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EPITOPES AS TAXONOMIC CHARACTERS: A NOVEL APPROACH FOR THE IDENTIFICATION OF *ANOPHELES GAMBIAE* AND *AN. ARABIENSIS*

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INTRODUCTION

Anopheles gambiae has the dubious distinction of being the most efficient vector of malaria in the world. The most dangerous form of malaria is caused by the parasite *Plasmodium falciparum*, which is estimated to cause approximately 1 million infant deaths annually in subsaharan Africa. To compound an already complicated problem, chloroquine resistance of *P. falciparum* malaria has become wide spread with epicenters in both Africa and Southeast Asia. Despite a long history of clinical research, malaria remains the major disease of the tropics. It is a challenge for entomologists to expand on the knowledge of the ecological, systematic and evolutionary aspects of mosquito vector biology.

Anopheles gambiae Giles, 1902 was initially described as a single species. Studies have since revealed *An. gambiae* to be a species complex, containing at least six sibling species that are extremely difficult to differentiate based on morphological characters. Two salt water species found in west and east African coast were named *An. melas* (Theobald, 1902) and *An. merus* (Donitz, 1903) respectively. Freshwater species were originally designated non-Linnean nomenclature as species A, B, C, and D (Muirhead-Thomson, 1948; Davidson, 1956, 1962). However, new nomenclature was proposed by White (1975) to adopt *An. gambiae* Giles, 1902 for species A, *An. arabiensis* Patton, 1905 for species B, *An. quadriannulatus* (Theobald, 1911) for species C, and Species D was renamed *An. bwambi* (White, 1985). Hybridization tests contributed most of the background information on the identification of these species. *An. gambiae* and *An. arabiensis*, two of the most anthrophilic members of the complex, are sympatric while differing in vectorial efficiency and behavior (Coluzzi *et al.*, 1979). Correct species identification is crucial for the epidemiological studies of malarial transmission by these two medically important Anophelines.

Existing technology

While crossing experiments were important in identifying the breeding species of *An. gambiae* complex, a more practical alternative was needed for field research. Identification of the members of *An. gambiae* complex by the banding patterns on the polytene chromosome of ovarian nurse cells or salivary glands of 4th instar larvae (Coluzzi, 1966; Coluzzi and Sabatini, 1967, 1968, 1969; White, 1973; Davidson and Hunt, 1973) emerged as an important means for identifying field-collected specimens. However, the considerable technical experties required by this cytogenetic method probably prevented its popularization. Subsequent effects to develop a field-worthy technique included the use of electrophoretic studies of enzyme systems (Mahon *et al.*, 1976; Miles 1978), analysis of the differences in cuticular hydrocarbon composition (Carlson and Service, 1979, 1980; Hamilton and Service, 1983), and DNA probes (Panyim *et al.*, Collins *et al.*, 1988). Nevertheless, the special chemical, equipment and laboratory requirements render these procedures impractical for field studies, especially those involving hundreds to thousands of identifications.

A practical field test

To resolve the need for a practical field test, we developed an immunoassay targeted at detecting the subtle differences of a major yolk protein present in both *An. gambiae* and *An. arabiensis*. The main reasons for selecting the major yolk protein, vitellogenin or vitellin, as the marker molecule is its abundance after the female mosquito has taken a blood meal. Furthermore, Ma *et al.* (1984, 1986) have already demonstrated the feasibility of using ELISA to measure vitellogenin

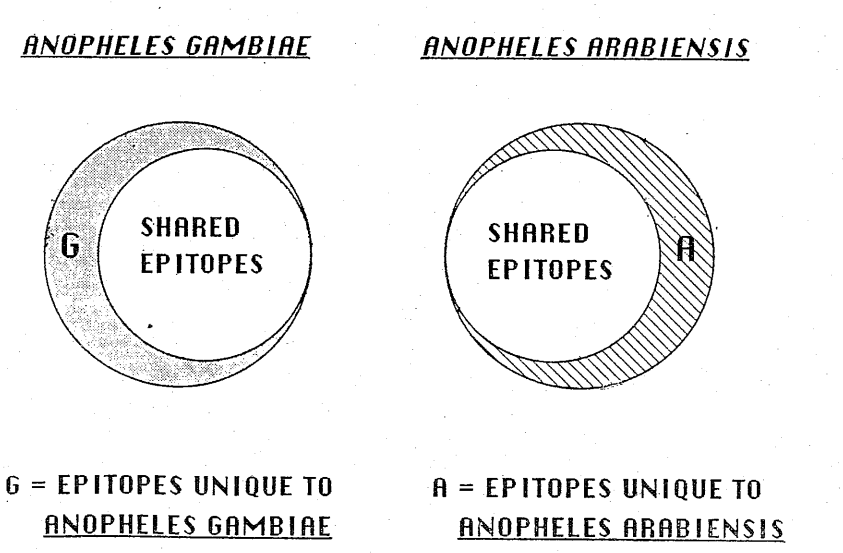

Fig. 1. Shared and species-specific epitopes of *An. gambiae* and *An. arabiensis* vitellogenins.

in individual insect samples from *Aedes atropalpus* and *Ae. aegypti.* The key to this immunoassay is the development of an antibody specific to those epitopes unique to the marker molecule of one species (Fig. 1; G or A) resulting in a positive colorimetric reaction, while no reaction would be detectable for the other species. Due to the close relateness of these two sibling species (Fig. 1; with a great number of shared epitopes), the polyclonal antibody generated against the marker molecule cannot differentiate the two species. In order to obtain a species-specific polyclonal antibody, crossreacting immunoglobulins can be removed by immunoaffinity chromatography using marker molecules of the other sibling species as ligand.

Development of a species-specific antibody

Before we can develop an immunochemical procedure for species identification, an immunoaffinity chromatography procedure was developed for obtaining a

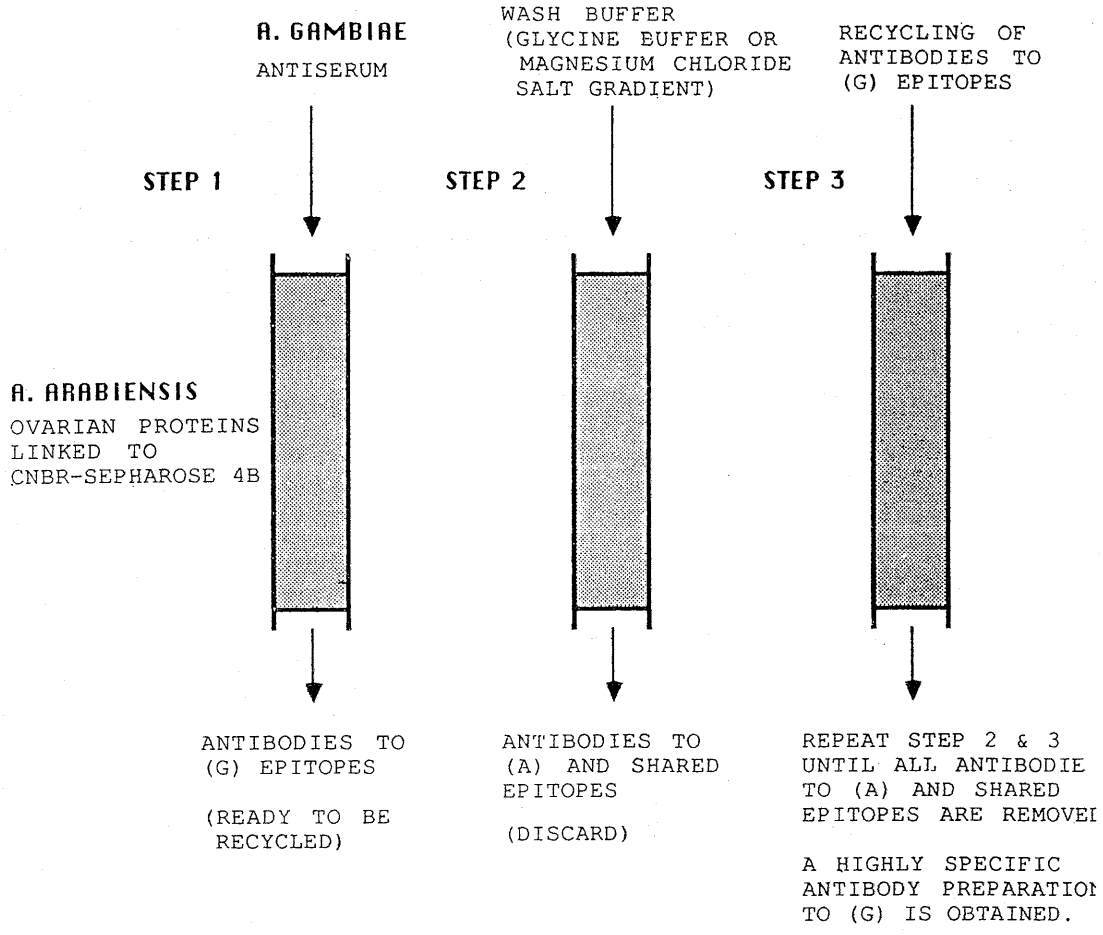

Fig. 2. Affinity purification of rabbit polyclonal antibody to *An. gambiae* for the purpose of obtaining a species-specific antibody.

polyclonal antibody that will bind to *An. gambiae* and not to *An. arabiensis* vitellin. A rabbit antiserum to a highly purified preparation of *An. gambiae* vitellin was obtained and purified by protein A to collect the class G immunoglobulins. This antibody was then eluted through the affinity column with *An. arabiensis* soluble yolk proteins bound as ligand (Fig. 2; step 1). The eluant represented the first cycle of purification. Antibodies that were selectively retained by the ligands were desorpted by washing with a low pH or a salt gradient buffer (step 2). After extensive washings with phosphate-buffered saline, the column was then cleared for a second cycle of purification (step 3) by re-introducing the first eluant into the column. With successive passages, the crossreacting antibodies were removed leaving an antibody specific to *A. gambiae* vitellin (G epitopes).

Enzyme-linked immunosorbent assay

The modified double antibody sandwich ELISA protocol was identical to the one described by Ma *et al.* (1986). Primary antibodies consisted of monoclonal antibodies developed against *An. gambiae* G3 vitellin, which also recognized vitellin obtained from *An. arabiensis* GMAL (Fig. 3). The coating concentration was 10 μg/ml in coating buffer. After blocking with BLOTTO for 1 h, 50 μl of the ovarian sample was introduced. With another 1 h incubation, the sample was discarded, washed 3 times with PBS-Tween, and the secondary antibody was introduced. Secondary antibodies were protein-A and affinity-purified rabbit polyclonal antibodies to *An. gambiae* G3 vitellin at 10 μg/ml in PBS. After another 1 h incubation, the wells were washed 3 times with PBS-Tween followed by the addition of the tertiary antibody. Tertiary or indicator antibodies were alkaline phosphatase-labeled goat anti-rabbit conjugates (Kirkegaard and Perry Laboratory). The

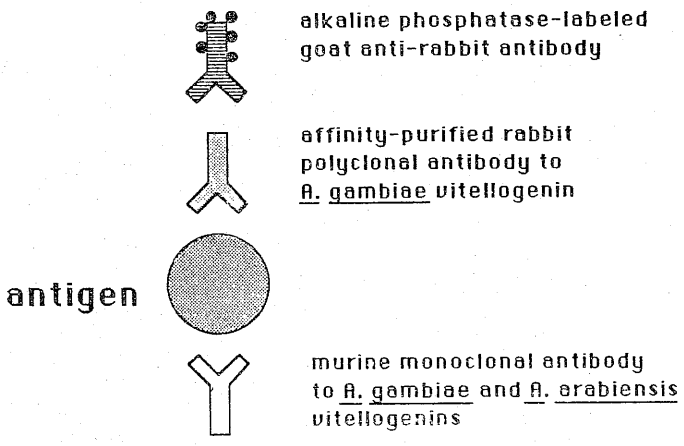

Fig. 3. Modified double antibody sandwich ELISA for the differentiation of *An. gambiae* from *An. arabiensis*.

enzyme substrate *p*-nitrophenyl phosphate (Sigma) was added in 10% diethanolamine-HCl (1 mg/ml). Colorimetric readings were recorded by a Dynatech Automatic ELISA reader.

The ELISA end point of the modified double sandwich test was 40 ng/ml (Fig. 4). The ovarian sample concentrations were adjusted to 0.1 μg to 1 μg per 50 μl. This dilution ensured that there was sufficient *An. gambiae* vitellin present to yield an unambiguous signal. At the same time, a maximum of 1 μg *An. arabiensis* vitellin was far below the detection limit of the ELISA. Thus, the negative ELISA results obtained for *An. arabiensis* samples were usually identical to background levels. (Optical density readings: *An. gambiae* 0.852 +/− 0.0415, *An. arabiensis* 0.0292 +/− 0.0015). We selected semi-gravid female mosquitoes for the test because usually microgram levels of proteins were present in the single ovary samples. Collecting enough protein for the ELISA test was never a problem with this assay because of the abundance of vitellogenin internalized at this particular stage of egg development.

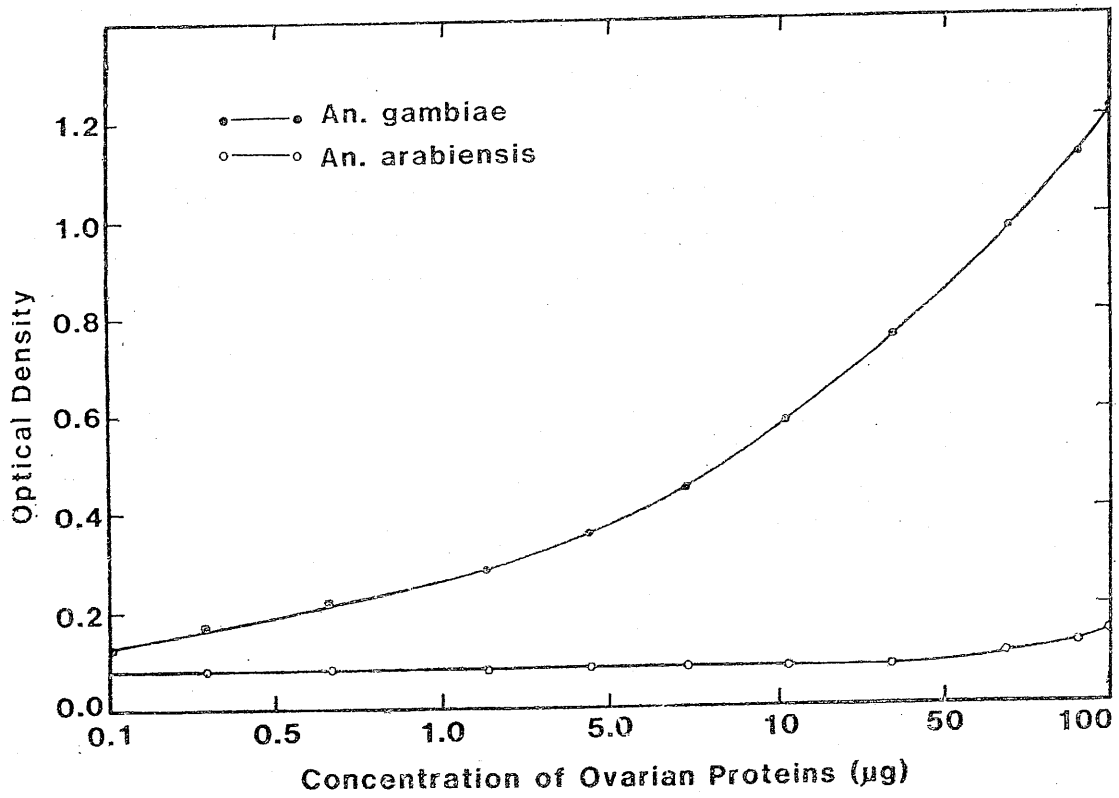

Fig. 4. Modified double antibody sandwich standard curves using monoclonal antibodies to vitellins of both *An. gambiae* and *An. arabiensis* as the coating antibodies, affintiy-purified rabbit polyclonal antibody to *An. gambiae* as the secondary antibody, and alkaline phosphatase-labelled goat anti-rabbit as the indiactor antibody.

Field trial

The accuracy of this ELISA test was verified by a field study staged at western Kenya. Field mosquitoes were collected for species identification by ELISA and the standard polytene chromosome method. The experimental set up was as following.

Adult females of the *A. gambiae* complex were collected from the villages of Saradidi and Kisian (Beier *et al*,, 1987), near Kisumu, in western Kenya. Collections were made during the short rainy season from October 28 to November 8, 1986. Selected collection sites included human dwellings and a cow-baited tent containing 12 bovids. Mouth-aspirator collected female mosquitoes were held at room temperature (20-24°C) until egg development reached the half-gravid stage. One half-gravid ovary was dissected, placed in 50 μl of phosphate-buffered saline and frozen to be processed for species identification by ELISA, the other was preserved in Carnoy's fixative and stored at 4°C for chromosome observations. The polytene chromosome preparations were obtained from the ovarian nurse cells using the method described by Hunt (1973). The species were identified and the different karyotypes were scored according to the nomenclature of Coluzzi *et al.* (1979).

For each mosquito collected, one half of the ovary was processed for ELISA, while the other was fixed and stained for polytene chromosomes. At Kisian, two groups of mosquitoes were collected from inside houses and a cow tent. Among 550 house-collected mosquitoes identified as *An. gambiae* by polytene chromosome morphology, the ELISA results showed 98.9% agreement (Table 1). Among 69 individuals identified as *An. arabiensis*, ELISA results showed 94.2% matching. Of the mosquitoes collected from cow tent, 18 *An. gambiae* and 97 *An. arabiensis*, there was 100% agreement with the ELISA method.

At Saradidi, all collections were from inside houses. Among the 59 identified

Table 1. Species identification by ELISA mosquitoes collected from the villages of Kisian and Saradidi, two small towns in Western Kenya. Percentages represent the agreement of the ELISA results with standard chromosome identifications.

Location	Collection technique	Species identified by chromosome			
		An. gambiae		*An. arabiensis*	
		N	% Positive	N	% Positive
Kisian	Inside houses	550	98.9%	69	94.2%
	Cow tent	18	100 %	97	100 %
Saradidi	Inside houses	59	98.3%	6	100 %
Total		627	98.6%	172	97.7%

as *An. gambiae*, there was 98.3% agreement, and the 6 identified as *An. arabiensis* in 100% concordance with the ELISA result (Fig. 5). If we assume that there was no misreading of the polytene chromosomes and no mishandling of ovarian samples, the accuracy of the ELISA with affinity-purified antibody was 98.6% (N=799) in its first legitimate field trial.

Future considerations

The concept of focusing on one major protein as the marker can be applied to other structural or physiologically important proteins for taxonomic purposes. For example, some cuticular proteins of the larval stages could be investigated as potential marker proteins. Another example is the use of hemolymph proteins such as the storage protein and lipophorin-like molecules. The ideal candidate protein must be abundant enough to be easily detectable by ELISA, found in a particular life stage, present in both sexes, have good storage properties and be present in an extended period of the mosquito's life history. An antiserum could then be made to such a protein, followed by affinity purification using the sibling species' protein as the absorptive ligand. The species-specific antibody could then be used in a ELISA procedure to detect the protein amongst homogenized tissues. Although the present ELISA is limited to identifying adult females, there is potential to employ this method in epidemiological and ecological studies where extensive sampling is required.

Another possibility is the development of monoclonal antibodies specific to (g) and (A) species-specific epitopes. Using conventional hybridoma protocol, it is difficult to generate this type of species-specific monclonals because most antibodies generated tend to recognize only a few immunodominant species. An alternative method is needed. "Immunological paralysis" by the injection of one antigen at high doses to newborn animals will induce a state of immunological unresponsiveness to the immunogen when they become adults (Felton and Ottinger, 1942; Hanan and Gyama, 1954; Smith and Bridges, 1958). Hockfield (1987) described a hybridoma procedure which involved the immunosuppression of Balb/c mice starting at birth with repeated injections of an immunogen until the mice reached adulthood. A similar immunogen with minor epitope differences was then injected before the splenocytes were harvested for PEG fusion. This procedure generated a high frequency of immunoglobulin G class antibodies that recognized the "novel" antigenic determinants even if they were present in small amounts. This protocol will be valuable for obtaining monoclonal antibodies for species-specific vitellogenin epitopes.

Another advantage of this type of ELISA is that it can be integrated with existing ELISA technology for malaria sporozoite and blood meal identifications

(Beier *et al.* 1987, 1988). With more field studies, this immunochemical method will find a role in malarial transmission studies.

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