FALSE-POSITIVE RESULTS OF A PLASMODIUM FALCIPARUM HISTIDINE-RICH PROTEIN 2-DETECTING MALARIA RAPID DIAGNOSTIC TEST DUE TO HIGH SENSITIVITY IN A COMMUNITY WITH FLUCTUATING LOW PARASITE DENSITY

DAVID R. BELL,* DANNY W. WILSON, AND LAURA B. MARTIN
Australian Centre for International and Tropical Health and Nutrition, The Queensland Institute of Medical Research and The University of Queensland, Brisbane, Queensland, Australia

Abstract. The persistence of parasite histidine-rich protein 2 (HRP2) in the circulation after parasite clearance has been considered a drawback for rapid diagnostic tests (RDTs) targeting HRP2 and a major cause of false-positive results. This paper reports results of a study into whether a proportion of RDT HRP2 false-positive cases carried parasites using polymerase chain reaction analysis as the gold standard rather than microscopy. The high rate of RDT false-positive parasitemia results in comparison with microscopy was shown to predominantly represent cases that had a parasite density below the threshold for detection by microscopy. Despite the generally low disease-endemic prevalence of malaria in the area, there was a high prevalence of chronic infections with low, fluctuating, parasite densities that were better detected by RDT. Our results suggest that in areas known to have low-density parasitemias, RDTs targeting HRP2 may increase diagnostic sensitivity in comparison with microscopy. While microscopy remains the standard for comparison of malaria diagnostic accuracy, the limitations of microscopy, and the possibility that RDTs may have superior accuracy in some circumstances, should be taken into account when interpreting results of diagnostic trials.

INTRODUCTION

Reports of wide variation in accuracy of malaria rapid diagnostic tests (RDTs) in published field trials, usually compared with light microscopy as a gold standard,1–5 are hinder- ing the introduction of this technology into routine malaria case management. High false-positive rates, particularly for RDTs targeting histidine-rich protein 2 (HRP2),6–9 are frequently attributed to prolonged persistence of HRP2 in the circulation after parasite death,10,11 and are assumed to compromise the utility of RDTs targeting this antigen.12 A study in the Philippines compared the results of the ICT Malaria Pf/Pv RDT with microscopy, and found a high discordant rate involving cases positive for Plasmodium falciparum by the RDT but negative by microscopy.11 This paper reports the results of polymerase chain reaction (PCR) analysis of blood samples collected from 250 suspected malaria cases from the previous Philippine study. In contrast to previous interpretations of false-positive HRP2 detection,12 the PCR results presented here suggest that the detection of persistent HRP2 in the circulation may significantly increase diagnostic sensitivity. The implications of increased diagnostic sensitivity on interpretation of field studies are also discussed.

METHODS

Blood sampling was performed in late 1998 in remote areas of the province of Agusan del Sur in the southern Philippines, a region with low-endemic P. falciparum and P. vivax malaria. Other Plasmodium species are rarely reported in this region.12,13 Clinical malaria is usually not treated here due to poor availability of drugs. Study subjects were selected on the basis of recent symptoms and signs consistent with malaria. The study site, population, and the correlation of clinical diagnosis with microscopy and RDTs have been previously described.11

The study was reviewed and approved by the ethics review boards of the Research Institute for Tropical Medicine, Manila, and Queensland Institute of Medical Research, Australia. Blood was collected after informed consent was obtained from all adult participants at the Philippine field site, and from guardians of all minors. Briefly, all patients had finger prick blood samples collected for microscopy and the RDTs. The ICT Malaria Pf/Pv RDT (Amrad-ICT, Melbourne, Victoria Australia) includes a P. falciparum-specific HRP2-binding band and a pan-specific aldolase-binding band. Two positive test bands indicate either a P. falciparum infection or a mixed species infection. Non-falciparum infections are detected by the presence of a pan-specific band in the absence of a positive HRP2 reaction. The RDT were conducted strictly according to manufacturer’s instructions, and the bands were graded from 1 (weak) to 3 (equal to the control band). Barely discernible grade 1 bands were noted as faint. Blood films were read by an experienced technician after delayed staining per local practice and later restained and read by an expert microscopist.11 Finger prick blood (50 μL) was also preserved in 450 μL of 6 M guanidine hydrochloride,14 and stored at 4°C until analysis for parasite DNA by PCR.

Blood samples were extracted using the Wizard DNA Miniprep Kit (Promega, Madison, WI), eluted in 100 μL of 10 mM Tris-HCl, pH 8.5, and stored at −20°C until use. A PCR using nested primer pairs was conducted to screen all extracted samples for the presence of P. falciparum and P. vivax parasites. The primers used encompassed previously described polymorphic regions of the P. falciparum genes for merozoite surface protein 1 (MSP1), MSP2, and glutamate-rich protein (GLURP), and the P. vivax genes for MSP1, apical membrane antigen 1 (AMA1), and MSP3.15–17 First-round reactions were conducted in 50-μL volumes containing 0.2 mM of each dNTP, 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 1.25 units of Taq Gold (Applied Biosystems, Foster City, CA), 100 ng of the forward and reverse primers, 0.6–1 μL of the sample DNA, and 1 mM MgCl2. An aliquot (1–1.5 μL) of the first-round product and 1 mM (for P. falciparum reactions) or 1.5 mM (for P. vivax reactions) MgCl2 were added to the second-round PCR mixture. Cycling conditions were the same as for the primer pairs. An initial 10-minute denaturation step at 94°C preceded all reactions.

The PCR products for P/MSP1, P/GLURP, Pv/MSP1, and PvAMA1 were subjected to electrophoresis on 3% agarose

* Address correspondence to David R. Bell, Malaria Diagnostics, Malaria, Vector-Borne, and Other Parasitic Diseases, Western Pacific Regional Office, World Health Organization, PO Box 2932, Manila, The Philippines. E-mail: beld@wpro.who.int

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gels; the Pf/MSP2 PCR products were subjected to electrophoresis on 2% agarose gels; and the Pv/MSP3 PCR products were subjected to electrophoresis on 0.8% agarose gels. DNA bands were visualized by staining with ethidium bromide under ultraviolet illumination. A finger prick blood sample was considered parasite positive if at least two species-specific malaria gene products (of a possible three) were observed. Each sample was run three or four times before being considered negative for a single PCR primer pair.

Data were analyzed using Epi-Info version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA). Prevalence is reported with exact binomial 95% confidence intervals; measurements of diagnostic accuracy also include 95% confidence intervals. Since the RDT cannot distinguish between P. falciparum and a mixed species infection when both HRP2 and aldolase bands are positive, the RDT P. falciparum-positive cases cannot be used in assessment of the accuracy of detection of P. vivax.

RESULTS

Of the 347 samples obtained from suspected malaria symptomatic subjects, 3 were unsuitable for PCR analysis. Of the remaining 344 samples, 250 were selected and analyzed by PCR. Samples analyzed by PCR included all 198 cases with parasitemia identified by RDT and/or microscopy, and 52 randomly selected samples from the 149 concordant negative samples. Of the 250 samples selected, PCR analysis showed 124 samples positive for P. falciparum mono-infections, 39 samples positive for P. vivax mono-infections, 38 positive for mixed species infections, and 49 samples negative for parasite DNA. The distribution of the PCR results in comparison with RDT results are shown in Table 1. There was good agreement between the number of P. falciparum-positive and P. vivax-positive results obtained by PCR and the RDT.

Analysis of false-positive RDT results. In the original sample analysis, 48 samples were parasite positive by RDT but negative by microscopy. When these same samples were tested by PCR, 92% of these false-negative samples were true positives as determined by amplification of parasite genes. Plasmodium falciparum single-species infections were detected in 39 of 47 samples with positive HRP2 RDT results, and infection with P. vivax was confirmed in a single false-positive non-falciparum case (Table 2). Mixed species infections were detected in four additional samples. Only four of the false-positive samples were negative by PCR, of which two had faint-positive HRP2 bands and two were strongly positive.

Analysis of negative RDT results. The four cases that were parasite negative by RDT but positive by microscopy, were shown by PCR to have amplifiable parasite DNA; three were

| Table 2 |
|------------------|---|---|---|---|---|
| PCR              | RDT No. | Pf | M | Pv | Neg |
| P. falciparum    | 47      | 39 | 4 | 0  | 4  |
| P. vivax         | 1       | 0  | 0 | 1  | 0  |

* RDT = polymerase chain reaction; RDT = rapid diagnostic test; Pf = Plasmodium falciparum; M = mixed; Pv = P. vivax; Neg = negative.

P. falciparum and one was P. vivax. Of the 52 samples negative by both RDT and microscopy, PCR detected parasites in nine samples; four were P. falciparum and five were P. vivax. When extrapolated to the 149 concordant negative cases in the original symptomatic study population, this would equal 11 P. falciparum cases and 14 P. vivax cases not detected by either RDT or microscopy.

Prevalence and mixed-species infections. Plasmodium falciparum parasites were detected in 162 of the 250 samples analyzed by PCR, and P. vivax was detected in 77 of the PCR analyzed samples. When extrapolated to the original 347 symptomatic samples, this would equal prevalences of 48.7% (43.3–54.0%) and 24.7% (20.3–29.7%) for P. falciparum and P. vivax, respectively. Notable among the parasite species detected by PCR in the samples is the high number of mixed P. falciparum and P. vivax infections (19%, n = 38), while only 3.7% had been observed by microscopy. Interestingly, mixed-species infections were detected in nearly half (49.4%) of the P. vivax cases.

Assessment of RDT accuracy. Sensitivity and specificity are dependent on parasite density and predictive values are dependent on prevalence. Thus, for these values to be useful predictors of RDT accuracy, they must be applied to the population on which the RDTs are to be used. Since only a random subset of negative cases (52 of 149) were assessed by PCR, the rate of false-negative cases detected (four P. falciparum and five P. vivax) is extrapolated to the 149 negative RDT cases in the original study. Resultant estimates for sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for detection of P. falciparum were 91.1% (85.5–94.8%), 96.1% (91.7–98.3%), 95.7 (90.9–98.1%), and 91.9% (86.8–95.3%), respectively.

Among the 38 mixed-species infections identified by PCR, 33 had a positive pan-specific aldolase band (i.e., the RDT failed to detect P. vivax aldolase in 13% of the cases). Excluding cases positive for P. falciparum by RDT (see Methods), the sensitivity, specificity, PPV, and NPV of the RDT for P. vivax extrapolated to the original study population was 81.4% (71.2–88.7%), 99.3% (95.6–100%), 98.6% (91.3–99.9%), and 90.0% (84.0–94.0%), respectively.

DISCUSSION

The results of PCR analysis of samples from Agusan del Sur in the Philippines illustrate the dependence of comparative malaria diagnostic studies on the choice of the gold standard determinant of parasitemia. In published studies, microscopy is commonly used as a means for comparison of accuracy because PCR analysis is frequently unavailable or impractical. In field assessment of RDT sensitivity, a direct comparison between the new field diagnostic tool and a commonly accepted standard method for demonstration of para-
sitemia (usually microscopy) is essential. Problems arise when established methods are used as a gold standard without allowing for inherent inaccuracies.

Published estimates of reproducible PCR sensitivity for the single copy genes used in this study range from 10 to 100 ring-stage parasites/μL (laboratory isolates) to as high as 400 parasites/μL in field samples. Conducting up to nine PCRs with both the *P. falciparum* and *P. vivax*-specific primers greatly increases the reproducibility of parasite detection. A greater number of subpatent infections in the Philippine samples may have been detected had *Plasmodium* species been identified by PCR specific for rRNA. However, estimates of HRP2 RDT sensitivity (60–100 parasites/μL) fall within the limits expected for reproducible detection by a single-copy gene PCR.

While malaria microscopy may be capable of detecting very low parasite densities in some specialized centers (10–50 parasites/μL), microscopy of blood films collected under conditions commonly present in many malaria-endemic areas (e.g., the present study) has been demonstrated to miss many cases with low-density parasitemias. Other studies have demonstrated that RDTs can sometimes detect parasite densities that only the best microscopists might reliably identify. Initial comparison of these samples by microscopy showed many apparent false-positive RDTs results, similar to other studies. The long persistence of HRP2 in the circulation is a consequence of low specificity and negative predictive values. However, comparative PCR results presented here demonstrate that a positive HRP2 band in the absence of microscopy confirmation does not necessarily indicate a failure of the RDT, but rather a failure of the comparator standard assay. Parasite density in peripheral circulation in chronic *P. falciparum* infections can fluctuate between levels above and below the threshold of detection expected of microscopy due to sequestration of parasites and the effects of host immunity. This is commonly addressed with hospitalized patients by repeated blood sampling before parasitemia is excluded. In contrast, HRP2 can remain detectable for more than 10 days after parasite death, and a recent peak in parasite density may therefore leave a detectable trace of circulating antigen days later. As a result, HRP2 may be present in higher concentrations than the current peripheral parasite density, shown in Figure 1, may also explain the few false-negative RDT results that field microscopy had detected. Although repeat sampling for microscopy is impractical in field trials, the limitations of single sampling are rarely addressed in subsequent interpretation of results. Since single-sample microscopy supports the diagnosis of malaria in parts of Agusan del Sur, it is disturbing that many subpatent infectious cases in this area are not treated and thus contribute to disease transmission.

Detection of persistent HRP2 in the blood may therefore be either an advantage or a disadvantage in an RDT, depending on the characteristics of the population concerned. In this study area where prophylactic drug treatment is rare, HRP2 persistence may aid diagnostic accuracy. Conversely, in populations where recent drug treatment is likely, an RDT detecting a less persistent antigen, such as parasite lactate dehydrogenase (pLDH), may be an advantage.

The remaining small number (n = 4) of false-positive *P. falciparum* RDT results confirmed by PCR may have resulted from a number of factors. HRP2 may persist after elimination of parasites, although persistence after recent treatment is unlikely because anti-malaria treatment was not widely available and was excluded by verbal and clinic records. Spontaneous resolution of untreated infections is a relatively rare event, given the expected duration for untreated *P. falciparum* infections of several months, but could fully account for the low false-positive rate observed here. Heterophilic antibodies and antibodies directed against animal antigens are also known to cause false-positive results in immunoassays. Cross-reactivity with rheumatoid factor has been reported with other products, but is unlikely with the IgM antibodies used in this RDT. Analysis by PCR may also have missed cases due to technical errors or PCR inhibition.

The difficulty presented by choice of gold standard for determining RDT accuracy is most clearly apparent when reporting sensitivity, specificity, PPV, and NPV. In this study, the sensitivity of the RDT for *P. falciparum* was low, which is consistent with the high sensitivity of the PCR and the high rate of chronic infections in the study population. If the comparison had been restricted to microscopy, the sensitivity for *P. falciparum* detection would have been much higher (97.9%). Although sensitivity comparable to the PCR would be ideal, it is unrealistic at present to expect this from a field RDT. In view of the high sensitivity of the PCR, the 90% comparative sensitivity of the RDT for *P. falciparum* in this population is surprisingly good. The PPV and NPV are also dependent on underlying parasite prevalence in the population, and thus will also vary widely geographically with an RDT of equal accuracy.

Sensitivity for *P. vivax* in this study is markedly lower than...
that for *P. falciparum*, which is consistent with poorer sensitivity of the aldolase antibody band noted elsewhere.\(^1,3,34,44,45\)

Assessment is complicated because the target antigen is also produced by *P. falciparum*. In clinical practice, the importance of this band lies mainly in detection of *P. vivax* infections in people without *P. falciparum* infections, ensuring that they are also treated. Since standard drug combinations for infections with *P. falciparum* are also active against blood-stage *P. vivax*, failure to distinguish mixed-species infections from *P. falciparum* infections will not hinder management of the immediate illness, Although it will hinder anti-relapse therapy against *P. vivax*.

The 38 samples positive by PCR for both *P. falciparum* and *P. vivax* indicate that mixed-species infections in the Philippines may be far more common than previously believed. Results of earlier microscopy-based analysis had shown only 13 mixed-species infections,\(^11\) which is consistent with other Philippine studies.\(^12,13\) High prevalence of mixed-species infections shown by PCR, but missed by microscopy, has also been observed in east Africa (Gnimig J, unpublished data), the Middle East, and Asia.\(^24,25,28\) Cross-species immunity may occur, which suppresses the density of *P. vivax* and masks it from microscopy. Thus, the failure of RDT targeting pan-specific antigens to distinguish mixed-species infections from *P. falciparum* infections imparts little disadvantage compared with microscopy in this case. However, the failure of microscopy to detect a large number of *P. falciparum* parasitemias is alarming in its potential to promote continued transmission through under-treatment. Infections with *P. ovale* and *P. malariae* are rare in the Philippines,\(^46\) and although primers specific for these species were not used the absence of cases who were aldolase positive but PCR negative confirms that these species play little role.

In view of the greater sensitivity for *P. falciparum* of the HRP2 band over the aldolase band,\(^1,3,34,44,45\) the detection of aldolase but not HRP2 in one sample shown to be *P. falciparum* by both PCR and microscopy is unexpected. One explanation is that this case represents *P. falciparum* with an HRP2 gene deletion (Traore I and others, unpublished data), or reduced HRP2 expression. If this occurs, it may be a further contributor to the other RDT false-negative *P. falciparum* cases.

The PCR analysis of these Philippine field samples emphasizes the importance of the characteristics of the comparative standard when interpreting both diagnostic and epidemiologic studies. Reliance on microscopy alone in disease-endemic areas such as Agusan del Sur may result in misleading interpretation of the values of alternative forms of diagnosis, and underestimate mixed-species infections. Likewise, comparison between different studies of RDTs, and the usefulness of statements on requirements for RDT sensitivity,\(^47,48\) are of limited value if detailed information on population and comparative standards is not included. Local epidemiologic characteristics, as well as diagnostic aims, should be considered when choosing the appropriate RDT. Antigen persistence, in particular, may offer advantages or disadvantages in different situations.

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Authors’ addresses: David R. Bell, Malaria Diagnostics, Malaria, Vector-Borne, and Other Parasitic Diseases, Western Pacific Regional Office, World Health Organization, PO Box 2932, Manila, The Philippines, Telephone: 63-2-526-801, Fax: 63-2-521-1036, E-mail: belld@who.int. Danny W. Wilson, Walter and Eliza Hall Institute, Melbourne, Victoria 3050, Australia. Laura B. Martin, Malaria Vaccine Development Branch, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852.

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