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Plasmodium knowlesi detection methods for human infections—Diagnosis and surveillance

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Abstract

Within the overlapping geographical ranges of *P. knowlesi* monkey hosts and vectors in Southeast Asia, an estimated 1.5 billion people are considered at risk of infection. *P. knowlesi* can cause severe disease and death, the latter associated with delayed treatment occurring from misdiagnosis. Although microscopy is a sufficiently sensitive first-line tool for *P. knowlesi* detection for most low-level symptomatic infections, misdiagnosis as other *Plasmodium* species is common, and the majority of asymptomatic infections remain undetected. Current point-of-care rapid diagnostic tests demonstrate insufficient sensitivity and poor specificity for differentiating *P. knowlesi* from other *Plasmodium* species. Molecular tools including nested, real-time, and single-step PCR, and loop-mediated isothermal amplification (LAMP), are sensitive for *P. knowlesi* detection. However, higher cost and inability to provide the timely point-of-care diagnosis needed to guide appropriate clinical management has limited their routine use in most endemic clinical settings. *P. knowlesi* is likely underdiagnosed across the region, and improved diagnostic and surveillance tools are required. Reference laboratory molecular testing of malaria cases for both zoonotic and non-zoonotic *Plasmodium* species needs to be more widely implemented by National Malaria Control Programs across Southeast Asia to accurately identify the burden of zoonotic malaria and more precisely monitor the success of human-only malaria elimination programs. The implementation

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of specific serological tools for *P. knowlesi* would assist in determining the prevalence and distribution of asymptomatic and submicroscopic infections, the absence of transmission in certain areas, and associations with underlying land use change for future spatially targeted interventions.

1. Introduction

The recent increase in zoonotic transmission of the Cercopithecinae Old World monkey malaria parasite *Plasmodium knowlesi*, endemic to Southeast Asia, exemplifies how intensive anthropogenic land-use change is driving emerging disease spill-over to humans (Brock et al., 2019). Although a single presumed anomalous human *P. knowlesi* infection was first reported in the 1960s in Peninsular Malaysia (Chin et al., 1965), it was not until 2004 that Singh and colleagues using molecular detection methods identified a large number of naturally acquired human knowlesi malaria cases in Sarawak, East Malaysia (Singh et al., 2004). Molecular analysis of archival samples in Sarawak (Lee et al., 2009a) and Thailand (Jongwutiwes et al., 2011) demonstrated that in this region *P. knowlesi* had been misreported on routine microscopy at least as far back as the mid-1990s. Regional under-reporting of *P. knowlesi* infections is likely occurring, with PCR confirmed infections now documented from all countries in Southeast Asia where its major natural macaque hosts (*Macaca fascicularis* and *M. nemestrina*) and *Anopheles* Leucosphyrus group vectors are present (Herdiana et al., 2016; Iwagami et al., 2018; Lubis et al., 2017; Moyes et al., 2014; Singh and Daneshvar, 2013).

Changes in human land use are thought to play a major role in driving the increase in *P. knowlesi* exposure to humans, with the relative influence of associated environmental factors such as forest fragmentation varying at different spatial scales (Brock et al., 2019; Fornace et al., 2019b). Increased interaction between humans, vectors and the monkey parasite reservoir results from adaptive behaviours in response to these changing landscapes (Fornace et al., 2019a; Stark et al., 2019). Differences in monkey and human host biting preferences between *An.* leucosphyrus group species may also influence spatial variation in *P. knowlesi* transmission patterns (Vythilingam, 2010). Vector adaptation includes features such as earlier outside peak biting times in the evening for *An. balabacensis*, an incriminated *P. knowlesi* vector in Sabah (Wong et al., 2015). Adaptation to human-to-human transmission as a potential underlying driver of the emergence of *P. knowlesi* has not been evident in studies to date comparing parasite genetic lineages between macaque and human hosts (Divis et al., 2015; Jeslyn et al., 2011; Jongwutiwes et al., 2011; Lee et al., 2011). However, vector competence for this transmission mode has been experimentally proven (Chin et al., 1968), with incriminated *P. knowlesi* vectors such as *An. dirus* and *An. balabacensis* also being the primary vector for non-zoonotic *Plasmodium* species in certain areas (Marchand et al., 2011; Vythilingam, 2010). A decrease in cross-protective human immunity from the reduction of malaria transmission due to *P. vivax* may also be contributing to the prevalence of symptomatic disease or patent *P. knowlesi* infections detectable by microscopy in endemic areas (Muh et al., 2020).

Technological advances in malaria detection methods, specifically the advent of highly sensitive and specific molecular tools, have allowed accurate confirmation of *P. knowlesi*

human infections across Southeast Asia (Singh and Daneshvar, 2013). However, first-line point-of-care testing for suspected malaria in most co-endemic settings in Southeast Asia primarily involves microscopy, which is inherently unreliable for diagnosing *P. knowlesi* due to morphological similarities with *P. malariae* and *P. falciparum*. Further complicating microscopic diagnosis, *P. knowlesi* co-infections particularly with *P. vivax* are common in endemic areas (Singh and Daneshvar, 2013). Current lateral flow malaria rapid diagnostic assays designed for non-zoonotic malaria species have shown poor sensitivity and specificity for *P. knowlesi* detection, particularly at the low parasite counts often seen even in symptomatic infections (Barber et al., 2013b; Grigg et al., 2014). Molecular tools including nested, real-time, and single-step PCR, and loop-mediated isothermal amplification (LAMP), have now been designed for *P. knowlesi* detection (Singh and Daneshvar, 2013). However, higher cost and inability to provide the timely point-of-care diagnosis needed to guide appropriate clinical management has limited their routine use in most endemic clinical settings.

Molecular tools used for research surveillance purposes, including in monkey hosts and mosquito vectors, have highlighted key knowledge gaps in estimating regional variation in the transmission and disease burden of *P. knowlesi* (Shearer et al., 2016). This includes the detection of underreported asymptomatic or submicroscopic infections (Fornace et al., 2016b; Grignard et al., 2019; Imwong et al., 2019; Jongwutiwes et al., 2011; Lubis et al., 2017; Marchand et al., 2011). In this context, understanding of *P. knowlesi* transmission has been further enhanced by the recent development of serological surveillance tools for *P. knowlesi* indicating past exposure. These multiplex sero-surveillance platforms also offer the potential for more detailed understanding of factors associated with exposure to *P. knowlesi* and other malaria species over time (Herman et al., 2018). Use of these sero-surveillance tools has already provided important insights into *P. knowlesi* transmission and specific environmental associations, including identifying infection exposure in demographic groups such as children and women thought to be at lower risk (Fornace et al., 2019b).

The emergence of *P. knowlesi* in Southeast Asia, with an intractable monkey parasite reservoir and ongoing misidentification with other *Plasmodium* species, threatens regional malaria elimination goals (Chin et al., 2020; Cox-Singh and Singh, 2008). In Malaysia, implementation of routine PCR for species confirmation of all malaria cases since 2012 has enabled public health authorities to demonstrate the near-elimination of *P. falciparum* and *P. vivax* malaria, alongside a concurrent increase in *P. knowlesi* cases in high-risk groups such as adult males with a history of forest exposure or agricultural work (Chin et al., 2020; Hussin et al., 2020; WHO, 2020). Molecular tools have also allowed accurate descriptions of the disease spectrum of *P. knowlesi*, ranging from asymptomatic infections, to severe malaria in 6%–9% (Grigg et al., 2018a; Singh and Daneshvar, 2013), and fatalities in 2.5 per 1000 cases (Rajahram et al., 2019). However, routine molecular confirmation of cases is not possible in many resource constrained settings, and WHO do not specifically include PCR-confirmed *P. knowlesi* case notifications in global yearly reporting (Barber et al., 2017b; WHO, 2020). Thus, the true regional prevalence and disease burden of *P. knowlesi* infections remains poorly understood, with limited use of accurate detection methods outside research settings.

In 2017 the WHO convened an evidence review group to guide research priorities in response to the public health threat posed by the emergence of *P. knowlesi*, which included a major recommendation to develop and improve laboratory detection methods (WHO, 2017). The following sections review current detection methods for both point-of-care diagnosis and surveillance of *P. knowlesi* human infections.

2. Point-of-care diagnosis

2.1 Microscopy

Microscopy remains the gold standard for malaria diagnosis in most endemic countries, as it is inexpensive, fast, sensitive and allows parasite quantification. Microscopy does however require trained laboratory technicians and equipment, an electricity supply and ongoing quality assurance procedures (WHO, 2015a). Older malaria microscopy teaching or reference tools generally in routine use in Southeast Asia do not include descriptions of *P. knowlesi* or comment on similarities with *P. malariae* or *P. falciparum*. Formal public health malaria reporting by microscopy in most countries outside Malaysia have not yet added provisions for suspected *P. knowlesi*. *P. malariae* remains the conventional reporting default, further limiting accurate regional estimates on the true burden of disease (Barber et al., 2013; Cox-Singh et al., 2008). Morphological similarities between *P. knowlesi* and *P. malariae* have allowed the existence of human *P. knowlesi* cases to previously remain undiagnosed. Misdiagnosis of *P. knowlesi* as the more benign *P. malariae* has also contributed to a lack of recognition of severe disease, with important adverse clinical and treatment outcomes (Rajahram et al., 2012; William et al., 2011), including case-fatalities (Rajahram et al., 2019). In contrast to *P. knowlesi, P. malariae* preferentially infects older senescent red blood cells (RBCs), which in addition to a slow 72-h replication cycle and host immunity results in chronic infections with low parasite counts (Collins and Jeffery, 2007). In *P. knowlesi* endemic areas, infections appearing morphologically similar to *P. malariae*, particularly with higher parasite counts, should be reported and treated as *P. knowlesi* with PCR confirmation for final reporting purposes where possible (Barber et al., 2017a,b).

2.1.1 P. knowlesi *morphology in human infections*—

The first descriptions of the morphology of *P. knowlesi* in experimental inoculated human infections by Knowles and Das Gupta in 1932 commented on the similarity with *P. malariae*, including little or no amoeboid activity, and normal size of infected red blood cells (Knowles and Gupta, 1932). Sinton and Mulligan more extensively detailed the 24-h life-cycle of the parasite, the distinctive stippling occasionally observed in schizont-infected cells (different from that seen in *P. vivax)* and the presence of an accessory chromatin dot (Sinton and Mulligan, 1933), originally thought to be diagnostic for *P. knowlesi* but subsequently found to be present in other simian *Plasmodium* species (Coatney et al., 1971).

More recently, Lee et al. in 2009 described detailed microscopy findings in 10 human *P. knowlesi* cases from Sarawak, Malaysia (as in Fig. 1). Early trophozoites resembled those of *P. falciparum*, with a ring-like cytoplasm, and double chromatin dots, multiply infected red blood cells and applique forms were all seen. Late trophozoites were almost indistinguishable from *P. malariae*, with dense irregular cytoplasm and band-like forms often

present. Schizonts were noted on average to contain 10 merozoites, and up to 16, in contrast to *P. malariae*, which has a maximum of 12 and are more regularly arranged (Lee et al., 2009b). Stippling was not observed in this study, although has been described in other case reports (Figtree et al., 2010; Jongwutiwes et al., 2004). Gametocytes have been described as being rare, spherical, and develop over 48h. Resembling *P. malariae* sexual stages, the mature female macrogametocyte stains blue and contains a small eccentrically placed nucleus, compared to the pink staining male microga-metocyte with a nucleus making up about half of the body of the parasite (Coatney et al., 1971).

2.1.2 P. knowlesi *parasite count measurements*—The majority of symptomatic *P. knowlesi* human infections consist of patent low-level asexual parasites when quantified on peripheral blood films. A large prospective study of 481 knowlesi malaria cases presenting to primary health facilities in Sabah, Malaysia demonstrated a median parasite count of 2480/μL (IQR 538–8481/μL; range 20–263,772/μL). Median parasitemia was lower than that seen in falciparum or vivax malaria in both adults and children. Children less than 12 years of age with *P. knowlesi* infections had a lower median parasitemia of 1722/μL (IQR 386–4830/μL) compared to adults (Grigg et al., 2018a).

Extremely high parasite counts of greater than 500,000parasite/μL (10% of infected red blood cells) can occur, in part attributed to the fast 24-h parasite life-cycle, although more definitely linked to alternative red blood cell invasion pathways such as the presence of *P. knowlesi* normocyte binding proteins (Ahmed et al., 2014). A lower parasite count threshold of 100,000/μL to define hyperparasitemia compared to falciparum malaria was determined from severe knowlesi malaria cases in Malaysia (Barber et al., 2013a) and subsequently adopted as the WHO epidemiological and definition for hyperparasitemia defining severe malaria for *P. knowlesi* (WHO, 2014). These guidelines defined a parasitemia >20,000/μL as the clinical definition requiring treatment as severe malaria in those in whom other laboratory measures of severe malaria cannot be measured (WHO, 2014).

2.1.3 P. knowlesi *life stages in human infections*—Similar to *P. malariae* and *P. vivax*, the majority of *P. knowlesi* infections (86%) are asynchronous in data from Sabah, with a mean proportion of early trophozoite ring stages relative to other parasite life stages of 44%, compared to late trophozoites at 54% and schizonts comprising on average 2% (Grigg et al., 2018a), consistent with other prospective tertiary hospital data (Barber et al., 2013a). In contrast to *P. falciparum* infections, which due to microvascular cytoadherence demonstrate almost exclusively synchronous ring stage infections in peripheral blood films, *P. knowlesi* synchronous ring stage-only infections were seen in a small proportion (5%) of cases (Grigg et al., 2018a). *P. knowlesi* asynchronous infections had a higher median parasite count of 2177/μL, compared to synchronous ring-stage infections of 461/μL. Schizonts were present in 38% (95% CI 33%–42%) of *P. knowlesi* infections in this study overall, with a schizont proportion >10% relative to other life-stages found to be associated with higher parasite counts and severe disease (Barber et al., 2013a; Grigg et al., 2018a). Gametocytes were noted to be present by microscopy in 14% (95% CI 11%—18%) of patients (Grigg et al., 2018a), lower than in Lee et al. where detailed research microscopy

demonstrated four (40%) patients had gametocytes, although also at very low levels of up to ~3% of infected red blood cells (Lee et al., 2009b).

2.1.4 *Diagnostic performance of routine malaria microscopy for* P. knowlesi *infections*—

Studies involving reference PCR diagnostics have consistently demonstrated the poor utility of thick blood film microscopy to correctly identify symptomatic *P. knowlesi* infections (Mahittikorn et al., 2021). In addition to misidentification with *P. malariae*, *P. knowlesi* is often misreported as both *P. falciparum* and *P. vivax*, and vice versa, despite the latter having distinct morphological differences including enlargement of infected red blood cells (Barber et al., 2013; Coutrier et al., 2018). In countries north of Malaysia, *P. knowlesi* has been most commonly detected as co-infections with *P. falciparum* or *P. vivax*, creating further challenges for the use of microscopy for accurate diagnosis and reporting (Jongwutiwes et al., 2011; Marchand et al., 2011). Co-infections also highlight the shared mosquito vectors between zoonotic and human-only *Plasmodium* species in co-endemic areas (Marchand et al., 2011). Implications of these findings for co-endemic settings in Southeast Asia include the lack of anti-relapse therapy for *P. vivax* and, historically, the inappropriate treatment of *P. falciparum* with chloroquine.

In Malaysia, Barber et al. described the limitations of microscopy to reliably distinguish between *Plasmodium* species in a co-endemic region of Malaysia in a prospective evaluation of PCR-confirmed *Plasmodium* infections (Barber et al., 2013a). In routine hospital-based microscopy of 130 malaria patients, only 72% of PCR confirmed *P. knowlesi* monoinfections were identified as '*P. malariae/P. knowlesi*', 13% as *P. falciparum* and 10% as *P. vivax* (Barber et al., 2013a). More recently in state-wide malaria notifications from Sabah over the period 2015–2017, despite nearing elimination of *P. falciparum* and *P. vivax*, the specificity of microscopy to correctly identify *P. knowlesi* remained low for at 68% (95% CI 62%–73%) against reference malaria PCR (Cooper et al., 2020).

In other countries where research studies have used appropriate PCR methods to detect *P. knowlesi* infections, routine microscopy has been shown to perform poorly in terms of differentiating all *Plasmodium* species. In Aceh Province, Indonesia, in 2014–2015, a small study of 41 microscopy positive malaria cases, including 3 cases reported as *P. malariae*, found 19 *P. knowlesi* monoinfections using PCR, with routine microscopy misidentifying 56% of all infections (Coutrier et al., 2018). In a study from North Sumatra, Indonesia, of 1169 participants with PCR confirmed *Plasmodium* species infections, 377 were found to be positive for *P. knowlesi* (including around half with mixed *P. vivax* infections) on PCR, with none of the 34 with patent microscopic infections reported as *P. knowlesi* (Lubis et al., 2017). In Thailand in a study conducted in 2008–2009, there were 3300 febrile patients with PCR confirmed malaria, nearly all of whom were diagnosed with either *P. falciparum* (52%) or *P. vivax* (48%) by microscopy. Of these patients 23 were subsequently confirmed as *P. knowlesi* (8 monoinfections, and 15 mixed infections) (Jongwutiwes et al., 2011). The monkey malaria species *P. cynomolgi*, also known to infect humans in Southeast Asia albeit more rarely, resembles *P. vivax* morphologically on microscopy, further complicating accurate diagnosis and reporting (Grignard et al., 2019; Imwong et al., 2019).

2.1.5 *Automated visualisation of blood films for detecting* P. knowlesi *infections*—

Novel diagnostic devices based on automated visualization of malaria microscopy blood films using machine learning approaches are increasingly being validated for detection of *Plasmodium* species infections (Torres et al., 2018). These approaches hold promise, with a recent malaria microscopy scanner device evaluating laboratory cultured *P. knowlesi* and *P. falciparum* parasites demonstrating higher precision than microscopy for parasitemia at a lower limit of 0.1% of infected RBCs, although further development to allow differentiation between species on clinical samples using thick blood films is required (Firdaus et al., 2021).

2.2 Rapid diagnostic tests

Point-of-care diagnostics such as immunochromatographic rapid diagnostic tests (RDTs) play an important role in parasite-based malaria diagnosis world-wide, as they can be performed by people with minimal training in areas where good quality microscopy cannot be maintained (WHO, 2021). Other advantages, particularly compared to molecular detection tools, include the limited amount of time needed to conduct the RDT and receive a result (usually less than 20 min) and relatively low cost. Commercially available sensitive RDTs to detect *Plasmodium* genus and/or species-specific tests for *P. falciparum* and *P. vivax* are widely used, including in returned travellers from endemic settings (WHO, 2020). Of the most commonly used RDTs, many utilise antigen capture for parasite lactate dehydrogenase (pLDH), with a variety of monoclonal antibodies targeting different epitopes associated with *P. falciparum* and *P. vivax* pLDH isoforms allowing species specific identification (McCutchan et al., 2008). Although no specific *P. knowlesi* RDTs have been designed to date, cross-reactivity between certain pLDH epitopes for *P. falciparum* and *P. vivax* with a subset of *P. knowlesi* parasites also expressing these epitopes allows their detection, with sensitivity associated with the degree of binding affinity. In addition, tests targeting non-specific *Plasmodium* species pLDH or aldolase enzyme are able to detect *P. knowlesi* (McCutchan et al., 2008); Table 1.

Importantly, *P. knowlesi* does not appear to cross-react with the highly specific *P. falciparum* histidine-rich protein-2 (HRP2), used in the most sensitive *P. falciparum*-based RDTs. The extensive use of *P. falciparum*-HRP2 based RDTs has resulted in selection of parasites with deletions in the *pfhrp2* and *pfhrp3* genes, rendering *P. falciparum* infections with these deletions undetectable. However, *pfhrp2/3* deletions have been reported only in countries outside Southeast Asia, and non-expression of pLDH or aldolase targets has not been documented. WHO recommends ongoing use of *P. falciparum*-HRP2 based tests in areas where <5% false negative rates for *P. falciparum* detection are present (WHO, 2021).

2.2.1 Pan-pLDH based RDTs—

The best performing RDT published to date for detecting *P. knowlesi* was the First Response Malaria pLDH/HRP2 Combo™ test (Premier Medical Ltd., India). Barber et al. demonstrated a sensitivity of the pan-pLDH component of 74% (95% CI 65%–80%) for detecting *P. knowlesi* infections, with a positive result in 95/129 patients. For samples tested prior to antimalarial treatment the sensitivity increased to 88% (95% CI 73%–95%), although with a higher median parasite count of 7493/μL (IQR 3823–19,895/μL) among those tested. Within this group, for those with parasite counts

greater than 1000/μL sensitivity further increased to 97% (95% CI 83%–99%), although had a corresponding low sensitivity of 25% below this parasite count cut-off. Sensitivity in severe malaria with higher parasitemias was 95% (36/38; 95% CI, 83–99), however the pLDH target failed to detect two moderately high individual parasite counts of 3486 and 48,833 parasite/μL. All *P. knowlesi* samples tested negative for the concurrent *P. falciparum* HRP2 target (Barber et al., 2013b).

The OptiMAL-IT™ malaria RDT (DiaMed, USA) includes a pan-pLDH based component and was evaluated by Foster et al., with positive tests reported in 18 freshly collected *P. knowlesi* samples, resulting in a sensitivity of 64% (95% CI 44%–81%). The median parasite count of samples tested was 9131/μL, however sensitivity was markedly reduced in those with lower parasitemias, with a single positive result in the small number of three total samples tested with parasite counts below 500/μL (Foster et al., 2014). The poor performance of the pan-pLDH component in the OptiMAL-IT™ test was consistent with a separate larger study of 190 *P. knowlesi* clinical samples, which comprised a lower median parasite count of 2301/μL among those tested. A poorer sensitivity of 29% (95% CI 22%–35%) for the OptiMAL-IT™ pan-pLDH target was demonstrated, despite a low minimum detectable parasite count of 30 parasites/μL (Grigg et al., 2014).

The Paramax-3 Rapid Test for Malaria Pan/Pv/Pf™ (Zephyr Biomedicals, India) was evaluated by Foster et al., who reported a poor sensitivity of the pan-pLDH test component of 32% (95%CI 17%–46%) in fresh *P. knowlesi* infected samples (Foster et al., 2014). Grigg et al. evaluated the CareStart™ Malaria HRP2/pLDH (Pf/VOM) Combo RDT (Access Bio Inc., USA), with the VOM pLDH component targeting non-falciparum species (*P. vivax*, *P. ovale* and *P. malariae*). This pLDH based target was also relatively poor for detecting *P. knowlesi*, with 77/178 clinical samples positive, resulting in a low sensitivity of 43% (95% CI 36–51) (Grigg et al., 2014). For the studies evaluating the Paramax-3™ and CareStart™ RDTs which also contain a *P. falciparum*-HRP2 test, a small number of false positive results, less than 0.04%, were recorded for *P. knowlesi* samples read by research staff blinded to the underlying PCR-confirmed *Plasmodium* species result (Foster et al., 2014; Grigg et al., 2014).

2.2.2 P. falciparum *and* P. vivax*–pLDH antibody cross-reactivity with* P. knowlesi—

The known *P. knowlesi* cross-reactivity with certain *P. falciparum* and *P. vivax*-pLDH epitopes has been exploited to evaluate RDTs containing these test components for detection of *P. knowlesi* infections. For *P. knowlesi* a high homology of 96.8% has been found with the pLDH ortholog for *P. vivax*, and over 90% for *P. falciparum*, *P. malariae* and *P. ovale* (Singh et al., 2012). Despite the inherent lack of specificity in this approach, cross-reactivity against *P. knowlesi* in clinical samples has been demonstrated. A *P. falciparum*-pLDH (rather than HRP2) target in the OptiMAL-IT™ RDT demonstrated positive test results in 18/28 *P. knowlesi* samples in Sarawak, Malaysia with a sensitivity of 64% (95% CI 44%–81%) (Foster et al., 2014). Positive results for *P. falciparum*-pLDH were also recorded among 190 patients with comparatively lower overall *P. knowlesi* parasite counts, resulting in a sensitivity of 29% (95% CI 22%–35%) in Sabah, Malaysia (Grigg et al., 2014). A *P. vivax*-pLDH target was evaluated in the Paramax-3™ RDT, with a lower sensitivity of 32% (95 %CI 15%–54%) for detecting *P. knowlesi* compared to the

pan-pLDH component (Foster et al., 2014). The Core Malaria Pan/Pv/Pf™ malaria RDT (Core Diagnostics Ltd., USA) demonstrated a positive *P. vivax*-pLDH result for a single returned traveller with PCR confirmed knowlesi malaria and a parasite count of 40,000/μL (Berry et al., 2011). The addition of the *P. falciparum*-pLDH or *P. vivax*-pLDH tests in the OptiMAL-IT™ and Paramax-3™ RDTs respectively did not confer any improvement in overall sensitivity when results were combined with the pan-pLDH test components for *P. knowlesi* diagnosis.

2.2.3 Pan-aldolase based RDTs—The BinaxNOW Malaria RDT™ (Inverness Medical Innovations Inc., USA) contains a pan-aldolase target validated for conventional major human *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*), in addition to a *P. falciparum* HRP2 test component. The pan-aldolase test performed poorly when conducted on *P. knowlesi* samples, with a reported sensitivity of 29% (95% CI 12%–26%), including negative results for all 10 *P. knowlesi* samples tested with a parasite count of less than 5000/μL (Foster et al., 2014). The ParaHIT Total Dipstick™ RDT was evaluated by Barber et al, which also contains a pan-aldolase target and *P. falciparum* HRP2 target. *P. knowlesi* detection was also poor, with the pan-aldolase target demonstrating a sensitivity of 23% (95% CI 16%–32%) and the lowest parasite count detected was 4412/μL (Barber et al., 2013b).

2.2.4 RDT use in returned travellers from endemic countries—Published case reports from returned travellers with *P. knowlesi* malaria who visited endemic countries in Southeast Asia including from the Netherlands, Sweden, Spain, Australia, France, Japan, Germany, and Italy most commonly described the use of the BinaxNOW™ RDT as a primary point-of-care diagnostic tool. The pan-aldolase test strip for *Plasmodium* species detection was positive in thtree case reports in patients with moderate to high parasite counts of 84,000/μL (van Hellemond et al., 2009), 40,000/μL (Berry et al., 2011) and 7700/μL (Ong et al., 2009), with the remainder negative. A number of *P. knowlesi* case reports from various non-endemic countries after the BinaxNOW™ evaluation was published in 2014 highlights the ongoing use of this RDT as a first line point of care malaria diagnostic tool in returned travellers. This is despite the poor performance demonstrated for detecting *P. knowlesi*, in addition to that seen for *P. vivax* at lower parasite counts in previous WHO malaria RDT product testing (WHO, 2015b). The OptiMAL-IT™ RDT has also been reported as having positive *P. falciparum*-pLDH results when conducted in Netherlands (van Hellemond et al., 2009) and Singapore (Ong et al., 2009) for *P. knowlesi* confirmed symptomatic infections. The Core Malaria Pan/Pv/Pf™ malaria RDT used in a single *P. knowlesi* case report demonstrated a positive pan-pLDH and negative *P. falciparum*-HRP2 test in addition to the positive *P. vivax*-pLDH result previously mentioned (Berry et al., 2011).

2.2.5 *RDT detection for* Plasmodium *species in monkey hosts—*Kawai et al. evaluated two malaria RDTs for the detection of different simian malaria species infections in non-natural Japanese macaque hosts. The study macaques were separately inoculated with *P. knowlesi* Hackeri strain, *P. gonderi*, *P. cynomolgi* B strain, and *P. coatneyi* CDC strain and monitored over the ensuing course of infection (Kawai et al., 2009). The Entebe Malaria Casette™ (Laboritorium Hepatika, Indonesia) was evaluated, which contains a *P.*

vivax-pLDH target found to be initially negative for the *P. knowlesi* infected macaque at a parasitemia of 0.04%, and subsequently positive at later higher parasitemias of 11.2% and 28.8% of infected red blood cells. The OptiMAL-IT™ RDT was also concurrently evaluated with positive *P. falciparum*-pLDH and pan-pLDH results at the same parasite count levels. Serial dilutions of the quantified *P. knowlesi* sample were used to demonstrate weakly positive results remaining present at a minimum of 100parasites/μL for both RDTs. Both the *P. vivax*-pLDH target for the Entebe Malaria Cassette™ and the OptiMAL-IT™ pan-pLDH test demonstrated positivity when tested on other Old World non-human primate malaria species infections including *P. gonderi*, *P. cynomolgi* and *P. coatneyi*. The *P. falciparum*-pLDH test component for OptiMAL-IT™ was positive for *P. gonderi*, however negative for *P. cynomolgi* and *P. coatneyi* (Kawai et al., 2009).

2.2.6 Summary and recommendations for RDT use—Commercially available RDTs evaluated to date have limited utility in accurately detecting *P. knowlesi* clinical infections and should not replace microscopy as an existing first line malaria diagnostic tool in endemic areas, or PCR detection methods for accurate final *Plasmodium* species reporting. Neither pan-pLDH or pan-aldolase based tests have demonstrated sufficient sensitivity for detecting *P. knowlesi*, particularly with low parasite counts of less than 1000/μL which are seen in around 33% (95% CI 32%–34%) of over 8000 *P. knowlesi* malaria cases in endemic areas such as Sabah, Malaysia over recent years (*unpublished data, Sabah Ministry of Health*). Cross-reactivity of *P. knowlesi* with *P. falciparum*-pLDH and *P. vivax*-pLDH test components are also insufficiently sensitive to diagnose *P. knowlesi* reliably. In co-endemic areas specificity for the original *P. falciparum* and *P. vivax* pLDH targets is potentially less than manufacturer's report (Grigg et al., 2014). Point-of-care malaria RDT use in returned travellers from *P. knowlesi* endemic areas requires the concurrent use of microscopy for initial diagnosis and clinical management, and validated PCR detection methods for accurate final *Plasmodium* species reporting.

RDTs remain a major point-of-care malaria diagnostic tool in Southeast Asia, with over 12.7 million distributed in the WHO regions encompassing *P. knowlesi* endemic areas in 2019 (WHO, 2020). Consistent with current WHO recommendations, in malaria endemic areas in Southeast Asia (or in returned travellers) where RDTs are used as a first line tool, a *P. falciparum*-HRP2 based test should be preferentially used to distinguish falciparum versus non-falciparum malaria. An additional pan-pLDH and *P. falciparum*-pLDH test component which cross-reacts with *P. knowlesi*, or a *P. vivax*-pLDH test utilising a specific epitope which has been definitively shown to lack any cross-reactivity with *P. knowlesi* (McCutchan et al., 2008), may theoretically assist in identifying *P. vivax* infections. However, in general current RDTs remain unable to distinguish mixed *P. vivax/P. knowlesi* infections, and the poor sensitivities demonstrated to date limit the possible use of any current combination of target antigens to accurately diagnose *P. knowlesi* monoinfections (Grigg et al., 2014).

Commercial appeal for developing and including *P. knowlesi* specific targets in malaria RDTs is constrained in part by the relatively low reported regional incidence of human infections, which in turn is underestimated by the lack of accurate point-of-care diagnostic tools. Current epitopes particularly for pLDH based tests are usually undisclosed by commercial manufacturers, with reported specificity for the major *P. falciparum* and *P.*

vivax RDT targets not known to be determined against *P. knowlesi* or other simian malaria species able to cause human infections, such as *P. cynomolgi* or *P. simium*, for which cross-reactivity may also exist. WHO malaria RDT product testing also report rigorously conducted panel detection scores only against *P. falciparum* and *P. vivax* (WHO, 2018), and ideally would expand validation protocols to encompass *P. knowlesi* and other clinically relevant human or simian malaria species. Future novel RDTs such as magneto-optical detection of hemozoin, an intrinsic by product of *Plasmodium* species haemoglobin digestion, hold promise for sensitive *P. knowlesi* detection at low parasite counts, although also need to be able to reliably differentiate major *Plasmodium* species (Arndt, 2021) (Box 1).

3. Molecular detection

3.1 PCR assays

3.1.1 Background—The development of molecular detection assays for *P. knowlesi* has delivered enhanced sensitivity and specificity compared to microscopy. A number of assays utilizing different gene targets and methods have been developed, including the use of nested PCR, real-time PCR, single-step PCR; summarized in Table 2.

SSU rRNA has been used as the most common target for all *Plasmodium* species identification as most *Plasmodium* parasites have two to three distinct genes that are differentially expressed during the parasite's life cycle (Waters, 1994). The first *P. knowlesi* oligonucleotide primers, Pmk8-Pmkr9, developed by Singh et al. targeted the ssu rRNA-S gene expressed during the sexual stages (Singh et al., 2004). This PCR assay enables simultaneous examination for other *Plasmodium* infections using initial well-established genus-specific primers (rPLU6 with either rPLU1 or rPLU5) in the primary PCR followed by species-specific detection (Snounou and Singh, 2002). In conjunction with initial confirmatory sequencing of part of the *P. knowlesi* ssu rRNA and csp genes, this PCR assay was instrumental in demonstrating that the majority of malaria cases in the Kapit division of Sarawak, Malaysia, were actually single-species or mixed *P. knowlesi* infections (Singh et al., 2004).

Further study in Thailand however subsequently confirmed the possibility of cross-reactivity of these primers with *P. vivax* isolates. When the *P. knowlesi* and *P. vivax* ssu rRNA-S gene sequences were aligned, they showed identical sequences corresponding to the Pmkr9 primer and also similarities in the first 19 bases of the Pmk8 primer despite the differences at the 3′ end. Therefore, Pmk8-Pmkr9 primers may cause false amplification of *P. vivax* ssu rRNA under certain amplification conditions (Imwong et al., 2009). In addition, these 18S rRNA gene primers were also reported to cross-react with four other malaria parasites present in forest macaques of SE Asia: *P. inui, P. hylobati, P. cynomolgi*, and *P. coatneyi* (Lucchi et al., 2012). New primers using a fragment of the *P. knowlesi* ssu rRNA gene expressed during the asexual stages were subsequently designed. The PkF1160, PkF1140 and PkR1150 primers were shown to be specific for *P. knowlesi* with a level of detection of 1 to 10 parasite genomes per sample (Imwong et al., 2009). Updated ssu rRNA gene primers (Kn1f and Kn3r) were also developed in Sarawak, with specificity validated against other endemic simian *Plasmodium* species (Lee et al., 2011).

The Pmk8 and Pmk9 primers initially developed for *P. knowlesi* have now been updated or superseded with alternative primer pairs in routine practice in Malaysia at state reference laboratories (Divis et al., 2010; Nuin et al., 2020). Cross-reactivity of *P. knowlesi* infections with older *P. vivax*-designed primers amplifying parts of the ssu rRNA gene (Kimura et al., 1997) and the mitochondrial Cox1 gene (Tham et al., 1999) have also been demonstrated in returned travellers (Berry et al., 2011; Tanizaki et al., 2013). Other *P. vivax* ssu rRNA gene primers (Perandin et al., 2004) have also been demonstrated to show cross-reactivity with the closely genetically related zoonotic monkey parasite *P. cynomolgi* in Cambodia (Imwong et al., 2019). The routine use of newer PCR assays where specificity against *P. knowlesi* and other simian *Plasmodium* species has been tested is highly recommended.

3.1.2 *Alternative target genes for* P. knowlesi *identification*—Target genes of *Plasmodium* species that provide an alternative to the ssu rRNA gene include circumsporozoite surface protein (csp), a nuclear gene encoding a cysteine protease, cytochrome *b* (Conway, 2007), and dihydrofolate reductase (Tanomsing et al., 2010). More recently, the high copy number gene family encoding SICAvar antigens has been used as an alternative target (Lubis et al., 2017).

The use of mitochondrial-encoded cytochrome *b* is well established for diagnostics and species-discrimination in *Plasmodium* (Conway, 2007). It is highly conserved and possesses multiple copy numbers (20–150) in each parasite (Feagin, 1992; Mercereau-Puijalon et al., 2002), compared to 4–8 copies of the 18S rRNA (Vaidya and Mather, 2005). It has also been used to determine the evolutionary history of parasites (Escalante et al., 1998).

Dihydrofolate reductase (DHFR) and thymidylate synthase (TS) of *Plasmodium* species are enzymes with an essential role in the intra-erythrocytic life-cycle. They are relatively conserved between different isolates of the same species. Amplification of these genes has been used to investigate point mutations implicated in parasite antifolate resistance (Grigg et al., 2016a,b). The sensitivity and specificity to detect *P. knowlesi* has been reported to be similar to those detected by ssu rRNA assays. Other advantages are the ability to detect mixed-species infections, as these loci are less prone to genetic variation than the non-protein coding ribosomal genes (Tanomsing et al., 2010).

The schizont-infected cell agglutination variant antigens (SICavar) genes encode an antigen family unique to *P. knowlesi*, with an estimated number of more than 100 members, including both multiexon and truncated forms randomly distributed across all 14 chromosomes (Pain et al., 2008). SICAvar was designed to address potential cross-reactivity between *P. vivax* and *P. knowlesi*, with higher sensitivity also reported in one study compared to ssu rRNA and cytb amplification (Lubis et al., 2017).

3.1.3 Comparative performance of PCR assays—PCR based amplification assays for identification of malaria parasites to the species level have been developed in various formats, including nested PCR, heminested, multiplex PCR, and real-time PCR. The nested PCR is time-consuming, labour-intensive, and requires a large number of reagents and disposable consumables. It also has high risk of cross-contamination, although this can be minimised by training of laboratory staff and well-designed laboratory layout. Real-time

PCR has the advantage of a rapid result, minimal contamination risk (as it is a sealed system) and also allows parasite quantification. However, it is more expensive due to the costs of fluorescent reagents and specialized assay platforms. A single-step multiplex PCR reduces the time needed for PCR amplification and reactions, therefore saving on costs. It allows simultaneous identification of all human-infecting *Plasmodium* species, however the primer design and optimisation steps are challenging to ensure a highly sensitive and specific assay (Chew et al., 2012).

Since its introduction in the 1990s, nested PCR targeting the ssu rRNA gene has been considered the gold standard method for malaria detection (Snounou et al., 1993). The nested Kn1f and Kn3r (Lee et al., 2011) and the heminested PkF1160, PkF1140 and PkR1150 (Imwong et al., 2009) PCR assays were developed with improved specificity against other zoonotic and non-zoonotic species, targeting the sexual and asexual stages of *P. knowlesi* ssu rRNA, respectively. Either primer set can be used as a nested reaction following primary amplification with broad genus-specific *Plasmodium* primers (Snounou et al., 1993), and have a reported level of detection of 1–6 parasites/μL. However, it takes six PCR amplifications to enable the identification of *P. knowlesi* and the four major human-only *Plasmodium* species, with further PCR amplifications required if necessary to distinguish other co-endemic macaque *Plasmodium* species such as *P. cynomolgi, P. coatneyi, P. inui* and *P. fieldi* (Lee et al., 2011). The latter macaque *Plasmodium* species have recently been described to naturally infect humans in Malaysia (Yap et al., 2021), with *P. inui, P. inui*-like, *P. coatneyi*, and also *P. simiovale* able to be described with the use of nested PCR (Lee et al., 2011) and additional sequencing of the ssu rRNA and COX1 genes (Yap et al., 2021).

In contrast to nested PCR, a significant advantage of qPCR is the straight-forward *Plasmodium* species detection analysis through the use of fluorophores, without the need for gel electrophoresis. Several assays for *P. knowlesi* detection using qPCR targeting either S-type (Babady et al., 2009; Divis et al., 2010; Link et al., 2012) or A-type (Oddoux et al., 2011) ssu loci have also been developed. Two of these incorporated a *P. knowlesi*-specific probe (Divis et al., 2010; Link et al., 2012), with the remaining assays using a multiplexed *P. knowlesi*-probe with the human-only *Plasmodium* probes to allow detection of all *Plasmodium* species in a single reaction (Babady et al., 2009; Oddoux et al., 2011). Babady et al. used four FRET probes to enable simultaneous detection of five *Plasmodium* species at two reading channels. Although this assay has the advantages of a qPCR assay and maintains the sensitivity and specificity for all *Plasmodium* species identification, the four probes used increased the cost of the reaction and remains of limited value for field deployment (Babady et al., 2009). Another approach to minimise the cost of all *Plasmodium* species identification by qPCR was the modification of a SYBR Green qPCR protocol to include a *P. knowlesi*-specific probe. However, the sensitivity of this assay is much poorer (100 copies/μL) than that offered by Babady (Oddoux et al., 2011).

These limitations led to the development of a single-step hexaplex PCR assay (Chew et al., 2012). This assay is comprised of a single reverse *Plasmodium* genus-specific and five forward species-specific primers of human malaria parasites (*P. knowlesi, P. falciparum, P. vivax, P. malariae, P. ovale curtisi* and *P. ovale wallikeri*). The single-step hexaplex

approach allows detection of single or mixed malaria infections within a shorter laboratory timeframe (~3 h). In addition, the limit of detection for *P. knowlesi* was reported to be lower than previous published PCR assays at 0.25 parasites/μL. Although primers were robustly designed for *Plasmodium* species specificity, when testing specificity against 171 clinical malaria isolates the reference nested PCR assay used to confirm *P. knowlesi* included the original Pmk8 and Pmk9 primers known to falsely amplify a proportion of *P. vivax* monoinfections. Specificity was also not reported against the epidemiologically relevant *P. cynomolgi*. Despite this, with further validation this assay has the potential to provide an ideal and more affordable molecular detection method for malaria diagnosis (Chew et al., 2012).

The cytochrome *b* assay allows the identification of all *Plasmodium* species with high sensitivity and has been used with samples of body fluids other than blood. The first nested PCR developed targeting cytochrome *b* was designed by Putaporntip et al. The *cytb* PCR detected malarial DNA from a higher proportion of blood samples compared to the reference 18S rRNA assay, resulting in an improved sensitivity by 16%, with a reported limit of detection of 10 parasites/μL. When using saliva and urine samples, *cytb* amplification demonstrated marked superiority compared to the ssu rRNA assay, which was not able to identify *P. knowlesi* (Putaporntip et al., 2011). Cytb-targeted primers were further adapted to detect *P. knowlesi* in urine and additional faecal samples. After an inoculation of *P. knowlesi* infected RBCs into a macaque, *P. knowlesi* DNA in urine samples was detected early at day 2 similar to the detection of DNA in blood samples, while the DNA in faecal samples were only detected on day 7. However, the parasite DNA in faecal samples was detected up to 37 days after blood samples were PCR-negative. This may be due to the accumulation of parasite DNA in the gallbladder, from where it is gradually excreted to the faeces. This offers an attractive approach for studies in macaque hosts (Kawai et al., 2014; Putaporntip et al., 2011; Tanizaki et al., 2013).

The SICAvar gene assay was developed as a heminested PCR amplification protocol and was tested against human and simian malaria parasites. It was reported to be specific for *P. knowlesi*, with the limit of detection of 0.2 parasites/μL, lower than most of other reported *P. knowlesi* PCR assays. This assay identified a significant number of sub-microscopic and asymptomatic *P. knowlesi* cases in a malaria endemic area of Indonesia, of which nearly half were co-infected with other *Plasmodium* species, most commonly *P. vivax*. However, the apparent presence of the many *P. knowlesi-P.vivax* co-infections in the study could not be confirmed by performing head-to-head PCR assays using the other target genes due to the limited material available from the samples, which had been collected as dried blood spots (Lubis et al., 2017).

3.1.4 Detection of submicroscopic or asymptomatic infections—The use of highly sensitive PCR assays in research settings has increasingly demonstrated the extent of submicroscopic or asymptomatic *P. knowlesi* infections in population-based surveys across Southeast Asia. This includes a proportion of documented *P. knowlesi* infections from molecular surveys in Thailand (Jongwutiwes et al., 2011), Vietnam (Marchand et al., 2011), East Malaysia (Fornace et al., 2016b; Grignard et al., 2019; Siner et al., 2017), West Malaysia (Jiram et al., 2016; Noordin et al., 2020), Indonesia (Lubis et al., 2017),

Myanmar (Ghinai et al., 2017) and Cambodia (Imwong et al., 2019). The potential role of asymptomatic or submicroscopic human *P. knowlesi* infections contributing to ongoing transmission in human or monkey host populations is not established, although these transmission modes have been experimentally demonstrated (Chin et al., 1968). It remains unclear what proportion of these low-level infections either spontaneously resolve, persist at levels sufficient to be transmitted, develop into symptomatic disease resulting in seeking treatment and detection, or progress further to high parasitemia and severe disease.

Different approaches to maximizing the sensitivity of molecular methods to detect very low-level *Plasmodium* species infections have been used. In a cross-sectional community malaria survey conducted in Cambodia, Imwong et al. initially used a previously validated high-volume *P. genus* quantitative assay (uPCR) able to detect to down to 22genomes/mL of blood (Imwong et al., 2014). Samples positive for malarial DNA on this screening assay were then evaluated using a novel nested PCR amplifying 18S ssu rRNA genes (both A and S type), which encompasses 14 zoonotic malaria species including the endemic macaque parasites *P. knowlesi, P. cynomolgi, P. coatneyi* and *P. inui*. The PCR products (233–298bp) were then sequenced and aligned against corresponding individual reference ssu rRNA sequences for final diagnosis. This method was able to detect 8 *P. knowlesi* monoinfections, and 11 *P. cynomolgi infections* (including 2 co-infections with *P. vivax*). The very low-level parasitemia of asymptomatic *P. knowlesi* or *P. cynomolgi* infections seen in this study (geometric mean 52 and 4parasite/μL respectively) means a large proportion of infections in this area are likely to be below the threshold of ~40 parasites/μL able to be detected using routine microscopy or pan-pLDH based RDTs (Imwong et al., 2019).

A study of household and village members of confirmed *P. knowlesi* cases in Sabah revealed a high proportion of submicroscopic *P. knowlesi* carriage, with an estimated prevalence of 6.9% using latent class analysis of multiple *P. knowlesi* species-specific PCR assays (Fornace et al., 2016a). Findings differed depending on the sensitivity of the molecular *P. knowlesi*-specific assay, with the ssu rRNA nested assay expected to identify 15% of these infections, compared to Cytb nested PCR (59.1%), ssRNA qPCR (87.9%), and Plasmepsin qPCR (81.3%) (Fornace et al., 2016a). A subsequent rigorously designed community cross-sectional survey of 10,100 participants conducted in the same area in Sabah used a pooled 10 × 10 matrix of samples for DNA extraction and PCR detection for submicroscopic *Plasmodium* species infections (Fornace et al., 2019b). A well-established nested *P. genus* and non-zoonotic species-specific PCR assay targeting the ssu rRNA was initially conducted (Snounou et al., 1993), followed by the SICAvar assay for *P. knowlesi* detection (Lubis et al., 2017), with 9 infections found: 2 *P. knowlesi*, 1 *P. knowles/P. vivax*, with the remainder non-zoonotic *Plasmodium* species. A further analysis using 876 selected high-risk samples from the same survey conducted the nested zoonotic malaria PCR and sequencing method developed previously (Imwong et al., 2019), demonstrating improved sensitivity with 54 (6.2%) submicroscopic *Plasmodium* species infections reported. This included 3 *P. knowlesi* infections, and 2 *P. cynomolgi* infections (previously misdiagnosed as *P. vivax* and mixed *P. knowlesi/P. vivax*), with the remainder non-zoonotic species infections (Grignard et al., 2019).

In a longitudinal molecular malaria survey in Sarawak, Siner et al. used a pooled sample of four individuals dried blood spots for initial nested ssu rRNA PCR *P. genus* screening in 2118 participants, before DNA re-extraction and individual participant screening on those positive with Pmk8-Pmkr9 primers for *P. knowlesi* (Singh et al., 2004), other zoonotic monkey *Plasmodium* species (Lee et al., 2011) and non-zoonotic malaria. A further 884 individuals underwent the same PCR assay protocol without initial pooling of four samples. There were seven *P. knowlesi* infections in total detected, including a single asymptomatic submicroscopic infection, and five asymptomatic infections with parasite counts <50/μL, with no apparent difference in detection between samples initially pooled vs non-pooled (Siner et al., 2017).

3.1.5 *Molecular detection of* P. knowlesi *gametocytes*—The 24-h replication cycle of *P. knowlesi* in human host RBCs is known to rapidly produce mature gametocytes within around 48h (Knowles and Gupta, 1932). A short period of gametocyte infectivity then follows, with the timing hypothesised to increase transmission efficiency by occurring mainly at night during peak blood-feeding of mosquitoes (Hawking et al., 1968), although other mechanisms related to circadian within-host immunity and RBC physiology have been proposed (Mideo et al., 2013). The ability to accurately detect and quantify the level of gametocyte production in human *P. knowlesi* infections would assist in exploring fundamental parasite biology questions related to adaptation and replication in human hosts. This includes more accurately defining the timing and degree of gametocytogenesis relative to asexual parasitemia, infectivity to mosquitoes, and the risk of sustained human-human transmission. Other major applications of sensitive gametocyte detection include the ability to identify or evaluate the effect of future transmission blocking vaccines or treatments.

Molecular methods to detect *P. knowlesi* sexual life-stage stages hold significant advantages over microscopy, with most *P. knowlesi* infections likely to have gametocyte densities below the threshold of microscopic detection (Grigg et al., 2018a). Microscopy is insensitive due to the scarcity of gametocytes present in peripheral blood films (Lee et al., 2009b), and the limited number of fields screened usually in thick films when concurrently quantifying asexual parasite infected RBCs by counting the presence of up to 200 white blood cells (WHO, 2015a). Targets for the design of molecular assays to detect gametocytes are based on the underlying sexual-stage parasite biology. In preparation for gametocyte activation after mosquito ingestion in the midgut, mRNA transcripts encoding stage-specific proteins are produced by gametocytes in the human host in order to repress translation for the subsequent rapid genome replication and nuclear division. For *P. falciparum* this includes *pfs*16 mRNA, present from the earliest gametocyte stage onwards, *pfpeg*3 and *pfpeg*4 present in stage II gametocytes, and *pfs*25 mRNA for which transcription only begins to occur in mature stage IV gametocytes (Bousema and Drakeley, 2011). Other female specific gametocyte surface proteins include *pfs*47, thought to be involved in immune evasion in the mosquito midgut, and their male gametocyte paralogs *pfs*48/45 (Molina-Cruz et al., 2020).

Molecular detection assays for *P. falciparum* gametocyte protein orthologs have been designed and evaluated for *P. knowlesi*, with various primers developed to detect *pks*25 mRNA from mature gametocytes (Armistead et al., 2018; Grigg et al., 2016a,b; Maeno et al., 2017). In a clinical trial of antimalarial treatment for patients with uncomplicated

knowlesi malaria in Sabah, Malaysia, Grigg et al. demonstrated the presence of *P. knowlesi* *pks*25 mRNA transcripts in 85/100 (85%; 95%CI 76%–91%) of participants tested at enrolment (Grigg et al., 2016a,b). Of those positive for *pks*25 mRNA only 16% had gametocytes detected by microscopy (Grigg et al., 2016a,b), consistent with a separate clinical trial where only microscopy was performed and 32/123 (26%) patent infections had gametocytes present (Grigg et al., 2018b). There was an association with higher parasitemia and detectable with a median parasite count of 2140 vs 704 parasite/μL respectively; $P = 0.019$. At day 7, there were 5/97 (5%) patients persistently positive for *pks*25 mRNA: 3 p25 mRNA: 3 patients initially treated with artesunate-mefloquine, and 2 with chloroquine (Grigg et al., 2016a,b).

Maeno et al. conducted a comparative study of gametocyte detection for four species in Khan Hoa Province, Vietnam in both humans and mosquitoes. A nested real-time PCR assay was developed targeting *pks*25 mRNA to detect *P. knowlesi* gametocytes, with 15/32 (47%) of those with *P. knowlesi* confirmed asexual stage infections also positive for *pks*25 transcripts. All *P. knowlesi* samples were co-infections with one or more non-zoonotic *Plasmodium* species. The proportion of *P. knowlesi* infections with detectable *pks*25 mRNA was comparable to that seen for the *P. falciparum* and *P. vivax* gametocyte specific targets. Serial dilution of gametocytes from a *P. knowlesi* infected monkey was used to demonstrate a lower limit of detection of 1 gametocyt/μL for the RT-PCR *pks*25 assay. In this study, 70% of mosquitoes with *P. knowlesi* in their salivary glands also carried human malaria parasites, supporting the possibility that mosquitoes are infected with *P. knowlesi* from human infections (Maeno et al., 2017).

Armistead et al. designed multiple *P. knowlesi* RT-qPCR detection assays across gametocyte developmental stages, including targets for *pks*16, *pks*25, and *pks*47 mRNA. Using *P. knowlesi* H-strain cultured samples, they demonstrated infectivity of *An. dirus* through membrane infected RBC feeders, however none of the quantified transcripts of the 3 targets correlated well with infectivity, and peaks in *pks*25 and *pks*47 transcription were not associated with gametocyte maturation as may have been expected (Armistead et al., 2018).

3.2 Loop-mediated isothermal amplification (LAMP)

More recently LAMP has been evaluated as a potentially simple and sensitive diagnostic tool compared to microscopy as an alternative molecular diagnostic option for *P. knowlesi* infections in resource limited areas without specialized facilities for PCR (Britton et al., 2016). First described by Notomi, briefly, this molecular approach relies on a *Bacillus stearothermophilus* (*Bst*) polymerase to perform DNA strand separation and amplification of target sequences of 300 base pairs, at a single temperature of 65°C for 1 h using a set of four primers that form a stem-and-loop structure that promotes ongoing DNA amplification (Notomi et al., 2000). Magnesium pyrophosphate, a by-product of the LAMP DNA amplification process, forms a white precipitate following successful target amplification that can then be visualized by eye, by turbidimetry (Mori et al., 2001) or colourimetrically using hydroxynaphthol blue (Britton et al., 2016), malachite green (Lucchi et al., 2016b),

phenol red (Lai et al., 2020) or SYBR green (Lai et al., 2021) to objectively confirm a positive result.

3.2.1 LAMP platforms—There are two commercially available LAMP platforms. The first, the Eiken Loopamp™ MALARIA detection kit (Eiken chemical company, Japan) offers a *Plasmodium* genus specific assay (panLAMP) for identifying parasites that cause human infection and one able to specifically detect *Plasmodium falciparum* (PfLAMP) infection. When used together to identify non-falciparum infections, this panLAMP platform was able to identify *P. knowlesi* from 50 patients with uncomplicated *P. knowlesi* infections, at a genus level, with 100% sensitivity and specificity for infections with mean parasitemia of 1197 parasites/μL (IQR 300-3920) and a limit of detection of 2parasite/μL (Piera et al., 2017). However, this platform does not have a *P. knowlesi* species-specific assay currently available. The second commercial platform, *Illumigene* Malaria LAMP (Meridian Biosciences Inc., Cincinnati, OH) has demonstrated good sensitivity and specificity for identifying *Plasmodium* species using undisclosed LAMP primers (Lucchi et al., 2016a; Rypien et al., 2017) but has not been validated using *P. knowlesi* clinical samples. Several non-commercial LAMP platforms have also been published, such as HTP-LAMP (Perera et al., 2017) and RealAMP (Patel et al., 2013) one of which, HtLAMP, has been evaluated in *P. knowlesi* samples (Britton et al., 2016).

3.2.2 P. knowlesi *specific LAMP primers*—Since its first use in identifying *P. falciparum* (Poon et al., 2006), several LAMP primers specific for detecting *P. knowlesi* have been developed (summarised in Table 2), each with different targets, advantages and drawbacks. The first *P. knowlesi*-specific LAMP primers targeting the beta-tubulin gene demonstrated good species specificity and limit of detection of 10^2 copies/μL of target DNA but was only validated in samples from infected macaque monkeys and not human clinical samples (Iseki et al., 2010). Lau et al. published *P. knowlesi* specific LAMP primers targeting the apical membrane antigen 1 (AMA-1) (Lau et al., 2011) followed by genus and species-specific primers targeting the 18S rRNA gene for each of the *Plasmodium* parasites that cause human infection (Lau et al., 2016). These LAMP primers demonstrated a reported 100% sensitivity and specificity on a limited number of clinical samples, n = 13 (Lau et al., 2011), n = 57 (Lau et al., 2016), and n = 71 (Lai et al., 2021), including validation against *P. vivax*, however lacked detail on the limit of detection for these primers. In keeping with other LAMP studies demonstrating superior analytical sensitivity of primers targeting mitochondrial genes (Polley et al., 2010), *P. knowlesi*-specific mitochondrial target LAMP primers have been shown to have a limit of detection of 0.2 parasites/μL (Britton et al., 2016). However, given the 97% sequence homology between *P. vivax* and *P. knowlesi* at the target gene, this assay demonstrated 96% sensitivity, 30% specificity overall, but 100% specificity only if *P. vivax* samples were excluded (Britton et al., 2016).

3.2.3 Challenges for LAMP as a diagnostic tool—The advantages of LAMP include less need for equipment and technical expertise compared with PCR, potential for high specificity and rapid turnaround time. However, the requirement for parasite DNA extraction processes, high potential risk of contamination given the large amount of DNA

amplification and cost per sample remain limitations to the widespread use of the LAMP for diagnosis of malaria in primary care settings compared with microscopy.

Much effort has been made to simplify the preparation of DNA template for LAMP. LAMP has been performed on DNA templates generated directly from heat-treated blood samples (Poon et al., 2006), boil and spin method (Hopkins et al., 2013) and closed-circuit PURE extraction technology (Eiken chemical company) (Polley et al., 2013) with the latter two processes showing 97.5% sensitivity compared with nested PCR (Hopkins et al., 2013). A comparison of different volumes of whole blood with chelex or chelex-saponin extraction protocol found lower limit of detection with LAMP with a 20 μL blood volume with chelex-saponin compared with 5 or 10μL (Britton et al., 2015) confirming that, similar to high volume ultrasensitive PCR (Imwong et al., 2014), blood volume impacts LAMP performance particularly at low parasitemia. Filter paper dried blood spots (DBS) have also been extracted using a multistep Chelex-based process (Han et al., 2007) from symptomatic and asymptomatic patients (Aydin-Schmidt et al., 2017) or sodium dodecyle sulphate (SDS) (Polley et al., 2010). LAMP performed on whole blood extracted by SDS was able to detect 2–5 parasites/μL compared with 10 parasites/μL from DBS extracted with SDS (Polley et al., 2010). Commercial DNA extraction kits with closed systems such as PURE (Eiken Chemical company, Japan) for either small number of samples (Polley et al., 2013) or high throughput (Perera et al., 2017) minimize the risk of potential contamination found in the multistep DNA extraction protocols but with associated increased cost. LAMP performed on blood samples appeared to be more sensitive than when performed on saliva (Singh et al., 2013) or urine (Najafabadi et al., 2014) for identifying *P. falciparum* and *P. vivax*. However, no direct comparison of DBS with different volumes of whole blood or on different sample types has been performed using LAMP in the detection of *P. knowlesi*.

3.2.4 *The future of LAMP for* P. knowlesi *diagnosis*—

Overall, the optimal role for LAMP, as either a point of care diagnostic for case detection or a molecular tool for surveillance, remains unresolved. Platforms such as non-instrumented nucleic acid amplification by LAMP (NINA-LAMP) (Sema et al., 2015), lateral-flow device LAMP (Mallepaddi et al., 2018) and LAMP MinION nanopore sequencing (Imai et al., 2017) combined with appropriately sensitive species-specific primers may improve its applicability as a point-of-care diagnostic test. However, these technologies remain constrained by need for DNA extraction, risk of contamination, low sample throughput capacity and cost. LAMP also has been incorporated into several lab-on-a-chip platforms such as multiplex microfluidic arrays (Mao et al., 2018) and microchambers on cell microarrays (Ido et al., 2019) which hold much promise for improved field applicability of LAMP technology. However, except for LFD-LAMP, all of these platforms are yet to be fully validated in large numbers of *P. knowlesi* clinical samples.

In terms of surveillance, two high throughput platforms have been published to overcome the limited number of samples and prohibitive costs associated with existing commercial platforms for this application. The colourimetric high-throughput LAMP (HtLAMP) platform based on a 96 well plate required minimal infrastructure, was low cost and was applied in a resource limited setting (Britton et al., 2015). However, this assay required a separate DNA extraction process, whereas an alternative high-throughput LAMP (HTP

LAMP) was able to combine a 96 well plate platform with a closed DNA extraction process (Perera et al., 2017) albeit at likely high cost. However, only the HtLAMP platform has been evaluated with *P. knowlesi* specific primers (Britton et al., 2016).

Therefore, the applicability of LAMP for *P. knowlesi* diagnosis remains constrained by two significant challenges: analytical sensitivity to detect sub-microscopic parasitemia without compromising species specificity, and cost-effective applicability in resource limited settings as an alternative to PCR either as a point of care diagnostic tool or for purposes of *P. knowlesi* malaria surveillance.

4. Serology

4.1 Importance of serology for malaria surveillance

The use of serology as a tool to survey and monitor transmission and exposure to infection, in addition to measuring the success of interventions has been well documented for *P. falciparum* (Plucinski et al., 2018; Wu et al., 2020) and *P. vivax* (Edwards et al., 2021; Longley et al., 2020). Measuring antibodies provides a fast and simple means for evaluating transmission intensity (Stewart et al., 2009) and in combination with parasite prevalence data can be used to monitor changes in malaria transmission over time (Drakeley et al., 2005). In this context serological tools have a major advantage compared to molecular methods, as they can capture exposure from asymptomatic or transient symptomatic infections in those not seeking treatment (Corran et al., 2007). However, to date less focus has been afforded to understanding transmission for the other causes of human malaria including *P. knowlesi* using serological methods, meaning neglected infections from species such as *P. knowlesi* remain excluded from the elimination narrative (Barber et al., 2017b; Chin et al., 2020).

4.2 Development of serologic assays for *P. knowlesi* detection

To complement existing approaches for assessing exposure to infection, species-specific serological reagents are an essential part of the toolbox. The ability to accurately assess the prevalence of each infecting species and monitor the impact of control and intervention approaches on each could help to provide better guidance for country-level programmatic decision-making. Serology has a number of obvious advantages, specifically the ability to accurately measure recent and historical exposure to infection (Edwards et al., 2021; Helb et al., 2015). Increasingly serology assays are also being designed using multiplex platforms (Chan et al., 2021). These platforms have allowed for high-throughput biomarker discovery and evaluation which in turn has led to an expansion in both the number and utility of the protein targets available (Tran and Crompton, 2020). As more serology tools are discovered and developed, the utility of the individual, or panel of, targets need to be properly assessed. Early seroepidemiological studies aimed at assessing non-specific malaria exposure through cross-reactivity to *P. knowlesi* antigens were conducted using indirect haemaglutination tests (Kagan et al., 1969). This approach was based on crude parasite preparations generated from lysed infected erythrocytes taken from splenectomised rhesus monkeys. The unrefined nature of the antigen preparation would suggest there were antigens conserved across the *Plasmodium* spp. present in the assay, highlighted by the wide geographical distribution of

seropositive responses in non-*P. knowlesi* endemic settings seen in military personnel when using this technique (Kagan et al., 1969).

Designing recombinant proteins provides a more focused means for developing specific targets for use in serology assays. However, previous approaches have focused on targeting proteins such as MSP1 and AMA1 that shared moderate to high levels of sequence homology, and or exhibited cross-reactive responses with other *Plasmodium* spp. (Muh et al., 2020; Narum and Thomas, 1994; Waters et al., 1990). This includes other previous *P. knowlesi* antigens designed mainly to interrogate underlying parasite biology rather than for use as sero-surveillance tools in co-endemic areas such as *Pk*CSP (Sharma and Godson, 1985), *Pk*DPB (Singh et al., 2002), *Pk*SPATR (Mahajan et al., 2005), *Pk*LDH (Singh et al., 2012), *Pk*1-Cys peroxiredoxin (Hakimi et al., 2013), *Pk* knowpains (Prasad et al., 2012), *Pk*MSP1-42 (Cheong et al., 2013a,b), *Pk*MSP1-33 (Cheong et al., 2013a, 2013b), *Pk*MSP1-19 (Lau et al., 2014), *Pk*Trags (Tyagi et al., 2015), *Pk*MSP3 (Silva et al., 2016) and *Pk*SBP1 (Lucky et al., 2016). Such reagents likely would lack the required specificity to accurately define exposure to dissect *P. knowlesi* infections in regions co-endemic for other species, increasing the potential for false positive responses.

In order to facilitate *P. knowlesi* sero-surveillance in areas of Southeast with co-endemic *Plasmodium* species, Herman et al. used an in silico modelling approach to design four novel recombinant *P. knowlesi* antigen constructs from three genes: *Pk*SERA3-antigen 1, *Pk*SERA3-antigen 2, *Pk*SSP2/TRAP, and *Pk*TSERA2-antigen 1 (Herman et al., 2018). Initial target candidate genes were selected from known seroreactive *P. falciparum* orthologues, with protein sequences processed using analytical in silico tools aiming to maximise specificity for *P. knowlesi* against other *Plasmodium* species. This included iterative BlastP interrogation of target sequences against *Plasmodium* specific and other relevant databases, domain prediction, exclusion of signal peptides and transmembrane domains and glutathione-S-transferase tagging to aid expression solubility, and construction of phylogenetic trees to exclude high identity cross-species amino acid alignments. An *Escherichia coli* expression system was used to produce soluble, multimerised and stable antigen products with final yields from 11.9 to 20.5 mg/L. Blood-stage transcription of SERA3 and TSERA2 candidate genes was also confirmed, in contrast to the sporozoite stage SSP/TRAP gene. Finally, non-synonymous SNPs associated with the three known distinct *P. knowlesi* genetic subpopulations were characterized and shown to predominantly exist outside regions covered by the recombinant constructs, highlighting their potential utility for all *P. knowlesi* strains (Herman et al., 2018).

Validation of the four new recombinant antigens was subsequently conducted using ELISA to detect IgG responses for 97 individuals with acute *P. knowlesi* infections at three time points (day 0, day 7 and day 28) following treatment in a clinical trial in Sabah, Malaysia. Negative controls consisted of 26 Ethiopian non-*P. knowlesi* and 29 UK malaria-naïve sera, with cut-off values to define reactivity for *P. knowlesi* antigens taken as the mean OD (±3 SD) from the latter group. A single weakly positive sample was recorded for both SERA3 ag 1 and SSP2/TRAP in the Ethiopian negative controls. The best performing antigen was the *Pk*SERA3 antigen 2, which was positive in 67% of all time-points in *P. knowlesi* cases, with maximum positivity at day 7 and up to 50-fold increases compared to day 0 recorded.

Antibody prevalence for *Pk*SERA3 antigen 1, *Pk*SSP2 and *Pk*TSERA2 antigen 1 also peaked at day 7, however remained relatively low at 18%, 33% and 43% respectively. Using data adaptive boosted tree regression models, the combined ability of all four antigens to classify *P. knowlesi* exposure was 89% (IQR 43%–61%), defined as the cross-validated area under the receiver operating curve, with the highest relative variable importance contributed by *Pk*SERA3 antigen 2 at 50% (Herman et al., 2018).

A separate panel of 9 recombinant *P. knowlesi* antigens was generated by Müller-Sienerth and colleagues, based on the most highly expressed *P. falciparum* and *P. vivax* protein orthologues known to produce high antibody responses in endemic areas: CyRPA, GAMA MSP10, MSP4, MSP5, P12, P38, P41, and P92 (Müller-Sienerth et al., 2020). This study differed in approach by utilizing a mammalian expression system in order to improve the likelihood that appropriate post-translational modifications such as disulfide bonds are present (Crosnier et al., 2013). This in turn allows correct protein folding to recreate the original conformation of epitopes and therefore improve antibody recognition (Forsström et al., 2015). Malaysian sera from PCR confirmed *P. knowlesi* infections initially demonstrated the highest antibody responses in P12, MSP10 and P38 when quantified by ELISA. Cross-species seropositivity of these three *P. knowlesi* antigens was minimal when tested as part of a larger panel including 19 selected antigens for *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* using sera from small numbers of European returned travellers with malaria. Cross-reactivity was not shown to be related to the degree of sequence identity between protein orthologues, including for the P12 antigen despite a high sequence identity of 72% between *P. knowlesi* and *P. vivax*. Logistic regression classification models combining the three *P. knowlesi* antigens were then used to demonstrate on a larger dataset of 50 *P. knowlesi* sera and 66 uninfected controls from Malaysia a high AUC of the median ROC curve of 0.91. Using a model score of greater than 0.5 was shown to accurately diagnose 82% of *P. knowlesi* infections with a 3% false positive rate.

4.3 Potential use case scenarios for *P. knowlesi* serologic tools

In 2017 a meeting was convened in Paris to review the state-of-theart with antibody-based assays in the context of malaria surveillance (Greenhouse et al., 2019). A key aim was to define potential use-case scenarios in terms of use and evaluation of serological biomarkers in control and elimination programmes. Although focused on *P. falciparum* and *P. vivax*, many of the use cases could be applied to *P. knowlesi,* including: (i) documenting the absence of transmission over a given geographic space; (ii) stratification of transmission level; (iii) measuring the impact of interventions and monitoring changes in malaria transmission; and (iv) decentralized immediate public health response. The summary provided a detailed understanding of what tests would be required for a given situation/population, establishing target product profiles for the future development of diagnostics and surveillance tools (Greenhouse et al., 2019).

Using recently developed *P. knowlesi* antigens designed as species-specific reagents (Herman et al., 2018). Fornace and colleagues conducted a survey of 2503 participants to determine the level of exposure and infection within case-study communities across Sabah, Malaysia and neighbouring Palawan, Philippines (Fornace et al., 2018). This was the first

study to assess *P. knowlesi* infection and associated risk using antibodies, demonstrating a seroprevalence of 7.1% to *Pk*SERA3 antigen 2, including comparable seroreactivity between adult men and women (despite passive case detection of acute infections mainly consisting of men), and 4.2% seroprevalence in children <15 years. Despite a very low acute infection prevalence of 0.2% using PCR, the use of serological tools was able to demonstrate marked spatial heterogeneity in transmission intensity between sites (i.e., higher intensity in Malaysia compared to Philippines), and associations with *P. knowlesi* exposure and agricultural work, high forest cover or clearing around houses (Fornace et al., 2018).

This was followed by a large population-based cross-sectional malaria survey of 10,100 individuals to assess exposure and environmental risk to *P. knowlesi* across Northern Sabah, Malaysia (Fornace et al., 2019b). In this study, a multiplex Luminex platform was used to concurrently evaluate IgG responses for *P. knowlesi* SERA3 ag 2 and SSP2, in addition to 8 other antigens for *P. falciparum* and 5 for *P. vivax*. Classification of seropositive individuals for *P. knowlesi* (exposure in the last year), *P. falciparum*, and *P. vivax* was conducted using a data adaptive meta-learning algorithm (Super Learner, van der Laan et al., 2007). The overall seroprevalence of *P. knowlesi* was 5.1% (95% CI 4.8–5.4), with high antibody responses seen across all age groups. However, individual-level associations with higher *P. knowlesi* exposure risk in the final Bayesian hierarchical model were seen with increasing age and male sex, in addition to macaque contact and forest activities, with personal insecticide use protective. Household-level associations with exposure included raised level house construction, lower geographical elevation and fragmented land types classified at varying spatial scales around the household (intact forest, irrigated farming land, pulpwood and oil-palm plantations). The latter are consistent with separate modelling demonstrating the relative importance of different environmental variables at small scales of <1 km around households (proportion of cleared land, aspect, slope), compared to larger spatial scales of 4-5 km (fragmentation of deforested areas) (Brock et al., 2019).

The implementation of serological tools in national malaria control programmes would result in several potential benefits. Using serological exposure to understand the mechanistic links between land use change and *P. knowlesi* transmission highlights their utility in the possible design of spatially-targeted interventions, particularly for low-resource settings with scarce or absent occurrence data. Future use of serological tools outside highly studied areas in Malaysia are also vital to understand regional differences, including demonstrating the potential absence of transmission in certain areas. However, implementation of serology for *P. knowlesi* will require guidance on validated and consistent data analysis methods to define seropositivity cut-offs (Chan et al., 2021). This is particularly the case in multiplex platforms, which ideally need to take into account known antibody titers and kinetics, regional variation in transmission, and population-level immunity for other human-only *Plasmodium* species that may be providing some cross-protective effect (Muh et al., 2020) (Box 2).

5. Conclusion

P. knowlesi is an emergent public health threat across Southeast Asia. Implementation of improved detection methods and reporting practices for point-of-care diagnostics are vital

to understanding the disease burden and distribution of symptomatic human infections and allowing the timely administration of appropriate treatment. Newer lateral flow or novel RDTs with the ability to accurately differentiate *P. knowlesi* and other non-*P. falciparum* species are ideally required for this to occur. Molecular methods incorporating validated PCR assays for *P. knowlesi* can improve accurate regional case reporting to monitor progress towards elimination of other human-only species, with additional utility in detection of low-level infections to understand broader transmission dynamics. However, molecular tools remain expensive in low-resource settings and public health programmes may require further input to understand the background prevalence or predicted transmission risk in order to guide their optimal targeted deployment. The implementation of multiplex serological tools with *Plasmodium* species specific recombinant proteins allows improved understanding of geographically heterogenous *P. knowlesi* transmission intensity and associated underlying environmental drivers, in addition to the ability to guide both the design and evaluation of future spatially targeted interventions.

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BOX 1

Point-of-care summary and diagnostic reporting recommendations

These summary guidelines refer to *P. knowlesi* endemic areas in Southeast Asia.

Microscopy

- Microscopy remains a sufficiently sensitive first-line tool for *P. knowlesi* detection for the majority of low-level symptomatic infections.
- Routine microscopy is unable to differentiate *P. knowlesi* infections from *P. malariae*, or early ring stage infections with *P. falciparum*, and misidentification with *P. vivax* also occurs.
- In knowlesi-endemic areas where microscopy is used as a first-line tool, blood films in which parasite morphology appears similar to *P. malariae* and the parasite count is less than 5000/μL should be reported as *'P. knowlesi/P. malariae'* (or 'suspected *P. knowlesi'* in relevant national guidelines).
- Blood films in which parasite morphology appears similar to *P. malariae* and the parasite count is greater than 5000/μL should be reported as *P. knowlesi*, and for any infections with a parasite count greater than 15,000/μL consider early administration of intravenous artesunate due to the high risk of severe disease.

Rapid diagnostic tests

- Current RDTs (pLDH or aldolase-based) remain insufficiently sensitive to detect low-level *P. knowlesi* infections and should not replace microscopy as a first-line diagnostic tool where possible.
- Cross-reactivity of *P. knowlesi* with *P. falciparum* and *P. vivax*-pLDH based test components occurs, however is insufficiently sensitive and specific for diagnostic purposes.
- Where RDTs are used as a first-line diagnostic tool, they should include a *P. falciparum*-specific HRP2 test component.
- RDTs should additionally include the highest performing pan-pLDH test possible to detect non-falciparum malaria.
- RDTs containing a pan-aldolase test component should not preferentially be used.
- Positive RDT results should where possible have subsequent PCR conducted for final *Plasmodium* species confirmation.

BOX 2

Molecular and serology methods summary and diagnostic reporting recommendations

These summary guidelines refer to *P. knowlesi* endemic areas in Southeast Asia.

PCR and LAMP

- A number of PCR assays have been developed which are highly sensitive and specific for *P. knowlesi* detection.
- A validated *P. knowlesi* species-specific PCR assay should be included routinely as part of any PCR assay conducted for suspected or microscopy positive malaria from Southeast Asia.
- Nested PCR assays targeting the 18S ssu rRNA gene are the most commonly used for *P. knowlesi* detection. Compared to real-time PCR they have lower sensitivity and are more time consuming, although are less expensive.
- The most well-validated nested PCR assays include an initial *P. genus* amplification round, followed by species specific detection for *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi.* Where possible, species-specific detection for *P. cynomolgi* should also be considered.
- Early *P. knowlesi* designed nested primers, Pmk8-Pmkr9, can cross-react with *P. vivax* monoinfections to produce a false-positive mixed *P. vivax/P. knowlesi* result.
- Updated and more specific nested ssu rRNA primers for *P. knowlesi* include PkF1160, PkF1140 and PkR1150, or Kn1f and Kn3r, which have been validated against zoonotic and non-zoonotic *Plasmodium* species.
- Some older nested PCR assays contain *P. vivax* species-specific primers which have been shown to cross-react with *P. knowlesi* and *P. cynomolgi.* The well-established VIV1-VIV2 primers do not.
- PCR has been successfully used in Malaysia, a pre-elimination setting for non-zoonotic malaria, for accurate species reporting of all zoonotic and non-zoonotic malaria cases, and for research surveillance purposes across Southeast Asia including the detection of submicroscopic or asymptomatic infections.
- Reference laboratory molecular testing of malaria cases for both zoonotic and non-zoonotic *Plasmodium* species needs to be more widely implemented by National Malaria Control Programmes across Southeast Asia to accurately identify the burden of zoonotic malaria and to more accurately monitor the success of human-only malaria elimination programmes.
- *P. knowlesi* mature gametocytes are able to be detected by real-time qPCR assays targeting *pks*25 mRNA, with validation and implementation

in naturally infected human clinical studies demonstrating much higher sensitivity compared to microscopy.

- LAMP based platforms for *P. knowlesi* diagnosis remain constrained by two main challenges: the ability to detect submicroscopic infections while retaining high species-specificity, and any significant cost benefit compared to PCR for diagnostic species-confirmation or surveillance.

Serology

- Antigenic reagents have been identified that can distinguish exposure to *P. knowlesi* infection from human-only malaria with a reasonable degree of specificity.
- Antibody responses to these antigens can be used at the population-level to assess the degree of exposure, including the absence of exposure.
- Antibody responses can also be used to identify demographic and spatial risk factors which can support targeting of control approaches.
- Population-level evaluation of novel targets would allow the identification of antigens for development of species-specific lateral flow devices to help with rapid case management.

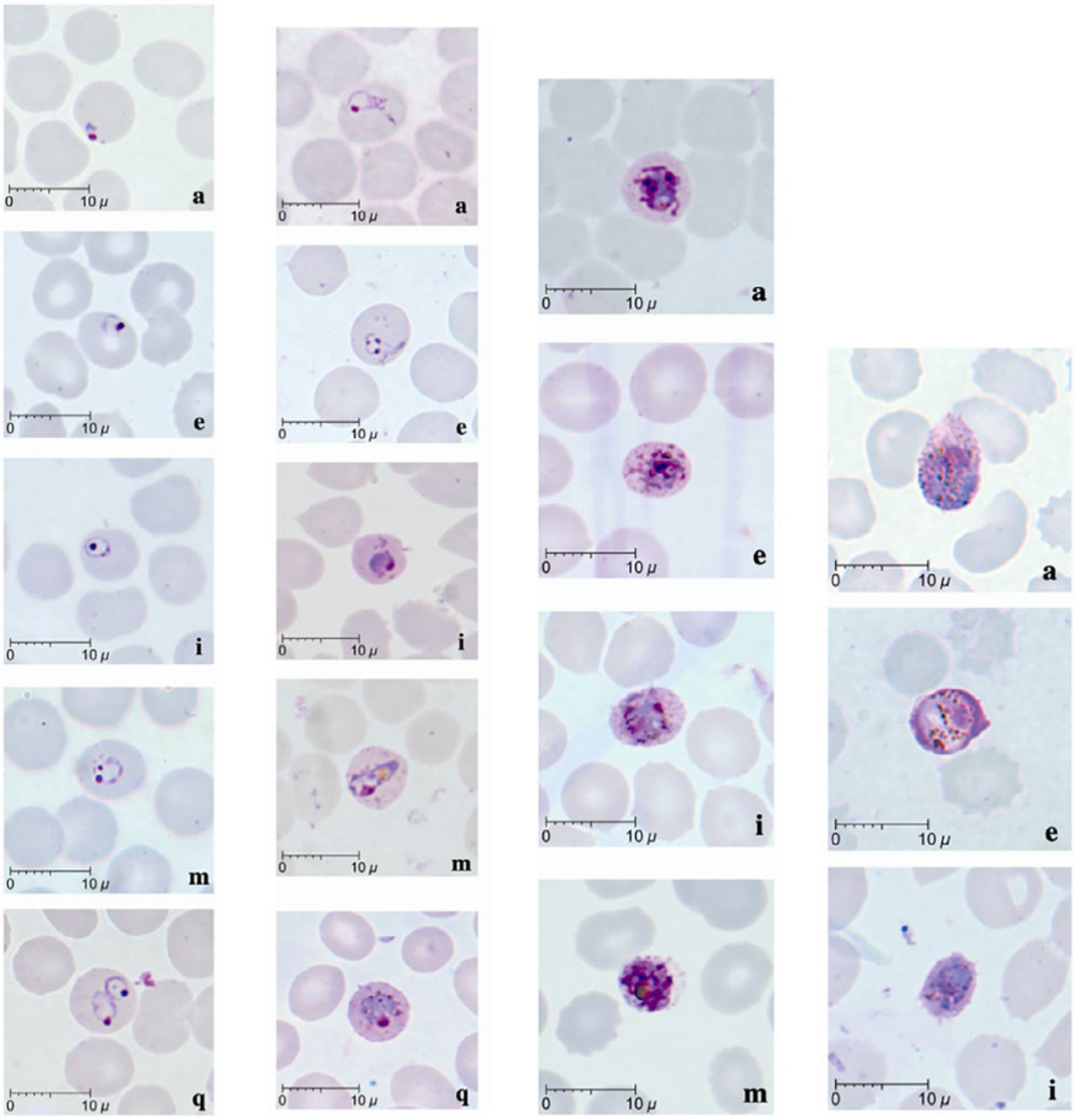

Fig. 1.

Morphology of *P. knowlesi* life-stages. L-R columns: 1. Early trophozoites (rings): note normal sized RBC, multiply infected RBCs, double chromatin dots; 2. Late trophozoites (including band forms); 3. Schizonts; 4. Gametocytes (a) macrogametocyte, (e) microgametocyte, (i) young gametocyte. *Adapted from* Lee, K.S., Cox-Singh, J., Singh, B., 2009b. *Morphological features and differential counts of Plasmodium*

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Table 1

Sensitivity of individual antigen/antibody targets in commercially available RDTs evaluated to date for detecting *P. knowlesi*.

RDT name (manufacturer; product code)	WHO RDT testing round/ product code	Target antibody (epitope)	Target *Plasmodium* species	Sensitivity for *P. knowlesi*, n/N, % (95% CI)	Parasitaemia Median parasites/ µL, (IQR) [range]	Lowest parasite count/µL detected	Location	Year	References
First Response Malaria Antigen pLDH/HRP2 Combo Card Test Premier Medical Corporation Ltd., Mumbai, India	1, 2 I16FRC25	Pan-pLDH	*P. genus*	30/34, 88 (73–95)	7493 [907–584,018]	907	Kota Kinabalu, Sabah, Malaysia	2013	Barber et al., (2013)
		Pf-HRP2	*P. falciparum*	0/34, 0 (0)					
ParaHIT Total Dipstick SPAN Diagnostics, Ltd., Surat, India	1, 2 55IC204-10	Pan-aldolase	*P. genus*	22/96, 23 (16–32)	50,794 [395–340,954]	4412	Kota Kinabalu, Sabah, Malaysia	2013	Barber et al., (2013)
		Pf-HRP2	*P. falciparum*	0/96, 0 (0)					
BinaxNOW Malaria Inverness Medical Innovations, Inc., FL, USA	1, 2 IN660050	Pan-aldolase	*P. genus*	8/28, 29 (12–26)	9131 [159–911,616]	1/12 for <500/ µL	Sarikei, Sarawak, Malaysia	2014	Foster et al., (2014)
		Pf-HRP2	*P. falciparum*	0/28, 0 (0)	9131 [159–911,616]	NA			
Paramax-3 Rapid Test for Malaria Pan/Pv/Pf Zephyr Biomedicals, India	1, 2 50320025	Pan-pLDH	*P. genus*	10/25, 40 (21–59)	9131 [159–911,616]	2/6 for <5000/ µL	Sarikei, Sarawak, Malaysia	2014	Foster et al., (2014)
		Pv-pLDH	*P. vivax*	8/25, 32 (15–54)	9131 [159–911,616]	1/8 for <500/ µL			
		Pf-HRP2	*P. falciparum*	1/25[a], 4 (0–20)	9131 [159–911,616]	—			
OptiMAL-IT DiaMed, CA, USA	1, 3 710024	Pf-pLDH (17E4)	*P. falciparum*	18/28, 64 (44–81)	9131 [159–911,616]	1/3 for <500/ µL	Sarikei, Sarawak, Malaysia	2014	Foster et al., (2014)
				55/190, 29 (22–35)	2301 [30–641,464]	30parasite/µL	Kota Kinabalu, Sabah, Malaysia	2014	Grigg et al., (2014)
		Pan-pLDH (19G4)	*P. genus*	20/28, 71 (54–88)	9131 [159–911,616]	1/3 for <500/ µL	Sarikei, Sarawak, Malaysia	2014	Foster et al., (2014)
				57/189, 30 (24–37)	2301 [30–641,464]	30parasite/µL	Kota Kinabalu, Sabah, Malaysia	2014	Grigg et al., (2014)
CareStart™ Malaria HRP2/ pLDH (Pf/VOM) Combo Access Bio, Inc. NJ, USA	2, 4 G0171	VOM-pLDH	*P. vivax, P. ovale, P. malariae*	77/178, 43 (36–51)	2301 [30–641,464]	50 parasites/ µL	Kota Kinabalu, Sabah, Malaysia	2014	Grigg et al., (2014)
		Pf-HRP2	*P. falciparum*	4/183[a], 2 (0–4)	2301 [30–641,464]	—			

[a]False positive Pf-HRP2 result.

Systematic evaluations, pre-treatment, fresh whole blood samples only.

Table 2

Molecular detection methods for *P. knowlesi*.

Type of assay	Gene target	Primers or probe(s) sequence	Limit of detection	Advantages	Disadvantages	References
Nested PCR	SSU rRNA	Pmk8 Pmkr9	1–6 parasites/μL	Highly conserved; can be combined with nested non-zoonotic malaria	Can spuriously amplify *P. vivax*	Singh et al. (2004)
		PKF1160 PKR1150 PKF1140	1–10 parasite genomes/sample	Sensitive/specific; can be combined with nested non-zoonotic malaria	Lengthy amplification rounds (105 mins each nest)	Imwong et al. (2009)
		Kn1f Kn3r	1–6 parasite/μL	Sensitive/specific; can be combined with nested non-zoonotic malaria		Lee et al. (2011)
		PK18SF PK18SRc	NR			Jongwutiwes et al. (2011)
	DHFR-thymidylate synthase	Pk-Lin-F Pk-Lin-R	Not reported	1 μL of genomic DNA; 0.4 U Taq; heminested; amplification efficiency and specificity less prone to genetic variations within species or cross-hybridization between species	Lengthy amplification rounds (90 and 105 mins)	Tanomsing et al. (2010)
	Mitochondrial cytochrome B	PKCBF PKCFR	1–10 copies/μL	Detected on saliva and urine; highly sensitive - more copies in the mtDNA than 18S rRNA; highly conserved with limited variation; Cytb saliva detection better than microscopy	Secondary PCR has to be performed separately for each species	Putaporntip et al. (2011)
		PKCBF PkCBR-ed	Not reported			Tanizaki et al. (2013)
		PKCBF PkCBR-ed	Not reported	1μL DNA; *Pk* DNA from urine and faeces is detected for a longer period than from blood	*P. knowlesi* only	Kawai et al. (2014)
	SICAvar	SICAf1 SICAf2 SICAr1	0.1 parasites/μL	Sensitive	*P. knowlesi* only; specificity not cross-checked using *P. vivax* PCR on clinical samples	Lubis et al. (2017)
Real-time PCR	SSU rRNA	Pk1 probe Pk2 probe	10 copies/μL	Simultaneous detection of all *Plasmodium* species; no cross-reactivity with other pathogens	Cannot differentiate mixed *P. vivax* and *P. knowlesi*; four probes; expensive	Babady et al. (2009)
		NVPK-F NVPK-R NVPK-Probe	5 copies/reaction (<1 parasites/μL)	Sensitive	*P. knowlesi* only	Link et al. (2012)
		Pke'F Pkg'R	100 copies/μL	Detects all species; low cost compared to other RT-PCR	Still expensive; two probes; different melting temperatures	Oddoux et al. (2011)
		Pk probe	10 copies/μL (<3 parasites/μL on clinical samples)	Rapid results; low contamination risk; high throughput; quantification of parasites	Standard 5 μL DNA used	Divis et al. (2010)

Type of assay	Gene target	Primers or probe(s) sequence	Limit of detection	Advantages	Disadvantages	References
				No cross-reactivity-including *P. cynomolgi* and *P. inui*; needs Plasmo1 and 2 primers		
	Plasmepsin	Pk (probe)	1–6 parasites/μL	Five species High reported sensitivity and specificity 4.5 h from DNA preparation to PCR amplification 96-well (for 5 species)	Limited to BioRad iQ5 multicolor RT PCR; no lower limit of detection tested with clinical samples	Reller et al. (2013)
Single step PCR	SSU rRNA		0.25 parasites/μL	1.5 μL DNA; faster, less labour intensive; Detects all species; very sensitive; can detect co-infections; less expensive	Inconsistent results due to primers and DNA competition effects; minimal samples of *P. ovale* and *P. malariae*	Chew et al. (2012)
	[a]Consensus repeat sequences	Pkr140	1 parasite/μL	Non-nested; 1 μL DNA; sensitive	*P. knowlesi* only; specificity not well validated with lack of clinical samples tested; bioinformatics approach to sequence target selection	Lucchi et al. (2012)
	Cytochrome c oxidase subunit 1	PlasmoCOI (FDB+RDB)	Not reported		Validated on a single *P. knowlesi* sample	Setiadi et al. (2016)
LAMP	β-tubulin	F3, B3, FIP, BIP, FLP, BLP	100 plasmid copies/ sample			Iseki et al. (2010)
	AMA-1	F3, B3, FIP, BIP, FLP, BLP	10 plasmid copies/ sample			Lau et al. (2011)
	18S rRNA	F3, B3, FIP, BIP, FLP, BLP	10plasmid copies/ sample			Lau et al. (2016)
	Mitochondrial	Pk 101	0.2 parasites/μL			Britton et al. (2016)

[a]Consensus repeat sequences: chromosome ends and interiors; not protein-encoding.

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