A comparison of three diagnostic techniques for malaria: a rapid diagnostic test (NOW® Malaria), PCR and microscopy

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Malaria is a common, life-threatening infection in endemic tropical areas and one that presents a diagnostic challenge to laboratories in most non-endemic countries. A rapid and accurate diagnosis is a prerequisite for effective treatment, especially for the potentially fatal cases of *Plasmodium falciparum* infection. In the present, multi-centre study, the performances of a rapid diagnostic test (NOW® Malaria) and several, commercial, PCR-based assays (AMS61®, AMS42®, AMS43®, AMS44® and AMS45®) were compared against the results of microscopical examination of bloodsmears (the current ‘gold standard’). The subjects were either non-European immigrants (*N* = 135) or international travellers (*N* = 171).

There was good concordance between the results of all the detection methods, with kappa values of >0.8. Although the NOW Malaria rapid test was both sensitive (100%) and specific (100%) in detecting *P. falciparum* infections, it was less specific (93.1%) and sensitive (90.7%) in identifying the other *Plasmodium* species.

The results from the AMS61 assay, designed to detect any malarial infection, generally paralleled those of the microscopy (kappa = 0.89), giving a specificity of 98.2% and a sensitivity of 91.0%. Although the use of species-specific molecular primers to identify pure infections with *P. falciparum* and *P. vivax* gave results that were in good agreement with the microscopy results, they were less accurate in identifying mixed infections.

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agreement with those of the microscopy, the subjects who had apparently pure infections with P. ovale or P. malariae were always found PCR-negative. Compared with the standard microscopy, both the NOW Malaria test and the PCR-based assays were therefore poor at identifying mixed infections.

The NOW Malaria test and the PCR-based assays clearly need to be improved, particularly for the correct identification of infections with Plasmodium spp. other than P. falciparum, including mixed infections. For now, expert microscopy must remain the mainstay of the laboratory diagnosis of malaria.

Malaria is generally considered to be the most important parasitic infection of humans, especially in the many endemic tropical and sub-tropical areas in which the morbidity and mortality associated with this disease continue to increase. Even in developed countries where the disease is not endemic, increasing immigration and worldwide travel to endemic regions have led to an increased incidence of imported malaria. Immigrants from less developed areas slowly lose any immunity they have to malaria and are then at high risk of contracting the symptomatic disease when they visit their native countries, especially if, as often occurs, they fail to take any antimalarial chemoprophylaxis (Franco-Paredes and Santos-Preciado, 2006). Since intercontinental travel now only takes a few hours by plane and the duration of a trip to endemic areas is normally limited to a few days or weeks, most travellers do not develop any symptoms of malaria until they return home (Playford and Walker, 2002).

The prompt treatment of malaria, which may save the patient’s life, requires the rapid and accurate diagnosis of the infection. For an optimal diagnosis, each Plasmodium species present in the patient’s blood must be detected and identified and, especially in cases of P. falciparum infection, the level of parasitaemia must be evaluated (Moody, 2002; Haditsch, 2004). For nearly a century, the diagnosis of malaria has been centred on the microscopical detection of the causative parasites on stained thick and/or thin bloodsmears, and the careful examination of well-prepared and well-stained bloodsmears remains the ‘gold standard’ in clinical practice (Murray et al., 2003). Such microscopy does, however, have several disadvantages: it is time-consuming and requires both an experienced microscopist (especially when the parasite burden is very low) and a microscope. Over the last few decades, several attempts have been made to develop more rapid, simple, sensitive and non-microscopical methods for the detection of malaria. They include techniques for the identification of antigens released from parasitised erythrocytes, PCR-based assays for the detection of DNA from the parasites, and immunofluorescence (Moody and Chiodini, 2000; Hänscheid, 2003).

The malarial antigens targeted by some current rapid diagnostic tests (RDT) are histidine-rich protein 2 (HRP-2), parasite lactate dehydrogenase (pLDH) and Plasmodium aldolase. Although HRP-2 is a water-soluble protein expressed only by P. falciparum, the pLDH antigen is present, as a species-specific isomer, in each of the four Plasmodium species that are human pathogens (Moody and Chiodini, 2002; Jelinek et al., 2000; Craig et al., 2002). The aldolase enzyme has been suggested as a target antigen for the detection of the species other than P. falciparum (Moody, 2002; Grobusch et al., 2003; Murray et al., 2003).

Several PCR-based assays have been developed for the detection of malarial parasites, with most based on the parasites’ 18S ribosomal RNA (18S rRNA) genes. The PCR-amplified products are usually detected by gel electrophoresis or by Southern blotting following hybridization with DNA probes. PCR-based assays offer various advantages over rapid immunochromatographic tests and microscopy, especially very high specificity and sensitivity (down to as few as five parasites/μl blood; Haditsch, 2004).
In the present study, the performances of a commercial RDT and a commercial diagnostic system based on several PCR-based assays were compared against the ‘gold standard’ results of the microscopical examination of bloodsmears. The subjects were residents of Italy who were at risk of malaria because they were non-European immigrants from malaria-endemic areas or Italians who had travelled to such areas. Overall, 13 north Italian units for the study and treatment of tropical and infectious diseases participated in the study.

SUBJECTS AND METHODS

Subjects
Overall, 306 subjects were investigated: 171 (55.9%) Italians (IT) who had travelled to malaria-endemic regions and 135 (44.1%) non-European immigrants (nEi) from malaria-endemic areas. All had presented, in northern Italy, with suspected malaria.

The recent epidemiological data recorded, in a separate medical-history questionnaire for each subject, included country visited or country of origin, length of trip, use of antimalarial prophylaxis and self-reported adherence to it, onset of specific symptoms other than fever (e.g. headache, abdominal pain, or diarrhoea), and any self-treatment prior to diagnosis.

Diagnostic Methods
Blood samples from each subject were investigated by microscopy (at the hospital at which the subject presented), in an RDT (again at the unit at which the subject presented), and in a series of PCR-based assays (at the Laboratory of Parasitology, IRCCS S. Matteo, Pavia).

MICROSCOPY
The microscopy involved the examination of Giemsa-stained thin and thick bloodsmears, with the level of any parasitaemia detected being recorded as the percentage of erythrocytes infected. Each Giemsa-stained thick smear was checked by an experienced microscopist, at ×1000, and only considered negative if no malarial parasites were seen after a search of at least 30 min. The thin smear corresponding to each positive thick smear was examined, to identify the Plasmodium species present.

RDT
The RDT employed, on blood samples collected in EDTA tubes, was the NOW Malaria test (Binax Inc., Portland, ME), which was used according to the manufacturer’s instructions. This immunochromatographic assay detects *P. falciparum* HRP-2 and the aldolase isomer of each of the *Plasmodium* species infecting humans (the so-called ‘pan-malarial’ antigen). Blood (15 µl) was applied directly onto the purple sample pad and two drops of test reagent were added to the lower absorbent white pad, located immediately below the sample pad. After the blood mixture had run along the strip, the card was folded to rinse the strip. The result was read 10 min later. The uppermost coloured line is the control and a test result was only considered valid if this band appeared. Of the two test bands, the bottom one indicates whether any malarial parasites are present while the top one is *P. falciparum*-specific. When only the top test band appeared, the test was considered to be positive for a *P. falciparum* mono-infection. The appearance of both test bands indicated that the test was positive for *P. falciparum* alone or a mixed infection (of *P. falciparum* with *P. vivax*, *P. ovale* and/or *P. malariae*). When only the bottom test band appeared, the test was considered to be negative for *P. falciparum* but positive for *P. vivax*, *P. ovale* and/or *P. malariae*.

PCR
Conventional PCR, using five commercial kits (AMS61®, AMS42®, AMS43®, AMS44® and AMS45®; Clonit srl, Milano, Italy), was also carried out on blood samples
collected in EDTA tubes. Although each kit is based on the 18S rRNA gene of the parasites, AMS61 is designed to detect any of the *Plasmodium* species infecting humans while the other kits are designed to be specific for *P. falciparum* (AMS42), *P. ovale* (AMS43), *P. malariae* (AMS44) or *P. vivax* (AMS45). The kits were used according to their manufacturer’s instructions.

The cells in each blood sample (0.5 ml) were washed, centrifuged, re-suspended in 200 \( \mu l \) extraction solution, incubated for 30 min at 60°C, and then either used directly in the PCR or stored at -20°C until they could be used. Each amplification was run in a 50-\( \mu l \) reaction mixture containing 0.5 \( \mu M \) of each primer, 100 \( \mu M \) of each deoxyribonucleotide triphosphate (dNTP), 60 mM Tris–HCl and 1.5 mM MgCl\(_2\). The 9700 thermocycler used (Applied Biosystems, Foster City, CA) was set to give an initial denaturation at 94°C for 5 min, followed by 40 cycles, each of 30 s at 94°C, 30 s at 50°C, and 45 s at 72°C, and then a final extension at 72°C for 5 min. The amplification products were separated by electrophoresis in pre-stained agarose gels (Elchrom, Cham, Switzerland).

**Data Analysis**

Descriptive statistics, calculated (separately) for the Italian and non-European subjects, were reported either as means and S.D. (continuous variables) or as absolute and relative (%) frequencies (categorical variables). To be considered a true case of malaria, a subject had to be smear-positive for malarial parasites. The results of the microscopy were taken as the ‘gold standard’. The agreement between diagnostic methods was assessed by calculating the kappa statistic (\( \kappa \)) and corresponding S.E. (Landis and Koch, 1977); \( \kappa \)-values of >0.6 and >0.8 were considered indicative of substantial and almost perfect agreement, respectively.

The sensitivity (%) and specificity (%) of the RDT and PCR, compared with the gold standard, were determined, together with their 95% confidence intervals (CI).

Version 9 of the Stata software package (Stata Corporation, College Station, TX) was used for all the statistical analyses.

**RESULTS**

**Epidemiology**

The mean (S.D.) age of the 306 subjects was 37.3 (16.2) years. Most (65.0%) of the subjects examined had each returned from a brief trip (of \( <1 \) month) to a malaria-endemic area. Most (86.4% — 81.7% of the IT and 92.4% of the nEi) had visited sub-Saharan African countries, while the others had returned from South–east Asia (9% — 12.4% of the IT and 4.5% of the nEi) or Central and South America (3.6% — 4.1% of the IT and 3.0% of the nEi). In addition, one Italian had come back from Turkey and two from Oceania (Papua New Guinea).

Not only almost all (98.6%) of the non-European immigrants but also most (65.7%) of the Italian subjects stated that they had not taken any antimalarial chemoprophylaxis during their trip. In addition, 44.8% of the Italians and both non-European immigrants who claimed they had taken antimalarial chemoprophylaxis had not taken the correct chemoprophylaxis recommended for the particular areas they had visited.

Adequate information on presenting symptoms were collected for 282 of the subjects. Of these, 159 (56.4%) — 54.0% of the IT and 46.0% of the nEi — complained of fever (>38°C) on hospital admission.

**Diagnosis**

Table 1 summarizes the results of all the diagnostic methods used. Direct microscopic examination of bloodsmears was positive in 142 cases (28.1% of the IT and 70.9% of the nEi). Levels of parasitaemia, estimated for 126 of the smear-positive patients, varied from 0.0007% to 16.0% (mean=1.12%).
Of the 142 smear-positive samples, 126 (88.7%; 38 IT and 88 nEi) were found positive by RDT, nine (eight IT and one nEi) were RDT-negative, and the other seven (one IT and six nEi) were not tested by RDT.

Of the 159 smear-negative subjects tested by RDT, seven (4.4%; three IT and four nEi) were found RDT-positive. Four of these seven had been treated with antimalarial drugs during their stay in tropical areas or had treated themselves with such drugs a few days prior to being tested.

Among the subjects investigated by both microscopy and RDT, 51 (30.5%) of the IT and 99 (76.1%) of the nEi were found positive by one or both techniques.

Table 2 reports the *Plasmodium* species detected in each subject by direct microscopy. Four (3.3%) of the 118 cases microscopically identified as pure *P. falciparum* infections (with parasitaemias ranging from 0.0007% to 0.2%) were RDT-negative; one of these four was a non-European immigrant returning from Ghana, with only gametocytes visible in his bloodsmears.

One (9.0%; parasitaemia=0.5%) of the 11 samples which appeared to be *P. vivax* mono-infections on microscopical examination, three (60%; mean parasitaemia=0.01%) of the five samples which appeared to be *P. ovale* mono-infections, and the single sample which appeared to be a pure *P. malariae* infection were found RDT-negative.

Five of the six mixed infections detected by microscopy, all at low parasitaemias (0.01%–0.05%), were found RDT-positive, although the band indicating the presence of pan-malarial antigen failed to develop in two of these cases (one identified, by microscopy, as *P. falciparum*–*P. malariae* and one as *P. falciparum*–*P. ovale*). The exception was one case with mixed infections by both techniques.

<table>
<thead>
<tr>
<th>Method</th>
<th>Italian international travellers</th>
<th>Non-European immigrants</th>
<th>All subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct microscopy</td>
<td>47/167 (28.1)</td>
<td>95/134 (70.9)</td>
<td>142/301 (47.2)</td>
</tr>
<tr>
<td>Rapid diagnostic test (NOW Malaria)</td>
<td>42/170 (24.7)</td>
<td>92/126 (73.0)</td>
<td>134/296 (45.3)</td>
</tr>
<tr>
<td>PCR (AMS61 kit)</td>
<td>34/124 (27.4)</td>
<td>69/105 (65.7)</td>
<td>103/229 (44.9)</td>
</tr>
</tbody>
</table>

**TABLE 1. Positive results in each of the diagnostic tests investigated**

<table>
<thead>
<tr>
<th>Species</th>
<th>No. and (%) of smear-positive:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Plasmodium falciparum only</em></td>
<td>33 (71.7)</td>
</tr>
<tr>
<td><em>P. vivax</em> only</td>
<td>5 (10.9)</td>
</tr>
<tr>
<td><em>P. ovale</em> only</td>
<td>4 (8.7)</td>
</tr>
<tr>
<td><em>P. malariae</em> only</td>
<td>1 (2.2)</td>
</tr>
<tr>
<td><em>P. falciparum</em>-<em>P. vivax</em></td>
<td>0</td>
</tr>
<tr>
<td><em>P. falciparum</em>-<em>P. ovale</em></td>
<td>1 (2.2)</td>
</tr>
<tr>
<td><em>P. falciparum</em>-<em>P. malariae</em></td>
<td>1 (2.2)</td>
</tr>
<tr>
<td><em>P. vivax</em>-<em>P. malariae</em></td>
<td>1 (2.2)</td>
</tr>
<tr>
<td><em>P. ovale</em>-<em>P. malariae</em></td>
<td>0</td>
</tr>
</tbody>
</table>

**TABLE 2. The *Plasmodium* species detected by direct microscopy, in the 46 Italian international travellers and 95 non-European immigrants found smear-positive**
TABLE 3. The Plasmodium species detected by PCR, in the 28 Italian international travellers and 69 non-European immigrants found PCR-positive

<table>
<thead>
<tr>
<th>Species*</th>
<th>No. and (%) of PCR-positive:</th>
<th>No. of PCR-positive subjects not giving same result by microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Italian international travellers</td>
<td>Non-European immigrants</td>
</tr>
<tr>
<td><strong>Plasmodium falciparum only</strong></td>
<td>26 (92.9)</td>
<td>62 (89.9)</td>
</tr>
<tr>
<td><strong>P. vivax only</strong></td>
<td>0 (0.0)</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td><strong>P. ovale only</strong></td>
<td>2 (7.1)</td>
<td>4 (5.8)</td>
</tr>
<tr>
<td><strong>P. falciparum–P. vivax</strong></td>
<td>0 (0.0)</td>
<td>1 (1.4)</td>
</tr>
</tbody>
</table>

*Blood from the subject found, by microscopy, to have a mixed infection of *P. vivax–P. malariae* was not tested in the PCR-based assays.

†Identified by microscopy to be a mixed infection of *P. falciparum* with either *P. malariae* (one case) or *P. vivax* (one case)

‡Identified by microscopy to be a mixed infection of *P. malariae* with *P. ovale* (one case) or mono-infections with *P. falciparum* (one), *P. malariae* (one) or *P. vivax* (one).

§Identified by microscopy to be a mixed infection of *P. falciparum* with *P. ovale*.

**P. falciparum–P. ovale** infection, who appeared RDT-negative.

**PCR V. MICROSCOPY**

The PCR kits were used to test 229 blood samples (Table 1) and gave positive results for 103 (44.9%) of them (Table 1). These results show general concordance with those obtained by direct microscopy (Table 3).

Only three smear-negative subjects were found PCR-positive (two IT found PCR-positive for *P. falciparum–P. malariae* and one nEi found PCR-positive for *P. falciparum* only), whereas eight smear-positive cases (three IT and five nEi) were found PCR-negative. Overall, 110 cases (82 IT and 28 nEi) were found negative both by direct microscopy and PCR.

In terms of the identification of the *Plasmodium* species present, the PCR-based assays gave generally good agreement (96.6% for the IT and 92.3% for the nEi) with the results of the direct microscopy (Table 4). The molecular assays showed a

TABLE 4. The levels of agreement between the results of the rapid diagnostic test (RDT) or those of the PCR-based assays and those of the direct microscopy used as the ‘gold standard’

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Variable</th>
<th>RDT (NOW Malaria)</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Italian international travellers</strong></td>
<td>No. of subjects</td>
<td>165</td>
<td>119</td>
</tr>
<tr>
<td>Agreement with microscopy (%)</td>
<td>93.3 (0.83)</td>
<td>96.6 (0.92)</td>
<td></td>
</tr>
<tr>
<td>κ and (S.E.)</td>
<td>0.83 (0.08)</td>
<td>0.92 (0.09)</td>
<td></td>
</tr>
<tr>
<td>Sensitivity (and 95% confidence interval) (%)</td>
<td>82.6 (76.8–88.4)</td>
<td>91.7 (86.7–96.6)</td>
<td></td>
</tr>
<tr>
<td>Specificity (and 95% confidence interval) (%)</td>
<td>97.5 (95.1–99.9)</td>
<td>98.8 (96.8–100.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Non-European immigrants</strong></td>
<td>No. of subjects</td>
<td>124</td>
<td>104</td>
</tr>
<tr>
<td>Agreement with microscopy (%)</td>
<td>96.0 (0.90)</td>
<td>92.3 (0.82)</td>
<td></td>
</tr>
<tr>
<td>κ and (S.E.)</td>
<td>0.90 (0.09)</td>
<td>0.82 (0.10)</td>
<td></td>
</tr>
<tr>
<td>Sensitivity (and 95% confidence interval) (%)</td>
<td>98.9 (97.0–100.0)</td>
<td>90.7 (85.1–96.3)</td>
<td></td>
</tr>
<tr>
<td>Specificity (and 95% confidence interval) (%)</td>
<td>88.6 (83.0–94.2)</td>
<td>96.5 (93.0–100.0)</td>
<td></td>
</tr>
</tbody>
</table>
very good level of agreement with microscopy for the subjects found, by microscopy, to have pure *P. falciparum* or pure *P. vivax* infections but they failed to identify two of four cases of *P. ovale* mono-infection. In addition, as shown in Table 3, the species-specific PCR ‘wrongly’ identified as *P. ovale* mono-infections three cases who had been identified by microscopy as mono-infections with *P. falciparum* (one case), *P. vivax* (one case) or *P. malariae* (one case), respectively, and one case who had been identified by microscopy as a mixed infection (*P. ovale–P. malariae*). Similarly, concordance was not perfect for *P. malariae*.

Of the six cases identified as mixed infections by direct microscopy, only one was ‘correctly’ identified by PCR as *P. falciparum–P. vivax*. Four others appeared to be mono-infections by PCR and one appeared PCR-negative.

**Diagnostic Accuracy Compared with the Gold Standard**

For the detection of malarial infection in the Italian subjects, the results of both the RDT and PCR demonstrated almost perfect agreement (i.e. $\kappa$-values of $>0.8$) with those of the microscopical gold standard (Table 4). The PCR gave a higher level of agreement with the microscopy and a higher sensitivity than the RDT.

The corresponding results for the immigrant subjects were equally satisfying, although, with these cases, it was the RDT that gave a higher level of agreement with the microscopy and a higher sensitivity than the PCR.

Compared with the results of the direct microscopy, both the PCR and RDT gave almost perfect agreement and near-perfect sensitivities and specificities in the detection of *P. falciparum*, the clinically most important species of *Plasmodium* (Table 5).

**DISCUSSION**

The current gold standard and the recommended technique used for the laboratory diagnosis of malaria is the microscopical examination of stained bloodsmears, with the additional determination of parasite concentrations. With this method, an expert microscopist should be able to detect about 50 parasites/$\mu$l blood (i.e. a parasitaemia of about 0.001%) and to identify to the species level 98% of the malarial parasites seen (Moody, 2002; Haditsch, 2004). This procedure is time-consuming, however, and requires special training to gain sufficient microscopical expertise.

Recently, efforts have been made to develop more rapid and simple procedures that offer sensitivities equivalent to, or better than, those of direct microscopy. These assays involve the use of fluorescence microscopy, immunochromatographic RDT or PCR (Moody, 2002; Murray et al., 2003; Haditsch, 2004).

The present study was designed to evaluate the diagnostic performance of an RDT

<table>
<thead>
<tr>
<th>Variable</th>
<th>Diagnostic test</th>
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<tbody>
<tr>
<td></td>
<td>RDT (NOW Malaria)</td>
</tr>
<tr>
<td>No. of subjects</td>
<td>116</td>
</tr>
<tr>
<td>Agreement with microscopy (%)</td>
<td>99.1</td>
</tr>
<tr>
<td>$\kappa$ and (S.E.)</td>
<td>0.96 (0.09)</td>
</tr>
<tr>
<td>Sensitivity (and 95% confidence interval) (%)</td>
<td>100 (100.0–100.0)</td>
</tr>
<tr>
<td>Specificity (and 95% confidence interval) (%)</td>
<td>92.9 (88.2–97.5)</td>
</tr>
</tbody>
</table>
and a qualitative, PCR-based method in two groups of subjects at relatively high risk of malarial infection — Italians and non-European immigrants who, having visited malaria-endemic areas, were seeking medical care. The results of these techniques were compared against direct microscopy as the ‘gold standard’.

When used to test samples from the two groups of subjects for any malarial infection, both the RDT and PCR showed almost perfect agreement with the results of the gold-standard microscopy, with only slight differences in sensitivity and specificity (Table 4). Both the RDT and PCR appeared at least as good as microscopy in detecting (mixed or single) infection with *P. falciparum* (Table 5). As previously reported (Craig *et al.*, 2002; Playford and Walker, 2002; Marx *et al.*, 2005), the NOW Malaria rapid test was both sensitive (100%) and specific (100%) in detecting *P. falciparum* infections. It was, however, less specific (93.1%) and sensitive (90.7%) in identifying the other malarial species, presumably because of the low specificity, for the pan-malarial antigen, of the monoclonal antibodies used in the test and/or the test’s relatively poor sensitivity in cases with low parasitaemias (Jelinek *et al.*, 2001; Grobusch *et al.*, 2002; Iqbal *et al.*, 2002). More recently, three-band tests, which detect additional malarial parasites by using HPR-2 and aldolase or parasite LDH as targets, have been developed (Pattanasin *et al.*, 2003; Marx *et al.*, 2005; WHO, 2005) but there have been few studies, to date, to evaluate the performance of these ‘improved’ assays.

Only 3.3% of the subjects who appeared, by microscopy, to have pure *P. falciparum* infections were found RDT-negative. These false-negatives in the RDT may have been the result of very low parasitaemias, the exclusive presence of circulating gametocytes (with no asexual stages) or variability in the target antigen (Iqbal *et al.*, 2002; Baker *et al.*, 2005).

The seven cases who, though recorded as smear-negative, were found RDT-positive could reflect the prolonged persistence of antigen reactivity after effective therapy (Gatti *et al.*, 2002), or subpatent infection, with parasitaemias below those detectable by microscopy (Tjitra *et al.*, 2001; Iqbal *et al.*, 2004; WHO, 2005). The PCR-based assay for malarial parasites of any species (i.e. the AMS61 kit) gave results that were in almost perfect agreement with those of the microscopy (κ=0.89), with a specificity of 98.2% and a sensitivity that was slightly lower (91.0%).

The use of species-specific molecular primers to identify pure *P. falciparum* infection (kit AMS42) or pure *P. vivax* infection (kit AMS45) gave results that were in a good agreement with those of the microscopy. Disappointingly, however, no samples were found positive in the PCR that employed specific primers for *P. ovale* or *P. malariae*, even though both of these species were recorded by microscopy. Poor sensitivity in the PCR-based detection of these species may indicate that the corresponding species-specific primers are not very efficient. Further studies will be performed to evaluate the minimal levels of *P. ovale* or *P. malariae* parasitaemias that are detectable using, respectively, the AMS43 and AMS44 kits. The diagnostic accuracy of the molecular technique in detecting *P. falciparum* infections was almost perfect, with a sensitivity of 98.9% and a specificity of 100% (Table 5). Similar results have been recorded in earlier investigations (Patsoula *et al.*, 2003; Perandin *et al.*, 2004; Farças *et al.*, 2006).

In terms of the mixed infections that were identified by the microscopy, both the NOW Malaria test and the PCR-based assays showed poor concordance with the ‘gold standard’. Such poor performance in the identification of mixed infections has been described before for the newer diagnostic tests, especially those based on immunocapture (Jelinek *et al.*, 2001; Iqbal *et al.*, 2002). In the present study, however,
it was difficult to assess reliably the ability of the RDT and PCR to recognise true mixed infections accurately — not only the lack of a precise methodology to calculate the contribution of each species to the total parasitaemia in a mixed infection but also the small number of such infections detected by direct microscopy made an extensive evaluation impossible. The present results do indicate that both the RDT and PCR need to be improved, particularly for the correct identification of species other than P. falciparum and mixed infections.

The inter-study variation seen in the apparent performance of PCR-based assays for the detection of malarial infection (Moody, 2002; Haditsch, 2004; present study) probably mostly reflects methodological differences in the various study protocols. The development of standardized techniques for the assessment of test performance would help make inter-study comparisons more meaningful. Ideally, PCR-based methods should be evaluated within a clinically relevant time-frame, mimicking their use for the immediate screening of febrile patients in both field and laboratory settings (Haditsch, 2004).

In conclusion, the present results confirm that immunochromatographic techniques can usefully supplement microscopy, especially for quick primary checks of patients, although the use of RDT for self-diagnosis (Haditsch, 2004) is not recommended. Although the PCR-based assays shows the best sensitivity and specificity for the detection of malarial parasites, especially P. falciparum and P. vivax, the technology continues to be limited to reference laboratories. Expert microscopy should not be disregarded when evaluating the parasite burden in P. falciparum infections. Parasitologists should still have adequate and regular training to help them develop and maintain proficiency in the morphological diagnosis of the most common human blood parasites, in particular those causing malaria.

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REFERENCES


Iqbal, J., Khalid, N. & Hira, P. R. (2002). Comparison of two commercial assays with expert microscopy for


