Evaluation of Anti-Malaria Potency of Wild and Genetically Modified Enterobacter Cloacae Expressing Effector Proteins in *Anopheles Stephensi*

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Research

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Abstract

Background: Malaria is one of the most lethal infectious diseases in tropical and subtropical areas of the world. To fight the disease, paratransgenesis using symbiotic bacteria offers a sustainable and environmentally-friendly strategy. Here we evaluated the disruption of malaria transmission in the *Anopheles stephensi-Plasmodium berghei* assemblage, using wild and modified insect gut bacterium, *Enterobacter cloacae*.

Methods: The assay was carried out using *E. cloacae dissolvens* wild-type (WT) and its three engineered strains expressing GFP-defensin (GFP-D), scorpine-HasA (S-HasA), and HasA, The 3-5 day-old female mosquitoes were supplemented overnight with the studied bacteria [1×10^9cells/mL of 5% (wt/vol), fructose and red dye (1/50 ml)] soaked on cotton-wool. Each group of sugar-fed mosquitoes was then starved for 4-6 hours and fed on a *P. berghei*–infected mouse for 20 min in the dark at 17-20°C. The blood-fed mosquitoes were kept at 19±1°C and RH 80±5, and parasite infection was measured by midgut dissection and oocyst counting 10 days post-infection (dpi).

Results: Both wild-type and genetically modified bacterial strains significantly (P< 0.0001) disrupted the *P. berghei* development in the *An. stephensi* midgut, in comparison with the control group. The mean parasite inhibition of *E. cloacae*^{WT}, *E. cloacae*^{HasA}, *E. cloacae*$^{S\text{-}HasA}$, and *E. cloacae*$^{GFP\text{-}D}$ was measured as 72, 86, 92.5 and 92.8 respectively.

Conclusions: The wild and modified *E. cloacae* might abolish oocyst development by providing a physical barrier or by excretion of intrinsic effector molecules. These findings reinforce the case for the use of either wild or genetically modified *E. cloacae* bacteria as a powerful tool to combat malaria.

Background

Of the world's vector-borne diseases, malaria causes the greatest health concern, with 229 million cases and 409000 deaths globally in 2019 [1]. The *Plasmodium* parasite is the causative agent of malaria and the female *Anopheles* mosquito is the vector of the disease. The *Anopheles stephensi* species is the main malaria vector from Asia to the Horn of Africa [2–5], and therefore programs to control the *A. stephensi* population and to limit the ability of the mosquitoes to transmit *Plasmodium* (refractory mosquitoes) have the potential to reduce the malaria disease burden [6].

Currently, the most common methods of mosquito control are indoor residual spraying (IRS) and insecticide-treated nets (ITN) [1, 7]. But due to emerging insecticide resistance in malaria vectors, particularly in *A. stephensi* [8–10], and considering the eco-environmental concerns about off-target effects of insecticide use, it is imperative to find innovative control measures [11–15]. Transmission blocking strategy (TBS) has been recently proposed as a potential means of malaria control, with more emphasis on inhibiting the development of the *Plasmodium* parasite in the vector mosquito [16–18]. Gametocytocidal drugs, transmission-blocking vaccines, and replacement of wild mosquitoes with refractory mosquitoes are currently the most important methods used in TBS. The last method consists

of the genetic manipulation of *Anopheles* mosquitoes to render them refractory to *Plasmodium* parasite development. This is done using anti-plasmodium molecules (transgenesis) [19], naturally refractory mosquitoes [20], artificial gene-drive mechanisms [21, 22], or micro-symbionts genetically modified by effector molecules, that are reintroduced into the wild mosquito population (paratransgenesis) [6, 12, 23, 24].

The *Plasmodium* parasite, during development in its invertebrate hosts (vectors), undergoes a decreasing population trend, from 10^3-10^4 gametocytes to 10^2-10^3 motile ookinetes and, finally, to five or fewer oocysts [25, 26]. This bottleneck could be considered a prime target for intervention and blocking the parasite transmission [27, 28]. The main factors creating the bottleneck include gut digestive enzymes, the mosquito's immune responses and intestinal microbial flora. The intestinal microbial flora plays a vital role in blocking parasite development in the *Anopheles* midgut. This effect is exerted directly by the proliferation of bacteria after a blood meal, simultaneously with the development of the ookinete stage, and indirectly via expression of anti-microbial genes [29–35].

To date, a number of different symbiotic bacteria have been suggested for use in a paratransgenesis strategy for fighting malaria. *Serratia* AS1 (isolated from *Anopheles spp.*), *Asaia sp.* (isolated from *An. gambiae*, *An. stephensi*, *Aedes albopictus* and *Ae. aegypti)* and *Pantoea agglomerans* (isolated from *An. stephensi, An. gambiae* and *An. funestus*) are the bacterial species most used for the paratransgenic control of malaria [11, 24, 26, 30, 36–40]. These genetically modified bacteria could potentially abolish *Plasmodium* parasite development in the *Anopheles* midgut by expressing anti-*Plasmodium* molecules, however, due to convergent evolution, the wild-type bacteria have shown limited intrinsic antiparasitic activities in the mosquito midgut [26, 41].

Enterobacter cloacae, a gram-negative, facultative anaerobic, rod-shaped bacterium, was found as a component of the microflora of *An. stephensi* [42, 43], *An. albimanus* [44], *Ae. albopictus* and *Ae. aegypti* [45], as well as other medically important insects [46, 47]. This bacterium has shown an innate blocking effect on *Plasmodium* development and could potentially limit *P. berghei* and *P. falciparum* development in *An. stephensi* through a marked population increase leading to stimulation of the mosquito immune system and expression of immune response compounds such as serine protease inhibitors (SRPN6) [48]. These innate features suggested *E. cloacae* as a suitable candidate for paratransgenesis studies against malaria parasite. This study was intended to evaluate the transmission-blocking potential of wild type and engineered *E. cloacae* expressing defensin and scorpine effector molecules to block *Plasmodium berghei* development in *An. stephensi*.

Methods

Mosquito rearing

Anopheles stephensi, Beech strain was used in this study; this strain was originally collected in Pakistan as an additional type of the SDA500 strain, and was kindly provided in 2005 by Professor P.F. Billingsley,

Sanaria, Inc [49]. Mosquitoes were maintained on 5% (wt/vol) fructose solution at 27 ± 1°C and 65 ± 5% relative humidity (RH), under 12:12 dark:light (D:L) photoperiodic conditions. All mosquito rearing facilities were provided by Tehran University of Medical Sciences, School of Public Health.

Maintenance of parasite life cycle

BALB/c mice (6 weeks old and 18–20 g weight) were used in this project. The mouse colonies were maintained in an animal house with 40–50% RH and 24 ± 1°C. The *P. berghei* ANKA strain was used, specifically clone 2.34 (a gift from Prof. Marcelo Jacob-Lorena of Johns Hopkins Bloomberg School of Public Health, Department of Molecular Microbiology and Immunology, Malaria Research Institute, Baltimore, USA). Parasites were maintained using the protocols previously described by Sinden et al. [50] and Dehghan et al. [51]: briefly, the parasites were maintained in female BALB/c mice by serial mechanical passages (3 or 4 passages). To maintain the gametocyte infectivity to mosquitoes, in direct passages, hyper-reticulocytosis was induced 3 days before infection, by treating mice with 100 µL intraperitoneal (i.p.) 1% phenylhydrazinium chloride (Sigma®) (PH; 10 mg/mL in PBS) per 10 g mouse. Parasitemia was monitored in Giemsa-stained tail-blood smears. Five to six days post-infection (dose of injected parasite ≈ 10^4), exflagellation was examined by mixing a drop of infected blood (4–5µL) with ookinete culture medium 20–25 µL (RPMI 1640 medium containing L-glutamine and 25 mM HEPES, 2 g/L NaHCO, 50 mg/L hypoxanthine, 50,000 U/L penicillin and 50 mg/L streptomycin; pH 8.3, filter sterilized). Mice with ≈ 3 exflagellation centers in each field of 40X microscopic magnification were used in the transmission blocking assay (Fig. 1).

Transformation of bacteria

Enterobacter cloacae dissolvens bacterium was originally isolated by sampling the midgut microflora of the sand fly *Phlebotomus papatasi*, found in the main zoonotic cutaneous leishmaniasis foci in central Iran [46]. We have genetically engineered two different *E. cloacae* strains to produce 1) Defensin (a peptide isolated from Radish seeds, Rs-AFP [52]), plus green fluorescent protein (GFP) which is called here *E. cloacae*$^{GFP-D}$, and 2) Scorpine-HasA (scorpion *Pandinus imperator* venom) plus HasA, a heme-binding protein as an exporting system [53] which is called here *E. cloacae*$^{S-HasA}$. Defensin and Scorpine proteins are antimalarial effector molecules with different killing mechanisms. As a control, we also genetically engineered a strain of *E. cloacae* to produce only HasA which is named *E. cloacae*^{HasA}. The transgenic *E. cloacae*$^{GFP-D}$ strain with originally manipulated pBR322 plasmid was used in this study. The engineered pBR322 plasmid containing the Defensin gene as effector molecule, plus a GFP marker and tetracycline resistance genes, called pBR/DG plasmid, is maintained in Tehran University of Medical Sciences. This strain is distinguishable from other similar bacteria colonies by green fluorescence microscopy.

We used two plasmids including PDB47-Scorpine-HasA and PDB47-HasA (hosted in *Serratia AS1* a gift from Prof. Marcelo Jacob-Lorena of Johns Hopkins Bloomberg School of Public Health, Department of

Molecular Microbiology and Immunology, Malaria Research Institute, Baltimore, USA. The plasmids were extracted and transferred into the wild type (WT) *E. cloacae dissolvens*.

Transmission blocking assay

The transmission blocking assay was carried out using wild/engineered bacteria, including the ampicillin-resistant *E. cloacae* wild type (WT), the ampicillin and tetracycline resistant *E. cloacae*$^{GFP-D}$, and the ampicillin and apramycin resistant *E. cloacae*^{HasA} and *E. cloacae*$^{S-HasA}$. The bacterial strains were cultured in BHI broth at 37°C and antibiotics, including ampicillin (100 µg/mL), tetracycline (12.5 µg/mL) and apramycin (80 µg/mL) were added into the media based on their antibiotic resistance patterns. After overnight growth, bacteria were harvested by centrifugation (3,000×g, 10 min), washed twice in sterile PBS, and resuspended in 5% (wt/vol) sterile fructose and red dye (1/50ml)] (Nilgoon®, Iran) to obtain 10^9 cells/mL.

The 3–5 day-old female mosquitoes, in five groups, were fed on sterile cotton wool soaked in fructose solution, with or without bacterial cells for 24 h. As each mosquito became fully sugar-fed, as identified by the red dye, they were separated via sucking tube and transferred into another cage and starved for 4-6h prior to the infected blood feed. The infected blood feeding process was carried out at 17–20°C for 20 min and infected blood-fed mosquitoes were maintained on 5% fructose/0.05% para-amino-benzoic acid (PABA) at 19 ± 1°C and 75 ± 5% RH, with a 12:12 photoperiodic D:L.

The mosquito's midguts were dissected 10 days post-infected blood feed (dpi), and stained with 0.5% (wt/vol) mercurochrome (Sigma®). *Plasmodium* oocyst development was examined by light microscopy and the oocysts counted (Fig. 1).

A subset of the bacterially-infected sugar-fed females, as well as the blood-fed specimens, were tested for the presence and proliferation of the bacteria by midgut dissection at 0, 12, 18, 24, and 36 hours post-blood meal (Fig. 2). The population dynamics of the engineered bacteria colony-forming units (CFUs) were defined by plating serially diluted homogenates of midguts on LB agar plates containing 100 µg/ml of the appropriate antibiotics.

Statistical Analyses

Significant differences in oocyst intensities between two samples were analyzed using the Mann–Whitney test. Multiple-sample comparisons were analyzed using the nonparametric Kruskal–Wallis test, and medians were compared using Dunn's test. All statistics were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software). $P < 0.05$ was considered to be statistically significant.

Results

Proliferation of bacteria in mosquito midgut

Generally, dynamics of the engineered bacteria showed that the bacteria could easily be established in the mosquito midgut via sugar meals laced with bacteria. The bacteria proliferated strongly in the mosquito midgut after ingestion of a blood meal and became the dominant microflora of the midgut, based on the CFUs in the plates. *E. cloacae* dynamics were monitored at different times after a blood meal and midgut homogenates were plated on selective antibiotic-containing plates. The numbers of engineered bacteria increased dramatically, by more than ≈ 10000-fold, 24h after ingestion of a blood meal (Fig. 2a and 2b), and the rapid propagation of transgenic *E. cloacae* was simultaneous with the development of the ookinete stage of the plasmodium parasite in the mosquito gut (Fig. 2c and Fig. 3).

Transmission blocking assay

The oocyst numbers in the mosquito midguts were counted on day 10 post-infection. All strains of wild-type or transgenic bacteria significantly ($P < 0.0001$) impaired the development of *P. berghei* in the *An. stephensi* midgut, in comparison with the control group (Table 1).

Table 1. P-value and significance level of *P. berghei* development inhibition in paratransgenic *An. stephensi* harboring different *E. cloacae* strains. P-value was considered at < 0.05 as statistically significant. *Abbreviations*: WT, *E. cloacae* Wild Type; HasA, *E. cloacae* expressing HasA (*E.cloacae*^{HasA}); Sco, *E. cloacae* expressing scorpine and HasA (*E. cloacae*$^{S\text{-}HasA}$); Def, *E. cloacae* expressing defensin and green fluorescent protein (*E.cloacae*$^{GFP\text{-}D}$).

	-Bact	WT	HasA	Sco.	Def.	Significant level
-Bact		***	***	***	***	
WT	<0.0001		No	**	**	
HasA	<0.0001	0.176		No	*	
Sco.	<0.0001	0.007	0.051		No	
Def.	<0.0001	0.003	0.020	0.323		
P-value						

The wild-type strain of *E. cloacae* inhibits oocyst formation by ≈ 72%, in comparison with the control group (without bacteria). The transgenic *E. cloacae* strain expressing the HasA protein alone inhibits oocyst formation by 86%, while the transgenic *E. cloacae* strain expressing both HasA and scorpine proteins inhibits oocyst formation by 92.5%. Finally, the transgenic *E. cloacae* strain expressing the

defensin protein had the greatest inhibitory effect (92.8%) on oocyst formation. Importantly, the infection prevalence (the percentage of mosquitoes that have one or more oocysts) was 86.3% in the control group, and this was reduced to 47.1%, 25% and 20% in paratransgenic mosquitoes with wild type, GFP-D and S-HasA strains, respectively. The transmission-blocking potential (TBP) index was determined in paratransgenic mosquitoes with wild type, GFP-D and S-HasA strains to be 45.4, 71 and 76.8, respectively (Fig. 4).

Discussion

The *Plasmodium* parasite is very vulnerable in the mosquito midgut and consequently components of the midgut microbiome could negatively affect parasite development in several ways: by impairing its development through secreting anti-parasitic compounds; by activating the host immune system; and by competing with the parasite for available space in the midgut [6, 26]. *E. cloacae* bacterium, a known symbiont of the gut microflora of most *Anopheles* species, has been suggested as a good candidate for the paratransgenic control of malaria [54]. The rapid propagation of transgenic *E. cloacae* 18-24h after the mosquito consumes a blood meal can block the parasite development by competing for the same space as the parasite (Fig. 2c and Fig. 3), by the greatly increased expression of anti-plasmodium molecules that lyse the *Plasmodium* parasite in the *Anopheles* midgut (Fig. 4); and by activating the mosquito immune system against the bacteria, which also leads to parasite control [48].

The present study was designed to investigate the efficacy of different strains of *E. cloacae* in disrupting *P. berghei* development, while previous studies have investigated different aspects of *E. cloacae* [44, 48, 54, 55]. In our study, we showed that *E. cloacae* bacteria multiply rapidly in the mosquito midgut, 18-24h after ingestion of a blood meal, to become the dominant species in the midgut microflora. This was shown by the GFP marker and by culturing the mosquito's midgut contents at different times after the blood meal. Similarly, Pumpuni et al. [30] showed that the midgut bacterial load of *An. gambiae* and *An. stephensi* increased by 11–40 times, 24h after blood feeding. Demaio et al. [56] also obtained similar results in *Ae. triseriatus, Culex pipiens*, and *Psorophora columbiae*, and Wang et al. [26] reported that the bacterial load of *P. agglomerans* increased 200-fold in the *An. stephensi* midgut, 24–48 h after blood meal ingestion. The finding of Dehghan et al. [54], that *E. cloacae* was highly stable in sugar solution, suggested that using sugar bait stations to introduce the transgenic bacteria in the field could be a feasible paratransgenic approach.

Furthermore, we know that development of the *Plasmodium* parasite could be affected by the presence of certain bacteria in the microflora of the mosquito midgut. In this study, the interaction of *E. cloacae* and *P. berghei* in vivo leads to a significant inhibition of oocyst formation, relative to the control group (P-value < 0.0001). This correlates well with the findings of Pumpuni et al. [29] that the presence of 100,000 *Ewingella americana* in the mosquito midgut reduced the *P. falciparum* infection rate to zero and those of Gonzalez-Ceron et al. [44], who reported a reduction in *P. vivax* infection rate in *An. albimanus* in the

presence *S. marcescens, E. cloacae* and *E. amnigenus*,. In this regard, the coincidence of bacterial multiplication with the ookinete stage in *Anopheles* gut will affect the bacteria-parasite interaction directly and indirectly. We show that transgenic bacteria could overcome the harsh environment and barriers in the *Anopheles* midgut, such as digestive enzymes, to become the dominant component of the gut microflora, leading to an increase in the expression of antiparasitic molecules. This correlates well with the findings of Dong et al. [31], who showed that when the *Chryseobacterium meningosepticum* bacterium enters the *An. gambiae* midgut, it rapidly becomes a dominant species, indicating the competitive nature of this bacterium in the midgut environment.

The results of this study showed that all the bacterial strains disrupted the development of *P. berghei* to a significant degree, compared with the control group ($P < 0.0001$). Even the *E. cloacae* WT led to significantly impaired parasite development ($P < 0.0001$), indicating the inherent effect of these bacteria in parasite control. The transgenic *E. cloacae* $^{GFP-D,}$ expressing Defensin, further inhibited parasite development compared with WT ($P = 0.003$), indicating the suppressive effect of defensin, which lyses the parasite inside the mosquito gut [57–59]. The inhibitory effect of Scorpine was very similar to that of Defensin, and we saw no significant differences in inhibition of oocyst formation between *E. cloacae*$^{S-HasA}$ and *E. cloacae*$^{GFP-D}$ ($P = 0.051$). Similarly, Kokoza et al. [57] expressed Cecropin A and Defensin A in *Ae. aegypti* mosquitoes to control *P. gallinaceum*, and reported that *Plasmodium* transmission was completely blocked.

Scorpine is an antimalarial peptide from the venom of the *Pandinus imperator* scorpion and its amino acid sequence is very similar to those of Cecropin and Defensin, which led to the suggestion that Scorpine might have a similarly inhibitory effect on the *P.berghei* [60]. Indeed, Conde et al. (2000) [60] found that it completely inhibited *P. berghei* fertilization and oocyst formation. Wang et al. [11, 26] reported that symbiotic bacteria, *P. agglomerans* and *Serratia* AS1, transgenically expressing Scorpine, could inhibit the *P. falciparum* development in *An. gambiae* by 98% and 93% accordingly. The additional expression of HasA protein in the *E. cloacae*$^{S-HasA}$ strain was found to enhance the anti-*Plasmodiun* effectiveness of Scorpine. It is possible that HasA could create a membrane pore in the *E. cloacae* wall to allow the direct export of Scorpine protein from the bacterial cytoplasm into the mosquito midgut.

Three bacterial species have previously been proposed as candidates for paratransgenetic malaria control: *Serratia* AS1, *Asaia* sp. and *P. agglomerans* bacteria, transgenically expressing anti-*Plasmodium* proteins had been demonstrated to be suitable micro-symbionts in the mosquito midgut [26, 41]. Here, we evaluated a new candidate bacterium, *E. cloacae*, and showed that it has a strong innate control effect on the *Plasmodium* parasite in the mosquito midgut, and that this effect could be enhanced by the transgenic expression of anti-*Plasmodium* proteins.

Previously, the symbiotic bacterium *Asaia*, transgenically expressing Scorpine, was shown to inhibit *P. berghei* development by 63% in *An. stephensi* midgut [41]; while we found that, when expressed in *E. cloacae* in this study, Scorpine causes a 92.5% inhibition of oocyst formation. This remarkable difference can thus most probably be attributed to the inherent anti-parasitic activity of the *E. cloacae* bacterium. In

addition, Wang et al. [26] reported that the expression in *P. agglomerans* of HlyA protein (which, like HasA, causes pore formation in the bacterial wall) has a negligible effect (21.2%) on parasite development. Therefore, *E. cloacae*, owing to its intrinsic antiparasitic properties could be preferred to other paratransgenesis candidates such as *Asaia* sp. and *P. agglomerans*. This advantage can be attributed to the stimulation of the mosquito's immune system and the secretion of serine protease inhibitors, which are produced by mosquitoes to control bacteria, but are not specific to the target organism and are suppressed if the *Plasmodium* parasite is present in the midgut [48]. The *E. cloacae* bacterium is found in the normal gastrointestinal micro-flora of humans and many other animals and is generally reported to be widespread in insect midguts [42, 44, 55, 61, 62], thus alleviating any potential safety concerns concerning its release in the field.

Conclusions

In conclusion, we consider an alternative strategy for control of the *Plasmodium* parasite, through the use of bacterial symbionts of the mosquito, genetically engineered to express anti-*Plasmodium* effector molecules. The paratransgenesis strategy converts the proven mosquito vector into an ineffective disease vector. This approach could be effective for multiple mosquito and parasite species, concomitantly. The present findings provide the foundation for the use of either wild-type or genetically modified *E. cloacae* bacteria as a powerful tool to combat malaria. However, further studies are needed to determine how effectively these bacterial strains can be established in the field and the conditions required to do this.

Abbreviations

dpi
days post infection
ITN
insecticide treated nets
IRS
indoor residual spraying
TBS
Transmission blocking strategy
SRPN6
serine protease inhibitors
ip
intra-peritoneal
PABA
para-amino-benzoic acid
CFUs
colony forming units

WT
wild type
S-HasA
Scorpine-HasA
GFP-D
GFP-defensin.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HD: design, conceptualization, methodology, carrying out of project, writing original draft

MAO: Supervision, design of the work, writing, review & editing, interpretation of data, revising the final manuscript, funding acquisition, resources

SHMK: Formal analysis, supervisor of project,

BY: genetically manipulation of bacteria, formal analysis

NM: Methodology, revision of the manuscript

HV: Formal analysis, data analysis

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Figures

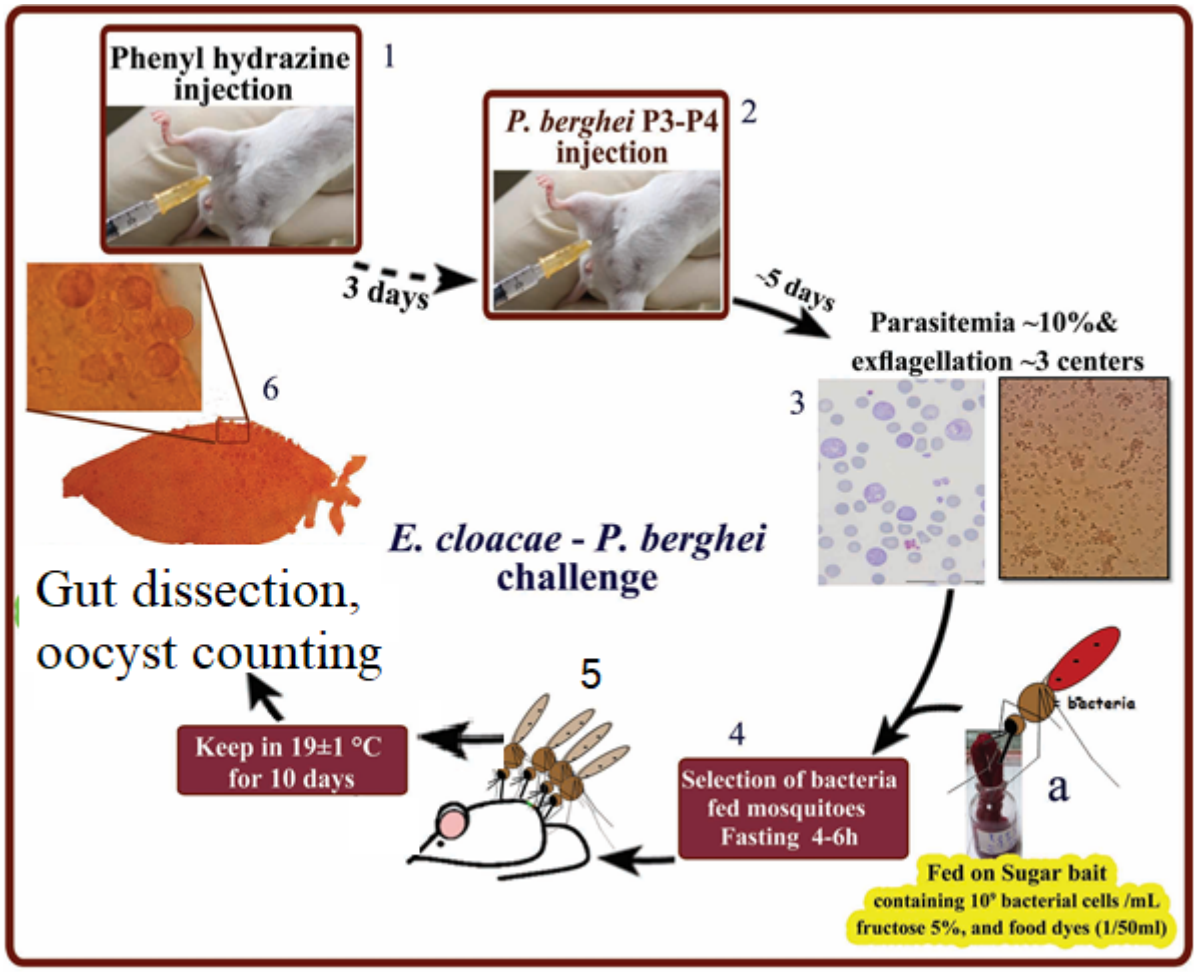


Figure 1

Schematic illustration of transmission blocking assay. (1-3) Infection of mouse with P. berghei ANKA strain clone 2.34. (a) introduction of bacteria into the mosquito midgut via sugar feeding. (4) Fasting sugar-fed mosquito for 4-6 hours. (5) Mosquito feeding on P. berghei-infected mouse (6) Dissection of the mosquito midgut, staining with 0.5% mercurochrome and counting the infective oocysts by light microscopy.

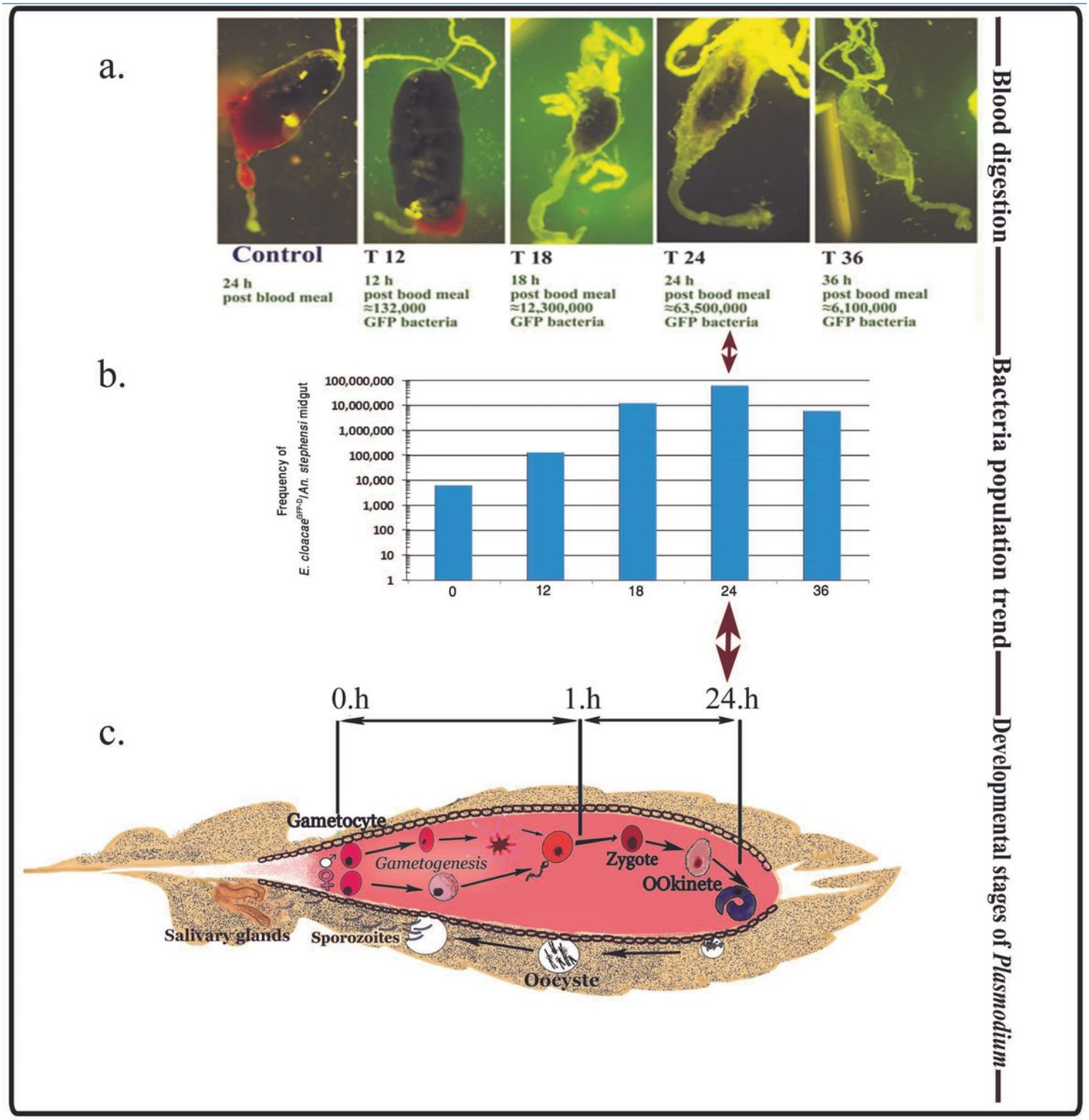


Figure 2

Trends of E. cloacae proliferation in mosquito midgut. (a) Digesting process of blood meal in paratransgenic An. stephensi over time. (b) The frequency of E. cloacaeGFP-D in the An. stephensi midgut at different times after ingestion of a blood meal. (c) Schematic development of Plasmodium parasite in Anopheles midgut shows the simultaneous bacterial proliferation and ookinete formation at 18-24 h after a blood meal.

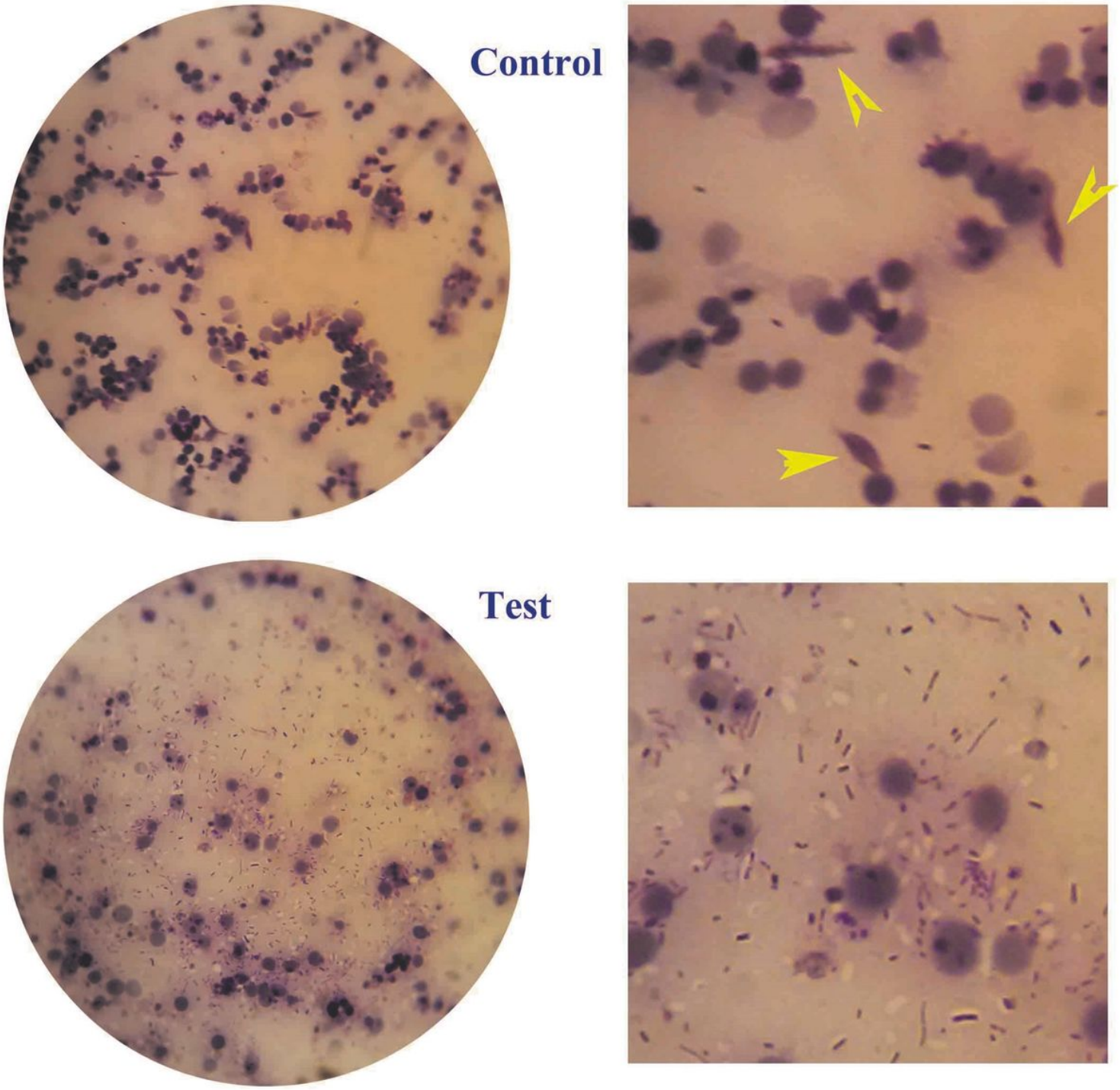
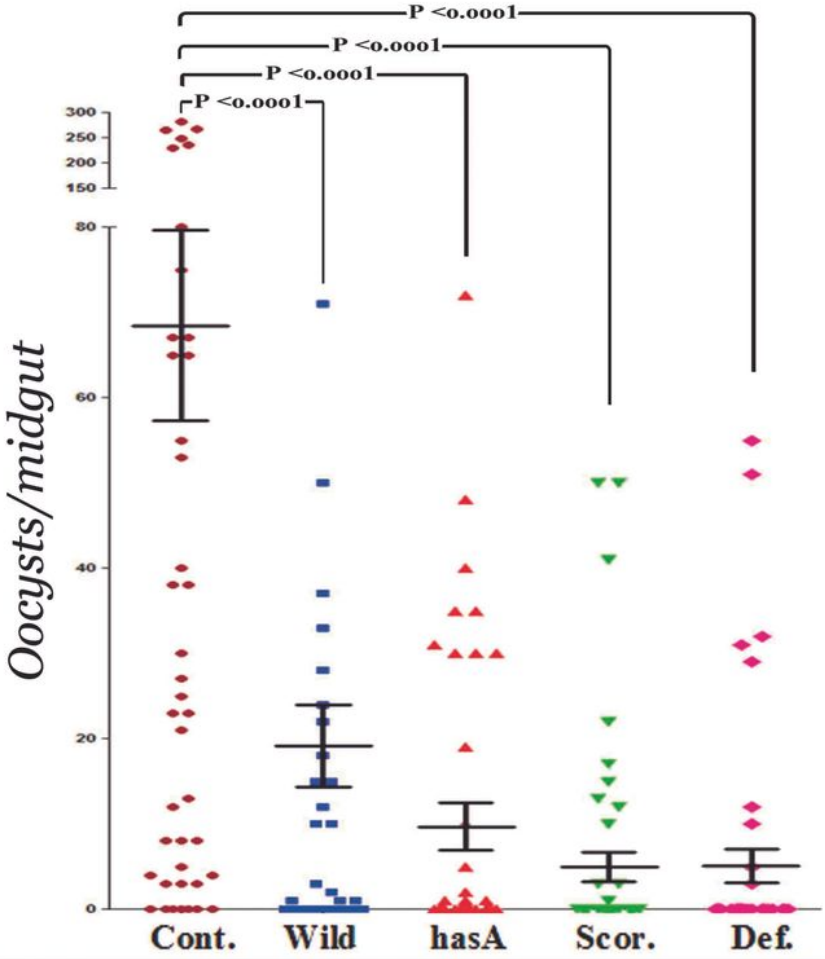


Figure 3

The presence or absence of motile ookinetes (barrel-shaped, shown by arrows) of P. berghei in the An. stephensi mosquito midgut in the control and E. cloacaeGFP-D test group 20-24 h after ingestion of an infected blood meal. Upper panels: Presence of P. berghei ookinetes are seen in the remains of digested RBC in mosquito midgut in the absence of E. cloacae bacteria. Lower panels: Strong proliferation of E. cloacaeGFP-D bacteria correlates with a lack of ookinetes 20 h after ingesting an infected blood meal with 104-105 parasites/µl in midgut. The thick smear was stained by Giemsa.



	Cont.	Wild	hasA	Scor.	Def.
N	51	51	50	45	48
Range	0-281	0-116	0-90	0-55	0-50
Prevalence	86.3	47.1	42	20	25
TBP*	-	45.4	51.3	76.8	71
Mean	68.4	19.2	9.7	5.1	4.9
%inhibition	-	71.9	85.8	92.5	92.8
Median	38	0	0	0	0
%inhibition	-	100	100	100	100
P-value	-	<0.0001	<0.0001	<0.0001	<0.0001

Figure 4

Inhibition of P. berghei development in An. stephensi by wild and transgenic E. cloacae strains. An. stephensi mosquitoes were fed on 5% (wt/vol) fructose solution and red food dye supplemented with either PBS (Control) or with wild or transgenic E. cloacae strains in five groups. After 8h, the five groups of mosquitoes were fed on the same P. berghei-infected mice. Oocyst numbers were determined 10 days after the infected blood meal. Inhibition: inhibition of oocyst formation relative to the control; Mean: mean

oocyst number per midgut; Median: median oocyst number per midgut; N: number of mosquitoes analyzed; Prevalence: percentage of mosquitoes carrying at least one oocyst; Range: range of oocyst numbers per midgut; TBP: transmission blocking potential: 100 − {(prevalence of mosquitoes fed with transgenic E. cloacae)/[prevalence of control (−Bacteria) mosquitoes] × 100}. Inhibition: inhibition of oocyst formation relative to the control. Abbreviations: Wild, E. cloacae WT; HasA, E. cloacaeHasA; Scor, E. cloacaeS-HasA; Def, E. cloacaeGFP-D; Cont, control group without bacteria.

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