THE SPOROZOITE ENZYME-LINKED IMMUNOSORBENT ASSAY : APPLICATION IN MALARIA EPIDEMIOLOGY

*Michael J. Bangs**

ABSTRACT

Recent biotechnological breakthroughs have led to the development of various methods for detection and identification of human pathogens in their vectors. Monoclonal antibodies produced against malaria sporozoite antigens have permitted the development of several sensitive, species specific immunological tests (IFA, IRMA, ELISA). One of these, a two-site enzyme-linked immunosorbent assay (ELISA) has been developed as a useful epidemiological tool in the identification of malaria-infected mosquitoes. This method employs highly species specific monoclonal antibodies that recognize the repetitive immunodominant epitope of the circumsporozoite (CS) protein. Monoclonal antibodies have been developed for all four species of human malaria. The key feature of the ELISA technique is the use of an enzyme indicator for an immunological reaction. The antigen capture or "sandwich" ELISA configuration uses the purified monoclonal both as the solid phase and, conjugated to enzyme, as a marker for the presence of CS protein in a mosquito homogenate incubated in the wells of a microtitration plate. This technology has shown advantages over other methods for epidemiological data collection. Mosquitoes can be caught, dried and stored until a time convenient for examination. The sporozoite rate by *Plasmodium* species can be identified easily, and when combined with the man-biting rate provides the sporozoite inoculation rate, an important entomologic estimate of the number of potential infective bites a person could expect over a given period of time. Presently, mosquitoes can be tested individually or pooled up to 20 anophelines. The assay is sensitive enough to detect 1 infected mosquito per pool or as few as 25 sporozoites per 50 µl of mosquito extract. Basic principles and procedures are covered concerning solid substrate, adsorption to solid substrate, buffers and wash solutions, conjugates and enzyme substrates. The advantages and limitations of this technique in present malaria studies is discussed.

INTRODUCTION

Recent biotechnological breakthroughs have lead to the development of various methods for detection and identification of human and animal pathogens in their vectors. One of these, a two-site or double sided enzyme-linked immunosorbent assay (ELISA) has been developed as a useful epidemiological tool in the identification of malaria-infected mosquitoes[1,2,3]. This method employs highly species-specific monoclonal antibodies[4] that recognize and capture the repetitive immunodominant epitope of the circumsporozoite (CS) protein of plasmodia parasites[5]. To date, monoclonal antibodies have been developed for all four sporozoite species of human malaria. Monoclonal antibodies produced against malaria sporozoite surface membrane antigens have permitted

* Head, Entomology Division, U.S. Naval Medical Research Unit No. 2 Jakarta Detachment APO San Fransisco, CA 96356

species-specific immunological tests, namely the immunofluorescent and immunoradiometric assays[6,7]. DNA hybridization assays, a competitive and potentially powerful technique, have received attention as well[8]. However, one significant drawback is that the assay is not stage specific. DNA probes need to be developed that can discriminate between stages in the life cycle. This involves detection of gene transcripts, messenger RNAs that are stage specific and sufficiently abundant. No such assays are yet available. This paper will review only the sporozoite ELISA and its potential use in malaria epidemiologic studies.

VECTOR-PARASITE RELATIONSHIP

In order to fully appreciate the sporozoite ELISA technique and its use in field malaria epidemiology, an understanding of the plasmodia extrinsic (sexual) life cycle within the vector is essential[9]. In the normal course of events, a female anopheline mosquito will imbibe blood as a protein source for egg development while at the same time ingesting infective male and female gametocytes from the patient individual. Fertilization occurs in the midgut of the mosquito which results in a zygote (ookinete). This stage invades the midgut epithelium and begins oocyst development. Depending on temperature, parasite species and strain and vector competence, the oocyst will complete maturation and rupture in 8 to 20 days emptying into the hemocoel of the mosquito hundreds, if not thousands of sporozoites. When released the sporozoites move throughout the body of the mosquito. If successful, many invade the salivary glands where they await injection into a susceptible host when the female attempts her next blood meal. Competent mosquitoes are capable of producing a range of 100 to greater than 10,000 sporozoites from a single infective feeding. The species specific monoclonal antibodies produced by a hybrid cell line and used in the ELISA express the surface coat antigen or circumsporozoite protein (CSP) of the sporozoite.

ELISA PRINCIPLES AND METHODOLOGY

The key feature of the ELISA technique is the use of an enzyme indicator for an immunological reaction. A great deal of time and effort goes into the development and refinement of an ELISA according to the objectives of the test. A wide variety of substrates, buffers, wash solutions and conjugates need to be screened in order to obtain the best possible reaction desired. Enzyme-substrate combinations have a range of options that require careful investigation to arrive at the optimal configuration for the task[10].

If necessary, a battery of produced monoclonals must be screened eliminating those of low sensitivity and cross-reactivity[11]. In general, plastics with a range and variety of adsorptive properties have become almost universally accepted as the solid substrate of choice. Antigens or antibodies may be passively adsorbed to the solid substrate or they may be linked using a variety of techniques[10]. Choice of blocking buffer may have a profound effect on the outcome of the assay and it should be thoroughly evaluated. Most wash solutions contain weak detergents which help reduce non-specific binding reactions. A wide variety of enzymes and substrates are available and should be tailored to the optimal configuration. If results are to be

read objectively, plate readers can be set at specific wavelengths based on the absorption profile of the substrate being used.

A variety of configurations are available depending on the specific purpose of the test. Common configurations in use include direct (antigen detection), indirect (antibody detection), antigen capture, antibody capture, and competitive or blocking ELISAs. Amplification systems, for example, peroxidase anti-peroxidase, or biotin-streptidin, are also available to help increase the specific signal or reaction. The sporozoite ELISA is an antigen capture or "sandwich" configuration which is a useful tool for the rapid detection of specific CSP antigens.

THE SPOROZOITE ELISA

This system uses the purified monoclonal antibody (MAb) both as the solid phase and, conjugated to enzyme, as a marker for the presence of CS protein. There is no need to purify the antigen nor attach it to the solid phase. The test described herein was developed by investigators at the Walter Reed Army Institute for Research. The sandwich ELISA is begun by adsorption of the capture MAb to the wells of a plastic microtitration plate. After 30 minutes incubation at room temperature the well contents are aspirated and the remaining active binding sites on the plate are blocked with blocking buffer. After species identification, mosquitoes to be tested are ground in blocking buffer containing NONIDET P-40, diluted with blocking buffer, and 50 µl aliquots added to the wells. The remaining mosquito triturate should be frozen for later assays if necessary. It has also been shown that freezing and thawing the triturate causes increased CS antigen disruption which results in greater test specificity. Positive and negative controls are also added to specific wells at this time. Measured amounts of recombinant proteins or actual NP-40 treated sporozoites are used for the quantitative positive controls.

When CS antigen is present it will form an antigen-antibody complex with the capture MAb. After 2 hours incubation, the mosquito triturate is aspirated and the wells are washed. The respective peroxidase-linked MAb is then added to the wells. If CS antigen has been captured, this step will complete the formation of the "sandwich". After 1 hour incubation the well contents are aspirated, the plate is washed and the clear peroxidase substrate solution is added. As the peroxidase enzyme reacts with the substrate a dark green product is formed. The intensity of the colorimetric reaction (signal) is in proportion to the amount of CS antigen present in the test sample. Results are read visually or at 414 nm using an ELISA plate reader 30 or 60 minutes after the substrate is added. Positive (= reactive) samples must be retested for confirmation and, if desired, an estimation of sporozoite load per mosquito can be made by making quantitative comparisons with recombinant CS protein optical density readings (reproduced from ELISA kit instructions, R.A. Wirtz).

A 96 well U-bottom polyvinyl microtitration plate is used for the solid substrate. Typically, the outer wells of the plate are not used because of variability in binding and non-specific reactions. A reactive or "positive" control is placed in the upper left. The five wells directly under this positive control contain laboratory-reared non-reactive or "negative" controls. Mosquitoes can be tested individually or can be pooled into 5 or 10 head-thoraxes per well allowing up to 540 assayed mosquitoes per plate. This could be warranted in areas where the expected sporozoite rate is less than 1%. A favorable

property of pooling is the robustness against the unwanted effects of variations in the specificity of the test. Generally, a balance between pool size, sensitivity and specificity has to be found with respect to the loss of information on the one hand and the gain in time and labor on the other[12]. Several important modifications have occurred recently, the most significant being a reduction in required incubation time. The incubation time of capture antibody to the solid substrate has been reduced from overnight to 30 minutes, which has resulted in a significant saving of time and an increase in assay sensitivity. The entire test can now be performed within 5 hours.

The current advantages and limitations of this assay are listed as follows :

Advantages :

- *Plasmodium* species-specific detection of infected and infective mosquitoes.
- Capable of detecting 100 sporozoites per mosquito (25 sporozoites per 50 µl triturate).
- Potentially a more reliable method for sporozoite detection where mosquito infection rates and/or sporozoite densities are low or very low.
- Use of stable reagents, most are commercially available.
- Ability to test dry, frozen or freshly caught mosquitoes.
- Less time consuming and labor intensive than standard microscopic dissection methods.
- Results can be read visually or quantitatively
- Increased ability of identifying primary and secondary vectors.

Limitations :

- Positive control materials and monoclonal antibodies (MAb) are not available commercially.
- Refrigeration requirements for prepared PBS, blocking buffer, mosquito grinding solution, wash solution, MAbs and positive control stock solutions.
- Increased costs for reagents and supplies over standard dissection method.
- Possible existence of non-reactive human parasite strains to standard MAbs because of natural phenotypic heterogeneity in the repetitive domain (epitope).
- A positive ELISA on a mosquito is not proof that the species is a vector (i.e. results are not synonymous with salivary gland sporozoite rates). It must be remembered that CS antigen is present in developing oocysts (Beier et al, 1988) as well as free sporozoites that can be found nearly anywhere in the body of the mosquito[13].

This last limitation is an important one with regards to malaria epidemiology. How accurate is this technique in establishing sporozoite rates when compared to standard dissection methods ?

A recent study by Robert et al[13] investigated the distribution of circumsporozoite antigen in *Anopheles gambiae* infected with *Plasmodium falciparum*. They found the existence of free antigen associated with sporozoites 14 days post-infection in the head, salivary glands, thorax, midgut, legs, ovaries, malpighian tubules and other sites using a similar CS ELISA. Ponnudurai et al[14] found that less than 50% of the *P. falciparum* sporozoites were present in the salivary glands of mosquitoes three weeks post infection. The remainder were found distributed

throughout the rest of the mosquito body. They also observed the number of sporozoites found in the thorax was always lower than that predicted by ELISA, concluding that large quantities of CS antigen is shed in infected salivary glands. These findings confirm the possibility of detecting hemocoel sporozoites by ELISA which do not enter the salivary glands even when restricting the assay to the head-thorax portion of mosquito. The presence of CS antigen in the thorax of one mosquito and the absence of sporozoites and CS antigen from its salivary glands show that using thoraxes, the ELISA cannot discriminate between infected and infective mosquitoes.

Why is this distinction important ? The sporozoite rate is considered the most important entomological factor in the epidemiology of human malaria[11]. It is a measure of the proportion of *infective* mosquitoes from the total anophelines examined[15]. Epidemiologically, we are interested in knowing the sporozoite rate at particular points in time. Unfortunately, what percentage of infected mosquitoes become infective is dependent on many factors, principle of which is the daily survival rate. Will the mosquito live long enough to become infective ? If we extrapolate carelessly, we could greatly overestimate the vectorial capacity in the anopheline population based on exaggerated sporozoite rates. It would also be possible for a nonvector species to test positive because some anophelines are capable of supporting the development of sporozoites that are unable to enter the salivary glands[16]. In short, infection does not necessary lead to infectivity.

The World Health Organization has recommended the use of ELISA and immunoradiometric techniques for sporozoite detection in field caught mosquitoes, however, there exists conflicting information in the literature regarding its accuracy and applicability in epidemiologic work. Wirtz et al[11] in Papua New Guinea found excellent correlation between the ELISA and standard dissection results; no significant difference was seen between the two methods. In neighboring Irian Jaya (Indonesia), we compared the two methods but in a less rigorous way (Bangs et al, unpublished). Based on our results we found a significant difference between the two methods. The ELISA (head-thorax only) detected 6-fold more sporozoite antigen positive mosquitoes than dissection methods observing actual sporozoites in the salivary glands. Koros et al[17] from Kenya showed the ELISA tests overestimated the proportion of *Anopheles* with salivary gland sporozoites by 44.7 % and that dissection techniques were more sensitive than ELISA for detecting low-grade infections.

These results indicate that it is essential that newly described vectors, especially secondary ones, need to be confirmed through experimental vector competence studies to actually detect sporozoites in the salivary glands.

Applications in Field Malaria Epidemiology :

What about the practical use of this technique in malaria epidemiology ? It is essential that all results be reported and interpreted with respect to the limitation that an ELISA positive is not conclusive evidence for establishing an individual mosquito as a vector, but rather shows that CS antigen can develop in that species.

The primary limitation of the sporozoite ELISA is that it cannot distinguish between infected and infective mosquitoes. This produces some inherent problems with estimation of the true sporozoite rate. Even

when restricting the assay to the head-thorax portion of the mosquito, salivary gland false-positives are possible because of circulating CSP. This drawback must be kept in mind when interpreting assay results.

The sporozoite rate bears a direct relationship to the risk of malaria infections in the human population. Accurate estimation of this rate plays a fundamental role in malaria epidemiology and helps us to estimate the intensity of transmission and vectorial capacity at a given time and area[18].

The development of the ELISA has dramatically increased our ability for the detection, identification and quantitation of sporozoites in mosquitoes over standard dissection methods. A number of studies have been made in evaluating the applied use of the sporozoite ELISA[19,20,21,22,23]. The ELISA has also played an important part in recent epidemiological studies in Papua New Guinea[24,25,26]. Increased awareness of entomologic transmission dynamics have helped towards a better understanding of the complex inter-relationship between vector, parasite and host. This test has allowed us to examine a greater number and variety of anopheline species, determine their importance, and direct specific control and surveillance activities towards them.

The sporozoite rate, in combination with other parameters, such as biting rate and host preference, permits an estimation of other important transmission indexes, such as the entomological or daily sporozoite inoculation rate (EIR). The EIR is a function of the sporozoite rate and human-biting rate (average number of bites/human/night). This index is considered the most important determinant of malaria prevalence[27]. It reflects the expected number of infective bites/human/night. From the EIR, calculations on the number of expected sporozoite infective bites one could expect over the entire year can be made by multipying the average EIR for all sampling periods by 365 days. The inversion function of the EIR (I/EIR) is the time to inoculation exposure, which represents the theoretical time required for an individual to be exposed to one infective bite. The days to infective inoculation can illustrate the seasonal difference in transmission rates correlated with rainfall or other variables. Relationships between feeding preference (human blood index) and sporozoite rate provides information on vectorial capacity and for predicting the response change imposed during intervention programs[28].

To illustrate some examples of what the ELISA can provide, Burkot et al[24] have shown correlation between the total EIR and parasite prevalence in children. They also found increased efficiency of transmission of *P. falciparum* which they attributed to heavier sporozoite densities in naturally infected mosquitoes compared to mosquitoes infected with *P. vivax*. Burkot et al[25] were able to show variation in average sporozoite and inoculation rates among different villages of close geographic proximity as well as varying rates within a village over time.

Biotechnologic advances that are both appropriate and applicable for field use will always be welcomed by investigators and control personnel to more accurately measure malaria incidence and intensity of transmission. Further laboratory and field evaluations and refinements of the sporozoite ELISA will hopefully help to overcome many of its current limitations. In the event of phase IIb or III malaria vaccine field trials, background information on transmission dynamics and exposure rates will be invaluable for site selection, timing and vaccine evaluation[29]. It is hoped that one day multiple tests can be performed on individual mosquitoes that can

accurately identify it, determine infectivity and host preference with use of DNA probes, sporozoite and blood meal ELISAs.

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QUESTIONS AND ANSWERS :

1. Question: Do you use your own production of monoclonal Ab anti-sporozoit (Namru 2 laboratory) or ELISA kit commercially available for your work ?

   Answer : Namru-2 does not produce its own sporozoite Ag, all are derived from WRAIR - all within the "investigational" policy. The ELISA kit should become available from WHO sometime in future. Many of the limitations need to be worked out.

2. Question: - Regarding making your ELISA more "field applicable" could you use simple preservation procedures for buffers such as adding NaN_3 (sodium azide) to buffers ?
   - Have you considered a spacer added to the capture mAb to improve sensitivity (detection limits) of the BLDSA ?

   Answer : - I would have to refer that question to someone, Bob Wirtz, (WRAIR) who has and is responsible for the development and refinements of the current test.
   - Detection limits (i.e. ZS sporozoites per 50 ul triturate) are I feel more than sensitive enough. A detection threshold of N 100 sporozoite per mosquito when natural infective mosquitoes. Normally have far in excess than number I believe makes the test acceptable.