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THE USE OF IMMUNORADIOMETRIC ASSAYS FOR THE DETECTION OF THE INVERTEBRATE HOST (ANOPHELES) INFECTION AND OF SYNTHETIC PEPTIDE FOR THE STUDY OF THE LEVEL OF ANTI-SPOROZOITE ANTIBODIES: A REVIEW

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General procedures for two IRMA's and their applicability to field studies have been discussed. The 2-side IRMA can be used with specificity and sensitivity to determine the Plasmodium *species being transmitted by any given mosquito vector, and it also allows quantitation of the sporozoite load. The synthetic peptide-based IRMA allows specific determination of the* Plasmodium *inducing the antibody response in the sera of individuals living in malaria endemic areas, and will also allow quantitation of antisporozoite anbibody levels. Both of these assays will most certainly be used to determine the effectiveness of malaria vaccine field studies.*

This paper reviews the use of an immunoradiometric assay (IRMA), in conjunction with monoclonal antibodies (moABs), as an epidemiological tool for surveying mosquito populations for their ability to transmit malaria to man. It also discusses the use of an IRMA, based on synthetic peptides corresponding to the repetitive immunodominant epitopes of circumsporozoite (CS) proteins (Nussenzweig & Nussenzweig, 1984), for detecting antisporozoite antibodies in the sera of individuals living in malaria endemic areas.

Two-site immunoradiometric assay – Prior to the development of immunoassays based on moABs specific for the sporozoite stage of malaria parasites, the only methodology available for the determination of mosquito infection rates was salivary gland and midgut dissection combined with microscopic examination. This procedure is very laborious and since it requires living mosquitoes, it must be performed close to collection sites. In addition, the plasmodial species of the sporozoites detected by dissection cannot be determined by morphology, a severe shortcoming of this method in areas where multiple species of Plasmodia are present.

The 2-site IRMA, described by Zavala et al. (1982, 1983), uses species specific antisporozoite moABs for the detection of CS antigen in dried field collected mosquitoes. This assay is simple to perform and permits large numbers of mosquitoes to be efficiently processed. In addition, it allows specific determination of the infecting *Plasmodium* species and quantitation of the sporozoite load.

The methodology for the IRMA is briefly summarized. CS antigen is extracted by triturating dried mosquitoes in PBS containing BSA, protease inhibitors, and the detergent Nonidet P-40. Mosquitoes can be processed separately, or in areas where mosquito infection rates are known to be low, mosquitoes can be pooled together for processing. The presence of large amounts of contaminating material from uninfected *Anopheles* does not interfere with the specific interaction of the assay. Mosquito extracts can be frozen at -20°C and retain their reactivity for months.

MoAB is bound to wells of microtiter plates by overnight incubation at room temperature, and the following morning, the plates are washed with PBS-BSA prior to the addition of mosquito extracts. We have found that for all washes, BSA can be substituted for by non-fat powdered milk which is much cheaper than BSA. After a two hour incubation of mosquito extracts with the antibody coated wells, the plates are washed and ^{125}I-labeled moAB is added for an additional one hour. The labeled moAB is the same as the cold capture moAB. Finally the plates are washed, dried, and counted in a gamma counter.

Non-specific binding of microbial and mosquito antigens is measured by adding extracts of mosquitoes infected with an unrelated plasmodial parasite to the wells.

We recently used this assay to obtain information on vectors and potential vectors of malaria in northern Brazil (De Arruda et al., 1986). Our study area was the state of Pará where there is extensive year-round transmission of both *Plasmodium falciparum* and *P. vivax*. We were

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interested in looking at malaria transmission in this area because no major study had been done on malaria transmission since the extensive studies of Deane et al. (1946). In recent years, the ecology of the state of Pará has been dramatically changed by man's exploitation of the land for its natural resources and the development of hydro-electric energy. The resulting influx of large numbers of non-immune migrant workers has undoubtedly greatly increased the incidence of both *P. vivax* and *P. falciparum* malaria.

Six districts were chosen for study and there was a total of 30 collection sites, consisting mainly of small riparian villages of 25 to 50 families. Mosquitoes were caught biting volunteers either indoors or outdooors.

11,002 Anophelines were randomly divided into three groups and taxonomically identified. One group was processed by dissection. The other mosquitoes, after drying over chemical dessicant, were processed using either the IRMA or the ELISA.

A total of 4,730 *Anopheles*, representing seven species, was assayed for *P. falciparum* or *P. vivax* sporozoite antigen, using the respective monoclonals in a 2-site IRMA (Table I). *A. darlingi* was the only species found to be infected with *P. falciparum*. *P. vivax* had a broader host range and was found in four of the *Anopheles* species listed. For both *P. vivax* and *P. falciparum*, the mosquito infection rates were unexpectedly high.

TABLE I

Prevalence of *Plasmodium vivax* and *P. falciparum* infected *Anopheles* as determined either by the IRMA or the ELISA

	Number mosquitoes (%) infected				No. mosquitoes assayed	
	P. vivax		*P. falciparum*			
Anopheles sp.*	IRMA	ELISA	IRMA	ELISA	IRMA	ELISA
darlingi	25 (1.3)	18 (0.9)	53 (2.7)	85 (4.2)	1929	2043
albitarsis	83 (4.5)	5 (2.2)	0	0	1858	227
triannulatus	9 (12.0)	12 (8.9)	0	0	75	135
nuneztovari	9 (1.5)	5 (1.2)	0	0	593	433
oswaldoi	0	1 (0.2)	0	10 (2.3)*	116	442

*A. *intermedius* (86) and *A. braziliensis* (73) were not found infected using the IRMA. *A. aquasalis* (981), *A. intermedius* (19), *A. minor* (2), *A. braziliensis* (17), and *A. peryassui* (11) were not found infected using the ELISA.

+ All these *Anopheles* were collected in the district of Capanema.

(Taken from De Arruda et al., 1986)

These data are interesting for a number of reasons. In the case of *P. vivax*, none of the four mosquito species in which we found CS antigen, with the exception of *A. darlingi*, had been implicated as malaria vectors of any significance in northern Brazil. Our data do, in fact, indicate a higher *P. vivax* infection rate in *A. albitarsis*, *A. triannulatus*, and *A. nuneztovari* than in *A. darlingi*.

The failure to detect *P. falciparum* sporozoite antigen in *A. albitarsis*, *A. triannulatus*, and *A. nuneztovari* is striking, since many of these mosquitoes were collected along with *A. darlingi* in districts where both *P. vivax* and *P. falciparum* were present in a high percentage of blood films of the local populations. The data strongly suggest the possibility that either *A. albitarsis*, *A. triannulatus*, and *A. nuneztovari* are totally refractory to infection with *P. falciparum* or that the oocysts abort early in their development before the appearance of the CS antigen.

As shown in a study done by Collins et al. (1984) on the transmission of *P. falciparum* in the Gambia, West Africa, the IRMA can also be used to quantitate the number of sporozoites in mosquitoes, that is, to determine the sporozoite load. To do this, a standard curve is prepared using known numbers of sporozoites (Fig. 1). In this assay, CS antigen extracted from 47 sporozoites was detected. The counts obtained using the 47 sporozoites were above those obtained using negative control mosquitoes infected with an unrelated *Plasmodium*. 7.3% (94 out of 1,280) of the field collected mosquitoes tested positive. In this particular study, the mosquito extracts were brought up to a volume of 300μl before being tested. Only 30μl were used for the assay, that is, 1/10th of the total mosquito extract was used, an thus the threshold of sensitivity of the assay

for infections in the field-collected mosquitoes was around 500 sporozoites. By extrapolation, one can determine the sporozoite load of each of the field-collected mosquitoes.

A couple of points should be stressed regarding the IRMA. If the field collected mosquitoes are considered to have light sporozoite loads, then the mosquito extracts should be diluted as minimally as possible before testing, or else the CS antigen may be diluted out and the mosquito will test negative. Because laboratory conditions vary from one assay to the next, standard curves should be prepared each time field collected mosquitoes are assayed. In addition, the sensitivity of the IRMA depends on the moAB that is used and its binding affinity.

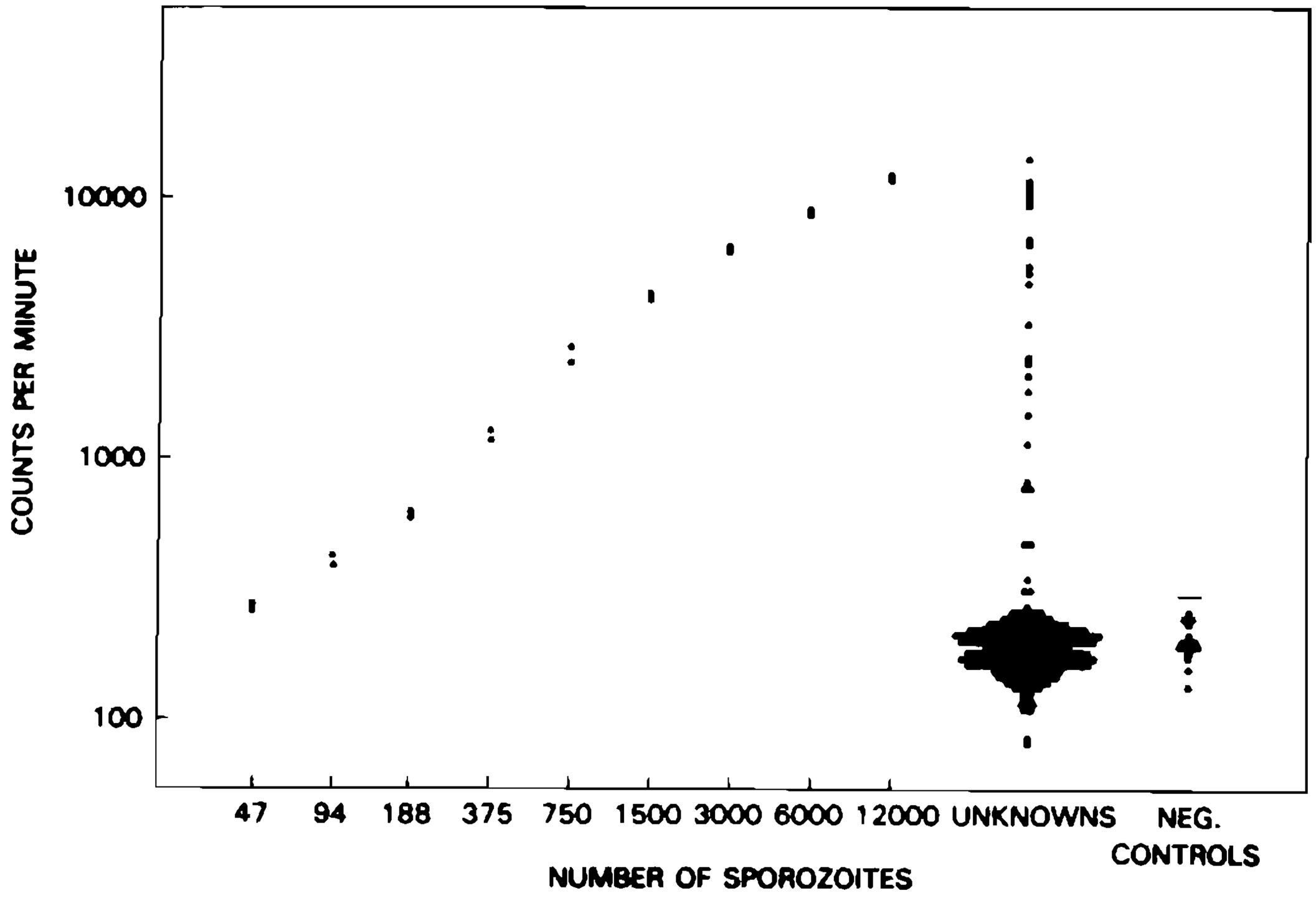

Fig. 1: Results of a typical immunoradiometric assay run. The standard curve, control and unknown mosquitoes were prepared and processed by the procedure described in the text. Each point shows the number of counts recorded over 1 min for a single 30 μl aliquot. The small horizontal line above the negative control group represents the point below which 99.5% of the control counts fall when such counts are fitted to a t-distribution. (Taken from Collins et al., 1984).

One shortcoming of the two-site IRMA for determining plasmodial infections in mosquitoes is that if the whole mosquito is used for the assay, it does not discriminate between sporozoites derived from an oocyst infection and a salivary gland infection. Separation of abdomens and thoraces and assaying the two parts of the mosquitoes separately in part alleviates this problem. However, even using this approach, one cannot say with certainty that sporozoites have invaded and localized in the salivary glands and that the mosquito is therefore fully capable of transmitting the parasite.

Synthetic peptide based IRMA for detecting humoral response to sporozoites – The IRMA can also be used to detect antisporozoite antibodies in the sera of individuals living in malaria endemic areas. The antigen for this assay is synthetic peptide corresponding to the repeated immunodominant epitope of the CS protein. These epitopes are strictly species and stage specific, and in the case of *P. falciparum* and *P. vivax*, it has been shown that the epitopes are conserved among large numbers of geographic isolates (Zavala et al., 1985a).

The data presented are taken from a paper published by Zavala et al. (1985b). The antigen for the assay was the synthetic peptide asparagine-alanine-asparagine-proline repeated three times – $(NANP)_3$. A series of studies had shown that the dominant epitope of the *P. falciparum* CS protein was contained in the $(NANP)_3$ sequence.

The methodology for the assay is briefly summarized. Antigen conjugates are prepared by incubating peptide in PBS containing BSA and glutaraldehyde for five hours at room temperature. 2M lysine is added, and after an additional 30 minutes of incubation, the preparation is aliquoted and stored at - 20°C.

Antigen conjugate is bound to the wells of microtiter plates by overnight incubation at room temperature. This is followed by a second overnight incubation of the wells with PBS containing BSA and ethanolamine. These plates can be stored frozen for a period of at least several months and still retain their reactivity.

For testing the sera are diluted in the PBS-BSA-ethanolamine buffer containing also 0.5% Tween-20 and 0.1% NP-40. Following centrifugation, the sera are incubated with the peptide-coated plates, and bound antibody is detected by adding ^{125} I-labeled anti-human immunoglobulin. Finally, the wells are washed, dried, and counted in a gamma counter.

Using this protocol, 58 blood samples obtained in the Gambia, West Africa, were assayed for the presence of antibodies directed against *P. falciparum* sporozoites. The results indicated that the immune response of humans to sporozoites is age dependent, as was previously shown by Nardin et al. (1979) using an indirect immunofluorescent assay. Using the IRMA, the percentage of positive sera increased with age, ranging from 21% in children 1 to 14 years old to 85% in adults older than 34 years.

In the study of Zavala et al. (1985b) an indirect immunofluorescent assay was used to detect antibodies to sporozoites in randomly selected IRMA-negative and IRMA-positive sera. It was found that there was a highly significant correlation between the IRMA results and immunofluorescent titers (Table II). Of the eight IRMA-negative sera that were examined, six were also negative by immunofluorescence, and two had very low immunofluorescent titers. It should be pointed out that G.Z. is a human volunteer who was vaccinated with x-irradiated *P. falciparum* sporozoites and who was protected against sporozoite challenge.

TABLE II

Specificity of anti-*P. falciparum* sporozoite antibodies in the sera of randomly selected individuals older than 20 years of age and living in a malaria endemic area

Identification of serum	IRMA with $(NAMP)_3$ as antigen (Δcpm)*	IFA with glutaraldehyde fixed sporozoites as antigen	
		Serum Titer	Serum Titer in the presence of $(NAMP)_3$ **
G.Z.+	9201	4096	320
IDA	4851	1280	<20
8017	3539	640	<20
7930	3501	640	<20
7979	3311	640	<20
7973	2735	320	<20
P-2	2473	320	<20
P-5	2024	320	<20
8012	1765	640	<20
Normal	163	<10	N.D.
7981	168	<10	N.D.
8074	133	20	N.D.
7878	96	<10	N.D.
P-12	75	<10	N.D.
8312	72	<10	N.D.
P-11	-13	<10	N.D.
8286	-91	20	N.D.
7907	-103	<10	N.D.

*When the results of IFA and IRMA were compared by a non-parametric method (Spearman Rank Correlation), the r_s was 0.87 ($p < 0.001$).

**Serum samples were incubated with 50 μg/ml $(NANP)_3$ for 2 hours at room temperature before performing the IFA. N.D. = not done.

+ G.Z. is a human volunteer vaccinated with irradiated *P. falciparum* sporozoites, and protected against malaria infection.

(Taken from Zavala et al., 1985b).

When the field-collected positive sera and the G.Z. serum were preincubated with soluble $(NANP)_3$ peptide, the immunofluorescent response was greatly reduced or totally abolished (Table II), indicating that in these sera, most or all of the antisporozoite antibodies were directed against the epitope defined by the $(NANP)_3$ sequence.

Titers reached by the field-collected and G.Z. sera in the IRMA were also determined. At all dilutions, the laboratory-induced G.Z. serum gave higher counts than the field-collected sera with their antisporozoite antibodies induced under natural conditions by the bites of infected mosquitoes. Most of the field-collected sera were, however, still positive in the IRMA at a 1:160 ·r, and some were positive at a dilution of 1:320.

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