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Short Report: *Plasmodium*-Specific Molecular Assays Produce Uninterpretable Results and Non-*Plasmodium* spp. Sequences in Field-Collected *Anopheles* Vectors

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Abstract. The Malaria Research and Reference Reagent Resource–recommended PLF/UNR/VIR polymerase chain reaction (PCR) was used to detect *Plasmodium vivax* in *Anopheles* spp. mosquitoes collected in South Korea. Samples that were amplified were sequenced and compared with known *Plasmodium* spp. by using the PlasmoDB.org Basic Local Alignment Search Tool/n and the National Center for Biotechnology Information Basic Local Alignment Search Tool/n tools. Results show that the primers PLF/UNR/VIR used in this PCR can produce uninterpretable results and non-specific sequences in field-collected mosquitoes. Three additional PCRs (PLU/VIV, specific for 18S small subunit ribosomal DNA; *Pvr47*, specific for a nuclear repeat; and GDCW/PLAS, specific for the mitochondrial marker, *cytB*) were then used to find a more accurate and interpretable assay. Samples that were amplified were again sequenced. The PLU/VIV and *Pvr47* assays showed cross-reactivity with non-*Plasmodium* spp. and an arthropod fungus (*Zoophthora lanceolata*). The GDCW/PLAS assay amplified only *Plasmodium* spp. but also amplified the non-human specific parasite *P. berghei* from an *Anopheles belenrae* mosquito. Detection of *P. berghei* in South Korea is a new finding.

The Malaria Research and Reference Reagent Resource[1] recommends a nested polymerase chain reaction (PCR) specific for the 18S small subunit ribosomal DNA (ssrDNA) gene fragment for the detection of human *Plasmodium* species in *Anopheles* spp. mosquito vectors.[2] This assay was first designed to screen human blood for *Plasmodium* spp.,[3,4] and was later modified to screen mosquito vectors for *Plasmodium* spp. DNA.[2] The modifications were based on the results of a comparison of extraction techniques designed to mitigate the issue of inhibitors to PCR[2,5] in the DNA extraction method.[1] The same primers (PLF/UNR/VIR) were used in both studies.[2,3]

The PLF/UNR/VIR assay was used to test for *P. vivax* in mosquito vectors (Table 1). *Anopheles* mosquitoes were collected bi-weekly (3–4-day intervals) by using Mosquito Magnet traps (Pro-Model; American Biophysics Corp., Greenwich, RI) during August–September 2010 in South Korea. Mosquito collections were conducted in the Demilitarized Zone adjacent to the Military Demarcation Line separating North Korea from South Korea and at Warrior Base and Tongilchon located approximately 3 km from the Demilitarized Zone where malaria transmission was suspected.[6] *Anopheles* spp. females were placed individually in 2-mL cryovials, dried, and shipped to the Walter Reed Biosystematics Unit (Suitland, MD).

The head and thorax were separated from the abdomen from individual mosquitoes to isolate only sporozoite-infected (salivary glands) mosquitoes. Total genomic DNA was extracted by using phenol-chloroform extraction using the Autogen automated DNA extraction robot (AutoGen Inc., Holliston, MA) and eluted in 50 μL of buffer in a 96-well plate format. Mosquitoes were identified to species by sequencing the internal transcribed spacer region 2 and a sequence comparison to voucher specimens available in the National Center for Biotechnology Information (NCBI) (Bethesda, MD) database. Of the mosquitoes tested, 56% were *An. kleini*, 27% were *An. belenrae*, 15% were *An. sinensis*, and 2% were *An. pullus*. For *P. vivax* detection, the PCR master mixture contained 1× buffer, 0.4 μM of each primer, 0.1 mM of each dNTP, 1.5 mM $MgCl_2$, 5% dimethyl sulfoxide, 1 unit of Biolase Taq, and 1 μL of DNA template. The total reaction volume was 20 μL. The same master mixture was used in both rounds of amplification (nested PCR), and 1 μL of PCR template was used in the second reaction. For each PCR, a new master mixture was created to mitigate issues with a single batch and to minimize contamination of the master mixture. The cycling parameters were 94°C for 2 minutes; followed by 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1 minute; and a final extension at 72°C for 7 minutes. The same cycling parameters were used in the second PCR with an increase to 40 cycles.

The PCR amplicon was subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide in the same 96-well format as the plate layout of the DNA extraction for quick interpretation. Gels were photographed to record the results. Of the 94 individual mosquitoes tested for *P. vivax*, 20 produced amplicons after PCR, although results were ambiguous (Figure 1). Only six of the amplified samples produced bands that were approximately 499 basepairs, the expected size for this assay. All amplified samples were cleaned by using ExoSapIT (Affymertix Inc., Santa Clara, CA) and sequenced in both directions. Sequences were trimmed, cleaned, and aligned using Sequencher software (Sequencher V5.1; Gene Codes Co., Ann Arbor, MI). Sequences in which 90% of the base calls were quality calls were run through the *Plasmodium* full genome database PlasmoDB (http//:PlasmoDB.org). The quality of a base call is defined by peak height and peak separation.[7] Samples that did not significantly match any *Plasmodium* species in the database were then run through a standard Basic Local Alignment Search Tool (BLAST)/n search at the NCBI website.

Only 9 of the 20 samples produced a quality sequence. Of these samples, none of the sequences were of the expected

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TABLE 1
Primers, targets, and fragment sizes used for detecting *Plasmodium* species

Primer	Locus	Sequence 5′→3′	Specificity	Size of product, basepairs
GDCW 2	*cytB*	CGGTCGCGTCCGGTAGCGTCTAATGCCTAGACGTATTCCTGATTATCCAG	*Plasmodium* sp.	–
GDCW4	*cytB*	CGCATCACCTCTGGGCCGCGTGTTTGCTTGGGAGCTGTAATCATAATGTG	*Plasmodium* sp.	–
PLAS1	*cytB*	GAGAATTATGGAGTGGATGGTG	*Plasmodium* sp.	815
PLAS2	*cytB*	TGGTAATTGACATCCAATCC	*Plasmodium* sp.	815
PLF	18S	AGTGTGTATCAATCGAGTTTC	*Plasmodium* sp.	821 for *P. vivax*; 787 for *P. falciparum*
UNR	18S	GACGGTATCTGATCGTCTTC	*Plasmodium* sp.	–
VIR	18S	AGGACTTCCAAGCCGAAGC	*P. vivax*	499
PLU5	18S	CTTGTTGTTGCCTTAAACTTC	*Plasmodium* sp.	1,200
PLU6	18S	TTAAAATTGTTGCAGTTAAAACG	*Plasmodium* sp.	–
VIV1	18S	CGCTTCTAGCTTAATCCACATAACTGATAC	*P. vivax*	120
VIV2	18S	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	*P. vivax*	–
Pvr47-F	*Pvr47*	CTGATTTTCCGCGTAACAATG	*P. vivax*	333
Pvr47-R	*Pvr47*	CAAATGTAGCATAAAAATCYAAG	*P. vivax*	–

size of 499 basepairs once they were trimmed (Table 2). When sequences were run against the known *Plasmodium* spp. in the PlasmoDBs BLAST database, none of the nine sequences matched any species of *Plasmodium* with a percent match > 70%. When the same samples were run through the NCBI BLAST/n tool, no significant matches were identified. However, a positive control of *P. vivax* extracted from *An. dirus*[8] significantly match *P. vivax* in the PlasmoDBs BLAST/n tool (100% match), as well as in the NCBI BLAST/n tool (100% match), showing a successful amplification and sequencing reaction. The negative control of water produced no amplicon, suggesting that amplification products were not caused by laboratory contaminants.

When the primers were run through the NCBI database, the PLF primer had a 100% query coverage (QC) and 100% match identity (MI) to a *Hepatocyst* sp. (HQ605039.1). The UNR primer aligned with species in multiple phyla including Lophotrochozoa (AB679345), Rhodophyta (JX828192), Alveolata (JQ178269), Cnidaria (AB693054), and uncultured fungus (KC218924) with 100% QC and 100% MI. The VIR primer was also significantly similar to species in many phyla, which included Chordata (XM002609218) and Viridiplanta, with 100% MI with 89% QC; several types of fungus, including *Myceliophthora thermophila* (XM003662658) and *Metarhizium anisopliae* (AJ251965) with 100% MI and 89% QC; Ecdysozoa (XM002633807) with 84% QC and

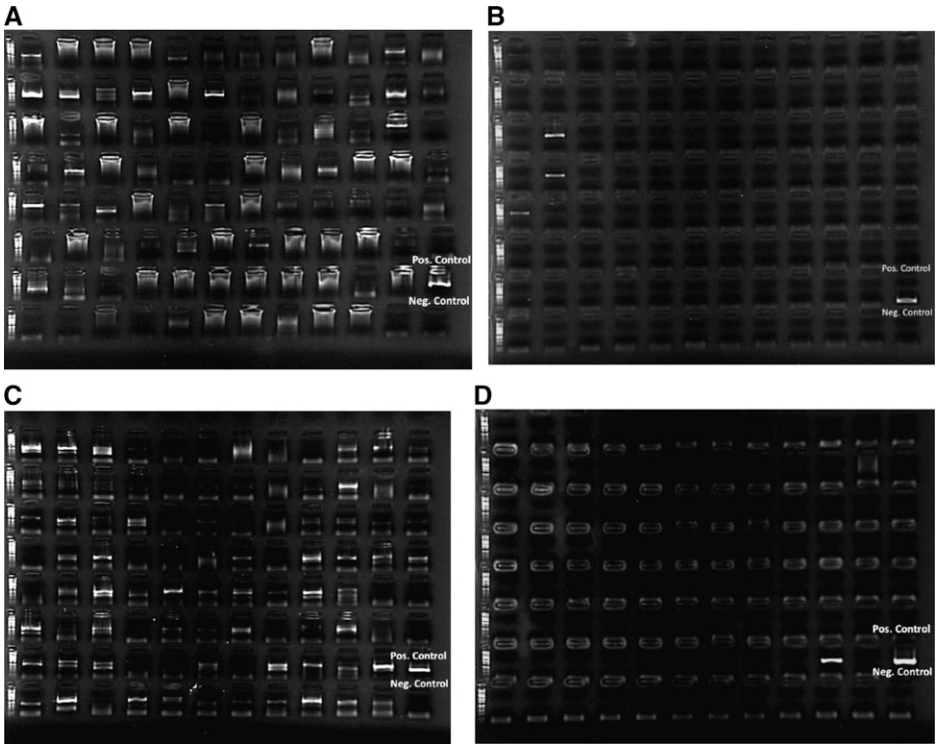

FIGURE 1. Agarose gel electrophoresis showing polymerase chain reaction amplification by **A**, semi-nested primers PLF/UNR/VIR; **B**, nested primers PLU5/PLU6/VIV1/VIV2; **C**, single-round Pvr47 F/R primers; and **D**, *cytB* nested primers GDCW2/GDCW4/PLAS1/PLAS2, for field collected *Anopheles* spp. mosquitoes. Pos. = positive; Neg. = negative.

TABLE 2
Sequencing results of amplified bands in field-collected *Anopheles* spp. mosquitoes*

Primer	Sample	Length, nucleotides	QC, %	Result PlasmoDB	% Match	*P*	Result NCBI	%	Coverage, %
PLAS 1/2	+ Control	629	99	*Plasmodium vivax*	100	**2.30×10^{-9}**	*P. vivax*	100	100
PLAS 1/2	10G	663	100	*P. berghei*	98	9.20×10^{-5}	*P. berghei*		
VIV1/2	+ Control	210	98	*P. vivax*	99	**9.50×10^{-34}**	*P. vivax*	99	100
VIV1/2	1E	170	92	*P. vivax*	64	0.057	*Zoophthora lanceolata*	99	100
VIV1/2	2C	241	93	*P. vivax*	86	0.042	*Z. lanceolata*	97	100
VIV1/2	2D	238	90	*P. vivax*	72	0.015	*Z. lanceolata*	98	100
PLF/VIR	+ Control	593	99	*P. vivax*	100	**5.90×10^{-9}**	*P. vivax*	100	100
PLF/VIR	3E	522	97	*P. vivax*	67		None		
PLF/VIR	6C	119	95	None			None		
PLF/VIR	10B	283	98	None			None		
PLF/VIR	16	352	100	*P. vivax*	67	0.00024	None		
PLF/VIR	1B	371	100	None			None		
PLF/VIR	1E	240	95	*P. chabaudi*	63	0.76	None		
PLF/VIR	2B	318	98	None			None		
PLF/VIR	2C	271	99	None			None		
PLF/VIR	2D	246	98	*P. knowlesi*	67	0.9999	None		
Pvr47 F/R	+ Control	116	86	*P. vivax*	99	**1.50×10^{-13}**	NA		
Pvr47 F/R	1C	498	95	None			NA		
Pvr47 F/R	1H	299	98	None			NA		
Pvr47 F/R	2H	543	97	*P. falciparum*	54	0.2	NA		
Pvr47 F/R	5E	474	96	None			NA		
Pvr47 F/R	8H	360	96	*P. berghei*	62	0.0067	NA		
Pvr47 F/R	9G	146	91	*P. yoelii*	69	0.95	NA		

*QC = query coverage; NCBI = National Center for Biotechnology Information; NA = not available. Values in **bold** are statistically significant.

100% MI; and several bacterium, including *Cryptococcus neoformans* (XM771947) and several uncultured bacterium (JQ818134, JQ818132, and JQ818120) with a 100% MI and 78% QC.

Three additional PCRs were then tested for detection of *P. vivax* in field-collected female mosquitoes. We tested a second 18S ssrDNA assay (PLU/VIV) that was originally designed to screen human blood for parasites,[9] a nested PCR specific for mitochondrial *cytB* (GDCW/PLAS),[10] and a single-step PCR specific for the 14–41 copy nuclear tandem repeat region *Pvr47* (Pvr47F/R)[11] (Table 1).

The PCR master mixtures and cycling parameters for the PLU/VIV and *Pvr47* assays were the same as the protocol used for the PLF/UNR/VIR assay above. The PCR mixtures were made and run independently in each assay. The *Pvr47* assay was a single round of amplification. The nested GDCW/PLAS assay consisted of a 10-μL reaction with a master mixture comprised of 0.3 μM of each primer, 2 mM $MgCl_2$, 0.125 mM dNTP, 1× buffer, 5% dimethyl sulfoxide, 1 unit of Biolase Taq, and 1 μL of DNA. The cycling parameters consisted of an initial temperature of 94°C; followed by 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute and 30 seconds; and a final extension at 72°C for 10 minutes. The nested PCR used the same cycling parameters for the second round, but the number of cycles was increased to 40.

DNA from the same 94 samples, which were previously tested by using the PLF/UNR/VIR primers, was tested again with the three assays listed above (Figure 1). A positive control of *P. vivax* extracted from *An. dirus* was included, as well as a negative control of nuclease-free water. The resulting amplicons were again sequenced in both directions, and the sequences were run through PlasmoDB BLAST and NCBI BLAST/n tools, with the exception of the *Pvr47* amplicons. *Pvr47* is a novel locus, whose sequences are not available in the NCBI database; therefore, sequences were only run through PlasmoDB BLAST/n.

Pvr47 produced amplicons in 60 of the 94 samples, but only 10 of these were of the approximate expected size of 333 basepairs. Most amplicons was either of the wrong size, smears, or produced multiple-banding patterns. Of the 60 amplified samples, only six produced clean sequences that were obtained from the *Pvr47* assay. None of the sequences matched any of the *Plasmodium* spp. found in the PlasmoDB.org database > 70%. For the PLU/VIV assay, 3 of the 94 samples amplified, but all were approximately 1,000 basepairs instead of the expected 120 basepairs. All samples had a quality score > 90%. However, none of these samples matched any species of the *Plasmodium* spp. found in the PlasmoDB.org database > 85%. When run through the NCBI database, all three matched *Zoophthora lanceolata* strain ARSEF (Accession nos. EF392550.1, EF392550.1, and EF392550.1) > 97%. *Zoophthora* is an arthropod fungus that is sometimes used as an insecticide.[12,13] The PLU5/6 primers were aligned with the 18S sequence of *Z. lanceolata.* The PLU5 primer matched the sequence with only a 1-basepair difference; the PLU6 primer matched the sequence with a 7-basepair difference. This finding might suggest that running a nested PCR with a total of 75 cycles could increase the probability of non-specific binding and explain the amplification of this fungus although the primers are not an exact match.

The GDCW/PLAS was the most successful of the assays because it did not result in smearing or non-specific binding. Amplification was observed in one *An. belenrae*, which was the approximate correct size of 815 basepairs. This sample was a 100% match to the rodent malaria parasite *P. berghei* in the PlasmoDB database, as well as in NCBI database (100% MI and 100% QC; accession no. DQ414645). The GDCW/PLAS assay is used to amplify human and primate

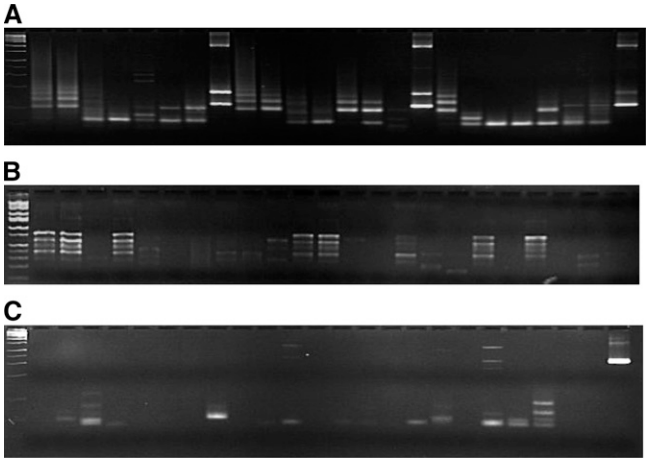

FIGURE 2. Agarose gel electrophoresis showing multiple banding patterns produced by polymerase chain reactions PLU/VIV (**A**), PLF/UNR/VIR (**B**), and *Pvr47* (**C**) used to detect *Plasmodium* spp. in *Anopheles* spp. mosquitoes.

malaria pathogens, but exclude *Apicomplexa* and human blood. To verify that this assay amplifies *P. berghei*, seven *An. stephensi* with known *P. berghei* infections were tested by using this PCR. All seven samples produced amplicons. However, before the testing of *An. stephensi*, no *P. berghei* had been used in this laboratory, which excluded on-site contamination. The discovery of *P. berghei* in South Korea and in *An. belerae* is novel. Also, the head and thorax was separated from the abdomen, suggesting that the sporozoites were in the salivary glands, and that the infection had not merely been ingested but had also propagated. Because *P. berghei* can infect multiple rodent vectors, and is a temperate species, it may have a wider range than previously expected.

Over the course of this research genomic DNA was extracted from several thousand mosquitoes for vector identification, and many of these mosquitoes were also screened for *P. vivax*. The *Pvr47*, PLF/UNR/VIR, and PLU/VIV assays continually produced uninterpretable results and multiple banding patterns, leading to this sequencing work. Samples that produced amplicons were subjected to electrophoresis on agarose gels for one hour to illustrate these results (Figure 2). The GDCW/PLAS assay did not produce any amplicons, illustrating that these primers are more specific than those used in the other three assays.

Our results showed that the PLF/UNR/VIR, PLU/VIV, and *Pvr47* assays produce non-specific sequences and uninterpretable results and have primers that could potentially bind to species in multiple phyla. Also, the PLU/VIV assay detects *Z. lanceolata*, an arthropod fungus. The GDCW/PLAS assay is more specific, but detects *P. berghei*, which is a rodent pathogen. Therefore, amplicons must be verified by sequencing. Although there is always potential for DNA degradation during shipping that could also result in smearing and irregular banding patterns, the clean sequences for the mosquito and *Plasmodium* amplicon is evidence that not only is the DNA viable, it is intact enough to provide quality sequences (Table 2). Our positive controls were infected mosquitoes, showing that our DNA extraction and detection methods are optimized for *in vivo* detection despite using an extraction protocol different from those used in the original publications. Also, if inhibitors were an issue, amplification of the mosquitoes or positive control would not have occurred. The strongest evidence that there may be issues with this detection method is their BLAST results for primers, which were independent of any laboratory work.

The conclusions of this study cast doubt on previously reported infection rates from South Korea and elsewhere where these assays were used.[6] Many samples produced clear amplicons, sequences multiple pea for a single base pair, we suspected was caused by co-amplification of multiple organisms. Although one could argue that a band of the incorrect size is not a positive result *per se*, an assay by definition should produce a binary band or no band result. With the exception of the GDCW/PLAS primers, these assays do not produce such a result. Although these assays may work well with human blood, confounding factors from field-collected mosquitoes, such as sample degradation, exposure to environmental contaminants, and low levels of mosquito infections, may reduce the utility of these assays, and therefore render them unsuitable for use for the detection of specific *Plasmodium* spp. The *cytB* GDCW/PLAS assay is the most promising technique for future studies, despite the fact that it is not limited to human-specific *Plasmodium* spp. detection. Because the interpretation of results when PCR is used can be ambiguous and somewhat subjective, the use of real-time assays in which a present diagnostic negative/positive result is pre-programmed (based on cycle threshold) would produce more robust results in future studies. However, the primers used in a real-time assay must be thoroughly tested with field-collected mosquitoes to ensure they do not amplify non-*Plasmodium* species because many also target the 18S ssrDNA region.[14]

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