A DIPSTICK ELISA FOR RAPID DETECTION OF HUMAN BLOOD MEALS IN MOSQUITOES[1]

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ABSTRACT. A dot-enzyme-linked immunosorbent assay, dot-ELISA, that allows for identification of human blood meals in mosquitoes in less than 2 h is presented. Strips of mylar-backed nitrocellulose paper, the dipstick, may be inoculated with anti-human capture antibody, blocked, dried and stored for at least 3 months before use. The Dipstick ELISA consistently detected human blood meals at 24 h post-blood meal in frozen *Anopheles* mosquitoes and at 32 h post-blood meal on samples eluted off filter-paper smears. This ELISA detects human IgG at dilutions of 1:25,600, and produces strong positive results at dilutions between 1:400 and 1:12,800. The Dipstick ELISA is highly specific, and no false positives were detected when tested against cow, horse, goat, dog, cat, pig, rabbit, mouse, rat, chicken, raccoon and opossum sera. All reagents for the assay are commercially available. The Dipstick ELISA meets requirements for a rapid and simple assay for the identification of human blood meals and should have particular application to short-term field studies and emergency epidemiological investigations. A modified protocol, the Trough ELISA, which treats dipsticks jointly in disposable pipet troughs during the conjugate and substrate steps, was developed and produced as a kit. The Trough ELISA produces 1–4% random false positives, and we recommend that the Trough ELISA not be used.

INTRODUCTION

Identification of blood meals in hematophagous arthropods is an important tool in epidemiological investigations of vector-borne diseases. In particular, an assessment of the proportion of feeds taken on man, or the Human Blood Meal Index (HBI), is an essential variable in epidemiological investigations of vector-borne diseases such as malaria, and is needed to assess the vectorial capacity of each vector population. A number of biochemical and immunological techniques have been developed and successfully used to identify blood meal sources in mosquitoes (Washino and Tempelis 1983). Among these methods, enzyme-linked immunosorbent assay (ELISA) techniques have been demonstrated to provide both high sensitivity and specificity (Anonymous 1987). The World Health Organization (1987) and others recognized the demand for a rapid, accurate blood meal identification assay that could be conducted under field conditions or in simply equipped laboratories. A simple, rapid assay could provide entomologists and epidemiologists with real time data, and circumvent the major criticism of the standard procedure of shipping filter smears to a central processing center (World Health Organization 1987).

Herein, we present a new dot-ELISA method for the identification of human blood meals in mosquitoes that can be completed in less than 2 h when using preblocked dipsticks. This technique is an indirect or sandwich type ELISA that is performed on a mylar-backed nitrocellulose paper which can be cut into strips to be used as dipsticks. All reagents needed to conduct this ELISA are commercially available, and both the capture and conjugated antibodies are affinity purified, which reduces cross-reactivity and limits the need to adsorb antiserum with cross-reacting blood sera.

Based upon initial blind testing of 14 different blood meal assays, the ELISA presented below was selected by the World Health Organization for further testing and development. Selection criteria included sensitivity, specificity, simplicity and minimum-time required to conduct the test under field conditions and/or in simply equipped laboratories located in disease endemic areas (V. Houba, personal communication, 1987; World Health Organization 1987).

Unlike other ELISAs used in blood meal analysis (Beier et al. 1988, Lombardi and Esposito 1986, Service et al. 1986), the dipsticks provide a permanent record of test results. This Dipstick ELISA meets requirements for a fast and simple assay for the identification of human blood meals when testing a relatively small number of specimens, and should have particular application to field studies and to short-term or emergency epidemiological investigations.

MATERIALS AND METHODS

Dipstick ELISA protocol: The basic protocol is outlined below and presented schematically in Fig. 1. All procedures are conducted at room

[1] The views of the authors do not purport to reflect the positions of the U.S. Department of Defense. The mention of proprietary products does not constitute endorsement by the U.S. Department of Defense.

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temperature. Dipsticks must be processed individually throughout the procedure. Dipsticks may be exposed to antigen, conjugate and substrate solutions in separate 1.5-ml microcentrifuge tubes (Eppendorf #2236380-8, Thomas Scientific, Philadelphia, PA) or in wells of styrene microtiter plates (for example, Dynatech Immulon 2, Dynatech Inc., Alexandria, VA). However, polyvinyl chloride microtiter plates are unsuitable.

1) Marking the mylar-backed nitrocellulose (NC) sheets. The mylar-backed 0.45-μm nitrocellulose membrane (BA85) is supplied in 15 × 15 cm sheets (Schleicher & Schuell Inc., Keene, NH). The use of plastic gloves when handling nitrocellulose is recommended to keep body oils off the membrane. However, normal handling of the membrane with clean hands will not affect the results of the assay. The nitrocellulose surface of the sheets may be marked with a high quality ballpoint pen and ruler to produce dipsticks of the desired size. We prefer dipsticks that are approximately 3.75 cm long, and 0.4–0.5 cm wide with a line drawn 0.5 cm from one end to produce a small box. Sheets should be marked such that the small boxes (the site where the capture antibody will be placed) are not located along the sheet margins. These dimensions allow for 4 rows of dipsticks per sheet, or about 148 dipsticks per sheet. The desired number of dipsticks may then be cut from the sheet as a single unit or block (*not individually*). Dipsticks may be numbered immediately if your testing needs are known, or the dipsticks may be numbered as they are needed after blocking. Only number dipsticks when they are thoroughly dry.

2) Preparation of stock capture antibody solution. Reconstitute a 1.0-mg vial of lyophilized, unlabeled, goat anti-human IgG (H + L) (Kirkegaard & Perry Lab. Inc., Gaithersburg, MD) with 1.0 ml of a 50% glycerol solution. The reconstituted capture antibody remains stable for at least 3 months at 4–8°C; however, aliquots of the stock capture antibody solution may be stored at −20°C for 1 year.

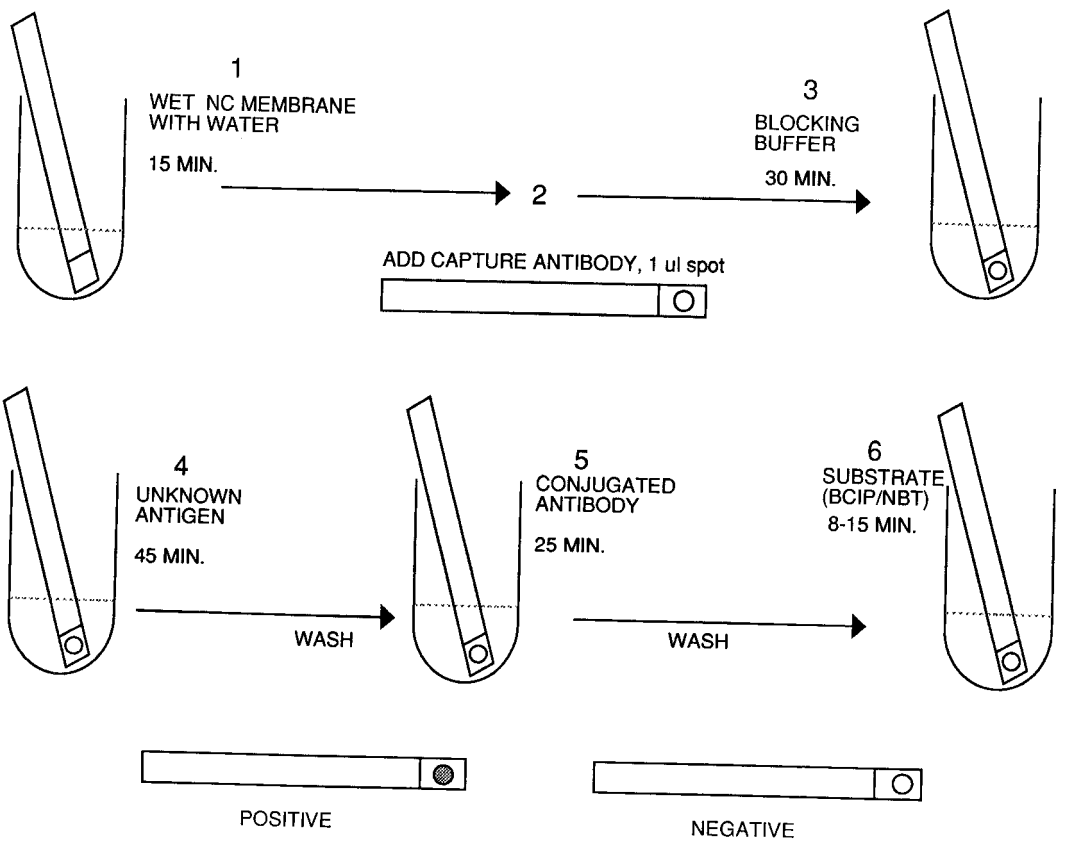

Fig. 1. Schematic of steps in Dipstick ELISA protocol with positive and negative dipsticks. See Materials and Methods for details.

3) Wetting the nitrocellulose membrane. Cut a section from the marked sheet that includes the desired number of dipsticks and wet, or soak, in distilled water for 15 min to prepare the membrane for the adsorption of capture antibody. After 15 min, remove the membrane from the water and lightly blot with a clean paper towel to remove excess moisture.

4) Adsorption of capture antibody. The working capture antibody solution is a 0.2 mg/ml solution, or a 1:5 dilution of the reconstituted capture antibody in phosphate saline buffer (PBS), pH 8.0–8.3. The working solution should be made immediately before use. A small number of dipsticks, 38 or less, should be treated at one time because the dipsticks must be prepared immediately after blotting in the wetting step above. After blotting, but before the nitrocellulose membrane becomes dry, place a 1.0-μl drop of the capture antibody solution in the middle of the small boxed end of each dipstick. The capture antibody will bind and dry in less than 60 sec. Capture antibody may be applied with disposable 5-μl microcapillary displacement pipettes (Cole-Parmer Instrument Co., Chicago, IL), or a variety of repeating pipettes. Allow dipsticks to dry for 2–10 min.

5) Blocking. Submerge dipsticks in a blocking solution [a 1:5 dilution of a 2% milk diluent/blocking concentrate (Kirkegaard & Perry Lab. Inc.) in PBS] for 30 min. Remove dipsticks and air dry. Dipsticks may be used immediately or stored desiccated in a refrigerator or at room temperature (less than 22°C) for 90 days. Although blocking is not absolutely necessary, it reduces background color and also serves to stabilize the capture antibody if dipsticks are to be stored.

6) Preparation of mosquito samples and controls. Whole mosquito specimens or mosquito abdomens may be prepared by triturating an individual specimen in 0.5 ml of PBS in a 1.5-ml microcentrifuge tube with a plastic pestle (Kontes Scientific Glassware/Instruments, Vineland, NJ). Freshly blooded mosquitoes should be held at room temperature for at least 2 h before processing or smeared onto filter paper and punches of filter paper used to prepare the antigen source. When using filter-paper smears as the antigen source place one or 2 punches of the filter paper in a microcentrifuge tube with 300 μl of a diluent solution (1:20 dilution of a 2% milk diluent/blocking concentrate in PBS). Allow the solution to stand at room temperature for 2 h before testing. For each test, at least one negative and one positive control must be run. A 1:201 dilution of serum in PBS from one or more nonhuman sources should be used as negative controls, and a 1:201 to 1:401 dilution of human serum as the positive control. Alternatively, mosquitoes blooded on known hosts could be used as controls. Male mosquitoes should not be used as negative controls in this or any other blood meal ELISA, except as reagent controls.

7) Antigen. Label or number each dipstick for permanent association with a mosquito specimen or control. Cut the desired number of dipsticks, usually 24 per test, from a treated nitrocellulose sheet. Place the treated end of each dipstick in a mosquito homogenate or antigen source for 45 min. Dipsticks may be placed directly into the microcentrifuge tubes from step 6 or into plate-wells containing 200–250 μl of antigen source.

8) Washing and blotting. Remove dipsticks from antigen source and wash by holding them *individually* under flowing distilled water or clean tap water for 10–30 sec. Do not place dipsticks together in a tube for washing. Shake or blot excess water from dipsticks.

9) Preparation of enzyme-conjugated antibody solutions. The stock conjugate solution is prepared by reconstituting a 0.1-mg vial of lyophilized, alkaline phosphatase conjugated, goat anti-human IgG (H + L) (Kirkegaard & Perry Lab. Inc.) and storing as described for the capture antibody in step 2. The working conjugate solution is a 0.2-μg/ml solution, or a 1:501 dilution of the stock conjugated antibody in a diluent solution [diluent solution = 1:20 dilution of a 2% milk diluent/blocking concentrate in PBS], and should be prepared immediately before use.

10) Conjugate step. Place dipsticks individually into microtiter plate-wells or tubes with 200–250 μl of working conjugate solution for 20–25 min.

11) Wash and blot as in step 8.

12) Substrate. Place dipsticks individually into wells containing 200–250 μl of the 3 part BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium) substrate solution (Kirkegaard & Perry Lab. Inc.). Place the required amount of Tris buffer from the substrate kit into a test tube before use so that the buffer may warm to room temperature. The BCIP and NBT concentrate solutions should be added to the Tris buffer immediately before use. A small purple dot will develop in 8–15 min on positive dipsticks. Reaction time should be judged by inspection of the positive control dipstick. All dipsticks should be removed simultaneously when a purple dot is easily visible on the positive control dipstick. As a general rule, it is better to remove the dipsticks a little too early rather than a little too late, because with excessive exposure, all dipsticks that have been exposed to animal IgG will develop to some extent. Air dry dipsticks and attach to a sheet

of heavy paper or a collection form as a permanent record of your assay.

13) Interpretation of results. Test results are interpreted by comparing the reaction on each unknown dipstick with the positive and negative control dipsticks. Test resolution improves after the dipsticks are thoroughly dried, 45–60 min, because background color fades upon drying which increases the contrast between positive spots and surrounding areas.

Evaluation of Dipstick ELISA by World Health Organization (WHO)-interlaboratory trial: In 1987, the above ELISA was evaluated by blind testing of mosquito smears and whole blood samples on filter paper in an interlaboratory trial, sponsored by WHO-Vector Biology Control Division (WHO/VBC) and organized by WHO-Immunology Research and Training Center (WHO-IRTC), Geneva, Switzerland (World Health Organization 1987). The unknowns included filter-paper smears of *Anopheles stephensi* Liston killed at 0, 8, 24, 32 and 48 h after feeding, and a mosquito fed on glucose only. Whole blood, filter-paper samples were prepared by placing a 50-μl dot of each blood sample on a Whatman No. 1 filter paper and drying at 37°C for 1 h before storage and shipment. Whole blood samples included: cow, dog, pig, goat, chicken, 2 pure human samples, one 1:10 mixture of human:cow, and one 1:100 mixture of human:cow. Samples were tested, and the results and dipsticks were forwarded to WHO for evaluation.

Final evaluation: The experiments described in this final evaluation are only those used to document the final version of the ELISA, and the testing of various parameters are not reported on herein.

Specimens of *An. albimanus* Wied. were given their first blood meal on human volunteers, examined for the presence of a blood meal and sorted. Blooded specimens were maintained in an insectary at 27 ± 1°C. After 24 h, mosquitoes were provided with a sugar solution. At 1 h and at 6 h post-blood meal, and every 6 h thereafter, 9 specimens were killed and placed individually into 1.5-ml tubes, and stored at −70°C until analyzed by Dipstick ELISA. Specimens were analyzed by Dipstick ELISA with a 1:401 dilution of human serum in PBS used as positive controls, and a 1:201 dilution of cow serum in PBS used as negative controls.

To investigate specificity and sensitivity of the Dipstick ELISA, we conducted a series of ELISAs, each of which included a 12-step serial dilution of human serum in PBS starting at 1:100 and running through 1:204,800 (12 dipsticks), and 1:200 dilutions of the following animal sera in PBS (12 dipsticks): cow, horse, goat, dog, cat, pig, rabbit, mouse, rat, chicken, raccoon and opossum.

A modified version, the Trough ELISA, for field kits and its evaluation by WHO and the Uniformed Services University of the Health Sciences (USUHS): In 1988–89, we developed a modification of our basic dipstick protocol that employed disposable multichannel pipet troughs (Costar, Cambridge, MA) for joint treatment of dipsticks in the conjugate and substrate steps. We produced 7 kits, each of which included 1,000 preinoculated and preblocked dipsticks and all items needed to conduct the Trough ELISA. These 7 kits were evaluated in a field trial, sponsored by WHO/VBC and organized by WHO-IRTC, Geneva, Switzerland, in collaboration with 5 institutions in malaria endemic areas (Brazil, Cameroon, India, Malaysia and Tanzania) and by WHO-IRTC, Geneva, Switzerland. A description of the kits with detailed instructions for the Trough ELISA prepared by us and included in each kit will be published by WHO (V. Houba, personal communication, 1990) and will not be repeated herein.

RESULTS AND DISCUSSION

Evaluation of Dipstick ELISA by WHO-interlaboratory trial: Filter-paper smears from the digestion series and a glucose-fed specimen were prepared and divided into 3 aliquots each. Dipsticks from all 3 aliquots were positive for samples from 0, 8, 24 and 32 h post-blood meal (World Health Organization 1987, Anonymous 1987). The results from the 48-h post-bloodmeal sample were ambiguous, with 2 dipsticks scored as weak positives and one dipstick scored negative. Tests of the glucose-fed mosquito resulted in 3 negatives.

The Dipstick ELISA correctly identified the following blood samples as nonhuman: cow, dog, pig, goat and chicken. The assay correctly identified the following blood samples as human: 2 separate pure human samples, and a 1:10 mixture of human and cow blood. The single error observed during this blind evaluation was a failure to identify the 1:100 mixture of human:cow blood as positive for human (World Health Organization 1987). For whole blood samples, the efficiency of the ELISA was 90%. [Efficiency = ((true positives + true negatives)/(true positives + false positives + true negatives + false negatives)) × 100].

Final evaluation and troubleshooting: Specimens of *An. albimanus* engorged on humans were tested at time intervals post-blood meal to investigate the sensitivity of the Dipstick ELISA with respect to blood meal digestion. Results indicate that the assay consistently produces strong positive reactions at digestion periods of

Table 1. Sensitivity of Dipstick ELISA, see Materials and Methods section for protocol, to human blood meal after digestion by *Anopheles albimanus* at 27 ± 1°C. Specimens engorged on human subjects at time 0.

Hours postblood meal	Digestion series—replicate number								
	1	2	3	4	5	6	7	8	9
1	++	++	++	++	++	++	++	++	++
6	++	++	++	++	++	++	++	++	++
12	++	++	++	++	++	++	++	++	++
18	++	++	++	++	++	++	++	++	++
24	+	±	+	+	++	+	++	++	+
30	±	+	±	±	±	±	±	−	±
36	+	±	±	+	+	++	+	±	±
42	−	−	±	−	−	−	−	−	−
48	−	−	−	−	−	−	−	−	−
54	−	−	−	−	−	−	−	−	−
Negative control	−	−	−	−	−	−	−	−	−
Positive control	++	++	++	++	++	++	++	++	++

++ = strong positive; + = positive, or definite positive but light; ± = ghost image of uncertain status, scored as negative; − = negative.

18 h or less on frozen specimens (Table 1). At 24 h post-blood meal, 8 of the 9 specimens gave positive to strong positive results, whereas one specimen produced a ghost image of uncertain status and was scored negative. Most dipsticks from tests conducted on specimens of 30 and 36 h post-blood meal developed ghost images that were scored as negatives. At 42, 48 and 54 h post-blood meal, all dipsticks except one displayed clear negative results. These results compare favorably with those of a direct, liquid-phase ELISA for blood meal identification (Beier et al. 1988). The liquid-phase ELISA (Beier et al. 1988) consistently detected human blood meals in frozen specimens up to 23 h post-blood meal with detectability falling off appreciably after 23 h.

The sensitivity and specificity of the assay were documented by 8 separate replicates of the Dipstick ELISA, each of which included a 12-step serial dilution of human serum starting at a 1:100 dilution and running through 1:204,800, and 1:200 dilutions of 12 different animal sera (Table 2). Results indicate that the Dipstick ELISA is sensitive to human IgG at serum dilutions of 1:25,600, and that the assay provides strong positive results at dilutions between 1:400 and 1:12,800. Results from the 1:100 dilution indicate that very concentrated serum samples may yield inconsistent results with about 50% of dots appearing light enough to be considered strong ghost images, rather than positives. To reduce the likelihood of obtaining false negatives from newly engorged specimens we recommend (see step 6, Materials and Methods): that all freshly engorged specimens be held for 2 h before being processed; or that freshly engorged specimens be smeared onto filter paper and punches of the filter paper be used to prepare the antigen source. Additionally, human IgG in insect blood-meals appears to be better preserved by the filter-paper smear technique and by simple drying than by freezing. This effect is apparent in our Dipstick ELISA and also in the liquid-phase ELISA of Beier et al. (1988). In the Dipstick ELISA, human blood meals in frozen specimens were detectable to 24 h post-blood meal, and human blood meals were consistently detected in smears of *An. stephensi* at 32 h post-blood meal (see *Evaluation of Dipstick ELISA by WHO-interlaboratory trial*). In the liquid-phase ELISA (Beier et al. 1988), blood meals from frozen specimens were detectable to 23 h post-blood meal, and human IgG was consistently detected at 32 h post-blood meal in specimens of *An. gambiae s. lat.* and *Anopheles funestus* Giles dried in a desiccator jar at room temperature.

The Dipstick ELISA correctly identified all 12 animal sera as nonhuman in all 8 tests. The distribution of ghost images among the 12 animal sera appears random. It is unlikely that the ghost images represent true cross-reactivity as the animal species known to be most highly cross-reactive, e.g., dog, produced consistently negative results. However, in other modifications of this ELISA we have observed limited cross-reactivity between the human antibody and cow, cat, dog, mouse and rat. These and other nontested host species may cross-react to produce strong ghost images. Ghost images in negative control dipsticks will result in reduced specificity of the human test because each test dipstick is compared with the positive and negative controls to interpret test results. If cross-reactivity appears to be a problem in a study area, it may be reduced by adding sera of cross-reacting species to the anti-human conjugate solution at a 1:501 to a 1:1,001 dilution.

Strong ghost images and even weak positive

Table 2. Results of 8 replicates of the Dipstick ELISA, see Materials and Methods section for protocol, conducted on serial dilutions of human serum and 1:200 dilutions of 12 animal sera.

Dilution	Antigen	Replicate number							
		1	2	3	4	5	6	7	8
1:100	Human	+	+	±	+	±	±	+	±
1:200	Human	+	+	+	+	++	++	+	+
1:400	Human	++	++	++	++	++	++	+	++
1:800	Human	++	++	++	++	++	++	++	++
1:1,600	Human	++	++	++	++	++	++	++	++
1:3,200	Human	++	++	++	++	++	++	++	++
1:6,400	Human	++	++	++	++	++	++	++	++
1:12,800	Human	++	++	++	++	++	++	++	++
1:25,600	Human	++	++	++	++	+	±	+	++
1:51,200	Human	+	+	++	+	±	−	−	±
1:102,400	Human	−	±	±	−	−	−	−	−
1:204,800	Human	−	±	−	−	−	−	−	−
1:200	Cow	−	±	−	−	−	−	−	−
1:200	Horse	−	±	−	−	−	−	−	−
1:200	Goat	−	−	−	−	−	−	−	−
1:200	Dog	−	−	−	−	−	−	−	−
1:200	Cat	−	−	−	−	−	±	−	−
1:200	Pig	±	−	−	−	−	−	−	−
1:200	Rabbit	−	−	−	−	−	−	−	−
1:200	Mouse	−	−	−	−	−	−	−	−
1:200	Rat	−	−	−	−	−	−	−	−
1:200	Chicken	−	−	−	−	−	−	−	−
1:200	Raccoon	−	−	−	−	−	−	−	−
1:200	Opossum	−	−	−	−	−	−	±	−

++ = strong positive; + = positive, or definite positive but light; ± = ghost image of uncertain status, scored as negative; − = negative.

results may appear on known negative dipsticks for reasons other than cross-reactivity. The most common reasons for poor results are inadequate individual washing of dipsticks, joint washing of dipsticks, or allowing the dipsticks to come into direct contact with each other. Another common mistake is the use of too many dipsticks in a single test. This assay is designed for the rapid testing of a relatively small number of mosquitoes, 20–28, at a time. The processing of large numbers of dipsticks during a single test is difficult because dipsticks must be individually handled and washed. We have obtained consistently good results when using 24 dipsticks per test, which allows for 22 unknowns, one positive control and one negative control. Overexposure to substrate or incorrectly mixing the substrate solution may also lead to difficulties in interpreting test results. Time in substrate solution depends on the temperature and the age of the substrate solution. We typically allow the Tris buffer to come up to room temperature before use. However, the BCIP and NBT concentrate solutions should be added to the Tris buffer in the substrate tube only immediately before use. Dipsticks should be removed from the substrate solution simultaneously when a purple circular spot appears on the positive control dipstick. In our experience, this occurs between 8–20 min and commonly from 8–14 min. As a general rule, it is better to remove the dipsticks a little too early rather than a little too late, because with excessive exposure all dipsticks that have been exposed to animal IgG will develop to some extent.

A modified version, the Trough ELISA, for field kits and its evaluation by WHO and USUHS: The field trial of the Trough ELISA by WHO and cooperating laboratories has been completed and the results will be published separately by WHO personnel.

As a means of comparison with the basic protocol outlined in the methods section (Table 2), we conducted 8 separate Trough ELISAs employing the same antigen sources (Table 3). Four false positives (4.17%) were observed among the 96 nonhuman antigen sources in the Trough ELISA (Table 3), versus no false positives in the basic dipstick protocol (Table 2). Additionally, 22 ghost images (22.9%) were observed among the 96 nonhuman antigen sources in the Trough ELISA, versus 5 ghost images (5.2%) in the basic protocol (Table 2). Ghost images in and of themselves are not a serious problem as they can typically be recognized as such and scored as negatives. However, among dipsticks exposed to human serum, note that ghost images (±) and positives (+) are more

Table 3. Results of 8 replicates of the Trough ELISA, a modified Dipstick ELISA that treats dipsticks jointly in troughs during the conjugate and substrate steps, conducted on serial dilutions of human serum and 1:200 dilutions of 12 animal sera.

Dilution	Antigen	Replicate number							
		1	2	3	4	5	6	7	8
1:100	Human	+	±	±	+	±	+	+	+
1:200	Human	+	+	+	+	+	+	+	+
1:400	Human	+	+	+	+	+	++	++	+
1:800	Human	+	+	+	+	+	++	++	++
1:1,600	Human	+	+	+	++	++	++	++	++
1:3,200	Human	++	+	++	++	++	++	++	++
1:6,400	Human	++	++	++	++	++	++	++	++
1:12,800	Human	++	++	++	++	++	++	+	+
1:25,600	Human	+	++	++	+	+	±	−	+
1:51,200	Human	±	±	++	±	+	±	−	−
1:102,400	Human	±	±	±	±	±	−	−	−
1:204,800	Human	±	±	±	±	±	±	−	−
1:200	Cow	−	−	±	+	−	−	−	−
1:200	Horse	±	±	−	−	−	−	−	−
1:200	Goat	−	−	−	+	±	−	−	−
1:200	Dog	−	−	±	−	±	−	−	−
1:200	Cat	−	±	+	−	±	±	−	−
1:200	Pig	±	−	−	−	−	−	−	±
1:200	Rabbit	−	−	−	−	−	−	−	−
1:200	Mouse	−	−	−	±	−	−	−	−
1:200	Rat	−	±	−	−	±	−	−	−
1:200	Chicken	±	−	−	±	±	−	−	−
1:200	Raccoon	±	±	−	+	±	−	−	−
1:200	Opossum	±	±	−	−	−	−	−	−

++ = strong positive; + = positive, or definite positive but light; ± = ghost image of uncertain status, scored as negative; − = negative.

common in the Trough ELISA (Table 3) than in the basic dipstick protocol (Table 2), and that strong positives (++) are less common in the Trough ELISA than in the basic protocol. In total, the contrast between positives (+ and ++) and ghost images is less distinct in the Trough ELISA than in the basic protocol, which increases the uncertainty of scoring for ghost images and light positive dipsticks.

We have experimented extensively with other parameters of the Trough ELISA during the past year. Originally, we believed that it was possible to remove excess antigen by washing and by cutting the time in conjugate to a minimum, and to develop a test that allowed joint treatment of dipsticks in the conjugate and substrate steps. The number of ghost images and false positives can be reduced to less than half the levels observed in Table 3 by extensive washing and by reducing the time in conjugate to 15–20 min. However, it is not possible to consistently remove all the excess antigen by washing, and antigen from one dipstick with excess antigen may contaminate the conjugate solution and produce ghost images or false positives on nearby dipsticks. We documented the presence of antigen contaminate in used conjugate solutions by employing used conjugate solutions as the antigen source in subsequent basic protocol Dipstick ELISAs, while unused conjugate solutions were used as control antigen sources. In each test, which was repeated 5 times, there was sufficient antigen in the used conjugate solutions to produce false positives or ghost images on all exposed dipsticks, whereas an equal number of dipsticks exposed to unused conjugate solutions prepared at the same time gave consistently negative results as would be expected. Obviously, there was a sufficient amount of free antigen contaminant in the used conjugate solution to produce false positives. Therefore, we recommend that the Trough version of the Dipstick ELISA not be used.

CONCLUSIONS

After considerable effort, we have come to the conclusion that each dipstick must be kept separate in all steps. If a microtiter plate-well is employed in the antigen, conjugate and substrate steps outlined in the basic protocol, then false positives will be eliminated, ambiguous ghost images will be rare and the Dipstick ELISA will produce consistently reliable results. The Dipstick ELISA meets requirements for a rapid (less than 2 h when using preblocked dip-

sticks) and simple assay for the identification of human blood meals in a relatively small number of specimens (24 dipsticks per test). The Dipstick ELISA should have particular application to discrete field studies and to short-term or emergency epidemiological investigations. Investigators wishing to conduct a large number of blood meal assays for human and other host species, or those wishing to establish a laboratory for the processing of a large number of specimens on a routine basis would be advised to set up a liquid-phase ELISA (Beier et al. 1988, Service et al. 1986).

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