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J. Clin. Microbiol. 2014, 52(6):2144. DOI: 10.1128/JCM.00565-14.

Published Ahead of Print 16 April 2014.

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A Quality Control Program within a Clinical Trial Consortium for PCR Protocols To Detect *Plasmodium* Species

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Malaria parasite infections that are only detectable by molecular methods are highly prevalent and represent a potential transmission reservoir. The methods used to detect these infections are not standardized, and their operating characteristics are often unknown. We designed a proficiency panel of *Plasmodium* spp. in order to compare the accuracy of parasite detection of molecular protocols used by labs in a clinical trial consortium. Ten dried blood spots (DBSs) were assembled that contained *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*; DBSs contained either a single species or a species mixed with *P. falciparum*. DBS panels were tested in 9 participating laboratories in a masked fashion. Of 90 tests, 68 (75.6%) were correct; there were 20 false-negative results and 2 false positives. The detection rate was 77.8% (49/63) for *P. falciparum*, 91.7% (11/12) for *P. vivax*, 83.3% (10/12) for *P. malariae*, and 70% (7/10) for *P. ovale*. Most false-negative *P. falciparum* results were from samples with an estimated ≤ 5 parasites per μl of blood. Between labs, accuracy ranged from 100% to 50%. In one lab, the inability to detect species in mixed-species infections prompted a redesign and improvement of the assay. Most PCR-based protocols were able to detect *P. falciparum* and *P. vivax* at higher densities, but these assays may not reliably detect parasites in samples with low *P. falciparum* densities. Accordingly, formal quality assurance for PCR should be employed whenever this method is used for diagnosis or surveillance. Such efforts will be important if PCR is to be widely employed to assist malaria elimination efforts.

Molecular detection methods for malaria parasites, including PCR assays, detect low-level malaria parasitemias that are missed by microscopy and rapid diagnostic tests (RDTs). In many settings, these “submicroscopic” infections usually far outnumber patent infections (1). Because they are not identified by routine clinical or research diagnostics and therefore remain untreated, they constitute a reservoir of parasites for ongoing transmission (2). Thus, efforts to reduce transmission and eliminate malaria may need to use PCR in order to eliminate the malaria reservoir (3).

PCR-based methods to detect malaria parasites are diverse in design and operation. These factors have the potential to introduce substantial variability in the operating characteristics of methods (4). The definition of “submicroscopic” parasites is further complicated by inconsistencies between operators in assessing parasites by microscopy (5). Collectively, these microscopic and molecular considerations can produce inconsistencies in measurement and undermine the generalizability of findings related to PCR-detectable parasites.

Quality control procedures have been endorsed by the WHO for parasite detection by both microscopy (6) and RDTs (7), likely owing

to their clinical use. Although RDTs have been advanced as adequate tools to capture the submicroscopic parasite reservoir (8), their sensitivity of parasite detection is generally lower than that achieved by PCR methods or by expert microscopy (9). Therefore, PCR methods are increasingly employed for parasite detection in research studies, and there exist nascent efforts to standardize quantification (10) and reporting (11) of assays. Nevertheless, the comparative, qualitative performance of PCR assays used to detect the major species of malaria parasites is largely unknown.

Received 28 February 2014 Returned for modification 24 March 2014

Accepted 8 April 2014

Published ahead of print 16 April 2014

Editor: M. J. Loeffelholz

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doi:10.1128/JCM.00565-14

TABLE 1 Constituents of the proficiency panel

Sample	Component(s) ^a
1	<i>P. ovale</i> from a patient admitted with microscopic <i>P. ovale</i>
2	<i>P. falciparum</i> strain 3D7 from culture (approximately 5%, or 4×10^6 parasites) added to <i>P. malariae</i> and trace <i>P. falciparum</i> from a patient admitted with microscopic <i>P. malariae</i> (submicroscopic <i>P. falciparum</i>)
3	<i>P. vivax</i> strain Brazil I from an <i>Aotus</i> monkey (approximately 5×10^5 parasites)
4	Noninfected fresh whole blood
5	<i>P. falciparum</i> strain 3D7 from culture (approximately 5%, or 1.6×10^7 parasites)
6	<i>P. falciparum</i> strain 3D7 from culture (approximately 5%, or 9×10^6 parasites) and <i>P. vivax</i> strain Brazil I from an <i>Aotus</i> monkey (approximately 5×10^5 parasites)
7	<i>P. falciparum</i> strain 3D7 from culture (approximately 5%, or 1.5×10^6 parasites) added to <i>P. ovale</i> from a patient admitted with microscopic <i>P. ovale</i>
8	<i>P. falciparum</i> strain 3D7 from culture (trace, or 1.5×10^5 parasites)
9	<i>P. falciparum</i> strain 3D7 from culture (approximately 0.5%, or 2.6×10^5 parasites)
10	<i>P. malariae</i> and trace <i>P. falciparum</i> from a patient admitted with microscopic <i>P. malariae</i> (submicroscopic <i>P. falciparum</i>)

^a All samples included whole blood, either from an infected patient (samples 1, 2, 7, and 10) or uninfected whole blood from a single donor (samples 3, 4, 5, 6, 8, and 9). Estimated quantities of input 3D7 were computed from input parasitemias and output quantitation.

The Malaria in Pregnancy Consortium is a global consortium of research groups conducting clinical studies investigating the impacts of current and novel interventions to prevent pregnancy-associated malaria (<http://www.mip-consortium.org/>). Two factors necessitated the evaluation of PCR assays in this complex clinical trial consortium: (i) the use of multiple laboratories with various methodologies for molecular parasite detection, and (ii) the requisite need to quantify the impact on birth outcomes of the low-level parasitemias that are typically observed in pregnancy. Therefore, we assembled a “proficiency panel” of *Plasmodium* parasites and distributed this panel to associated molecular laboratories for masked testing. Herein, we describe the design of the panel and the results of testing at 9 molecular laboratories from four continents.

MATERIALS AND METHODS

Panel design. A panel of 10 dried blood spots (DBSs) was designed that contained the four most common human malaria parasites: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. We designed the panel to address several common issues with molecular detection methods: (i) the ability to detect all four of the most common human malaria species in Africa; (ii) the sensitivity of *P. falciparum* detection, taking into account three different parasite densities (5%, 0.5%, and trace); (iii) the ability to discriminate species within mixed-species infections that would most likely include *P. falciparum* (thus, we included samples of each of the other species mixed with *P. falciparum*).

Additionally, we included a DBS with uninfected whole blood as a control. The sources of each parasite are summarized in Table 1.

Panel assembly. For DBSs with *P. falciparum*, *P. falciparum* line 3D7 (MRA-102, MR4; ATCC, Manassas, VA) was cultivated in continuous *in vitro* culture in O⁺ human red blood cells (RBCs) to 5% to 13% parasite density (as confirmed by light microscopy), centrifuged to remove serum and concentrate RBCs, and split with uninfected fresh whole blood collected in a tube with EDTA to obtain the targeted parasite densities. Because of variations in the density of cultivated parasites and hematocrits, varied final densities of *P. falciparum* 3D7 were considered approximate. For DBSs with *P. falciparum* mixed with other species, *P. vivax*, *P. ovale*, or *P. malariae* was added to the 3D7 aliquot.

For *P. vivax*, a 500- μ l specimen of 40,000 parasites/ μ l at 50% hematocrit was used. This was diluted with fresh uninfected whole blood with or without additional *P. falciparum* strain 3D7 from culture to obtain *P. vivax*-containing DBSs. For *P. malariae* and *P. ovale*, we blotted fresh venous blood from admitted patients onto filter paper, with or without additional *P. falciparum* strain 3D7 from culture, to obtain DBSs.

All DBSs consisted of 50 μ l of blood aliquoted from the single preparations as described above. These were placed onto pre-labeled Whatman 3MM filter paper in duplicate and left to air dry overnight. All DBSs were produced in a laminar flow hood. The 10 DBSs that comprised each panel were placed into a single sealable plastic bag with desiccant and separated within by standard weighing paper.

Panel internal quality control. Genomic DNA (gDNA) was extracted within 2 weeks of preparation and storage at 4°C from each DBS of a single set of 10 DBSs after punching a 5-mm hole from each DBS by using a QIAamp DNA minikit. gDNA samples were tested for *P. falciparum*, *P. malariae*, *P. ovale*, and the human gene glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), using duplex real-time PCR assays targeting the *Plasmodium* 18S rRNA gene (12). Cycle thresholds (C_T) were set manually by personnel masked to input gDNA. Samples were also tested in a separate SYBR green real-time PCR assay targeting only *P. vivax* (13); this 25 μ l reaction mixture consisted of 12.5 μ l of SYBR green master mix, 900 nM (each) primers targeting the *P. vivax* 18S rRNA, and 5 μ l of template.

In order to estimate the quantities of *P. falciparum* parasites in the samples, gDNA samples were tested in a *P. falciparum*-specific real-time PCR assay targeting the single-copy gene *P. falciparum* lactate dehydrogenase (*Pf**ldh*) (14). The reactions were performed with a set of 10 standards of *P. falciparum* 3D7 gDNA from 0.1 ng/ μ l to 5×10^{-5} ng/ μ l, which were used to generate a standard curve and estimate parasite quantities in the unknowns.

All real-time PCRs were prepared in a laminar flow hood with filtered pipette tips, tests were performed in duplicate, and samples were tested on plates that included appropriate negative and positive controls.

Distribution of panels. We offered the panel to collaborating laboratories within the Malaria in Pregnancy Consortium and to others who requested it. The panels were posted at room temperature and received within 7 days of sending. Each filter paper was labeled only 1 to 10. A letter accompanying the panel informed recipients of the following: “The samples represent *P. falciparum*, *vivax*, *ovale*, and *malariae*, some in mixed fashion, and some as pure species. All samples were prepared with whole parasites, so they should accommodate any genes targeted by your molecular assays.” Therefore, recipients were blinded to the constituents of the DBSs. The panels were tested per each laboratory’s standard procedures for parasite detection.

Collaborating laboratories were located in Gambia, Benin, Gabon, Kenya, Tanzania, Mozambique, India, The Netherlands, and Italy. After the collaborating laboratory tested the panel, feedback was provided on the accuracy of results to both the laboratory staff and the principal investigator of the associated clinical study. The detection rate was defined as the number of correct species detected divided by the number of species tested for; therefore, these rates were computed only for species that were

targeted by the laboratory's assay. False-positive results were defined as the detection of a species that was absent; false-negative results were defined as the failure to detect a species that was present.

Ethics statement. The collection and use of *Plasmodium* parasites from patients at the University of North Carolina Hospital were approved by the UNC Institutional Review Board.

RESULTS AND DISCUSSION

Panel assembly and quality control. We produced 20 panels of 10 DBSs each. The constituents of each panel are outlined in Table 1. Internally, a panel was first tested with real-time PCR assays that collectively detected all 4 *Plasmodium* species that are known to be transmitted in Africa as well as a human gene. In this testing, human DNA was detected in all 10 DBSs. All four species were detected from each DBS on which they were known to be present, in both mono- and mixed-species samples. The DBS with only uninfected blood was positive only for the human control gene. There were no false-positive results.

A second assay capable of estimating *P. falciparum* quantity was also 100% sensitive and specific, although the two samples containing "trace" *P. falciparum* returned discordant results between the two replicates. Based upon standard curves, this assay returned quantity estimates of 0.5 parasites/μl of gDNA (sample 8) and 2 parasites/μl of gDNA (sample 10) for these two trace *P. falciparum* samples. Because each DNA extraction yielded 50 μl of gDNA from approximately 20 μl of blood, we estimated that these samples contained approximately 1.25 and 5 parasites/μl of whole blood, respectively.

Results by *Plasmodium* species. We distributed the panel to 9 collaborating molecular laboratories for testing. The protocols employed by these labs are outlined in Table 2. These laboratories were blinded to the true constituents of each DBS. Overall, 68 of 90 (75.6%) results were correct (Table 3). Of the 22 incorrect results, 20 were false-negative results and 2 were false positives.

The detection rate was 77.8% (49/63) for *P. falciparum*, 91.7% (11/12) for *P. vivax*, 83.3% (10/12) for *P. malariae*, and 70% (7/10) for *P. ovale* (Fig. 1). Among *P. falciparum* samples, the detection rate for *P. falciparum* was 77.8% both for monospecies samples (21/27) and for multispecies samples (28/36). Among *P. falciparum* monospecies samples, the detection rate was 100% (9/9) for those with 5% parasite density, 100% (9/9) for those with 0.5% density, and 33.3% (3/9) for those with trace parasites (Fig. 1). Therefore, overall, the diverse protocols employed were largely able to accurately detect *P. falciparum* when present at densities that are typically identified in clinical infections in monospecies infections and when mixed with other *Plasmodium* spp.

Detection of *P. falciparum* was reduced at the lowest density. In the sample containing only trace amounts of *P. falciparum* from *in vitro* culture, only 3/9 labs detected the parasite. Similarly, in the sample with microscopically detected *P. malariae* and submicroscopic *P. falciparum*, only 1/9 labs detected *P. falciparum*. This low detection rate (22.2%; 4/18) of trace *P. falciparum* suggests that some PCR protocols require optimization to improve sensitivity and that this sensitivity should be routinely quantified and reported, as has been suggested for real-time PCR protocols (15).

Among *P. vivax* samples, the detection rate was 100% (6/6) for monospecies *P. vivax* and 83.3% (5/6) for *P. vivax* when mixed with *P. falciparum*. Only 6 of the 9 labs included methods to detect *P. vivax* in their protocols. The only false-negative *P. vivax* result originated from lab B, which failed to report any mixed infections.

TABLE 2 Protocols employed to detect *Plasmodium* spp. parasites at an internal laboratory and 9 collaborating laboratories

Lab	gDNA extraction	PCR chemistry	No. of cycles	Positive criterion	Gene target	No. of replicates	No. of targets	Species targeted	Reference(s)
UNC	QIAamp DNA minikit	Real-time PCR with TaqMan probes	40	$C_T < 40$	<i>Plasmodium</i> 18S rRNA	2	Duplex/monoplex	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	22
UNC	QIAamp DNA minikit	Real-time PCR with TaqMan probe	40	$C_T < 40$	<i>P. falciparum</i> lactate dehydrogenase	2	Monoplex	<i>P. falciparum</i>	14
A	QIAamp DNA blood kit	Nested PCR	30/30	Agarose gel electrophoresis	<i>Plasmodium</i> 18S rRNA	1	Monoplex	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i>	25, 26
B	QIAamp DNA minikit	Real-time PCR with TaqMan probe	40	$C_T < 40$	<i>Plasmodium</i> 18S rRNA	2	Duplex	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	25, 26
C	Chelex protocol	Real-time PCR with TaqMan probes	40	$C_T < 40$	<i>Plasmodium</i> 18S rRNA	2	Multiplex	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	27
D	QIAamp DNA minikit	Real-time PCR with XS probes	50	$C_T < 50$	<i>Plasmodium</i> 18S rRNA	2	Multiplex	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	28, 29
E	QIAamp DNA minikit	Real-time PCR with TaqMan probes	40	$C_T < 40$	<i>P. falciparum</i> 18S rRNA	2	Monoplex	<i>P. falciparum</i>	30
F	Invitrogen PureLink genomic DNA kit	Real-time PCR with TaqMan probes	50	$C_T < 45$	<i>Plasmodium</i> 18S rRNA	2	Multiplex	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	13, 27
G	QIAamp DNA minikit	Real-time PCR with TaqMan probe	40	$C_T < 40$	<i>P. falciparum</i> 18S rRNA	2	Monoplex	<i>P. falciparum</i>	25
H	QIAamp DNA minikit	Real-time PCR with TaqMan probe	40	$C_T < 40$	<i>P. falciparum</i> 18S rRNA	2	Monoplex	<i>P. falciparum</i>	30
J	QIAextractor	Nested PCR	24/29	Capillary gel electrophoresis	<i>Plasmodium</i> 18S rRNA	1	Monoplex	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	22, 23

After revising their workflow, both *P. falciparum* and *P. vivax* were successfully detected. Therefore, the diverse protocols employed in these labs were largely able to detect *P. vivax* in mono- and mixed-species parasitemias.

P. ovale had the lowest detection rate of the four species (70%), though it was assayed the least frequently, in only 10 tests. The three false-negative results occurred in labs that reported other false negatives; therefore, it is unclear if the errors resulted from laboratory procedures or from an inability to detect the dimorphic *P. ovale* (16).

There were only 2 false-positive results: one lab reported *P. falciparum* in the negative DBS, and another lab reported *P. falciparum* in the *P. vivax* monospecies sample. Among the 20 false-negative results, 14 resulted from the failure to detect *P. falciparum* in trace quantities either in a monospecies sample or mixed with *P. malariae*.

Results by laboratory. Accuracy ranged from 100% (Lab A) to 50% (Labs B and F). In five of the labs (C, E, G, H, and J), the only incorrect results were the failure to detect *P. falciparum* in trace amounts, either alone or when mixed with *P. malariae*.

Detection of mixed infections was challenging. Lab B returned 5 incorrect results: one false positive (*P. falciparum* detected in an uninfected sample) and four false negatives (all in mixed-species infections). These results prompted discussions with the laboratory head and with a reference laboratory regarding the protocol used. The real-time PCR protocol consisted of a single set of *Plasmodium* primers multiplexed with four species-specific probes, suggesting that, in mixed-species infections, only the dominant species was being amplified and detected. This competitive inhibition can reduce the sensitivity of parasite PCR assays (17). The protocol was modified to include species-specific forward primers, and a fresh panel was retested with the updated protocol. Using this updated protocol, the assay was newly able to detect *P. vivax*, *P. ovale*, and *P. malariae* when each was mixed with *P. falciparum*, thus improving the assay's ability to detect mixed infections (Lab D). The updated protocol subsequently went live in Labs B and D.

Future directions and recommendations. Our observations suggest several practices that will help to assure high quality in molecular detection protocols.

- (i) A DBS panel of *Plasmodium* spp. should be tested and interpreted by standard protocols by routine laboratory staff blinded to sample constituents.
- (ii) This testing should be repeated at planned intervals or when procedures are altered by the addition of new reagents, hardware, or personnel.
- (iii) For protocols that are employed to detect low-level, "sub-microscopic" parasitemias to aid in malaria elimination efforts, the lower limit of detection of the assay should be formally quantified and reported. This can be achieved by using a panel of *P. falciparum* samples across a range of parasite densities.

PCR methods and laboratories vary in their sensitivities and specificities to detect malaria parasites. Most protocols tested in this study consistently detected the major species *P. falciparum* and *P. vivax* at densities that typically manifest symptoms. However, sensitivities and specificities of *P. falciparum* detection were much more variable for low-level and submicroscopic parasitemias. Submicroscopic infections are important because they constitute reservoirs of parasites that can sustain transmission (18) and are targeted by elimination campaigns (2). Additionally,

TABLE 3 Results of molecular testing of the proficiency panel at the source laboratory and 9 collaborating laboratories

Lab	Species targeted	Species detected when sample contained ^a									
		<i>P. ovale</i>	<i>P. falciparum</i> and <i>P. malariae</i>	<i>P. vivax</i>	Negative	<i>P. falciparum</i> (5%)	<i>P. falciparum</i> (5%) and <i>P. vivax</i>	<i>P. falciparum</i> (5%) and <i>P. ovale</i>	<i>P. falciparum</i> (trace)	<i>P. falciparum</i> (0.5%)	<i>P. falciparum</i> (trace) and <i>P. malariae</i>
UNC	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	<i>P. ovale</i>	<i>P. falciparum</i> and <i>P. malariae</i>	<i>P. vivax</i>	Negative	<i>P. falciparum</i>	<i>P. falciparum</i> and <i>P. vivax</i>	<i>P. falciparum</i> and <i>P. ovale</i>	<i>P. falciparum</i> (trace)	<i>P. falciparum</i> (0.5%)	<i>P. falciparum</i> and <i>P. malariae</i>
UNC	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i>	Negative	<i>P. falciparum</i> and <i>P. malariae</i>	Negative	Negative	<i>P. falciparum</i>	<i>P. falciparum</i> and <i>P. vivax</i>	<i>P. falciparum</i> and <i>P. ovale</i>	<i>P. falciparum</i> *	<i>P. falciparum</i>	<i>P. falciparum</i> and <i>P. malariae</i>
A	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i>	Negative	<i>P. falciparum</i> and <i>P. malariae</i>	<i>P. vivax</i>	Negative	<i>P. falciparum</i>	<i>P. falciparum</i> and <i>P. vivax</i>	<i>P. falciparum</i>	<i>P. falciparum</i> *	<i>P. falciparum</i>	<i>P. falciparum</i> and <i>P. malariae</i>
B	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	<i>P. ovale</i>	<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. falciparum</i>	<i>P. falciparum</i>	<i>P. falciparum</i>	<i>P. falciparum</i>	<i>P. falciparum</i>	<i>P. falciparum</i>	<i>P. malariae</i>
C	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	<i>P. ovale</i>	<i>P. falciparum</i> and <i>P. malariae</i>	<i>P. vivax</i>	Negative	<i>P. falciparum</i>	<i>P. falciparum</i> and <i>P. vivax</i>	<i>P. falciparum</i> and <i>P. ovale</i>	Negative	<i>P. falciparum</i>	<i>P. malariae</i>
D	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	<i>P. ovale</i>	<i>P. falciparum</i> and <i>P. malariae</i>	<i>P. falciparum</i> and <i>P. vivax</i>	Negative	<i>P. falciparum</i>	<i>P. falciparum</i> and <i>P. vivax</i>	<i>P. falciparum</i> and <i>P. ovale</i>	Negative	<i>P. falciparum</i>	<i>P. malariae</i>
E	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	Negative	<i>P. falciparum</i> and <i>P. malariae</i>	Negative	Negative	<i>P. falciparum</i>	<i>P. falciparum</i> and <i>P. vivax</i>	<i>P. falciparum</i>	Negative	<i>P. falciparum</i>	Negative
F	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	Negative	<i>P. falciparum</i>	<i>P. vivax</i>	Negative	<i>P. falciparum</i>	<i>P. falciparum</i> and <i>P. vivax</i>	<i>P. falciparum</i>	Negative	<i>P. falciparum</i>	Negative
G	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	Negative	<i>P. falciparum</i> and <i>P. malariae</i>	Negative	Negative	<i>P. falciparum</i>	<i>P. falciparum</i> and <i>P. vivax</i>	<i>P. falciparum</i>	Negative	<i>P. falciparum</i>	Negative
H	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	Negative	<i>P. falciparum</i> and <i>P. malariae</i>	Negative	Negative	<i>P. falciparum</i>	<i>P. falciparum</i> and <i>P. vivax</i>	<i>P. falciparum</i>	Negative	<i>P. falciparum</i>	Negative
J	<i>P. falciparum</i> , <i>P. vivax</i> , <i>P. malariae</i> , <i>P. ovale</i>	<i>P. ovale</i>	<i>P. falciparum</i> and <i>P. malariae</i>	<i>P. vivax</i>	Negative	<i>P. falciparum</i>	<i>P. falciparum</i> and <i>P. vivax</i>	<i>P. falciparum</i> and <i>P. ovale</i>	Negative	<i>P. falciparum</i>	<i>P. malariae</i>

^a False-positive results are shaded; false-negative results are shown in bold. *, one of two replicates was positive for *P. falciparum*.

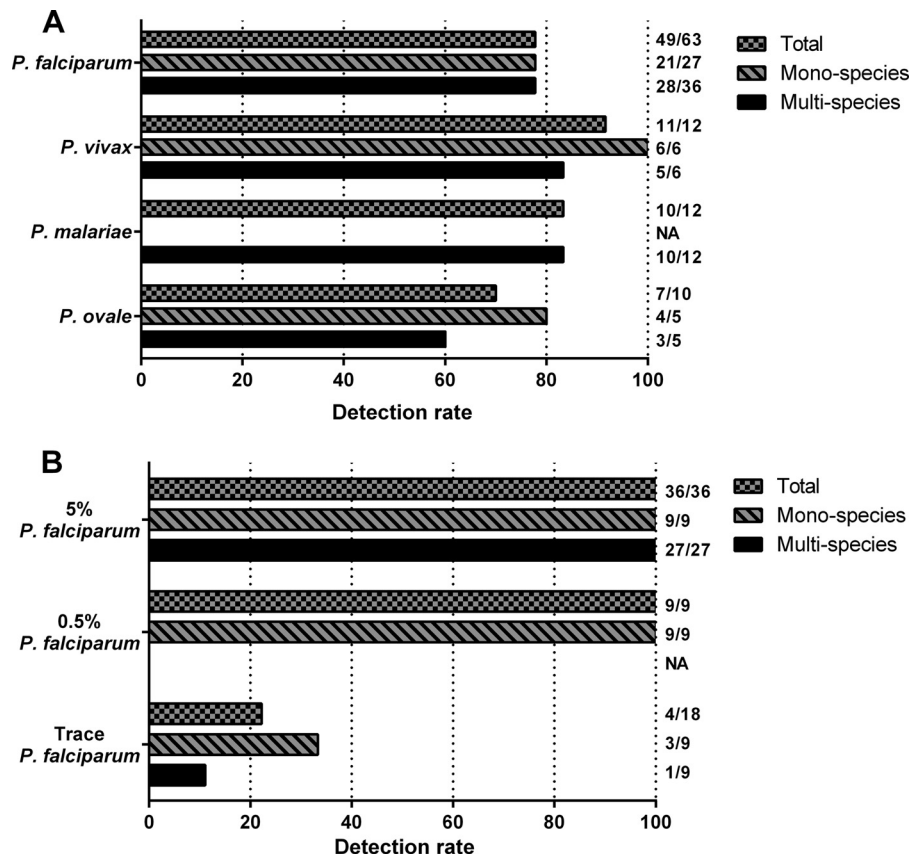


FIG 1 Detection rate at nine collaborating laboratories of each *Plasmodium* spp. (A) and *P. falciparum* at different parasite densities (B). The detection rate was defined as the number of correctly detected species divided by the number of *Plasmodium* spp. for which the samples were tested. 5%, 0.5%, and trace indicate the approximate parasite density of the sample tested; based upon testing in a real-time PCR assay, the “trace” specimens included between 1.25 and 5 parasites/ μ l of whole blood (see Results). (A) Results for each of the four species tested. (B) Results only for *P. falciparum* at the three approximate densities included in the panel. NA, not tested. Values to the right of the graph are the number of positive tests per total number of tests.

quantitative PCR methods are increasingly used to measure parasite clearance in drug efficacy studies (19).

Our study had several limitations. First, we included a limited number of strains of each species owing to restricted availability, and genetic diversity of the targets could have produced false negatives. Nevertheless, we did include reference standards of both *P. falciparum* and *P. vivax*, and all assays used by collaborating labs targeted common, conserved sequences and were previously validated against multiple *P. falciparum* strains. Additionally, as noted above, we included only a single *P. ovale* sample of indeterminate geographic origin, which may not reflect global species diversity owing to the recent recognition of its dimorphism. Finally, with only 10 DBSs, we did not comprehensively test the limits of detection and the quantitative performance of the assays. Future efforts should include a greater range of parasite densities to assist in defining this measure for molecular parasite assays.

In order to allow comparability between sites, PCR assays should be subject to strict ongoing quality control programs. Such efforts can utilize new panel preparations, such as those described here, including preparations of parasites obtained from central repositories, such as the Malaria Research and Reference Reagent Resource Center (MR4) or a *P. falciparum* preparation available through the UK National Institute for Biological Standards and Control, which has been endorsed by the WHO as a standard

quantity of *P. falciparum* DNA (10). Notably, the WHO currently sponsors external quality assurance programs for the molecular detection of influenza virus (20) and HIV drug resistance mutations (21). Similar centralized efforts may be required if molecular detection assays for malaria parasites become increasingly vital for malaria control programs.

ACKNOWLEDGMENTS

This work was supported by the Malaria in Pregnancy Consortium, which is funded through a grant from the Bill & Melinda Gates Foundation to the Liverpool School of Tropical Medicine and the European & Developing Countries Clinical Trials Partnership. Tests at Istituto Superiore di Sanità in Rome were performed in the framework of the PregVax Consortium; the PregVax project received funding from the European Union Seventh Framework Programme (FP7/2007–2013) under grant agreement 201588.

We thank John Barnwell (Centers for Disease Control and Prevention, Atlanta, GA) for providing the *P. vivax* DNA, Melissa Miller and Peter Gilligan (University of North Carolina) for assisting with clinical specimen collection, and MR4 for providing us with the 3D7 strain of *P. falciparum* that was originally contributed by D. J. Carucci. We appreciate the input provided by two reviewers within the Malaria in Pregnancy Consortium. We also thank Mireia Piqueras and Azucena Bardaji (both of the Barcelona Centre for International Health Research) for their assistance with trial coordination and the lab technicians who participated in the study process at each participating laboratory.

All authors declare that they have no relationships that may constitute a conflict of interest.

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