RAPID DIAGNOSTIC DEVICES FOR MALARIA: FIELD EVALUATION OF A NEW PROTOTYPE IMMUNOCHROMATOGRAPHIC ASSAY FOR THE DETECTION OF PLASMODIUM FALCIPARUM AND NON-FALCIPARUM PLASMODIUM

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Abstract. The NOW® ICT Malaria P.f./P.v. for Whole Blood (Binax, Inc., Portland, ME) is a new malaria rapid diagnostic device that represents a technical advance over previous assays, such as ICT™ Malaria P.f./P.v. and ICT™ Malaria P.f. We evaluated this device in March 2001 in symptomatic patients at malaria clinics in Maesod, Thailand. Microscopic examination of Giemsa-stained blood smears was the reference standard. In 246 patients, microscopy showed 32 (13.0%) infected with Plasmodium falciparum, 63 (25.6%) with P. vivax, 6 (2.4%) with mixed infections of P. falciparum and P. vivax, 5 (2.0%) with P. malariae, and 140 (56.9%) negative. Sensitivity for P. falciparum was 100% and specificity was 96.2% (200 of 208; 95% confidence interval [CI] = 92–98). For P. vivax, sensitivity was 87.3% (55 of 63; 95% CI = 77–93) and specificity was 97.7% (173 of 177; 95% CI = 95–99), but all the four false-positive results were microscopically positive for P. malariae; thus, specificity for non-falciparum Plasmodium was 100%. These results suggest improved performance over NOW® ICT predecessors.

INTRODUCTION

The ICT™ Malaria P.f. test (ICT Diagnostics, Brookvale, New South Wales, Australia) was the first malaria rapid diagnostic device (MRDD) designed for convenience of use in a booklet format with the test strip mounted on cardboard.1 Like other non-microscopic malaria rapid tests, ICT™ Malaria P.f. was an immunochromatographic assay. The original ICT assay detected only Plasmodium falciparum histidine-rich protein-2 (HRP-2). By 1999, AMRAD (Frenchs Forest, New South Wales, Australia) acquired ICT Diagnostics, and continued manufacturing the product. About the same time, the product was further developed by adding the capability to detect non-falciparum malaria. The refined assay was renamed ICT™ Malaria P.f./P.v. This improvement was achieved by using monoclonal antibodies to capture Plasmodium aldolase, in addition to the HRP-2 test.2 In July 2000, AMRAD ceased the production of ICT, sold its ICT division to Binax, Inc. (Portland, ME), where further developmental work was done to refine the test. The new ICT test was released under the name NOW® ICT Malaria P.f./P.v. for Whole Blood (referred to as NOW® ICT hereafter). In this study, we performed a preliminary evaluation of the NOW® ICT prototype for its operational characteristics and test accuracy. With respect to diagnostic accuracy, we sought to determine the test sensitivity and specificity in detecting P. falciparum and P. vivax, at specified ranges of parasitemia, in the blood of patients presenting with possible clinical malaria in Thailand.

MATERIALS AND METHODS

Subjects. Patients seeking diagnosis and treatment at the malaria clinics under the responsibility of the Maesod Vector Borne Diseases Control Unit in Tak Province, Thailand from March 12 to 30, 2001 were screened for eligibility. Eligible subjects were those ≥20 years old with symptoms of an oral temperature ≥38°C, headache, or a history of fever in the past 72 hours. The malaria clinics serve mainly ethnic Karen and Burmese migrants from Myanmar (formerly Burma). Subjects were recruited regardless of their sex, ethnic background, occupation, or previous malaria episodes. The protocol was approved by the Thai Ministry of Public Health’s Ethical Review Committee for Research in Human Subjects, and the Walter Reed Army Institute of Research Human Use Review Committee.

Specimen collection. Two milliliters of blood was drawn into an EDTA Vacutainer® tube (Becton Dickinson, Franklin Lakes, NJ). Two slides, each with thick and thin blood smears, and one slide with only a thick smear were immediately prepared. The thick smear was stained and examined by the clinic’s microscopist, not by study investigators, according to the clinic’s routine procedures. Based on this microscopic result, treatment was provided by the clinic staff according to the standard guidelines established by the Thai Malaria Control Program. The tubes containing the remaining blood samples were briefly held and transported in a cooler with ice to a separate testing station where MRDD assays and a white blood cell (WBC) count were done. On average, blood specimens were tested within 2 hours 29 minutes (range = 8 minutes to 6 hours 51 minutes). An aliquot of each blood specimen in the EDTA tube was also set aside for molecular diagnosis. It was transported on dry ice to the Armed Forces Research Institute of Medical Sciences (AFRIMS) and stored at −20°C.

White blood cell count. A sample from the blood specimen was processed for a WBC count before it was passed on to the NOW® ICT technician. The WBC count was done using an automated blood cell counter (Coulter® T-890; Beckman-Coulter, Inc., Fullerton, CA) within three hours after blood collection.

NOW® ICT Malaria P.f./P.v. The prototype assay kits evaluated in this study (Lot no. 030611) look similar to the earlier ICT™ Malaria P.f./P.v. test. Despite this external similarity, the formulation and manufacture of this assay differ significantly from the earlier ICT tests. The new manufacturer has introduced new materials, modified reagents, and altered the assembly of the assays. The precise nature of these
changes is proprietary information, and beyond the scope of this report, which describes assay performance. Similar to earlier tests, this assay detects *Plasmodium falciparum* HRP-2 and aldolase, a pan-*Plasmodium* antigen. It was performed according to the manufacturer’s instructions using EDTA blood collected as described. Following application of 10–15 μL of blood onto the purple sample pad, two drops of test reagent were added to the lower absorbent white pad, located immediately below the sample pad. Lysed blood wicked up to the base of the upper white absorbent pad in a few minutes, when the booklet was closed. The result was read through the viewing window of the device 10 minutes later. Test line intensity was graded visually according to reference test cards provided by the manufacturer, on a scale of 0 (negative, no test line), 0.25 (barely visible), 0.5 (faint), 1.0, 2.0, and 3.0 (most intense). Similar to the previous ICT Malaria P.f./P.v. test, the uppermost line is the control line and of the two test lines, the lower one is *Plasmodium* genus, or pan-malaria-specific, and the upper one is *P. falciparum*-specific. When a NOW ICT test shows a positive result for non-*P. falciparum*, only the lower test line is reactive. When it shows a positive result for *falciparum*, either both test lines or only the upper test line may be reactive.

**Reference microscopy.** Immediately after blood was drawn from the patient, aliquots were drawn from the collection tube to make the blood smears. Using a micropipetter, 6 μL of blood was placed on a slide for the thick smear and 4 μL of blood from the same sample was placed on the same slide for the thin smear. Two such slides were made from each sample. The blood was immediately spread on the slides, and put aside to dry, protected from dust and insects. A third blood smear was prepared for the clinic staff, and provided to them for staining and interpretation for clinical diagnosis. Approximately 3–6 hours after collection, the two study thick and thin smears were stained for 25 minutes using Giemsa (Original Azure Blend Type, Thomas Scientific Catalog no. C363G33; ACCRA Laboratory /Brand of Biochemical Sciences, Inc., Swedesboro, NJ) diluted with distilled water immediately before use to obtain 5% working solution. One slide was kept for reference. The other was read by two experienced AFRIMS microscopists blinded to each other’s readings and NOW® ICT results. The thick film was screened for 200 oil-immersion fields (1,000× magnification) before declaring the film to be negative. If positive, parasite species were recorded and the numbers of asexual and sexual parasites were separately counted against 200 WBC, or against 500 WBC if less than 10 asexual parasites were found after reaching the 200th WBC. Parasite density was calculated by multiplying the mean number of asexual parasites counted/WBCs counted by the total WBC per microliter. Any discrepancies in detection of parasitemia or species classification between the two microscopists were resolved by the third, expert AFRIMS microscopist, whose reading was accepted as final.

**Polymerase chain reaction (PCR).** In a small number of samples in which there was species discrepancy between microscopy and NOW® ICT, molecular assays were done a month later from the whole EDTA blood that had been kept frozen at −20°C. We used a nested PCR as well as a quantitative PCR–enzyme-linked immunosorbent assay (PCR-ELISA) for the diagnosis of four human malaria species as previously described. The PCR technician was blinded to the microscopy and NOW® ICT results.

## RESULTS

A total of 246 patients were enrolled in the study. The NOW® ICT tests had to be repeated in 39 of 285 assays used (13.7%) because of poor wicking of the lysed blood or, in a few cases, because of poor background clearance, which caused an inability to complete or interpret the test. A successful test was eventually completed for each sample. Analysis of the 246 valid tests showed that lysed blood reached the edge of the absorbent pad at the top end of the strip in an average of 2.9 minutes (95% confidence interval [CI] = 2.6–3.2, range = 1–16). Control lines were easily visible with an intensity grade of 1 in all except for five samples, which were fainter.

We defined a true positive result for *P. falciparum* in the NOW® ICT test, for samples with a microscopic finding of *P. falciparum* monoinfection, as a positive *P. falciparum* test line of any detectable visibility, with or without a positive pan-*Plasmodium* line. We defined a true positive result for *P. vivax* if the non-*P. falciparum* test line showed any detectable visibility, with a negative *P. falciparum* line, for a sample with a microscopic finding of *P. vivax* monoinfection. Samples with mixed infection by microscopy were excluded from the calculation of sensitivity and specificity.

Microscopy showed 32 (13.0%) with *P. falciparum* monoinfection, 63 (25.6%) with *P. vivax* monoinfection, 6 (2.4%) with mixed *P. falciparum*-/*P. vivax* infections, 5 (2.0%) with *P. malariae*, and 140 (56.9%) negative (Table 1). The sensitivity of the assay for detecting malaria parasites (any species at any parasitemia) was 94.3% (100 of 106; 95% CI = 88–98). The assay detected malaria in 100% of samples with parasitemia exceeding 250 asexual parasites/μL. The sensitivity for *P. falciparum* was 100%. The lowest level of *P. falciparum* parasitemia recorded in this series was 20 asexual parasites/μL. The specificity for *P. falciparum* was 96.2% (200 of 208; 95% CI = 92–98).

The overall sensitivity for detecting *P. vivax* was 87.3% (55 of 63; 95% CI = 77–93). When stratified by level of parasitemia, *P. vivax* sensitivities were 95.9% (47 of 49; 95% CI = 86–99) for >500 parasites/μL, 66.7% (6 of 9; 95% CI = 30–93) for 101–500 parasites/μL, and 40% (2 of 5; 95% CI = 5–85) for ≤100 parasites/μL. Five *P. vivax* samples gave false-negative results by the NOW® ICT, but all had parasite densities <250/μL. The other three NOW® ICT false-negative results were test results for *P. falciparum*, and not

### TABLE 1

Cross-tabulation of NOW® ICT Malaria *Plasmodium falciparum*/ *Plasmodium vivax* (NOW ICT) results by Giemsa microscopy results*

<table>
<thead>
<tr>
<th>Microscopy</th>
<th>Negative</th>
<th>Pf only</th>
<th>Non-Pf only</th>
<th>Pf with/ without non-Pf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>135</td>
<td>5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pf asexual</td>
<td>–</td>
<td>3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pf asexual plus gametocytes</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pv asexual</td>
<td>4</td>
<td>1</td>
<td>18</td>
<td>–</td>
</tr>
<tr>
<td>Pv asexual plus gametocytes</td>
<td>1</td>
<td>0</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>Mixed Pf and Pv</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pm</td>
<td>1</td>
<td>–</td>
<td>4</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>141</td>
<td>9</td>
<td>59</td>
<td>37</td>
</tr>
</tbody>
</table>

*Pf = *Plasmodium falciparum*; Pv = *P. vivax*; Pm = *P. malariae.*
negative results. In one of these samples, with parasitemia of 129 parasites/μL, a nested PCR and a PCR-ELISA both indicated the presence of P. vivax (PCR-ELISA absorbance value = 3.6, cut-off value = 0.1), P. falciparum (absorbance = 1.8), as well as P. ovale (absorbance = 2.6). The other two samples, both with P. vivax parasitemias >250/μL, were misdiagnosed as P. falciparum, by the NOW® ICT, i.e., the assay detected the presence of parasites but characterized the species differently from the microbiologist. Subsequently, a nested PCR showed one of these samples to contain a mixed infection (P. falciparum and P. vivax).

Specificity for P. vivax was 97.7% (173 of 177, 95% CI = 95–99), but four of four false-positive results were in samples microscopically and PCR positive for P. malariae. Therefore, specificity for non-falciparum Plasmodium was 100%. One P. malariae case was negative by the NOW® ICT, but the parasite density was very low (38/μL). Five samples gave false-positive results by the ICT, showing the P. falciparum-specific test lines. However, a subsequent PCR-ELISA indicated that two of these were actually a mixed infection of P. vivax and P. falciparum with low-positive absorbance values (0.135 and 0.208, respectively), suggesting the possible detection of remnant HRP-2 by the NOW® ICT in these cases.

For P. falciparum, 88.9% (8 of 9) with an upper test line intensity grade of 3 and 61.9% (13 of 21) with an intensity grade of 2 had >5,000 parasites/μL (Table 2). For P. vivax, only two samples showed very intense test line intensity (grade 3); both had >5,000 parasites/μL. Ninety percent (18 of 20) with a lower test line intensity grade of 2 and 30.8% (4 of 13) with an intensity grade of 1 had >5,000 parasites/μL. All test and control lines maintained their initial intensity when re-examined 4–8 hours later.

The sensitivity of the lower, pan-specific test line for P. falciparum was found to be 100% (26 of 26) for parasite densities >500/μL. However, for parasite densities ≤500/μL, the line appeared only in three of six specimens. The lines of these three samples were all very faint (two with grade 0.5 and one with grade 0.25). All 10 samples with test line intensities of grades 2 and 3 had >5,000 parasites/μL. Among 17 with pan-Plasmodium line intensity grades of 0.5–1.0, 11 (64.7%) had >5,000 parasites/μL (Table 3).

**DISCUSSION**

Like its predecessor, the NOW® ICT does not always distinguish P. falciparum monoinfection from a mixed infection with both P. falciparum and non-falciparum species. The test does not distinguish among P. vivax, P. malariae, and P. ovale. An earlier ICT version, ICT™ Malaria P.f./P.v., was shown to detect P. malariae and P. ovale in addition to P. falciparum and P. vivax.4,5 Since the test is not specific for P. vivax, the name of the test is known to be misleading.

The field accuracy and availability of non-microscopic, rapid test devices for malaria diagnosis have been extensively reviewed.6-9 The NOW® ICT tests evaluated in this study have proved to be sensitive, specific, convenient, and rapid. Test lines were stable. Control lines were easily visible, but should be slightly intensified. The problem of poor wicking in a substantial proportion of these assays must be rectified in future versions. The reason for this problem is unclear, but it may have to do with irregularity in the nitrocellulose membrane used as the test strip or with the bridging between the sample pad and the strip.

As in most earlier studies of the ICT test, both sensitivity and specificity for P. falciparum were excellent.9,10 In many endemic areas outside Africa, P. vivax can be as prevalent, or more frequently found, than P. falciparum. The MRDDs capable of detecting only P. falciparum are of limited value in such areas. The addition of a second antibody directed against a pan-genus antigen such as aldolase, allowing differentiation between P. falciparum and non-falciparum Plasmodium species, can be useful in such settings. Earlier versions of ICT tests were known for their limited sensitivity for P. vivax.9,10 The high sensitivity and specificity for P. vivax observed in this study suggests that significant improvements have been made in this assay. Our findings have also confirmed the previous observation that the basic antigen detection system used by NOW® ICT is capable of detecting P. malariae. This study also documented the detection of P. falciparum by the proprietary, pan-specific monoclonal antibodies used for NOW® ICT. This issue was questioned earlier in relation to ICT Malaria P.f./P.v. tests.2 Our data suggested that the assay (as indicated by the lower test line) was also sensitive for P. falciparum and that test line intensity also varied with parasite density. However, these findings must be considered very preliminary because of the limited number of blood samples con-
taining ≤5,000 parasites/μL in our series. Given the current test design, the weaker intensity of the pan-specific test line (in comparison to the HRP-2 test line at a similar parasitemia level) helps to avoid incorrect diagnosis of species, since a low-level *P. falciparum* parasitemia could otherwise generate a positive pan-specific test line but negative HRP-2 line.

Based on this study of limited sample size, our results suggest that the performance of the NOW® ICT is improved over its predecessors and that the accuracy of this MRDD is above the minimum requirements for field diagnosis of malaria. However, these results pertain only to the kit lot we have evaluated and are not necessarily indicative of future product quality. This point is noteworthy because, in the last few years, variation in product quality of some MRDDs was observed and poor sensitivities for both *P. falciparum* and non-*P. falciparum* were noted in assays that had performed well in the past.11–14 This study was not designed to address other issues pertaining to the limitations of MRDDs such as the persistence of HRP-2 post-treatment and the extent of false positivity that may be caused by gametocytemia and rheumatoid factor.15–18 Therefore, studies in different patient populations, field conditions, and endemic areas using different test kit lots will be needed to definitively define the performance of this assay.

By original intent, application of MRDD was for a clinical diagnostic adjunct to supplement microscopy as a rapid means of diagnosing malaria. In practice, the precise clinical uses of the assays will depend on their sensitivity, specificity, ease of use, stability when stored, and on the manufacturer’s ability to sustain reproducible performance over time. Until specific MRDDs are shown to meet strict criteria in each of these aspects, their actual clinical value remains speculative. However, if stable, reproducible assays can be developed to the point that they consistently match or exceed the accuracy of clinical microscopy, then they could come to play an important role in clinical settings. In this regard, it is important to remember that the accuracy of clinical microscopy is, in a field setting, in a very important role in clinical settings. In this regard, it is important to remember that the accuracy of clinical microscopy is, in a wide variety of settings, often deficient.19–22

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