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Protocols for the In Vitro Culture of the Zoonotic Malaria Parasite *Plasmodium knowlesi*

Amel O.A. Ahmed, PhD; Ioana Brasov, BS; Sujatha Rashid, PhD; Rebecca Bradford, MS, MBA; Robert E. Molestina, PhD
ATCC, Manassas, VA 20110. Correspondence: aahmed@atcc.org

Abstract

Plasmodium knowlesi is an emerging zoonotic malaria parasite causing severe human infections in Southeast Asia, characterized by high parasitemia, a rapid 24-hour erythrocytic cycle, and frequent coinfection with *P. falciparum* and *P. vivax*. Continuous in vitro culture of non-falciparum human malaria parasites remains technically challenging, limiting experimental reproducibility and downstream applications. Here, we describe protocols for adapting two *P. knowlesi* strains to long-term in vitro culture using host-specific erythrocytes under defined hematocrit and gas conditions. Parasites were cultured at 2-4% hematocrit in RPMI-based media supplemented with horse serum and/or AlbuMAX II under low-oxygen conditions. One strain was successfully adapted to human erythrocytes, demonstrating sustained asexual replication, stable growth across serial passages, parasitemia exceeding 11% by day 20, and the successful generation of cryopreserved seed stocks. In contrast, the second strain exhibited host-cell restriction. It could not be maintained in human erythrocytes but was successfully propagated in rhesus macaque erythrocytes under reduced oxygen conditions. These findings highlight strain-dependent host specificity and define optimized culture conditions that support robust parasite growth, long-term maintenance, and preservation. Optimized culture conditions expand available resources for *P. knowlesi* research and enable its application in downstream experimental systems. Given the comparatively high reported transfection efficiencies of *P. knowlesi* relative to *P. falciparum*, these culture systems provide a robust platform for advancing molecular studies of zoonotic malaria parasites.

Background

- Long-term in vitro culture of *P. knowlesi* remains technically challenging, limiting reproducible experimentation. This restricts genetic, functional, and drug-screening studies
- Additional in vitro human malaria parasite models that are genetically tractable and that complement the *P. falciparum* system have tremendous potential.
- The objective of this study was to adapt two *P. knowlesi* strains to long-term in vitro culture and to evaluate host erythrocyte specificity by comparing growth in human versus rhesus macaque erythrocytes under defined hematocrit and gas conditions.

Methods

***Plasmodium knowlesi* strains used**

- Two *P. knowlesi* strains were evaluated for continuous in vitro growth*: Plasmodium knowlesi* A1-H.1 and *Plasmodium knowlesi* YH-1 (BEI Resources; MRA-1283 www.beiresources.org).

Culture media, blood, and in vitro conditions (Figure 1)

- Human blood was tested for the presence of Duffy antigen (Fy^a) using Anti-Fy^a monoclonal IgG in an indirect agglutination test.
- Seed stocks were cryopreserved at ≥ 5% parasitemia containing ≥50% ring-stage parasites.

Methods

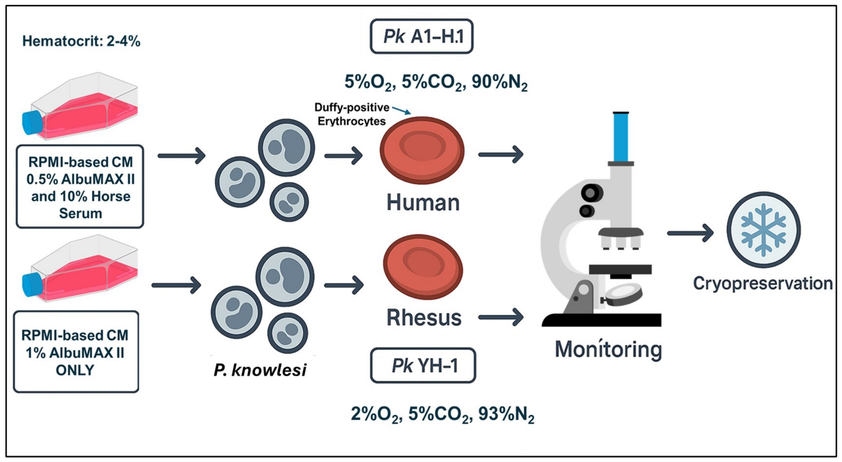

Figure 1: Schematic overview of in vitro culture conditions used to evaluate strain- and host-erythrocyte specificity of *Plasmodium knowlesi*. Parasites were cultured at 2–4% hematocrit in leukocyte-depleted erythrocytes using RPMI-based media supplemented with horse serum and/or AlbuMAX II. Cultures were maintained under low-oxygen gas conditions. Strain A1-H.1 was adapted to Duffy-positive human erythrocytes under 5% O_2, whereas strain YH-1 (MRA-1283) exhibited host restriction and required rhesus macaque erythrocytes under lower oxygen conditions.

Results

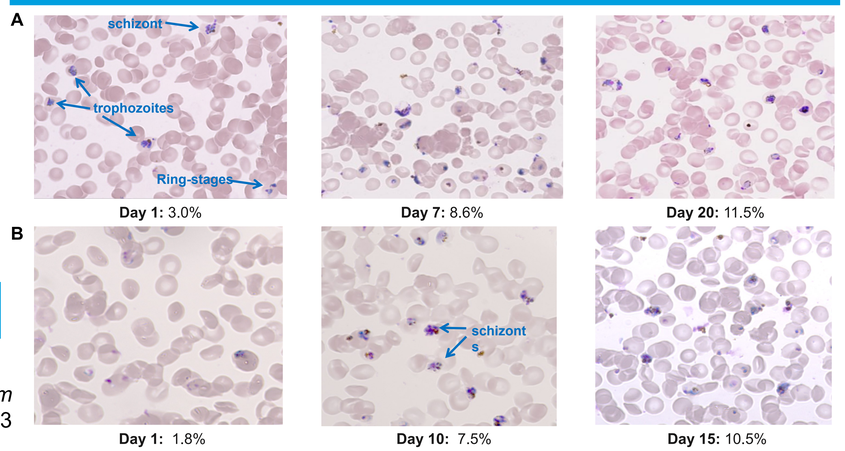

Figure 2: Giemsa-stained blood smears. (A) *P. knowlesi* A1-H.1 parasite showing successful adaptation to Duffy-positive human erythrocytes; sustained asexual replication was achieved and parasitemia exceeded 11% by day 20. Stable growth was maintained across passages. (B) *P. knowlesi* YH-1 (MRA-1283) appears to be host-restricted. This strain demonstrated strain-specific erythrocyte dependence. It was successfully propagated in rhesus macaque erythrocytes at a lower oxygen content, and parasitemia exceeded 10% by Day 15.

Results

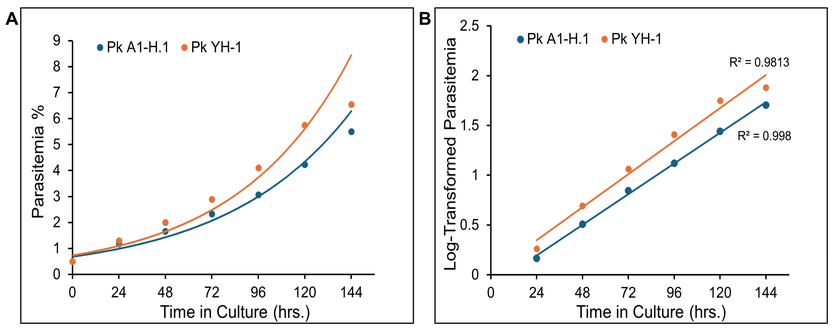

Figure 3: Growth kinetics of *P. knowlesi* strains A1-H.1 and YH-1. (A) Exponential growth curve showing rapid replication with a 24-hour erythrocytic cycle. (B) Linear regression of log-transformed parasite counts across six asexual cycles, where the slope provides an empirical estimate of parasite growth rate ($R^2 \geq 0.90$).

Conclusions

- Successful long-term in vitro culture of *P. knowlesi* requires a compatible combination of parasite strain and host erythrocyte.
- Optimized conditions support high parasitemia, culture stability, and cryopreservation.
- These culture systems expand access to *P. knowlesi* for genetic manipulation, functional genomics, and antimalarial drug discovery.
- *P. knowlesi* exhibits higher reported transfection efficiencies than *P. falciparum*; these protocols enable expanded molecular and translational studies of zoonotic malaria.

Future Planned Work

Adaptation of *Pk YH-1* parasite cultured in rhesus macaque erythrocytes to human erythrocytes is underway to harmonize *Plasmodium knowlesi* in vitro culture conditions; preliminary data are promising

Acknowledgments

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