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Mosquito ingestion of antibodies against mosquito midgut microbiota improves conversion of ookinetes to oocysts for *Plasmodium falciparum*, but not *P. yoelii* ☆

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ABSTRACT

The mosquito midgut is a site of complex interactions between the mosquito, the malaria parasite and the resident bacterial flora. In laboratory experiments, we observed significant enhancement of *Plasmodium falciparum* oocyst production when *Anopheles gambiae* (Diptera: Culicidae) mosquitoes were membrane-fed on infected blood containing gametocytes from *in vitro* cultures mixed with sera from rabbits immunized with *A. gambiae* midguts. To identify specific mechanisms, we evaluated whether the immune sera was interfering with the usual limiting activity of gram-negative bacteria in *An. gambiae* midguts. Enhancement of *P. falciparum* infection rates occurred at some stage between the ookinete and oocyst stage and was associated with greater numbers of oocysts in mosquitoes fed on immune sera. The same immune sera did not affect the sporogonic development of *P. yoelii*, a rodent malaria parasite. Not only did antibodies in the immune sera recognize several types of midgut-derived gram-negative bacteria (*Pseudomonas* spp. and *Cedecea* spp.), but gentamicin provided in the sugar meal 3 days before an infectious *P. falciparum* blood meal mixed with immune sera eliminated the enhancing effect. These results suggest that gram-negative bacteria, which normally impair *P. falciparum* development between the ookinete and oocyst stage, were altered by specific anti-bacterial antibodies produced by immunizing rabbits with non-antibiotic-treated midgut lysates. Because of the differences in developmental kinetics between human and rodent malaria species, the anti-bacterial antibodies had no effect on *P. yoelii* because their ookinetes leave the midgut much earlier than *P. falciparum* and so are not influenced as strongly by resident midgut bacteria. While this study highlights the complex interactions occurring between the parasite, mosquito, and midgut microbiota, the ultimate goal is to determine the influence of midgut microbiota on *Plasmodium* development in anopheline midguts in malaria endemic settings.

1. Introduction

Successful transmission of malaria parasites is dependent on the completion of the early stages of sporogonic development in the anopheline mosquito midgut. These early stages are comprised of 2 distinct life-stage transitions: 1) the gametocyte–ookinete transition and 2) the ookinete–oocyte transition (reviewed by [1]). While the dynamics of this developmental progression varies among parasite species [1,2] as well as mosquito species [3–6], disruption at any point can compromise oocyst and ultimately, sporozoite development. Disruption of sporozoite development may considerably reduce overall parasite transmission by mosquitoes.

As the parasites progress through these two developmental transitions, they are influenced by a variety of host and mosquito factors that either reduce or enhance their maturation in the midgut. The gametocyte–ookinete transition is influenced strongly by gametocyte maturity [7] or extrinsic factors either from the environment (e.g. temperature) [8,9] or the host (e.g. cytokines, complement, toxic nitrogen oxides, and drugs [10,11]). In contrast, the ookinete–oocyst transition is influenced by complex interactions between the innate immunity of the mosquito midgut [12–15], resident midgut microbiota [16–20], the peritrophic matrix [10,21–23], and specific interactions with carbohydrates on the midgut inner surface [24,25]. Currently, these complex interactions are an active field of study.

The purpose of this study was to investigate the effect of anti-mosquito midgut antibodies on the vector competence of anopheline mosquitoes for malaria. This was examined by comparing the effects that anti-sera raised against crude homogenates of *Anopheles gambiae* mosquitoes had on the early sporogonic development of the human

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malaria parasite, *Plasmodium falciparum* and that of the rodent malaria parasite, *Plasmodium yoelii*.

2. Materials and methods

2.1. Mosquito rearing

Standard procedures were used to rear *A. gambiae* (G-3 strain) in our laboratory setting [17,26]. All life stages were reared in three different insectaries under controlled environmental conditions (27°C and 70% RH and LD 12:12 h photoperiod). Larvae were fed powdered dog chow and adult mosquitoes received a daily change of 3% Karo syrup (Best Foods, Englewood Cliffs, NJ).

2.2. Antigen preparation and rabbit immunization

Two lots of anti-midgut sera were produced from a total of eight young (6 weeks old) New Zealand white rabbits. One lot was produced in 6 rabbits (indicated hereafter as rabbit sera #3 to 8). The immunizing source for this lot was comprised of whole midgut lysates dissected from newly emerged, day old female *A. gambiae* mosquitoes that had not been offered sugar or carbohydrate source upon eclosion. A second sera lot was produced in 2 rabbits (indicated hereafter as rabbit sera #9 and 10). The immunizing source for this lot was comprised of midguts dissected from three to five day old female *A. gambiae* mosquitoes that had been maintained on a sterile carbohydrate source (3% Karo syrup-soaked cotton pads). Midguts were excised in chilled phosphate buffered saline (PBS), pH 7.4 (Dulbecco, Sigma, St. Louis, MO) and stored at −70 °C in 100 μl of PBS. Prior to inoculation, midguts were homogenized in chilled PBS (pH 6.9) containing 2 mM phenylmethylsylfonylflouride (PMSF) (Sigma, St. Louis, MO). Immunization procedures followed the protocol outlined in Noden et al. [27]. Briefly, each rabbit received an initial subcutaneous injection of 150 homogenized midguts mixed 1:1 in complete Freund's adjuvant (Sigma). Subsequent immunizations were delivered at 7–10 day intervals using the same concentration of crude midgut antigen mixed in incomplete Freund's adjuvant (Sigma). Rabbits received one primary plus 6 booster immunizations. Sera were collected weekly and frozen at −70 °C. Sera were obtained from each rabbit prior to beginning the immunizations in order to serve as negative control sera for each rabbit. An ELISA was used to determine the anti-midgut antibody titer in rabbit sera [27]. The anti-midgut IgG titers of rabbits 3 through 8 ranged from 1/32,000 to 1/256,000 and the anti-midgut IgG titers in rabbits 9 and 10 were 1/128,000 and 1/64,000, respectively.

2.3. Characterization of midgut microbiota

To partially characterize the bacterial flora inhabiting the intestinal tract of teneral female mosquitoes (*i.e.*, midgut microbiota), day-old non-sugar-fed female mosquitoes were surface-sterilized in 70% ethanol for 5–10 min, and then rinsed three times in sterile saline (0.9% NaCl solution) [17]. Mosquito midguts were dissected using sterile technique. Two midguts were pooled in a 1.5-ml Eppendorf (Madison, WI) tube containing 20 μl of sterile saline and triturated with a plastic grinder. One microliter of each triturated sample was streaked on trypticase soy agar (TSA) supplemented with 5% sheep blood (Becton-Dickinson, Cockeysville, MD). All cultures were maintained at 27 °C under aerobic conditions. Bacterial colony-forming units (CFU) were enumerated after 48 h to allow maximum growth potential for bacterial isolation [17]. Individual bacterial colonies with distinct morphologies were subcultured. All subcultured isolates were screened for Gram stain morphology, reactivity to oxidase and indole tests, and fermentation on triple sugar iron slants. Pure cultures were identified to species using the Becton Dickinson Crystal Enteric/Nonfermenter Identification System (Becton Dickinson, Cockeysville, MD). These isolation techniques may not have detected all species of bacteria present within the mosquito alimentary tract.

2.4. Sera reactivity against midgut microbiota

An indirect fluorescent antibody (IFA) assay was used to determine whether the sera from immunized rabbits contained antibodies that recognized the bacteria isolated from the midguts of newly-emerged female *A. gambiae* mosquitoes. Ten microliters of bacterial suspension were placed into each well of multispot IFA slides, air-dried, and stored at −20 °C until the time of assay. Twenty microliters of pooled immune sera, diluted 1:50 in PBS, were added to half of the IFA wells while pooled pre-immune sera from the same rabbits, also diluted 1:50 in PBS, were added to the remaining wells, thereby serving as the negative controls. After incubating at 37 °C for 30 min in a moist chamber (Fisher, Pittsburgh, PA), the slides were rinsed twice in PBS (pH 7.4)(Sigma) to remove any unbound rabbit IgG. Slides were air-dried and 20 μl of fluorescein-conjugated goat anti-rabbit IgG (H & L) (Kirkegaard & Perry, Gaithersburg, MD) diluted 1:100 in PBS (Sigma) and rhodamine counterstain (Difco Lab, Detroit, MI) were added to each IFA well. The slides were incubated at 37 °C for 30 min in a moist chamber then washed twice in PBS, air dried, stored at 4 °C and examined by epifluorescence microscopy.

2.5. Effects of gentamicin and immune sera on midgut microbiota

To determine the effect of gentamicin on midgut derived bacterial isolates, suspension of 10^6, 10^4 and 10^2 bacteria per ml of nutrient broth were incubated for 6 h at 37 °C in solutions containing increasing concentrations of gentamicin (0.2 μg/ml, 2 μg/ml, and 20 μg/ml; Elkins-Sinn, Cherry Hill, NJ). Following incubation, 1 μl from each sample was streaked onto TSA plates containing 5% sheep blood (BRL, Baltimore, MD). Plates were incubated overnight at 37 °C, and bacteria colonies were counted. To determine the effect of the immune sera on the bacterial isolates, suspensions of 10^2, 10^3 and 10^4 cells/ml of each bacterial species were incubated at 37 °C for 6 h in 500 μl of nutrient broth along with 100 μl of each of the antisera pools. After a 6 h incubation, 1 μl from each sample was streaked on to a TSA plate containing 5% sheep blood. Plates were incubated overnight at 37 °C, and bacteria colonies were counted. In both assays, a Gram-positive bacteria (*Micrococcus*) was used as a control.

2.6. P. falciparum infections using immune sera

A. gambiae were fed cultured *P. falciparum* NF54 gametocytes following the protocol described by Ponnudurai et al. [28]. The protocol was modified by substituting rabbit sera for human sera. Initially, normal rabbit sera obtained prior to rabbit immunization (pre-immune sera) were fed in parallel with human sera in 13 *P. falciparum* infection experiments to test the effect of normal rabbit sera on *P. falciparum* fertility. No differences in oocyst infection rates were observed between the two groups (data not shown) indicating that normal rabbit sera did not alter *P. falciparum* gametocyte infectivity within our model system. Thereafter, the pre-immune sera were used as negative controls [29,30]. To test the effect of immune sera on *P. falciparum* infectivity to *A. gambiae*, high titered immune sera were fed in parallel with pre-immune sera from the same groups of rabbits. Ookinete and oocyst densities and infection rates were determined as described by Vaughan et al. [3].

2.7. P. yoelii infections using immune sera

P. yoelii 17XNL parasites were maintained by serial blood passage in outbred ICR mice [31]. Three to 4 days after blood passage, exflagellation (wet mount) was assessed [32] to gauge the maturity of the male gametocyte populations. For membrane feeding of mosquitoes, blood

with mature male gametocytes was obtained from infected, anesthetized mice via cardiac puncture. The blood was washed in 2.5 ml of pre-warmed incomplete media (RPMI 1640, Hepes, and hypoxanthine) [28] and centrifuged at 2000 rpm for 30 s in order to separate infected mouse erythrocytes from native sera. The supernatant was discarded and 200 μl of washed erythrocytes were added to each of two vials, one containing 200 μl of pre-immune sera and the other containing 200 μl of pooled immune sera. This mixture was then pipetted into water-jacketed membrane feeders and 3–5-day-old *A. gambiae* were allowed to feed. Blood-fed mosquitoes were held at 24 °C and 50% RH with free access to sugar solution. After 8–10 days, mosquito midguts were dissected and examined (400×) for oocysts. Encapsulated oocysts were included in the oocyst counts because they represent ookinetes which successfully penetrated the midgut epithelium. Sera pools from rabbits #6–8 and #9–10 were used in these experiments.

2.8. Effect of immune sera on *P. falciparum* development within aseptic versus septic mosquitoes

To determine if serum antibodies specifically directed against midgut microbiota may have facilitated the passage of *P. falciparum* ookinetes across the midgut, groups of mosquitoes were cleansed of their midgut microbiota prior to infection by maintaining them for three days on a 10% sucrose solution containing 0.05% (*i.e.*, 50 μg/ml) gentamicin [20,26]. Dissection of gentamicin-treated mosquitoes and plating of midgut lysates on TSA blood agar plates confirmed that gentamicin treatment rendered midguts free of cultivable bacteria (Vaughan, unpublished results). Control groups (=septic mosquitoes) were maintained on normal sucrose solution and allowed to retain normal midgut microbiota. On the day of infection, the control (septic) and the gentamicin-maintained (aseptic) mosquitoes were each divided into two cages, making a total of four treatment groups. Two of the cages (a septic and an aseptic group) were fed *P. falciparum*-infected blood supplemented with the pre-immune sera, while the other septic and aseptic cages were fed *P. falciparum*-infected blood supplemented with higher-titered immune sera.

2.9. Effect of immune sera on *P. falciparum* development within *A. gambiae* mosquitoes reared in different insectaries

The raw material used to immunize rabbits originated from different places. Rabbits #3 to 8 were immunized with midguts from *A. gambiae* (G-3 strain) reared in a small, antiquated insectary, whereas rabbits #9 and 10 were immunized with *A. gambiae* (G-3 strain) reared in a larger, more modern insectary located within the same building. The insectaries were managed by different personnel, used separate supplies and employed slightly different husbandry techniques. To determine if the immune sera generated from *A. gambiae* mosquitoes reared in one insectary would exert the same effect on *Plasmodium* infectivity of *A. gambiae* mosquitoes reared in heterologous insectaries, groups of mosquitoes from these insectaries were membrane-fed *P. falciparum* gametocyte cultures and, in separate experiments, *P. yoelii* gametocytemic blood, both supplemented with either immune sera or pre-immune sera. After 8–10 days, lots of mosquitoes were dissected and midguts were examined for oocysts as described above.

2.10. Data analysis

Infection prevalence was expressed as percentage of mosquitoes infected and statistical comparisons were performed using Chi-square (X^2) analyses and Fisher exact tests (Epi-Info 6.0, CDC, Atlanta, GA). Parasite densities were expressed as the geometric mean numbers of parasites per mosquito, including the zero counts. Parasite densities were compared among treatments using analyses of variance (ANOVA) (SPSS, SPSS Inc., Chicago, IL) following appropriate data transformations (e.g., $\log_{10}x+1$). Because some of the data in Table 2 were not normally distributed, the sign test (a non-parametric equivalent of the paired *t*-test) was used for paired comparisons of means. Conversion efficiencies of ookinetes to oocysts were calculated using the methods of Zollner et al. [6].

3. Results

3.1. Effects of gentamicin and immune sera on midgut microbiota

Three Gram-negative bacterial species were isolated from the midguts of one day-old, non-sugar-fed female *A. gambiae* mosquitoes—*Pseudomonas aeruginosa*, *Ps. vesicularis* and *Cedecea lapagei*. Gentamicin concentrations greater than 2 μg/ml eliminated all *Ps. aeruginosa* and *C. lapagei* growth *in vitro*, indicating that these bacterial species were fully susceptible to antibiotics. Results for *Ps. vesicularis* were inconclusive based on the observation that there were differences in killing effect between the gentamicin test replicates. Rabbits immunized against whole midgut lysates of *A. gambiae* produced specific IgG against midgut lysates at titers ranging from 1/32,000 to 1/256,000, as measured by ELISA. Incubating the bacterial isolates with immune sera produced a pattern of immunostaining covering the outer surface of both *P. aeruginosa* and *C. lapagei* (Fig. 1). Pre-immune sera did not produce immunofluoresence. This indicated that immune sera contained IgG against bacteria present within the mosquito midguts used to immunize the rabbits. Despite this, there were no differences in the bacterial counts of *P. aeruginosa*, *P. vesicularis* or *C. lapagei* isolates (range=400 to

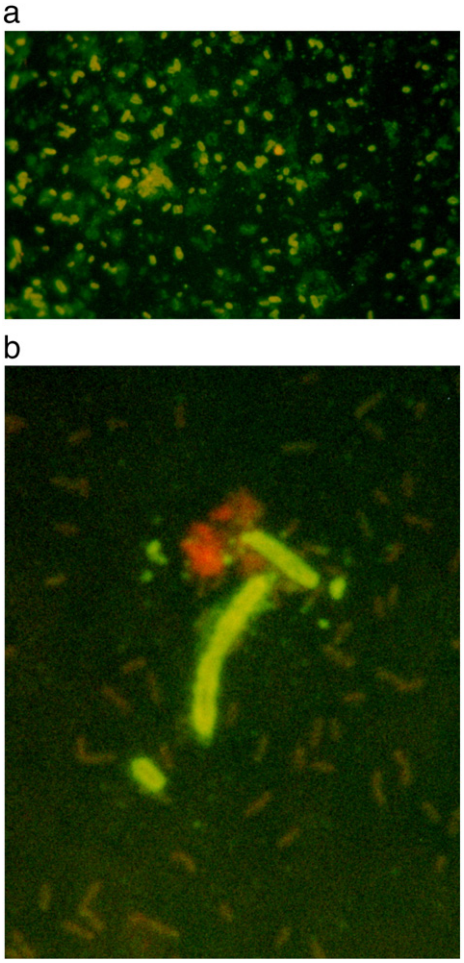

Fig. 1. IFA of polyclonal antibody on fixed slide with a) *Pseudomonas aeruginosa* and b) *Cedecea lapagei* isolated from *A. gambiae* midguts.

600 CFU) when the isolates were incubated for 6 h *in vitro* in the presence of immune versus pre-immune sera. This indicated that the immune sera were neither bactericidal nor bacteristatic.

3.2. P. falciparum infections using immune sera

In 12 of 12 paired *P. falciparum* infectious feedings, there were significantly higher oocyst prevalences in mosquitoes fed gametocyte cultures mixed with the immune sera than in the corresponding control mosquitoes (X^2, $p<0.05$) (Table 1). The same result was true for 11 of the 12 paired infectious feedings, with respect to oocyst abundance (ANOVA, $p<0.05$) (Table 1). To gain a clearer understanding of the site of action for this unanticipated effect, additional infections were conducted to include ookinete sampling (Table 2). Among eight paired infections, the mean densities of *P. falciparum* ookinetes within mosquitoes fed immune sera did not differ significantly from the ookinete densities in pre-immune fed mosquitoes (sign test, $p=0.14$). Yet one week later, the same infectious feedings yielded consistently more oocysts within the immune fed than in the pre-immune fed mosquitoes (sign test, $p=0.004$). As a result, the *per capita* loss of ookinetes was significantly lower in immune-fed versus pre-immune-fed mosquitoes (sign test, $p=0.004$). Thus, the immune sera did not affect the gametocyte-to-ookinete development transition (e.g., gamete fertilization) but somehow facilitated the transition of ookinetes to oocysts.

3.3. P. yoelii infections using immune sera

In contrast, the same high-titered immune sera did not affect *P. yoelii* oocyst development in *A. gambiae* mosquitoes (Table 3). In 7 of 8 paired *P. yoelii* infectious feedings, there were no significant differences between the oocyst prevalences of mosquitoes fed *P. yoelii* gametocyte preparations containing the immune sera versus those of matched mosquito cohorts fed the same gametocyte preparations but supplemented instead with the pooled pre-immune sera from the same rabbits (X^2, $p>0.05$). Likewise, in 6 of the 8 paired infectious feeding there were no significant differences in oocyst abundance between the immune fed and pre-immune fed groups (ANOVA, $p>0.05$) (see sera pools 6–8, Table 3). In those experiments where statistical differences among oocyst abundance occurred, in one experiment immune-fed mosquitoes yielded *lower* oocyst abundance ($p=0.04$) whereas in the other experiment immune-fed mosquitoes yielded *higher* oocyst abundance ($p=0.004$) than did mosquitoes in the corresponding control groups. This supports the conclusion that the immune sera had no real biological effect on the early sporogony of *P. yoelii*.

Table 2
Effect of immune sera containing anti-*Anopheles gambiae* midgut antibodies on *Plasmodium falciparum* ookinete and oocyst densities developing within *Anopheles gambiae* mosquitoes.

Infection #[a]	Treatment	Ookinete density	Oocyst density	Per capita loss of ookinetes
1[b]	Pre-immune[c]	875	0.5	1750-fold
	Immune[d]	696	12.5	56-fold
2	Pre-immune	159	0.1	1590-fold
	Immune	423	0.4	1058-fold
3[b]	Pre-immune	151	0.4	378-fold
	Immune	665	3.3	202-fold
4	Pre-immune	174	0.1	1740-fold
	Immune	218	0.9	242-fold
5[b]	Pre-immune	240	0.2	1200-fold
	Immune	885	0.9	983-fold
6[b]	Pre-immune	253	0.7	361-fold
	Immune	354	5.4	66-fold
7	Pre-immune	160	0.04	4000-fold
	Immune	109	0.2	545-fold
8[b]	Pre-immune	1296	0.7	1851-fold
	Immune	1368	2	684-fold

[a] Sera contains antibodies against day-old, non-sugar-fed *An. gambiae* midguts (titer:1/32,000–256,000).
[b] Numbers of mosquitoes dissected and oocyst densities from 5 of the 8 infections included in this table were previously reported in Table 1.
[c] Pre-immune sera = sera containing no antibody against *An. gambiae* midguts.
[d] Immune sera = pooled sera from immunized rabbits.

3.4. Effect of immune sera on P. falciparum development in aseptic versus septic mosquitoes

In two separate experiments, septic mosquitoes (*i.e.*, not pre-treated with gentamicin) had a higher prevalence of *P. falciparum* oocyst infections when fed gametocyte cultures mixed with immune sera than did septic mosquitoes fed gametocyte cultures mixed with pre-immune sera (X^2, $p<0.05$). This was similar to that observed in our previous experiments (Table 1). However, in aseptic mosquitoes

Table 1
Plasmodium falciparum NF54 oocyst prevalence (%) and abundance (GM[a]) within *Anopheles gambiae* mosquitoes fed gametocytes mixed with pre-immune sera or immune sera from rabbits immunized against mosquito midgut tissue and associated microbiota. Experimental feeds were replicated 12 times, each using a different *P. falciparum* gametocyte culture.

Sera pool[d]	Pre-immune sera[b]		Immune sera[c]		Level of significance	
	Oocyst prevalence (n)	Oocyst density (range)	Oocyst prevalence (n)	Oocyst density (range)	Oocyst prevalence (X^2 tests)	Oocyst density (ANOVA)
3–5[e]	41% (49)	0.5 (0–5)	100% (28)	12.4 (0–80)	<0.0001	<0.0001
	36% (47)	0.4 (0–4)	71% (51)	3.3 (0–29)	0.0001	<0.0001
	19% (86)	0.2 (0–4)	46% (50)	0.9 (0–7)	0.0001	<0.0001
	20% (50)	0.3 (0–8)	92% (13)	4.2 (0–19)	<0.0001	<0.0001
6–8[e]	55% (20)	0.7 (0–5)	90% (21)	5.4 (3–27)	0.03	<0.0001
	15% (54)	0.2 (0–6)	35% (41)	0.4 (0–4)	0.049	0.14
	40% (45)	0.7 (0–13)	66% (38)	2.0 (0–25)	0.03	0.003
	20% (50)	0.3 (0–8)	64% (39)	2.0 (0–47)	<0.0001	<0.0001
9–10[f]	35% (20)	0.03 (0–4)	94% (18)	5.4 (0–19)	0.0005	<0.0001
	42% (36)	0.6 (0–5)	72% (43)	1.9 (0–24)	0.01	0.001
	43% (49)	2.0 (0–63)	86% (43)	7.8 (0–86)	<0.0001	0.0001
	11% (27)	0.1 (0–4)	54% (22)	1.7 (0–15)	0.003	0.0001

[a] GM = geometric mean.
[b] Pre-immune sera = sera containing no antibody against *An. gambiae* midguts.
[c] Immune sera = pooled sera from immunized rabbits.
[d] Sera pool = identifies the rabbits from which the sera was pooled.
[e] Sera contains antibodies against day-old, non-sugar-fed *An. gambiae* midguts (titer:1/32,000–256,000).
[f] Sera contains antibodies against 3–5 day old, sugar-fed *An. gambiae* midguts (titer:1/64,000–128,000).

Table 3
Plasmodium yoelii 17XNL oocyst prevalence (%) and abundance (GM[a]) within *Anopheles gambiae* mosquitoes fed gametocytes mixed with pre-immune sera or immune sera from rabbits immunized against mosquito midgut tissue and associated microbiota. Experimental feeds were replicated 8 times, each using *P. yoelii* infected erythrocytes harvested from different sets of mice.

Sera pool[d]	Pre-immune sera[b]		Immune sera[c]		Level of significance	
	Oocyst prevalence (n)	Oocyst density (range)	Oocyst prevalence (n)	Oocyst density (range)	Oocyst prevalence (X^2 tests)	Oocyst density (ANOVA)
6–8[e]	48% (27)	3.3 (0–102)	51% (47)	2.9 (0–78)	1.01	0.77
	68% (28)	7.0 (0–159)	45% (33)	2.4 (0–49)	0.13	0.045
	69% (42)	9.4 (0–210)	80% (30)	16.3 (0–120)	0.44	0.26
	85% (52)	27.3 (0–240)	94% (48)	51.1 (0–250)	0.25	0.06
9–10[f]	16% (45)	0.1 (0–3)	35% (48)	0.8 (0–24)	0.05	0.004
	36% (47)	0.7 (0–21)	32% (50)	1.0 (0–92)	0.83	0.46
	75% (16)	3.0 (0–29)	65% (23)	1.7 (0–9)	0.76	0.23
	52% (27)	2.6 (0–75)	64% (22)	4.0 (0–70)	0.59	0.46

[a] GM = geometric mean.
[b] Pre-immune sera = sera containing no antibody against *An. gambiae* midguts.
[c] Immune sera = pooled sera from immunized rabbits.
[d] Sera pool = identifies the rabbits from which the sera was pooled.
[e] Sera contains antibodies against day-old, non-sugar-fed *An. gambiae* midguts (titer 1/32,000–256,000).
[f] Sera contains antibodies against 3–5 day-old, sugar-fed *An. gambiae* midguts (titer 1/64,000–128,000).

(*i.e.*, gentamicin-treated), there were no differences in *P. falciparum* oocyst prevalences between mosquitoes ingesting gametocytes mixed with immune sera versus mosquitoes ingesting gametocytes mixed with pre-immune sera (Table 4). Regardless of whether they had fed on immune or pre-immune sera, the aseptic mosquitoes produced statistically greater oocyst infection prevalences than did septic mosquitoes fed pre-immune sera (X^2, $p<0.05$). Conversely, oocyst development in aseptic mosquitoes was statistically equivalent to that in septic mosquitoes fed on immune sera.

3.5. Effect of immune sera on *P. falciparum* development within *A. gambiae* mosquitoes reared in different insectaries

During the course of this study, *A. gambiae* mosquitoes were reared in two different insectaries. Normally, the mosquitoes used for *P. falciparum* infections originated from the same insectary used to generate the immune sera. When such was the case, *P. falciparum* oocyst prevalences were always significantly greater in the immune fed versus the pre-immune fed mosquitoes (see Table 1). However, when the mosquitoes used for *P. falciparum* infections originated from an insectary different from that used to raise the mosquitoes for immunogens, significantly higher oocyst prevalences in immune fed versus pre-immune fed mosquitoes occurred in only two of the five paired feeding experiments conducted (Table 5). Statistically, the proportion of feeds with increased *P. falciparum* oocyst prevalences due to the addition of immune versus pre-immune sera was significantly greater in experiments that utilized mosquitoes originating from the same insectary used to produce the immunogen (*i.e.*, 12 of 12 feeds; Table 1) than in experiments that utilized mosquitoes originating from a different insectary (*i.e.*, 2 of 5 feeds; Table 5) (Fisher's exact test, $p=0.01$). Similar insectary differences were not observed for *P. yoelii* infections (data not shown).

Table 4
Plasmodium falciparum NF54 oocyst prevalence (n) within *Anopheles gambiae* mosquitoes fed gametocytes mixed with pre-immune sera or immune sera from rabbits immunized against mosquito midgut tissue and associated microbiota. Prior to infection, mosquitoes were either maintained for three days on gentamicin antibiotic (aseptic) or left untreated (septic). Experimental feeds were replicated twice, using different *P. falciparum* gametocyte cultures.

Pre-treatment	Replicate	Pre-immune-fed	Immune-fed	p-value
Aseptic (Gentamycin)	1	79% (39)	89% (36)	0.43
Septic		42% (36)	72% (72)	0.004
Aseptic (Gentamycin)	2	97% (34)	93% (30)	0.91
Septic		43% (39)	86% (43)	<0.001

4. Discussion

The original impetus for this study was an investigation into mosquito-based transmission blocking immunity. During their escape, *Plasmodium* ookinetes utilize specific ligands on the luminal surface of midgut epithelial cells to recognize and penetrate the midgut [33]. Mosquito ingestion of antibodies against such ligands can hinder molecular recognition and prevent the escape of ookinetes from the midgut, thus terminating *Plasmodium* infection of mosquitoes and blocking malaria transmission [34]. Exploratory studies on mosquito-based transmission blocking immunity have met with some success [25,35,36]. In our study, we immunized a series of rabbits with whole midgut lysates from female *A. gambiae* mosquitoes. High-titered immune sera were produced, mixed with *Plasmodium*-infected erythrocytes and fed back to *A. gambiae* mosquitoes. Although the outcomes of these feeding assays were highly reproducible, results were opposite of what we had initially predicted and hoped for. Instead of reducing oocyst development, co-feeding the immune sera with *P. falciparum* gametocytes increased oocyst prevalence and density when compared to pre-immune fed mosquitoes (Table 1). Increased oocyst production for *P. falciparum* in immune-fed mosquitoes was the result of an increased *per capita* conversion of ookinetes to oocysts—*i.e.*, formation of ookinetes was equivalent between immune-fed and nonimmune-fed mosquitoes but the ookinetes in immune-fed mosquitoes were more successful in crossing the midgut and developing into oocysts (Table 2). However when the same immune sera were mixed with the rodent parasite, *P. yoelii*, and fed to *A. gambiae* mosquitoes, there was no effect on the outcome of oocyst infection (Table 3).

Table 5
The efficacy of rabbit anti-midgut immune sera on *Plasmodium falciparum* oocyst prevalence in *Anopheles gambiae* mosquitoes when the insectary facility used to rear mosquito stocks for antigen production (*i.e.*, rabbit immunizations) differed from the insectary facility used to rear mosquitoes for bioassay testing (*i.e.*, *P. falciparum* infections). Values indicate oocyst prevalences among lots of co-infected mosquitoes (n).

Sera Pool[a]	Pre-immune Sera[b]	Immune Sera[c]	p-values
6–8	57% (30)	59% (29)	0.91
6–8	20% (50)	64% (39)	<0.0001
6–8	16% (19)	50% (32)	0.03
9–10	38% (58)	22% (58)	0.11
9–10	86% (22)	78% (32)	0.68

[a] Identifies the rabbits from which the sera were pooled.
[b] Sera containing no demonstrable IgG against *A. gambiae* midguts and midgut microbiota.
[c] Sera containing demonstrable IgG against *A. gambiae* midguts and midgut microbiota.

In addition to being immunoreactive against *A. gambiae* midgut tissue, the immune sera were also immunoreactive against bacteria isolated from within the midguts (Fig. 1). Experiments, whereby mosquitoes were cleansed of cultivable midgut bacteria prior to testing, suggested that antibodies against these bacteria (and possibly non-cultivable species as well)—but not the midgut tissues—were responsible for these unanticipated results (Table 4). This was substantiated by our findings that the enhancing effect of the immune sera was insectary-specific. That is, as long as the origin of mosquitoes used for production of anti-sera and the infectious feeds were derived from the same insectary, inclusion of immune sera in a gametocytemic blood meal increased *P. falciparum* oocyst prevalence. This was repeated 12 times with the same result (Table 1). But this high degree of reproducibility faltered once the origin of mosquitoes for anti-sera production and parasite infections were derived from different insectaries (Table 5). The reasons underlying this curious finding are speculative. Perhaps the different insectaries produced mosquitoes with different midgut microbiotas (see [20]). Nevertheless, these data indicate that the apparent enhancing effect of immune sera on *P falciparum* oocyst prevalence was not due to antibodies directed against mosquito midgut tissue. If that were the case, then the enhancing effect of immune sera would have been observed no matter what the origin of the mosquito stocks. Thus we concluded that antibodies directed against midgut microbiota (but not midgut tissue) enhanced the success of *P. falciparum* ookinetes crossing the midgut and developing to the oocyst stage.

Curiously, the immune sera did not enhance oocyst production in *P. yoelii* (Table 3). The most plausible explanation for this involves the kinetics of events occurring within the midgut following an infectious bloodmeal. After blood is ingested, the midgut undergoes enormous changes in cellular morphology and metabolism, including stretching, diuretic removal of fluid from the blood, secretion of digestive enzymes, and peritrophic matrix formation. Likewise, the midgut microbiota undergoes dynamic changes. Unfed mosquitoes typically harbor a variety of bacteria within their midguts [18,19]. With the influx of blood, midgut bacteria enter exponential growth phase, peaking at about 18 to 24 h [17,18]. If the ingested blood also contains viable *Plasmodium* gametocytes, the parasites will undergo their own developmental kinetics involving exflagellation, fertilization, ookinete formation and motility. However, the kinetics of these early developmental events differs among *Plasmodium* species [1]. Ookinete formation for *P. falciparum* NF54 peaks at 24 to 32 h [3]—a time when the bacterial density is greatest. In contrast, ookinete formation for *P. yoelii* peaks at 8 to 12 h [4]—well in advance of the exponential growth of midgut microbiota. Thus it seems reasonable that midgut microbiota probably exert a greater influence on the ookinetes of late-developing species such as *P. falciparum* when bacterial densities are high than for early-developing species such as *P. yoelii* when bacterial densities are low. It is important to note that despite their immunoreactivity against midgut microbiota (Fig. 1), the immune sera did not inhibit bacterial growth *in vitro*. Thus, it is unlikely that ingestion of immune sera by mosquitoes appreciably altered the normal growth kinetics of midgut microbiota *in vivo*.

Although the precise mechanism(s) remains unknown, recent studies suggest that the enhancing effect that anti-bacterial antibodies had on *P. falciparum* ookinete passage through the mosquito midgut probably involves antibody-mediated interference with either 1) the mosquito innate immune response to gram-negative bacteria, or 2) bacterial secretions that directly or indirectly lower parasite success. With regard to the former, innate immune responses of *Anopheles* midguts are activated by molecular recognition of certain moieties of gram-negative bacteria, resulting in the up-regulation of several effector genes possessing anti-*Plasmodium* activity [12,14,15,20]. However, these immune responses fail to activate fully when the midgut microbiota is removed by antibiotics administered prior to blood feeding [14,20]. This implies that midgut bacteria must be present in order for the innate immune response of the midgut to respond vigorously. It is notable that specific antibodies in our immune sera covered the entire surface of midgut bacteria. Therefore, it is possible that anti-bacterial IgG in the immune sera bound to immunostimulatory moieties of bacteria and masked their presentation to mosquito immune cells, thereby dampening the innate immune response [14,15,20,37]. By coating the bacteria and dampening the innate immune response of the mosquito midgut, antibacterial antibodies in the bloodmeal may have allowed more ookinetes to penetrate the midgut without sustaining harm from mosquito immune effectors. This would result in a greater prevalence and density of oocysts in immune-fed mosquitoes.

Alternatively, polyclonal antibodies to midgut microbiota may neutralize bacterial secretions that hinder parasite development. For example, *Enterobacter* bacteria cultured from the midguts of wild *A. arabiensis* mosquitoes from Zambia secrete reactive oxygen species (ROS) that block *P. falciparum* ookinete development [38]. Conceivably, specific anti-*Enterobacter* antibodies could hamper bacterial secretion of ROS and thus allow for more successful ookinete formation. In our experiments, antibody-mediated enhancement only occurred during the egress of *P. falciparum* ookinetes from the midgut (e.g., 18 to 36 h) when midgut bacteria are in late log phase or stationary phase growth [17]. As bacterial populations enter stationary phase, they often undergo quorum sensing, whereby rising concentrations of constitutively-secreted signal molecules (e.g., N-acetylhomoserine lactone) reach a threshold and trigger a coordinated shift in gene expression away from replication and towards the formation of biofilms. Quorum sensing and biofilm formation are well studied in *Ps. aeruginosa*[39,40]—a species that was isolated from *A. gambiae* midguts during this study. Although the effect on ookinete motility is unknown, bacterial biofilms have been shown to adhere to and inhibit *in vitro* growth of other protozoan parasites, namely trypanosomatid and leishmania (promastigote) parasites *in vitro*[41,42]. Interestingly, Tashiro et al. [43] demonstrated that rabbit polyclonal antibodies against *P. aeruginosa* surface antigens inhibited biofilm formation *in vitro*. Thus it is plausible that the rabbit polyclonal anti-*P. aeruginosa* antibodies present within our immune sera (see Fig. 1) may have had a similar inhibitory effect on biofilm formation by *P. aeruginosa* within mosquito bloodmeals, thereby relaxing potential 'biofilm barriers' to *P. falciparum* ookinete passage and increasing their successful transition to oocysts (Table 2).

5. Conclusions

This study emphasizes the role of gram-negative bacteria in altering the efficiency of *P. falciparum* development in *A. gambiae*. Not only are bacteria, including those isolated in this study, common in the midguts of laboratory-raised and field-caught anophelines [17,19,20,44,45], bacteria have been shown to reduce *Plasmodium* infections of *Anopheles* in laboratory and natural conditions [15–17,20,38,46,47]. The common thread uniting our study with previous studies is the idea that the normal bacterial flora found within mosquito midguts may play an important regulatory role in the survival of *Plasmodium*. Our studies suggest that this is particularly true for those species such as *P. falciparum*, whose developmental timing within the mosquito midgut may lag behind that of the exponential growth of bacterial populations after a blood meal. As progress proceeds towards the development of mosquito midgut-based intervention strategies, it will be important to continue to define the influence of midgut microbiota on *Plasmodium* development in the midguts of wild anophelines within malaria endemic settings [38].

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