Methods for Detection and Identification of Plasmodium Knowlesi: A Review Article

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ABSTRACT

Background: *Plasmodium knowlesi* is a simian malaria parasite that causes malaria in human also. *P. knowlesi* detected in human population of many South East Asian countries. It causes life threatening malaria complications. Early detection is very crucial for successful clinical management of *knowlesi* malaria.

Aim: To provide insight into the detection methods used for diagnosis of *knowlesi* malaria.

Method: Detailed reviews of the previous studies on malaria caused by *Plasmodium knowlesi* with the particular emphasis on diagnostic methods, especially polymerase chain reaction-based molecular detection methods and loop mediated isothermal amplification technique (LAMP) was performed.

Result: Due to the morphological similarities between *P. knowlesi* and other malaria parasites infecting humans, blood film microscopy is not a reliable method for the detection of *P. knowlesi*. Rapid diagnostic methods based on *P. knowlesi*-specific antibodies have not been available yet. For half a decade, Nested PCR is in use as the standard method for identification of *P. knowlesi*. While requiring sophisticated equipment, real-time PCR provides rapid results with higher specificity and sensitivity, so it used in reference laboratories. Loop-mediated isothermal amplification is a promising method for diagnosis of *knowlesi* malaria in the field. These methods detect small subunit ribosomal RNA as the molecular target. However, recent studies demonstrated the use of multicity, repetitive sequence as the molecular target in single-step PCR detection of *P. knowlesi*.

Conclusion: *P. knowlesi* misguided for *P. malariae* by microscopy, which is responsible for high fatalities due to *P. knowlesi* infections because *P. knowlesi* produces more severe malaria as compared to *P. malariae*. Although molecular diagnostic methods are sensitive and specific for *P. knowlesi*, but these methods are not reliable as compared to gold standard PCR Technique.

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1. Introduction:

Plasmodium knowlesi found in macaques (long-tailed and pig-tailed) monkeys, which is a malarial parasite. Humans infection specifically malaria holds a key focus throughout Southeast Asia and in Malaysia since 2004. *P. knowlesi* is one of the major five species of *Plasmodium* which can cause severe malaria in humans. Identification of different species of *Plasmodium* can be confirmed by molecular detection techniques. More than 100 *Plasmodium species* are infecting rodents, reptiles, birds, primates and other mammals, it has long been recognized that *Plasmodium* parasites are species-specific. Four species of *Plasmodium* (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*) are responsible for causing malaria in humans (Garnham, 1966).

The parasite causes infections in humans and a wide range of animals. Zoonotic malaria–needs global overview

of policy and system research. Malaria among human is prevalent in tropical and sub-tropical regions, including Asia, Africa and South America (World Health Organization, 2014). World Malaria Report 2014 pointed out that recent studies have revealed that *Anopheles latens* of *Leucosphyrus* mosquitos' group is the main vector responsible for transmission of *P. knowlesi* among monkeys as well as from monkeys to humans (Antinori et al., 2013). The parasite that is transmitted to human beings through the bite of an infected female Anopheles mosquito caused malaria that is a life-threatening disease. It is a preventable and curable disease. There were 228 million cases of malaria in 90 countries and death toll reached 405,000 in 2018 **(**World Health Organization, 2020).

Even though natural *P. knowlesi* infection of human was first reported in 1965 in a US army surveyor who was stationed in jungle area of West Malaysia (Chin et al., 1965). *knowlesi* malaria was not considered a public health problem until 2004 when Singh *et al.* discovered a large number of human *knowlesi* infections in Kapit Division of Sarawak, Malaysia (Singh et al., 2004). Since that time, human cases of *knowlesi* malaria have been reported in other countries in South East Asia region including Singapore (Ng et al., 2008), Philippines (Luchavez et al., 2008), Vietnam (Van et al., 2009), Thailand (Putaporntip et al., 2009) and Indonesia (Figtree et al., 2010), and even named the fifth type of human malaria.

2. Diagnostic Techniques:

2.1. Microscopy

In high endemic areas, the gold standard for diagnosis of malaria is microscopic examination of the peripheral blood (William et al., 2011). The species are identified by observing the morphological differences between the species of malaria parasite. The screening of large number samples is not possible only 10-30 parasites per microliter of blood can be detected at a time by expertise. *P. knowlesi*, early blood forms are closely resembled with those of *P. falciparum* and late blood forms are similar with *P. malariae*, making it difficult to distinguish *P. knowlesi* from other human malaria parasites by microscopy. It was demonstrated that 116 of 126 *knowlesi* malaria cases (92%) were misdiagnosed as *P. malariae* infections based on microscopy results (Singh et al., 2004).

2.2. Rapid diagnostic test

Rapid diagnostic tests are useful for diagnosis in field areas because diagnostic results are achieved within very short period of time requiring no laboratory well equipped facilities. Although given the fact that P. knowlesi could infect humans and cause life-threatening infections, care must be taken when using these tests for malaria diagnosis in P. knowlesi-endemic areas (Kawai et al., 2009) Rapid diagnostic test based on P. knowlesi-specific antibody would be a valuable tool in endemic areas where malaria due to P. knowlesi has significant threat (McCutchan et al., 2008).

2.3. Nested PCR assays

For specific detection of *P. knowlesi* Nested PCR assays have been used. A nested PCR assay was developed for the detection and species differentiation of human malaria parasites including *P. knowlesi*. This assay targets the small subunit ribosomal RNA (SSU rRNA) and involves the amplification of DNA in each blood sample with *Plasmodium*-specific primers and subsequent amplification of the resulting amplicon with species-specific primers. In Malaysia *P. knowlesi*-specific primers Pmk8 and Pmkr9 were designed that were based on SSU rRNA gene sequences of *P. knowlesi* isolates. By microscopy, these isolates were misidentified as *P. malariae*, but by sequencing analysis of SSU rRNA gene these were found to be *P. knowlesi* six parasites per microliter of blood is the lower limit of detection for this assay.

A large number of human cases of *knowlesi* malaria are discovered with nested PCR technique, that showed zoonotic transmission of *P. knowlesi* from monkeys to humans. Two new primer sets PkF1060-PkR1550 and PkF1040-PkR1550 were designed to target SSU rRNA of *Plasmodium* parasites (Imwong et al., 2009). These primers were demonstrated to be specific to *P. knowlesi* without producing amplicon with any other parasite species. The nested PCR assays with these primers could detect 1 to 10 parasite genomes.

In 2009, the species identification of the *Plasmodium* parasites causing malaria in Thailand using their own nested PCR assay was carried out. The primers PK18SF and PK18SR were used for detection of *P. knowlesi* (Daneshvar et al., 2009).

2.4. Real-time PCR assays

P. knowlesi can be detected with two real-time PCR assays. Both assays are modified versions of previously developed assay for the detection of malaria parasites that infect humans. These also target the gene coding for the 18S ribosomal RNA subunit of *Plasmodium* species.

The first one involves amplification of the 18S ribosomal RNA with primers PF1 and PF2 and employs two sets of fluorescent resonance energy transfer (FRET) probes, PF3 and PF4 for the detection of genus-specific sequence in the amplicon and PK1 and PK2 for detection of *Plasmodium knowlesi*-specific sequence, which are distinguished by640 nm and 705 nm wavelengths. However, this assay has not been validated using clinical samples (Babady et al., 2009). So, another *P. knowlesi*-specific real-time PCR assay using Taqman hydrolysis probe. This assay consists of two reactions - the screening reaction for detection of *Plasmodium* DNA and the specific reaction for detection of *P. knowlesi*

specific DNA. The same set of primers Plasmo 1 and Plasmo 2 were used for both reactions but different probes were used: *Plasmodium* screening probe Plasprobe and *P. knowlesi*-specific Pk probe the 5' ends were labeled with the fluorophore and 3' ends with the quencher. Plas probe recognizes the conserved DNA sequence within 18S ribosomal RNA genes in all four species of human malaria parasite as well as *P. knowlesi*. The Pk probe was designed to bind to *P. knowlesi*-specific 30 base pair sequence within 18S ribosomal RNA gene (Divis et al., 2010). This real time PCR-assay was found to detect *P. knowlesi* DNA even if the parasitaemia level is as low as 3 parasites per microliter of blood.

2.5. Loop-mediated isothermal amplification (LAMP) assays

There are two LAMP assays for the detection of *P.* knowlesi infection. These assays used the different targets: the first one targeting species-specific β tubulin gene and the second one targeting apical membrane antigen-1 (AMA-1) gene of *P. knowlesi* (Iseki et al., 2010).

Both assays use six sets of primers that bind to eight regions of the target sequence. In both assays, the amplicon was detected by assessment of turbidity, the ladder pattern of amplified products on gel electrophoresis or direct visualization of amplified products in the presence of fluorescent nucleic acid stain. Both assays can detect *P. knowlesi* whole blood even if parasitaemia level is 0.01% (Lau et al., 2011). The advantage of these assays is that detection of *knowlesi* malaria was accomplished under isothermal condition within one hour without requiring expensive thermal cyclers.

2.5. Single-step PCR assay

Although ribosomal RNA gene is the target in PCR-based detection of *Plasmodium* species infecting humans including *P. knowlesi*, this target occur in only 4 - 8 copies per genome. As the low copy number of the target limits the sensitivity of the PCR assay, the bioinformatics analysis of whole genome sequence of *P. falciprum* and *P. vivax* was performed to identify the new multicopy target DNA sequences for PCR based detection of malaria parasites. As the result, one putative diagnostic high-copy number target was identified in each species: Pfr364 occurring in 41 copies in *P. falciparum* and Pvr47 occurring in 14 copies in *P. vivax* (Demas et al., 2011).

Fourteen sets of primers were designed to amplify these target sequences from *P. knowlesi* genome. Only three sets of primers, namely Pkr140-3, Pkr140-4 and Pkr140-5, produce the amplicon and all of these primers recognize the Pkr140 repeat sequence which occurs in 7 copies in *P. knowlesi* genome. However, two of these three primers set Pkr140-3, Pkr140-4 are not specific for *P. knowlesi* and yielded non-specific amplicon when tested with simian malaria parasites (Lucchi et al., 2012). The remaining primer set Pkr150-5 is 100% specific for *P. knowlesi* and does not show cross-reactivity with non *knowlesi* human and simian malaria parasites. Furthermore, non-nested PCR assay using this new primer set could detect the parasitaemia level as low as one parasite per microliter of blood, indicating that the limit of detection for this assay is lower than reported for previous nested PCR assay.

In contrast to the previously identified repeat sequence target in *P. falciparum* and *P. vivax* that are confined to the chromosome end, Pkr140 repeat sequence targeted by this new primer set is distributed throughout the entire length of chromosomes. The chromosome ends are rich in multicopy, species-specific genes and are therefore the good place to search for new diagnostic targets.

3. Conclusions

Malaria caused by plasmodium *Knowlesi* is a life-threatening disease and early diagnosis of this disease is essential. Although conventional microscopy is the routine method used for diagnosis of malaria, it has many limitations specially, it is not reliable for the diagnosis of *knowlesi* malaria because of the morphological similarity of *P. knowlesi* with other malaria parasites infecting humans. PCR-based detection methods targeting specific rRNA genes and multicopy repeat sequences are more sensitive, specific and reliable than the conventional microscopy for diagnosis of malaria. It is impractical to use these methods in the field for application and is limited to reference laboratories because these assays require sophisticated equipment and trained personnel. In contrast, detection methods based on LAMP are simple and easy to perform and would be useful in places where expensive thermal cyclers are not available Rapid diagnostic test specific for *P. knowlesi* is still lacking but such test would be of great use for the diagnosis of *knowlesi* malaria in remote areas, the area most likely to be affected by *knowlesi* malaria.

4. Recommendations

Based on detailed reviews of the deaths due to malaria it is reported that deaths resulting from *knowlesi* malaria were misidentified among *P. knowlesi* and *P. malariae* on blood film examination by microscopy as both of these organisms have close resemblance. Patients diagnosed as having *P. malariae* infections are treated with oral antimalarial therapy and less likely to receive parenteral antimalarial therapy because *P. malariae* infection have relatively benign course (Rajahram et al., 2012). This report highlights the importance of molecular diagnostic methods in the diagnosis of *P.*

knowlesi infections and the need to develop molecular diagnostic tests that can be applied in the field. The health care providers should be educated that if severe illnesses in non-falciparum malaria especially in *P. knowlesi* endemic areas they must initiate parenteral antimalarial therapy as early as possible in suspected cases of *knowlesi* infections. A high index of suspicion, and use of molecular diagnostic methods, fatalities due to *P. knowlesi* infections, can be prevented.

5. List of abbreviations

PCR: Polymerase chain reaction; SSU rRNA: Small subunit ribosomal RNA; FRET: Fluorescent energy transfer; LAMP: Loop-mediated isothermal amplification; AMA-1: Apical membrane antigen-1.

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7. Conflict of Interest

The authors have no conflict of interest to declare.

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