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A new Plasmodium falciparum field isolate from Benin in continuous culture: Infectivity of fresh-produced and cryopreserved gametocytes to Anopheles gambiae

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Abstract

Transmission-blocking vaccines and drugs are likely to be key interventions in efforts to achieve malaria elimination. However, transmission-blocking studies are reliant upon a limited number of culture-adapted strains of *Plasmodium falciparum* with limited genetic variability, or on field isolates which are only maintained transiently in the laboratory and therefore not amenable to replication studies. Herein, we investigated the gametocytogenesis capacity and infectivity to *Anopheles gambiae* mosquitoes of a *P. falciparum* field isolate collected from a malaria patient from Benin compared to those of NF54 strain. The intraerythrocytic developmental cycle (IDC) was similar in both *P. falciparum* strains (ranges of parasitaemias were respectively 0.02–11.53% and 0.035–10.5% throughout twelve days of culture). The culture-adapted parasites displayed a significant higher infectivity to *An. gambiae* compared to that of NF54 (mean oocyst prevalence and intensity: 16.94%, $CI_{95\%}$= [15.15–18.73] vs. 3.13%, $CI_{95\%}$= [2.30–3.96], $p < 0.0001$ and 3 vs. 1 oocysts/infected mosquito, $p = 0.002$ respectively). Even after cryopreservation for up to 14 days, gametocytes from the field isolates were capable of infecting *An. gambiae* mosquitoes at a prevalence of up to 30% with an average of 12 oocysts/ midgut. This new *P. falciparum* strain will enhance malaria transmission-blocking studies in endemic countries.

Background

Since the beginning of the twenty-first century, substantial strides in malaria control have been made through the use of long-lasting insecticide-treated bed nets (LLINs), indoor residual-insecticide spraying (IRS) and artemisinin-based combination therapies (ACTs) [1]. However, as insecticide-based interventions have been scaled-up, a concomitant spread of insecticide resistance has been reported in all major *Anopheles* mosquito species across malaria-endemic areas [2–6]. Similarly, with the increased treatment of malaria cases, recent emergence of *Plasmodium falciparum* resistance against ACTs has been noted [7], threatening malaria control and elimination efforts. *Plasmodium falciparum* remains the most widespread *Plasmodium* species across tropical regions of the world, causing the highest malaria morbidity and mortality rates [8]. During their asexual intraerythrocytic developmental cycle (IDC) in the human host, *P. falciparum* parasites invade the red blood cells (RBCs) resulting in the occurrence of malaria symptoms. Thereafter, some asexual parasites form gametocytes, the sexually-differentiated transmission stages. Following feeding on gametocyte-infected human blood, *Anopheles gambiae* mosquitoes can become infected and transmit malaria parasites after biting another host [9,10]. Studies have reported that carriers of subpatent *Plasmodium falciparum* might have future periods of increased parasite density [11,12]. These low-density infections have been shown to contribute to and sustain malaria transmission in endemic settings [13]. The transmissible stage (gametocyte) of *Plasmodium* parasite has now become an attractive target for malaria elimination using either gametocytocidal drugs [14,15] or vaccines against the sporogonic stages of the parasite within mosquito vectors [16,17].

To better protect their citizens from malaria, the African Union, in partnership with the Roll Back Malaria (RBM), launched a continent-wide campaign in July 2018 called "Zero malaria starts with me" [18].

Additionally, a new initiative called "Civil Society for Malaria Elimination (CS4ME)" from civil society organisations was launched in 2018. This coalition aims to encourage grassroots movements in malaria control initiatives and ensure that decision-making is inclusive for those communities most affected by this fatal disease [19]. Community engagement is key to malaria elimination but requires that the research community continues to develop effective control technologies. Blocking the transmission of *Plasmodium* parasite from humans to *Anopheles* vectors remains a promising strategy for malaria control and elimination [10,20,21]. In order to evaluate this strategy, *in vitro* transmission-blocking studies targeting gametocyte-stage parasites have been developed. However, these studies are often difficult to perform in the laboratory; particularly in malaria endemic countries. This is due to the difficulty of viable (capable of infecting mosquitoes) *in vitro* gametocyte production [22,23]. This underlines the need for promoting experimental feeding assays for cryopreservation of *Plasmodium* gametocytes to strengthen malaria transmission-blocking assays.

Studies on the transmission of malaria parasites are commonly restricted to a few *P. falciparum* reference laboratory strains maintained in *in vitro* culture for decades [24,25]. The unavailability of viable cultured gametocytes, coupled with financial and ethical issues raised by the recruitment of gametocyte carriers, and variations in the infectivity of gametocytes from different carriers [26] hamper the establishment of these experiments in several malaria endemic areas especially sub-Saharan Africa. Moreover, the use of standard laboratory strains of *P. falciparum* as NF54 for investigating parasite biology may have limited applications in the field because the genetic composition of these laboratory isolates often deviate from their original source [27]. In addition, it is possible that long-term culturing of *P. falciparum* NF54 strain can lead to genetic changes as observed in some clones of *P. falciparum*[28] and therefore, all the NF54 parasite-lines in culture in different laboratories may not be genetically identical. Such changes in the genetic composition of laboratory-adapted *P. falciparum* clones could lead to the appearance of differences in transcriptomes [27] that could directly influence the interactions of parasites with their vectors. Recently, there has been a drive to use fresh culture-adapted *P. falciparum* field isolates (which are genotypically and phenotypically close to those in the field) for antimalarial drug screening rather than long-term laboratory strains which are heavily selected for *in vitro* growth [29–31]. It is also important not to use only freshly collected *P. falciparum* gametocyte-positive blood samples from patients to study parasite-vector interactions for malaria transmission-blocking assays given that, the samples are usually not enough to perform multiple assays, which make it hard to distinguish between technical and biological sources of variation in infectivity experiments.

In this study, we implemented *in vitro* continuous culturing of *P. falciparum* field isolates freshly collected from a malaria-infected individual until gametocytogenesis as well as the laboratory-standard reference strain NF54. We compared the dynamics of the IDC and the propensity to generate gametocytes between the parasite strains. Further, we evaluated the infectivity to *An. gambiae* of gametocytes from the field isolates compared to that of NF54 through standard membrane feeding assays. Finally, we determined mosquito infectivity of cryopreserved gametocytes from the culture-adapted field isolates.

Results

P. falciparum**NF54 and field isolates in routine culture and gametocytogenesis**

Both *P. falciparum* strains showed similar IDC through merozoite, ring, trophozoite and schizont parasite stages. During the 12 days of *in vitro* culture, an increase in blood-stage parasitaemia was recorded in both *P. falciparum* strains (ranging from 0.035 to 10.5% for NF54 and 0.02 to 11.53% for Ben229, $p = 0.57$) (Fig. 1). On day 14 post-thawing, Ben229 displayed significantly higher V-stage mature gametocytaemia (1.8%) compared to that of *P. falciparum* NF54 (0.9%) ($p = 0.003$) as illustrated in **Fig. 2a**.

Infectivity to An. gambiae**of**P. falciparum**NF54 and field isolates**

Mature gametocytes were generated from both NF54 and Ben229 strains. Abilities of male gametocytes to exflagellate were similar ($p = 0.15$) in both *P. falciparum* strains (Fig. 2b). The NF54 gametocytes only infected 3 of the 8 *An. gambiae* strains used with mean oocyst prevalence varying from 3.9 to 21.7% and a median intensity of 1 oocyst/midgut (Figs. 3a, **b**). In contrast, the culture-adapted *P. falciparum* field isolates (Ben229) infected all 8 *An. gambiae* strains with a mean oocyst prevalence ranging from 9.7 to 40.5% and a median oocyst intensity up to 6 oocysts/infected mosquito midgut (Figs. 3c, **d**). The pyrethroid-resistant *An. gambiae* s.s. KisKdr strain exhibited the highest mean oocyst prevalence (40%) and median oocyst intensity (6 oocysts/midgut) with Ben229 (Figs. 3c, **d**). When mosquito specimens from all strains were pooled, the mean oocyst prevalence and intensity of Ben229 were significantly higher (16.94%, $CI_{95\%}$= [15.15–18.73], $p < 0.0001$ and 3 oocysts/infected mosquito midgut, $p = 0.002$) compared to those of NF54 (3.13%, $CI_{95\%}$= [2.30–3.96] and 1 oocyst/infected mosquito midgut) (Figs. 3e, **f**). The images presented in **Fig. 4** show the morphologies of asexual, gametocyte and oocyst stages of *P. falciparum* Ben229.

Viability and infectiousness of cryopreserved gametocytes from field isolates

Interestingly, we observed that mature stage-V gametocytes of Ben229 remained viable (morphologically) and stable (in term of density) over 2 weeks of cryopreservation. Overall, no difference was observed between pre- and post-cryopreservation gametocytaemias for any of the four gametocyte culture replicates (Fig. 5a). Two of the strains of *An. gambiae* (KisKdr and TSB) were used to evaluate infectiousness of post-cryopreserved gametocytes; chosen because they displayed the highest permissiveness to infection (in terms of oocyst prevalence recorded in the first experiments). After thawing, both *An. gambiae* strains were infected with mean oocyst prevalence ranging from 6.25 to 30% and median oocyst intensity varying from 3 to 12 oocysts/midgut (Figs. 5b, **c, d**).

Discussion

Effective malaria control relies on several approaches including transmission-blocking strategies. However, few studies have been performed to evaluate these strategies by using near-field isolates of *P.*

falciparum. In the present work, we compared the intraerythrocytic developmental cycle (parasitaemias) over time, and infectivity to *An. gambiae* of culture-adapted field isolates to those of a reference laboratory *P. falciparum* strain NF54. *Plasmodium falciparum* field isolates (Ben229) parasitaemias were similar to those of NF54 over time, indicating that the field isolates seemed to have normal phenotypic characteristics with regard to their growth and maintenance under standard *P. falciparum* continuous culture conditions. Other studies reported phenotypic stability and subsequent red blood cell invasion in culture-adapted *P. falciparum* field isolates from Kenya, China-Myanmar and South America [32–34]. In addition, our findings indicate that both of the *P. falciparum* strains produced functionally mature gametocytes which successfully exflagellated, although the Ben229 strain generated subtantially higher mature stage V gametocytaemias. Thus, the culture-adapted Ben229 strain could be more suitable for the screening of vaccines and drugs against asexual and sexual blood stages of *P. falciparum*.

For malaria elimination in endemic countries, one of the most promising measures remains the blocking of transmission of *Plasmodium* from human host to *Anopheles* mosquito [35] since the passage through the malaria vector is a fundamental step of parasite life cycle [10]. Laboratory reference *P. falciparum* strains such as NF54 and 3D7 are still commonly used in *Plasmodium* transmission-blocking assays [20,36,37]. Carrying out such studies using freshly culture-adapted field isolates could provide more realistic parameters reflecting malaria transmission as occurring in natural settings. By comparing the overall mosquito infection success (prevalence and intensity of oocyst infection), we observed a higher infectivity of *P. falciparum* Ben229 to both laboratory and near-field *An. gambiae* compared to that of NF54. Since both parasite strains were cultured in the same conditions and used for the feeding assays in one hand, and they displayed similar number of exflagellation centers on another hand, the difference of infectivity observed may, in part, be explained by the differences in the Ben229 performance to develop in these mosquito strains. This superior performance developed by Ben229 in infecting malaria vectors could be related to the several gametocyte features such as biology, physiology, genoytpe, behaviour, plasticity, sex ratio and mating patterns that favoured their infectivity to mosquitoes. Morlais and colleagues [38] demonstrated that, during their within-mosquito development, *P. falciparum* field isolates use various strategies to reproduce according to the parasite genotypes. They found that genetic polymorphism in the parasite diploid stages positively correlated to the oocyst loads in infected mosquitoes. In addition, other studies reported that, a gene in laboratory *P. falciparum* strain NF54 allowed parasites to evade the immune system of *An. gambiae* mosquitoes favouring their within-mosquito development [39,40]. Since the *P. falciparum* field isolates Ben229 has been freshly adapted to culture in 2018, it would have maintained a genetic polymorphism that allowed them to reproduce more successfully in mosquito vectors than long-term laboratory NF54 parasites. We plan to explore in further studies, the genetic factors that conferred the observed enhanced performance of *P. falciparum* Ben229.

A model-based evaluation of intervention strategies demonstrated that existing tools and the unrealistical deployement of current control strategies are unlikely to achieve the goal of reducing malaria transmission in most African settings [41]. This finding has highligthed the need for additional and new tools and strategies that specifically target malaria transmission by reducing the vectorial capacities of

the main malaria vectors in sub-Saharan Africa [35]. Among these tools/strategies, vaccines that interrupt malaria transmission (VIMT) could be a crucial strategy which will considerably contribute to the effort to eradicate malaria in African settings [42]. Progress has been made in development of transmission-blocking vaccines (TBVs) that have shown promising safety and functional activity during clinical assays [43–45]. Once the candidate antigens that prevent the onward transmission of malaria parasite to its vector are discovered, both direct and standard membrane feeding assays are used to assess their efficacy [46,47].

The evaluation of the candidate antigens for TBV discovery faces a number of peculiar challenges that related to the type of parasite and mosquito colony, the parasite exposure and the availability of viable gametocytes [48]. In the aim to predict the efficacy of these candidate antigens in natural settings, field circulating parasites and local mosquito colonies would have to be the experiment materials. Moreover, the level of parasite exposure (relies primarily on gametocytes density in the infected blood) would need to be mastered technically. The result obtained in the present work overcomes this requirement and will allow different candidate antigens to be evaluated using naturally occurring parasites isolates with local mosquito colonies in African settings where the availability of viable cryopreserved gamatocytes has become possible. We report that cryopreserved V-stage mature gametocytes of *P. falciparum* field isolates (Ben229) maintained in gassed tissue culture flasks, remained stable (in terms of density) and infectious to both laboratory and near-field *Anopheles gambiae* mosquitoes after been cryopreserved. Furthermore, mosquito infectivity level produced was almost similar to that of the fresh-produced gametocytes. Cryopreserved Ben229 gametocytes could help to strenghten the production of infectious *P. falciparum* gametocytes which could boost inter laboratory replication studies in malaria endemic regions because they can be transported in liquid nitrogen to other laboratories.

So far, technical platforms for successful *P. falciparum* gametocyte culture followed by experimental infections are lacking in Africa. Empirically, the *P. falciparum* strains such as NF54 and 3D7 are the parasite lines most available and used for *in vitro* routine culture in some African laboratories [49–51]. But production of infectious gametocyte from these parasite strains for experimental infections of mosquitoes is still an arduous task. The present work proposes the *P. falciparum* field isolates (Ben229) collected and adapted in *in vitro* routine culture in Benin (West Africa) showed the higher performances in terms of gametocyte generation and infectivity to malaria vector *An. gambiae* over NF54 line. This provides a glimmer of hope for promoting these field isolates of *P. falciparum* and therefore, facilitate expansion of the platforms for experimental infections in malaria-endemic countries. Thus, Ben229 could be an interesting asset for evaluation of the VIMT candidates in endemic zones through membrane feeding assays.

Plasmodium falciparum field isolates adaptation in *in vitro* culture for infectious gametocytes production could undoubtedly improve cellular studies towards effective malaria control and elimination. The present work highlights infectivity to *An. gambiae* of the fresh-produced and cryopreserved V-stage mature gametocytes from freshly culture-adapted *P. falciparum* field isolates. Further studies are required to (i) explore the genomic profile of *P. falciparum* Ben229, (ii) expand the number of field isolates and (iii)

investigate how long the infection capacity of V-stage mature gametocytes from culture-adapted field isolates will be maintained during cryopreservation.

Methods

Plasmodium falciparum**NF54 and field isolates in**in vitro**continuous culture**

In this study, we used two *P. falciparum* strains (laboratory line NF54 isolated from Netherlands in 1981 [24,25] and field isolates (encoded Ben229) collected in Benin in 2018. The NF54 strain was obtained from MR4, BEI Resources, USA. The *P. falciparum* field isolates Ben229 were collected from a symptomatic patient in the framework of a cross-sectional study we have conducted in 2018 among school children aged ≥ 4 years in Ouidah, South-westhern Benin.

In vitro culturing of both NF54 and the field isolates strains was implemented following standard protocols with minor modifications [52,53]. Briefly, after centrifugation of 3 4 mL of blood, at 800 g for 5 minutes, plasma was removed and the pellets washed and then adjusted to 5% haematocrit with RPMI-1640 (with 2 mM L-glutamine, 25 mM HEPES, 0.85 g/L Sodium bicarbonate) supplemented with 50 mg/L Hypoxanthine (Gibco), 0.25% (w/v) Albumax II (Gibco) as human AB^+ serum substitute [54–57] without gentamycin. Cell culture flasks (T-75 cm^2 Nunclon^{TM}, Denmark) were used under a reduced oxygen environment of 3% O_2, 5% CO_2 and 92% N_2 (AIR LIQUID France Industrie) and incubated at 37°C. The parasites were maintained in uninterrupted culture with periodic supplement of washed O^+ RBCs and media, over at least four weeks (culture flasks were changed weekly) and parasitaemias were quantified daily by microscopy based on rapid Giemsa staining of thin smears [58].

Plasmodium falciparum**NF54 and field isolates gametocytogenesis and exflagellation tests**

Gametocytes of *P. falciparum* NF54 and field isolates were generated as previously described with slight modifications [59,60]. Cultures were maintained until approximately 8% of RBCs showed ring-stage parasitaemia before culture synchronization using 5% D-sorbitol (Sigma). Fresh O^+ RBCs were added to the synchronized cultures, which were maintained below 30°C for up to 30 minutes in order to stimulate gametocyte production. Each culture was kept at 37°C, and the medium was changed daily during formation of the sexual stages until V-stage mature gametocytes were harvested, 14 to 15 days later [61–63]. All gametocyte developmental stages were monitored daily under a microscope and the V-stages especially were identified and quantified according to previously described method [64].

To assess the ability of male *P. falciparum* gametocytes to exflagellate, 200 µL of the gametocyte culture from both *P. falciparum* strains were aliquoted into microfuge tubes and subjected to reduced temperature (30°C) by placing in tap water for 10 minutes according to protocol previously described [65].

Assessment of mosquito infectivity of NF54 and Ben229 fresh-produced gametocytes

The *P. falciparum* standard membrane feeding assays (SMFA) were performed as described previously [66] to explore the infectiousness of both NF54 and Ben229. The different *An. gambiae* strains used, their insecticide resistance profiles are summarized in Table 1. The near-field strains of *An. gambiae* s.l. collected in 2018 were maintained under pressure of the discriminating dose of two insecticides (0.05% deltamethrin and 0.1% bendiocarb) according to WHO test procedures [67].

Table 1
Known resistance mutations and phenotypic resistance profiles for each An. gambiae strain.

***An. gambiae* strains**	**Type of the strains**	**Known resistance mutations**	**Resistance profiles**	**References**
Kisumu (Ki)	Laboratory colonies	None	Susceptible for all insecticides	69
KisKdr (Kd)		Homozygous for *kdr*^{R}(L1014F) mutation	Pyrethroids and DDT resistant	70
AcerKis (Ac)		Homozygous for *ace-1*^{R}(G119S) mutation	Carbamates and organophosphates resistant	71
AcerKdrKis (Ak)		Homozygous for both *kdr*^{R}(L1014F) and *ace-1*^{R}(G119S) mutations	Pyrethroids and DDT, carbamates and organophosphates resistant	72
ASD	Near-field strains	Unknown	Deltamethrin resistant	73,74
ASB		Unknown	Bendiocarb resistant	
TSD		Unknown	Deltamethrin resistant	3,75
TSB		Unknown	Bendiocarb resistant	

ASD, Accra selected with deltamethrin; ASB, Accra selected with bendiocarb; TSD, Tiassalé selected with deltamethrin; TSB, Tiassalé selected with bendiocab.

During SMFA, all materials were pre-warmed and maintained at 37°C to prevent early exflagellation. The NF54 and Ben229 gametocytes from the culture flasks were confirmed to be free for any bacterial contamination by microscopic observations. The mosquitoes used for the feeding experiments were 3 to 4-day old *An. gambiae* females that had been sucrose-starved for 24 hours. Prior to infection, the gametocyte cultures were centrifuged at 700 g for 3 minutes. Pellets were immediately resuspended with AB^{+} heat-inactivated serum to 50% haematocrit and with O^{+} blood to 0.4% gametocytaemia. Mosquitoes were fed on the gametocyte-infected blood up to 20 minutes to allow the majority of them to be fully engorged. After 24 hours, fed mosquitoes were separated and maintained at 27 ± 2°C and 70 ± 8% relative

humidity with access to 10% honey solution until midgut dissection. Each infection experiment was performed three times.

Cryopreservation of Ben229 gametocytes and experimental infections

To assess whether cultured stage V (mature) gametocytes from the *P. falciparum* field isolates could successfully infect *Anopheles* mosquitoes after a round of cryopreservation, a subset of the gametocytes produced was transferred into the vapour phase liquid nitrogen (AIR LIQUID Cryogenic Division, France) according to the standard protocol [68] with slight modifications. Four gametocyte cultures with different gametocytaemias (encoded respectively GC1 (1.27%), GC2 (0.87%), GC3 (0.73%) and GC4 (1.53%)) were centrifuged separately as described above and supernatants were removed. Pellet volume was estimated and equal volumes of the glycerin-based solution (Sigma) consisting of glycerin, D-sorbitol and NaCl were added and the cell suspension was aliquoted in 2 mL individual cryovials (Nunc™, China). After gentle agitation, the cryovials were rapidly transferred in liquid nitrogen for 14 days.

The procedures described in Keister *et al.*[68] with minor modifications were used to thaw the cryopreserved gametocytes. The frozen gametocytes were thawed in a 37°C water batch for 1 minute. Following centrifugation at 700 g for 3 minutes, supernatants were removed and the gametocyte cultures were reconstituted with 3.5% NaCl solution. The NaCl solution was removed after centrifugation and a pellet sample obtained from each gametocyte culture was taken to prepare the thin smear and the remaining was suspended in complete medium for 3 hours prior to experimental infections. Giemsa-stained thin smears were examined under light microscopy for morphological evaluation of gametocytes and assessment of their densities before cryopreservation and post thawing. Experimental infections using both a laboratory colony (KisKdr) and a near-field strain (Tiassalé selected with bendiocarb) of *An. gambiae* were performed three times as described above.

Oocyst counting

The midguts of blood-fed mosquito females were dissected in 0.05% mercurochrome on 7th day following experimental infections. Intensity of malaria parasite infection in each individual female was assessed by counting oocysts under a light miscroscope.

Statistical Analysis

All data analysis was carried out using GraphPad Prism version 8.02 (San Diego, California USA). Parasite (asexual stages and gametocytes) densities were determined by counting the parasitized RBCs from examination of at least 20,000 RBCs and expressed as percentages. The number of exflagellation centers observed per field was used as the ability of male gametocytes to exflagellate. Oocyst prevalence was defined as the number of mosquitoes presenting at least one oocyst in their midguts divided by the total number of dissected mosquitoes. Oocyst intensity is the average number of oocysts developed by infected individual mosquitoes. To determine significance of the difference in parameters such as dynamics of parasitaemias, stage V gametocytaemia, exflagellation ability of male gametocytes and

infectivity (oocyst prevalence and intensity) between NF54 and Ben229, we used Mann-Whitney tests with $p < 0.05$ accepted as statistically significant.

Availability of data and materials

The datasets are available from the corresponding author on reasonable request.

Declarations

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Author contributions

A.A.M., L.S.D., D.W. and M.J.D. conceived and designed the study. A.A.M., L.S.D., F.K.A. and L.D. performed the experiments. A.A.M. and L.S.D. analysed the data. A.A.M., L.S.D., L.E.A., E.M.O., D.W. and M.J.D drafted the manuscript. All authors read and approved the final version of manuscript.

Additional information

Competing interests: The authors declare that they have no competing interests.

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