DEN 1 (West Pacific '74 strain) and DEN 2 (16681 and New Guinea C strains) viruses were compared. Crude antigens included cell lysates and tissue culture supernatants from DEN virus-infected Vero cells (1, 19) and DEN-infected suckling mouse brain virus preparations (American Type Culture Collection, Rockville, Md.). Purified antigens included glycerol gradient-purified virus (18) and sucrose gradient-purified virus (Microbix Biosystems, Inc., Ontario, Canada), both obtained from DEN-infected tissue culture supernatants. The final antigen selected for manufacturing dipsticks at Integrated Diagnostics (Baltimore, Md.) was the formalin-inactivated, sucrose gradient-purified DEN 2 virus (Microbix). For the IgG microplate ELISA, lysates prepared by sonicating DEN virus-infected Vero cells were used as coating antigen (1). These lysates consisted of either DEN 2-infected cells only or a cocktail containing all four DEN serotypes prepared by mixing cell lysates from different serotype-infected cultures. Uninfected Vero cell lysate was used as a control antigen. For the IgM antibody capture ELISA, supernatant from Vero cells infected with either DEN 2 only or a supernatant cocktail containing a mixture of all four DEN virus serotypes was used as the antigen (19). Uninfected Vero cell supernatant was used as a control antigen.

Human sera. A panel of 125 serum samples previously collected during a cross-sectional DEN antibody survey in an area of Peru where DEN 1 is known to be endemic was tested to compare the sensitivity and specificity of the microplate and dipstick ELISAs in detecting DEN IgG antibody (10). These samples had been tested for DEN IgG antibody in an earlier study by the microplate ELISA and were selected for the dipstick ELISA comparison to include a wide range of reactivities from negative to strongly positive (Table 1).

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TABLE 1. Range of DEN antibody OD values in the microplate ELISA for human sera used to evaluate the dipstick assay

Microplate ELISA OD range[a]	No. of serum samples	
	IgG	IgM
0.000–0.099	20	27
0.100–0.499	18	27
0.500–0.999	37	14
≥1.000	50	7
Total	125	75

[a] Adjusted ELISA OD values of ≥0.100 were considered positive at a serum dilution of 1:100.

Another panel of 75 serum samples that had been collected during acute fever studies in Peru and Somalia was tested to compare the IgM antibody capture microplate ELISA and the dipstick ELISA for detecting DEN IgM antibody (9a, 19). These sera had previously been tested by the IgM antibody capture microplate ELISA and were selected, as were the IgG samples, to represent a wide range of reactivities (Table 1). Sera that were known to contain IgM antibody to yellow fever (YF) virus and to several non-DEN-related viruses as well as to malaria were included to evaluate the specificity of the dipstick ELISA.

Paired serum samples from 20 patients were tested for both IgM and IgG antibody to compare the dipstick and microplate ELISAs for the diagnosis of acute DEN infections. DEN virus previously had been recovered from all of the acute-phase serum samples, including seven DEN 1, four DEN 2, seven DEN 3, and two DEN 4 isolates. For the DEN 1 specimens, the acute-phase samples were collected during patient visits to an outpatient clinic, and the convalescent-phase samples were collected from 18 days to as long as 96 days later (9a). The acute-phase samples for the DEN 2 cases were collected on the day of hospitalization, and follow-up samples were collected from 13 to 50 days later following discharge (19). The DEN 3 and DEN 4 specimens also were from hospitalized patients, but serum samples were generally collected on the day of admission and day of discharge, resulting in intervals of only 1 to 5 days between most paired samples (21a).

DEN IgG antibody dipstick ELISA. The dipstick ELISA kits were produced by Integrated Diagnostics, Baltimore, Md., and were used according to the manufacturer's instructions. For each sample assay, the kit provided a dipstick prespotted with DEN antigen, four liquid reagents (serum diluent, enhancer, alkaline phosphatase-conjugated goat-anti-human IgG [H + L chains] and IgM [μ chain] antibodies, and alkaline phosphatase substrate), and four reaction cuvettes. The antigen sticks contained six wells (Fig. 1). The first well, closest to the handle, was the positive reagent control consisting of human IgG and IgM. The second well was the negative control consisting of phosphate-buffered saline (PBS). These served as internal reagent controls. Wells 3 to 6 contained four decreasing concentrations of DEN 2 sucrose-gradient-purified antigen to mimic antibody dilution. The dipstick test was performed in 2-ml reaction cuvettes at 50°C in a water bath. Each dipstick was run through a series of four reaction cuvettes, including a 1:200 dilution of test serum (10 μl in 2 ml of diluent) (5 min), enhancer (5 min), enzyme conjugate (15 min), and enzyme substrate (5 min), with washings with distilled water in between each of these steps as described previously (14). The entire procedure was completed in approximately 45 min. After a final wash, each dipstick was blotted and allowed to dry prior to being read. A positive reaction produced a blue dot in the center of the reaction well (Fig. 1). The blue dot had an easily seen, distinct border, with the outer perimeter of the well colored white to pale grey. Antibody-positive dipstick ELISA results were defined as one or more positive DEN antigen dots. Known DEN antibody-positive and -negative control sera were included with each assay run.

DEN IgM antibody dipstick ELISA. Test kits were also provided by Integrated Diagnostics for detection of IgM antibody to DEN; these were similar to the IgG test kits described above but had alkaline phosphatase-conjugated goat anti-human IgM (μ chain) instead of anti-human IgG plus IgM conjugate. Each serum sample tested for DEN IgM antibody was first absorbed with recombinant protein G bound to Sepharose (MINI Rapi.Sep.M; Integrated Diagnostics) to remove IgG antibody as recommended by the manufacturer. Each dipstick was run through a series of four reaction cuvettes as described for the IgG assay with the exception of three modifications. First, the incubation times for the serum samples and conjugate were extended to 90 and 45 min, respectively. Second, alkaline phosphatase-conjugated goat anti-human IgM (μ chain-specific) antibody was used. Third, since protein G treatment resulted in a 1:8 dilution of the serum, 80 μl of diluted serum was added to the first reaction cuvette instead of 10 μl of undiluted serum. The entire procedure for the DEN IgM dipstick ELISA took approximately 3 h to complete.

DEN IgG microplate ELISA. Sera were tested for DEN IgG antibody by a microplate ELISA as described previously (1). Microtiter plate wells (Immunlon II; Dynatech Laboratories, Chantilly, Va.) were coated with either DEN-infected or uninfected Vero cell lysates diluted in phosphate-buffered saline (PBS [pH 7.4]) overnight at 4°C. Optimum coating dilutions of the DEN 2 and DEN cocktail antigens were determined by block titration with known positive sera. After rinsing with wash buffer (0.1% Tween 20 and 0.001% thimerosal in PBS), blocking buffer (5% dry milk in PBS) was added to each well, and the mixture was allowed to incubate for 30 min at 37°C. The wells were washed again, and serum samples diluted 1:100 in serum dilution buffer (5% dry milk, 0.1% Tween 20 in PBS) were added in duplicate to the DEN antigen-coated and uninfected antigen-coated wells. After an hour of incubation at 37°C, the plates were washed, and horseradish peroxidase-conjugated goat anti-human IgG (γ chain specific) (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was added to each well to detect bound antibody. After another hour of incubation at 37°C, the wells were washed, a chromogenic substrate {ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] Kirkegaard and Perry} was added, and the mixture was allowed to incubate for 30 min at 37°C. The plates were then read at 410 nm with an ELISA microplate reader (Dynatech MR 5000). Adjusted optical density (OD) values were calculated by subtracting the OD of the uninfected antigen-coated well from the corresponding DEN antigen-coated well. Based on extremely low OD values obtained for the negative control sera, samples with an adjusted OD greater than or equal to 0.10 were considered positive for DEN IgG antibody.

DEN IgM microplate ELISA. Serum samples were tested for DEN IgM antibody by an IgM antibody capture microplate ELISA (19). Briefly, microtiter plate wells (Linbro Titertek, ICN Biomedicals, Aurora, Ohio) were coated with goat anti-human IgM, μ chain-specific, antibody (Biosource, Camarillo, Calif.) overnight at 4°C. The wells were washed, incubated with blocking buffer (0.5% bovine serum albumin and 3% dry milk in PBS) at 37°C for 1 h, and washed again, and a 1:100 dilution of each serum sample was added to each of four wells on the plate and incubated for 1 h at 37°C. After another wash, DEN 2 or DEN cocktail Vero cell supernatant-derived antigen diluted in PBS containing 5% dry milk, 0.1% Tween 20, and 1% normal human serum was added to each of two wells for each serum sample, and uninfected Vero cell culture supernatant was added to each of the other two wells. Optimum coating dilutions of the infected cell supernatant antigen were determined by titration with known positive sera. After incubation for 1 h at 37°C, the plates were washed as described before and diluted hyperimmune mouse ascitic fluid with antibodies to DEN was added. Following another hour of incubation at 37°C, plates were washed, and horseradish peroxidase-conjugated goat anti-mouse IgG plus IgM plus IgA (Kirkegaard and Perry), diluted in serum dilution buffer, was added to each well. The plates were incubated for 1 h at 37°C and washed, and ABTS substrate was added to each well. Following a final incubation at 37°C for 30 min, the microplates were read at 410 nm with an ELISA microplate reader. Results were calculated as noted for the IgG microplate ELISA.

Comparison of assays. Reactivities of the selected panels of sera in the IgG microplate ELISA and the IgM antibody capture microplate ELISA with the DEN 2 antigen preparation were compared to reactivities in the dipstick IgG ELISA and IgM ELISA, respectively, to determine sensitivity and specificity. The association between the number of reactive dots in the dipstick ELISA and microplate ELISA OD values was determined with the Spearman rank correlation coefficient. For comparisons of the dipstick ELISA and microplate ELISA for the detection of DEN IgG or IgM antibody in paired sera, the DEN cocktail antigens were used to coat the microplates, since the patients were known to have been infected with different serotypes of DEN virus.

RESULTS

Comparison of DEN antigen preparations for use in dipstick ELISA. Comparison of different DEN antigen preparations for use in developing the dipstick ELISA to detect IgG antibody indicated that the infected tissue culture supernatants with or without subsequent gradient purification were strongly

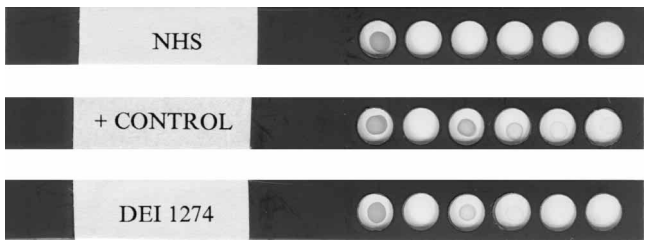

FIG. 1. Representative dipstick ELISA containing positive reagent control (dot 1 [closest to handle]), negative control (dot 2), and serial dilutions of DEN antigen (dots 3 to 6). The negative control (normal human serum [NHS]) is shown at the top (score of 0). Positive control (DEN immune serum) is shown in the middle (score of 4+). A positive test serum (DEI 1274) from Peru is shown at the bottom (score of 2+).

TABLE 2. Comparison of different DEN virus antigen preparations for use in the IgG dipstick ELISA

Antigen preparation	Antigen reactivity[a]	
	Immune serum[b]	Control serum[c]
Glycerol gradient-purified DEN 2	256	<2
Glycerol gradient-purified DEN 1	256	<2
Sucrose gradient-purified DEN 2	512	<2
Vero cell supernatant DEN 2	128	<2
Vero cell supernatant control	<2	<2
Concentrated cell supernatant DEN 1	256	<2
Vero cell lysate DEN 2	1,024	64
Vero cell lysate control	8	16
Suckling mouse brain DEN 2	256	64
Suckling mouse brain control	32	64

[a] Reciprocal of the greatest antigen dilution giving a positive reaction. Antigen was spotted on dipsticks in serial twofold dilutions and tested against a constant (1:200) serum dilution.
[b] Pooled convalescent-phase sera from secondary DEN infections diagnosed by the HI test (20).
[c] Control serum was known DEN IgG antibody negative (Integrated Diagnostics, Baltimore, Md.).

reactive with a positive control serum and showed minimal reactivity to a negative control serum (Table 2). Vero cell lysate and suckling mouse brain preparations of DEN antigen exhibited a high reactivity with the negative control serum. Because the sucrose gradient-purified DEN antigen produced the best signal/noise ratio when the positive and negative serum samples were tested, it was used in the final production of the IgG and IgM dipstick ELISA kits produced by Integrated Diagnostics.

Reproducibility of the dipstick ELISA. To determine the reproducibility of the assay, 8 to 10 separate 1:200 dilutions were prepared with each of two known IgG-positive serum samples and tested in the dipstick ELISA on the same day. This procedure was repeated on four separate occasions. The coefficients of variation (CVs) of the test results [(standard deviation/mean) × 100%] for each sample on each day were calculated. The mean CV was 18.5% (range, 10.3 to 21.7%).

Comparison of the dipstick ELISA and the microplate ELISA. Comparison of the dipstick and microplate ELISA tests for detection of IgG and IgM antibody to DEN showed high sensitivities and specificities and overall agreements of 96% or better (Table 3). For DEN IgG detection, five samples that were positive in the IgG microplate ELISA were negative in the IgG dipstick ELISA. The antibody OD values for these samples ranged from 0.100 to 0.233 (mean of OD, 0.16 ± 0.05). The number of positive antigen dots in the IgG dipstick ELISA correlated significantly with the IgG microplate ELISA OD values ($r = 0.857$, $P < 0.0005$) (Fig. 2). For DEN IgM detection, the single false-antibody-negative sample by the IgM dipstick ELISA had a low positive OD value of 0.20 in the IgM antibody capture microplate ELISA. DEN antigen titers determined by the IgM dipstick ELISA correlated significantly with OD values from the DEN IgM antibody capture microplate ELISA ($r = 0.764$, $P < 0.0005$) (Fig. 3).

None of the samples with IgM antibody to the non-DEN-related viruses or to the malaria parasite, *Plasmodium falciparum*, reacted in the dipstick ELISA or the IgM antibody capture microplate ELISA; however, 9 of the 10 known YF IgM

TABLE 3. Comparison of dipstick ELISA (test method) with the microplate ELISA (reference method)

Assay	*n*[a]	% Sensitivity[b]	% Specificity[c]	% Agreement[d]
IgG	125	95.2 (100/105)	100.0 (20/20)	96.0 (120/125)
IgM	75	97.9 (47/48)	100.0 (27/27)	97.7 (74/75)

[a] *n*, total number of sera tested.
[b] Capacity to define true positives [(number of dipstick-positive sera that were also microplate positive/total number of microplate-positive sera) × 100%].
[c] Capacity to define true negatives [(number of dipstick-negative sera that were also microplate negative/total number of microplate-negative sera) × 100%].
[d] (Number of sera positive by both methods + number of sera negative by both methods/total number of sera tested) × 100%.

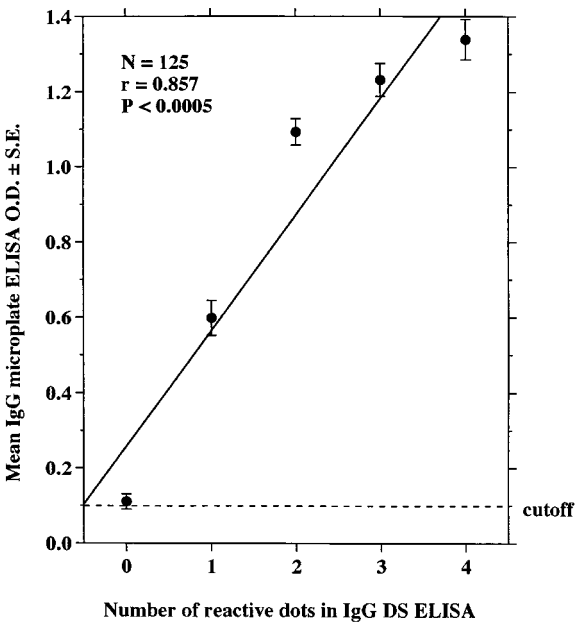

FIG. 2. Correlation of DEN IgG microplate ELISA OD values with the number of reactive dots in the DEN IgG dipstick (DS) ELISA.

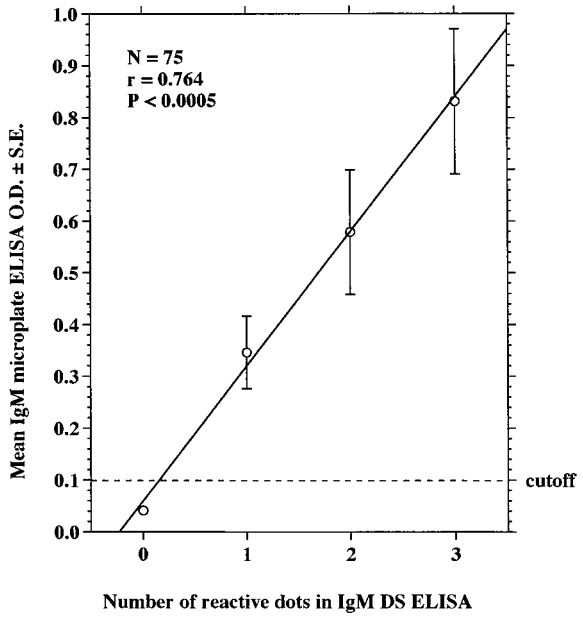

FIG. 3. Correlation of DEN IgM antibody capture microplate ELISA OD values with the number of reactive dots in the DEN IgM dipstick (DS) ELISA.

TABLE 4. Reactivity of IgM control sera from patients with other diseases by DEN IgM antibody capture microplate ELISA and DEN IgM dipstick ELISA[a]

Serum specificity[b]	Total no. of serum samples	No. of serum samples positive by ELISA:	
		Microplate	Dipstick
Malaria	11	0	0
Varicella-zoster virus	1	0	0
Rubella	1	0	0
Epstein-Barr virus	1	0	0
Cytomegalovirus	1	0	0
Hepatitis B core	1	0	0
YF	10	9	9[c]

[a] All sera were screened at a 1:100 dilution.
[b] IgM antibody-positive non-DEN viral disease sera were provided by Integrated Diagnostics, Inc. IgM antibody-positive malaria and YF sera were obtained from Peru (21b).
[c] Five serum samples were cross-reactive for one dot, four serum samples were cross-reactive for two dots, and the second dots were all weakly positive.

antibody-positive serum samples reacted with DEN antigen in both of these assays (Table 4).

In both the dipstick ELISA and the microplate ELISA, 10 of the 20 acute-phase samples from virus isolation-confirmed DEN patients were negative for DEN IgG antibody (Table 5). The second samples from nine of these patients were positive for DEN IgG antibody, and one remained negative in both assays. Three other acute-phase samples were negative in the dipstick ELISA, but showed a low level of reactivity (OD values of 0.103, 0.150, and 0.247) in the microplate IgG ELISA. The second samples from these three patients converted to positive in the dipstick IgG ELISA and showed at least a twofold increase in OD values in the microplate IgG ELISA. The remaining seven acute-phase samples were positive for DEN IgG in both assays.

Acute-phase samples from 12 patients were negative for DEN IgM antibody in both the IgM antibody capture microplate ELISA and the dipstick ELISA. The second samples from all 12 of these patients were positive in the IgM antibody capture microplate ELISA, but only 9 patients seroconverted to positive in the IgM dipstick ELISA (Table 5). The three patients that did not seroconvert in the latter assay all had DEN 1 infections, and the OD values of their second samples in the IgM antibody capture microplate ELISA were only weakly reactive (0.177, 0.187, and 0.204). An additional two patients had negative acute-phase samples in the IgM dipstick ELISA, but had weakly positive OD values (0.163 and 0.175) in the IgM antibody capture microplate ELISA. Second samples from both of these DEN 3 patients were positive in the IgM dipstick ELISA and showed between a 7- to 10-fold increase in OD values in the IgM antibody capture microplate ELISA. The first and second samples from the remaining six patients were positive for DEN IgM antibody in both the IgM dipstick ELISA and the IgM antibody capture microplate ELISA.

DISCUSSION

The DEN dipstick ELISA developed by Integrated Diagnostics was shown to be sensitive and specific for the detection of DEN IgG and IgM antibody when compared to the standard microplate ELISA. There were no false positives for either DEN IgG or IgM antibody, and the few false negatives all had low OD values in the microplate ELISA. In addition, the results with the 20 serum pairs from virus isolation-confirmed DEN infections showed that the dipstick ELISA could be used for the diagnosis of acute DEN infection. The dipstick formatted with DEN 2 antigen was able to detect both IgM and IgG antibodies from patients infected with each of the four different serotypes of DEN virus. All of the DEN IgG antibody seroconversions and most of the DEN IgM antibody seroconversions detected in the microplate ELISA were also detected by the dipstick ELISA. The three seroconversions diagnosed in the IgM antibody capture microplate ELISA that were missed in the IgM dipstick ELISA were all examples in which the second serum had very weak reactivity in the microplate ELISA. These were from DEN 1 patients whose paired samples were collected 66 to 96 days apart. Since previous studies have shown that DEN IgM antibody usually only persists for 2 to 3 months following the acute disease, the long period between the collection of the first and the second samples from these patients would explain the low IgM reactivity (12). The dipstick ELISA also did not detect DEN antibody in some of the acute-phase samples that were weakly reactive for IgM or IgG in the microplate ELISA; although all of these cases of infection were subsequently diagnosed as DEN antibody seroconversions when the second samples were tested in the dipstick ELISA.

The Integrated Diagnostics dipstick ELISA that was compared to the microplate IgG ELISA in this study used the standard commercial format designed to detect both IgG and IgM antibody with alkaline phosphatase-conjugated goat anti-human IgG (H + L) and IgM (μ). This format would have allowed the same dipstick ELISA kit to be used for the detection of total DEN antibody with untreated serum and the specific detection of IgM antibody after IgG removal with protein G. Method validation studies showed, however, that with the incubation period of 5 min for the test serum and 15 min for the alkaline phosphatase conjugate, predominately IgG antibody to DEN was detected in unseparated serum (16a). These standard incubation times were based on previously validated studies with the dipstick ELISA to detect antibody to *Rickettsia typhi* (14) and *Rickettsia rickettsii* (15) and with other antibody assays in commercial distribution. For example, IgM antibody to leptospiral antigens can be easily detected with the standard incubation times and without IgG removal. Removal of IgG only increased the IgM titers marginally, even with a stronger IgM conjugate (16a). This observation suggested that DEN IgM antibody is of lower avidity and is more sensitive to IgG interference when binding to the viral epitopes.

The sensitivity of the dipstick ELISA for detecting DEN IgM antibody was increased by lengthening the incubation times for the test serum and conjugate steps to 90 and 45 min, respectively. Additionally, the conjugate was modified to have only goat anti-human IgM (μ chain specific) antibody (at a higher concentration than in the IgG dipstick kit) to further increase the IgM sensitivity of the assay. The specificity and sensitivity of the assay were also increased by removal of IgG from the sera by a recombinant protein G treatment. The removal of the DEN IgG antibody from the sera reduced the competition for viral binding sites with the DEN IgM and therefore increased the sensitivity of the assay (13, 17, 23). The protein G treatment is quantitative in its binding, removing a known quantity of IgG from sera. The binding capacity is 16 to 22 mg/ml, satisfying the IgG range of most clinical samples. This is of particular concern because sera from DEN secondary infections have very high titers of IgG which are rapidly produced because of immunological memory induced by DEN complex and flavivirus cross-reactive epitopes. Additionally, the IgM antibody response in secondary DEN infections appears to be truncated compared to that in primary DEN in-

TABLE 5. Comparison of microplate and dipstick ELISAs for detection of IgM and IgG antibodies in paired serum samples from virus isolation-confirmed DEN infection

Patient no.	Disease onset date (mo/day/yr)	Date sample taken (mo/day/yr)	Result by ELISA:				DEN virus serotype[c]
			Microplate[a]		Dipstick[b]		
			IgM	IgG	IgM	IgG	
1	—[d]	10/07/91	0.018	0.150	0	0	1
		12/12/91	0.177	0.398	0	1	
2	—	10/15/91	0.075	0.071	0	0	1
		12/30/91	0.336	0.684	1	2	
3	—	10/15/91	0.088	0.032	0	0	1
		12/30/91	0.204	0.357	0	1	
4	—	10/16/91	0.042	0.161	0	2	1
		11/04/91	0.368	1.049	1	3	
5	—	09/24/91	0.076	0.071	0	0	1
		12/27/91	0.187	0.512	0	2	
6	—	12/19/91	0.042	0.000	0	0	1
		01/06/92	0.825	0.486	3	2	
7	—	01/10/92	0.036	0.064	0	0	1
		01/29/92	1.439	0.979	1	2	
8	01/14/92	01/19/92	0.050	0.009	0	0	2
		02/01/92	0.647	1.020	2	2	
9	01/09/92	01/14/92	0.345	0.370	3	1	2
		02/22/92	0.529	0.762	3	2	
10	01/10/92	01/12/92	0.055	0.247	0	0	2
		02/22/92	0.168	0.992	2	1	
11	01/06/92	01/07/92	0.043	0.048	0	0	2
		02/26/92	0.545	0.872	3	2	
12	10/04/93	10/07/93	0.175	1.077	0	2	3
		10/10/93	1.208	1.090	1	2	
13	08/01/93	08/04/93	0.163	0.008	0	0	3
		08/08/93	1.566	0.037	2	0	
14	08/24/93	08/26/93	0.030	0.069	0	0	3
		09/23/93	0.974	0.363	1	1	
15	09/06/93	09/09/93	0.045	0.056	0	0	3
		09/14/93	1.618	0.997	3	2	
16	10/12/93	10/16/93	1.069	0.993	2	2	3
		10/18/93	1.741	1.134	2	2	
17	01/21/94	01/25/94	0.787	1.072	1	2	3
		01/28/94	1.819	1.069	2	3	
18	—	07/14/94	1.430	0.103	1	0	3
		07/15/94	1.690	0.237	2	1	
19	03/05/94	03/11/94	0.104	1.100	1	2	4
		03/13/94	0.410	1.171	1	2	
20	06/30/94	07/05/94	0.324	0.813	1	2	4
		07/07/94	0.264	1.148	1	2	

[a] Adjusted ELISA OD value at 1:100 dilution of serum; adjusted ELISA OD values ≥0.100 were considered positive.
[b] Number of reactive dots at 1:200 dilution of serum.
[c] DEN virus was isolated from the first serum sample of each pair.
[d] —, onset date not available.

fections (12). Removal of the IgG antibody also reduces the interference of IgM rheumatoid factor when present in the sera, which binds to DEN-specific IgG antibody and can lead to false-positive IgM tests (13, 17, 23).

Other dot-blot ELISA formats also have been reported for either total DEN antibody (4) or specific IgM antibody (3, 5) detection. The total DEN antibody dot ELISA used a protein A conjugate to detect DEN antibody bound on a nitrocellulose membrane to a mixture of all four serotypes of DEN antigen and required less than 3 h to complete. This test correlated well with the HI test for the diagnosis of secondary DEN cases of infection based on testing a single acute-phase serum or paired sera at a 1:1,000 dilution. However, since more than 50% of the secondary cases of infection were reported to remain positive in the total DEN antibody dot ELISA for at least 6 months, any diagnosis based on a single acute-phase sample would have to be interpreted with caution. This dot ELISA was not sensitive for the diagnosis of primary DEN infections. The dot ELISA for detecting DEN IgM antibody used an IgM antibody capture format on a nitrocellulose membrane (3, 5). This dot ELISA was reported to show good sensitivity and specificity with serum samples collected from clinically suspected hospitalized patients with DEN fever or DHF when compared to an IgM antibody capture microplate ELISA, but required an overnight incubation step compared to 3 h for the Integrated Diagnostics DEN IgM dipstick ELISA.

A problem common to all serological assays for the diagnosis of DEN virus infections is the broad crossreactivity among members of the *Flavivirus* genus, as shown by our results with the YF sera. The DEN viruses cocirculate with other human pathogenic flaviviruses, such as YF virus and Japanese encephalitis virus, in many areas of the world. Tests such as the HI and microplate IgG ELISA with whole-virus antigen preparations detect broadly cross-reactive epitopes shared by the different

flaviviruses, complicating the definitive diagnosis of the virus responsible for an infection (21). Even so, by determining end-point titers against the known human pathogenic flaviviruses that occur in an area, the HI test and microplate IgG ELISA can still be used for the serological diagnosis of DEN with properly timed samples unless the patient has previously been exposed to a different flavivirus. In the latter case, the anamnestic IgG response is so broadly cross-reactive that these IgG tests cannot be used for a specific DEN diagnosis; however, a diagnosis may still be possible by determining the IgM comparative end-point titers (2). Both the IgG dipstick ELISA (Fig. 2) (14, 22) and the IgM dipstick ELISA (Fig. 3) provided an estimate of antibody titer; whether this feature would be discriminating enough to specifically identify an infecting flavivirus with dipsticks made with different flavivirus antigens remains to be determined.

Compared to the standard serological assays currently in use in most laboratories for the detection of DEN IgG and/or IgM antibody such as the HI test and the microplate ELISA, the dipstick ELISA is faster and simpler to perform. The DEN IgG and IgM dipstick ELISAs can be completed in about 45 min and 3 h, respectively, and the only equipment required is a 50°C water bath or heating block. Thus, the dipstick ELISA would be particularly suitable for health facilities or laboratories without expensive microplate readers and washers or that may need to test only small numbers of clinically suspect DEN patients and would like to have same-day diagnostic capability.

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