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

LIZETTE L. KOEKEMOER,1 ELIZABETH M. RANKOE,1 JACOBUS P. LA GRANGE,2 JOHN GOVERE2 AND MAUREEN COETZEE1

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. demeillonii* and *An. marshallii* s.l. These 2 species are not recognized malaria vectors and thus it is possible that the ELISA results are misleading.


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malaria cases in South Africa has increased from 8,786 in 1995 to 59,774 in 2000 (Department of Health, South Africa, unpublished Malaria Reports).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 Komatiport (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'-TTAAATTTGTTGAGTTAAAAACG-3'; and rPLU5 antisense: 5'-CCTGTGTTGCTAAATACCTC-3'. The *P. falciparum*-specific primers used in the 2nd PCR reaction were rFAL1 sense: 5'-TTAAATCTGGTTGGGAAAACCCATATTATTT-3'; and rFAL2 anti-sense: 5'-ACACAATGAACCTCACTCATGACTACCCGTC-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.

**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'-GACCCGTTCGCACTATTTTG-3'; and D3B antisense: 5'-TCGGAAGGACCAGCTACTA-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 1. Specimens analyzed for enzyme-linked immunosorbent assays (ELISAs).

<table>
<thead>
<tr>
<th>Date</th>
<th>Number</th>
<th>Positive ELISA</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan.-Feb.</td>
<td>46</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>March</td>
<td>24</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>April</td>
<td>64</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>May</td>
<td>73</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>June</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>July</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sept.</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Oct.</td>
<td>26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nov.</td>
<td>52</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dec.</td>
<td>70</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1997

<table>
<thead>
<tr>
<th>Date</th>
<th>Number</th>
<th>Positive ELISA</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan.</td>
<td>529</td>
<td>1</td>
<td>0.18</td>
</tr>
<tr>
<td>Feb.</td>
<td>556</td>
<td>1</td>
<td>0.18</td>
</tr>
<tr>
<td>March</td>
<td>272</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>April</td>
<td>1,231</td>
<td>46</td>
<td>3.73</td>
</tr>
<tr>
<td>May</td>
<td>412</td>
<td>80</td>
<td>19.4</td>
</tr>
</tbody>
</table>

Total 3,380 128 3.786
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.

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

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.

Properties 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.

![Fig. 1. Banding patterns observed after single-strand conformation polymorphism electrophoresis. Lane 1: Anopheles marshallii group, 2: An. demeilloni, 3: An. leesonii Evans (another member of the An. funestus group), 4: An. rivulorum, 5: An. vaneedi, 6: An. funestus.](image)

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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of specimens positive by ELISA (%)</th>
<th>Number of positive ELISA-specimens showing amplification with <em>Plasmodium falciparum</em> primers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anopheles demeilloni</em></td>
<td>44 (81)</td>
<td>3 (5)</td>
</tr>
<tr>
<td><em>An. marshallii</em></td>
<td>6 (11)</td>
<td>1 (17)</td>
</tr>
<tr>
<td><em>An. rivulorum</em></td>
<td>4 (7)</td>
<td>2 (50)</td>
</tr>
</tbody>
</table>
Anopheles marshallii  
An. demeilloni

electrophoresis.

tifications of El-lSA-positive samples after SSCP

of these gave amplification products (Table 4).

ELISA and PCR tests are so obviously different
other salivary gland dissections or ELISA data.

DISCUSSION

Enzyme-linked immunosorbent assays have been
in the salivary glands of female mosquitoes (Beier
be used in detecting Plasmodium parasites in

to be reliable in detecting Plasmodium parasites in

Table 4. Species identification and results of enzyme-

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample size tested</th>
<th>ELISA-positive (%)</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anopheles marshallii</td>
<td>639</td>
<td>4 (0, 63%)</td>
<td>0</td>
</tr>
<tr>
<td>An. demeilloni</td>
<td>121</td>
<td>0 (0%)</td>
<td>0</td>
</tr>
</tbody>
</table>

that the ELISA method overestimates the number of
of infected mosquitoes. The possibility that very

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 incor-
rectly and resulted in false positives. However, the
same procedure used in this study was also fol-
lowed 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 pre-
sent 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 speci-
mens 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 con-
tamination was the reason, one would have expect-
ed 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 in-
dicate that they are not involved in malaria trans-
mission (Gillies and De Meillon 1968). It is inter-
esting to note that none of the Mpumalanga
Department of Health employees that were respon-
sible for the routine mosquito collections during
this time contracted malaria, lending credence to
the hypothesis that these species are not in fact bi-
ological vectors of P. falciparum. An infection rate of
almost 20% (see Table 2 during May 1997) found
within these mosquitoes would also have re-
sulted 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 holo-
endemic.

The ELISA detects proteins and a protein within
the mosquito itself might cross-react with the an-
tibody 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 pos-
itive result. Also, if a mosquito protein was respon-
sible for positive results, the number of false posi-
tives 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. vortex 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.

ACKNOWLEDGMENTS

This project was funded through research grants obtained from the University of the Witwatersrand and the Medical Research Council (M.R.C.). We would like to thank G. Snounou for comments on the P. falciparum PCR assay used for this study. Sporozoite identification was carried out using P. falciparum-positive controls and monoclonal antibodies PF2A10 from R. A. Wirtz, Centers for Disease Control—Entomology Branch, Atlanta, GA.

REFERENCES CITED


