Robust continuous in vitro culture of the *Plasmodium cynomolgi* erythrocytic stages

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INTRODUCTION

The development of a protocol for the routine continuous in vitro culture[1] of the erythrocytic stages of *Plasmodium falciparum* revolutionized research on this parasite responsible for the highest mortality rates globally. However, efforts to maintain the widespread and difficult to treat *P. vivax*[2] in vivo are hindered by a strict preference for invaded reticulocytes, a minor short-lived fraction of peripheral blood. Cultivation of *P. cynomolgi*, a macaque-infecting species phylogenetically close to P. vivax, was briefly reported in the early 1980s, but not pursued further. Here, we define the conditions under which P. cynomolgi can be adapted to long term in vitro culture to yield parasites that retain the key morphological and phenotypic features of P. vivax.

METHODS

Ethical committees and animal welfare

All animals were housed in accordance with the Guide for the Care and Use of Laboratory Animals and the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) Standards. All studies were approved by the Novartis Ethical Review Council and Novartis Institutional Animal Care and Use Committees prior (IACUC) to study initiation.

Parasite culture and investigations

Three P. cynomolgi strains were used in this study, the Berok strain[3], the B strain (P. cynomolgi bastianelli)[4] and M strain (P. cynomolgi Mulligan strain)[5,6]. Strains were used for continuous culture (Fig. 1) and studies on transmission, sporozoites, and infectivity were conducted.

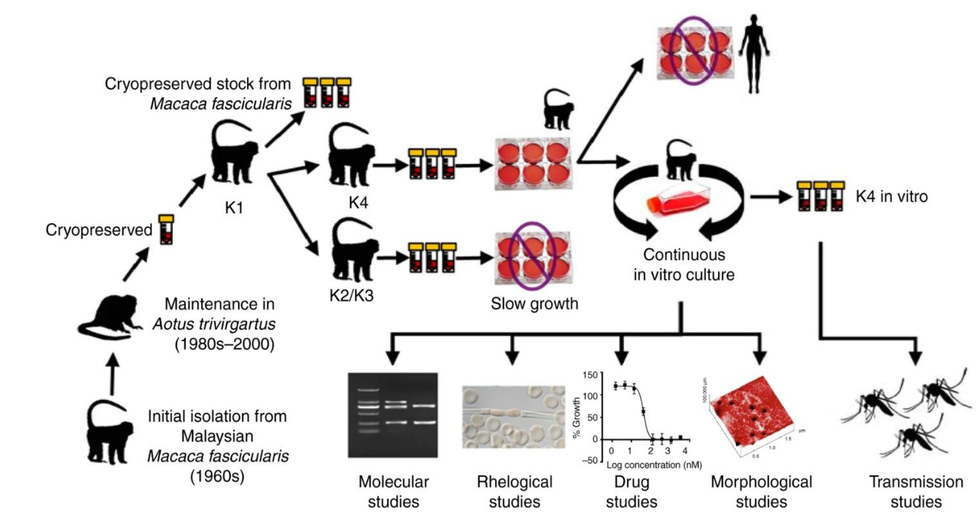

Fig. 1: Diagrammatic representation of the adaptation of P. cynomolgi Berok K4 line to continuous culture

RESULTS

In vitro-cultured Berok K4 line retains transmission ability

Two rhesus monkeys were infected with 100,000 P. cynomolgi Berok K4 sporozoites (Fig. 2a). Both monkeys became blood-stage patent on day 11 post infection (dpi). Monkey 1 relapsed 31 dpi and 52 dpi, after which radical cure was administered (chloroquine and primaquine), while the second remained negative for the whole duration of follow-up (102 dpi).

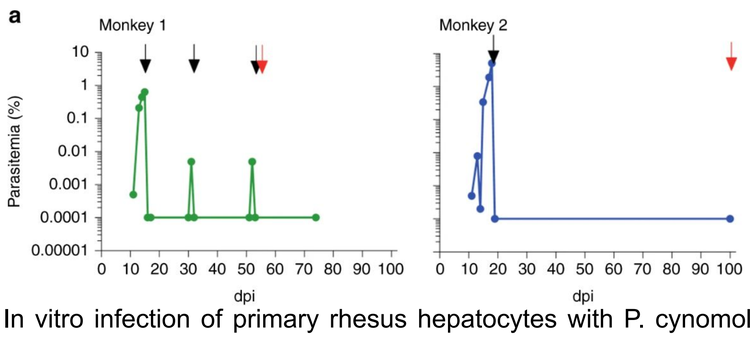

Fig. 2a: In vivo blood-stage parasitaemia in two rhesus monkeys infected with 100,000 P. cynomolgi Berok K4 sporozoites. Arrows indicate drug treatment (black arrows: 5-day chloroquine treatment, red arrow: 7-day primaquine treatment).

In vitro infection of primary rhesus hepatocytes with P. cynomolgi Berok K4 sporozoites produced both hypnozoites and developing liver-stage schizonts after 6 days (Fig. 2b). Average infectivity of sporozoites derived from the P. cynomolgi Berok K4 line was lower than that of those derived from the P. cynomolgi M strain (Fig 2c). P. cynomolgi Berok K4 line generated half the number of hypnozoites compared with that produced by P. cynomolgi M strain (Fig. 2d).

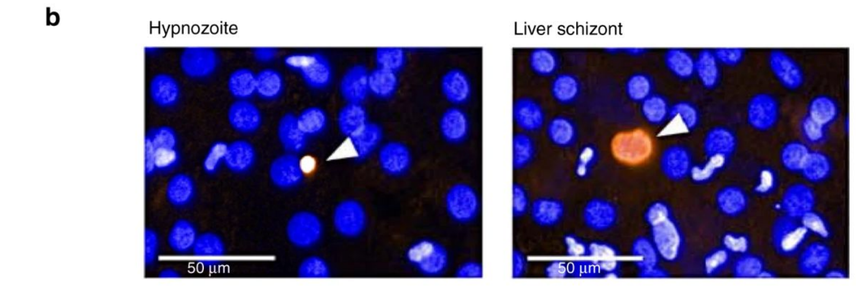

Fig. 2b: Hypnozoites (left panel) and developing liver-stage schizonts (right panel) under anti-PcHsp70 stain, Alexa 568 fluorescent dye and DAPI for nuclei.

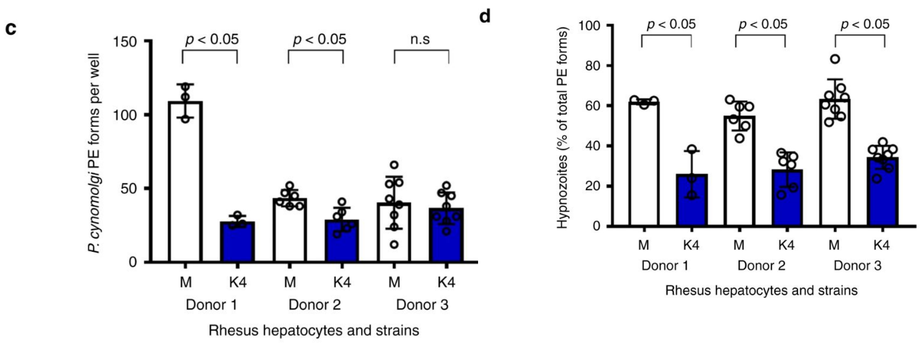

Fig. 2c: Around 16 pre-erythrocytic forms per 10,000 inoculated sporozoites (K4 line) and 23 PE forms (M strain).
Fig. 2d: 30% hypnozoites (K4 line) and 60% (M strain) observed in vitro using primary rhesus hepatocytes.

REFERENCES

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4. Garnham, P. C. A new subspecies of Plasmodium cynomolgi. Rivista Di Parassitologia 20, 273–278 (1959).
5. Coatney, G. R. et al. Transmission of the M strain of Plasmodium cynomolgi to man. Am. J. Trop. Med. Hyg. 10, 673–678 (1961).
6. Mulligan, H. W. Descriptions of two species of monkey Plasmodium isolated from Silenus irus. Arch. f. Protist 84, 285–314 (1935).

RESULTS (CON'T)

Nanostructure and rheology of Berok K4 line-infected RBCs

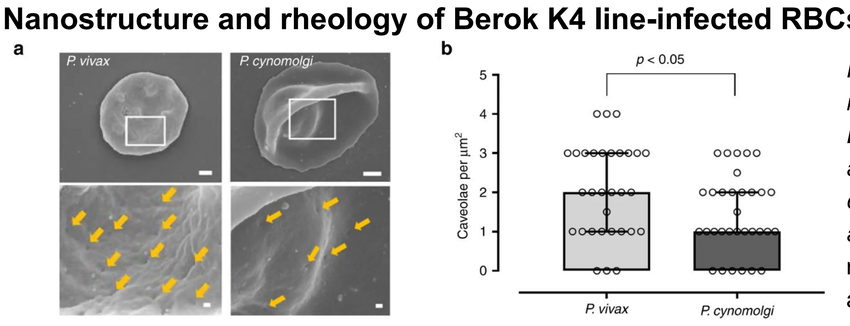

Fig. 3a-d: Phenotypic and rheological characteristics of the Berok K4 line from in vitro culture are similar to P. vivax, including caveolae structures (yellow arrows in SEM, scale bars represent 1 µm and 100 nm for area shown at higher magnification in white box) *and their median (+/- IQR) dimensions in infected RBCs, but at lower frequency in P. cynomolgi.*

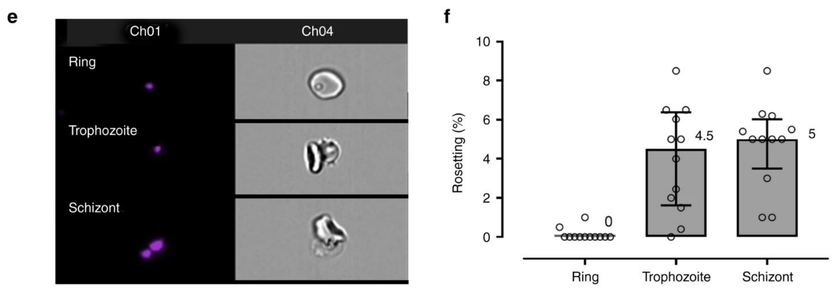

Fig. 3e-f: Amnis flow imaging shows that the mature erythrocytic stages P. cynomolgi Berok K4 readily formed rosettes with uninfected red blood cells, also a key feature P. vivax.

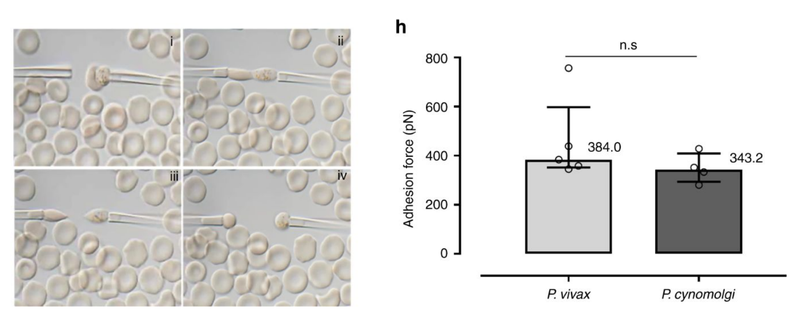

Fig. 3g-h: Rheological stability of the rosettes is demonstrated by the dual micropipette aspiration method.

High-throughput drug susceptibility assay using the Berok K4 line

SYBR green I proliferation assay was validated for use with the P. cynomolgi Berok K4 line. Dose response and single-point screens based on 96- and 384-well-plate assay formats were established (Fig. 4a–c).

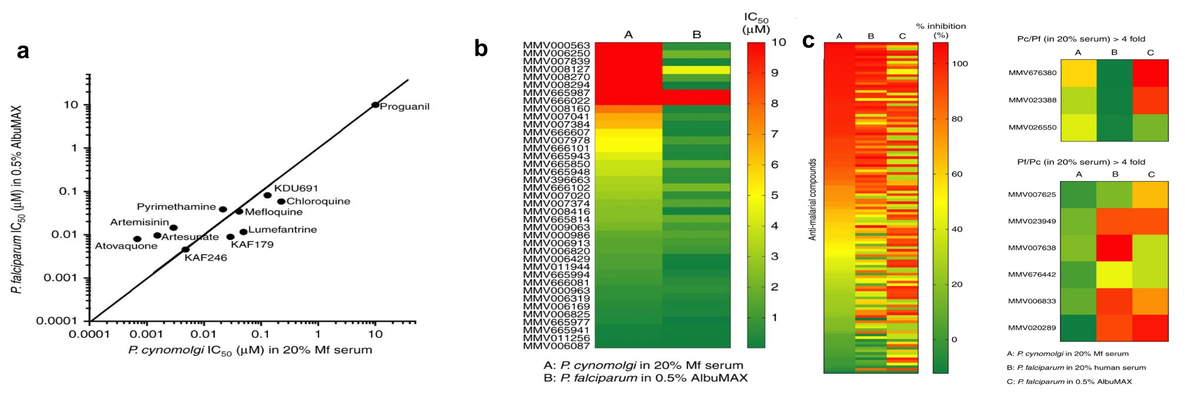

Fig. 4: Drug susceptibility testing using P. cynomolgi Berok K4 in vitro culture. 4a: Correlation of P. cynomolgi Berok K4 and P. falciparum IC50 values of common antimalarial reference compounds in a SYBR green I proliferation assay; 4b: Heatmap showing IC50 of a representative set of compounds from the Malaria Box; 4c: Heatmap showing percentage of inhibition of the 125 antimalarial compounds from the Pathogen Box.

Majority of the compounds showed activity against both P. falciparum and P. cynomolgi, except for six compounds—MMV008127, MMV006250, MMV008270, MMV000563, MMV007839 and MMV008294 which displayed an IC50 > 10 µM for P. cynomolgi, and < 5 µM for P. falciparum.

Nine compounds (MMV676380, MMV023388, MMV026550, MMV007625, MMV023949, MMV007638, MMV676442, MMV006833 and MMV020289) showed more than fourfold difference in inhibition between P. cynomolgi Berok K4 and P. falciparum in their equivalent serums.

Schizont maturation assays in the Berok K4 line and P. vivax

IC50 values for chloroquine were similar for *P. cynomolgi* in both assays (Fig. 5a,b), and equally comparable with the IC50 values obtained from the *P. vivax* clinical isolates (Fig. 5c).

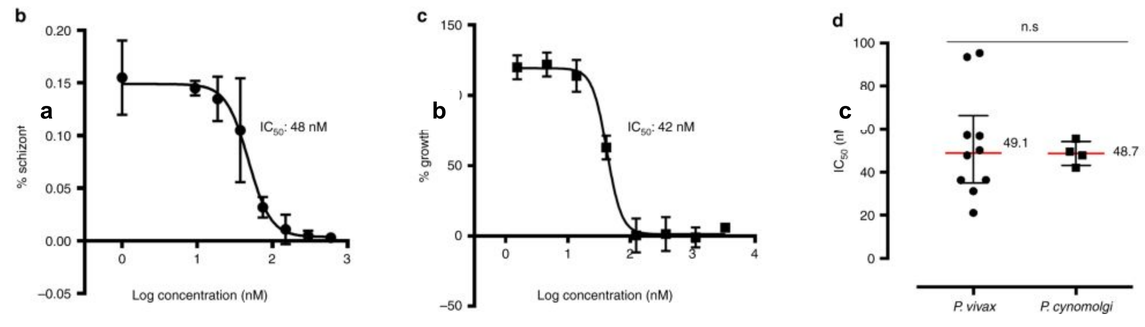

Fig. 5a: IC50 determination of chloroquine in P. cynomolgi Berok K4 continuous culture using schizont maturation assay, 5b: C50 determination of chloroquine in P. cynomolgi Berok K4 continuous culture using SYBR green I proliferation assay, 5c: IC50 of chloroquine in P. cynomolgi Berok K4 continuous culture and P. vivax clinical isolates using schizont maturation assay.

CONCLUSION AND FUTURE WORKS

The fact that the in vitro-maintained Berok K4 line parasites retained infectiousness to mosquitoes subsequent to cultivation augurs well for the eventual use of in vitro cultures to support mosquito infections. We are further exploring modifications to our culture protocols for high-throughput screening to accelerate critical fundamental and translational research to develop drugs and vaccines against *P. vivax*, a widespread species whose control will determine the success of current efforts to eradicate malaria.