IJPSR (2019), Volume 10, Issue 6 **(Review Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 04 September 2018; received in revised form, 18 December 2018; accepted, 04 April 2019; published 01 June 2019

TOWARDS SUCCESSFUL ADAPTATION OF *PLASMODIUM KNOWLESI* TO LONG-TERM *IN-VITRO* CULTURE IN HUMAN ERYTHROCYTES

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Keywords:

Plasmodium knowlesi, CD34+ hematopoietic stem cells, Reticulocytes, Erythrocytes, Parasite invasion

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ABSTRACT: *Plasmodium knowlesi*, a simian malaria parasite, is widely used as a malaria model and now characterized as a clinically significant parasite that can lead to outbreaks throughout most countries in Southeast Asia. Despite its importance, both fundamental and clinical studies of *P. knowlesi* have been impeded by the lack of a convenient *in-vitro* culture system partly due to the parasite's preference for reticulocytes. Due to a limited number of reticulocytes that could be collected from fresh sources such as umbilical cord, bone marrow, and peripheral blood, scientists have opted reticulocytes generated from CD34+ hematopoietic stem cells (HSCs) that have been cultured in an *in-vitro* system. With sufficient growth factors (GFs) and differentiation factors (DFs), a higher number and more homogenous populations of reticulocytes could be obtained from HSCs *in-vitro*. Here, we review an approach to produce massive expansion of CD34+ HSCs and their differentiation into reticulocytes that could be used for the establishment of a continuous *in-vitro* culture of *P. knowlesi* or *P. vivax*. The successful development of the long-term *in-vitro* culture of this parasite could ultimately provide an ideal system for forwarding diagnostic, anti-malarial drug and vaccine studies in malaria.

INTRODUCTION: Despite years of tremendous elimination efforts and progress, malaria remains as one of the most significant life-threatening diseases affecting people in tropical and subtropical regions. The World Malaria report 2017 reported that about 3.2 billion people were at risk of malaria, which accounts for nearly half of the world's population. In 2016 alone, there were roughly 216 million malaria cases, an increase of 5 million cases over the previous year, an estimated 445,000 malaria deaths [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.10(6).2663-69

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.10(6).2663-69

In the same year, Malaysia reported 2,327 malaria cases, which occurred mostly in Malaysian Borneo; Sabah and Sarawak, as well as in other states in Peninsular Malaysia such as Kelantan, Perak and Selangor [2].

Malaria is caused by hemoprotozoa from *Plasmodium species* and spread to humans through the bites of infected female Anopheles mosquitoes. *P. falciparum* is the most prevalent malaria parasite in sub-Saharan Africa, but outside of Africa, *P. vivax* is the predominant parasite in the region of the Americas, the Eastern Mediterranean and Southeast Asia [1]. In most endemic areas in Southeast Asia, *P. knowlesi* has been reported to cause a majority of malaria cases [3]. According to the WHO Meeting report 2017, Malaysia recorded the highest burden of *P. knowlesi* (69% of total reported cases, mostly mono-infection) in 2016, which mostly affected men than women (about 80%) with more cases occurred among adults aged over 55 years old [2]. *P. knowlesi* cases were mostly reported in Sabah and Sarawak of Malaysian Borneo and other states of Peninsular Malaysia including Kelantan, Pahang, Johor, Terengganu, Selangor, Kedah, Penang, Perak and Melaka [1, 4, 5, 6, 7, 8]. The natural hosts of *P. knowlesi* are long-tailed (*Macaca fascicularis*) and pig-tailed (*Macaca nemestrina*) macaques [9, 10, 11, 12]. Warren *et al*., (1970) stated that knowlesi malaria was a rare zoonosis of humans after the first description of *P. knowlesi* human infection was revealed in 1965 by Chin *et al*., (1965) [13, 14].

However, more human cases of this simian parasite have been identified after a large focus of apparent *P. malariae* cases in Kapit, Sarawak was investigated by Singh *et al*., (2004) using a molecular technique [15]. By microscopic examination, a band form appearance of late trophozoites of *P. knowlesi* has been frequently misdiagnosed as *P. malariae*, and its early trophozoites have frequently been misidentified as *P. falciparum* due to similar characteristics such as the presence of double chromatin dots, multiple - infected erythrocytes and appliqué forms [16, 17, 18, 19]. Since *P. knowlesi* is now the dominant malaria parasite infecting humans in Southeast Asia and two times more prevalent than *P. falciparum* or *P. vivax* [7], if left untreated, it can result in a severe or fatal disease characterized by acute renal failure, acute respiratory distress syndrome and shock [20, 21]. Therefore, knowlesi malaria has become one of the healthcare emergencies in most Southeast Asian countries including Malaysia [22].

Studies of many aspects of *P. knowlesi* biology have posed an increasingly important challenge. This is partly due to the need to grow the parasite in its macaque host or *in-vitro* using macaque blood [17, 23, 24]. These requirements have restricted studies to laboratories with access to macaques or macaque blood. The recent attempt to grow and proliferate *P. knowlesi in-vitro* in human red blood cells (RBCs) is, therefore, a substantial step towards expanding research on *P. knowlesi* [17, 25, 26]. In this review, we discuss technical developments of the *P. knowlesi in-vitro* culture system and how this system can be developed based on a few current approaches that have been used thus far. We also highlight future potentials of the available human-adapted *P. knowlesi* parasite lines.

Establishment of the Continuous *in-vitro* Culture of *P. knowlesi*: Since the maintenance of *P. knowlesi in-vitro* in rhesus RBCs for several intraerythrocytic cycles was achieved in 1945, [27] attempts to develop a continuous *in-vitro* culture for this malaria parasite have never ended [28]. The long-term *in-vitro* culture system would allow full analysis of parasite-host cell interactions [29], improve understanding of the biology of *P. knowlesi* such as the mechanism of invasion [30, 31] and resistance markers [32], and contribute to the development of new anti-malarial drugs and vaccines [31, 33, 34, 35]. The study by Ball *et al*., (1945) indicated that target cells of *P. knowlesi* are not restricted to a certain age in macaques, however, in humans, it was found that *P. knowlesi* mainly invades reticulocytes [17, 27]. Previous attempts showed that *P. knowlesi* failed to be maintained exclusively in human blood [25, 29].

The direct culture in 100% human RBCs resulted in very low growth rates and a complete loss of parasites within a week [25]. Similarly, Lim *et al*., (2013) showed that *P. knowlesi* was poorly proliferated in pooled human blood and had a decreased invasion efficiency into older RBCs [36]. Thus, the predilection for reticulocytes has been a major obstacle to establish a continuous *in-vitro* culture for *P. knowlesi* as these young RBCs circulate in peripheral blood at a very low concentration (0.5-1% of total RBCs) and for a very short time (24 h) [26]. Besides, a large quantity of reticulocytes is required to maintain a continuous culture and obtain an efficient parasite invasion. It was evidenced when *P. knowlesi* was cultured in blood enriched with more than 8% reticulocytes, increasing parasitemia and propagation [36]. The continuous culture was also successfully established and maintained for up to six months.

Potential Use of $CD34^+$ Hematopoietic Stem Cell (HSC)-Derived Reticulocytes for Continuous *in-vitro* Culture of *P. knowlesi*: As reticulocytes represent only 0.5-1% of total RBCs in the bloodstream and have a short lifespan prior to maturation [26], collecting sufficient reticulocytes from peripheral blood (PB) to maintain a *P.*

knowlesi or *P. vivax* culture *in-vitro* is nearly impossible [28]. *P. vivax* has been shown to have a close phylogenetic relationship to *P. knowlesi* [37]. Furthermore, techniques to concentrate reticulocytes collected from PB using percoll centrifugation, several washing and concentration steps could damage the cells, thus affect invasion efficiency [34, 38].

In an attempt to overcome this hurdle, several approaches have used hematopoietic stem cells (HSCs) isolated from either umbilical cord blood (UCB), bone marrow (BM) or PB as potential resources to generate higher production of reticulocytes *in-vitro* for parasite invasion [26, 34, 39, 40]. The culture systems include the application of specific cytokines and either co-culture the HSCs with feeder cells (*i.e.*, human mesenchymal stem cells) [41, 42, 43, 44] to mimic the medullar micro-environment or without feeder cells [43, 45]. With sequential addition of specific growth factors (GFs) such as stem cell factors (SCF), interleukin-3 (IL-3), hydrocortisone and erythropoietin (EPO) in $CD34^+$ HSC differentiation medium, the parasite culture could be maintained for a longer period in the presence of reticulocytes derived from $CD34^+$ HSCs [34, 39].

Although HSC - derived reticulocytes need approximately two weeks to mature rather than the use of immediately available reticulocytes, the former resource assures a more homogeneous and standardized cell population [34, 46, 47]. Furuya and his colleagues have improved the method of HSC culture to produce a higher percentage of reticule-cytes by using cryopreserved erythroblasts frozen after 8-day cultivation of UCB-isolated HSCs. The method allows the recovery of reticulocytes in a shorter time than with continuous stem cell culture. Moreover, a substantial number of reticulocytes (up to 0.8% of the total cells) are successfully invaded by *P. vivax* following 24 h post-cultivation [48]. Due to the similarities between *P. knowlesi* and *P. vivax* regarding their invasion pathways [33], genetics [49, 50], hosts and target cells [51, 52], these approaches would provide insights into the development of long-term *in-vitro* culture system for *P. knowlesi*.

Cryopreservation of Reticulocytes and Malaria Parasite Isolates: Cryopreservation is a process of preserving the biological function of intact living cells or tissues by freezing and storing the material below -80 °C typically at or near the temperature of liquid nitrogen (-196 °C) [53]. This technique has been used in *Plasmodium* studies to allow research to be carried out in laboratories with limited access to fresh parasite isolates [54]. This technique facilitates more research groups working on this field and increases chances for major discoveries [55]. Among the cryopreservation solutions that have been used in previous studies are glycerolyte solution, medium containing glycerol and sorbitol, and IMDM/10% DMSO/40% FCS solution [48, 56].

Noulin *et al*., (2012) reported a reliable cryo-preservation protocol for HSC-derived reticulocytes that could be invaded by cryopreserved *P. vivax* or *P. falciparum* isolates. More than 70% of cryopreserved cells were remained viable and stable compared to the control cells measured before cryopreservation [34]. Another study by Borlon *et al*., (2012) showed that the invasion efficiency of both cryopreserved parasite isolates and reticulocytes was similar to those obtained with fresh samples. They were also able to maintain the culture for up to 10 days [54]. These findings show that cryopreservation of reticulocytes did not affect cell stability as they matured normally and were still viable and able to support parasite growth and invasion [34, 54]. The cryopreservation method could be easily replicated in laboratories outside endemic areas and substantially contribute to the development of a continuous *in-vitro* culture of *P. knowlesi* in the future.

Invasion Mechanism of *P. knowlesi*: The invasion mechanism of *P. knowlesi* involves the interaction of a specific ligand presented on the merozoite surface known as Duffy binding proteins (DBPs) [57] and a receptor available on human RBCs namely Duffy antigen receptor for chemokines (DARC) [58]. The invasion depends largely on the availability of DARC because no invasion was observed in Duffy-negative RBCs [25]. The level of DARC on mature RBCs is reduced compared to that on reticulocytes [25], which possibly explains the predilection of *P. knowlesi* for reticulocytes. Besides Duffy binding protein, the reticulocyte binding-like proteins (RBLs), another important ligand family, also contribute to the successful invasion of human RBCs [49, 59].

The RBL proteins expressed by *P. knowlesi* merozoites have been identified as normocyte binding proteins, PkNBPXa and PkNBPXb [57, 60, 61]. Unlike PkNBPXb, PkNBPXa binds to not only macaque cells but also human RBCs [60]. Therefore, the availability of human-adapted *P. knowlesi* line would provide a platform to elucidate the invasion mechanism and hence, facilitates in the improvement of strategies to control knowlesi malaria.

The Similarities of *P. knowlesi* and *P. vivax*: *P. knowlesi* is an ideal model for better understanding the various biological aspects of *P. vivax* (*e.g*., genetics, invasion and relapse mechanism, drug resistance) [17, 62]. Historically, *P. knowlesi* shares a few common biological aspects with *P. vivax*; both parasites were found to invade mainly human reticulocytes [63] and use DARC to invade the reticulocytes [62]. The gene sequence of *P. knowlesi* and *P. vivax* showed a near perfect synteny scattered with expansions of species-specific genes [37, 64]. Thus, in-depth study of *P. knowlesi* could provide an insight into *P. vivax* biology and the subsequent discovery of diagnostic tools, drugs and vaccines [17, 62].

Adaptation of *P. knowlesi* to Continuous *in-vitro* Culture in Human Erythrocytes: Many attempts have been made to adapt *P. knowlesi* to stably proliferate in human RBCs *in-vitro*. However, due to its low replication rates, *P. knowlesi* has failed to stably grow in human RBCs alone [65]. This obstacle has been overcome by slowly growing *P. knowlesi* in a mixture of macaque and human RBCs [25, 36]. Following an initial adaptation in human RBCs, the parasites proliferation rates increased further from 2 fold to 5 fold per day [25]. In their report, Moon and his colleagues managed to produce one human-adapted *P. knowlesi* line known as *P. knowlesi* A1.H1 (PkA1-H.1) line that has been successfully adapted to continuous culture *in-vitro*, providing an *in-vitro* model suitable for further study on *P. knowlesi*. A major step towards adaptation involves a change in host cell preference for invasion [25].

While not restricted to cells of a certain age in macaques, *P. knowlesi* was found to invade mainly young RBCs in humans. In contrast, the human-adapted line had increased invasion efficiency into older RBCs, providing access to a wider range of suitable host cells [17, 25]. The invasion of adapted *P. knowlesi* lines were still DARC dependent [25]. However, further investigation is necessary on the exact receptors involved in the later stage of invasion as the DARC level on blood cells diminishes as they mature [17]. Therefore, the availability of human RBC-adapted *P. knowlesi* lines and the analyses of adaptation mechanism would enable the understanding of the parasite biology and the pathophysiology of knowlesi malaria in humans.

The Implications of Adaptation Success to Malaria Study: A successful establishment of continuous *P. knowlesi in-vitro* culture and adaptation in human RBCs would offer an ideal system for various implications to malaria research. The development of several advanced tools and technologies for use in other *Plasmodium* studies such as *P. falciparum* could be readily adapted for use in *P. knowlesi* [17]. The application of advanced microscopy technologies such as real-time imaging of invasion 66, super-resolution microscopy [67] and long-term live microscopy [17] to the *P. knowlesi* system will be particularly fruitful. The combination of high transfection efficiency and the ability to culture *P. knowlesi* in human RBCs will be ideal for the establishment of high-throughput analysis of gene functions [29] and novel reverse genetics, which can be used to investigate the role of RBC receptors in *P. knowlesi* invasion [68].

The application of several knockout/knockdown systems such as ligand-regulatable FKBP protein destabilization domains (ddFKBP) [69, 70] and the DiCre conditional recombinase system [71] in *P. knowlesi* will be beneficial in determining the functions of essential blood stage proteins. Likewise, high-efficiency genome editing tools such as TALEN (transcription activator-like effector nucleases) [72], CRISPR (clustered regularly interspaced short palindromic repeat)-Cas system [73] and zinc-finger nucleases [74] could be used to facilitate genomic integration of DNA constructs in *P. knowlesi* [17].

Also, human RBC-adapted *P. knowlesi* would allow a comprehensive study of invasion mechanism hence would prevent infection and suppress re-emergent blood stage parasite *via* the development of new anti-malarial drugs and vaccines [36, 75, 76]. Taken together, malaria research could be expanded further following successful adaptation and development of a long-term *P. knowlesi in-vitro* culture.

ACKNOWLEDGEMENT: We would like to thank Universiti Sains Malaysia (USM) for the Research University Grant given (USM RUI Grant No. 1001/PPSK/812201).

CONFLICT OF INTEREST: The authors declare no conflict of interest.

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How to cite this article:
Mohamad FS and Abu-Bakar N: Towards successful adaptation of *Plasmodium knowlesi* to long-term *in-vitro* culture in human erythrocytes. Int J Pharm Sci & Res 2019; 10(6): 2663-69. doi: 10.13040/IJPSR.0975-8232.10(6).2663-69.