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Use of a rapid, single-round, multiplex PCR to detect malarial parasites and identify the species present

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A new, rapid assay, based on a single-round, multiplex PCR, can be used to detect *Plasmodium falciparum*, *P. vivax*, *P. malariae* or *P. ovale* in human blood. The PCR, which targets the conserved 18S small-subunit RNA genes of the parasites, not only permits a malarial infection to be detected but also allows each *Plasmodium* species present to be identified, even in cases of mixed infection.

Each year, >200 million individuals become infected with malarial parasites and there are more than 1 million malaria-attributable deaths (WHO, 2000). Rapid diagnosis and the early treatment of clinical cases are important in reducing the mortality. Light microscopy has been the 'gold-standard' method for the laboratory diagnosis of malaria for more than a century — since 1891, when Romanowsky developed a polychrome staining technique to demonstrate malarial parasites in bloodsmears (Gilles, 1993). Good microscopy, however, requires highly trained personnel and is time-consuming, labour-intensive and sometimes insufficient for the accurate identification of the one or more species of *Plasmodium* that may be present in a blood sample (Milne *et al.*, 1994). Although rapid immunochromatographic tests are now available for malaria diagnosis, the sensitivities of these assays are generally lower than that of the microscopical examination of thick smears, and their usefulness in identifying the malarial species present is limited (Moody *et al.*, 2000).

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Several PCR-based assays for the detection of the *Plasmodium* species infecting humans, in which nested or semi-nested primers are employed for the amplifications, have been described (Snounou *et al.*, 1993*a*, *b*; Roper *et al.*, 1996; Singh *et al.*, 1997; Kain *et al.*, 1998; Rubio *et al.*, 1999; Ciceron *et al.*, 1999; Gal *et al.*, 2001). The results of evaluating these molecular tests have been encouraging, indicating specificities and sensitivities higher than those achievable by routine microscopy. For example, the lowest level of parasitaemia usually detectable by the microscopical examination of thick smears is about five to 20 parasites/μl blood (Bruce-Chwatt, 1984; Ciceron *et al.*, 1999) whereas the corresponding limit of detection for the PCR-based assays has been reported to be as low as 0.02 parasite/μl (Ciceron *et al.*, 1999). Most of the molecular assays available are based on nested (Snounou *et al.*, 1993*b*; Laoboonchai *et al.*, 2001; Parkes *et al.*, 2001; Toma *et al.*, 2001; Zakeri *et al.*, 2002) or hemi-nested PCR (Rubio *et al.*, 1999; Schindler *et al.*, 2001). A single round of amplification may reveal that malarial parasites are present or absent but identification of the parasite species requires a further step: a second round

of amplification (as used in nested PCR); blotting and hybridization of the amplification products with specific probes (Ciceron *et al.*, 1999; Schindler *et al.*, 2001); or investigation of the amplified products by ELISA (Laoboonchai *et al.*, 2001). Recent advances in PCR technology (semi-automation, rapid nucleic-extraction methods and one-tube, reverse-transcriptase PCR) now offer the possibility of using PCR-based assays routinely as a diagnostic method for the detection of malarial parasites. For diagnosis of malaria, especially in individuals with acute infection, a rapid method, that does not rely on a second round of amplification or hybridization method to achieve high sensitivity and accurate species identification, is preferable. A method based on a single round of amplification is also less prone to cross-contamination. The aim of the present study was to develop and evaluate a new, rapid assay which uses one set of primers in a single-round, multiplex PCR targeted at species-specific sequences in the small-subunit, ribosomal-RNA (SSUrRNA) genes of the four *Plasmodium* species infecting humans.

MATERIALS AND METHODS

Samples

All samples were routine, clinical, EDTA-anticoagulated specimens of whole blood submitted to the Department of Clinical Parasitology of the Hospital for Tropical Diseases in London (U.K.) to be examined for the presence of malarial parasites. Each sample was coded prior to transport (according to international regulations) to the U.K.'s National Institute for Biological Standards and Control, in South Mimms, for testing. No blood samples were taken specifically for this study.

To explore the sensitivity of the PCR-based assay, 10-fold serial dilutions of the DNA extracted from blood samples containing known numbers (parasites/μl) of one or two *Plasmodium* species were also tested in the assay. Mixed infections were also simulated, by mixing blood infected solely with *P. falciparum* with blood infected with *P. vivax*, to give varying ratios of *P. falciparum* to *P. vivax*. DNA was then extracted from each mixture and tested in the assay (see below).

PCR

DNA was extracted from a 200-μl aliquot of each blood sample, using a commercial kit (QIAamp DNA Mini Kit; QIAGEN, Crawley, U.K.), and eluted in a final volume of 60 μl water.

In the PCR, a single reverse primer (5′-GTA TCT GAT CGT CTT CAC TCCC), which was conserved in all four *Plasmodium* species, was used with four, species-specific, forward primers: one each for *P. falciparum* (5′-AAC AGA CGG GTA GTC ATG ATT GAG), *P. vivax* (5′-CGG CTT GGA AGT CCT TGT), *P. ovale* (5′-CTG TTC TTT GCA TTC CTT ATGC) and *P. malariae* (5′-CGT TAA GAA TAA ACG CCA AGCG). The reaction mix for the PCR, which was mostly based on another commercial kit (HotStarTaq Master Mix Kit: QIAGEN), contained 3.0 mM $MgCl_2$, 400 μM of each dNTP, 2.5 units HotStartTaq DNA polymerase, 15 pmol of the reverse primer, 15 pmol of the *P. falciparum* forward primer, 17.5 pmol each of the *P. ovale* and *P. malariae* forward primers, 7.5 pmol of the *P. vivax* forward primer and 5 μl of extracted DNA, in a final volume of 25 μl. The reaction was carried out on a RoboCycler Gradient 96 thermal cycler (Stratagene, Amsterdam). An initial, 15-min incubation at 95°C (to activate the HotStart polymerase) was followed by 43 cycles, each of 45 s at 95°C followed by 90 s at 60°C, and then a final step at 72°C for 5 min. Stringent precautions were taken to avoid possible cross-contamination. The amplified products were visualized by electrophoresis on a 2%-agarose gel and staining with ethidium

bromide. The lengths of the expected products were 276 bp for *P. falciparum*, 300 bp for *P. vivax*, 375 bp for *P. ovale* and 412 bp for *P. malariae*.

Sequencing

The amplified DNA products were separated on a 2%-agarose gel, extracted from the gel using a commercial kit (QIAquick Gel Extraction Kit; QIAGEN) and sequenced using a 310 DNA automated sequencer (Applied Biosystems, Foster, CA).

RESULTS

In initial experiments, DNA was extracted from blood containing known *Plasmodium* species and amplified using the multiplex PCR. Amplified DNA from each species of *Plasmodium* gave a band of the expected size when run on a 2%-agarose gel (Fig. 1). The identity of the amplified DNA was then confirmed by sequencing.

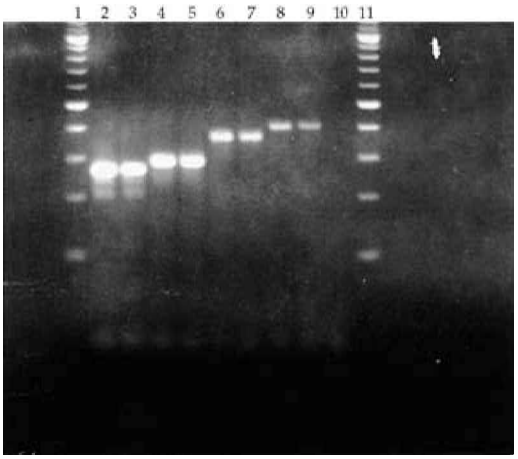

FIG. 1. The results of subjecting the PCR products (lanes 2–10) or 100-bp markers (lanes 1 and 11) to electrophoresis on 2.0% agarose and staining with ethidium bromide. The samples investigated were of blood infected with *Plasmodium vivax* (lanes 2 and 3; giving a 276-bp product), *P. falciparum* (lanes 4 and 5; giving a 300-bp product), *P. ovale* (lanes 6 and 7; giving a 375-bp product) or *P. malariae* (lanes 8 and 9; giving a 412-bp product) or, as a negative control, the sample-free reaction mix (lane 10; giving no product).

Of 32 microscopically-positive blood samples examined by PCR, 21 tested positive for *P. falciparum*, four for *P. malariae*, three for *P. vivax* and four for *P. ovale*. No mixed infections were detected. These results agreed with those obtained by microscopy (results not shown).

Sixteen microscopically-negative samples were also tested by PCR. Each of these samples had been split into two equal aliquots, one of which had been stored at 4°C and one, frozen, at −20°C, prior to testing. When all 32 aliquots were tested in the PCR, two were found positive for *P. falciparum* (Fig. 2). The two positive aliquots were of the same blood sample. The remains of the once-frozen aliquot of this sample were used to make a fresh thick smear, which was then carefully examined under the light microscope; one gametocyte of *P. falciparum* was then observed.

In the tests of the assay's sensitivity, *P. falciparum* was detected down to a 10^{-4} dilution of a mixed sample containing 500 *P. falciparum* and 4120 *P. ovale* parasites/μl, whereas the *P. ovale* in this mix was still detected after a dilution of 10^{-6}. The

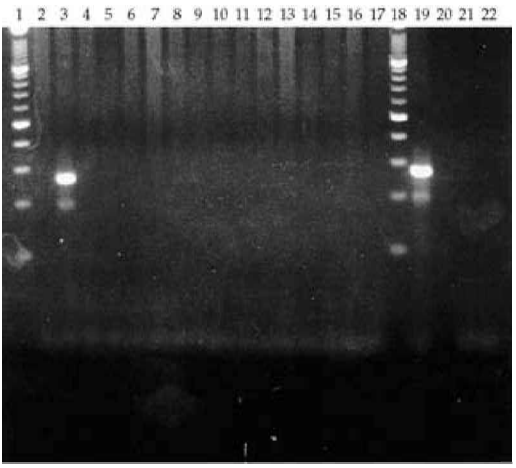

FIG. 2. The samples run to create these banding patterns were 100-bp markers (lanes 1 and 18), the products produced from smear-negative (lanes 2–17) or *Plasmodium falciparum*-positive (lane 19) blood samples or, as negative controls, sample-free reaction mixes (lanes 21 and 22).

P. falciparum in a blood sample containing 20,000 parasites of this species (but no *P. vixax*)/μl was still detectable after the sample was diluted to 10^{-6} (Fig. 3). These results correspond to a detection limit of 0.02–0.05 parasite/μl (0.3–0.8 parasite/reaction) for *P. falciparum* and 0.004 parasite/μl (0.06 parasite/reaction) for *P. ovale.* (There was insufficient material for a similar sensitivity determination for *P. vivax* or *P. malariae*.)

In the absence of any natural mixed infections, four mixtures of *P. falciparum* and *P. vivax* were created. These contained 700 *P. vivax* parasites and 1000, 100, 10 or one *P. falciparum* parasite(s)/μl, giving *P. vivax*:*P. falciparum* ratios of 1:1.4, 1:0.14, 1:0.014, and 1:0.0014, respectively. In the subsequent PCR-based assays, both species were detected in all four mixtures (Fig. 4). All four species were detected in other simulated mixtures (data not shown).

DISCUSSION

Although there have been many previous publications on the detection of malaria and identification of *Plasmodium* species by PCR,

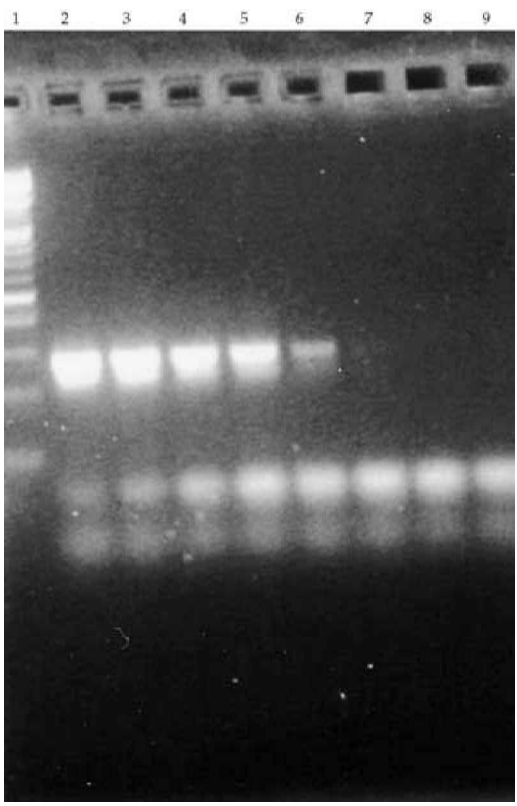

FIG. 3. Apart from those in lane 1 (which are of the 100-bp makers), all of the bands shown here were produced using a blood sample containing 20,000 *Plasmodium falciparum* parasites/μl. The DNA tested in the PCR-based assay came from the same volume of a 10^{-1} (lane 2), 10^{-2} (lane 3), 10^{-3} (lane 4), 10^{-4} (lane 5), 10^{-5} (lane 6), 10^{-6} (lane 7) or 10^{-8} (lane 8) dilution of this sample.

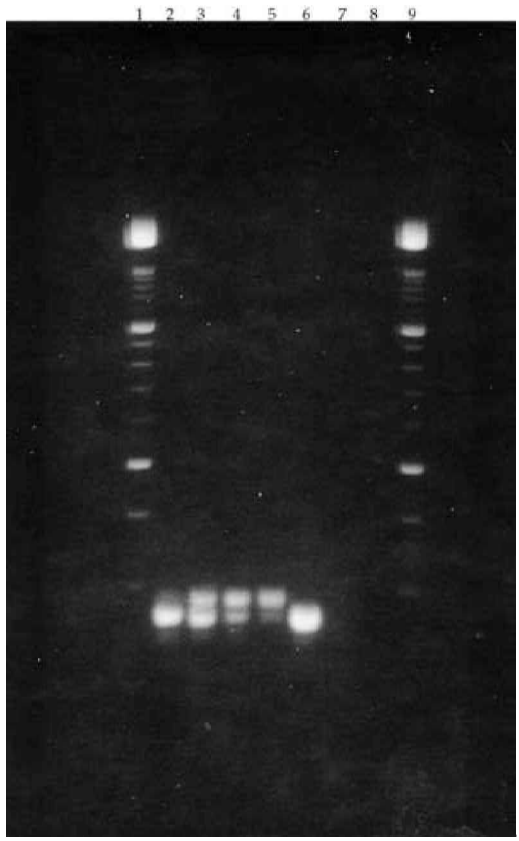

FIG. 4. Most of the bands shown here were produced from blood samples (simulated mixed infections) containing 700 *Plasmodium vivax* and 1000 (lane 2), 100 (lane 3), 10 (lane 4) or one (lane 5) *P. falciparum* parasite(s)/μl. Also included in the run were 100-bp markers (lanes 1 and 9), a *Plasmodium falciparum*-positive control (lane 6), and, as negative controls, sample-free reaction mixes (lanes 7 and 8).

all relate either to the use of some form of nested PCR, using nested or hemi-nested primers (Snounou *et al.*, 1993*a*, *b*; Rubio *et al.*, 1999; Gal *et al.*, 2001), or to the detection of amplified DNA using labelled, specific probes or ELISA (Laoboonchai *et al.*, 2001), for species identification. Single-round PCR has only been used alone to detect a particular species, usually *P. falciparum* (Tham *et al.*, 1999; Barker *et al.*, 1994; Hang *et al.*, 1995), or simply to detect malarial infection, without species identification (Ciceron *et al.*, 1999). In contrast, the PCR-based assay described here uses a single round of PCR to detect **and** identify the *Plasmodium* species present in blood samples. The method is relatively quick, compared with nested or hemi-nested PCR, and can be completed within 3 h of specimen receipt. Thus, a same-day diagnosis of malaria could be provided, and the assay would clearly be useful in a reference-laboratory setting. The risks of cross-contamination, which can be a problem with assays based on nested PCR unless suitable, stringent precautions are observed, are relatively low for single-round assays. The test could provide a powerful back-up to bloodsmear examination (which is still far from redundant and is not replaced by this technique) and should prove very useful in the detection of parasitaemias which are too low to be detected in bloodsmears (Babiker *et al.*, 1999). In a malaria-vaccine study, Cheng *et al.* (1997), using a PCR-based method, were able to detect asexual malarial parasites in peripheral-blood samples from two infected volunteers 6 or 7 days before they detected the parasitaemias by thick-smear microscopy and symptoms appeared.

The facilities for the PCR-based detection of a growing list of pathogens are now widely available in many diagnostic laboratories; malaria is very likely to be added to the list. However, the reported sensitivities of the available PCR-based assays, for malaria and other infections, vary widely. From the published data it is not possible to determine whether these differences represent intrinsic variability in assay sensitivities or are a result of calibration using different reference reagents that are poorly characterized and standardized. The sensitivity of any PCR-based assay will also depend on the volume of sample from which DNA is extracted and the proportion of extracted DNA that is amplified.

The accuracy with which the parasites in the samples used in sensitivity determinations are counted (or with which the amount of parasite DNA in the samples is estimated) is important for overall accuracy and the valid comparison of data. Most researchers record parasitaemias as the number of infected cells/100 erythrocytes (estimated from thin bloodsmears) or by counting parasites, in thick smears, against a fixed number of leucocytes (typically 200). In a few cases, laboratories have used purified DNA from *P. falciparum* grown *in vitro* to calculate the sensitivity of the assay (Ciceron *et al.*, 1999; Schindler *et al.*, 2001). When they used such DNA as a reference, Ciceron *et al.* (1999) estimated the detection limit of their assay to be 0.02 parasite/μl, whereas the results of testing dilutions of blood from three patients with *P. falciparum* indicated a limit varying, from 0.005–0.1 parasite/μl, depending on which patient served as the blood donor (for all three patients, the parasitaemia was adjusted to 1% and the haemocrit to 50%). The assay described was able to detect down to 0.02–0.05 parasite/μl for *P. falciparum* and 0.004 parasite/μl for *P. ovale*.

Some current research is focused on the production of appropriate standard reagents, accurately calibrated and characterized, for use in assays of malarial nucleic acids. It is just as vital that appropriate quality control and standardization are applied to the introduction of nucleic-acid testing for malaria, as to the microscopical diagnosis of malaria (Edwards *et al.*, 1999) and the PCR-based detection of other disease organisms, such as blood-borne viruses (Anon., 1997; Nübling *et al.*, 1998; Saldanha, 2001).

The present assay offers the capability of distinguishing mixed infections from single infections and of identifying all of the *Plasmodium* species present in a mixed infection. As *P. falciparum* is the most virulent and dangerous of the four species causing human malaria, it needs prompt and appropriate therapy. It is particularly important, therefore, that infections with this species are detected, especially in the many areas where the first-line treatment for infections with the other *Plasmodium* species is no longer effective against *P. falciparum*. Some PCR-based assays appear to be much better than conventional microscopy at revealing all the species present in mixed infections (Snounou *et al.*, 1993*b*; Rubio *et al.*, 1999; Toma *et al.*, 2001; Zakeri *et al.*, 2002; Myjak *et al.*, 2002), which may be common in endemic areas. *Plasmodium falciparum* is most likely to go undetected on bloodsmears when another *Plasmodium* species is present. Encouragingly, *P. falciparum* could still be detected in the simulated mixed infections of the present study, even when present at a low parasitaemia (one parasite/µl) and swamped with many more parasites of another *Plasmodium* species. The PCR-based method described here, which could be easily automated, is now being used routinely in London, to confirm the microscopical diagnosis of malaria (unpubl. obs.).

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