



Evaluation of immunological markers in serum, filter-paper blood spots, and saliva for dengue diagnosis and epidemiological studies

Angel Balmaseda^a, Saira Saborio^a, Yolanda Tellez^a, Juan Carlos Mercado^a,
Leonel Pérez^a, Samantha N. Hammond^b, Crisanta Rocha^c,
Guillermina Kuan^d, Eva Harris^{e,*}

^a Departamento de Virología, Centro Nacional de Diagnóstico y Referencia, Ministerio de Salud, Apartado Postal 2900, Managua, Nicaragua

^b Sustainable Sciences Institute-Nicaragua, C/S Socrates Flores Vivas, Managua, Nicaragua

^c Unidad de Infectología, Hospital Infantil Manuel de Jesús Rivera, Managua, Nicaragua

^d Centro de Salud Socrates Flores Vivas, Ministerio de Salud, Managua, Nicaragua

^e Division of Infectious Diseases, School of Public Health, University of California, Berkeley, 1 Barker Hall, Berkeley, CA 94720-7354, United States

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ABSTRACT

Background: Numerous immunological approaches exist to diagnose dengue or detect dengue virus (DENV) infections.

Objectives: To determine the best immunological markers and specimen types for dengue diagnosis and for measuring incidence of DENV infection in community-based studies.

Study Design: In one study, acute- and convalescent-phase samples were collected from hospitalized suspected pediatric dengue cases in Managua, Nicaragua, from September 2003 to February 2004. A second study examined specimens collected in a community setting in Managua before and after the 2003–2004 dengue season to measure incidence of DENV infection. In both studies, detection of anti-DENV IgM, IgA, and IgG in serum, filter-paper blood spots, and saliva was compared to a gold standard performed on serum samples.

Results: For dengue diagnosis, the highest sensitivity and specificity was obtained by measuring IgM or IgA in serum or filter-paper blood spots; intermediate and poor results were obtained in saliva for IgM and IgA, respectively. Detection of IgG alone in serum, filter-paper blood spots, or saliva functioned best for measuring DENV infection.

Conclusions: Detection of IgM and IgA in serum and filter-paper blood spots yielded optimal results for diagnosis of dengue cases, whereas IgG was the best marker for measuring incidence of DENV infection.

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1. Introduction

The four serotypes of dengue virus (DENV1–4) cause the most important mosquito-borne viral disease affecting humans world-

wide, with tens of millions of dengue cases annually.¹ DENV infection can be asymptomatic, can manifest as classic dengue fever (DF), or can evolve into the most serious form, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). Traditionally, diagnosis and epidemiologic surveillance relies on detection of DENV-specific IgM antibodies in serum of suspected dengue cases,^{2,3} while the incidence of DENV infection is determined by detecting DENV-specific IgG or total antibody in serum via ELISA or the time-consuming and cumbersome hemagglutination inhibition or plaque reduction neutralization test (PRNT) assays.^{4–8} A few studies have evaluated the use of anti-DENV IgA as a dengue diagnostic marker^{9–11}; others have assessed alternatives to serum

Abbreviations: DENV, dengue virus; DHF/DSS, dengue hemorrhagic fever/dengue shock syndrome; Ig, immunoglobulin; ELISA, enzyme-linked immunosorbent assay; PPV, positive predictive value; NPV, negative predictive value; ROC, receiver operator characteristic; OD, optical density.

* Corresponding author. Tel.: +1 510 642 4845; fax: +1 510 642 6350.

E-mail address: eharris@berkeley.edu (E. Harris).

as diagnostic specimens.^{9,11–13} However, no comprehensive studies of multiple immunoglobulin classes and different types of biological samples have been reported, especially in relation to measuring incidence of DENV infection. This study evaluates the utility of different immunological markers for diagnosis of acute illness and for measuring the incidence of DENV infection. For dengue diagnosis, IgM and IgA in acute- and convalescent-phase serum, filter-paper blood spots, and saliva were studied; for determining DENV infection over a 6-month period of time, changes in levels of IgM, IgA and IgG in serum and filter-paper blood spots, as well as IgM and IgG in saliva, were evaluated.

2. Methods

2.1. Study population

Two distinct populations participated in this study. For assessing immunological markers for diagnosis of symptomatic dengue cases, children and infants ages 0–14 years old who presented to the Hospital Infantil Manuel de Jesús Rivera (HIMJR) from September 2003 to February 2004 with suspected acute dengue within 4 days of symptom onset were invited to participate in the study. Inclusion criteria included meeting the WHO criteria for a suspected case of dengue,¹⁴ less than or equal to 4 days since symptom onset, consent from a parent or legal guardian, and assent for children over 5 years of age. Exclusion criteria included the presence of a hematologic or other illness that increased risk to study participants. Acute-phase samples (days 1–4 post-symptom onset) and convalescent-phase samples (days 10–14 post-symptom onset) were collected from each participant.

The second population was comprised of healthy children from non-contiguous homes living in District II of Managua, whose families were participating in an entomological study from July 2003 to January 2004. Consent to participate in the serological survey was required from a parent or guardian, and assent was required from all subjects 5 years of age and older. Samples evaluated in this study were collected in July 2003 and January 2004 (6 months apart).

These studies were approved by the Institutional Review Boards at the University of California Berkeley and the Nicaraguan Ministry of Health.

2.2. Sample collection

To minimize inconvenience to participants, blood obtained via standard venipuncture was used both for the serum sample and for the filter-paper blood spot. Blood was collected into a glass tube (without anti-coagulants), and prior to its coagulation, 0.2–0.4 cc of blood was transferred via microcapillary to 20-weight Whatman 3 paper. The paper, cut into the shape of microscope slides, was placed upright in a slide case at ambient temperature until dry (up to 2 h). The filter-paper samples were then placed in ziplock bags and kept at 4 °C until processing. After the blood was removed for the filter-paper specimen, the glass tube was maintained at ambient temperature until coagulation and then kept at 4 °C for a maximum of 24 h; during this time, serum was collected into Eppendorf tubes and frozen at –20 °C until processing. Samples from the same participant were processed simultaneously and within 12 months of their collection. For saliva, participants dripped saliva from their mouths into a 1-in. diameter specimen cup. For very young children, nurses used plastic disposable pipettes to gently suction saliva from under the tongue. After the specimen containers were sealed, samples were immediately placed at approximately 4 °C for a maximum of 24 h, at which time they were transferred into Eppendorf tubes and frozen at 20 °C

until processing. Again, samples from the same participant were processed simultaneously and within 12 months of their collection.

2.3. Laboratory methods

For all assays, serum and saliva from previously laboratory confirmed dengue and non-dengue cases were used as positive and negative controls, respectively.

2.3.1. Capture ELISA—IgM and IgG in serum, filter-paper blood spots and saliva; IgA in serum

To perform capture ELISA of anti-DENV IgM, IgA or IgG,⁹ polystyrene wells were fixed with 100 µl of anti-human IgM, IgA or IgG antibodies diluted in carbonate–bicarbonate buffer and were incubated overnight at 4 °C. After one wash with phosphate-buffered saline/5% Tween–20 (PBS-T), wells were incubated at 37 °C for 30 min with 50 µl of serum diluted 1/20 in PBS-T, 50 µl of PBS eluted overnight from filter-paper blood spots (approximately a 1:20 dilution; one 6-mm diameter circle in 200 µl PBS-T), or 50 µl of undiluted saliva. Wells were washed four times with PBS-T after each incubation. After a 1-h incubation with 50 µl of a mixture of antigens from all four DENV serotypes obtained from infected C6/36 cells,⁹ wells were incubated for 30 min at 37 °C with 50 µl of pooled polyclonal human anti-DENV immunoglobulin (with high inhibition ELISA titer directed against all serotypes) conjugated to horseradish peroxidase and diluted in PBS-T plus 5% normal human serum. This was followed by incubation with 50 µl of tetra-methyl-benzidine (TMB) substrate for 10 min, and the reaction was stopped with 50 µl of 12.5% sulfuric acid. Optical density (OD) was measured at 450 nm in a Humanreader ELISA reader (Human Gesellschaft, Taunstein, Germany). For diagnosis of suspected cases, samples with OD greater than 2 times (IgM ELISA) or 1.8 times (IgA ELISA) the mean of the OD of negative controls were considered positive. For measuring incidence, a >twofold increase in IgM, IgA (serum only), and IgG antibody units between pre- and post-season samples was considered positive; antibody units were calculated as follows:

$$\text{units} = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD positive control} - \text{OD negative control}} \times 100.$$

2.3.2. Sandwich ELISA—IgA in saliva

To measure anti-DENV IgA in saliva, wells were fixed with 100 µl of human anti-DENV immunoglobulin at 10 µg/ml in carbonate–bicarbonate buffer, pH 9.5, and incubated overnight at 4 °C. After one wash with PBS-T, wells were incubated at 37 °C for 30 min with PBS-T containing 1% bovine serum albumin, then incubated at 37 °C for 1 h with 100 µl of a mixture of antigens from all four DENV serotypes obtained from infected C6/36 cells. Duplicate wells incubated with antigen prepared from uninfected C6/36 cells were also prepared for each sample tested. After four washes with PBS-T, 100 µl of each undiluted saliva sample was added to duplicate wells containing antigen from DENV-infected C6/36 cells or antigen from uninfected C6/36 cells. Wells were incubated for 1 h at 37 °C, washed four times with PBS-T, then incubated for another hour with 100 µl of anti-human IgA conjugated to horseradish peroxidase. After four final washes with PBS-T, 100 µl of TMB substrate was added for 10–20 min, and the reaction was stopped with 12.5% sulfuric acid. OD was measured at 450 nm in a Humanreader ELISA reader. Samples where the value of the OD obtained using antigen prepared from DENV-infected cells minus the OD obtained using antigen prepared from uninfected cells was greater than 0.2 were considered positive.

Table 1
Sensitivity and specificity of IgM and IgA in different specimens as a dengue diagnostic test

Markers	Total (n)	Positive ^a	Positivity ^b (%)	Sensitivity ^c (95% CI)	Specificity ^c (95% CI)	PPV (95% CI)	NPV (95% CI)	ROC area
Gold standard	356	313	87.9					
IgM serum	356	291	81.7	93 (89.5, 99.5)	93 (80.9, 98.5)	99 (97.0, 99.8)	64.5 (51.3, 76.3)	0.930
IgA serum	356	291	81.7	93 (89.6, 95.5)	86 (72.1, 94.7)	98 (95.7, 99.3)	62.7 (49.1, 75.0)	0.895
IgM FP ^d	169	153	90.5	95.6 (91.1, 98.2)	88.9 (51.0, 99.7)	99.4 (96.4, 100)	53.3 (26.6, 78.7)	0.923
IgA FP ^d	159	140	88.1	93.3 (88.1, 96.8)	88.9 (51.8, 99.7)	99.3 (96.1, 100)	44.4 (21.5, 69.2)	0.911
IgM saliva	208	155	74.5	78.7 (72.3, 84.2)	86.7 (59.5, 98.3)	98.7 (95.5, 99.8)	23.6 (13.2, 37.0)	0.827
IgA saliva	76	50	65.8	67.6 (55.7, 78.0)	100 (15.8, 100)	100 (92.9, 100)	7.7 (0.9, 25.1)	0.838

^a Positive by the gold standard (IgM seroconversion; \geq fourfold increase in inhibition ELISA titer; or RT-PCR/viral isolation).

^b Positivity (%) was calculated in relation to the total number of samples (positive and negative cases) of the indicated sample type analyzed by a given technique.

^c Sensitivity and specificity were calculated relative to the gold standard.

^d FP, filter-paper blood spots.

2.3.3. Inhibition ELISA—total serum Ig

The inhibition ELISA¹⁵ was used for determining total antibody as described in prior studies⁹; this technique had been previously validated against hemagglutination inhibition.^{16,17} The percent inhibition (PI) was calculated as $[1 - (\text{OD sample}/\text{OD negative control})] \times 100$. The titer of a sample was considered as the last dilution with PI ≤ 50 .

2.3.4. Gold standard definition and assays

A “gold standard” was established for laboratory confirmation of dengue cases using paired acute- and convalescent-phase serum samples, consisting of IgM seroconversion, a \geq fourfold increase in total anti-DENV antibodies by inhibition ELISA, detection of viral RNA by RT-PCR,^{18,19} and/or virus isolation.¹⁸ For dengue cases, a primary DENV infection was defined when the titer of the convalescent sample was <5120 by inhibition ELISA (equivalent to <2560 by hemagglutination inhibition),^{15–17} whereas a secondary infection consisted of a convalescent serum with a titer ≥ 5120 by inhibition ELISA.

The “gold standard” for detecting DENV infection in pre- and post-season serum samples was the inhibition ELISA; a person was considered to have experienced a DENV infection when a \geq fourfold increase was observed in the inhibition ELISA titer between paired samples. Statistical analyses were conducted in STATA, Version 8 (StataCorp, College Station, TX).

3. Results

3.1. Evaluation of diagnostic assays in different specimen types

Acute and convalescent samples from 356 participants were obtained. Participant age range was 0–14 years of age (mean 6.7, mode 6, S.D. 4.2). In terms of gender, 50.3% of participants were male and 49.7% were female (1:1 ratio). Acute and convalescent-phase samples from 356 participants were analyzed by the gold standard assays. Serum IgM and IgA assays were performed on all 356 (100%) samples. A representative number of samples (45–58%) were processed for filter-paper blood spots and IgM saliva. Because the results were not promising for IgA sandwich ELISA, only 76 (21%) samples were assayed.

Anti-DENV IgM and IgA in different sample types were assessed for their utility as a diagnostic test in suspected hospitalized dengue patients, as compared to the “gold standard” as defined in Section 2 (Table 1). The number of samples positive by each assay comprising the gold standard is detailed in the Supplemental table. In serum, both IgM and IgA behaved similarly, with high sensitivity, specificity, positive predictive value (PPV), and receiver operator characteristic (ROC) area; the lower negative predictive value (NPV) is due to the low numbers of negative cases. Interestingly, the sensitivity of detection of IgA in serum and filter-paper blood spots was higher in secondary than in primary DENV infections; whereas in saliva, the sensitivity was higher in primary infections (Fig. 1).

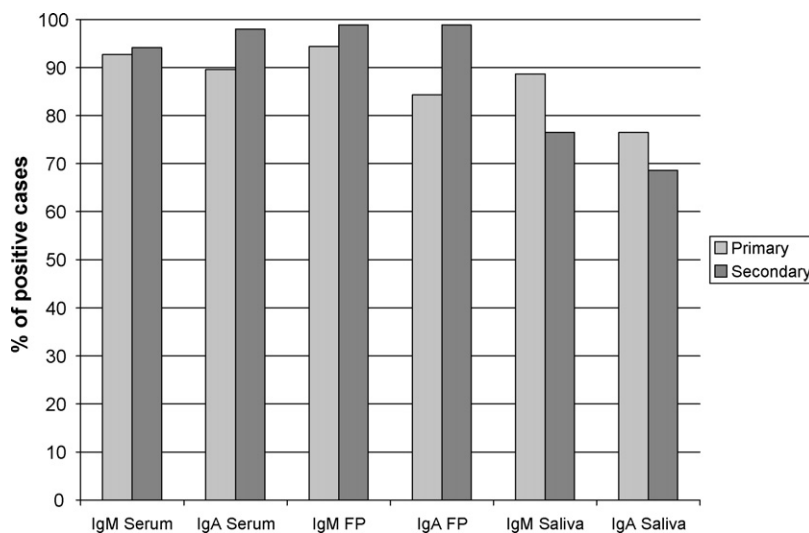


Fig. 1. Detection of positive dengue cases according to immune status. The percent of laboratory-confirmed dengue cases is shown for each immunoglobulin (IgM, IgA) and sample type (serum, filter-paper blood spots, saliva) divided into primary and secondary DENV infections. Classification of primary and secondary infection was performed based on inhibition ELISA titer in convalescent samples.

Table 2
Evaluation of different serological markers and biological specimens for measuring incidence of DENV infection

	Total	TP ^a	TN ^a	FP ^a	FN ^a	Sensitivity ^b	Specificity ^b	PPV ^{b,c}	NPV ^{b,c}	ROC area
Gold standard	322	57	265							
Serum										
IgG	321	51	246	18	6	89.5 (78.5, 96.0)	93.2 (89.4, 95.9)	73.9 (61.9, 83.7)	97.6 (94.9, 99.1)	0.913
IgM	314	22	190	69	33	40 (27.0, 54.1)	73.4 (67.5, 78.6)	24.2 (15.8, 34.3)	85.2 (79.9, 89.6)	0.567
IgA	259	34	196	14	15	69.4 (54.6, 81.7)	93.3 (89.1, 96.3)	70.8 (55.9, 83.0)	92.9 (88.5, 96.0)	0.814
IgG + IgM	322	54	184	81	3	94.7 (85.4, 98.9)	64.9 (63.5, 74.9)	40.0 (31.7, 48.8)	98.4 (95.4, 99.7)	0.821
IgG + IgA	321	52	236	28	5	91.2 (80.7, 97.1)	89.4 (85.0, 92.8)	65.0 (53.5, 75.3)	97.9 (95.2, 99.3)	0.903
IgG + IgM + IgA	322	54	175	90	3	94.7 (85.4, 98.9)	66 (60.0, 71.7)	37.5 (29.6, 45.9)	98.3 (95.2, 99.7)	0.804
Filter-paper										
IgG	288	43	220	18	7	86 (73.3, 94.2)	92.4 (88.3, 95.5)	70.5 (57.4, 85.5)	96.9 (93.7, 98.8)	0.841
IgM	276	29	184	43	20	59.2 (44.2, 73.0)	81.1 (75.3, 85.9)	40.3 (28.9, 52.5)	90.2 (85.3, 93.9)	0.701
IgA	289	38	213	23	15	71.7 (57.7, 83.2)	90.3 (85.7, 93.7)	62.3 (49.0, 74.4)	93.4 (89.4, 96.3)	0.810
IgG + IgM	305	50	125	125	5	90.9 (80.0, 97.0)	50.0 (43.6, 56.4)	28.6 (22.0, 35.9)	96.2 (91.3, 98.7)	0.705
IgG + IgA	305	53	140	110	2	96.4 (87.5, 99.6)	56.0 (49.6, 62.2)	32.5 (25.4, 40.3)	98.6 (95.0, 99.8)	0.762
IgG + IgM + IgA	305	54	112	138	1	98.2 (90.3, 100)	44.8 (38.5, 51.2)	28.1 (25.9, 35.1)	99.1 (95.0, 100)	0.715
Saliva										
IgG	318	45	212	51	10	81.8 (69.1, 90.9)	80.6 (75.3, 85.2)	46.9 (36.6, 57.3)	95.5 (91.9, 97.8)	0.812
IgM	318	22	186	76	34	39.3 (26.5, 53.2)	71.0 (65.1, 76.4)	22.4 (14.6, 32.0)	84.5 (79.1, 89.1)	0.551
IgG + IgM	319	45	118	145	11	80.4 (67.6, 89.8)	44.9 (38.8, 51.1)	23.7 (17.8, 30.4)	91.5 (85.3, 95.7)	0.626

^a TP, TN, FP, FN stand for true positive, true negative, false positive, and false negative, respectively, as compared to the gold standard.

^b Sensitivity, specificity, concordance, PPV and NPV were calculated with respect to positive DENV infections as defined by a fourfold or greater increase in inhibition ELISA titer.

^c PPV and NPV stand for positive and negative predictive values, respectively.

Regarding specimen type, the highest sensitivity, specificity, PPV, and ROC area were obtained using IgM and IgA ELISAs from both filter-paper blood spots and serum (Table 1). Detection of DENV-specific IgM in saliva yielded intermediate results. Poor sensitivity was obtained from measurements of IgA in saliva, even though an ELISA system was developed that involved initial capture of DENV-specific antibodies in the test specimen followed by detection of bound IgA in an attempt to decrease the background due to other IgA antibodies in the saliva that occurred with the original IgA capture ELISA tested.

3.2. Measuring incidence of DENV infection

Pre- and post-dengue season samples were obtained from 322 participants. Participant age range was 1–14 years old (mean 8,

mode 12, S.D. 4). The male to female ratio was 51:49%. Immunological markers were evaluated individually and in combination (Table 2). The “gold standard” in this comparison study was the inhibition ELISA performed on serum samples. The incidence of infection based on the gold standard was 18% and was consistent across age (data not shown). The highest sensitivity, specificity, and concordance was achieved via detection of anti-DENV IgG in serum; comparable results were obtained with a combination of anti-DENV IgG and IgA (Table 2 and Fig. 2). Detection of IgG alone also yielded the best results in filter-paper blood spots and in saliva samples. In general, while detection of IgM in combination with other immunological markers increased sensitivity somewhat, it also decreased the specificity to an unacceptable extent. Because IgA in saliva yielded such low sensitivity in a diagnostic assay (Table 1), it was not used to measure incidence of DENV infection.

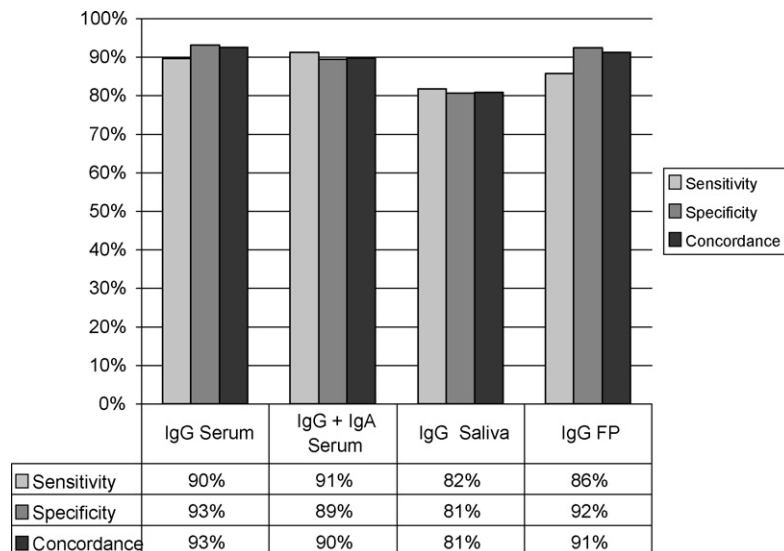


Fig. 2. Detection of anti-DENV IgG in serum, filter-paper blood spots, and saliva. The sensitivity, specificity and concordance is shown for the serological markers that yielded the best results for measuring incidence of DENV infection, using samples from 322 children collected in July 2003 and January 2004. Total anti-DENV antibody as determined by inhibition ELISA was used as the reference technique. FP, filter-paper blood spots.

4. Discussion

These studies together provide a comprehensive analysis of both immunological markers and specimen type in the context of dengue diagnostic assays for acute cases and measurement of DENV infection incidence in field studies. Our results demonstrate that IgA is a reliable marker for dengue diagnosis. Previous studies have shown the diagnostic utility of IgA in serum principally in secondary cases^{3,9,10,20}; here, sensitivity was greater in secondary cases as well (98% in secondary versus 90% in primary cases). Interestingly, in contrast to serum and filter-paper blood spots, detection of IgM and IgA in saliva was greater in primary than in secondary dengue cases. It is not clear why this occurs; future studies are needed to address this point and investigate whether salivary anti-DENV Igs result only from serum transudation or whether they derive from proximal lymph nodes as well. The lower sensitivity of IgM in saliva in this study compared to prior studies^{9,21} is likely due to the fact that different gold standards were used. The previous papers analyzed the diagnostic utility of IgM detection in saliva samples compared to IgM in serum, whereas this study compares numerous markers, including IgM in saliva, to a gold standard consisting of IgM ELISA, inhibition ELISA, RT-PCR and/or virus isolation. In terms of specimen type, filter-paper blood spots and serum yielded the highest sensitivity, specificity, PPV, and ROC area in both IgM and IgA ELISAs. This is important because filter-paper blood spots are easier to obtain than venous blood in infants and young children. Although filter-paper specimens have been used for detecting anti-DENV IgM,^{22,23} no reports describe measurement of anti-DENV IgA in this sample type. Even though the sensitivity of anti-DENV IgM detection in saliva was lower than that of IgM and IgA in serum and filter-paper blood spots, it could potentially be useful for epidemiological surveillance and in confirmation of dengue in an outbreak situation in a particular community. In contrast, detection of anti-DENV IgA in saliva yielded poor sensitivity and thus is not recommended for dengue diagnosis.

With respect to measuring the incidence of DENV infection, anti-DENV IgG antibody alone and anti-DENV IgG plus IgA proved to be the best markers in serum, yielding the highest sensitivity, specificity and concordance; however, the combination of IgG and IgA did not provide any additional advantage over IgG alone. In filter-paper blood spots and saliva, anti-DENV IgG alone yielded the best results. IgM antibody alone was not useful for measuring the incidence of DENV infection; combination with IgG and/or IgA increased the sensitivity but resulted in a concomitant decrease in specificity. Although detection of IgG in saliva was less sensitive than in serum or filter-paper blood spots, it is an acceptable and attractive marker for community-based studies because of its non-invasive nature. For instance, it was the method of choice for monitoring DENV infection in children in a large study of the impact of community participation in mosquito control and dengue prevention in Managua, Nicaragua over the past four years. Since there is IgG cross-reactivity between DENV and other flaviviruses, this must be kept in mind when interpreting the IgG results; when the study was performed, no other flaviviruses were known to be circulating in Managua (data not shown). In summary, this extensive analysis of specimen type and immunoglobulin class indicates which samples and markers function best in dengue diagnostic assays and epidemiological surveys.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2008.07.016.

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