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# A TaqMan real-time PCR assay for the detection and quantitation of *Plasmodium knowlesi*

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## Abstract

**Background:** The misdiagnosis of *Plasmodium knowlesi* by microscopy has prompted a re-evaluation of the geographic distribution, prevalence and pathogenesis of this species using molecular diagnostic tools. In this report, a specific probe for *P. knowlesi*, that can be used in a previously described TaqMan real-time PCR assay for detection of *Plasmodium* spp., and *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale*, was designed and validated against clinical samples.

**Methods:** A hydrolysis probe for a real-time PCR assay was designed to recognize a specific DNA sequence within the *P. knowlesi* small subunit ribosomal RNA gene. The sensitivity, linearity and specificity of the assay were determined using plasmids containing *P. knowlesi* DNA and genomic DNA of *P. falciparum, P. knowlesi, P. malariae, P. ovale* and *P. vivax* isolated from clinical samples. DNA samples of the simian malaria parasites *Plasmodium cynomolgi* and *Plasmodium inui* that can infect humans under experimental conditions were also examined together with human DNA samples.

**Results:** Analytical sensitivity of the *P. knowlesi*-specific assay was 10 copies/μL and quantitation was linear over a range of 10-$10^6$ copies. The sensitivity of the assay is equivalent to nested PCR and *P. knowlesi* DNA was detected from all 40 clinical *P. knowlesi* specimens, including one from a patient with a parasitaemia of three parasites/μL of blood. No cross-reactivity was observed with 67 *Plasmodium* DNA samples (31 *P. falciparum*, 23 *P. vivax*, six *P. ovale*, three *P. malariae*, one *P. malariae/P. ovale*, one *P. falciparum/P. malariae, one P. inui and one P. cynomolgi)* and four samples of human DNA.

**Conclusions:** This test demonstrated excellent sensitivity and specificity, and adds *P. knowlesi* to the repertoire of *Plasmodium* targets for the clinical diagnosis of malaria by real-time PCR assays. Furthermore, quantitation of DNA copy number provides a useful advantage over other molecular assays to investigate the correlation between levels of infection and the spectrum of disease.

## Background

The sensitivity and specificity of a diagnostic test define the extent to which a pathogen can be effectively identified in a patient specimen. For malaria, the examination of thin and thick blood smears by microscopy has been the gold standard diagnostic method for over a century. This test is simple to perform, requires only a microscope and has a sensitivity of 50 parasites/μL [1]. The parasitaemia can be quantified and the species of *Plasmodium* identified based on parasite morphology. When read by an experienced microscopist, the four major species of human malaria (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*) can usually be discriminated.

However, a major pitfall of microscopy was recently identified in the failure of this method to distinguish between the benign *P. malariae* species and the potentially lethal primate species *Plasmodium knowlesi* [2]. Zoonotic transmission of *P. knowlesi* from monkeys to humans was previously only observed in sporadic cases [3,4] and by blood passage from monkeys to humans in laboratory controlled experiments [5-7] but was not routinely detected by microscopic analysis of patient specimens due to morphological similarities between *P. knowlesi* and *P. malariae* [8]. As such, *P. knowlesi* was not recognized as a cause of malaria in human populations, until recently. Using molecular diagnostic tools,

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