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TWO-SITE IMMUNORADIOMETRIC ASSAY (IRMA): DETECTION, EFFICIENCY, AND PROCEDURAL MODIFICATIONS

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ABSTRACT. A 2-site immunoradiometric assay (IRMA) has been done using *Anopheles stephensi* fed on *Plasmodium vivax* blood through parafilm to ascertain at what time in the sporogonic cycle circumsporozoite (CS) antigen can be detected, and to detect CS antigen in mosquitoes squashed on filter paper (FP) and cellulose acetate membrane (CAM). The CS antigen was detectable only in mature oocysts, a day prior to sporozoite liberation, and in salivary gland sporozoites. Dissected sporozoites adsorbed on FP/CAM also can be analyzed by IRMA for *Plasmodium* species identification.

A highly sensitive 2-site immunoradiometric assay (IRMA) (Zavala et al. 1982) has been used for the detection of sporozoite antigen in field-collected and dried mosquitoes in several studies. This study was carried out to: 1) ascertain at what time during the sporogonic cycle could circumsporozoite (CS) antigen be detected by IRMA, and 2) detect the sporozoite antigen in mosquitoes squashed on filter paper (FP) and cellulose acetate membrane (CAM), and in sporozoite-positive glands adsorbed on FP and CAM. Samples were also tested to determine if the same squash could be used for the detection of blood meal source.

Anopheles stephensi Liston, type form, were fed on *Plasmodium vivax*-infected human blood through a parafilm membrane and kept in an insectary maintained at 28 ± 1°C and 75 ± 5% RH for parasite development. For objective 1), *An. stephensi* fed on 4 *P. vivax*-infected blood samples were used. Of the fed mosquitoes, 3–4 mosquitoes each day were dried and stored in a plastic tube for the IRMA and the same number examined under a microscope for midgut and salivary gland infection up to 10 days postfeeding. For objective 2), the head and thorax, and abdomen of 4–5 mosquitoes and the same number of whole mosquitoes on days 5, 8, and 11 postfeeding were squashed onto a strip of FP (No. 1 Whatman) and CAM (Cellagram III, Shandon) with the help of a glass rod. Sporozoite-positive glands were dried on 1-cm^2 pieces of FP or CAM. Also, some mosquitoes from batches showing sporozoite positivity were refed on human blood through parafilm before squashing on FP/CAM. These were stored at room temperature (25 ± 5°C) in a plastic box for the IRMA. Mosquitoes squashed on FP/CAM were assayed after storing for different time periods at room temperature. From the same batches, a few mosquitoes were stored in plastic tubes for use as controls.

The IRMA was carried out as per Zavala et al. (1982) with some modifications (Subbarao et al. 1988) using 2F2 (Pv) and 2A10 (Pf) monoclonals. Dry mosquitoes were frozen with PBS buffer containing proteases, *viz.*, antipain, leupeptine, aprotinin, and NP40, and then ground with a glass rod to release the CS antigen. Elution of FP/CAM with squashes was done in PBS buffer containing proteases by freezing and thawing; grinding was not required. However, in assays where mosquitoes were analyzed for blood meal source, elution of FP/CAM was done in PBS buffer without proteases and after taking the aliquot for blood meal analysis, proteases were added for IRMA. Blood meal analysis was carried out on agarose gels using countercurrent immunoelectrophoresis (CCIE) (Bray et al. 1984) with some modifications.

Extrinsic development of *P. vivax* took about 7–9 days at 28 ± 1°C. Oocysts were visible in the gut on day 3 after the infective blood meal in all replicates. The oocysts increased in size progressively and sporozoites were detected in the salivary glands on day 7. Sporozoite antigen was detected by IRMA from day 7 onwards in all the infected mosquitoes except in one, where it was detected on day 6 (Table 1). Immunoelectron microscopic studies by Nagasawa et al. (1988) and the immunostaining procedure of Golenda et al. (1990) have shown that the sporozoite surface antigens appear in the early oocyst stage and their quantity increases progressively as sporozoite development proceeds. In our experiments the CS antigen was detectable on day 7 onwards after feeding. In one of the batches head plus thorax and abdomen were assayed separately, CS antigen was detected in the abdomen only on day 7. This confirms that the CS antigen is detectable only in sporozoites or in oocysts of *P. vivax* that are ready for sporozoite liberation. Similar observations were made by Collins et al. (1984) with *Plasmodium knowlesi* and *Plasmodium cynomolgi,* where they found that CS antigen could be detected in infected mosquitoes approximately 24 h before sporozoites appear in the salivary glands. Thus,

Table 1. Comparison of batch positivity by immunoradiometric assay (IRMA) and dissection in *Anopheles stephensi*.

Days after feeding on *Plasmodium vivax*-infected blood	IRMA		Dissection		
				No. positive	
	No. tested	No. positive (%)	No. tested	Oocysts (%)	Sporozoites (%)
Days 1 and 2	24	0	0	0	0
Days 3, 4, and 5	30	0	33	22 (66.7)	0
Day 6	8	1 (12.5)	13	10 (76.9)	0
Day 7 onwards					
Mosquitoes stored dry	26	9 (34.6)	26	12 (46.2)	8 (30.8)
Squashed on FP	42	23 (54.8)	20	6 (30.0)	10 (50.0)
Squashed on CAM	42	16 (38.1)	20	6 (30.0)	10 (50.0)
Refed on human blood and squashed on FP[1]	17	10 (58.8)	36	9 (25.0)	20 (55.5)
Refed on human blood and squashed on CAM[1]	16	8 (50.0)	36	9 (25.0)	20 (55.5)

[1] All samples showed human antigen positivity by countercurrent immunoelectrophoresis (Bray et al. 1984).

it appears that the sporozoite rates estimated by IRMA are primarily due to gland-positive mosquitoes, although a fraction of positive mosquitoes may be attributed to the detection of CS antigen in mature oocysts.

The results obtained for mosquitoes squashed on FP and CAM were comparable to those for dried mosquitoes stored in plastic tubes (Table 1). For the same batch of mosquitoes, counts per minute (cpm) observed for sporozoite-positive mosquitoes adsorbed on FP/CAM were in the range of 2,200–13,500, whereas cpm for dried mosquitoes were in the range of 1,450–5,350. This suggests efficient elution of CS antigen bound to the FP or CAM by the method used. In both matrices, CS antigen was not detected in day-5 mosquitoes both in head plus thorax and abdomen, and in day-8 and -11 mosquitoes, it was detected in both body portions. This supports our earlier mentioned findings that the IRMA detects CS antigen only in matured sporozoites. Squashing of mosquitoes on FP/CAM instead of storing them as such did not affect the specificity, as none of the mosquitoes tested showed positivity against *Plasmodium falciparum* monoclonals (2A10). The CS antigen was detected in mosquito squashes stored for 1½ years at room temperature. Our results also indicated that a single smear can be used both for sporozoite detection and to determine the source of a blood meal (Table 1).

The CS antigen was also detected in strips of CAM and FP that had sporozoite-positive salivary glands dried onto them. Samples stored for 5–6 months at room temperature showed 69.6% positive (16 positives out of 23 tested) on FP and 61.5% positive (8 positives out of 13 tested) on CAM. Beier et al. (1991) reported 50.4% positivity by ELISA on microslides having dried *P. falciparum* sporozoites tested after a month. The more than 30% negativity for CS antigen (Pv 210) in sporozoite-positive samples may be due to the presence of another variant of the CS antigen, Pv 247. Kain et al. (1991) have reported the presence of the Pv 247 variant in Indian isolates.

Collection of samples on FP/CAM would simplify the procedures of labelling, storing, transporting, and processing mosquito samples. In laboratories where dissection of salivary glands for sporozoite positivity under the light microscope is the only means of incrimination, dissected sporozoites could be adsorbed on FP/CAM and sent to a reference laboratory where facilities for IRMA are available for sporozoite species identification.

Circumsporozoite antigen was detected in 5–6-year-old *P. vivax*-infected mosquitoes stored at 28–35°C, indicating the stability of CS protein at room temperature.

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