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Nested multiplex PCR for identification and detection of human *Plasmodium* species including *Plasmodium knowlesi*

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ABSTRACT

Objective: To develop a new technique for diagnosis of *Plasmodium knowlesi* and at the same time to be able to discriminate among the diverse species of *Plasmodium* causing human malaria.
Methods: In this study the nested multiplex malaria PCR was redesigned, targeting the 18S rRNA gene, to identify the fifth human *Plasmodium* species, *Plasmodium knowlesi*, together with the other human *Plasmodium* (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*) by amplified fragment size using only two amplification processes and including an internal reaction control to avoid false negatives.
Results: The technique was validated with 91 clinical samples obtained from patients with malaria compatible symptoms. The technique showed high sensitivity (100%) and specificity (96%) when it was compared to the reference method employed for malaria diagnosis in the Instituto de Salud Carlos III and a published real-time PCR malaria assay.
Conclusions: The technique designed is an economical, sensitive and specific alternative to current diagnosis methods. Furthermore, the method might be tested in *knowlesi*-malaria endemic areas with a higher number of samples to confirm the quality of the method.

1. Introduction

The latest World Malaria Report stated that malaria affects approximately half of the world's population, with an estimated 214 million cases of malaria (range: 149–303 million) and 438 000 malaria deaths globally (range: 236 000–635 000) [1].

The known causative agents of human malaria include *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax* (*P. vivax*), *Plasmodium malariae* (*P. malariae*) *and Plasmodium ovale* (*P. ovale*). Moreover, a fifth species, *Plasmodium knowlesi* (*P. knowlesi*), which is still considered a zoonosis, produce several cases in Southeast Asia [2] being the first cause of malaria in Malaysia counting for the 71% of the cases [1,2].

P. knowlesi was discovered in 1927 by Franchini in its natural host, the long-tailed macaque (*Macaca fascicularis*). The first natural infection in humans was reported in 1965 [3] in a man from the United States after his visit to Peninsular Malaysia but it was not until 2004 when a large focus of naturally acquired *P. knowlesi* infections in human beings was identified [4] and it began to be considered as the fifth human malaria species.

Probably human infection with *P. knowlesi* is not recent, as evidenced by the frequent misdiagnosis that is recorded using molecular identification tools such as PCR [4–6]. Prospective studies performed in Malaysia, where the five different *Plasmodium* species that infect humans coexist, concluded that microscopy does not reliably distinguish among *P. falciparum*, *P. vivax* and *P. knowlesi* [7–9]. Morphologically, *P. knowlesi* resembles *P. falciparum* in the early blood ring stage and is impossible to distinguish from *P. malariae* in other stages [10].

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Knowlesi-malaria cases have been largely concentrated in the forested areas of Southeast Asian countries coinciding with the distribution of its natural vectors *Anopheles latens* and *Anopheles cracens* [11] but traveler's cases have been reported elsewhere in Europe, America, Asia and Australia [5,12].

P. knowlesi has the shortest erythrocytic replication period, just 24 h, and the high parasite loads which lead to severe malaria and death, for that reason have to include *P. knowlesi* detection in the current malaria diagnosis [5,12].

To date, conventional light microscopy examination of Giemsa stained thin and/or thick smear films still remains the gold standard for routine laboratory diagnosis. Human malaria *Plasmodium* species have clear differences to be identified, but species such as *P. knowlesi* may be easily confused [7]. Alternatively, rapid diagnostic immunochromatographic tests (RDT) detecting *Plasmodium* antigens are being widely used in most countries [13]. These test presents limitations, as microscopy, because it does not detect asymptomatic patients and low parasitemias [6]. Furthermore, sensitivity in the absence of *P. falciparum* infection is low, missing between 11% and 22% of non-*falciparum* cases [14] or until 57% when just *P. ovale* or *P. malariae* are analysed [15]. The RDT for *P. knowlesi*-infected blood samples are poor sensitivity and specificity, some test *P. knowlesi* could be misdiagnosed as *P. falciparum* meanwhile in others the sensitivity was between 29% and 40% without discriminating the species except as non-*falciparum* [16].

Molecular tools such PCR has provided more specific, sensitive, and reliable molecular techniques for the diagnosis of malaria [17–19].

The more extended PCR assay for detection of *P. knowlesi* was a nested PCR [4,20] which in the first round of PCR amplification used genus-specific primers, followed by species-specific primers in separate second rounds of PCR amplification for each human malaria species. Nested PCR assays are very sensitive and can detect *P. knowlesi* parasitemia even when it is less than 10 parasites/μL. Despite being considered a highly sensitive and specific for the diagnosis of malaria technique has drawbacks, as it involves of 5–6 separate PCR reactions to detect the five species of *Plasmodium*, which consumes time, labour and materials, with high potential to produce cross-contamination. Furthermore, several reports shows that *P. knowlesi* specific primers, described by Singh *et al* [4], have cross hybridization with *P. vivax* [12,21,22] and *P. vivax* primers with *Plasmodium cynomolgi* and other monkeys malaria [23].

Several real-time PCR have been designed for the identification of the different *Plasmodium* species that can infect humans [12,24–26]. Real-time PCR assays have the advantage over nested PCR assays that are faster and with less contamination risk but generally are less sensitivity [12,27] and need more expensive reagents and equipment which are the major drawbacks faced by the introduction of these tests in laboratories with resources constraints [28]. Also, new methods as loop mediated isothermal amplification (LAMP) were developed for *P. vivax* [29], *P. falciparum* [30] or *P. knowlesi* [31] with the inconvenient to be single specific methods and with a sensitivity similar to the conventional PCR methods [32].

The aim of this report is to describe and validate a molecular diagnostic method with suspicious malaria patients' samples in order to improve the diagnosis of the *Plasmodium* species which can affect humans, including *P. knowlesi*. The method is a modification of the nested malaria PCR (NM-PCR) accredited (UNE-EN ISO 15189:2013) for molecular malaria diagnosis performing in the Spanish Malaria Reference Laboratory [23], and an improvement of the semi-nested multiplex malaria PCR [17].

2. Materials and methods

2.1. Control samples

Twenty blood malaria infected samples, ten *P. falciparum*, three *P. vivax*, two *P. ovale*, two *P. malariae* and one *P. knowlesi* were used to optimize the method, together with blood samples infected with *Trypanosoma cruzi*, *Leishmania infantum* and *Babesia microti*. Uninfected human blood was used as negative control. All samples used for this study came from the sample collection of the Malaria and Emerging Parasitic Diseases Laboratory (Spanish National Register BioBank no C.0001392).

2.2. Clinical samples

A total of 91 anonymous whole-blood samples diagnosed by PCR from the sample collection of the Malaria and Emerging Parasitic Diseases Laboratory in collaboration with the Parasitological Department of the Institute for Medical Research (Spanish National Register BioBank no C.0001392) were used for this study.

Samples used were sixteen *P. falciparum*, eighteen *P. vivax*, fourteen *P. ovale*, seven *P. malariae* and eleven *P. knowlesi*, besides twenty-four negatives malaria samples.

2.3. DNA extraction

DNA extraction was performed using the QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions started with 200 μL of whole blood.

2.4. Malaria detection and identification

2.4.1. NM-PCR

The NM-PCR was performed as previous published [23]. Five μL of DNA was used as template in the first amplification process. This method is the method accredited (UNE-EN ISO 15189:2013 no 175/LE1213) as reference molecular method for malaria diagnosis in the Spanish Reference Laboratory which characterizes, by a double (nested) multiplex PCR, the human *Plasmodium* species except for *P. knowlesi* [23].

2.4.2. Specific knowlesi-PCR

The specific characterization of *P. knowlesi* was performed adding a parallel nested PCR to the first amplification of the NM-PCR with the same reaction mix characteristic and with the primer sequences, concentration and annealing temperature described in Table 1.

2.4.3. Nested multiplex malaria for the five human Plasmodium (NM5-PCR)

ssrRNA gene sequences between primers PLF and REV from first amplification process of the NM-PCR from different *Plasmodium* species present in Genbank database were aligned with Clustal W software [33] to locate specific differential regions for

Table 1
List of primers including name, sequence, final concentration, PCR step, fragment size, and specificity of the NM5-PCR and nested *P. knowlesi* PCR.

Name	Sequence 5′-3′	Final concentration (μM)	PCR (T[a])	Size[a] (pb)	Specificity
PLF	AGTGTGTATCAATCGAGTTTC	0.075 0	1° PCR 58 °C	783–821[b]	*Plasmodium*
REV	GACGGTATCTGATCGTCTTC	0.075 0	1° PCR 58 °C	–	Universal
HUF	GAGCCGCCTGGATACCGC	0.012 5	1° PCR 58 °C	231	Human
NewPLFshort	CTATCAGCTTTTGATGTTAG	0.150 0	2° PCR 53 °C	–	*Plasmodium*
Malshort	TCCAATTGCCTTCTG	0.250 0	2° PCR 53 °C	215	*P. malariae*
Falshort	GTTCCCCTAGAATAGTTACA	0.150 0	2° PCR 53 °C	344	*P. falciparum*
Vivshort	AAGGACTTCCAAGCC	0.100 0	2° PCR 53 °C	457	*P. vivax*
OvaNew	CCAATTACAAAACCATG	0.360 0	2° PCR 53 °C	176	*P. ovale*
NewPKrev	CGCGGAGGCATC	0.100 0	2° PCR 53 °C	389	*P. knowlesi*
PkForw4	CCACATAACTGATGCCTCCG	0.500 0	*P. knowlesi* PCR 58 °C	278	*P. knowlesi*
PLR-L3	CTACTCCTATTAATCGTAACTAAGCCAA	0.250 0	*P. knowlesi* PCR 58 °C		*Plasmodium*

[a] Expected size of the PCR product between the corresponding Universal/*Plasmodium* primer with the species specific primer. [b] Size depending upon species.

P. knowlesi primer design. Three zones were characterized, the first around 390 to 415 bp position from the PLF primer (Position 1 of on the published sequence18S rRNA of *P. knowlesi* isolate ISCIII/P569/2009 access number HM106521), the second at the level of nucleotides 454 and 550 bp and the third from 560 to 580 bp. The second area was ruled out for to be an AT-rich region and the third area was excluded for to be the same area where Singh *et al* [4], designed its primer for *P. knowlesi* which cross with *P. vivax* [12]. Then just in the first area was possible to design a specific primer for *P. knowlesi* following the general primers design rules [17], with the inconvenient that the theoretical amplification fragment size coincides closely with the size of *P. ovale* amplification so designing a new specific primer for *P. ovale* amplification was also necessary using the same alignment (Table 1).

Detection of *Plasmodium* infection and identification of species was done by a modification of the second amplification of the NM-PCR adding the new primers designed (NewPkRev and Ovanew) and removing the Ovashort primer from the original second PCR reaction. The primer concentrations were determined empirically following the same steps in the original method [17]. The best primer concentrations were those where all the templates as single and double infections were perfectly amplified (Table 1).

The first reaction incorporated primers PLF, REV and HUF and was expected to yield two products; an amplification fragment of 231 bp from UNR-HUF, which was a positive control expected in all samples (infected and not infected samples) and the second band of 783–821 bp, depending on the species, from UNR-PLF which should detect the presence of any *Plasmodium* species [23]. This fragment was just visible when the reaction was optimized (see below) and the parasitaemia was over 1%–3%.

The second PCR reaction was used for the *Plasmodium* species identification and incorporated the products of the first reaction along with primers NewPLFshort, Malshort, Falshort, OvaNew, Vivshort and NewPKrev. Infection with different malaria species yielded products of different sizes (Table 1, Figure 1).

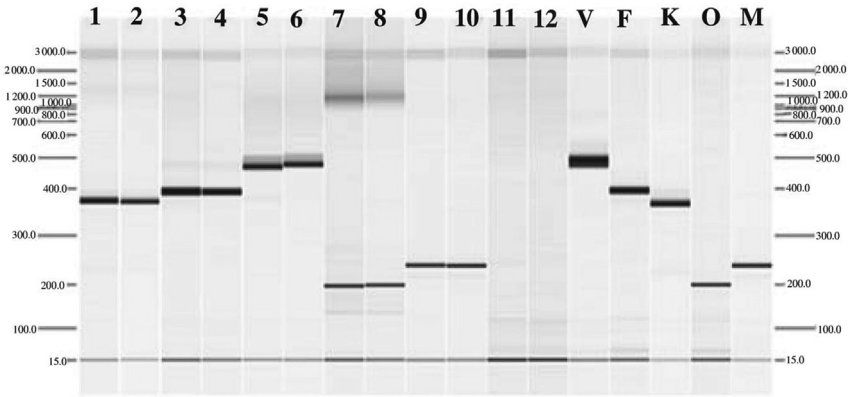

Figure 1. NM5-PCR amplification products.
Gel representation of QIAxcel (BioRad, Hercules, California, USA) capillary electrophoresis second amplification. Lines 1–2 and K= *P. knowlesi*; Lines 3–4 and F= *P. falciparum*; Lines 5–6 and V= *P. vivax*; Lines 7–8 and O= *P. ovale*; Lines 9–10 and M= *P. malariae*; and Lines 11–12= negative samples.

The PCR mix in both reactions consisted of 1× Biotools buffer, 2.0 mM $MgCl_2$, 200 μM each of dNTPs, the PCR primers, *Tth* DNA polymerase (Biotools SA, Madrid, Spain) and template DNA. The first reaction was performing in a final volume of 50 μL using 5 μL of template DNA and 2 units of *Tth* polymerase. The second reaction was carried out in a volume of 25 μL with 2 μL of the PCR product of the first reaction as template and one unit of *Tth* polymerase. For both reactions a GeneAmp® PCR System 2700 thermal cycler (Applied Biosystems Laboratory, Waltham, Massachusetts, USA) was used, beginning with 7 min at 94 °C, followed by (first-round) 40 cycles of 20 sat 94 °C, 20 sat 58 °C, and 30 s at 72 °C, or (second-round) 35 cycles of 15 s at 94 °C, 15 s at 53 °C, and 20 s at 72 °C. The final cycle was followed by an extension time of 10 min at 72 °C.

Amplified fragment size were estimated in an automatic capillary electrophoresis system (QIAxcel: QIAGEN, Hilden, Germany) or by 2.5% agarose gel electrophoresis with Pronosafe (Pronadisa, Madrid, Spain) staining in a Gel Doc 2000 (BioRad, Hercules, California, USA).

2.5. Statistical analysis

The Winepiscope® program was used to determine the values of sensitivity, specificity, positive and negative predictive value and Cohen's kappa coefficient [34].

3. Results

No amplification was obtained from samples infected with others parasites and with uninfected human blood except the human internal amplification control in the first PCR when the NM5-PCR was used.

The twenty positive *Plasmodium* samples give the expected results after the optimization of the NM5-PCR conditions, a fragment of 176 bp for *P. ovale* samples, one of 215 bp for *P. malariae* samples, one of 344 bp for *P. falciparum* samples, one of 389 for *P. knowlesi* sample and one of 457 bp for *P. vivax* samples (Figure 1). Mixed samples used as templates gave the expected fragments.

The validation of the technique was carried out by analyzing 91 blindness samples by the NM5-PCR in comparison with the NM-PCR and the specific *knowlesi*-PCR. When results were discordant, samples were being repeated for each technique. All samples, but one gave identical results, 24 negatives, 16 *P. falciparum*, 18 *P. vivax*, 14 *P. ovale*, 7 *P. malariae* and 11 *P. knowlesi*, in this case using the *knowlesi*-PCR. The discordant sample was negative by the NM-PCR and *P. falciparum* positive by the NM5-PCR.

The sensitivity of the technique was 100%, meanwhile, the specificity was 96.00%. The predictive positive and negative values were 98.51% and 100% respectively. The Cohen's kappa coefficient, a statistic which measured inter-rater agreement for qualitative items, was 97.21%.

4. Discussion

Tests with various experimental infections and controls always yielded the expected results. It is very difficult to assess the accuracy of the NM5-PCR method because any evaluation must be done with meaningful natural infections and involves comparing with others methods of diagnostic which might themselves be wrong. The gold standard for the clinical diagnosis of malaria is microscopy but often is a challenge to identify *Plasmodium* species, especially when the parasitemia is low, in cases of mixed infections or when *P. knowlesi* is evolved in the infection [7,35]. Moreover, it is a very subjective and laborious method and requires experienced staff. The RDT-based immunochromatographic detection of antigens by monoclonal antibodies is used in addition to microscopy [13], but these methods lack sensitivity and specificity for the identification of *P. knowlesi* [16]. These methods despite being quick, simple and easy to interpret, only identifies specifically *P. falciparum* infection and in some cases *P. vivax*.

The emergence of numerous cases of malaria caused by *P. knowlesi* has led the scientific community to develop new diagnosis methods based on PCR to identify this parasite [4,24,25,28]. Conventional PCR assays, such as seminested-PCR [17], nested-PCR [18,20], and nested multiplex-PCR [23] have shown high sensitivity and specificity compared with RDT or microscopy, as they can detect low levels of parasitemia [19,24,36] but also looks more sensitivity than real time PCR methods [37,38]. In general, multiplex PCR systems face difficulties in designing primers and in the search of the most optimal conditions for a highly sensitive and specific assay. The specificity of the primers for each species is essential in order to get good results in the identification of each species and avoid false negatives and misidentifications [12,22,39,40]. The comparison of the NM5-PCR with the NM-PCR, which was independently tested by other authors with high valour of specificity and sensitivity [39,41,42], and the *knowlesi*-PCR shows maximum concordance suggesting high specificity of the new design primers without cross reactivity between species and high sensitivity.

There was no clear evidence of false positive for NM5-PCR method. All control samples with other infections as Chagas diseases, leishmaniasis and babesiosis give negative results as all not-infected samples, but one negative sample by the reference method (NM-PCR) in the comparison test became positive for *P. falciparum* with the new method. There are some possibilities of cross-contamination of samples during PCR preparation but several measures in the laboratory are always taken to avoid these circumstances.

The new method has a sensitivity and specificity of 100% and 96% respectively compared with NM-PCR and *knowlesi*-PCR methods, with a Cohen's kappa coefficient well above 75% (97.21%) that indicate an excellent correlation and almost a perfect agreement [43]. The nested multiplex PCR, modified from the nested multiplex malaria PCR [22], described here (NM5-PCR) shows to be suitable for detecting the five species of *Plasmodium* that cause malaria in humans including *P. knowlesi*.

Showing high levels of sensitivity and specificity, just need two amplification process, include an internal amplification control and it is relatively inexpensive and fast. Furthermore, this method might be tested in *knowlesi*-malaria endemic areas with a higher number of samples to determinate the real capacity of the method and its suitability in these areas with limited resources to apply other methodologies.

Conflict of interest statement

We declare that we have no conflict of interest.

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