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# Investigation on possible transmission of monkeys' Plasmodium to human in a populations living in the equatorial rainforest of the Democratic republic of Congo

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ABSTRACT

Plasmodiums are protozoa that may infect various hosts. Only five species are now recognized as naturally parasitizing humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi.* This fifth species, *P. knowlesi*, previously identified as naturally parasitizing the monkey *Macaca fascicularis*, has been microscopically confused for a long time with P. malariae or *P. falciparum* and it was not possible to correctly differentiate them until the advent of molecular biology. To date, natural human infections with *P. knowlesi* only occur in Southeast Asia and a similar phenomenon of natural transmission of simian plasmodium to humans has not been reported elsewhere. This study was conducted to investigate a possible transmission of African small monkey's plasmodium to humans in populations living near the rainforest of the Democratic Republic of Congo (DRC) where several species of non-human primates are living. Two successive real-time PCRs were identified in the literature and used in combination for purpose. Only *P. falciparum* was found in this study. However, studies with larger samples and with more advanced techniques should be conducted.


## 1. Introduction

The first report on the existence of simian *Plasmodium* was published in 1908 by Daniels (1908). It was believed that simian *Plasmodium* spp. infect only monkeys and that human *Plasmodium* spp. infect only humans. Eyles et al. (1960) showed that a *Plasmodium vivax*-like species, *Plasmodium cynomolgi*, could infect humans in the laboratory. Warren et al. (1970) reported the first case *Plasmodium knowlesi* in humans, which had been detected in 1965. This species has long been known as a natural parasite of the macaque (*Macaca fascicularis*) and prior to the advent of molecular techniques was misidentified in humans as *Plasmodium malariae* or *Plasmodium falciparum*, which it resembles morphologically (Singh et al., 2004; Lee et al., 2009). In a large study in Sarawak, Malaysia, Singh et al. (2004) detected a high prevalence (57.7%) in humans. Since then, natural infections with this species, confirmed using molecular techniques, have been reported from many other countries in South-East Asia (Jongwutiwes et al., 2004; Vythilingam et al., 2006; Zhu et al., 2006; Cox-Singh et al., 2008; Luchavez et al., 2008; Ng et al., 2008; Khim et al., 2011). Thus, *P. knowlesi* has been declared to be the fifth species of *Plasmodium* infecting humans (White, 2008). More recently, Ta et al. (2014) have reported the first case of naturally acquired human infection with *P. cynomolgi*, in a woman in Malaysia. An initial microscopic diagnosis of *P. malariae/P. knowlesi* was established, followed by a second microscopic re-examination and a nested PCR that identified

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that species as *P. vivax*. However, a different PCR method and sequencing confirmed the infection as *P. cynomolgi*. These reports suggest that human infections by simian *Plasmodium* are probably under-reported because of misidentification. If *P. knowlesi* had been misdiagnosed for such a long time in Asia and if *P. cynomolgi* identification has been so difficult, it may be possible that in Africa, where many non-human primates live, natural transmission of simian *Plasmodium* spp. to humans has not been recognized. Indeed in Africa where malaria diagnosis is essentially microscopy-based, it is likely that some *Plasmodium* spp. in people have been misidentified.

Many studies have been reported that explore the presence of simian malaria parasites in human or human malaria parasites in other primates, but almost all of these studies were based on great apes, e.g., *Pan paniscus* (bonobo), *Pan troglodytes* (chimpanzee) or *Gorilla gorilla* (gorilla) (Contacos et al., 1970; Ollomo et al., 2009; Duval et al., 2010; Kaiser et al., 2010; Krief et al., 2010; Liu et al., 2010; Prugnolle et al., 2010). Very few studies included monkeys. For example, Prugnolle et al. (2011) analyzed blood samples from 338 monkeys of ten species and Pourrut et al. (2009) examined blood samples from 82 small monkeys (e.g., *Chlorocebus aethiops* and *Erythrocebus patas*) for *Plasmodium*. Many species of monkeys are present in the Democratic Republic of Congo (DRC) (Ladnyj et al., 1972; Colyn, 1994; Grubb et al., 2003) and some of these have been listed as hosts for *Plasmodium* species, especially *P. gonderi*, *Plasmodium petersi*, *Plasmodium georgesi* (Poirriez et al., 1993, 1995) and *Plasmodium* sp. DAJ- 2004 (Prugnolle et al., 2011). As *P. knowlesi* and *P. cynomolgi* naturally infect monkeys, and have been reported as able to also infect humans, the present study investigated the possible transmission of monkey *Plasmodium* to humans in a populations living in the equatorial rainforest of the DRC where several species of non-human primates are found.

## 2. Materials and methods

### 2.1. Sample collection

Three villages of the Equatorial Province of the DRC, Bolenge (N: 00° 24′ 89″, E: 18° 22′ 29″), Bongonde (N: -0.15° 42′ 42″, E: 18° 22′ 35″) and Wendji-Secli (N: -00° 06′ 56″, E: 18° 22′ 35″), were selected based on geographical location near the equatorial rain forest. One hundred individuals without symptoms of malaria were randomly selected from each village without consideration of age or sex. People with fever were not selected. A drop of blood from each individual using a finger prick and absorbed on to filter paper Whatman 3 MM (Sigma–Aldrich, St. Louis, MO, USA) was made in duplicate for each individual. People in these villages practice daily forest activities such as hunting and gathering. Monkeys are captured for commerce and for food.

### 2.2. DNA extraction

Parasite DNA was extracted using QIAamp® DNA mini kits (Qiagen, Hilden, Germany) according to manufacturer's instruction for filter paper and in a final elution volume of 150 μL.

### 2.3. Plasmodium species identification

Two real-time PCR were identified in the literature and were successively applied to the DNA templates. The first one allows the detection of all *Plasmodium* spp. and the second one allows the detection of the four common *Plasmodium* species that infect humans in Africa (*P. falciparum*, *P. malariae*, *P. vivax* and *Plasmodium ovale*).

As the species specific Real-Time PCR was more sensitive than the pan primer one, we hypothesized that if the first Real-Time PCR was positive and the second negative, it would suggest that a *Plasmodium* species different from the four human species was detected. In that case, sequencing would be considered.

### 2.4. First RT-PCR

This RT-PCR includes a pair of primers (Plasmo 1 and Plasmo 2) and one probe (plasprobe), designed to detect genes of the small subunit 18S rRNA of *Plasmodium* species, that allow the detection of all *Plasmodium* spp. (Table 1) .The mix consists of 12.5 μl of 2X Taqman Universal PCR Master Mix (Applied Biosystems, Waltham, MA, USA), 200 nM of each primer, 50 nM of probe and water for a total volume of 25 μl including 5 μl of DNA. PCR conditions were as follows: an initial step at 50 °C for 2 min, 95C° for 10 min, and 45 cycles of 95 °C for 15 s followed by 1 min at 60 °C (Rougemont et al., 2004).

### 2.5. Second RT-PCR

Four species-specific forward primers, one universal reverse primer (Plasmo 2) and four species-specific probes were used in two simultaneous duplex reactions (Pfal + Pviv and Pmal + Pova) (Cnops et al., 2010). The probes and primers were designed to detect genes of the small subunit 18S rRNA of the four *Plasmodium* species. The mix consisted of 12.5 μl of 2X Taqman Universal PCR Master Mix (Applied Biosystems), 200 nM of each primer and probes except for *P. vivax* at 100 nM in a final volume of 25 μl including 5 μl of DNA. The following conditions were used: 2 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 60 s at 60 °C.

Both RT-PCR were run on a Stratagene Mxp 3000 Instrument (SABiosciences) and in the presence of positive controls (human *Plasmodium*) provided by the Parasitology Unit, Institute of Tropical Medicine, Antwerp and the Laboratory of Clinical Microbiology, University Hospital of Liège, Belgium.

To assess our methodology, *P. berghei* and *P. vinckei* cultures (provided by the Interfaculty Drug Research Center, University Hospital of Liege) have been used as non-human *Plasmodium* templates. Unfortunately, we were not able to test for the other monkey parasites *P. gonderi*, *P. georgesi*, *P. petersi or Plasmodium* sp. DAJ 2004.

## 3. Ethical considerations

This study has received the ethical approval of the Ministry of Public Health of the DRC and of the Institutional Committee of the Faculty of Medicine, University of Kinshasa.

## 4. Results and discussion

### 4.1. Assessment of the methodology

As template, *P. vinckei* and *P. berghei* were used together with the four human *Plasmodium* as positive controls for the two successive RT-PCRs. The first one detected all *Plasmodium* but the second only detected *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* (Table 2).

As we were not able at the time of this study to get *P. gonderi*, *P. petersi* or *P. georgesi* samples, we aligned the primers and the probes used for both real-time PCRs with their genome to explore if they could match. Only primers and probe of the first RT-PCR matched.

### 4.2. Parasite identification

Of the three hundred samples analyzed, 139 (46,3%) were