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REVIEW

Plasmodium knowlesi malaria: current research perspectives

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Abstract: Originally known to cause simian malaria, *Plasmodium knowlesi* is now known as the fifth human malaria species. Since the publishing of a report that largely focused on human knowlesi cases in Sarawak in 2004, many more human cases have been reported in nearly all of the countries in Southeast Asia and in travelers returning from these countries. The zoonotic nature of this infection hinders malaria elimination efforts. In order to grasp the current perspective of knowlesi malaria, this literature review explores the different aspects of the disease including risk factors, diagnosis, treatment, and molecular and functional studies. Current studies do not provide sufficient data for an effective control program. Therefore, future direction for knowlesi research is highlighted here with a final aim of controlling, if not eliminating, the parasite.

Keywords: zoonosis, epidemiology, diagnosis, treatment, molecular, functional studies

Introduction

Plasmodium knowlesi, originally known to cause simian malaria, is now recognized as the fifth human malarial parasite.[1] Human knowlesi infections have been reported in nearly all the countries in Southeast Asia and in travelers returning from these countries.[2–4] The cumulative cases of knowlesi malaria in the Southeast Asia region from 2004 to 2015 is 3413 cases with 91.47% of these found in Malaysian Borneo.[5]

The parasite's natural reservoir hosts are the long-tailed macaque (*Macaca fascicularis*),[6] pig-tailed macaque (*Macaca nemestrina*),[7] and the banded-leaf monkey (*Presbytis melalophos*).[8] Thus far, several mosquito species belonging to the Leucosphyrus group have been incriminated as vectors for knowlesi malaria, namely, *Anopheles hackeri*,[9] *Anopheles latens*,[10] *Anopheles cracens*,[11] *Anopheles balabacensis*,[12] *Anopheles dirus,*[13] and *Anopheles introlatus*.[14] It is not surprising that the geographical distribution of *P. knowlesi* is confined to Southeast Asia since it follows the limits of natural distribution of both its natural hosts and vectors.[15] To date, the pattern of knowlesi infection in humans does not indicate that they are transmitted via human–mosquito–human.[16–18]

Several factors have led to an increased reporting of *P. knowlesi* cases. These include better diagnostic capacity, decreasing human malaria cases which in turn reduces relative immunity, increased awareness to *P. knowlesi*, and close proximity of humans with natural reservoir hosts or infected vectors due to changes in human land use.[5,18] Many studies have been carried out on *P. knowlesi* ever since it was first described in 1932 by Knowles and Das Gupta.[6] This has led to important findings in malarialogy, which include the discovery of antigenic variation in malaria[19] and demonstration of an absolute requirement for the Duffy receptor for the invasion of

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red cells by the parasite.[20] Furthermore, since the publishing of report of Singh et al[21] that largely focused on naturally acquired *P. knowlesi* infection in humans, the disease and the parasite have been further characterized, both clinically and molecularly. In order to grasp the current perspective of knowlesi malaria, this literature review explores the different aspects of the disease including risk factors, diagnosis, treatment, molecular and functional studies, mainly focusing on publications from the past five years.

Ecological and individual-level factors associated with infection

Risk factors associated with *P. knowlesi* infection have been an area of interest over the past few years as researchers have begun to investigate the links between environmental, occupational, sociodemographic, and domestic factors that may contribute to an increased risk of infection. The understanding and identification of these risk factors would be invaluable in designing appropriate and effective public health interventions for knowlesi malaria.

With the advancement of technology, including the use of unmanned aircraft systems or drones, the task of mapping spatial and geographic data to identify environmental factors has been greatly improved.[22] Fornace et al studied the association between *P. knowlesi* incidence and various environmental variables via satellite-based remote sensing data in Kudat and Kota Marudu, Sabah.[23] The study found that factors such as more than 65% forest cover in a 2 km radius, higher historical forest loss, and lower elevations were significantly associated with higher incidence of *P. knowlesi* infection. The authors postulated that the association may be a result of shifts in human settlement as well as changes in the macaque and mosquito habitat due to deforestation and agricultural activities.

Sociodemographic and individual-level factors may also carry increased risk for knowlesi malaria infection. A two-year case–control study was carried out by Grigg et al from 2012 to 2015 in Sabah, Malaysia.[24] From a demographic standpoint, participants with ages over 15 years were found to have increased risk of *P. knowlesi*, whereas other *Plasmodium* infections were found to be higher in individuals who were younger than 15 years of age. There was also a strong gender bias toward male participants indicating that males over the age of 15 years carried a higher risk of *P. knowlesi* infection. This suggests that health intervention programs focusing on maternal and child health may be inadequate for *P. knowlesi* malaria intervention. Other studies have also indicated higher parasitemia and increased *P. knowlesi* disease severity in association with age.[25–27] Higher risk in association with age may relate to occupational risk, as older individuals seek job opportunities in the area. Farming and palm oil plantation work were also identified to be associated with higher risk of infection.

Domestic and peri-domestic factors, such as having open eaves and gaps in the walls, having long grass around the house, sleeping outside of the house, and recent clearing of vegetation, were also associated with a high risk of *P. knowlesi* infection. A reduction of risk was observed in households with indoor residual spraying being practiced, although bed nets only proved to have a marginal effect on risk.

It was noted that recent presence of monkeys was a strong predictor for risk indicating a high likelihood that monkey to human transmission is still the main transmission pathway rather than human to human transmission. Separately, it was also noted that G6PD deficiency conferred some form of protection against *P. knowlesi*, with it being associated with decreased risk of *P. knowlesi* infection, similar to what has been observed in *Plasmodium vivax* malaria.[28] The presence of young sparse forest and rice paddy around the house similarly were significantly associated with reduced *P. knowlesi* risk.[24]

A study by Herdiana et al in 2016 also looked at malaria risk factor assessment using both active and passive surveillance in Indonesia.[29] Initial screening revealed a total of 19 *P. knowlesi* cases which was an unexpected discovery in the study. A comparison of *P. knowlesi* cases to non-cases indicated risk factor findings similar to the study by Grigg et al, with increased risk for *P. knowlesi* infection: male gender, participants of adult age, and forest exposure or forest-related work, as well as spending time overnight in the forest. When comparing *P. knowlesi* cases with *Plasmodium falciparum* and *P. vivax* infections, it was noted that *P. knowlesi* cases were more likely linked to forest exposure and peri-domestic factors.

A study by De Silva et al compared the distribution of the different Duffy genotypes among *P. knowlesi*-infected patients and healthy donors to determine if there was an association between Duffy genotypes and susceptibility to *P. knowlesi* infection.[30] The authors argued that due to the overwhelming homogeneity of the Duffy distribution in the region, assessment of susceptibility was not feasible. However, further studies into the Duffy distribution between *P. knowlesi* patients in Peninsular and Borneo Malaysia may allow for *P. knowlesi* susceptibility studies, especially if the Duffy distribution between both regions is markedly different.

Diagnosis

Microscopy is the gold standard for malaria diagnosis, but it has its limitations as ring forms of *P. knowlesi* resemble *P. falciparum* and trophozoites and schizonts resemble those of *P. malariae* and, hence, cannot be reliably differentiated.[2,31,32] Most *P. knowlesi* infections have been identified as infections of more benign *P. malariae*, which has been associated with failure to diagnose severe malaria and consequent delayed parenteral artesunate, with fatal outcomes.[33] In *P. knowlesi* endemic areas, microscopic diagnosis of *P. malariae* should be reported and treated as *P. knowlesi* to reduce *P. knowlesi* case-fatality rates.[33] Limitation of miscroscopic diagnosis has been reported in Sabah, where 21% and 38% of blood films of *P. malariae* and *P. knowlesi* were diagnosed as *P. falciparum* and *P. vivax*, respectively, by PCR.[34] Low parasitemia is fairly common in knowlesi malaria and can cause fever.[35] Knowlesi malaria with low parasitemia may not be detected by microscopy. Better methods for the diagnosis of knowlesi malaria such as molecular methods are needed.

To date, there are no immunochromatographic rapid diagnostic tests (RDTs) specifically designed for *P. knowlesi* detection. Currently available malaria RDTs have been mainly used for detection of *P. falciparum* and *P. vivax* infections. OptiMAL-IT, one of the first RDT that could detect *P. knowlesi*, could mistakenly identify it as *P. falciparum*, as the monoclonal antibody used to detect *P. falciparum* histidine rich protein II (HRP-2) cross-reacts with *P. knowlesi*. Among all the other RDTs, OptiMAL-IT has the highest sensitivity for detecting *P. knowlesi*, although the percentage is still low (32%–72%).[36–38] Other RDTs include BinaxNow that measures antibody response to nonspecific pan malarial aldolase, and Paramax-3 and Entebe Malaria Cassette specific for *P. vivax* lactate dehydrogenase, which can detect *P. knowlesi* as *P. vivax*, is mostly still unable to distinguish *P. knowlesi* from *P. vivax*.[37,38] Sensitivity of RDTs to knowlesi malaria is poor, particularly at low parasite densities,[36,37,39] and they are not currently suitable for clinical use. However, RDTs are so far the only commercially available point-of-care diagnostic tool due to it being user-friendly, rapid, and cost-effective. Other limitations of RDTs include HRP2 deletion, inadequate quality control for RDT, and limited heat stability to be used on the field.[40]

While microscopy remains the gold standard for malaria diagnosis and RDTs as supplementary test, diagnostic method for clinical purposes must consider the potential inaccuracies of these two; thus, molecular methods such as PCR are needed to further confirm and differentiate the human *Plasmodium* species. Confirmation of species by PCR will also allow appropriate administration of primaquine to patients with *P. vivax* or *P. ovale* infection.[36]

Molecular methods, such as PCR and real-time PCR, are more accurate in detecting *P. knowlesi* and are valuable for species identification in cases of mixed malaria infection.[41] Nested and real-time PCR based on 18S rRNA gene can detect *P. knowlesi* in as low as 1 parasite/μL of blood.[42,43] However, due to lack of facilities and the lengthy procedure, PCR is not widely used in all endemic areas. Loop-mediated isothermal amplification (LAMP) is another promising molecular diagnostic technique that is applicable for bedside use as it is sensitive, specific, rapid (60–90 min), and easy to use. LAMP does not require costly machine and expertise.[44] The Eiken Loopamp™ MALARIA Pan Detection kit targeted at *Plasmodium* genus can detect all malaria species. It has been shown to be highly sensitive to *P. falcip*arum, *P. vivax*,[45–48] and *P. knowlesi*.[49] However, this method is unable to identify species of infecting *Plasmodium* and quantification of positive samples is also impossible. Species-specific LAMP assays for all five human malaria species have been developed and were highly sensitive and specific.[50] However, due to its high sensitivity nature of LAMP, cross contamination can occur easily.[50]

Point-of-care molecular tools for rapid malaria diagnosis have also been reported. A lab-on-chip PCR diagnostic platform for malaria, the Accutas system, can detect five species of human malaria with high sensitivity (97.4%) and specificity (93.8%), and it can be performed directly with unprocessed blood (time saving).[51] Truelab Uno, based on TaqMan chemistry, has 100% sensitivity and specificity compared to the nested PCR.[52] Other point-of-care molecular tools for malaria include Illumigene Malaria LAMP workflow, nanomal, and nucleic acid lateral flow immunoassay DIAGMAL.[53] However, these molecular methods were not tested specifically for *P. knowlesi*.

Although molecular methods are the most efficacious in diagnosing *P. knowlesi* infection, most of these tests, especially nested PCR, can produce a false-positive *P. knowlesi* result in *P. vivax* infections.[54] On top of that, it requires expensive equipment and clean facility. An ideal molecular diagnostic test for point-of-care diagnosis of all five human malaria species that is cost-effective and suitable for the resource-limited setting is yet to be developed.

Treatment and drug resistance

Due to its short asexual cycle of 24 h, infection with *P. knowlesi* can rapidly progress into severe malaria that can be fatal.[55] The treatment guidelines developed by the World

Health Organization (WHO) for all human malaria including knowlesi malaria is based on four core principles: early diagnosis and prompt effective treatment, rational use of antimalarial agents, combination therapy, and appropriate weight-based dosing.[56] The proposed choice of treatment depends mainly on whether the patient presents with uncomplicated or severe infection. In 2015 guidelines for the treatment of malaria, WHO recommends that adults and children with uncomplicated knowlesi malaria be treated with artemisinin-based combination therapy (ACT) (except for pregnant women in their first trimester) or chloroquine. However, the latter is not recommended in areas with chloroquine-resistant *Plasmodium* species. Whereas in severe malaria, WHO suggests the administration of parenteral artesunate for at least 24 h followed by ACT in both adults and children.[56]

Since the history of *P. knowlesi* infection in humans is relatively short compared to other human malaria, only few studies have been done to assess the sensitivity of *P. knowlesi* toward different antimalarials. A prospective observational study in adults demonstrated that chloroquine together with primaquine was successful in treating uncomplicated knowlesi malaria.[57] In a randomized control trial, Grigg et al found both artesunate–mefloquine combination therapy and chloroquine monotherapy to be very effective in treating adults and children with uncomplicated *P. knowlesi* infection. However, the clearance of parasite and fever was notably faster in those receiving artesunate–mefloquine combination therapy.[58] A more recent randomized controlled trial comparing the efficacy of arthemeter–lumefantrine and chloroquine concluded that the former was effective in treating uncomplicated knowlesi malaria with good tolerability and rapid therapeutic response, thus supporting its use as a first-line ACT treatment policy for malaria in Malaysia.[27] In severe knowlesi malaria, intravenous artesunate has been shown to be effective with reduction in fatality rate.[25,59,60] Additionally, deaths from knowlesi malaria have been linked to the delay in administering intravenous artesunate.[61,62]

An ex vivo drug sensitivity assay using clinical *P. knowlesi* isolates revealed that they were sensitive to artemisinins and chloroquine but were less sensitive toward mefloquine.[63] Despite no evidence of mefloquine resistance from these studies and its monotherapy being reported to successfully treat uncomplicated knowlesi malaria,[64,65] using mefloquine as monotherapy for knowlesi malaria is discouraged.[55] This is likely due to concerns arising from treatment failures reported in rhesus monkeys[66,67] and in a single human knowlesi case.[68]

In studying the drug resistance mutations, Tiyagi et al found that the orthologs for known *P. falciparum* drug resistance genes, namely, chloroquine resistance transporter (*Pkcrt*) and dihydrofolate reductase (*Pkdhfr*) of their *P. knowlesi* isolates were all wild type.[69] Grigg et al did a similar study and found moderately diversed *Pkdhfr* sequence among their *P. knowlesi* isolates. Nevertheless, there was no evidence of selective drug pressure in humans.[70] In addition to crt and dhfr, other orthologs of *P. falciparum* drug resistance genes, including multidrug resistance-1 (mdr1), dihydropteroate synthase (dhps), and kelch K13, were also looked at, with no signs of positive selection.[71] Since only human hosts would be expected to have antimalarial drug exposure and as long as the transmission of *P. knowlesi* remains zoonotic, the absence of drug selection pressure will make it unlikely to develop antimalarial drug resistance.

Although current state of *P. knowlesi* treatment shows no resistance toward antimalarials, research on new therapeutic candidates should not be halted as multiple antimalarial-resistant *Plasmodium* sp. strains are emerging in Southeast Asian countries particularly in Cambodia, Myanmar, and Thailand.[72] These therapeutic candidates could aid in resolving antimalarial-resistant problems in other *Plasmodium species* or could be used as synergists and drug combinations to increase the efficiency of currently available antimalarials.

Molecular epidemiology and diversity

Genetic polymorphism studies not only guide malaria vaccine design[73] but also help us understand the population history and structure of a parasite and thus its adaptive potential. Hitherto, proteins of *P. knowlesi* involved in invasion of erythrocytes are generally under purifying (negative) selection (Table 1), in most cases different from those of *P. vivax* and *P. falciparum*.[73–83] Negative selection may imply that the genes are under functional constrains. At the same time, the mutations are deleterious to the parasite and the *P. knowlesi* population is screening for best-adapted variants.[75] Long-term population expansion of *P. knowlesi* in Malaysia has been suggested to be the cause of this selection.[73,79,83] There are, however, some differences in natural selection of these proteins in different geographical regions and different parts of the protein.[71,78,84] These studies have also demonstrated that most of these genes are genetically diverse and to some extent more polymorphic than their counterparts in *P. falciparum* and *P. vivax.* Whole genome sequencing of *P. knowlesi* clinical isolates from Sarawak revealed them to have much higher nucleotide diversity than *P. falciparum* and *P. vivax.*[71]

Table 1 Selection pressure on proteins of *P. knowlesi* involved in erythrocyte invasion

Proteins	Origin of analyzed samples	Selection pressure in *P. knowlesi*	References
Duffy binding protein alpha region II	Clinical samples from Peninsular Malaysia and Borneo	Purifying	73, 77
Normocyte binding protein Xa	Clinical samples from Peninsular Malaysia and Borneo	Purifying	74
Apical membrane antigen-1			
Whole protein	Clinical samples from Sabah	Purifying	75
	Clinical samples from Sarawak	No evidence of diversifying selection	85
Domain I	Clinical samples from Peninsular Malaysia	Purifying	80
Merozoite surface protein 3 (C-terminal)	Clinical samples from Peninsular Malaysia	Purifying	76
Circumsporozoite protein (nonrepeat regions)	Clinical samples from Peninsular Malaysia and samples from long-tailed macaques	Purifying; Slight positive at the C-terminal Th2R/Th3R region	78
Gamma protein region II	Clinical samples from Peninsular Malaysia and Borneo	Purifying	79
Merozoite surface protein 1			
Conserved domains	Clinical samples and samples from pig-tailed macaques and long-tailed macaques in Thailand	Purifying	81
MSP-1_{42}	Clinical samples from Selangor and Sabah	Purifying	83
Rhoptry-associated protein 1	Clinical samples from Peninsular Malaysia	Purifying	82

Furthermore, the gamma protein region II (γRII)[79] and circumsporozoite protein (CSP)[78] of different isolates from varying regions and isolation time exhibit different lengths.

Assuming that these sequenced proteins from various sources (human and macaque) are representative of the gene pool in macaques, the high genetic diversity and deviation of selection pressure on these proteins from those of *P. vivax* and *P. falciparum* demonstrate intrinsic differences in the immunological targets used by the respective host species (macaques and humans).[85] The *P. knowlesi* proteins' immunogenic roles have placed them under selection pressure to generate high polymorphism for immune evasion.[79] These polymorphisms are probably results of recombination and reproduction cycle of *P. knowlesi* in multiple hosts.[75] Indeed, the *Anopheles* vector may exert strong evolutionary pressure on vector-related genes of the parasite, which is an evidence of adaptation of the parasite to the vector distribution or vectorial capacity.[86]

These genetic diversity studies also uncovered the existence of multiple *P. knowlesi* lineages. It began with the observation of consistent dimorphisms (two distinct groups) in many of the studied proteins originating from humans and macaques. This distinction is not only associated with two separate geographical regions, that is, Peninsular Malaysia/Thailand and Borneo,[73,76,77,79,82,84,87–90] but also correlated with macaque host types, that is, with the long-tailed and pig-tailed macaque.[91] Further genome sequencing revealed three distinct clusters. The two major sympatric clusters are associated with the long-tailed or pig-tailed macaque, and one cluster consisted of isolates from Peninsular Malaysia.[71,86,91] This was also evident from the study on NBPXa protein, with the third cluster found only in Peninsular Malaysia. This led Ahmed et al to postulate that the negative selection on this gene could be the driving force in the evolution and separation of the protein into three clusters.[74]

The data show that genetic divergence of *P. knowlesi* resulted not only from long-term geographic isolation (between Peninsular and Bornean isolates) but also through extended, isolated transmission cycles within different macaque hosts but with evidence of recombination when coming in contact with each other.[86] These phenomena, clearly driven by evolution, are pertinent to understanding knowlesi infection in humans and macaques. The genomic diversity seen in *P. knowlesi* is likely caused by geography, as well as the myriad of hosts, vector distribution, and ecological changes.

When studying the adaptation of *P. knowlesi* in vitro to different host erythrocytes, deletion of 13 genes in clones adapted to cynomolgus blood and deletion of two and duplication of four genes in clones adapted to human blood were observed. In vitro adaptation from macaque erythrocyte to human erythrocyte shows that there is a gradual increase in

human erythrocyte invasion efficiency. Hence, *P. knowlesi* shows immense ability to adapt via improved ability to invade human erythrocytes, thereby increasing virulence and multiplication rates.[92]

Currently, we know very little of the differences in characteristics and pathogenesis of the three subpopulations of *P. knowlesi*.[91] There are some early evidences that they may exhibit differential pathogenesis.[87,88,93] Moreover, the dimorphisms seen in MDR2 and MRP1 (transporter genes related to antimalarial drug resistance)[89] should not be overlooked as their phenotypic characteristics and how they react to current antimalarials remains obscure to us. Isolation and investigation of these three subpopulations will be needed to shed more light on these. More importantly, we do not know if human to human transmission happens naturally. The genetic diversity in MSP1 and apical membrane antigen 1 (AMA1) observed among isolates of humans than those from monkeys in Thailand and non-human isolates of Malaysia suggests that it is possible.[81,85]

Functional studies in *P. knowlesi*

In 2013, *P. knowlesi* was reported to be successfully adapted to continuous culture in human erythrocytes by Moon et al[94] and Lim et al.[95] This breakthrough serves as an important model for *P. knowlesi* studies especially in validation of vaccine and drug targets. Several *P. knowlesi* vaccine/therapeutic candidates with their recent findings are discussed in this review.

An important parasite adhesin, the reticulocyte binding-like (RBL) family, is found to be involved in host cell erythrocyte receptor binding to facilitate merozoite invasion. NBPXa is one of the members in *P. knowlesi* RBL family and is expressed within the microneme organelles.[96] With the human erythrocytes-adapted *P. knowlesi* strain, Moon et al further identified NBPXa as a key mediator for knowlesi infection in human, as this protein is crucial for invasion of human erythrocytes but not cynomolgus erythrocytes.[92] By disrupting the *NBPXa* gene, in vitro multiplicative growth of parasites in human erythrocytes is prevented through impaired merozoite invasion, which support the potential of NBPXa in vaccine development.

Erythrocyte binding-like (EBL) family is another group that mediates interaction with host cell erythrocyte receptors.[97] One of the important members in *P. knowlesi* EBL family, Duffy binding protein α (PkDBPα), interacts with the Duffy antigen receptor for chemokines to invade human erythrocytes.[98,99] Genetic polymorphisms in PkDBPα have been postulated to lead to improved parasite binding ability to erythrocytes, thus enhancing the disease severity. This postulation was supported by Lim et al, who in their recent finding showed that two genetically distinct PkDBPα haplotypes from different geographical area demonstrated a different binding activity level to human erythrocytes by using erythrocyte rosetting assay.[93]

Tryptophan-rich antigens (TRAgs) are involved in rosetting formation and merozoite invasion, thereby contributing to disease severity.[100–102] *P. knowlesi* TRAgs are mostly expressed in its blood stage.[103] By using erythrocyte binding assay and parasite growth inhibition assay, Tyagi et al identified three TRAgs in *P. knowlesi* that were able to bind to human erythrocytes.[104] Two of them competed with *P. vivax* TRAgs for receptors on human erythrocytes. Besides, all of these PkTRAgs were found to inhibit the *P. falciparum* growth in vitro, further demonstrating the biological significance of this TRAgs-receptor interaction in heterologous parasites by sharing host receptors.

AMA1 and its interaction with rhoptry neck protein 2 (RON2) are essential for merozoite invasion. *P. knowlesi* AMA1 (PkAMA1) has been tested in vaccine trials, and the results revealed that immunized macaques were protected against infection with controlled parasitemia.[105] In 2015, the crystal structure of ectoplasmic region of PkAMA1 and its invasion-inhibitory monoclonal antibody R31C2 were developed.[106] R31C2 binds to the hydrophobic groove and interferes with the exposition of complete binding site on AMA1. This monoclonal antibody is found to be cross-strain reactive as it targets on a non-polymorphic epitope. Besides, no polymorphism near RON2-binding site of AMA1 was detected. The lack of polymorphism suggests that *P. knowlesi* has not developed a mechanism to evade the host's humoral response.

Proteins that demonstrated different mechanisms in parasite metabolic pathways from those found in human host are also of particular interest. Garg et al have carried out a study on *P. knowlesi* enzyme phosphoethanolamine methyltransferases (PkPMTs), which regulates the synthesis of phosphatidylcholine. Structural, biochemical properties, and inhibition profile of PkPMTs were evaluated using X-ray crystallography, enzyme kinetics, and mutant gene expression studies. PkPMTs enzyme activity could be inhibited by amodiaquine, chloroquine, and NCI compound NSC158011, whereas the yeasts carrying mutant PkPMT genes are unable to carry out phosphatidylcholine biosynthesis from phosphoethanolamine.[107] Disruption of phosphatidylcholine synthesis in *Plasmodium* would lead to failure of intact *Plasmodium* membrane formation and thus inhibit parasite proliferation.

These findings suggest that PkPMTs are suitable targets for chemotherapy, and the data from this study could further contribute to a better design of more selective antimalarial drugs against *P. knowlesi.*

In infected erythrocytes, *P. falciparum* skeleton-binding protein 1 (PfSBP1) is required to transport the erythrocyte surface ligand, erythrocyte membrane protein 1, to the surface of erythrocyte for mediating the surface ligands exposition.[108] Its ortholog in *P. knowlesi*, PkSBP1, has been recently localized to the "Sinton and Mulligan" stipplings in the cytoplasm of infected erythrocytes.[109] By using immunofluorescence assay and immunoelectron microscopy, transgenic *P. knowlesi* expressing the tagged recombinant PkSBP1 demonstrated an analogous trafficking pattern as in *P. falciparum*, supporting the hypothesis that *Plasmodium* has evolutionarily conserved protein export pathways. These pathway-related proteins could be used as malaria intervention targets as the disruption of the protein export structures would then interfere with the exposition of virulent ligands at the surface of infected erythrocytes.

Hitherto, *P. knowlesi* vaccine target antigens have not been evaluated in human trial, as the use of non-human primate models involves serious ethical concerns. Despite higher physiological similarity between human and primate compared to other animal models, vaccine studies of *P. knowlesi* in primate models are unable to completely represent the efficacy and safety of the targets in human due to the differences in host immunity regulations and pathogenic responses toward malaria. Taking into account that majority of *P. falciparum* single antigen was unable to raise specific antibodies up to protective levels in human vaccine trials,[110–112] the importance of multi-antigens combination and incorporation of immunostimulants should be considered in *P. knowlesi* vaccine development.

Future directions and conclusion

There remains a clear risk of continuous *P. knowlesi* infections, especially when land-use change and human behavior may have driven behavioral changes in the reservoir and vector, enabling closer contact and higher chances of spillover to the human population.[113] The current literature on risk factors associated with *P. knowlesi* has identified significant key risks that will prove helpful for the development of effective intervention programs and has highlighted the important environmental factors that intermingle with individual risk factors to *P. knowlesi* transmission. Further research into the way these risk factors associate with each other and further spatial mapping and geographic monitoring of *P. knowlesi* hotspots will prove to be an essential part of *P. knowlesi* research as a whole going forward.

WHO (2018) recommends to perform parasite-specific diagnosis in malaria-suspected patients before treatment to prevent unnecessary drug wastage and to halt antimalarial drug resistance.[114] Therefore, efforts should be invested to develop a point-of-care tool that will be able to detect and differentiate all human *Plasmodium* species to be used in limited-resources environment.

In-depth population genetic studies for both human and primate isolates are needed to shed light on possible human-to-human transmission of *P. knowlesi* and more importantly for us to understand the disease epidemiology and to guide knowlesi infection control. Knowlesi malaria cases have been showing an upward trend in Malaysian Borneo for the past decade and is also forecasted to increase.[34,115,116] Experimental transmission of *P. knowlesi* from monkey to man, from man to man, and from man back to monkey has been shown by Chin et al.[117] Besides, gametocytes can be found in infected patients,[118,119] and most vectors of *P. knowlesi* (e.g. *An. dirus*, *An. balabacensis*, and *An. cracens*) are also vectors of human malarias.[120] Although there is no direct evidence of natural human-to-human transmission of *P. knowlesi*, the likelihood of it occurring cannot be ignored until proven otherwise. Experimental studies on the vectors that transmit this parasite and knowing the distribution of vectors harboring this parasite will also lend credence to solving this issue.

Rapid development in genome editing tools such as clustered regularly interspaced short palindromic repeats highly increase the efficiency to engineer the genome.[121,122] Transfections for labeling, knockout/knockdown, and gene editing can be carried out in in vitro *P. knowlesi* parasites to understand its pathogenesis, drug susceptibility, and gene functions.[94] These in vitro culture-transfection systems coupled with suitable animal models could overcome the bottleneck that is hampering the translational malaria research.

The WHO Global Malaria Program has appropriate interventions focused on the malaria caused by *P. falciparum* and *P. vivax*, which cause major global morbidity and mortality. However, efforts for malaria control and elimination in certain Southeast Asia countries, such as Malaysia, are greatly impeded by the uncontrollable passage of *P. knowlesi* in macaque populations, which could lead to human malaria outbreak via zoonotic transmission. In conclusion, different strategies and interventions are needed to prevent *P. knowlesi* transmission from macaques to human. Effective vaccines are in need to control, if not eliminate the parasite.

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