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FALSE DETECTION OF *PLASMODIUM FALCIPARUM* SPOROZOITES IN *ANOPHELES MARSHALLII* GROUP MOSQUITOES

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ABSTRACT. A study was conducted to determine the role of members of the *Anopheles funestus* group in malaria transmission in the Mpumalanga Province, in the northeastern region of South Africa. Female anopheline mosquitoes were collected between January 1996 and November 1997 by means of human landing catches and tested for salivary gland *Plasmodium falciparum* infections by means of the enzyme-linked immunosorbent assay (ELISA) method with PF2A10 antibodies. Infection rates from April and May 1997 collections were 3.73% and 19.4%, respectively. None of the nonimmune collectors became infected with malaria. The ELISA-positive mosquitoes were tested with a polymerase chain reaction (PCR) malaria detection assay based on sequence variation present in the small subunit ribosomal RNA gene. Only 1.09% of ELISA-positive mosquitoes were PCR-positive for malaria. Initially, all mosquitoes were assumed to belong to the *An. funestus* group but subsequent molecular taxonomy showed this assumption to be false. The use of a single-strand conformation polymorphism (SSCP) assay revealed only 1 member of the *An. funestus* group, *An. rivulorum.* All other specimens produced banding patterns not seen before. Those samples were identified morphologically as *An. demeilloni* and *An. marshallii* s.l. These 2 species are not recognized malaria vectors and thus it is possible that the ELISA results are misleading.

KEY WORDS Enzyme-linked immunosorbent assay, *Plasmodium falciparum,* sporozoites, *Anopheles marshallii, Anopheles demeilloni,* mosquitoes

INTRODUCTION

The total number of reported malaria cases in South Africa has increased from 8,786 in 1995 to 59,774 in 2000 (Department of Health, South Africa, unpublished Malaria Reports). Various factors, for example, an increase in rainfall, have contributed to the increase in malaria incidence. South Africa has maintained a malaria vector control program based on indoor house spraying with residual insecticides since 1958, when total coverage was achieved (Sharp and le Sueur 1996). Historically, *Anopheles funestus* Giles was the major vector in the northeastern provinces and was eradicated when the vector control program was established. A number of species of anophelines in the region readily feed outdoors on humans. Two of these, *Anopheles vaneedeni* Gillies and Coetzee and *Anopheles rivulorum* Leeson, are members of the *An. funestus* group (Gillies and De Meillon 1968, Gillies and Coetzee 1987). Presently the main vector species in South Africa is considered to be *Anopheles arabiensis* Patton. However, this species is found in very low numbers (unpublished data) and probably is not responsible for all the malaria transmission in South Africa (Smith et al. 1997). *Anopheles rivulorum* has been incriminated as a vector in Tanzania (Wilkes et al. 1996). *Anopheles vaneedeni,* limited to southern Africa, has been shown to be fully susceptible to *Plasmodium falciparum* in the laboratory (De Meillon et al. 1997). To date, no scientific data have been produced implicating *An. vaneedeni* and *An. rivulorum* as vectors in South Africa.

The prevalence of malaria in Mpumalanga Province, South Africa, has increased dramatically over the last few years. During December 1995, 331 cases were reported, in comparison to January 1996 when this figure increased to 1,475 (Department of Health, unpublished Malaria Reports). This increase led to great concern. The aim of this study was to establish whether secondary mosquito vectors in Mpumalanga Province played a supplementary role in the malaria transmission.

MATERIALS AND METHODS

Mosquito specimens: All specimens used in this study were sent to the South African Institute for Medical Research (SAIMR) for sporozoite analysis by the Mpumalanga Department of Health, Malaria Control Programme, where the specimens were identified morphologically as belonging to the *An. funestus* group. Mosquitoes were collected from January 1996 to November 1997 using outdoor human landing catches (HLC) at 8 localities in the Komatipoort (25°26′S, 31°57′E) area (Table 2).

Mosquito sample preparation: The head and thorax were dissected from the abdomen, legs, and wings of individual mosquitoes. Scalpel blades and forceps used for dissections were rinsed in 5 N NaOH solution, followed by distilled water, and wiped dry after each mosquito was dissected to prevent contamination between specimens. The head and thorax were placed in a separate tube, and the abdomen, wings, and legs were returned to the original tube containing silica gel and stored at −70°C.

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The head and thorax were either stored at −70°C or homogenized immediately in 50 μl of buffer (blocking buffer with NP-40 or BB-NP-40). The pestle used to homogenize the sample was rinsed with 150 μl of blocking buffer (BB) giving a total volume of 200 μl of homogenate for analysis. The pestle and forceps were washed 3 times in washing solution, dried, and exposed to ultraviolet light for 30 min before reusing to eliminate any traces of DNA (1 N HCl can also be used for this purpose but we found that HCl tends to rust the forceps). Homogenized specimens were tested immediately or stored at −70°C for later use. Fifty microliters of mosquito triturate per microtiter well was used.

Enzyme-linked immunosorbent assay (ELISA) procedure: Monoclonal antibody (MAB) Pf-2A10 designed to detect *P. falciparum* circumsporozoite protein was used in this study (supplied by R. A. Wirtz, Centers for Disease Control and Prevention, Atlanta, GA). The ELISA procedure was done according to Burkot et al. (1984) and Wirtz et al. (1987). The results were analyzed using a microtiter plate reader at a wavelength of 405 nm. The absorbency cutoff value for positive specimens was calculated as twice the mean values of 7 negative controls. Positive samples were tested for confirmation. Negative controls consisted of *An. arabiensis* mosquitoes colonized in the Botha De Meillon Insectary at the SAIMR. The positive control consisted of a synthetic peptide supplied with the antibodies.

Polymerase chain reaction (PCR) confirmation of ELISA-positive specimens: Fifty microliters of ELISA-positive sample triturate were used for preparation of template for the PCR assay. Phenol–chloroform (Sambrook et al. 1989) was used to extract DNA from the ELISA homogenate. The DNA was resuspended in 50 μl of 1X TE buffer and 1 μl was used for PCR (Snounou et al. 1993). A negative control without DNA was run as for the other samples and extracted *P. falciparum* DNA from blood smears was used as positive control. The PCR primers were based on the sequence of the small subunit ribosomal RNA (ssrRNA) genes (Snounou et al. 1993). The *Plasmodium*-specific primers were rPLU6 sense: 5′-TTAAAATTGTTGCAGTTAAAACG-3′; and rPLU5 antisense: 5′-CCTGTTGTTGCCTTAAACTTC-3′. The *P. falciparum*–specific primers used in the 2nd PCR reaction were rFAL1 sense: 5′-TTAAACTGGTTTGGGAAAACCAAATATATT-3′; and rFAL2 anti-sense: 5′-ACACAATGAACTCAATCATGACTACCCGTC-3′. The cycles for amplification were step 1: 95°C for 5 min for 1 cycle; step 2: denaturing at 94°C for 1 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min; step 2 was repeated 25 times; step 3: 72°C for 5 min for 1 cycle. The 1st round of PCR used the *Plasmodium*-specific primers. The 2nd round of PCR used 1 μl of PCR product from the 1st round of PCR as template with the *P. falciparum*–specific primers.

Table 1. Specimens analyzed for enzyme-linked immunosorbent assays (ELISAs).

Date	Number	Positive ELISA	%
1996			
Jan.–Feb.	46	0	0
March	24	0	0
April	64	0	0
May	73	0	0
June	8	0	0
July	2	0	0
Sept.	15	0	0
Oct.	26	0	0
Nov.	52	0	0
Dec.	70	0	0
1997			
Jan.	529	1	0.18
Feb.	556	1	0.18
March	272	0	0
April	1,231	46	3.73
May	412	80	19.4
Total	3,380	128	3.786

Single-strand conformation polymorphism (SSCP) assay: Mosquito identification was done with the SSCP method of Koekemoer et al. (1999). Primers amplified the variable D3 domain in the 28S gene. Primer sequences were D3A sense: 5′-GACCCGTCTTGAAACACGGA-3′; and D3B antisense: 5′-TCGGAAGGAACCAGCTACTA-3′. The PCR cycling was repeated 30 times as follows: 94°C for 30 s, 49°C for 30 s, and 72°C for 30 s. A final autoextension for 5 min at 72°C was done for 1 cycle.

RESULTS

No molecular assay was available to identify members of the *An. funestus* group when this study was initiated and specimens were initially identified as belonging to the *An. funestus* group. The ELISAs conducted on mosquito specimens from January to December 1996 used whole specimens and no positive results were obtained over this period (Table 1). Because of the high cost of the assays and failure to identify positive specimens, samples were pooled the following year. Approximately 10 specimens were pooled and 51 pooled samples (n = 500) were assayed in January–February of 1997. One positive sample was found (Table 1). Specimens from mid-February were tested individually using only the head and thoraxes, whereas the remaining samples were stored on silica until a molecular assay was available for identifying the samples.

The ELISAs during April and May of 1997 showed that 3.73% and 19.4% of the samples were positive, respectively (Table 1). The positive sam-

Table 2. Localities and polymerase chain reaction (PCR) results for confirmation of enzyme-linked immunosorbent assay (ELISA) results for samples collected during April and May 1997.

Date (1997)	Locality	*n*	ELISA positive (%)	PCR positive (%)
April	Coopersdal	1	1 (100)	0 (0)
	Janulet Farm	85	18 (21)	3 (3.5)
	New Caravan Park	294	7 (2.38)	3 (1)
	Cheetahs Nursery	864	20 (2.31)	7 (0.8)
May	Janulet Farm	152	52 (34.2)	3 (1.9)
	Mangweni	1	1 (100)	0 (0)
	Martiens Farm	12	1 (8.3)	—
	New Caravan Park	17	1 (5.8)	0 (0)
	Cheetahs Nursery	186	18 (9.6)	1 (0.53)
	Oompie	12	1 (8.3)	0 (0)
	Sommereg	25	6 (24)	1 (4)
Total		1,649	126 (7.6)	18 (1.09)

ples during April of 1997 were retested by PCR to verify the positive results obtained by ELISA. Positive specimens from both April and May of 1997 were tested by PCR to confirm the presence of *P. falciparum* DNA and revealed that 1.09% of the samples were positive for both months (Table 2).

Seventeen percent of all positive ELISA specimens were bloodfed. No blood-meal analysis was performed, but because HLC were used to collect the specimens it is likely that the mosquitoes had fed on the collectors themselves. The possibility that the 1.09% infection rate detected by PCR could be due to DNA contamination between specimens during dissection and homogenizing of specimens was also investigated. Therefore, subsequent samples were homogenized as before, but an additional step was included to ensure prevention of DNA contamination. Pestles and forceps were washed and exposed to ultraviolet light for 30 min to destroy all traces of DNA. Cleaned pestles were used for homogenization of subsequent specimens.

An SSCP assay was developed in 1998 to identify 4 members of the *An. funestus* group (Koekemoer et al. 1999). The PCR-SSCP assay uses conserved primers to amplify a variable domain in the 28S gene. The conserved nature of the primers ensures amplification in various mosquito species. Mutation differences between the amplified fragments can be visualized on a nondenaturing polyacrylamide gel matrix. Positive ELISA specimens were identified using this assay. Legs or abdomens from positive mosquitoes were used in the PCR-SSCP assay and resulted in banding patterns that were not diagnostic for members of the *An. funestus* group (Fig. 1). Subsequent morphological analysis of 2 of these specimens, on the remains of wings and legs, identified them as *Anopheles demeilloni* Evans and *Anopheles marshallii* Theobald group. Neither of these 2 species are members of the *An. funestus* group. To reconfirm that the morphological identifications done on the remains were correct, preidentified specimens of *An. demeilloni* and *An. marshallii* group stored in liquid nitrogen were analyzed with the PCR-SSCP assay. The same SSCP

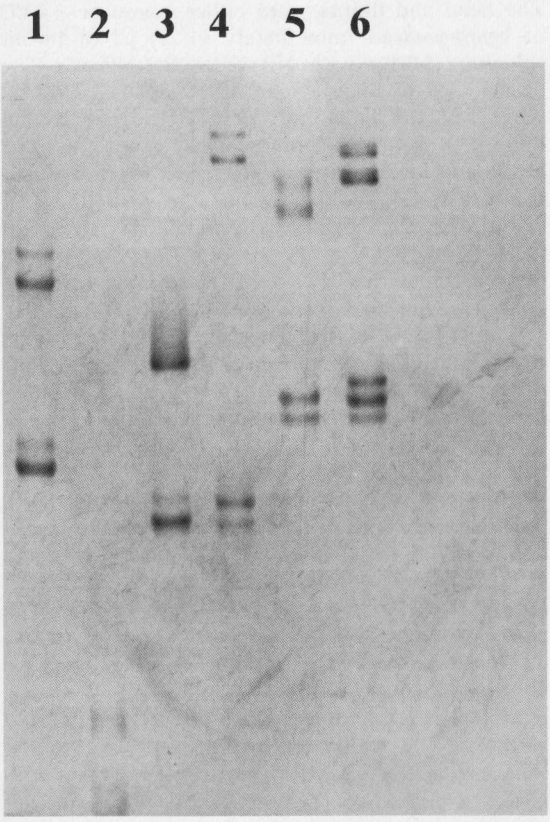

Fig. 1. Banding patterns observed after single-strand conformation polymorphism electrophoresis. Lane 1: *Anopheles marshalii* group, 2: *An. demeilloni,* 3: *An. leesoni* Evans (another member of the *An. funestus* group), 4: *An. rivulorum,* 5: *An. vaneedeni,* 6: *An. funestus.*

Table 3. Enzyme-linked immunosorbent assay (ELISA)-positive specimens analyzed for species identification linked to positive polymerase chain reaction (PCR) results. Contamination of DNA might explain the high percentage of positive rates after PCR analysis.

Species	Number of specimens positive by ELISA (%)	Number of positive ELISA-specimens showing amplification with *Plasmodium falciparum* primers (%)
Anopheles demeilloni	44 (81)	3 (5)
An. marshallii	6 (11)	1 (17)
An. rivulorum	4 (7)	2 (50)

Table 4. Species identification and results of enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) analyses for *Plasmodium falciparum* with pestles sterilized by ultraviolet light.

Species	Sample size tested	ELISA-positive (%)	% confirmation using nested PCR
Anopheles marshallii	639	4 (0, 63%)	0
An. demeilloni	121	0 (0%)	0

banding patterns were observed as was found for the infected specimens (Fig. 1). Table 3 gives identifications of ELISA-positive samples after SSCP electrophoresis.

Table 4 summarizes more recent ELISA results (collections from August to November 1997) where morphological identification was performed before ELISA. All pestles were exposed to ultraviolet light in between homogenizing of specimens, unlike the data from Table 3. Legs and wings, or abdomens, or legs, wings, and abdomens from these ELISA-positive specimens were tested by PCR and none of these gave amplification products (Table 4).

DISCUSSION

Enzyme-linked immunosorbent assays have been successfully used for the detection of *P. falciparum* in the salivary glands of female mosquitoes (Beier et al. 1991, Temu et al. 1998). The ELISA technique is used in our laboratory for routine detection of *P. falciparum* in the *An. gambiae* complex and there is no reason to believe that the results are not reliable (Temu et al. 1998). At the same time, the PCR assay (Snounou et al. 1993) has been shown to be reliable in detecting *Plasmodium* parasites in *An. gambiae* s.s. in West Africa (Arez et al. 1997).

It is difficult to explain why the results of the ELISA and PCR tests are so obviously different when using *An. demeilloni* and *An. marshallii* group specimens. Arez et al. (1997) used the nested PCR described by Snounou et al. (1993) in Guinea Bissau to determine the infection rate in *An. gambiae* s.s. caught indoors and concluded that PCR-based assays can be used in transmission studies. However, their results were not correlated with either salivary gland dissections or ELISA data. However, Snounou et al. (1993) found that the PCR assay is more sensitive than microscopical diagnosis because lower levels of parasites can be detected. The ELISA and microscopical method have been compared in *An. gambiae* s.s. (Wirtz et al. 1987), but other scientists have also described discrepancies when using ELISA (Esposito et al. 1986, Beier et al. 1990, Sylla et al. 2000). Sylla et al. (2000) compared the ssrRNA PCR with a stevor gene family PCR and found detection limits in the same range between the 2 assays. They also found that the ELISA method overestimates the number of infected mosquitoes. The possibility that very stringent PCR conditions can result in negative PCR results was discounted because the positive controls included in our PCR assays always came out positive.

Other factors that might influence the results were considered to explain the differences between the 2 assays described here. The 1st factor is that the technique might have been performed incorrectly and resulted in false positives. However, the same procedure used in this study was also followed with specimens from Ethiopia (n = 360), Mozambique (n = 73), and Côte d'Ivoire (n = 100). These assays were conducted twice on the same microtiter plates as the samples from the present study, and infection rates were as expected for these countries. This suggests that the technique was correctly performed but does not explain the high infection rate in the mosquitoes analyzed from Mpumalanga Province.

The 2nd factor is contamination between specimens during dissection and homogenization. This is unlikely because the negative controls throughout the study never gave positive results even though they were handled with the other specimens. If contamination was the reason, one would have expected to encounter the same degree of contamination in specimens analyzed from Ethiopia, Côte d'Ivoire, and Mozambique.

Another explanation could be that *An. marshallii* and *An. demeilloni* are in fact secondary vectors of *P. falciparum*. However, historical data analyses indicate that they are not involved in malaria transmission (Gillies and De Meillon 1968). It is interesting to note that none of the Mpumalanga Department of Health employees that were responsible for the routine mosquito collections during this time contracted malaria, lending credence to the hypothesis that these species are not in fact biological vectors of *P. falciparum*. An infection rate of almost 20% (see Table 2 during May 1997) found within these mosquitoes would also have resulted in a significant increase in malaria cases from this area, resulting in holoendemic malaria. Although the situation in Mpumalanga Province at the time of this study was considered to be worse than normal, it could not be classified as holoendemic.

The ELISA detects proteins and a protein within the mosquito itself might cross-react with the antibody giving a false-positive result. Legs, wings, and abdomens tested for 4 positive specimens failed to give positive ELISA results. This suggests that a mosquito protein is not responsible for a positive result. Also, if a mosquito protein was responsible for positive results, the number of false positives would be expected to be greater, because proteins between individuals of the same species in a population are relatively conserved. The high ELISA-positive results were more prevalent during

April and May than the rest of the year than would be expected if a mosquito protein was present.

A further possibility is the presence of another parasite species within the mosquito. Because both *An. demeilloni* and *An. marshallii* group mosquitoes are mainly exophilic and zoophilic, they conceivably may have been infected with another *Plasmodium* species. Such a parasite may have a similar protein to that detected by *P. falciparum* monoclonal antibodies, giving a positive result. Because the Mabs have only been tested against the most commonly found *Plasmodium* parasite species (*P. berghei, P. cynomolgi, P. knowlesi, P. vivax,* and *P. yoelii*), it is possible that they might react with a different *Plasmodium* parasite found in *An. marshallii* group or *An. demeilloni* mosquitoes.

Also, "false"-positive ELISA results might be associated with mosquito blood meals, as was found by Somboon et al. (1993) and Lochouarn and Fontenille (1999). Somboon et al. (1993) reported positive *P. falciparum* CSP-ELISA results from zoophilic *Anopheles* species (*An. vagus* Doenitz and *An. kochi* Doenitz) not implicated as malaria vectors in Thailand. These studies found that 25% of whole blood samples from cows were positive for 2A10 (*P. falciparum*) and NSV3 (*P. vivax*). Whole blood samples tested from pigs resulted in a positive rate of 8.33% for *P. falciparum* and *P. vivax*. The false-positive results were associated with the plasma fractions of the blood (Somboon et al. 1993). Lochouarn and Fontenille (1999) found false-positives of *P. malariae* and *P. ovale* in *An. gambiae* s.l. in Senegal associated with bovine blood meals. However, in the present study only 17% of the total sample of ELISA-positive individuals were blood fed and this does not explain the high infection rate in the remaining 83% of the specimens. Furthermore, the area is not used for cattle farming.

Beier et al. (1991) found that the ELISA test is not always as sensitive as originally postulated. In a study in Kenya, microscopic techniques were found to be more sensitive in detecting low-grade sporozoite infections in the salivary glands than the ELISA method. This might be because the sporozoites contained epitopes not recognized by the monoclonal antibodies. Epitope variation has yet to be described and remains a factor. Because the Kenyan mosquitoes were mainly anthropophilic the likelihood that the sporozoite infections were non–human-source *Plasmodium* species is small. However, *An. gambiae* s.l. and *An. funestus* were collected by all-night human-biting catches inside houses, outdoors, inside large tents, and by aspiration inside houses and species composition was not determined.

This study illustrates the importance of correctly identifying mosquito species in a malaria control program and the value of molecular methods in species identification. It still is not clear if other members of the *An. funestus* group other than *An. funestus* play a supplementary role in malaria transmission in Mpumalanga Province in South Africa. In addition, future studies need to be conducted to determine the reason for the high level of positive ELISA results found in *An. marshallii* group and *An. demeilloni* specimens in this study.

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