IDENTIFICATION OF BLOOD MEALS IN *AEDES AEGYPTI* BY ANTIBODY SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT. A comparative study of 3 ELISA protocols was performed for host blood meal identification in *Aedes aegypti*. In the sandwich-B ELISA, which used a combination of heavy and heavy + light chain conjugates, specificity was improved to such a degree that conjugates no longer required cross-adsorption with heterologous sera. Using the sandwich-B assay, human blood meals in laboratory reared *Ae. aegypti* could be detected longer after feeding (100% at 32 h and 80% at 42 h) than with a direct assay (100% at 20 h). Efficacy of the sandwich-B and direct ELISAs in field analyses was studied in parallel using 80 field collected mosquitoes from Thailand. The sandwich-B assay was superior (88% detection rate) to the direct assay (41% detection rate) and was thus selected as the method of choice for future field studies.

INTRODUCTION

Previous investigators recognized the potential of the enzyme-linked immunosorbent assay (ELISA) technique for identification of hosts from which mosquitoes, and other important arthropod vectors, had obtained a blood meal. The versatility of ELISAs can be seen in the methods one may choose to use, depending on factors such as antigen concentration in test samples and the kind of information desired from the assay. ELISA protocols used to determine mosquito host blood meal source include: 1) direct (Edrissian et al. 1985, Beier et al. 1988), 2) indirect (Burkot et al. 1981, Burkot and DeFoliart 1982, Burkot et al. 1988, Tesh et al. 1988), 3) a combination of direct and indirect (Linthicum et al. 1985), and 4) sandwich (Service et al. 1986). In the direct ELISA, antigen present in the test sample is adsorbed directly onto the surface of a microtiter plate well. A primary antibody, conjugated to an enzyme, reacts directly with bound surface host antigen. The amount of bound protein can be visualized upon the addition of an enzymatic substrate. This method is employed when the enzyme labeled primary antibody is available and antigen to be detected is present in the sample in abundance. When a primary labeled antibody is not readily available, the indirect ELISA may be used. An unlabeled primary antibody reacts with the host antigen in the plate, and an enzyme labeled secondary antibody reacts with the primary antibody. The sandwich ELISA is one of the most sensitive and specific methods and is used when samples containing minute amounts of antigen need to be examined (Kirkegaard and Perry Laboratories 1990). Antibodies adsorbed onto the surface of a microtiter plate well selectively capture homologous antigen from the test sample, which in turn is directly detected by an antigen-specific enzyme conjugated antibody.

This study evaluates different ELISA protocols for identification of blood meals taken by *Aedes aegypti* (Linn.), the principle vector of dengue viruses (Gubler 1988). Based on these analyses the most sensitive and specific procedure would be selected for use in longitudinal studies on bloodfeeding patterns of *Ae. aegypti* collected from areas endemic for dengue virus transmission in Thailand and Puerto Rico.

MATERIALS AND METHODS

Antisera: Tesh et al. (1988) reported that within blood meals of mosquitoes and sand flies, IgG and IgM survived longer than albumin and were still detectable at low levels several days after ingestion. Therefore, commercially available antisera (Kirkegaard & Perry Laboratories, Gaithersburg, MD) against IgG from 8 potential host species were used for capture (unlabeled) and detection (horseradish peroxidase labeled).

The sandwich-A ELISA used only heavy + light (H+L) chain specific reagents against human, bovine, chicken, dog, horse, mouse and swine IgG. The direct assay similarly used only H+L chain specific reagents against human, bovine, cat, chicken, dog, rat and swine. The sandwich-B ELISA tested for the same hosts as the direct assay and used heavy (H) chain human, cat, dog and swine and H+L chain bovine, chicken and rat specific antisera.

Lyophilized antisera were reconstituted in a 1:1 mixture of distilled water:glycerine, which permits routine storage at −20°C without freeze-thawing. All unlabeled and labeled antibody

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stock solutions for sandwich assays were diluted 0.5 mg/ml. Conjugated antibody stock solutions used in the direct assay were diluted 1.0 mg/ml.

ELISA solutions: Coat: Dulbecco's (Gibco Laboratories, Grand Island, NY) phosphate-buffered saline (PBS) pH 7.0–7.4, containing 0.01% phenol red (Sigma Chemical Co., St. Louis, MO) and 0.1% thimerosal (Sigma Chemical Co.). *Blocking buffer (BB):* 0.5% casein (Sigma Chemical Co.) was suspended in 0.1N NaOH and boiled. After the casein dissolved, PBS was slowly added to bring up to final volume. After cooling to room temperature, pH was adjusted to 7.4 with HCl, and 0.02% phenol red and 0.1% thimerosal were added. *Grinding solution:* Mosquitoes were ground in PBS with 0.1% thimerosal. *Wash:* PBS with 0.05% Tween 20 (Sigma Chemical Co.) and 0.1% thimerosal.

Grinding of mosquito specimens: Polystyrene (PS) microtiter plates (NUNC "U" well, Roskilde, Denmark) were filled with BB and incubated at room temperature for 3 h to prepare them for mosquito grinding. Wells were then aspirated dry and air-dried plates were stacked, covered and stored at −20°C until used. One whole mosquito or mosquito abdomen was placed in each well of a pre-blocked plate. Twenty μl of grinding solution was added to the well, and the mosquito was ground with a pestle attached to a Foredom foot-actuated electric grinder (Foredom Electric Co., Bethel, CT). After grinding, the pestle was rinsed 2 times with 65 μl of grinding solution with the rinses collected in the well (total volume = 150 μl). The pestle was then wiped with a tissue, dipped sequentially into 2 rinses of wash solution and 1 rinse of distilled water and then wiped dry with a clean tissue to prevent contamination between wells. The color of each triturated mosquito (red, brown, clear) was recorded. Plates containing ground mosquitoes were sealed with pre-cut mylar plate sealing tape (Dynatech Laboratories, Chantilly, VA) and stored at −70°C.

Cross-contamination between wells during grinding was studied using unfed and fully engorged (human, chicken) laboratory reared *Ae. aegypti* that were ground in alternating wells of a plate. Blood meal analysis showed no detectable cross contamination between wells.

Optimization of the sandwich assay: Reagent concentrations were initially optimized by checkerboard titrations to determine the highest sensitivity and lowest background. For each host species system, absorbance values of simultaneous two-fold dilutions of capture antibody (down the plate) and conjugate (across the plate) were compared. Each dilution consisted of duplicate wells of positive control host sera (1:1,000) with corresponding negative control wells. Working dilutions for antisera of each host system were chosen based on highest positive to negative absorbance ratios.

Specificity of reactions with heterologous IgG was assessed by dilution of conjugate (sandwich-B) or adsorption with heterologous sera (sandwich-A, direct) until a concentration was attained in which cross-reactivity was minimized. Thus, optimal working dilutions for a specific reagent lot were determined initially by checkerboard titration of coating antibody, followed by further specificity analysis of conjugate concentration. Due to variability from lot to lot, it is essential that the standardization procedure be repeated for each lot of reagents.

To determine maximal sensitivity and specificity, comparative testing was done with all 3 ELISA protocols using: 1) PS versus "U" well polyvinyl chloride (PVC) microtiter plates (Dynatech Laboratories); 2) Miles Laboratories (Naperville, IL) versus Kirkegaard & Perry Laboratories antisera of different IgG fragments — Fc, Fab, H+L and H; 3) blocking versus no blocking; 4) BB containing bovine serum albumin (BSA) versus BB without BSA; 5) TMB (tetramethylbenzidine) versus ABTS (2,2′-azino-di[3-ethyl-benzthiazoline sulfonate (6)]) 2-component substrates (Kirkegaard & Perry Laboratories); and 6) different incubation periods and temperatures.

Assays were carried out with fully engorged mosquitoes to confirm sensitivity results in blood meal detection of commercially purchased sera. Laboratory reared *Ae. aegypti* (F_1 progeny of females collected from Hua Sam Rong, Thailand) were fed on the arm of a human and then maintained at 31–32°C with 10% sucrose. At various time intervals after feeding, 5 mosquitoes were killed by freezing and stored at −70°C until they were ground and assayed in the ELISA (Table 5).

General protocol for ELISAs: We evaluated 3 ELISA protocols in an initial set of laboratory experiments. Based on results from those studies, one protocol was chosen to assay field collected mosquitoes.

ELISAs were conducted using the inner 60 wells of PVC microtiter plates. The first 2 rows contained duplicates of: 1) IgG positive control host sera, 2) IgG negative controls for the remaining 6 host species, and 3) unfed laboratory reared female *Ae. aegypti* as negative mosquito controls; each microplate had the same template of positive and negative controls. In the remaining 4 rows, 40 specimens could be tested for one host species. The same 40 specimens were tested multiple times on different microtiter plates, each to detect blood from a different host. All specimens assayed on a given day were tested against all possible host species.

Klowden (1988) reported that *Ae. aegypti* im-

bibe blood meals of 3.5 to 5 μl. Each field collected specimen was ground in 150 μl of PBS (5:150 dilution assuming a 5 μl blood meal volume), of which a 5 μl aliquot was added to 45 μl of BB (further 1:10) for ELISA testing. Assuming 50% of the blood meal is serum, actual antibody content tested was estimated to be at a 1:600 dilution. A convenient serum antibody (Sigma Chemical Co.) dilution of 1:1,000 was employed in ELISA controls; this was done in effort to match antibody content in homologous and heterologous controls to actual antibody content in ELISA blood meal aliquots of test specimens.

An automatic microplate washer (Model 1550 Bio-Rad, Richmond, CA) was used to wash the wells in microplates—3 cycles, 300 μl/well/cycle, 4 sec soak/cycle. Plates were then inverted and tapped dry onto a paper towel after the third wash cycle. Unless indicated, incubations were at room temperature (20–23°C).

Sandwich ELISAs: Both sandwich ELISAs (Table 1) followed the 8 steps listed below with the following distinction to be made in step 5. The sandwich-A assay used H+L chain reagents only and included cross-adsorption (overnight at 4°C) of conjugate with heterologous sera, before conjugate was added to each well. The sandwich-B ELISA used a combination of H chain and H+L chain conjugates that were determined during preliminary trials to not require cross-adsorption. Prior to addition to each well, conjugate used in the sandwich-B assay was incubated for 3 h in BB diluent. 1) PVC micro-

Table 1. Antibody concentrations (μg/ml) used in 3 different ELISA protocols designed to detect host blood meals in *Aedes aegypti*.

Sandwich-A ELISA[a]

Host	Capture antibody	Conjugated antibody	Conjugated antibody cross-adsorbed overnight at 4°C
Human	1.00	0.13	Human, mouse 1:500
Bovine	1.00	0.05	Not cross-adsorbed
Chicken	0.25	0.13	Not cross-adsorbed
Dog	2.00	0.05	Human, mouse 1:500
Horse	0.50	0.25	Swine 1:1,000
Mouse	1.00	0.25	Not cross-adsorbed
Swine	0.25	0.25	Human, dog, mouse 1:500

Sandwich-B ELISA[b]

Host	Capture antibody	Conjugated antibody
Human	4.00	0.05
Bovine	1.00	0.05
Cat	0.50	0.05
Chicken	0.25	0.13
Dog	2.00	0.05
Rat	2.00	0.05
Swine	1.00	0.10

Direct ELISA[a]

Host	Conjugated antibody[c]
Human	0.08
Bovine	0.16
Cat	0.08
Chicken	0.16
Dog	0.16
Duck	2.50
Rat	0.16
Swine	0.16

All antisera from Kirkegaard & Perry Laboratories, Gaithersburg, MD.

[a] Only anti-species IgG (H+L) capture and conjugated antibodies were used.

[b] Anti-species IgG (H) capture and conjugated antibodies were used against human, cat, dog, swine and anti-species IgG (H+L) capture and conjugated antibodies were used against bovine, chicken and rat.

[c] Each host conjugate was cross-adsorbed with a 1:500 dilution of all heterologous sera and incubated for 1 h at room temperature before addition to the ELISA plate.

plates were coated with 50 μl/well of capture antibody diluted in PBS and incubated overnight at 4°C. 2) Wells were aspirated dry, filled with 300 μl BB/well and held for 1 h. 3) Wells were aspirated dry and 45 μl of BB added (except to homologous and heterologous IgG control wells), followed by the addition of 5 μl of field collected mosquito suspensions to test wells and 5 μl of a ground unfed mosquito suspension to negative mosquito control wells. Fifty μl of sera (1:1,000 in BB) were added to homologous and heterologous IgG control wells. Plates were covered and incubated for 2 h. 4) Plates were washed. 5) Fifty μl of conjugate were added to each well. Plates were incubated for 1 h. 6) Plates were washed. 7) One hundred μl of TMB substrate, adjusted to room temperature 1 h prior, were added to each well. Plates were incubated for 30–60 minutes. 8) Absorbance values were read at 630 nm (MR5000 Dynatech Laboratories) and transferred on-line to an IBM PC for storage and analysis.

Direct ELISA: The direct ELISA (Table 1) was a modification of the protocol published by Beier et al. (1988) and included the following 6 steps. 1) Forty-five μl of PBS were added to each well (except to homologous and heterologous IgG control wells), followed by addition of 5 μl of field collected mosquito suspensions to test wells and 5 μl of ground unfed mosquito suspension to negative mosquito control wells. Fifty μl of sera (1:1,000 in PBS) were added to homologous and heterologous IgG control wells. Plates were covered and incubated for 3 h. 2) Plates were washed. 3) One hundred μl of cross-adsorbed conjugate diluted in BB were added to each well and plates were covered and incubated for 1 h. Cross-adsorption included incubation of a 1:500 dilution of all heterologous sera with conjugate for 1 h at room temperature prior to addition to plates. 4) Plates were washed. 5) One hundred μl of ABTS substrate, adjusted to room temperature 1 h prior, were added to each well. Plates were covered and incubated for 30 minutes. 6) Absorbance values were read at 410 nm, as described above.

Analysis of results: Separate negative cut-off values were calculated for each host species system on each day that the assay was conducted. Absorbance values of test specimens that were greater than the mean plus 3 standard deviations of the highest negative control (heterologous IgG and negative mosquito wells) for an individual host species system were considered to be positive. Positive mosquitoes were retested on a subsequent day for the host(s) with which it reacted. Only if confirmational analysis yielded another positive result did we conclude that a mosquito fed on a particular host.

Mosquito collection: Wild *Ae. aegypti* were collected by aspiration with a modified vacuum cleaner one day each week between 0900–1530 h during February–March 1992 in Village 6, near the town of Hua Sam Rong, Chachoengsao Province, Thailand (Edman et al. 1992). Collectors spent a minimum of 10 min inside and 5 min outside each house. Dried specimens were stored at −70°C until they were ground and assayed for host blood meal identification by the sandwich-B and direct ELISAs conducted in parallel.

RESULTS

Specificity: Positive homologous control absorbance values of sandwich-A, sandwich-B and direct ELISA specificity tests ranged from 1.39–2.30, 1.17–1.41 and 0.96–2.75, respectively, when test sera where diluted 1:1,000 (Table 2). Negative heterologous reactions for all 3 assays are compared in Table 3. Seventy-three percent of heterologous background values ranged from 0.09–0.19 in sandwich-A compared with 16% in sandwich-B; 67% of heterologous background values were <0.07 in sandwich-B compared with 13% in sandwich-A. Between the 2 sandwich assays, sandwich-B was chosen for further comparison with the direct assay because the majority of heterologous background values for that ELISA were approximately half of those observed in the sandwich-A assay. Comparisons between sandwich-B and the direct assay indicated that sandwich-B was more specific. Sixty-four percent of heterologous background values ranged from 0.09–0.19 in the direct assay compared with 14% in sandwich-B; 69% of heterologous background values were <0.07 in sandwich-B compared with 0% in the direct assay.

Sensitivity: Sensitivity tests generated negative cut-off values of 0.37 for sandwich-A, 0.12 for sandwich-B and 0.14 for the direct ELISA. Reciprocal dilutions (×1,000) of sensitivity limits ranged from 512–2,048 for sandwich-A, 320–2,560 for sandwich-B and 64–1,024 for the direct assay (Table 4). Sensitivities of the 3 assays were not different except for dog, which was low, in the direct assay. The sandwich-B ELISA was selected for further evaluation because it was more specific than the sandwich-A or direct ELISA and no cross-adsorption was needed for the conjugates.

Additional sensitivity tests were performed using laboratory reared *Ae. aegypti* fed on a human. Table 5 summarizes the results of blood meal detection using the sandwich-B and the direct ELISAs. The direct assay detected 100% of human blood meals up to 20 h post-feed with an average absorbance value of 0.30. The sand-

Table 2. Absorbance values (homologous mean and heterologous range of means) of specificity tests for the sandwich-A, sandwich-B and direct ELISAs.

Antisera	Human	Bovine	Cat	Chicken	Dog	Mouse	Rat	Swine
			Sandwich-A ELISA[a] *(absorbance 630 nm)*					
Homologous	1.81	1.60	NT	2.02	1.39	2.30	NT	1.71
Heterologous	0.06–0.10	0.07–0.15	NT	0.07–0.11	0.09–0.15	0.10–0.12	NT	0.10–0.12
			Sandwich-B ELISA[b] *(absorbance 630 nm)*					
Homologous	1.31	1.17	1.41	1.32	1.37	NT	1.29	1.28
Heterologous	0.05–0.07	0.09–0.11	0.05–0.08	0.06–0.08	0.06–0.08	NT	0.05–0.06	0.06–0.07
			Direct ELISA[c] *(absorbance 410 nm)*					
Homologous	2.06	1.65	2.02	2.75	0.96	NT	2.72	1.82
Heterologous	0.07–0.09	0.08–0.10	0.11–0.13	0.11–0.22	0.09–0.12	NT	0.09–0.12	0.09–0.13

NT = Not tested.

[a] Anti-species IgG (H+L) adsorbed to wells of a microtiter plate selectively captured homologous species IgG in the test sera (commercially purchased) sample, which in turn was detected by anti-species IgG (H+L) conjugate previously incubated with the heterologous sera. TMB substrate used.

[b] Anti-species IgG (H) for human, cat, dog, swine and (H+) for bovine, chicken and rat adsorbed to wells of a microtiter plate selectively captured homologous species IgG in the test sera (commercially purchased) sample, which in turn was detected by anti-species IgG (H) conjugate for human, cat, dog, swine and (H+L) conjugate for bovine, chicken and rat. All conjugates contained no heterologous sera. TMB substrate used.

[c] IgG in test sera (commercially purchased) samples that were directly adsorbed to wells of a microtiter plate were detected by anti-species IgG (H+L) conjugate previously incubated with the 6 heterologous sera. ABTS substrate used.

Table 3. Comparison of mean heterologous specificity absorbance values (from Table 2) of the sandwich-A, sandwich-B and direct ELISAs.

Type of ELISA	Number (%) heterologous means[a] (absorbance 630 nm)							
	0.05	0.06	0.07	0.08	0.09	0.10	0.20	Total
Sandwich-A		4 (13)	3 (10)	1 (3)	9 (30)	13 (43)		30
Sandwich-B[b]	9 (30)	11 (37)	3 (10)	2 (7)	4 (13)	1 (3)		30
Sandwich-B	16 (38)	13 (31)	5 (12)	2 (5)	5 (12)	1 (2)		42
Direct[c]			4 (10)	9 (21)	10 (24)	17 (40)	2 (5)	42

[a] Heterologous means that are ≥ the indicated O.D. value and < the next succeeding O.D. value.

[b] Absorbance values from the cat system were not included in the comparison with sandwich-A because cat was not tested for in that assay.

[c] Absorbance for ABTS substrate was read at 410 nm.

wich-B assay proved to be more sensitive with 100% detection up to 32 h post-feed with an average absorbance value of 0.76; even at 42 h post-feed 80% (4/5) of blood meals were still detectable with a relatively strong average absorbance value of 0.53 compared with 0% detection at the same time interval in the direct assay. Absorbance values of positive mosquitoes in the sandwich assay were 2 to 5 times stronger than corresponding signals in the direct ELISA.

Field application: Eighty field collected mosquitoes were examined in parallel using the sandwich-B and direct ELISAs to determine the relative efficacy of each assay when applied to field analyses. Figure 1 shows ELISA plates on which 40 field specimens were tested for human IgG by both methods. Quantitative host blood meal identification results presented in Table 6 show quite clearly that the sandwich-B ELISA is superior to the direct ELISA in actual field performance. In the sandwich-B assay, positive mosquitoes that were retested for confirmational analysis of human blood meals show that more than 6 times as many positive signals (38% vs. 6%) were >1.00 and ≤1.50 OD, and almost twice as many (42% vs. 25%) were >0.50 and ≤1.00 OD when compared with the direct assay. Almost 3.5 times as many retested human positives (69% vs. 20%) in the direct assay had signals that were greater than the negative cut-off value and ≤0.50 OD. The direct ELISA failed to detect one of the 2 dog blood meals confirmed by the sandwich-B ELISA.

Using the sandwich-B assay (Table 7), 88% (70/80) of the total number of specimens assayed reacted to ≥1 of the 7 hosts for which we tested compared with only 41% (33/80) identi-

Table 4. Mean sensitivity absorbance values for the sandwich-A, sandwich-B and direct ELISAs. The mean + 3SD of the highest negative heterologous control of each assay generated a respective negative cut-off value. Bold-face values indicate limits of assay sensitivity for each host system.

Reciprocal dilution[a] (×1,000)	Human	Bovine	Cat	Chicken	Dog	Horse	Mouse	Rat	Swine
Sandwich-A ELISA (absorbance 630 nm); negative cut-off = 0.37									
256	1.35	0.81	NT	0.95	0.83	1.51	1.03	NT	1.08
512	0.86	**0.60**	NT	**0.58**	**0.48**	1.25	**0.52**	NT	0.94
1,024	**0.51**	0.35	NT	0.27	0.32	0.83	0.32	NT	**0.67**
2,048	0.27	0.18	NT	0.17	0.23	**0.49**	0.20	NT	0.35
4,096	0.18	0.13	NT	0.12	0.18	0.27	0.15	NT	0.22
Sandwich-B ELISA (absorbance 630 nm); negative cut-off = 0.12									
160	0.56	0.26	1.03	0.33	0.44	NT	NT	0.86	0.49
320	0.35	**0.18**	0.56	0.25	0.26	NT	NT	0.44	0.41
640	**0.19**	0.12	0.28	0.17	**0.16**	NT	NT	0.23	0.29
1,280	0.12	0.10	0.15	**0.16**	0.11	NT	NT	**0.13**	**0.18**
2,560	0.08	0.10	**0.13**	0.09	0.08	NT	NT	0.08	0.12
5,120	0.06	0.08	0.10	0.08	0.07	NT	NT	0.07	0.07
Direct ELISA (absorbance 410 nm); negative cut-off = 0.14									
32	1.62	1.93	1.45	2.00	0.25	NT	NT	2.33	2.06
64	1.30	1.46	0.97	1.46	**0.14**	NT	NT	1.58	1.55
128	0.94	0.91	0.60	0.96	0.11	NT	NT	0.94	0.98
256	0.51	0.46	0.33	0.62	0.10	NT	NT	0.58	0.46
512	0.29	0.27	**0.18**	0.33	0.09	NT	NT	0.28	0.24
1,024	**0.17**	**0.16**	0.12	**0.20**	0.08	NT	NT	**0.16**	**0.15**
2,048	0.12	0.13	0.12	0.13	0.08	NT	NT	0.11	0.12

NT = Not tested.
[a] Commercially purchased species test sera were used.

fied in the direct assay. Ninety-seven percent of all detectable meals in both methods (68/70 in the sandwich-B and 32/33 in the direct) were identified as being from a single host, human. The sandwich-B assay detected one human-dog double meal that was not picked up by the direct method.

DISCUSSION

The specificity of capture and conjugated antibodies is an issue that needs to be addressed when screening for any possible host(s), regardless of the ELISA method employed. In theory, only species specific host capture antibody should bind to respective host IgG in the test sample, which in turn should only be bound by species specific host conjugate. However, in practice, this does not always occur. It has been our experience that nonspecific recognition between species specific capture antibodies and respective conjugates with unrelated host IgG in the test sample often occur, the results of which are false positive results. Failure to address this issue may erroneously lead to the possible misidentification of host blood meals. In the sandwich-A and direct ELISAs, we found it was essential that conjugates were cross-adsorbed with heterologous sera, even though this is an added step during which an error could be made, in order to minimize nonspecific cross-reactions between host species.

Specificity tests of the 3 ELISA methods compared in this study indicated that sandwich-B, which did not require cross-adsorbing conjugate with heterologous sera, was the most specific protocol. Overall, average negative heterologous control absorbance values were lowest for the sandwich-B ELISA (Tables 2 and 3).

Initially, we standardized the sandwich assay using anti-species IgG H+L chain reagents for all host species systems. However, specificity tests revealed cross-reactivity with several host species. For host systems that displayed cross-reactions, the appropriate combination and concentration of heterologous sera had to be incubated with the optimum conjugate concentration, at the appropriate temperature and for the optimal time period in order for cross-reactions to be eliminated; much time and effort was spent in determining the successful combination of these parameters (Table 1, Sandwich-A).

To find a substitute for the cross-adsorbing step while maintaining specificity, we tried to develop an assay using more specific antisera

Table 5. Sandwich-B and direct ELISA sensitivity limits in detection of human blood meals in fully engorged laboratory reared *Aedes aegypti*. Five mosquitoes were frozen at various time intervals following bloodfeeding. The number and mean absorbance values of positive mosquitoes are shown for each time interval. Negative cut-off values of 0.13 and 0.17 were calculated (mean + 3SD of the highest negative control) for the sandwich-B and direct ELISAs, respectively.

	Sandwich-B		Direct	
Hours post-blood feed	No. detectable specimens	Mean absorbance (630 nm)	No. detectable specimens	Mean absorbance (410 nm)
0	5	1.06	5	0.57
2	5	1.03	5	0.55
4	5	1.01	5	0.51
6	5	0.94	5	0.45
8	5	0.98	5	0.38
10	5	0.94	5	0.34
20	5	0.95	5	0.30
24	5	0.82	1	0.29
27	5	0.91	2	0.20
32	5	0.76	1	0.24
42	4	0.53	0	0.10
48–76	0	0.07	0	0.09

(anti-species IgG H chain) only for those host systems that cross-reacted. After working dilutions of these reagents were determined and specificity tests had been conducted, cross-reactions were still observed, though to a lesser degree. Rather than proceeding with cross-adsorption, we attempted to further dilute conjugates and carried out specificity tests to determine the dilution at which nonspecific reactions were eliminated but strong positive homologous control signals were maintained. This approach led to a protocol (Table 1, Sandwich-B) that generated background absorbance values at such low levels that even 24 h after the addition of substrate, the color of negative heterologous control wells remained clear.

We conclude that our sandwich-B ELISA, which uses a combination of H+L and H chain specific reagents, is an improvement over the direct ELISA (Beier et al. 1988). As Service et al. (1986) noted, results using the direct and indirect methods do not seem to be as accurate as the sandwich assay and are affected by the amount of materials other than test antigen in the sample.

Determining the source of a mosquito's blood meal by ELISA depends largely upon the rate of blood digestion by the mosquito. Generally, tropical conditions, like those in Thailand, lead to blood meals being digested within 24–48 hours. In temperate regions, complete blood meal digestion may take a week or more, depending on temperature (Service 1968). The detection period may also vary depending on blood meal volume and mosquito species being studied.

Using a direct ELISA to assay *Anopheles stephensi* (Liston) held at 27 ± 2°C, Beier et al. (1988) reported that blood meals were accurately detected ≤23 h after blood feeding. Burkot et al. (1981) used an indirect ELISA with *Aedes triseriatus* (Say) held at 22°C, and found that 100% of the blood meals were detectable up to 8 h after ingestion, 75% up to 12 h, 40% at 16 h, and 0% at 20 h. Tesh et al. (1988) used an indirect ELISA and found that in *Aedes albopictus* (Skuse) maintained at 28°C, IgG and IgM were detectable up to 72 h after blood feeding; they did not report the specificity of their assay. Service et al. (1986) used a sandwich ELISA with *Ae. aegypti* held at 24°C and reported that 100% of blood meals were detectable ≤26 h, 75% at ≤31 h, and 50% at ≤40 h, after which 0% were detected.

Our results (Table 5) agree with the Service et al. (1986) study and indicate that the sandwich-B ELISA is a sensitive and specific assay for determining the host upon which a mosquito has fed. We detected 100% of blood meals in *Ae. aegypti* collected ≤32 h after feeding and 80% at ≤42 h when mosquitoes were held at 31–32°C.

After assaying the 80 field collected *Ae. aegypti* using the sandwich-B and direct ELISAs in parallel (Fig. 1, Table 6, Table 7), we concluded that the sandwich-B assay is significantly more sensitive and effective in field analyses when compared with the direct assay. This is supported by the Wilcoxon signed rank test ($P < 0.0001$) using raw absorbance values obtained in the initial analyses of all 80 specimens (Table 6). Absorbance signals of positive mosquitoes assayed using the sandwich-B method were 2 to 6 times as strong as corresponding positives in the direct assay. The sandwich-B ELISA detected 88% (70/80) of total specimens assayed

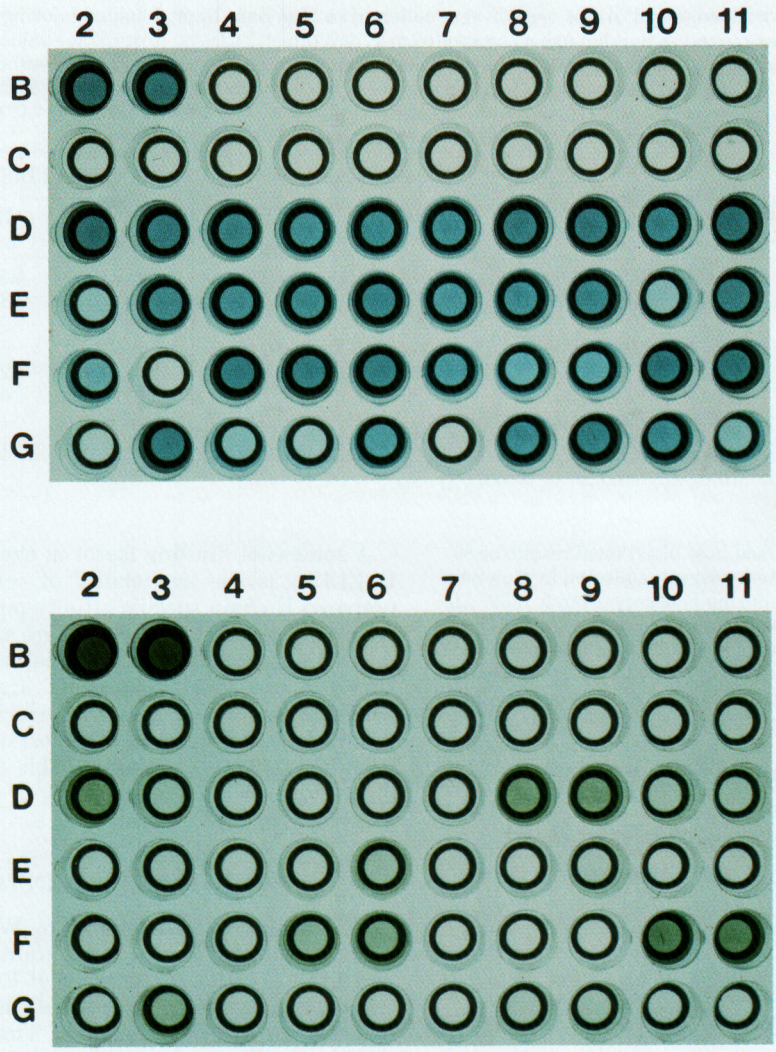

Fig. 1. Microplates from a parallel comparison of the sandwich-B (top) and direct (bottom) ELISAs[a] showing human blood meal identification results of 40 wild, engorged[b] *Aedes aegypti* collected in Hua Sam Rong, Thailand during February–March 1992. These plates were part of the analysis described in tables 6 and 7.

[a] The inner 60 wells of each ELISA microtiter plate designed to detect human IgG are shown. Each template consists of duplicate serum control wells of human (B2, B3), bovine (B4, B5), cat (B6, B7), chicken (B8, B9), dog (B10, B11), rat (C2, C3), swine (C4, C5) and unfed mosquitoes (C6, C7). Rows D through G contain 40 field *Aedes aegypti* identically arranged in both plates.

[b] Mosquitoes containing any amount of blood.

compared with only 41% (33/80) identified by the direct ELISA (Table 7).

At least one host was detected in all mosquitoes containing fresh red blood; all nonreacting specimens contained brown digested blood or were clear, indicating that little, if any, blood was left in the mosquito. Thus, we conclude that nonreacting mosquitoes contained blood meals digested beyond the limits of detection by our sandwich-B ELISA (Table 5) and that nonreacting specimens had not fed on hosts for which we did not assay.

Application of the sandwich-B ELISA in host blood meal identification of field collected mosquitoes was initiated as part of a longitudinal investigation of *Ae. aegypti* bloodfeeding behavior and dengue virus transmission in Thailand and Puerto Rico. Information was desired regarding the proportion of field collected *Ae. aegypti* that imbibe human blood and if that pro-

Table 6. Eighty wild, engorged[a] *Aedes aegypti* were collected in Hua Sam Rong, Thailand during February–March 1992 and examined in parallel using the sandwich-B and direct ELISAs. Absorbance values of positive mosquitoes using both methods are shown. All specimens were tested once and resultant absorbance values were used in the Wilcoxon signed rank test, the results of which show that the sandwich-B ELISA is significantly more sensitive and efficient at host identification than the direct ELISA ($P < 0.0001$). All positive mosquitoes were tested a second time to confirm initial results.

Host	Range of absorbance values for positive mosquitoes	Sandwich-B ELISA		Direct ELISA	
		Initial no. (%)	Retest no. (%)	Initial no. (%)	Retest no. (%)
Human	cut-off > OD ≤ 0.50	15 (20)	14 (20)	26 (74)	22 (69)
	0.50 > OD ≤ 1.00	15 (20)	29 (42)	8 (23)	8 (25)
	1.00 > OD ≤ 1.50	38 (51)	26 (38)	1 (3)	2 (6)
	1.50 > OD ≤ 2.00	6 (8)	0	0	0
		Total 74	69	35	32
Dog	0.50 > OD ≤ 1.00	2 (100)	2 (100)	1 (100)	0
	1.00 > OD ≤ 1.50	0	0	0	1 (100)
		Total 2	2	1	1

[a] Mosquitoes containing any amount of blood.

Table 7. Confirmed host blood meal results of 80 wild, engorged[a] *Aedes aegypti* collected in Hua Sam Rong, Thailand during February–March 1992. All specimens were examined in parallel using the sandwich-B and direct ELISAs to determine the relative efficacy of each assay in host blood meal identification of field collected mosquitoes. The number of mosquito host blood meals detected by each method are shown; corresponding percentages based on all detectable meals are indicated in parentheses.

Host	No. meals (%)	
	Sandwich-B ELISA	Direct ELISA
Human	68 (97)	32 (97)
Bovine	0	0
Cat	0	0
Chicken	0	0
Dog	1 (1)	1 (3)
Rat	0	0
Swine	0	0
Human and dog	1 (1)	0
Nonreactors	10	47
Total no. specimens	80	80

[a] Mosquitoes containing any amount of blood.

portion fluctuates seasonally. Variation in *Ae. aegypti* feeding behavior might be an important factor in seasonal changes of dengue virus related disease (Sheppard et al. 1969, Pant and Yasuno 1973).

In our laboratory, the sandwich-B protocol is quite efficient; an experienced technician can grind 1,000 specimens in 3 days and perform 1,000 assays in one work day. In addition, each ground specimen (150 μl) is enough for 25–30 assays; each assay requires only a 5 μl aliquot of the triturated mosquito.

A somewhat limiting factor in the sandwich-B ELISA is the availability of commercially prepared H chain specific affinity purified antibodies. These kinds of reagents are available for only a limited number of potential host species. Heavy chain specific reagents are currently available for 6 of the 7 hosts for which we tested (unavailable for chicken). The cost of the sandwich-B assay is essentially double that of the direct assay due to the necessity of purchasing unlabeled purified antisera.

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