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Identification of the five human *Plasmodium* species including *P. knowlesi* by real-time polymerase chain reaction

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Abstract (146 words)

Recently, *Plasmodium knowlesi* has been recognized as the fifth *Plasmodium* species causing malaria in humans. Hundreds of human cases infected with this originally simian *Plasmodium*-species have been described in Asian countries and increasing numbers are reported in Europe from travellers. The growing impact of tourism and economic development in South and Southeast Asia are expected subsequently to lead to a further increase in cases both among locals and travellers. *P. knowlesi* is easily misidentified in microscopy as *P. malariae* or *P. falciparum*. We developed new primers for the rapid and specific detection of this species by low cost real-time PCR and added this method to an already existing panel of primers used for the molecular identification of the other four species in one reaction. At the moment, reference laboratories should be able to identify undisputably and rapidly *P. knowlesi* as it is a potentially fatal pathogen.

Keywords: *P. knowlesi*, identification, real-time PCR, diagnosis.

INTRODUCTION

Until recently, human malaria was known to be caused only by the four species *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. Newly, retrospective studies with molecular tools have also shown hundreds of human cases infected with a simian *Plasmodium* species, *P. knowlesi* in numerous Southeast Asian countries, most in Sarawak and Sabah, Malaysian Borneo [1, 2], others in other parts of Borneo [3], in Peninsular Malaysia [4], Thailand [5], Myanmar [6], Philippines [7], Singapore [8] and Vietnam [9, 10]. Concomitantly, increasing numbers have been described in travellers returning from these areas [11-17]. This parasite has been recognized in 2008 by the WHO as the fifth human *Plasmodium* species potentially leading to fatal outcome [18-20]. The growing impact of tourism and economic development in South and Southeast Asia are expected subsequently to lead to a further increase in cases both among locals and travellers. Therefore, an early and accurate identification of suspected cases is warranted both in order to be able to start immediately the appropriate treatment and to improve the follow-up. Unfortunately, conventional diagnostic techniques such as microscopic analyses of asexual stages of *Plasmodium* on thick and thin blood films are unable to distinguish early trophozoites of *P. knowlesi* from *P. falciparum* and later erythrocytic stages from those of *P. malariae* [21, 22]. Molecular methods have therefore been described to help in detection of this life-threatening species. Babady et al. used a first set of FRET probes in real-time polymerase chain reaction to differentiate the four species *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* according to different melting temperatures [23]. Based on this analysis, *P. knowlesi* was indistinguishable from *P. vivax*. Therefore, the authors have designed a second specific set of FRET probes for *P. knowlesi*. This assay allows the detection of the five *Plasmodium* species in a same run but the two set of probes increase the cost of the reaction.

We have previously adapted with slight modifications the SYBR Green real-time PCR from de Montbrison et al. in order to identify and differentiate the four *Plasmodium* species: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* [24]. In the present study, we have expanded the method to the detection of the fifth human *Plasmodium* species, *P. knowlesi*.

MATERIALS AND METHODS

Bioinformatic study

In order to add the detection of *P. knowlesi* to the already used assay, a specific primer set was designed. A set of 87 small sub-unit gene sequences corresponding to the five *Plasmodium* species was recovered from two databases Genbank (NCBI; <http://www.ncbi.nlm.nih.gov/pubmed/>) and Plasmo DB (Malaria Parasite Genome Project; <http://plasmodb.org/>). Among them, fifty correspond to *P. knowlesi* with 37 sequences of A-type and 13 sequences of S-type. Ten sequences correspond to *P. falciparum* (5 A-type and 5 S-type), 14 to *P. vivax* (11 A-type and 3 S-type), 8 to *P. ovale* (7 A-type and 1 unidentified), and 5 to *P. malariae* (1 A-type and 4 unidentified).

The selected sequences were aligned using the BioEdit Sequence Alignment Editor® (version 7.0.5.3). Regions of conserved alignment for *P. knowlesi* but with enough polymorphisms with the four other *Plasmodium* species were then searched for to design of specific *P. knowlesi* primers.

DNA extraction and controls

Nucleid acids of blood samples were extracted by using the MagNA Pure LC automated system with the MagNA Pure LC Microbiology Kit M^{Grade} according to the manufacturer's instructions (Roche Diagnostics, France). Samples were kept at -80°C until used.

Two *P. knowlesi* samples taken from European tourists were used control the specificity of the primers [11, 15].

A positive control plasmid of *P. knowlesi* was constructed to evaluate the sensitivity of the method. DNA was amplified with the primers PkF1140 5'-GATTCATCTATTAAGAATTTGCTTC-3' [25] and PKg'R 5'-CAAATCCACTATATTCAATTCTACA-3', framing the specific region PKe'F -PKg'R and the PCR product was cloned into a plasmid (PCR Cloning kit®, Qiagen). Recombinant clones were selected and plasmid DNA was extracted, purified and quantified spectrophotometrically. Ten-fold serial dilutions in sterile water were prepared to obtain concentration from 10¹⁰ plasmid copies / µl to 10 copy / µl.

Real-time PCR

The already used analysis targets the A-type 18S ribosomal subunit gene of *Plasmodium* sp and is based on the fluorescent SYBR Green I dye used on a LightCycler (Roche Diagnostic, France). In this assay the primers PSP1AS (5'-CTACTCCTATTAATCGTAACT-3'), PFS1 (5'-CATTTAAACTGGTTTGGGAAAAC-3') or POS1 (5'-GCATTCCTTATCCAAAATGTG-3') are used in order to detect *P. falciparum* and *P. ovale*, respectively, and the primers PM1 (5'-ATAACATAGTTGTACGTTAAGAATAACCGC-3'), PM2 (5'-AAAATTCCCATGCATAAAAAATTATACAAA-3'), PV1 (5'-CGCTTCTAGCTTAATCCACATAACTGATAC-3') and PV2 (5'-CTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3') are used to detect *P. malariae* and *P. vivax* [24].

The reaction is performed in a final volume of 20 µl, containing five microliters of DNA extracted from blood samples, 5 µl of LightCycler FastStart DNA Master SYBR Green I Buffer

(Roche Diagnostics, France), 2.4 µl of MgCl₂ (4 mM), and 0.6 µl of each primer (initial concentration 10 µM, final concentration 0.3 µM).

The PCR program is similar for the five *Plasmodium* species: after an initial denaturation step of 8 minutes at 95°C, 40 cycles of denaturation at 95°C for 10 s., annealing at 58°C for 10 s. and extension at 72°C for 30 s. are performed. The melting curve is measured after 2 s. denaturation at 95°C followed by annealing (20 s. at 50°C) and slow increase in temperature (0.2°C/s. until 98°C) to detect the temperature needed for denaturation.

RESULTS

After the initial selection of several primer sets, the primers PKe'F (designed at the same location than the antisens primer PkR1150 from Imwong *et al.* [25]) (5'-TCTTTTCTCTCCGGAGATTAGAACTC-3') and PKg'R (5'-CAAATCCACTATATTCAATTCTACA-3') targeting the A type of SSU gene were chosen for specific amplification of *P. knowlesi* to obtain a 312 bp amplicon. These new primers were added to the above described protocol.

Results were analyzed with the threshold cycle (Ct), corresponding to the cycle at which the fluorescence signal becomes significantly different from the baseline signal and with the post amplification melting curve analysis that is related to the sequence of the PCR products. The quantity of DNA amplified is not directly equivalent to the parasitaemia present in the original blood sample. Amplifications of each five *Plasmodium* species with their specific primers result in specific melting temperatures: 78°C for *P. falciparum*, 79°C for *P. ovale*, 80°C for *P. vivax*, 74°C for *P. malariae* and 86°C for *P. knowlesi*. For each primer set, nonspecific amplification was excluded by the lack of amplification and melting curve with DNA of the four other species as with noninfected blood samples. The specificity of this assay was tested by using other common blood

pathogens as *Candida albicans*, *Toxoplasma gondii*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Cytomegalovirus and Hepatitis B virus. All these control pathogens resulted in negative amplification demonstrating the specificity of the primers (Fig 1).

The analytical sensitivity was determined with the concentration of the last positive amplicon obtained with a reliable reproducibility to be 10^2 copies / μ l (corresponding to a Ct = 34 cycles).

DISCUSSION

As the number of human *P. knowlesi* cases increases, clinicians and laboratory personnel should be alerted to this emerging - and potentially even lethal - cause of malaria, especially as it is easily misidentified as less dangerous *P. malariae* on microscopy [22]. Until now, the gold standard in malaria diagnosis is indeed based on microscopical examination of blood films but it remains difficult to detect mixed infections, low parasitaemia, atypical forms of *Plasmodium* sp. potentially modified by treatment as well as this fifth species, *P. knowlesi*. During last years, conventional methods have been improved by molecular tools. Therefore, laboratories should be able, now, to confirm or exclude the diagnosis of *P. knowlesi* malaria. Due to the uncertainty in identifying this species by microscopic analysis, and the absence of a reliable specific and sensitive rapid diagnostic test, molecular techniques are now the key for achieving this diagnosis.

In this study, we developed a real-time PCR analysis based on SYBR Green in order to identify *P. knowlesi* in addition to the four other human malaria species. Importantly, this technology remains low cost in comparison with the use of FRET probes. The method suits for use both in non-endemic areas such as Europe where only travellers with fever need to be tested and also in endemic areas in Southeast Asia where large-scale testing is required. The numbers of imported cases from travellers are certainly underestimated due to the difficulties in identifying

exactly the species. Moreover, the areas of potential spread in Southeast and South Asia where the vector mosquito has spread [18] should be explored more thoroughly to get accurate information on the distribution of the disease. Novel accurate tests such as the one presented here are needed as tools for such investigations. They are also needed for diagnostics in the ordinary laboratories investigating patient samples from endemic areas or from travellers returning with fever from such areas.

1453 words.

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Figure Legend

Figure 1. Specific detection of *Plasmodium knowlesi* with the primers PKe'F and PKg'R in SYBR Green real-time PCR.

A. Amplification curve of *P. knowlesi*. Samples containing DNA from *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* did not result in amplification of DNA with the primers PKe'F and PKg'R.

B. Melting curves analysis. The melting temperature obtained with *P. knowlesi* specific primers PKe'F and PKg'R is 86°C (+/- 0.5°C). No curve was obtained using the same primers with the other human *Plasmodium* species.