Plasmodium knowlesi: a relevant, versatile experimental malaria model

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SUMMARY

The primate malaria *Plasmodium knowlesi* has a long-standing history as an experimental malaria model. Studies using this model parasite in combination with its various natural and experimental non-human primate hosts have led to important advances in vaccine development and in our understanding of malaria invasion, immunology and parasite–host interactions. The adaptation to long-term *in vitro* continuous blood stage culture in rhesus monkey, *Macaca fascicularis* and human red blood cells, as well as the development of various transfection methodologies has resulted in a highly versatile experimental malaria model, further increasing the potential of what was already a very powerful model. The growing evidence that *P. knowlesi* is an important human zoonosis in South-East Asia has added relevance to former and future studies of this parasite species.

Key words: *Plasmodium knowlesi*, malaria, model, zoonosis, non-human primates, *in vitro*, vaccine, transfection, severe malaria.

INTRODUCTION

Plasmodium knowlesi is a non-human primate malaria from the *Plasmodium vivax* clade, that has split from *P. vivax* between 18 and 34 million years ago (Silva *et al*. 2015). The parasite can infect a wide range of non-human primates (Coatney *et al*. 1971), among which are the natural host, *Macaca fascicularis* and its close relative, the rhesus monkey (*Macaca mulatta*). The disease progression is quite different between the natural and experimental host, suggesting different parasite–host interaction mechanisms, which may be relevant to the variable disease outcomes observed in *falciparum* malaria in humans. Over the last decade it has become clear that *P. knowlesi* is not only a non-human primate malaria, but also an important zoonosis in South-East Asia, sometimes with fatal disease outcome (Singh & Daneshvar, 2013). *Plasmodium knowlesi* is now the most common cause of malaria in East and Peninsular Malaysia, both in travellers (Singh & Daneshvar, 2013) and in residents (Yusof *et al*. 2014). Thus, historical and current studies on *P. knowlesi* are relevant for human disease, with the parasite not only being a model malaria parasite, but importantly also a human pathogen. The classification of *P. knowlesi* as the fifth human malaria parasite has given research interest in this parasite a significant boost. Of the 1065 publications found in PubMed using '*knowlesi*' as keyword (query date October 31, 2016), 251 were published in the last 5 years (24%, first publication in 1935). In this review we will focus on the use of the animal and *in vitro* models, rather than on the human infections. An extensive overview of reported human *P. knowlesi* infections can be found in Siregar (Siregar *et al*. 2015).

Plasmodium knowlesi offers a versatile experimental system, as illustrated in Fig. 1. Rhesus monkeys form the most commonly used experimental host for *in vivo* studies (blue shaded area in Fig. 1) and can be infected by mosquito bite, isolated sporozoites, or blood stage parasites. Transmission, both to macaques and humans, occurs through mosquitoes from the leucosphyrus group of forest-dwelling Anopheline mosquitoes, including *Anopheles dirus* (Singh & Daneshvar, 2013; Moyes *et al*. 2016). Natural infections in wild macaques (Siregar *et al*. 2015) and *P. knowlesi* transmission dynamics from the natural host to humans (Vythilingam *et al*. 2006) have been the subject of extensive studies since macaques have been recognized as transmission reservoirs for human *P. knowlesi* malaria.

While in the wild infections exclusively occur through mosquito bites, in experimental conditions both mosquito bites and intravenous parasite injection can be used. Sporozoite infections by intravenous injection of 100 isolated sporozoites or by the bite of as little as 1 infected mosquito yield 100% infection in rhesus monkeys (Murphy *et al*. 2014). Experimental blood stage infections in vaccine challenge studies are generally performed with 10 000 blood stage parasites (Mahdi Abdel Hamid *et al*. 2011). Infected monkeys are used for parasite–host

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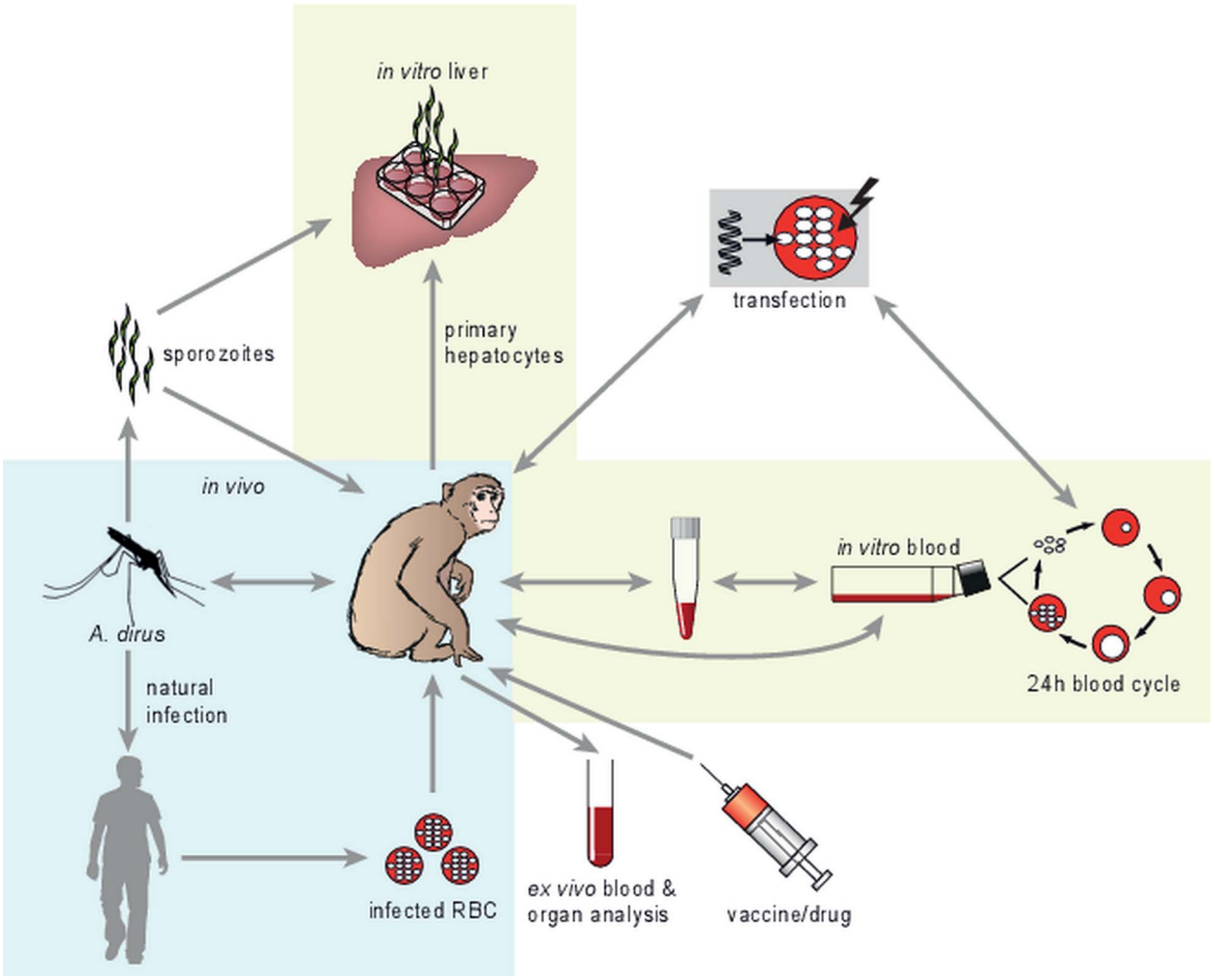

Fig. 1. The versatile *P. knowlesi* model. The blue-shaded area depicts the *in vivo* part of *P. knowlesi* work, with the monkey being the natural/experimental host, the mosquito providing transmission and the human as the zoonotic host. Different infection routes are shown, where parasite-infected human erythrocytes can be used to infect monkeys. Invasive sampling of organs and vaccine/drug application is also shown. The yellow-shaded areas depict *in vitro* work with *P. knowlesi*. Isolated sporozoites can be used to initiate *in vitro* liver stage cultures in primary macaque hepatocytes. Importantly, blood stage parasites have been adapted to continuous blood stage culture, in macaque or human erythrocytes. Transfection technology has been developed for *in vivo*- as well as *in vitro* derived parasites. Application of the various parts of this versatile model system is reviewed in detail in the text.

interaction studies and in vaccine- and drug efficacy studies. Importantly, invasive sampling of immunologically important organs is possible, either by needle biopsy during the study or by sampling larger parts of organs during necropsy at the end of vaccine efficacy studies. This allows the detailed study of immunological events that may lead to the discovery of correlates of protection (Epstein *et al*. 2011; Ishizuka *et al*. 2016). Such immunological studies are less relevant in rodent malaria models because of the large differences between human and rodent parasites and immune systems, and are not feasible in humans, where sampling is generally restricted to peripheral blood. Interestingly, to complete the arsenal of *in vivo* studies with *P. knowlesi*, a recent study has shown that parasites isolated from a human infection can be easily adapted to grow in monkeys, opening new possibilities to study human infection-adapted parasites e.g. at the level of red blood cell invasion characteristics (Amir *et al*. 2016).

Apart from *in vivo*/*ex vivo* studies, the *P. knowlesi* experimental model offers important *in vitro* possibilities, especially for liver and blood stage research (yellow shaded areas in Fig. 1). Isolated *P. knowlesi* sporozoites can be used for *in vitro* infection of primary rhesus hepatocytes, following similar protocols as described for the primate malaria *Plasmodium cynomolgi* (Zeeman *et al*. 2014). *In vitro* liver stage cultures provide the opportunity to study liver stage biology (Millet *et al*. 1990) and to perform immunological (sporozoite inhibition) and drug sensitivity studies (Fisk *et al*. 1989).

Plasmodium knowlesi is the only parasite, apart from *P. falciparum*, for which continuous long-term *in vitro* blood stage cultures have been

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developed, initially in rhesus monkey red blood cells (Kocken *et al*. 2002) and more recently also in human red blood cells (Moon *et al*. 2013). These advances provide easy access to blood stage parasite material for *in vitro* drug- and (inhibition of) invasion studies (Mahdi Abdel Hamid *et al*. 2011; Fatih *et al*. 2013), -omics studies facilitated by the availability of the annotated genome sequences of both parasite (Pain *et al*. 2008) and experimental rhesus monkey host (Zimin *et al*. 2014), and for *in vivo* and *in vitro* transfection studies (Fig. 1). In this review we will further illustrate the versatility of the *P. knowlesi* experimental model by highlighting advances in parasite–host interaction studies, vaccine development and technological developments in *P. knowlesi* studies.

PLASMODIUM KNOWLESI AS A MODEL FOR PARASITE–HOST INTERACTION STUDIES

After the discovery of *P. knowlesi* in the early 1930s (Sinton and Mulligan 1932), experimental *P. knowlesi* infections in various hosts (including human subjects (Knowles, 1935; Chin *et al*. 1965)) have extensively been used to model malaria host–parasite interactions. This has been useful to underpin some of the major discoveries in the fields of parasite invasion (Bannister *et al*. 1977), antigenic variation (Barnwell *et al*. 1982, 1983; Howard *et al*. 1983; Howard & Barnwell, 1985; al-Khedery *et al*. 1999; Barnwell, 1999; Corredor *et al*. 2004; Lapp *et al*. 2015) immunity and vaccine development (reviewed in the next section).

Plasmodium knowlesi *infections in experimental and natural hosts*

Plasmodium knowlesi infects a wide range of Old- and New World monkeys, with variable disease outcome (Table 1). In the natural host, *M. fascicularis*, parasitaemia levels are relatively low (1–3%) and short-lived, and disease is mild (reviewed in Butcher, 1996). However, the genetic background and origin of the monkey play an important role in disease outcome (Schmidt *et al*. 1977; Collins *et al*. 1992; Flynn *et al*. 2009). While mild disease is observed in *M. fascicularis* from the Philippines, *M. fascicularis* from West Malaysia developed severe disease, leading to death. Disease in other natural hosts like *Macaca nemestrina* (pig tailed macaques), *Presbytis* (Malayan leaf monkeys) and *Trachypithecus* has not been studied in detail. Other macaque species (*Macaca radiata, Macaca fuscata, Macaca mulatta*; Coatney *et al*. 1971) of which the rhesus monkey (*M. mulatta*) has been most widely used (Collins, 2012), are experimental hosts for *P. knowlesi*. Infection with blood stage parasites generally results in fulminant parasitaemia leading to death if left untreated, but infection with sporozoites leads to controllable blood stage parasitaemia in 30% of the monkeys (reviewed in Collins, 2012). The difference of disease outcome in *M. fascicularis* and *M. mulatta* is striking, given the fact that these two macaque species are closely related. A study with few animals showed that a primary infection in *M. fascicularis* leads to relatively fast development of parasite-inhibitory antibodies, as compared with rhesus monkeys (Butcher *et al*. 2010). Also, spleen cells from infected *M. fascicularis* have been shown to have anti-parasitic activity *in vitro* (Langhorne *et al*. 1977). Both studies suggest different immunological reactivity upon infection in two closely related macaque species, but further studies with larger groups are needed to confirm and expand these data.

The olive baboon (*Papio anubis*) (Ozwara *et al*. 2003*b*) has been used to study severe disease, which will be detailed below. The New World monkeys *Saimiri sciureus* (Collins et al. *1978*), *Callithrix jacchus* (Langhorne & Cohen, 1979) and *Aotus trivirgatus* (Collins *et al*. 1981) are also experimental hosts with self-curing or lethal infections in all species. *Plasmodium knowlesi* infections in experimental hosts may have a severe outcome (Table 1). In past studies *P. knowlesi*–host interactions were investigated in the light of severe malaria modelling, focusing on the comparison of characteristics observed in *P. falciparum*-cerebral malaria pathology (Jerusalem *et al*. 1983).

Severe malaria pathogenesis and animal models: a hotly debated topic

The latest WHO estimates suggest 214 million cases of malaria in 2015 and 438 000 deaths (World Health Organisation, 2015) mainly as a consequence of a series of complications termed severe malaria. Severe malaria is frequently identified as being synonymous with cerebral malaria but in reality this is only a subset of severe disease (Craig *et al*. 2012), which encompasses multi-organ failure (including kidney and liver), severe anaemia and metabolic derangement as well as pregnancy-associated malaria. While severe malaria has previously exclusively been ascribed to human infections with *P. falciparum*, recent investigations have highlighted similar disease in human infections with *P. knowlesi* (Daneshvar *et al*. 2009) and with lower frequency in *Plasmodium vivax* malaria (Price *et al*. 2007). Patterns of disease in *P. knowlesi* patients show several similarities to those seen with *P. falciparum*, such as multi-organ failure, albeit with the exemption of coma (Cox-Singh *et al*. 2010). In the context of disease, it is important to consider that *P. knowlesi* is unique among the primate and human malarias in that it has a 24 h erythrocytic cycle (reviewed in (Collins, 2012)), a characteristic that is likely to accelerate the development of

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Table 1. Experimental *P. knowlesi* infections in natural and experimental hosts. *Macaca radiata* is the only experimental host that uniformly yields a chronic, non-fatal infection

Host[a]	Aim of study	Disease outcome	Reference
M. mulatta; M. Fascicularis	Susceptibility/ immune mechanisms	Severe in rhesus; mild in *fascicularis*, with early inhibiting antibody production	Butcher *et al*. (2010)
M. mulatta	*P. knowlesi* transmission	~70% fatal	Collins *et al*. (1967)
M. mulatta; M. fascicularis of different origin	Susceptibility	Severe in rhesus; mild or severe in *fascicularis*, depending on origin	(Collins *et al*. 1992; Schmidt *et al*. 1977)
M. radiata	Susceptibility	Chronic, non-fatal	Dutta (1982)
P. anubis	Susceptibility	Acute, fatal disease or control of hyper-parasitaemia and chronic infection	Ozwara *et al*. (2003*b*)
S. sciureus	Susceptibility	Severe, fatal or low, controllable parasitaemia	Collins *et al*. (1978)
A. trivirgatus	Susceptibility	Non-fatal with complete recover or fatal with hyper-parasitaemia	Siddiqui *et al*. (1974)
C. jacchus	Susceptibility	Fulminating parasitaemia and death, or recovery and subsequently immune to infection	Langhorne & Cohen, (1979)

[a] *Macaca fascicularis* is a natural host for *P. knowlesi*.

complications (Cox-Singh *et al*. 2008) thus making accurate diagnosis and effective treatment an urgent priority. Until recently, human *P. knowlesi* infections were often misdiagnosed as *P. malariae*, which contributed to *P. knowlesi*-infected individuals not being treated appropriately (Rajahram *et al*. 2016).

The etiology of severe malaria is complex and involves an interplay between genetic background, immune response and parasite virulence, a hallmark of the latter being cytoadherence-mediated sequestration (the ability of parasite infected red blood cells to bind to surface markers of endothelial cells of the capillaries in critical organs e.g. brain, lungs). The pathogenesis of severe malaria is hotly debated: different phenomena are being considered as the overriding pathogenic mechanism, including cytoadherence-mediated sequestration (White *et al*. 2013), endothelial dysfunction (Kim *et al*. 2011) and inflammation (Clark & Alleva, 2009; Grau & Craig, 2012). However, recently (Cunnington *et al*. 2013) it has been postulated that a combination of the three proposed mechanisms with a different balance in each specific syndrome can best explain the variability in severe malaria pathogenesis observed across human cases.

Equally hotly debated are the animal models to be used for investigating the mechanisms behind the pathophysiological manifestations of severe disease (White *et al*. 2010; Langhorne *et al*. 2011; Craig *et al*. 2012). It is well documented that many of the key features of human malaria can be replicated in a variety of non-human primate models. However, despite their great potential, non-human primate models remain very under-utilized (Langhorne *et al*. 2011). While it may well be the case that no single animal model is able to reproduce all of the different features of severe malaria faithfully (Craig *et al*. 2012), it is important to bear in mind that severe malaria is highly heterogeneous (Cunnington *et al*. 2013) as its pathophysiological manifestations vary between parasite (*P. falciparum, P. knowlesi and P. vivax*) infections and even between human hosts infected by the same parasite (*P. falciparum*). It is therefore reasonable to assume that the heterogeneity of the pathophysiological presentations in animal models, if properly documented, could provide complementary insights on specific severe disease mechanisms. As compared with proposed larger field studies in humans (Cunnington *et al*. 2013), non-human primate studies on severe malaria offer the possibility to investigate severe disease in a controlled environment and to perform multivariate studies and invasive sampling that are not possible in human field studies. In terms of non-human primate models, different severe malaria models have been used, including *P. coatneyi-M. mulatta/fuscata, P. fragile-M. mulatta* (Craig *et al*. 2012) and *P. knowlesi* in a range of non-human primate hosts. The latter will be reviewed below in more detail.

Plasmodium knowlesi *animal models for severe disease*

New World monkeys, such as *Callithrix jacchus* and *Saimiri sciureus*, were shown to be differentially susceptible to disease caused by various *P. knowlesi* strains (Collins *et al*. 1978; Langhorne & Cohen, 1979). This characteristic makes them particularly interesting as a malaria model, as humans have also been shown to be differentially susceptible to *P. falciparum* (Andrade & Barral-Netto, 2011) and *P. knowlesi* disease, where – in the latter – a clear-cut correlation was established between parasitaemia levels and malaria disease patterns (Cox-Singh *et al*. 2010). Unfortunately, the studies on infections

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with *P. knowlesi* in New World monkeys are very limited and more well-designed experiments with a statistically significant number of animals per group are required to properly establish and validate these models for wider use.

Differential susceptibility to disease has also been observed in the *Papio anubis*-*P. knowlesi* H strain infection model (Ozwara *et al*. 2003*b*). The course of infection has been shown to be either acute or chronic. Animals with acute infection developed multiple system organ failure and cerebral involvement, while chronically infected animals presented with moderately enlarged spleens. The parasitaemia profiles of both groups were initially comparable with those observed in rhesus monkeys. However, animals that were able to control the initial hyper-parasitaemia developed chronic infection. In both cases, *P. anubis* individuals initially developed clinical symptoms such as apathy, raised fur and lethargy with dyspnea, which later disappeared in animals that controlled the hyper-parasitaemia. This model has also been used to study pregnancy-associated malaria, as infiltration of parasitized erythrocytes and inflammatory cells in the placental intravillous space, a key feature of human pregnancy-associated malaria, is observed in these animals (Onditi *et al*. 2015). Placental plasma and serum samples, assayed by enzyme-linked immunosorbent assay for cytokine profiles, appear to confirm findings from human pregnancy-associated malaria (Barasa *et al*. 2012), as elevated concentrations of tumour necrosis factor alpha (TNF-α) and interleukin 12 (IL-12) were found in both. A controlled study in the same *P. anubis* model addressed the question of whether co-infections with schistosoma enhance or attenuate severe malaria symptoms. This study clearly indicated that chronic *Schistosoma mansoni* infection attenuates the severity of *P. knowlesi* co-infection by mechanisms that may enhance innate immunity to malaria (Nyakundi *et al*. 2016).

The rhesus monkey is uniformly susceptible to disease and infections are generally lethal, if left untreated. However, a limited set of data shows that sporozoite-induced infections can be milder (Richards *et al*. 1977). While some papers have postulated that rhesus monkeys infected with *P. knowlesi* show no disease symptoms prior to death (Butcher *et al*. 2010), our own experience (unpublished) and other data (Ibiwoye *et al*. 1993; Chen *et al*. 2001) show that monkeys can display apathy, loss of appetite, lethargy, dehydration, fever and in some cases, display raised fur. Some hallmarks of severe disease in rhesus monkeys are discussed below.

Cytoadherence-mediated sequestration

While a critical factor in *P. falciparum* severe malaria in humans is accepted to be cytoadherence-mediated sequestration (White *et al*. 2013), this may not necessarily be the case for *P. knowlesi* human severe malaria (Daneshvar *et al*. 2009; Cox-Singh *et al*. 2010). A number of endothelial markers, such as ICAM-1, VCAM and CD36, have been linked to cytoadherence-mediated sequestration in human *P. falciparum* severe malaria studies (Helms *et al*. 2016). In *P. knowlesi* severe malaria, infected erythrocytes from five human subjects have been shown to bind in a specific but variable manner to the inducible endothelial receptors ICAM-1 and VCAM (three isolates bound to ICAM-1 and VCAM, one isolate bound to VCAM and one isolate did not bind to ICAM-1 or VCAM), while binding to the constitutively expressed endothelial receptor CD36 was not detected (Fatih *et al*. 2012).

In the *P. knowlesi*-rhesus monkey model, reports detailing the sequestration phenotype of infected erythrocytes in severe disease are limited and there has been debate on whether sequestration in this model is due to accumulation or cytoadherence. While all post-mortem light microscopy examinations documented in literature identified marked cerebral vascular congestion and widespread plugging of the brain capillaries and venules (micro-vessels) by heavily parasitized erythrocytes mixed with uninfected erythrocytes, light microscopy alone cannot properly document cytoadherence. Electron microscopy on rhesus monkey brains after infection with the *P. knowlesi* W1 strain showed major changes including adherence of large numbers of parasitized erythrocytes and macrophages to swollen microvasculature endothelial cells (Ibiwoye *et al*. 1993), increased number of fibroblasts and the deposition of collagen bundles in the extracellular matrix around damaged capillaries, parasite-packed micro-vessels and ischaemic hypoxia in several parts of the brain (Ibiwoye *et al*. 1995). Parasitic infiltration of all regions of the central nervous system and cytoadherence in some regions were further confirmed by an independent electron microscopy study (Mahdi & Ahmad, 1991). The latter already postulated in 1991 that a triad of mechanisms is involved in the etiology of cerebral malaria pathogenesis, namely mechanical obstruction, biochemical events promoted by free radicals and immunological dysfunction mediated by activated macrophages, which increase lipid peroxidation.

Early studies attributed *P. knowlesi* sequestration and the obstruction of cerebral capillaries in non-human primates to decreased deformability of *P. knowlesi*-infected erythrocytes (Miller *et al*. 1971). However, as reviewed in Galinski & Corredor (2004), studies with clonal populations of *P. knowlesi* expressing different variant surface antigens (SICAvar), indicate a role for some SICAvar antigens in sequestration. *SICAvar* genes in *P. knowlesi* and *P. falciparum* erythrocyte membrane

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protein 1 (*Pfemp1*) genes in *P. falciparum* malaria share a number of fundamental features relating to their role in antigenic variation mediated immune evasion. While the role of *P. falciparum Pfemp1* genes in sequestration has been detailed in a number of studies, the role of SICAvar in sequestration has been much less clear. The newly emerging data, suggesting a definite involvement of some SICAvar in sequestration, has led Galinski & Corredor (2004) to conclude that, while antigenic variation mediated immune evasion is a primary function in common between the *P. knowlesi* and *P. falciparum* variant antigens, the cytoadherent properties of such variant antigens could be viewed as a secondary adaptation, which has evolved as a much stronger characteristic in *P. falciparum* than in *P. knowlesi*, where sequestration is only partial.

Concluding remarks on studying parasite–host interactions using P. knowlesi*-non-human primate models*

While it is well accepted that both host and parasite factors influence severe malaria pathogenesis (Cunnington *et al*. 2013) and that the etiology of human severe malaria is dependent on the interplay between genetic background, immune response and parasite virulence, many *P. knowlesi*-rhesus monkey studies have been insufficiently documented to draw firm conclusions on the model. In a few cases the parasite strain is not mentioned and most often the origin of the rhesus monkeys is not well defined. Together with the parasite strain, the origin of the host represents one of the key factors in the characterization of a host–parasite interaction model. For example, the *P. knowlesi* H strain's sequestration profile in Indian rhesus monkeys is more pronounced than that of the Nuri strain (CHMK & AVW, unpublished observation). Similarly, *P. knowlesi* H and C strains infections in *M. fascicularis* of Malayan origin will result in death, while those of Philippine origin can control the parasitaemia (Schmidt *et al*. 1977; Flynn *et al*. 2009), indicating that the genetic background of the monkeys is important for the course of infection. For rhesus monkeys, mitochondrial typing characterized different origins (Burmese, Chinese and Indian) (Doxiadis *et al*. 2003) and differential pathophysiological manifestations for other infectious diseases have been recorded in rhesus monkeys from various origins (Langermans *et al*. 2001). Such studies for *P. knowlesi* infections would be very informative in relation to the display of parasite–host interactions, including severe malaria symptoms. Similarly, some early non-human primate pilot studies, e.g. severe malaria studies in *Callithrix jacchus* (Langhorne & Cohen, 1979), are worth following up with sufficient numbers of animals to reach statistical significance in case of differential disease course, using state-of-the-art technology and taking the current knowledge on severe *P. knowlesi* malaria in humans into account (Cox-Singh *et al*. 2010).

PLASMODIUM KNOWLESI AS A MODEL FOR VACCINE STUDIES

Gaining an understanding of immune responses against malaria using P. knowlesi

The *P. knowlesi*-rhesus monkey model has been extensively used in vaccine research. Besides testing vaccine concepts, it has also provided valuable information on the development of anti-malarial immune responses and parasite–host interactions following infection that formed the basis for human studies. In 1937, Coggeshall and Kumm showed that rhesus monkeys could be protected against a blood stage challenge with *P. knowlesi* upon passive immunization with serum from chronically *P. knowlesi*-infected rhesus monkeys (Coggeshall & Kumm, 1937). This paved the way for the classical work of Cohen *et al*. (1961), who demonstrated that passive transfer of antibodies from hyperimmune people living in endemic areas protected young children against malaria. In hyperendemic areas, people develop immunity only upon prolonged repeated exposure to malaria parasites. In part this slow development of immunity has been attributed to the ability of malaria parasites to evade the host's immune response by undergoing repeated antigenic variation. This important phenomenon was first described in *P. knowlesi*. In a seminal study by Brown & Brown (1965), it was shown that antigens expressed on the surface of infected red blood cells changed during the course of infection in chronically infected rhesus monkeys. These antigens, later termed schizont infected cell agglutination (SICA) antigens (Howard *et al*. 1983), also played a role in parasite sequestration, as described in the previous section. The identification and biochemical characterization of the *P. knowlesi* SICA antigens led to a similar line of experimentation to identify homologous antigens with similar characteristics in *P. falciparum*, which, in turn, led to the discovery of the *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) variant protein family (reviewed in (Galinski & Corredor, 2004)).

A non-human primate vaccine model relevant for humans

Rhesus monkeys have been widely used for vaccine studies, mainly because in line with their phylogenetic proximity to humans, they share many relevant immunological and biological features (Messaoudi *et al*. 2011). In rhesus monkeys, *P. knowlesi* produces a fulminating fatal parasitaemia if left untreated

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(Table 1), representing a rigorous model for vaccine testing. Many immunological reagents developed for human studies cross-react with rhesus monkey cells and are available to characterize the immune responses induced in these animals in detail. In addition, the genome of the rhesus monkey has been sequenced allowing for in-depth genome-wide analyses of protective immunological pathways (Zimin *et al*. 2014). Together with the opportunity to dissect immunological processes *in situ*, by examination of relevant organs, this renders the *P. knowlesi*-rhesus monkey model as a unique model in the search for correlates of protection. This model may even be further expanded in the future with the development of *in vivo* imaging approaches for non-human primates infected with malaria parasites to allow real-time characterization of interactions between host immune cells and parasites in different parts of the body (Beignon *et al*. 2014). As reviewed below, transfection technology for *P. knowlesi* is already well established, enabling the production of marker parasites for this purpose.

The availability of a primate-host combination with immunological features that are relevant for the human situation renders *P. knowlesi* in the rhesus monkey an attractive parasite for malaria vaccine studies.

Blood stage vaccination

Most of the early vaccination studies with *P. knowlesi* parasites were blood stage oriented. Taking advantage of the relatively large size and robustness of *P. knowlesi* merozoites, Dennis *et al*. (1975) developed a sieving method to isolate free live merozoites. The availability of *P. knowlesi* merozoites enabled groundbreaking studies on the invasion process of the red blood cell (Bannister *et al*. 1977; Aikawa *et al*. 1978). It also provided the opportunity for *P. knowlesi* vaccination studies using freshly isolated or freeze-dried merozoite preparations in Freund's adjuvant. Immunizations with these preparations elicited sterile protection against challenge with blood stage parasites (Mitchell *et al*. 1975, 1977) and this immunity was shown to involve merozoite blocking antibodies (Butcher *et al*. 1978), eventually leading to the identification and characterization of one of today's major blood stage candidate vaccines, the conserved Apical Membrane Antigen-1 (Deans *et al*. 1982; Peterson *et al*. 1989; Remarque *et al*. 2008). An early proof-of-concept vaccine study with PK66/AMA-1 affinity purified from *P. knowlesi* blood stage schizonts showed only marginal effects after challenge with blood stage *P. knowlesi*. However, following a booster vaccination, protective effects were observed after re-challenge with blood stage *P. knowlesi* (Deans *et al*. 1988). More recently, the PK66/AMA-1 antigen was expressed in yeast and vaccine efficacy of this antigen, formulated in a novel adjuvant co-vaccine HT, was assessed following *P. knowlesi* blood stage challenge (Mahdi Abdel Hamid *et al*. 2011). Similar to the earlier study, this showed only modest protective effects (1 out of 6 animals controlled the parasitaemia; 4 out of 6 animals showed a small delay in onset of parasitaemia and 1 animal was not protected). A subsequent booster vaccination followed by a re-challenge showed significant improvement of protective effects: 4 out of 6 monkeys controlled *P. knowlesi* parasitaemia, 1 monkey had a delayed onset of parasitaemia and 1 animal was not protected. Furthermore, it was shown that in order to achieve protection, high levels of inhibitory antibodies were required and purified IgG isolated from the vaccinated monkeys showed *in vitro* parasite growth inhibition that correlated with protection (Mahdi Abdel Hamid *et al*. 2011). This study showed that AMA-1, as a single subunit vaccine can confer protection against malaria in a highly stringent model, underlining the importance of AMA-1 as a malaria vaccine candidate, and thus supporting clinical development of this antigen from *P. falciparum* as *P. falciparum* vaccine component. However, in order to achieve sterile protection repeated infection and booster immunization were needed, indicating that a vaccination scheme that involves multiple immunizations is necessary. Alternatively, a combination vaccine involving multiple antigens, for example inclusion of another conserved vaccine candidate $MSP1_{19}$, may improve protective effects, a strategy that is currently investigated for *P. falciparum* (Faber *et al*. 2013).

Multi-stage vaccination strategies

Over the past 15 years a number of *P. knowlesi* vaccine studies have been performed in which combinations of antigens were used for vaccination, using prime-boost approaches. This strategy not only involved multi-antigen combinations, but also antigens targeting different stages of the parasite life-cycle using a DNA vaccination strategy that offers the flexibility to easily express a combination of antigens. Building on success in murine models (Sedegah *et al*. 1994, 1998, 2000), this strategy was also tested in the *P. knowlesi*-rhesus monkey model (Rogers *et al*. 2001). The vaccine strategy included three immunizations with a DNA vaccine containing two pre-erythrocytic stage antigens, the circumsporozoite protein (PkCSP) and sporozoite surface protein 2 (PkSSP2) and two blood stage antigens, apical membrane antigen 1 (PK66/AMA1) and merozoite surface protein 1 (PkMSP1p42), followed by a booster immunization with recombinant canarypox virus encoding the four antigens (ALVAC-4). This regimen did induce antibody and T-cell responses and provided partial protection against sporozoite challenge. One out of the 12 experimental monkeys

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was completely protected and the mean parasitaemia in the remaining monkeys was significantly reduced compared with control monkeys (Rogers *et al*. 2001). However, the levels of immune responses and protection were significantly lower than those observed in the *Plasmodium yoelii* mouse model (Sedegah *et al*. 1998, 2000). This could be the result of differences in the experimental set-up between the studies. In the murine model a single antigen was used, CSP, in contrast to the multistage cocktail that was used in the monkey study, potentially resulting in antigenic competition leading to lower protective effects. However, modest levels of immunity were also reported in human trials (Wang *et al*. 2001; Epstein *et al*. 2004) in which, analogous to the murine study, CSP was used as the single antigen. Different levels in responsiveness to DNA vaccination in mice and (non-)human primates may be a factor in this, but it may also be the result of the different vaccination procedures. Boosting in the murine model was performed with an attenuated vaccinia virus, in the rhesus monkey model with recombinant canarypox virus and in the human studies with either DNA or protein. Taken together, these data provided a rationale for further work with the *P. knowlesi*-rhesus monkey model aiming at dissection of the immunological mechanisms and optimization of the vaccination regimen. In a later study (Rogers *et al*. 2002), it was shown that the use of attenuated vaccinia virus rather than canarypox for boosting dramatically improved protection against challenge with *P. knowlesi* sporozoites. Two out of 11 monkeys showed sterile protection and 7 of the 11 monkeys spontaneously resolved their blood stage parasitaemia. In an attempt to further improve this, various vaccination strategies were tested in a head-to-head comparison (Jiang *et al*. 2009). However, none of the new vaccine components, including viral replicon particles and recombinant Adenovirus-5 could prime immune responses as effectively as DNA plasmid priming. Strikingly however, high levels of sterile protection were observed in the group that received the DNA prime/pox virus boost regimen with increased time intervals between the vaccination dosages. In this group 3 out of 5 monkeys never developed parasitaemia after sporozoite challenge and 1 out of the 5 monkeys spontaneously resolved its blood stage parasitaemia. Cellular (Jiang *et al*. 2009) and antibody responses (Hamid *et al*. 2011) were observed, but correlates of protection could not be found. Notwithstanding the impressive protective effects induced in this highly stringent model, all *P. knowlesi* DNA prime/pox virus booster vaccine studies failed to demonstrate protection in a second challenge 4 months later, indicating that further optimization is needed to obtain long-term protection. Alternatively, live-attenuated parasite vaccine strategies may be more suited to accomplish long-term sterile protection.

Plasmodium knowlesi *live attenuated whole-organism vaccination*

Over the past few years, significant progress has been made in the field of whole-organism malaria vaccine approaches (Hoffman *et al*. 2010; Epstein *et al*. 2011; Roestenberg *et al*. 2011) and some important hurdles in the development of a *P. falciparum* attenuated sporozoite vaccine for humans have been overcome (Richie *et al*. 2015). In order to dissect the immune responses elicited by vaccination with attenuated whole-organisms in a non-human primate model, Weiss and Jiang (Weiss & Jiang, 2012) immunized 9 rhesus monkeys with radiation attenuated *P. knowlesi* sporozoites. They found sterile protection in 5 out of 9 monkeys after challenge with *P. knowlesi* sporozoites. After treating 3 of these monkeys with a monoclonal antibody that removed CD8+ lymphocytes from the circulation, the monkeys all developed blood stage parasitaemia after re-challenge with sporozoites. After 4 months rest, CD8+ lymphocytes had re-appeared in the blood and upon a third challenge all monkeys were protected again. This indicates an important role for CD8+ T-cell responses in eliciting protection against malaria with live-attenuated pre-erythrocytic vaccine approaches. This type of response may be more prominent inside the liver as was recently shown by Ishizuka *et al*. (2016) who demonstrated that in rhesus monkeys vaccinated with attenuated *P. falciparum* sporozoites, *P. falciparum*-specific interferon-γ-producing CD8+ T-cells were present at ±100-fold higher frequencies in liver than in blood. *P. falciparum* does not develop into blood stage parasites in rhesus monkeys, precluding challenge studies that can directly correlate immune responses in the liver to *P. falciparum* vaccine efficacy. The *P. knowlesi*-rhesus monkey model is well suited to fill this knowledge-gap, because in this model both protective effects induced by vacci-nation as well as underlying immunological mechanisms inside the liver can be studied. The recent establishment of a protocol for using infective mosquitoes to challenge rhesus monkeys with *P. knowlesi* (Murphy *et al*. 2014) now allows for testing efficacy of *P. knowlesi* pre-erythrocytic vaccination strategies using the natural route of infection, thereby avoiding potential over/underestimation of vaccine effects due to artificial challenging.

The development of new technologies (such as transfection technologies and continuous long-term *in vitro* cultures) contributed to the use of *P. knowlesi* as a model parasite and dramatically increased the versatility of the *P. knowlesi*-non-human primate model for vaccine, drug and also more basic biological studies, as detailed below.

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TECHNOLOGICAL DEVELOPMENTS IN *PLASMODIUM KNOWLESI* STUDIES

Long-term continuous in vitro cultures

Short-term *in vitro* cultures of *P. knowlesi* blood stage parasites in rhesus monkey red blood cells were first described by Ball *et al*. (1945), but only in 2002 a *P. knowlesi* parasite line derived from *P. knowlesi* H strain (PkHcc), supporting continuous *in vitro* growth was developed (Kocken *et al*. 2002). When cultured parasites were injected back into a monkey, the parasitaemia did not exceed 0·2% in the first acceptor monkey. The secondary recipient developed fulminant parasitaemia again (Kocken *et al*. 2002), and these parasites could be grown *in vitro* without any adaptation period. This shows that this parasite can be shuttled from *in vitro* culture to *in vivo* infections with relative ease. The continuous blood stage culture reduced primate use for studying blood stage parasites, as rhesus monkey infections are not needed anymore as a source for blood stage parasites. However, during the adaptation to continuous *in vitro* culture, the PkHcc strain has lost its ability to form gametocytes, precluding transmission from *in vitro* cultures. PkHcc in monkeys did not regain gametocyte production, indicating that the gametocytogenesis pathway is irreversibly damaged in these parasites (Kocken *et al*. 2009). Thus, for transmission and liver stage studies, monkey infections with gametocyte forming *P. knowlesi* strains are still necessary (Fig. 1).

Although the *P. knowlesi* H strain parasite that is most extensively used for research was originally isolated from a naturally infected person in 1965 (Chin *et al*. 1965), the adaptation of *P. knowlesi* to *in vitro* blood stage cultures in human red blood cells has been a challenge, and *P. knowlesi* research was restricted to facilities that have access to macaque blood.

Recently, Moon *et al*. (2013) have achieved a breakthrough by adapting the *P. knowlesi* A1 strain to growth in human red blood cells (RBC). The researchers started with a stock taken from a rhesus monkey and adapted the parasite to *in vitro* culture in 100% *M. fascicularis* RBC. After 2 months of culture, the blood was changed to 80% human and 20% *M. fascicularis* RBC and the parasite was cultured in this RBC-mix for 10 months. The mixture of RBCs was then replaced with 100% human RBC and 17 months after the start of the experiment, a clonal parasite line was generated, capable of growing both in *M. fascicularis* as well as in human red blood cells. This made *in vitro* culture of *P. knowlesi* accessible to laboratories without access to macaque blood. Unfortunately *P. knowlesi* A1-H had already lost the ability to form gametocytes before adaptation to growth in human RBC, so this line is not suited for transmission and liver stage research. In parallel to Moon, Lim *et al*. (2013) adapted *P. knowlesi* H strain to *in vitro* culture with human RBC. Before adaptation, this strain had a strong preference for young human RBC and cultures could only be maintained when 8% of the human RBC consisted of reticulocytes. Adaptation of *P. knowlesi* H to long-term *in vitro* culture in human RBC was achieved by culturing the parasite in a mixture of 90% human RBC and 10% rhesus monkey RBC for a month, followed by culturing in 100% human RBC for 4 months, after which a stable parasite line was achieved that was able to grow at normal rate in human RBC. The preference for reticulocytes disappeared during the adaptation process. Gametocyte production is not reported in Lim *et al*. (2013). It will be very interesting to compare the genomes of the two strains to find which adaptations were needed for the parasites to survive in human RBC cultures and whether they have adapted along similar paths. With the *P. knowlesi in vitro* culture technology available, scientists now have the opportunity to perform functional analysis of especially the genes that are selectively found in the *P. vivax* clade (±90 genes) (Frech & Chen, 2011), to which *P. knowlesi* belongs. In that perspective, we will summarize the transfection technologies available for *P. knowlesi*.

Plasmodium knowlesi transfection technology

With the *P. knowlesi* genome fully sequenced (Pain *et al*. 2008) and the blood stage cultures in human RBC achieved, transfection technology is elemental to study the function of *P. vivax* clade-specific genes as well as the many hypothetical genes present in all malaria species including *P. knowlesi*.

The first *Plasmodium* transfection was performed in *Plasmodium gallinaceum* gametes and zygotes (Goonewardene *et al*. 1993), followed by episomal transfection of *P. falciparum* (Wu *et al*. 1995) and stable transfection of *Plasmodium berghei* blood stages (van Dijk *et al*. 1995). A versatile transfection system was also developed for *P. knowlesi*, initially *in vivo* (van der Wel *et al*. 1997) and subsequently using *in vitro* adapted parasites in rhesus monkey red blood cells (Kocken *et al*. 2002).

The first *P. knowlesi* transfection (van der Wel *et al*. 1997) was performed with infected red blood cells taken from a rhesus monkey, which were injected back into a recipient monkey after the transfection procedure (using Biorad electroporation in incomplete Cytomix (Wu *et al*. 1995)) (Fig. 1). Five years later, a long-term *P. knowlesi in vitro* culture was genetically modified side by side with *in vivo* parasites using double crossover recombination to target the CSP gene. Double crossover integration is preferred over single crossover integration, as a more stable genotype can be achieved because the insert can no longer loop out. The

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Table 2. Chronological overview of genetic modifications in *P. knowlesi*

Target-gene (in Pk)	Modification	Technique	Phenotype	Reference
	Heterologous expression TgDHFR	Episomal	Pyrimethamine^{R}	van der Wel *et al.* (1997)
CSP	KO	Double-CO	No oocysts	Kocken *et al.* (2002)
140 kDa locus	Rhesus IFN- γ overexpression	Double-CO	High rhIFN-γ level *in vitro*	Ozwara *et al.* (2003*a*)
140 kDa locus	Heterologous promoter activity	Episomal and double-CO	GFP-fluorescent parasites	Ozwara *et al.* (2003*c*)
rRNA-ssu locus	New pos and neg selectable markers	Episomal expression (hDHFR, Bsd, Neo, TK) and single-CO (hDHFR)	Pyr^{R} and WR99210^{R} (hDHFR) Neo^{R} (Neo) Blast^{R} (Bsd) Gancyclovir^{S} (TK)	Wel *et al.* (2004)
DBP-α	KO	Double-CO	No invasion of Duffy+ hRBC	Singh *et al.* (2005)
CDP-DAG synthase	Gene replacement with PfCDS-DAG Attempted KO	Double-CO	No phenotype of replacement. KO failed	Shastri *et al.* (2010)
P230p (PKH_041110)[a]	Knockout	Single-CO Episomal expression	Stable GFP expression	Moon *et al.* (2013)
PHISTb (PKH_103230)	GFP-tag	Episomal	Localisation of tagged protein in RBC periphery and parasite	Tarr *et al.* (2014)
MyoB[a] and MLC-B[a] (PKH_091610)	HA-tag	Single-co	PkMyoB and MLC-B localized at 'apical end' of parasite	Yusuf *et al.* (2015)
rRNA-ssu locus[b]	Introduce AttB site GFP-marker	Single-CO Integration using Bxb1 recombinase	Not mentioned	Dankwa *et al.* (2016)
NBPXa[a]	KO	Single-CO	No invasion of hRBC	(Moon *et al.* 2016)

Although the tools are available, only few genetic modifications have been reported.
[a] Performed in *P. knowlesi* adapted to culture in human red blood cells.
[b] Parasite modification is briefly mentioned in the section Methods, but no experiments described using the transgenic parasite.
TgDHFR, T. gondii-mutant dhfr gene conferring resistance to pyrimethamine; CSP, circumsporozoite protein; KO, knockout; CO, crossover; IFN-γ, interferon gamma; GFP, green fluorescent protein; rRNA-ssu D-type ribosomal RNA small subunit; hDHFR human mutant DHFR gene conferring resistance to pyrimethamine and WR99210; Bsd, Blasticydin S deaminase, confers resistance against blasticydin; Neo, neomycin phsphotransferase II, confers resistance against neomycin; TK, thymidine kinase, negative selection marker which sensitises for ganciclovir; DBP, duffy binding protein; hRBC, human red blood cells; CDP-DAG, cytidine diphosphate diacyglycerol synthase; Pf, *Plasmodium falciparum*; MyoB, myosin B; MLC-B, myosin light chain-B.

resulting transgenic parasite strains were analysed *in vitro* (Kocken *et al.* 2002) except for the transmission phenotype, which, due to lack of gametocyte production, could not be tested in the *in vitro* adapted strain. The transmission phenotype (lack of sporozoite production in mosquito midgut oocysts) confirmed the earlier observed *csp* knockout phenotype from rodent malaria (Menard *et al.* 1997; Kocken *et al.* 2002).One of the major advantages of *in vitro* cultures is the possibility to clone the parasite after transfection, to verify that any phenotype fits the genotype. To date only few genes have been genetically modified in *P. knowlesi*, either through knockout, overexpression or tagging. Table 2 provides a comprehensive overview, including observed phenotypes, if any. Limited use of the *P. knowlesi* system is most likely due to the fact that up to recently the *in vitro* culture was restricted to facilities that had access to macaque blood. Together with the adaptation of *P. knowlesi* to human red blood cells (Moon *et al.* 2013), transfection methodologies for *P. knowlesi* have been refined and optimised to obtain high efficiency, both for episomal and genome integration transfections (Moon *et al.* 2013). The transfection efficiency of *P. knowlesi* achieved by Moon is outperforming the transfection efficiency of *P. berghei*, which was by far the most efficient malaria transfection system available (Janse *et al.* 2006). For an excellent recent review covering the available molecular genetic systems in malaria see de Koning-Ward *et al.* (2015). In conclusion, the *P. knowlesi* transfection system is versatile, in terms of *vitro-vivo* shuttling, which can be essential for the detection of certain phenotypes that are not displayed *in vitro* (e.g. overexpression of IFN-γ (Ozwara *et al.* 2003*a*) could have impact on host immune response), and it is highly efficient and suitable for both episomal

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transfection as well as single and double crossover integration into the genome.

Although current transfection efficiencies for *P. knowlesi* parasites are relatively high, further improvements are expected to ensue from the application of the CRISPR/Cas tool for gene-editing of human cells that was developed by Doudna and Charpentier in 2012 (Jinek *et al*. 2012) and reviewed in Doudna & Charpentier (2014). Up to very recently, double crossover integration into the *P. falciparum* genome could only be achieved by positive and negative drug selection (Duraisingh *et al*. 2002), while currently, *P. falciparum* (Ghorbal *et al*. 2014; Wagner *et al*. 2014) and *P. yoelii* (Zhang *et al*. 2014) mutants can easily be generated using the CRISPR/Cas tool. Applying CRISPR/Cas technology in a high-throughput like fashion to *P. knowlesi* parasites could rapidly give insight into the ±90 genes that seem to be specific for *vivax*-type parasites (Frech & Chen, 2011).

Concluding remarks

In this review the *P. knowlesi*-rhesus monkey model was shown to be a valuable tool for the evaluation of vaccine approaches for *P. falciparum*. *Plasmodium knowlesi* is increasingly recognized as causative agent for disease in humans in South East Asia. Its transmission in humans has been reported in all countries in Southeast Asia except Laos. In fact, *P. knowlesi* is responsible for the majority of malaria cases in Malaysia (Singh & Daneshvar, 2013). The increasing incidence of *P. knowlesi* infections in humans demands measures aimed at reducing this burden. It should be possible to translate the obtained knowledge from animal models directly to human *P. knowlesi* vaccine development, speeding up this process.

The possibilities offered by the combination of what was already a powerful model system supplemented with innovative technologies (including the emerging CRISPR/Cas transfection technology), provide for the continuous attractiveness of the *P. knowlesi*-non-human primate/*in vitro* model. Well-designed and controlled studies will increase our knowledge on the biology of specific malaria parasite features, parasite–host interactions and on immunological mechanisms behind successful vaccination strategies. The exploitation of genomic (Pain *et al*. 2008; Zimin *et al*. 2014), proteomic (Pasini *et al*. 2010) and metabolomic (Salinas *et al*. 2014) methods as well as bio-imaging are key to further our understanding of malaria parasite–host interactions in general and the severe malaria syndrome in all its facets in particular (Cunnington *et al*. 2013).

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