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USE OF POLYMERASE CHAIN REACTION TECHNIQUE TO CONFIRM VECTEST™ SCREENING RESULTS IN *PLASMODIUM FALCIPARUM* AND *PLASMODIUM VIVAX* VK 210 LABORATORY-INFECTED *ANOPHELES STEPHENSI* MOSQUITOES

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ABSTRACT. We evaluated polymerase chain reaction (PCR) to confirm immunoassays for malaria parasites in mosquito pools after a failure to detect malaria with PCR during an outbreak in which pools tested positive using VecTest™ and enzyme-linked immunosorbent assay (ELISA). We combined VecTest, ELISA, and PCR to detect *Plasmodium falciparum* and *Plasmodium vivax* VK 210. Each mosquito pool, prepared in triplicate, consisted of 1 exposed *Anopheles stephensi* and up to 9 unfed mosquitoes. The results of VecTest and ELISA were concordant. DNA from a subset of the pools, 1 representative of each ratio of infected to uninfected mosquitoes, was extracted and used as template in PCR. All *P. vivax* pools were PCR positive but some needed additional processing for removal of apparent inhibitors before positive results were obtained. One of the pools selected for *P. falciparum* was negative by PCR, probably because of losses or contamination during DNA extraction; 2 remaining pools at this ratio were PCR positive. Testing pools by VecTest, ELISA, and PCR is feasible, and PCR is useful for confirmation of immunoassays. An additional step might be needed to remove potential inhibitors from pools prior to PCR.

KEY WORDS VecTest, enzyme-linked immunosorbent assay, polymerase chain reaction, *Plasmodium falciparum, Plasmodium vivax* VK 210

INTRODUCTION

Despite decades of interest, research, and control efforts, malaria is re-emerging as a major health problem. More than 2.1 billion people are estimated to live in endemic countries, with an estimated 200–300 million cases and 2 million deaths annually (WHO 1991). In addition, there is a growing threat of autochthonous transmission in countries that are currently free from the disease but harbor competent vectors. Such a situation occurred in the United States in the state of Virginia during 2002 (CDC 2002). As the risk of malaria outbreaks grows, it becomes important to identify appropriate diagnostic tools for identifying high-risk transmission areas and thereby efficiently target vector control. These tools include accurate methods for diagnosis of *Plasmodium* sp. infection in mosquito populations.

The gold standard for determination of mosquito infection is microscopic evaluation of dissected salivary glands, a laborious technique that requires experience and training. Immunological techniques, such as the enzyme-linked immunosorbent assay (ELISA), have been developed to detect *Plasmodium falciparum, Plasmodium vivax* VK 210, or *P. vivax* VK 247 (Burkot et al. 1984; Wirtz et al. 1985, 1992), and ELISA has been used successfully in the field (Acheé et al. 2000, Póvoa et al. 2001). Although ELISA is not a highly technical procedure, it does require equipment, time, and training. In response to the need for a more rapid and simple diagnostic tool, the VecTest™ Malaria Panel Assay was developed and can be used to qualitatively detect infections of *P. falciparum* and *P. vivax* VK 210 and VK 247 (Ryan et al. 2001).

The VecTest for malaria is a simple, rapid method appropriate to the field (Ryan et al. 2001). This test is based on the dual monoclonal antibody "sandwich" principle. If malaria antigens are present in the sample, they bind to the specific antibody with a gold sol particle label, migrate through the test zone, and bind to the corresponding immobilized malaria antibodies, forming a sandwich that is detected as a reddish-purple line in a predetermined area of the test zone.

The VecTest has been compared with the standardized ELISA, and both assays are concordant for detection of *P. falciparum* and *P. vivax* VK 210 and VK 247. In earlier studies, the efficacy, sensitivity, stability, and field use of VecTest were determined in a 16-center, collaborative international study, in which more than 40,000 VecTest assays were performed. There was 92% sensitivity, 98.1% specificity, 97.8% overall accuracy, and good stability of the kit (Ryan et al. 2001, 2002). Appawu et al. (2003) compared VecTest and ELISA in a malaria endemic site in Ghana; they found a relative specificity of 98.0% for the VecTest, but sensitivity was lower (88.8%). The accuracy ranged from 93.4 to 97.8% depending on the cut-off used for ELISA. They suggested that this variation has to be evaluated, depending on the entomological objectives of the test.

The polymerase chain reaction (PCR) is highly sensitive for detection of malaria parasites in clin-

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Table 1. Mosquito test pools and controls.

	Test pool[1]								
	A	B	C	D	E	F	G	H	I
Rate of infected/unfed mosquitoes	1+/1−	1+/2−	1+/3−	1±/4−	1+/5−	1+/6−	1+/7−	1+/8−	1+/9−
Total tested	3	3	3 (4)	3	3	3	3 (4)	3	3 (4)

[1] Additional pools prepared at the same ratios of infected: unfed mosquitoes that failed VecTest screening are noted in parentheses.
[2] N/A, not applicable.

ical samples (Snounou et al. 1993a, 1993b). PCR has been used to detect malaria infections in anopheline vectors and to confirm ELISA and microscopy results. Inhibitors in mosquito pools can negatively influence sensitivity of the PCR reaction, and level of inhibition varies with different protocols (Tassanakajon et al. 1993, Wilson et al. 1998, Vythilingam et al. 1999, Arez et al. 2000, Póvoa et al. 2000, Sylla et al. 2000).

The need for a test to confirm malaria diagnosis in mosquitos in nonendemic areas was recognized in an investigation of malaria in Virginia in 2002 (CDC 2002). Five pools of mosquitoes captured in the vicinity of cases were positive by both ELISA and VecTest for *P. vivax* malaria. However, because both ELISA and VecTest use the same monoclonal antibodies, concordant false positive reactions could not be excluded. In circumstances in which true positive immunoassay results are likely the same, the use of a confirmatory test based on an independent technology is desirable.

We report on a protocol to detect malaria parasites in laboratory-infected *Anopheles stephensi* involving VecTest and ELISA followed by confirmation with PCR. A critical aspect of this procedure entails the necessity of using a common pool for all 3 tests. The mosquito head is known to contain PCR inhibitors, but removal of heads prior to PCR is not an option because pooled specimens are usually processed intact. We simulate a scenario of low malaria endemicity with the use of pools composed of 1 potentially infected and various ratios of uninfected mosquitoes to determine whether the presence of uninfected mosquitoes interferes with sensitivity.

MATERIALS AND METHODS

Mosquitoes: Laboratory-infected *An. stephensi* females were acquired from either the Walter Reed Army Institute of Research (WRAIR; *P. falciparum* NF-54 strain, Forest Glen, MD) or from the Centers for Disease Control and Prevention (CDC; *P. vivax* VK 210 strain Salvador I, Atlanta, GA). Negative specimens consisted of unfed females. All mosquitoes were killed and stored at −80°C prior to processing. Mosquito pools were prepared from whole mosquitoes to simulate conditions used for screening by ELISA or VecTest.

VecTest: Three sets of 10 sample pools were processed in the 1.7-ml tubes provided in the kit (Medical Analysis Systems, Inc., Camarillo, CA). Tests were conducted at 23–26°C. Test pools consisted of 1 potentially infected mosquito and 1–9 negative females (Table 1). Before homogenization, the heads and thoraces were dissected from the bodies. Each positive control consisted of 1 infected salivary gland, whereas each negative control pool consisted of 5 negative (unfed) *An. stephensi.* Strip results were considered positive if a control line and either the *P. falciparum* or *P. vivax* VK 210 lines were visible 15 min after exposure to homogenate.

ELISA: Immediately after completing the VecTest, 50 µl of each pool was tested for sporozoite infection by ELISA (Wirtz et al. 1992, Acheé et al. 2000). ELISA was performed in Costar U-bottomed vinyl microtiter plates (Costar, Cambridge, MA). Plates were washed with Dulbecco's

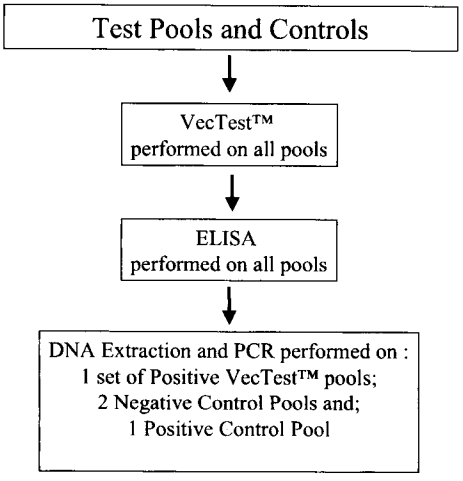

Fig. 1. A schematic of the study design used to test for potential PCR interference from varying numbers of negative mosquitoes in a sample pool. Study was repeated for both *Plasmodium falciparum* and *Plasmodium vivax* VK 210 laboratory-infected, colony-reared *Anopheles stephensi* female mosquitoes.

Table 1. Extended.

Negative controls	Positive controls[2]		
	VecTest™	ELISA	PCR
5−	Salivary gland dissection	*Plasmodium falciparum:* Recombinant DNA *Plasmodium vivax:* Synthetic Peptide	Blood
8	3	N/A	N/A

phosphate-buffered saline, pH 7.0–7.4, containing 0.05% Tween-20 (Sigma Chemical Co., St. Louis, MO). Peroxidase-conjugated monoclonal antibodies used were *P. falciparum* (2A10) and *P. vivax* VK 210 (NSV3) (Kirkegaard and Perry Laboratory, Gaithersburg, MD). Positive controls were either recombinant *P. falciparum* CSP (highest dilution = 100 pg/well) or synthetic peptide of *P. vivax* VK 210 (40 pg/well). Pools were considered positive if optical density readings (ODs) at 405 nm with an ELISA plate reader (Spectra MAX 250, Molecular Devices Corporation, Sunnyvale, CA) exceeded the mean absorbance OD plus 3 standard deviations of the negative pool OD 30 min after addition of substrate (ABTS Microwell Peroxidase Substrate System, KPL, Gaithersburg, MD).

DNA extraction: DNA was extracted from 100 μl of the VecTest lysate by the crude lysate protocol in the DNeasy tissue kit® (QIAgen, Valencia, CA). The final elution process was repeated, yielding ~200 μl of DNA extract.

Removal of PCR inhibitors: Because substances in mosquitoes can potentially interfere with PCR, inhibitors were removed from DNA extracts with the use of agarose-embedded blocks (Moreira 1998) followed by Gel Extraction (QIAquick gel extraction kit®, QIAgen): 1 volume of 1.6% melted low–melting point agarose (Invitrogen, Carlsbad, CA) was added to each DNA extract aliquot for a total of 100 μl. The mixtures were put in Parafilm and allowed to solidify. Blocks were transferred into 6-well tissue culture plates in excess TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0) and washed for 2 days with gentle agitation. After washing, DNA was extracted from the pellets with the QIAquick Gel Extraction Kit.

PCR amplification for parasite identification: A nested PCR protocol (Snounou et al. 1993a, 1993b) amplified the small-subunit ribosomal ribonucleic acid gene (SSU rRNA) of *P. vivax* and *P. falciparum.* The 1st 50-μl reaction amplified the SSU rRNA gene and contained 5 μl of purified template DNA, 3.6 mM of $MgCl_2$, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 10^{-3}% gelatin, 0.125 mM of each of the 4 dNTPs, 1.5 U of *Taq* DNA polymerase (TAq SuperPak DNA polymerase without $MgCl_2$, Sigma, St. Louis, MO), and 13 pm of each oligonucleotide (PLU5: 5′-CCT GTT GTT GCC TTA AAC TTC-3′ and PLU6: 5′-TTA AAA TTG TTG CAG TTA AAA CG-3′). The 2nd reaction, which amplifies both *P. vivax* and *P. falciparum,* was carried out in separate tubes with species-specific primers in a 20-μl reaction and the same conditions as the 1st reaction but with either 3 or 5 μl of the 1st reaction as a template, 2 mM of $MgCl_2$, and 5 pm of each oligonucleotide (FAL1: 5′-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3′ and FAL2: 5′-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3′; VIV1: 5′-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3′ and VIV2: 5′-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3′) (Snounou et al. 1993a, 1993b). Oligonucleotides were prepared on the Applied Biosystems 3948 synthesis and purification system. The cycling parameters on a PCT-200 thermocycler (MJ Research Inc., Waltham, MA) consisted of 95°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min and a final extension at 72°C for 5 min and reduction of the temperature to 4°C. PCR positive controls contained blood samples infected with either *P. falciparum* or *P. vivax* from clinical patients from Manaus, Amazonas, Brazil. Negative controls consisted of VecTest and ELISA negative pools.

RESULTS

Pools of *P. falciparum* and *P. vivax* VK 210 laboratory-infected, colony-reared *An. stephensi* females (or unfed negative controls) were analyzed by the VecTest and ELISA, and a subset of samples was tested by PCR (Fig. 1). For each type of *Plasmodium* parasite, there were 27 test pools, 3 positive control pools, and 8 negative control pools. The 27 test samples included 3 pools of each of 9 ratios of head + thorax from infected and unfed mosquitoes. The ratios varied from 1 infected:1 unfed to 1 infected:9 unfed. Mosquitoes were assumed to be infected after feeding on blood infected with *Plasmodium* sp. from culture (*P. falciparum* NF-54 strain, WRAIR) or monkeys (*P.vivax* VK 210 Salvador I strain, CDC).

All 3 *P. falciparum* positive control pools prepared by salivary gland dissection and 27 test pools had positive VecTest reactions, whereas the negative control pools were nonreactive. When tested by ELISA, ODs for all positive control and test pools exceeded the minimum threshold absorbance

Table 2. VecTest™, ELISA, and PCR results from *Plasmodium falciparum* test pools of laboratory-infected, colony-reared *Anopheles stephensi* female mosquitoes.

Assay	Samples tested	No. positive test pools/no. tested[1]										
		A	B	C	D	E	F	G	H	I	P	N
VecTest	All	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/2	0/8
ELISA[2]		3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/2	0/8
Mean absorbance		3.11	2.96	2.82	2.92	2.79	2.75	2.50	2.77	2.92	1.89	0.007
SSU rRNA PCR	Subset	1/1	1/1	0/1[3] [2/2][4]	1/1	1/1	1/1	1/1	1/1	1/1	1/1	0/2

[1] Letter designation refers to the number of potentially positive and negative mosquitoes in the sample pool: A, 1+/1−; B, 1+/2−; C, 1+/3−; D, 1+/4−; E, 1+/5−; F, 1+/6−; G, 1+/7−; H, 1+/8−; I, 1+/9−; P (salivary gland dissection), 1+; N (negative control), 5−.
[2] With 405-nm wavelength filter and read after 30 min. Pools were considered positive if absorbance values read >0.099 (i.e., the mean plus 3 SD above optical density of negative pools).
[3] After agarose purification method (see Materials and Methods).
[4] Number in brackets indicates reaction of the remaining *P. falciparum* C pools.

OD for a positive result. ODs of the negative control pools were below the critical minimum OD (Table 2).

Of the pools tested for *P. vivax* VK 210 reaction, 19 of 27 VecTest strips were nonreactive. VecTest results from 8 negative control pools were nonreactive, and all positive control pools were reactive. The lower frequency of positive reactions from *P. vivax* pools, compared with *P. falciparum* pools, probably reflects a lower intensity of infection. For pools with ratios corresponding to letters A, B, D, E, F, and H, at least 1 of the 3 pools at each of the ratios of infected to uninfected mosquitoes was positive. For pools lettered C, G, and I, additional pools were prepared at the same infected:unfed ratios and tested with VecTest strips. This was done to have ≥1 reactive pool at each ratio for the PCR tests. The number of additional tests is shown in Table 3.

When the pools were evaluated by ELISA for *P. vivax* VK 210, pools with positive test strips also had ODs above the minimum threshold. Pools from the negative control and pools with negative test strips had ODs below the minimum threshold. ELISA was not performed on the additional C, G, and I test pools (Table 3).

For PCR, we used 1 pool corresponding to each test ratio that was positive by VecTest and ELISA, in addition to 1 positive and 2 negative control pools (Fig. 1). Initially, *P. falciparum* test pool C and *P. vivax* VK 210 test pools C, G, and I yielded negative PCR results. After agarose purification, the 1st SSU rRNA PCR was positive in all *P. vivax* VK 210 pools, whereas the negative controls remained nonreactive. However, *P. falciparum* test pool C was still nonreactive after agarose purification (Fig. 2). It is likely that this negative result was caused by loss of DNA during the extraction procedures or destruction of DNA integrity by contaminants. The remaining C pools were subjected to DNA extraction and PCR, and both were positive, as were the positive controls (Fig. 2).

DISCUSSION

During vector surveillance, large numbers of mosquitoes need to be tested for the presence of parasites. Salivary gland dissection is impractical

Table 3. VecTest™, ELISA, and PCR results from *Plasmodium vivax* test pools of laboratory-infected, colony-reared *Anopheles stephensi* female mosquitoes.

Assay	Samples tested	No. positive test pools/no. tested[1]										
		A	B	C	D	E	F	G	H	I	P	N
VecTest	All	1/3	1/3	0/3 [1/4][3]	1/3	2/3	1/3	0/3 [1/4]	2/3	0/3 [1/4]	2/2	0/8
ELISA[2]		1/3	1/3	0/3	1/3	2/3	1/3	0/3	2/3	0/3	2/2	0/8
Mean absorbance		2.12	2.06	−0.01	2.24	1.57	1.08	−0.01	1.13	−0.005	1.69	0.001
SSU rRNA PCR	Subset	1/1	1/1	1/1[4]	1/1	1/1	1/1	1/1[4]	1/1	1/1[4]	1/1	0/2

[1] Letter designation refers to the number of potentially positive and negative mosquitoes in the sample pool: A, 1+/1−; B, 1+/2−; C, 1+/3−; D, 1+/4−; E, 1+/5−; F, 1+/6−; G, 1+/7−; H, 1+/8−; I, 1+/9−; P (salivary gland dissection), 1+; N (negative control), = 5−.
[2] With 405-nm wavelength filter and read and after 30 min. Pools were considered positive if absorbance values read >0.099 (i.e., the mean plus 3 SD above OD of negative pools).
[3] Number in brackets indicates additional pools prepared for PCR, not tested by ELISA.
[4] After agarose purification method (see Materials and Methods).

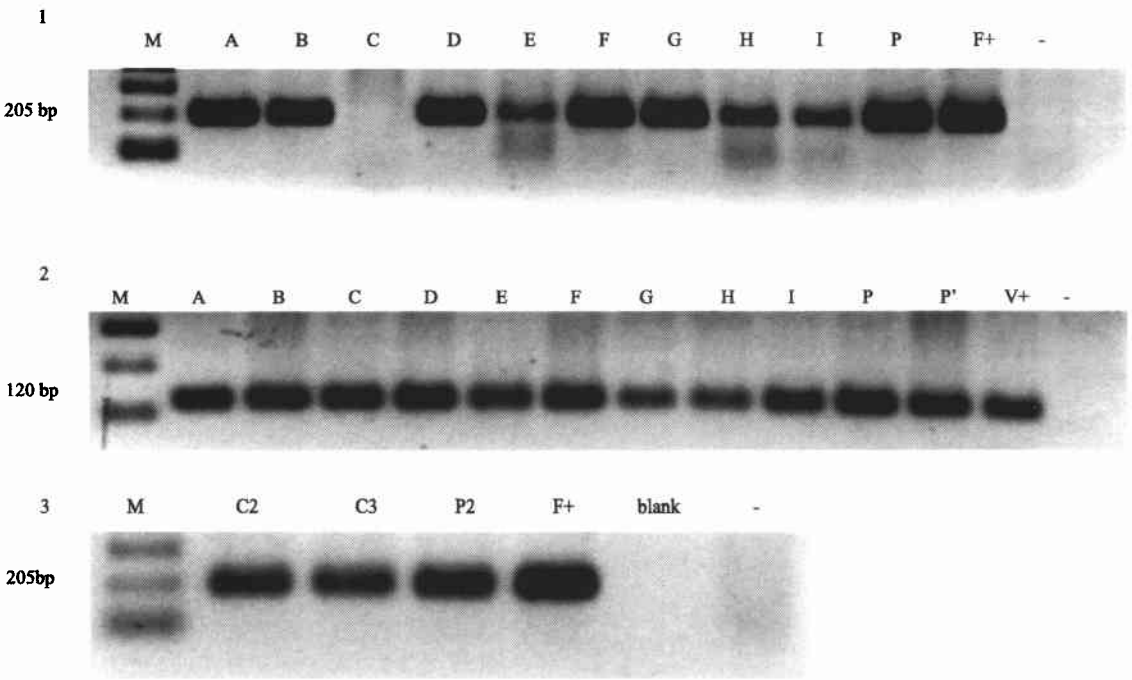

Fig. 2. Results from the nested PCR detection assay with the use of DNA extracted from *Plasmodium falciparum* test pools and controls (1) and from *Plasmodium vivax* test pools and controls (2). A–I, test pools as described in Table 1; P, positive control (salivary glands); P′, positive control after agarose purification; F+, blood sample infected with *P. falciparum*; V+, blood sample infected with *P. vivax*; −, negative control. (3) Results from the nested PCR detection assay with the use of DNA extracted from the remaining *P. falciparum* test pools. C2 and C3, 2 remaining pools from the 3 prepared for evaluation of the ratio lettered C (see Table 1); P2, positive control (salivary gland preparation); F+, blood sample infected with *P. falciparum*; blank, no product applied to the well; −, negative control. Electrophoresis was performed on 1.5% agarose gel in Tris acetate EDTA buffer. PCR product (10 μl) was used for each well, and gel was stained with ethidium bromide. M, 100 bp marker. Viewed as reverse images of agarose gels.

when processing large numbers of mosquitoes. Instead, ELISA has been used to test pools in the laboratory (Burkot et al. 1984; Wirtz et al. 1985, 1992; Acheé et al. 2000; Póvoa et al. 2001). VecTest was developed to test samples in the field. Both tests have high sensitivity and specificity, and there is potential for combining them into a primary screen and confirmatory test algorithm (Ryan et al. 2001, 2002). However, because both are based on solid-phase immunoassay technology and the same monoclonal antibody detection reagent, we evaluated the utility of PCR as an independent confirmatory test for malaria parasite detection in pools. Our application of a DNA extraction method to mosquito pools that had been lysed with the VecTest solution is novel.

There was 100% agreement between results with VecTest and ELISA. Variation in the number of unfed and uninfected mosquitoes did not interfere with the capability of either test to detect parasites (Tables 2 and 3). After DNA was extracted, a nested PCR protocol detected malaria DNA in the test samples. For 1 pool of *P. falciparum* and 3 pools of *P. vivax,* there was no amplification in the 1st PCR attempt. We hypothesized that the negative results on these pools could be due to interference by mosquito components in test samples (Arez et al. 2000). An agarose purification method (Moreira, 1998) was used to remove such components, and subsequent PCR of the *P. vivax* samples were positive (Fig. 2). The *P. falciparum* pool remained negative. We believe that DNA damage or loss occurred during extraction of this pool because all of the other infected:uninfected ratio pools were PCR positive (Fig. 2).

In ongoing studies, we have found that it is feasible to use the methodology described here for confirmatory testing of *P. vivax* immunoassay screening tests for surveillance purposes. These results indicate that samples prepared for assay by the VecTest are suitable for use in PCR, but careful handling and additional processing might be needed. Molecular confirmation of malaria infection in mosquitoes could be accomplished when there are as few as 1 infected mosquito in a pool with up to 9 negative specimens.

Low parasite levels within the vector can be a limiting factor in the detection of malaria infections by any method. After feeding mosquitoes with infected blood, we can only assume there is going to

be a successful salivary gland infection. In laboratory-infected *An. stephensi* mosquitoes, there was a difference in infection rate by the 2 parasite species. Mosquitoes exposed to *P. falciparum* were positive in the screening, whereas mosquitoes exposed to *P. vivax* had a lower infectivity rate and *P. vivax* could not be detected in the majority of pools.

Additional numbers of pools had to be prepared to obtain all ratios of infected:uninfected mosquitoes (Table 3). It is likely that there was a higher parasite load in the VecTest-positive *P. falciparum* samples than in those infected with *P. vivax.* Despite the difference, PCR detected parasite DNA in all the samples that were VecTest positive for *P. vivax.*

The mosquito species involved and the epidemic scenario in which samples are collected could influence quantities of parasites in the salivary glands and consequent sensitivity of the PCR assay. It has been demonstrated that detection of amplified PCR products with liquid-phase, nonisotopic hybridization ELISA increases the sensitivity of the method (Póvoa et al. 2000). However, it could be possible to use PCR for confirmation without these enhancements if inhibitory mosquito components are removed.

The literature and this study demonstrate the value of PCR as a confirmatory test for malaria-infected mosquitoes. As with any confirmatory test, a negative PCR result in the presence of a positive VecTest, ELISA, or both should not be interpreted as evidence of noninfection, especially if the mosquito parts testing are the head and thorax. PCR is a reasonably independent technology because parasite detection is based on genetic markers rather than immunoassay.

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