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Effect of *Carnobacterium Sp.* On Plasmodium Sporogony in *Anopheles Stephensi* Mosquito

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Abstract: **Malaria control is getting tougher each day due to development of resistance to anti-malaria drugs in the parasite and resistance to insecticides in malaria vectors. Alternate strategies are thus required to break the complex malaria parasite interaction. Malaria vectors like other living beings carries diverse flora in their gut, which may inhibit the malaria parasite development inside the mosquito. We earlier identified a natural gut residing lactic acid bacteria *Carnobacterium sp.*, isolated from lab-reared *Anopheles stephensi* mosquito. *An. stephensi* is known vector of malaria in Indian subcontinent. Thus, in the present manuscript efforts were made to understand the sporogonic development of *Plasmodium vinckei petteri* in presence of the bacterium. The bacterium was established in mosquito gut by feeding orally along with sugar meal to a group of gut sanitized mosquito. Groups of naïve, gut sanitized and *Carnobacterium sp*. fed mosquitoes were used to compare the difference in sporogony of *P. v. petteri.* This bacterium shows antagonistic effect on development of the malaria parasite.**

Keywords: **Mosquito, Gut bacteria, Malaria parasite, Sporogony.**

I. INTRODUCTION

Vector control is the main way to reduce malaria transmission at the community level. Through this intervention malaria transmission can be reduced from very high levels to close to zero. Insecticides treatment to mosquito vector is the main method of their control. The emergence of insecticide resistance necessitated the development of new strategies to reduce pathogen transmission in the field [1]. Malaria parasite needs to go through its developmental stages inside the mosquito mid-gut to complete its life cycle. Parasite bears a very high cost for infecting the vector as significant losses occur during its development inside the mosquito [2]. Inside Anopheles mosquito mid-gut, malaria parasite shares microenvironment with gut resident bacteria which can be a factor to parasite loss.

Insect gut harbors large communities of diverse microorganisms which outnumber the total cells in the insect itself [3]. Various studies have proven the contribution of microbial endosymbionts to the host's nutritional homeostasis and their role in digestion of blood [3], [4]. Many times mid-gut bacteria have been screened from laboratory colonized as well as from field populations of various mosquitoes [5], [6], [7], [8]. These studies reported the presence of both Gram negative as well Gram positive bacteria in mosquito mid-gut.

Few studies have shown that when *Anopheles* mosquitoes were treated with antibiotics, the level of *Plasmodium* oocysts production increased [9], [10]. These studies suggest that mid-gut biota provides a protective cover to *Anopheles* mosquito against malaria infections. The variation in diversity of mid-gut bacteria can also influence the development of malaria parasite inside the *Anopheles* mosquito [7], [11]. Cirimotich et al., (2011) identified a Gram negative bacterium from field populations of *Anopheles gambie* which can suppress the parasite up to a significant level. A recent study reported that a bacterium (*Chromo bacterium sp.*) isolated from the gut of an *Aedes* mosquito can reduce the malaria parasite and dengue virus infection in mosquitoes [13].

In earlier study we reported the gut bacterial diversity in laboratory colonised and in field collected *Anopheles stephensi* mosquito [14]. The *An. stephensi* is primary malaria vector in the area. *Carnobacterium sp.* was found the most dominating bacteria in the study. The dominant bacterium, *Carnobacterium sp.* is a Lactic Acid Bacteria (LAB), which

are known to secrete antimicrobial peptides, bacteriocins. In the present study the malaria vector *An. stephensi* were first sanitized with antibiotic to sanitize their guts, later the LAB *Carnobacterium sp.* was fed to the gut-sanitized mosquitoes to study their influence on the sporogony of rodent malaria parasite, *Plasmodium vinckei petteri*.

II. MATERIALS AND METHODS

Mice and rabbits were used in the present study. Rabbits were used for the blood (requires for egg laying) feeding to *Anopheles stephensi* mosquitoes to maintain their colony. BALB/c mice were used to maintain the life cycle of *Plasmodium v. petteri* parasite. Prior approval has taken from Animal Ethical Committee of the Institute before the use of animals for experimental purpose.

Cyclic colonies of the *Anopheles stephensi* mosquito were kept in an insectory maintained at a temperature of 28±1^{o}C and 70-80% relative humidity (RH) [17]. A simulated dusk and dawn machine with a photoperiod of 14 hour day and 10 hour night was fitted in the insectory. Adult mosquitoes were held in 30cm x 30cm x 30cm muslin cloth cages tied on iron frame. Mosquitoes were offered sterile 2% glucose soaked cotton pads as a source of energy and the cotton pads were changed every day. Rodent malaria parasite *Plasmodium v. petteri* 279BY was used to study the effect of mosquito mid-gut bacteria on its sporogony. Cryo-preserved stock of rodent malaria parasite was revived by inoculating the parasite into mice (BALB/c strain) and subsequently its life cycle was maintained through mice (primary host) and laboratory colonised *Anopheles stephensi* (vector) mosquito [18].

After emergence, adult *An. stephensi* were fed on cotton pad soaked in 2% glucose and 50mg/ml tetracycline for 4 consequent days to clear the gut biota. The tetracycline containing pad was then replaced by 2% glucose pad to allow the residual tetracycline in guts to degrade for two days [10]. One group of gut sterilised mosquito was fed with 10^4 bacteria/ml dilution of *Carnobacterium* in 2% glucose pads. Gut sanitized and *Carnobacterium* fed mosquitoes were kept at temperature of 28±1^{o}C and 70-80% RH till seventh day of their emergence. The establishment of the *Carnobacterium sp.* was confirmed by plating the guts of representative mosquitoes on LB Agar plates and sequencing the 16S rRNA as described earlier [8], [14].

Seven days after emergence of adults, gut-sanitized and *Carnobacterium sp.* fed mosquitoes were allowed to feed on a *Plasmodium v. petteri* infected Balb/c mice with nearly 4–6% parasitemia and 0.5–0.8% gametocytemia for each feeding. Immediately after infected blood feeding the exflagellation centers were counted. The malaria parasite infected mosquitoes were given 2% glucose for their further development. Malaria infected mosquitoes were maintained at 24± 1 ◦C and 60–70% relative humidity for sporogonic development of *P. v. petteri* inside the mosquito vector. On 8th day post-infection mid-guts were dissected and the mercurochrome stained oocysts were visually counted under 40x objective lens. Geometric mean of oocysts per mid-gut was calculated to see the infection intensity in each group. The differences in the geometric mean oocyst number per midgut were analyzed statistically using Mann Whitney Test on http://VassarStats.html site. Chi-square analysis was performed in Microsoft Excel software.

III. RESULTS AND DISCUSSION

Anopheles stephensi is an important malaria vector of Asia. Culturable gut bacteria profiling of the mosquito revealed, *Carnobacterium sp.* as dominant bacteria in the mid-gut of *An. stephensi* mosquitoes from Delhi region [14]. We studied the importance of this bacterium for sporogonic development of *Plasmodium vinckei petteri* in *Anopheles stephensi.* For the purpose the guts of the mosquitoes were first sanitized by feeding them orally with antibiotic, tetracycline to remove total gut bacteria and then the *Carnobacterium sp.* was established in the gut of the mosquitoes. The sanitization of mid-gut bacteria upon antibiotic feeding was confirmed as described earlier [10]. The gut sanitized mosquitoes were orally fed with *Carnobacterium sp.* and the establishment was confirmed by plating the guts on LB agar plates. Single type of colonies was obtained on the plates. Of which 10 colonies were randomly picked and for species identification, the total DNA was amplified using 16S rRNA specific primers and sequenced on ABI 3730 xl ABI sequencer. Sequencing confirmed reestablishment of *Carnobacterium sp.* in the mid-gut mosquito. Many workers have already established single bacteria in mosquito mid-gut using this method [13], [23].

The mosquitoes were fed with infective *P. v. petteri* mice. The exflagellation centre count was 0.05–0.15 per field at 1000× resolution for each feeding experiment. The number of oocysts in naïve, gut-sterilized and *Carnobacterium sp.* fed *An. stephensi* mosquitoes were recorded on Eighth day of infected feeding. Geometric mean number of oocysts produced

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in naïve blood fed and guts sanitized mosquitoes were 56.7 and 77.6 oocysts per mid-gut respectively against 59.9 oocysts per gut in *Carnobacteium sp.* fed group of mosquitoes (Fig 1). There was a significant difference in geometric mean number of oocysts in gut-sanitized and *Carnobacteium* fed groups (Mann Whitney test; $p < 0.01$).

Various studies have been carried out to see the effect of gut bacteria on sporogony of *Plasmodium* [9], [10]. These studies clearly indicated that the cumulative effect of total gut bacteria on sporogonic development of *Plasmodium* was inhibitory. Some studies also showed that individual bacteria from mosquito mid-gut can also reduce the parasite load in mosquito [12], [19]. In the present study, we noticed antiparasitic role of a Gram positive bacteria, *Carnobacterium sp.* against *Plasmodium v. petteri* infection. *Carnobacterium sp.* is a genus of lactic acid bacteria. These bacteria are known to have bio-preservative potential because of their bacteriocins producing ability [15]. This bacterium is able to tolerant high pressure and can grow at low temperatures; it can grow with increased CO2 concentrations and is used as protective cultures in order to inhibit growth of *Listeria monocytogenes* in fish and meat products [16]. Bacteriocins are both narrow spectrum and broad spectrum antimicrobial peptide. However, its role in killing *Plasmodium* has never been investigated, bacteriocin, *nisin* in active against protozoans as well [20]. The reduction in parasite load on mosquito mid-gut need not be result of secretion of bioactive compounds but also direct interaction with the bacteria but other factors such as immune system of the mosquito [21], [22].

IV. CONCLUSION

In conclusion, mosquito gut contains a large number of natural microbiota similarly to humans. The gut microbiota needs for adequate physiological functions and to maintain homeostasis. The natural gut biota negatively affects malaria parasite sporogony inside the mosquito vector. Individual bacteria isolated from mid-gut of *Anopheles* mosquito may inhibit the development of malaria parasite inside the mosquito vector [12]. We show that *Carnobacterium sp.* is also able to inhibit the sporogonic development of *Plasmodium v. petteri* up to certain extent in *Anopheles stephensi* mosquitoes. By changing the composition of mid-gut bacteria of wild *Anopheles* mosquitoes to increase the prevalence of naturally inhibitory bacteria can be a new strategy to control malaria in near future.

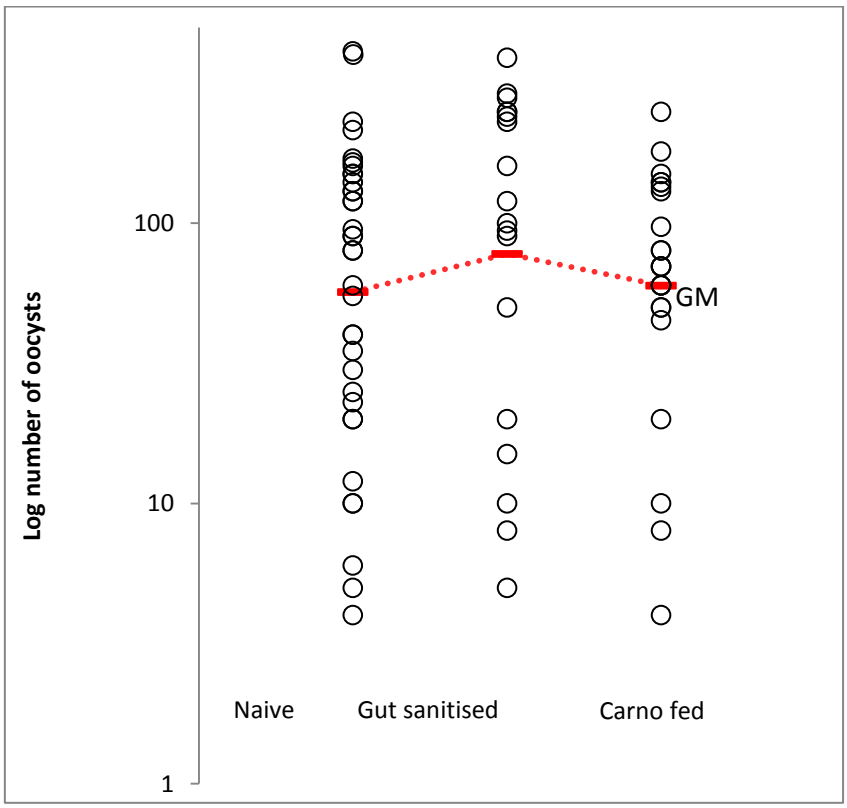

Fig1. Comparison of sporogonic development of *Plasmodium vinckei petteri* in naïve, gut sanitized and *Carnobacterium sp.* fed group of *Anopheles stephensi* mosquitoes. Oocyst in each mosquito gut was also counted (circle) and geometric mean (box) was calculated. The numbers of mosquitoes used in each treatment (n) are given as numbers against respective treatment. GM-geometric mean.

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