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Review

Diagnostic methods for *Plasmodium knowlesi*: performance, limitations and recommendations

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Abstract: Malaria is a life-threatening disease which causes an economic and public health burden in endemic regions. *Plasmodium knowlesi* is a zoonotic malaria caused by the bite of *P. knowlesi*-infected female *Anopheles* mosquitoes. The disease poses risks of complications owing to the short replication cycle of the parasite. Sensitive and specific diagnostic methods are needed to detect the presence of *P. knowlesi* infection in knowlesi-endemic regions. This paper reviews and compares the performance of various diagnostic techniques as well as highlights the limitations and challenges of the current diagnostic tools for *P. knowlesi*. Recommendations for further improvement of the *P. knowlesi* diagnostic tools are also included.

Keywords: Malaria, *Plasmodium knowlesi*, Point-of-care diagnosis, Loop-mediated isothermal amplification, CRISPR

Introduction

Malaria is a severe public health disease that affects 85 malaria endemic countries. According to the World Malaria Report 2021, the estimated number of malaria cases increased from 227 million in 2019 to 241 million in 2020. Malaysia reported zero indigenous human malaria cases for three consecutive years in 2020. However, a significant number of zoonotic malaria cases have been reported in the country with 2607 knowlesi malaria cases reported in 2020 [1]. *Plasmodium knowlesi* is the predominant aetiological agent for zoonotic malaria in Malaysia. The natural hosts of the parasite are long-tailed macaques (*Macaca fascicularis*), pig-tailed macaques (*Macaca nemestrina*), and banded-leaf monkey (*Presbytis melalophos)* [2-4]. *P. knowlesi* cross-species transmission occurs when a female anopheline mosquito infected with *P. knowlesi* injects the sporozoites into the human skin during a blood meal, whereby the sporozoites invade the host's circulatory system subsequently.

P. knowlesi replicates rapidly every 24 hours. The infection can become severe if left untreated [5]. Knowlesi malaria symptoms are often nonspecific with fever and chills seen in the majority of patients. Headache, rigors, malaise, anorexia, myalgia, cough, and other symptoms typical in falciparum and vivax malaria patients were also reported in *P. knowlesi* patients [6,7]. Complications of knowlesi malaria can result in respiratory distress, hyperparasitemia, jaundice, renal failure, hypotension, hypoglycemia, or multiorgan failure. Five cases of deaths due to knowlesi infection were reported in 2020 [1,7,8]. A systematic review on fatal knowlesi cases by Rajahram et al. (2019) [8] shows that 90% of fatal cases was due to *P. knowlesi* being misdiagnosed as other malaria species

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as well as the poor sensitivity and specificity of the rapid diagnostic tests in detecting the *Plasmodium* species. Therefore, a prompt and accurate diagnosis of *P. knowlesi* infection is required for immediate disease management and *P. knowlesi* surveillance. This paper seeks to evaluate the current diagnostic approaches for knowlesi as well as the limitations of these diagnostic methods.

Microscopic examination

Microscopic examination is the gold standard for malaria diagnosis. The method identifies the *Plasmodium* species based on the morphological characteristics of the parasite and parasite density which are crucial in the clinical management of malaria patients [9]. The most used stain for the preparation of blood films for the malaria parasite (BFMP) is Giemsa stain which is composed of eosin that stains the parasite nucleus red and methylene blue that stains the cytoplasm blue [10]. The method, however, requires highly skilled personnel to achieve a sensitivity of 50-500 parasites/µL and is often time-consuming [11,12].

The detailed microscopic examination shows that the early trophozoite stage of *P. knowlesi* has similar characteristics to the *Plasmodium falciparum* early trophozoite stage, such as double chromatin dots, multiply infected erythrocytes, and appliqué forms. The late stages of *P. knowlesi* have morphological characteristics that are almost identical to *Plasmodium malariae* in which 'band form' trophozoites, a distinct feature of *P. malariae*, have been observed in *P. knowlesi* [13]. Due to the indistinguishable characteristics between *P. knowlesi* and *P. falciparum* or *P. malariae*, microscopic examination of BFMP by microscopists who are unfamiliar with *P. knowlesi* may result in the misidentification of the parasite species.

A study by Mahittikorn et al. (2021) [14] shows that the misidentification rate of *P. knowlesi* as *P. malariae* by microscopic examination was approximately 57%. The highest rate of misidentification was observed in Sarawak, Malaysia through the subgroup analysis. This is likely because knowlesi malaria was not well-documented and known back in 2004 when many *P. knowlesi* cases were misdiagnosed as *P. malariae* in the Kapit Division of Sarawak, Malaysia [15]. In addition, misdiagnoses of *P. knowlesi* as *Plasmodium vivax* or vice versa were reported [16]. The former could result in an additional cost of treatment as primaquine is prescribed to remove hypnozoites in *P. vivax* infection while the latter could result in a lack of administration of primaquine. Therefore, molecular diagnostic methods are often required to confirm the infection.

Besides, there was evidence of knowlesi malaria which could not be detected by conventional microscopic examination due to the low parasite density in the clinical samples. Although sub-microscopic malaria rarely causes severe clinical manifestations of the infection, it must not be taken lightly as it is known to contribute to the transmission of the infection [17,18]. The same goes for asymptomatic knowlesi infection which was shown to be present at a substantial number within communities located in *P. knowlesi*-endemic areas [19]. This urges the use of the molecular identification method which has a higher sensitivity for the detection of submicroscopic infection.

Rapid Diagnostic Test (RDT)

RDT acts as an alternative for diagnosing malaria. Thus far, it plays an important role in the point-of-care diagnosis of knowlesi infection as the disease is more commonly reported in rural settings where accessibility to diagnostic facilities may be difficult. Furthermore, the test is easy to use, affordable, does not require trained personnel, and has a turn-around time of 15-30 min. The assay is based on the detection of protein produced by malaria parasites in *Plasmodium*-infected blood samples [20]. It is known that low parasite density influences the sensitivity of RDT and that RDT performance declines below 100 parasites/µL[12].

Lots of research has been conducted to evaluate the performance of RDTs in the detection of *P. knowlesi*. van Hellemond et al. (2009) [21] showed that the BinaxNOW malaria test (Binax, Inc., USA) could detect *P. knowlesi* at the pan-malaria test line that targets *Plasmodium* aldolase antigen. In the same study, positive reactions were observed at the pan-malaria LDH and *P. falciparum* LDH test lines of OptiMAL Rapid Malaria test (Bio-Rad Laboratories, USA), suggesting the cross-reactivity of *P. falciparum* LDH monoclonal antibody with *P. knowlesi* LDH. Comparison of the two RDTs suggested that the BinaxNOW malaria test could not detect *P. knowlesi* at the pan-malaria test line when samples with a low *P. knowlesi* parasitemia level (parasite density 1587/µL) were used [21]. This was further supported by the findings from Bronner et al. (2009) [22] and Link et al. (2012) [23] where the pan-malaria test line of BinaxNOW malaria showed negative results for *P. knowlesi* samples at 0.1% and 0.0005%, respectively. An unusual result was reported by Ong et al. (2009) [24] in which the BinaxNOW malaria test had both *P. falciparum* HRP2 and pan-malaria test lines positive for a PCR-diagnosed *P. knowlesi* sample. Nonetheless, the OptiMAL Rapid Malaria test from the study showed consistent results with van Hellemond et al. (2009) [21] in which the antibodies at the *P. falciparum* LDH test line cross-reacted with the *P. knowlesi* sample. Another study which evaluated Paramax-3 test reported cross-reactivity of *P. knowlesi* antigen with *P. vivax* LDH-detecting antibody at a low sensitivity (40%) [25].

Overall, cross-reactivity of *P. knowlesi* samples with *P. falciparum* and *P. vivax* LDH antibodies with a low sensitivity (< 75%) was observed. In addition, RDTs that

target *Plasmodium* aldolase antigen at the pan-malaria test line showed a poorer sensitivity for *P. knowlesi* samples (< 30%) [25,26]. A meta-analysis based only on a positive pan-malaria test line showed that the sensitivity of RDTs in detecting *P. knowlesi* ranged from 2% to 48% [27]. These findings highlight the necessity to develop an RDT that can detect *P. knowlesi* with high sensitivity and specificity for clinical management and epidemiological surveillance in resource-constrained settings where microscopy is not available.

Studies have been carried out to identify other potential biomarkers as the target antigen in malaria RDT. Two proteins which are involved in the glycolytic pathway of the parasite's life cycle have been evaluated for their use as target biomarkers in RDT which are glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoethanolamine-N-methyltransferase (PMT). An epitope specific for *P. knowlesi* GAPDH was identified using amino acid sequence alignment. However, the identified epitope has an identity of 79% with *P. vivax* orthologues. A further antibody detection approach is required to evaluate the use of this epitope as the target for *P. knowlesi*-specific detection [28]. An analysis of *Plasmodium* PMT proteins showed that the protein shared low percentage identities among the *Plasmodium* PMT orthologues. The concentration of PMT proteins was found to be similar to or slightly higher than that of pLDH proteins. Thus, it is a promising target antigen for malaria RDT [29].

Nested PCR assays

Nested PCR assays overcome the limitations of microscopic examination and RDTs for malaria diagnosis at the low parasitemia level. The method involves *Plasmodium*-specific primers for the first amplification and species-specific primers for the second amplification. Snounou et al. (1993) developed a nested PCR which targets the 18S ribosomal RNA (ssrRNA) gene that is present in high copy numbers and specific to *Plasmodium* parasites [30,31]. The nested PCR attained a high detection sensitivity for *P. falciparum*, *P. vivax*, *P. malariae*, and *Plasmodium ovale* [32]. In 2004, Singh et al. (2004) designed a primer pair (Pmk8 and Pmkr9) that targets the S-type ssrRNA gene (ssrRNA-S) which is expressed during the sexual stages for the detection of *P. knowlesi* in nested PCR. Many of the microscopically identified *P. malariae* samples were confirmed as *P. knowlesi* using the nested PCR assay [15]. Nevertheless, a further study by Imwong et al. (2009) [33] discovered that the Pmk8 and Pmkr9 primer pair could cross-react with *P. vivax*. The group then designed a new primer pair, PkF1140-PkR1550 primers, which amplifies a fragment of the *P. knowlesi* A-type ssrRNA (ssrRNA-A) gene which is expressed during the asexual stages. After primary amplification using rPLU1 and rPLU5 primers which are *Plasmodium* genus-specific, secondary reaction with PkF1140-PkR1550 primers shows specific detection of *P. knowlesi* and does not cross-react with other *Plasmodium* species [33,34]. Furthermore, the nested PCR assay using PkF1140-PkR1550 has a sensitivity of 1 to 10 parasite genomes per sample.

Despite its high sensitivity and specificity, the nested PCR is more expensive and time-consuming due to the need for expensive equipment and tedious PCR reaction preparation from DNA extraction to visualization of PCR result under a UV illuminator. Furthermore, the method is prone to cross-contamination due to the high sensitivity of the method and preparation of numerous reactions for species identification. To overcome this, nested multiplex PCR using newly designed primer pairs was developed by Miguel-Oteo et al. (2017) [35] to shorten the assay's turn-around time and reduce potential cross-contamination. The study shows that the technique has 100% sensitivity and 96% specificity for the detection of all five human malaria species. More studies are required to validate the reliability of this nested multiplex PCR as only a few samples were tested in the study (< 20 samples for each human *Plasmodium* species including *P. knowlesi*).

A study by Komaki-Yasuda (2018) [36] which aimed to reduce the nested PCR reaction time showed that the use of the "fast PCR enzyme" could reduce the reaction time to only 65 min for both the first and the second PCRs. DNA was extracted from diluted *P. falciparum* culture for analytical sensitivity testing of the assay, and it was shown that the assay was able to detect as low as 0.1 parasite/μL. Therefore, it was believed that this nested PCR system could detect submicroscopic *Plasmodium* samples. No analytical sensitivity for P. knowlesi detection was presented in the study. Nevertheless, the nested PCR system had a sensitivity and specificity of 100% when tested with 36 clinical samples. A larger number of samples should be evaluated to verify the performance of the nested PCR system using the "fast PCR enzyme".

Real-time PCR

Real-time PCR has several advantages over nested PCR, including a lower chance of DNA contamination due to closed-tube reactions, automated processing, and high throughput. The method is also able to detect the presence of *Plasmodium* parasites at low parasitemia levels as well as to determine the DNA copy number of the *Plasmodium* infection [37]. In a study by Babady et al. (2009) [38], a new set of *P. knowlesi*-specific fluorescence resonance energy transfer (FRET) hybridization probes (PK1 and PK2) were designed to distinguish *P. knowlesi* from *P. vivax* parasite in a real-time PCR assay. The newly designed *P. knowlesi* probes were labeled with fluorescein (PK1) and red-705 fluorophore (PK2) which allows detection of *P. knowlesi* at 705 nm when the specific hybridization event occurs. The assay showed an analytical sensitivity of 10 copies/μL *P. knowlesi* positive control plasmid and 100% specificity.

However, despite having a high analytical sensitivity, the use of another set of probes increases the reaction cost, and no *P. knowlesi* human clinical samples were tested in this study.

Another FRET-based quantitative real-time PCR (FRET-qPCR) assay was developed and the performance of the assay was evaluated using 56 reference samples from the United Kingdom National External Quality Assessment Services (UK NEQAS) for the detection of the *Plasmodium* species [39]. The assay targets the ssrRNA gene and produces amplicons of 157-165 base pairs. A primer pair (Plasmo 1 and Plasmo 2) and two target-specific hybridization probes (MalFL and MalLC640) were used to allow FRET-based detection of the amplicons. The results showed that the FRET-qPCR assay had a sensitivity of 100% which was higher than that of nested PCR (sensitivity = 96.43%) when compared to the report from UK NEQAS. Despite having a high sensitivity, the FRET-qPCR could not differentiate *P. knowlesi* from *P. vivax* because of identical melting temperature (Tm) values in the melting curve analysis. No reaction was observed in the assay when tested with UK NEQAS negative samples and DNA samples from *Leishmania*, *Babesia*, *Pneumocystis*, and *Toxoplasma*. Besides, the assay had a limit of detection (LOD) of 199.97 parasites/mL blood for *P. falciparum* but the LOD of *P. knowlesi* was not available from the study.

Divis et al. (2010) [40] adapted a TaqMan hydrolysis probe-based real-time PCR assay from Rougemont et al. (2004) [41] and designed a Pk probe for *P. knowlesi* detection. Validation of this real-time PCR assay showed an analytical sensitivity of 10 copies/µL *P. knowlesi* positive control plasmid which corresponds to approximately 1-2 parasite genomes. In a study by Calderaro et al. (2013) [42], the TaqMan probe-based real-time PCR obtained a LOD of 10 copies/µL synthetic DNA oligonucleotide of *P. knowlesi* ssrRNA gene, further validating that the assay has an excellent analytical sensitivity. When tested with clinical samples, the TaqMan real-time PCR assay was able to detect *P. knowlesi* infection at a parasitemia of 3 parasites/µL [40]. No cross-reactivity was observed in the TaqMan real-time PCR assay [40,42]. The TaqMan probe was also used in a qPCR-high resolution melting (qPCR-HRM) assay developed by Lamien-Meda et al. (2021) [43] which targeted a *Plasmodium* conserved mitochondrial DNA gene, *cox1* gene, for detection of all human *Plasmodium* species including *P. knowlesi*. The assay had a LOD of 42.47 copies/µL *P. knowlesi* positive control plasmid, whereas the specificity of the assay was 98.2% when compared to the results of the nested PCR.

SYBR green is also used as a reporter in real-time PCR assay. SYBR green binds to double-stranded DNA amplicons via intercalation between adjacent base pairs, emitting fluorescent signals following light excitation. Species-specific primer pairs that can bind to specific target sequences are therefore essential to avoiding non-specific amplification [44]. Oddoux et al. (2011) [45] designed PKe'F and PKg'R primers targeting the ssrRNA-A gene for specific amplification of *P. knowlesi* in SYBR green real-time PCR adapted from de Monbrison et al. (2003) [37]. The analytical sensitivity of the SYBR green real-time PCR was found to be 100 copies/µL *P. knowlesi* positive control plasmid with 100% specificity.

A high-throughput multiplex 5' nuclease quantitative PCR (qPCR) that can detect all human *Plasmodium* species including *P. knowlesi* was developed by Reller et al. (2013) [46]. The assay had a detection limit of 1-6 parasites/µL. The developed 5-plex qPCR showed a significant correlation between molecular parasitemia and microscopic parasitemia for *P. falciparum* samples but that was not significant for *P. vivax* infection. The study did not present correlation data between microscopic and molecular parasitemia levels for *P. knowlesi* infection. Nonetheless, the 5-plex assay showed 100% sensitivity and specificity for *P. knowlesi* detection when *plasmepsin* was selected as the target gene for the assay.

A retrospective study of two multiplex real-time PCR kits, RealStar Malaria S&T Kit 1.0 (altona Diagnostics) and "FTD Malaria differentiation" real-time multiplex PCR kit (Fast Track Diagnostics (FTD), Sliema, Malta) showed both the kits had similar performance characteristics, with 98.9% concordance between the assays. The RealStar Malaria S&T Kit 1.0 is species-specific to all four human *Plasmodium* species and *P. knowlesi*; however, the FTD assay is specific to all four human *Plasmodium* species only. The sensitivity of RealStar PCR and FTD assays were 95.1% and 96.8%, respectively, when compared to microscopic examination and genus-specific PCR results. Other performance parameters such as specificity, positive and negative predictive values, were not calculated in the study [47].

RealStar Malaria PCR (RealStar-genus) and RealStar Malaria Screen & Type PCR (RealStar-species) which are genus- and species-specific, respectively, were also evaluated by Ramírez et al. (2021) [48]. Out of 121 samples tested, RealStar-genus had a sensitivity of 98.9% with 1 *P. ovale* sample giving discordant results from the nested PCR method. RealStar-species assay, however, had a lower sensitivity of 97.8%. The LOD of RealStar-genus assay was calculated using two *P. falciparum* positive samples and showed a mean LOD value of 0.28 parasite/µL. Meanwhile, a similar approach was used to test the LOD of RealStar-species assay using two samples in duplicate of each *Plasmodium* species. It was shown that the RealStar-species assay had mean LODs of 0.42 parasite/µL, 1.60 parasites/µL, 0.18 parasite/µL, 0.34 parasite/µL and 0.25 parasite/µL for *P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae* and *P. knowlesi*, respectively.

Although real-time PCR assay is more costly, this detection method has been implemented for routine confirmation of malaria cases at the State Public Health Laboratory (Makmal Kesihatan Awam; MKA) in Sabah. A study by Nuin et al. (2020) [49] evaluated the performance of two commercial real-time PCR assays, QuantiFast™ Multiplex PCR kit (QIAGEN, Germany) and abTES™

Malaria 5 qPCR II Kit (AITbiotech, Singapore) for use in routine molecular diagnosis and surveillance of *P. knowlesi* and other human malaria infections in Sabah. It was found that the LOD of abTES™ real-time PCR assay was 0.125 parasite/µL which was lower than that of QuantiFast™ assay (LOD = 20 parasites/µL) for *P. knowlesi*. Evaluation of the assays showed that the sensitivities of QuantiFast™ and abTES™ were 98.1% and 100% for *P. knowlesi* clinical samples, respectively. Both assays showed a similar specificity which was 98.8%. Based on the study, the use of QuantiFast™ assay as the confirmatory detection method was favored whereas the abTES™ assay was employed as a second-line referral diagnostic tool for negative or mixed *Plasmodium* infections because abTES™ is substantially more expensive than QuantiFast™.

In 2021, van Bergen et al. [50] presented a study on the analytical performance of MC004 real-time PCR assay (MRC Holland, Amsterdam, Netherlands) for malaria diagnosis. MC004 is a single-tube multiplex assay which can detect all human *Plasmodium* species including *P. knowlesi* and *Plasmodium* cynomolgi. It detects mitochondrial *Plasmodium* DNA encompassing the cyclo-oxygenase 3 (COX-3), cyclo-oxygenase 1 (COX-1) and cytochrome b (CYTB) genes from *Plasmodium* species. Three different probes were used in the assay which were Texas red-labelled, Cy5-labelled, and Cy5.5-labelled. The study claimed that the occurrence of false-positive results was unlikely as three different probes were used in the assay, resulting in different melting curves at specific temperature. However, a discrepant result was observed for a *P. vivax* sample which was detected as *P. knowlesi* by MC004 assay and confirmed as *P. knowlesi* by Sanger sequencing. In this study, the LOD of the assay for each *Plasmodium* species was displayed as the highest dilution of the sample at which the *Plasmodium* species was consistently detected as the study found that the LOD depended on the starting concentration of the sample. It was shown that the LODs of the assay for *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi*, and *P. knowlesi* ATCC 30158 were comparable, which were 10^{-4} to 10^{-7} dilution from parasitemias of < 0.1% to 0.3%. Particularly, the LOD of the assay for *P. knowlesi* ATCC 30158 was 10^{-4} dilution from a sample with a parasitemia of 0.3%.

In summary, real-time PCR provides high sensitivity and excellent specificity for *P. knowlesi* detection as well as for other human *Plasmodium* species. The method can be used to identify different *Plasmodium* species and determine the parasite density of the infection which is important for adequate treatment and disease management. The method is also less time-consuming with a turn-around time of approximately 2-4 hours when compared to the nested PCR assay which has a turn-around time of 6 hours [43,48].

Other PCR-based assays

A semi-nested PCR (Sn-PCR) was described by Van Hong et al. (2013) [51] for the detection of *P. knowlesi*. The Sn-PCR targets ssrRNA genes in which the first reaction is *Plasmodium* genus-specific, followed by a Sn-PCR using a *Plasmodium*-specific forward primer (PLF) and a *P. knowlesi* species-specific reverse primer (PKR4). The study compared the performance of Sn-PCR with other existing nested PCR protocols: one using Pmk8-Pmkr9 primers (hereafter called PCR1) [15] and PkF1140-PkR1550 primers (hereafter called PCR2) [33]. The analytical sensitivity of the Sn-PCR was 100 fg/µL *P. knowlesi* positive control plasmid which was the same as PCR2 but lower than that of PCR1 which had a detection limit of 1 fg/µL. All the *P. knowlesi* reference samples (N = 13) tested were amplified successfully by the Sn-PCR. When tested with genomic DNA of non-*Plasmodium* origin and other non-*P. knowlesi Plasmodium* species, the Sn-PCR showed no cross-reactivity while PCR1 showed a false positive *P. knowlesi* for one *P. vivax* reference sample. The specificity of Sn-PCR was 100% when tested with 80 non-*P. knowlesi* malaria clinical samples. This Sn-PCR protocol would be advantageous if added to semi-nested multiplex PCR adapted by Rubio et al. (2002) [52] as it would become less costly compared to the existing nested PCR.

Besides targeting the *Plasmodium* ssrRNA gene, there was also Sn-PCR assay which was designed to target *P. knowlesi*-specific schizont-infected cell agglutination variant antigens (SICAvars), with more than 100 copies and randomly distributed across *P. knowlesi* chromosomes [53]. The LOD of the SICAvar PCR assay was determined to be 0.1 parasite/µL blood when tested with serially diluted *P. knowlesi* culture. In addition, the assay provided a higher sensitivity for *P. knowlesi* identification when compared to the ssrRNA nested PCR assay and was shown to be effective in detecting submicroscopic parasites. The assay was specific for *P. knowlesi* detection as no band was generated for other *Plasmodium* species.

On the other hand, droplet digital PCR provides direct quantification of target DNA without the need of generating standard curve [54]. Previously, this technique had been used for detecting four human *Plasmodium* species by targeting the 18S rRNA genes and the detection limit was 11 parasites/mL. Two years ago, Mahendran et al. (2020) [55] developed a duplex ddPCR for the detection of *P. knowlesi* and *P. vivax*. It was shown that the ddPCR assay had an analytical sensitivity of 10 copies/µL and 0.01 copy/µL for the constructed *P. vivax AMA-1* and *P. knowlesi plasmepsin* plasmids, respectively. This outperformed the qPCR assay which has an analytical sensitivity of 100 copies/µL and 10 copies/µL for the constructed *P. vivax AMA-1* and *P. knowlesi plasmepsin* plasmids, respectively. However, the concordance rate of ddPCR and nested PCR assay was only 69.3%. Clinical sensitivity and specificity of this assay for the detection of *P. knowlesi* were 90% and 81.08%, respectively, when compared to nested PCR. This indicates that the ddPCR assay can correctly identify

those who have malaria; however, low clinical specificity of the assay can lead to unnecessary treatments of the disease as false positive results were reported. Therefore, further optimization of the assay is required to support the use of ddPCR for *P. knowlesi* diagnosis.

Loop-mediated isothermal amplification (LAMP)

LAMP has been widely used for the diagnosis of several infectious diseases due to its ability to amplify nucleic acid with high sensitivity and specificity under isothermal conditions. The technique utilizes a set of four to six primers that bind to six to eight regions of the target gene, thus making the method highly specific [56]. Results of this assay are based on turbidity, gel electrophoresis, fluorescence or colorimetric detection [57,58]. A LAMP assay (*Pk*LAMP) was designed for *P. knowlesi* detection using six specific primers: a forward inner primer (FIP), a backward inner primer (BIP), two loop primers (Loop F and Loop B) and two outer primers (F3 and B3) which recognize eight regions on *P. knowlesi* β-tubulin gene sequence [59]. The results showed that *Pk*LAMP had a better sensitivity than single-PCR assay using the F3 and B3 primers by 100-fold. No cross-reactivity was observed from the assay. However, the sensitivity of *Pk*LAMP assay could only detect 10^2 copies/µL of the DNA template, highlighting the need for a more sensitive LAMP assay. Subsequently, LAMP assays designed by Lau et al. in 2011 and 2016 [60,61] which targeted the *apical membrane antigen-1 (AMA-1)* and *ssrRNA* gene, respectively, showed a lower LOD which was 10 copies of plasmid DNA for *P. knowlesi* specific detection. Superior to that, a high-throughput LAMP assay (HtLAMP) designed by Britton et al. (2016) [62] could detect *P. knowlesi* at 0.2 parasite/µL. The observed high sensitivity of HtLAMP assay could be due to the high copy number of the target mitochondrial gene. This shows the importance of target gene selection during the primer design.

In a study by Mallepaddi et al. (2018) [63], a LAMP-lateral flow device (LAMP-LFD) was developed for the detection of malaria including *P. knowlesi*. For this purpose, the loop primers of *Plasmodium genus*, *P. falciparum*, *P. vivax*, and *P. knowlesi* were specifically designed to consist of fluorescein-5,6-isothiocyanate labeled at the 5'-end of the Loop F primer and biotin labeled at the 5'-end of the Loop B primer. The presence of *Plasmodium* genomic DNA, regardless of species, will result in two colour lines on the test device as the amplified products will be captured by the antibodies on the test line and the presence of control line validates the results. It was found that the LAMP-LFD had a detection limit of 0.01 pg/µL DNA template for all five species whereas the sensitivity and specificity of the assay were 99% and 97.1%, respectively.

In general, the LAMP technique possesses great potential to be used as a point-of-care diagnostic tool as the method is highly sensitive and specific. However, it could also result in false positive results after parasites' clearance from the patient's bloodstream (Otten et al., 2009) [64]. Another major drawback of the assay is the risk of cross- and carry-over contamination of the assay owing to the highly sensitive nature of the assay. As such, precautions must be taken to avoid contamination. This includes establishing designated areas for various processes involved in the LAMP assay and the use of different sets of pipettes.

Recombinase polymerase amplification (RPA) assay

RPA assay is another novel diagnostic approach that uses isothermal amplification. Primers are designed to be able to bind to the DNA template with the guide of a recombinase that initiates strand-displacement synthesis. The method can amplify DNA targets in less than 30 min at 37 °C in a shaking incubator or heat block which is less expensive than a thermocycling-based assay. An established RPA assay for *P. falciparum* detection showed 100% specificity with a sensitivity of approximately 4-20 parasites per reaction [65]. In comparison to that, a *P. knowlesi* RPA assay developed by Lai et al. (2017) [66] showed a higher sensitivity in which it could detect one copy number of the plasmid after 20-23 min of incubation at 37 °C. Both sensitivity and specificity of the *P. knowlesi* RPA assay were 100%. Like LAMP, the results of the RPA assay can be visually analyzed with the incorporation of a colorimetric dye such as SYBR Green I as shown by Lai & Lau (2020) [67]. The change of dye colour from light orange to green indicates the presence of the amplified DNA amplicons. This can be advantageous in resource-limited settings owing to the simplicity and short turn-around time of the RPA assay. The LOD of the RPA assay combined with SYBR Green I had a LOD of one parasite/µL blood. Both sensitivity and specificity of the RPA assay were 100%.

Another detection approach that can be incorporated with RPA assay is employing the lateral-flow dipstick technology. The method employs antibodies that are immobilized on the strips and detects analytes containing antigenic labels [68]. In the case where HybriDetect-2 lateral flow strip (Milenia Biotec, Giessen, Germany) was used, Lai et al. (2018) [69] designed an RPA assay which included *P. knowlesi* specific detection and pan-*Plasmodium* detection. The LF probe used for both *Plasmodium* genus- and *P. knowlesi*-specific genes consists of an oligonucleotide backbone with a 5'-carboxyfluorescein (5'-FAM), a tetrahydrofuran (THF) residue, and a C3-spacer as the polymerase extension blocking group at the 3' end. Conventional primer and LF probe were used with a 5'-digoxigenin-tagged reverse

primer for *Plasmodium*-genus detection and a biotin-labelled reverse primer for *P. knowlesi*-specific detection. When amplicon analytes migrate along the strips, the labelled amplicons will be captured by the antibodies immobilized on the strips and the anti-FITC conjugated gold nanoparticles will react with the LF probe producing colour signals on the test and control lines. The study showed that the detection limit of the LF-RPA assay was 10 parasites/μL which is compatible with that of the nested PCR [33]. Hence, LF-RPA assay can be a potential point-of-care diagnosis tool for *P. knowlesi*. However, further validation of the assay's performance is needed.

Further recommendations

A variety of techniques for *P. knowlesi* diagnosis have been developed. However, most of the techniques still need to be validated before being implemented as a point-of-care tool for *P. knowlesi* infection. Such scenarios are seen in RPA and LAMP assays which show the potential for use in resource-limited settings. The LAMP-LFD developed by Mallepaddi et al. (2018) [63] can be improved by increasing the number of test lines for species-specific detection of *P. falciparum*, *P. vivax* and *P. knowlesi* as these species are known to cause complications and delayed or inaccurate treatment can lead to death [70-72]. In addition, multiplex LAMP reaction would be beneficial when incorporated with this lateral flow strip as it reduces the cost of reaction and eases the signal readout. However, the multiplex LF-LAMP assay requires careful design and optimization as multiple components are involved. Furthermore, these assays require extra precautions to prevent contamination particularly when a sterile environment is not accessible. This can be overcome by the addition of mineral oil to the LAMP assay and substitution of deoxythymidine triphosphate (dTTP) with deoxyuridine triphosphate (dUTP) as proven by Zen et al. (2020) [73]. This can significantly improve the robustness of the assay for use as a point-of-care diagnosis.

The CRISPR-based diagnostic method, which is also known as SHERLOCK, is an emerging diagnostic tool that comprises RPA, in vitro transcription, and RNA target detection using CRISPR RNA (crRNA) oligonucleotides and Cas13a/Cas12a. The assay was described in Lee et al. (2020) [74] and Cunningham et al. (2021) [75] for the detection of all *Plasmodium* infections and specific detection of *P. falciparum* and *P. vivax*. It was found that the analytical sensitivity of the CRISPR-based assay designed by Lee et al. had a lower detection limit of 0.36 and 1.2 parasites/μL when compared to 6.8 and 18.8 parasites/μL for *P. falciparum* and *P. vivax* specific detection, respectively. This could be explained by the choice of target genes which were subtelomeric repeat DNA (Pfr364) for *P. falciparum* detection and mitochondrial DNA for *P. vivax* detection which have a copy number of 41 and 20 copies, respectively. The target gene selected by Cunningham et al. was 18S rRNA gene which is present in a lower copy number in the *Plasmodium* genome. Hence, future CRISPR-based assay designed for *P. knowlesi* specific detection should target genes that have high copy numbers to achieve a high sensitivity. Moreover, additional optimization of the SHERLOCK assay is required to make it deployable in field settings.

Aptamer-based assay can be another method of interest for *Plasmodium* diagnosis. Aptamers are short, single-stranded oligonucleotides that bind to target molecules with high binding affinity and specificity. The synthesis of the molecules is less costly when compared to the conventional antibodies. Aptamers can be generated in vitro using systemic evolution of ligands by exponential enrichment of a vast pool of candidate sequences. The generated bio-recognition molecules have several advantages over antibody-based assay. Examples include its ease of synthesis, high stability, and ease of manipulation [76,77]. In a study by Cheung et al. (2018) [78], an aptamer-tethered enzyme capture (APTEC) assay was able to distinguish *P. falciparum* infection from *P. vivax* with the use of 2008s aptamer that reacted specifically to recombinant *P. falciparum* LDH protein and *P. falciparum* patient samples. Besides, Frith et al. (2018) [79] showed that the aptamers generated against species-specific epitope of *P. falciparum* LDH protein (LDHp 11) demonstrated greater binding specificity than the aptamers generated against the whole recombinant protein. Thus, this method can be employed for the generation of *P. knowlesi* specific aptamers against the highly conserved *P. knowlesi* LDH, or the *P. knowlesi* specific epitope identified in GAPDH and PMT proteins for *P. knowlesi* detection.

In addition, there is an increasing interest in the use of computing approach to improve malaria diagnosis. A cost-effective automated diagnostic system for malaria was proposed by Oliveira et al. (2017) [80] to aid laboratory personnel in remote locations by improving the accessibility, cost, rapidness, and accuracy. The mobile device-based automated system involves the combination of both digital processing image techniques and a learning process based on artificial intelligence algorithms for the specific identification of *Plasmodium falciparum* in the trophozoite ring stage. Another example of AI approach for malaria diagnosis is Yang et al. (2020) [81] in which deep learning was implemented to detect malaria parasites in thick smear images. The study showed that the model could be a promising alternative to manual parasite counting under microscopic examination. Thus far, automated parasite detection for *P. knowlesi* remains a gap to be filled as no study on that has been conducted before. The development of the automated diagnostic system would be advantageous for *P. knowlesi* diagnosis as most of the cases are reported in remote settings where experienced microscopists are not available.

Conclusion

P. knowlesi is the predominant malaria species in Malaysia which is life-threatening. Many *P. knowlesi* cases have been reported in several other Southeast Asian countries due to the advancement in diagnostic techniques and increased awareness of the disease. These advancements have contributed to increased reports of knowlesi malaria in travelers who return from *P. knowlesi*-endemic regions. As the parasite develops rapidly, early diagnosis is needed for prompt disease management. A versatile point-of-care tool is vital in overcoming the limitations of routine microscopic examination especially when submicroscopic infection is present. Meanwhile, sufficient training must be given to the laboratory personnel to avoid misidentification of the disease in microscopic examination. RDT for *P. knowlesi* specific detection is still essential as it can be used in remote settings, and does not require DNA preparation, sophisticated equipment, and skilled personnel. Nested PCR and real-time PCR assays will be used to confirm the identity of the *Plasmodium* species if RDT and microscopic examination fail to identify the *Plasmodium* species of the malaria infection. Most importantly, these molecular techniques play a significant role in detecting asymptomatic and submicroscopic infections especially in malaria-endemic communities. The LAMP, RPA, and CRISPR-based assays for *P. knowlesi* detection must be designed for applicability in remote settings.

Author Contributions

Jia Hui Tan searched the literature, drafted, and wrote the manuscript. Yee Ling Lau edited and approved the final version of the manuscript.

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Declaration of Competing Interests

The authors declare no conflict of interest.

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