International Scholars Journals

African Journal of Parasitology Research ISSN 2343-6549 Vol. 6 (3), pp. 001-006, March, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Fluorescence can be used to trace the fate of exogenous micro-organisms inside the alimentary tract of mosquitoes

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Accepted 04 January, 2019

There is a great deal of current research interest in utilising bacteria for the control of intractable arthropod-borne diseases such as dengue. Although there is accumulating evidence that bacterial infection is a promising control strategy, most studies on bacteria-insect interactions lacked useful markers for detecting pathogenesis. This provided the impetus to investigate bacterial infection in the dengue vector *Aedes albopictus*. The infection persistence patterns in key organs of the alimentary canal of females were examined using a GFP-expressing strain of *Escherichia coli* (Migula). Just after feeding with sugar meal containing the bacteria, the crop and midgut as well as parts of the Malpighian tubules showed fluorescence. From 1 h onwards, bacterial populations declined sharply in both the midgut and crop, with complete elimination in the former but persistence of bacteria at 7 h post-feeding in the latter. After 24 h, neither organ retained the fluorescent marker. However, culture of homogenates of these organs in Luria-Bertani medium revealed the presence of a bacterial population in the crop, but not in the midgut. These observations suggest a difference in the potential physiological actions expressible by the two organs. In fact, both are storage sites for ingested fluids, but the midgut has greater physiological activity. Presumably, one of these activities contributed to eliminating GFP-expressing *E. coli* from the *A. albopictus* midgut after 24 h. The results of the present study using a fluorescent marker to detect infection may be useful for developing strategies to fully characterise the main steps involved in the bacterial infection process in insects.

Key words: Bacteria infection, fluorescent marker, crop, midgut, persistence.

INTRODUCTION

There is growing concern regarding the eventual impact of global climate change on the evolution of arbovirus infections (Chastel et al., 2002), particularly dengue. Despite tremendous effort to control this disease, its transmission is increasing due to the combined effect of changing human demographics and the spread of pathogens and vectors (Gubler, 2004). Dengue is caused by a flavivirus transmitted by the mosquitoes *Aedes aegypti* (L.) and *A. albopictus*. The later mosquito, which is native to Southeast Asia, is ranked fourth on the list of the most invasive organisms worldwide (http://www.is-sg.org/data base/welcome). It is an important vector of several arboviruses, including those responsible for yellow fever and various types of encephalitis as well as a competent laboratory vector of at least 23 arboviruses (Mitchell, 1995). Its larvae emerge from the eggs in containers and ingest some of the microbial fauna (Sota et al., 1992) associated with organic detritus, their major carbon source (Clements, 1992). As with most insects, the adult

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stage has a relatively restricted diet and thus harbours a community of microbiota, in particular bacterial fauna, which participates in many types of interaction ranging from pathogenesis to obligate mutualism (Dillon and Dillon, 2004; Dharne et al., 2006). Many types of bacteria have been isolated from the midgut of field-collected mosquitoes (Straif et al., 1998). The principal route of the interactions between bacteria and the insect interior milieu is through the ingestion of contaminated food (Valley-Gely et al., 2008). In mosquitoes, there is evidence that bacteria can be transmitted transstadially from larvae to adults and in nectar/sugar-feeding at the adult stage (Pumpuni et al., 1996). On feeding, ingested food or blood meal and associated microorganisms and/or pathogens are transferred passively through the foregut and stored in the midgut, the site of digestion and absorption of nutrients (Clement, 1992) as well as infection (Wang et al., 2001). In nature, both sexes feed on nectar, which is stored temporarily in the crop and then gradually released into the midgut (Fisk and Shambaugh, 1954).

Ingested bacteria encounter the insect immune system and can often counteract host defences (Valley-Gely et al., 2008). There is evidence that most ingested bacteria are eliminated by mechanisms such as reactive oxygen species (ROS) and peristalsis, and persistence within the host requires ingested bacteria to be able to survive the conditions of the alimentary tract (Valley-Gely et al., 2008). As more bacteria survive in the midgut, their interactions with the epithelium molecules may increase and perturb gut physiology (Jackson et al., 2001). The degree to which these phenomena can occur may be dependent on bacterial persistence.

Recently, there has been renewed interest in characterising insect gut microorganisms because they are a potential source of novel bioactive compounds, such as antimalarial, antiviral, and antitumour peptides (Chernysh et al., 2002) as well enzymes (Zhang and Brune, 2004). In addition, studies on insect–bacteria interactions have attracted interest because manipulating microbial symbionts is thought to be an effective strategy for controlling the spread of pathogens that use insects as hosts (Dillon et al., 2005). Although bacterial infection has been reported to suppress vector competence in mosquitoes (Pumpuni et al., 1993; 1996; Lowenberger et al., 1996), there are still no satisfactory candidate vaccines or environmentally safe insecticides for controlling dengue (Chaturvedi et al., 2005). This situation in combination with the observation that bacterial infection can potentially suppress pathogen transmission provided an impetus to explore bacterial infection in *A. albopictus*. However, one problem faced by most studies on insect–pathogen interactions is the lack of means to identify pathogenesis. Here, the location and persistence of an orally ingested bacterium were investigated in key parts of the female digestive tract using fluorescent bacteria because monitoring of fluorescence allows detection of infection and its intensity can be used to determine persistence of infection.

MATERIALS AND METHODS

Rearing and producing sterile adult mosquitoes

A. albopictus used here originated from a colony maintained at the insectarium of Nagasaki University. To establish a new colony at the Microbiology Laboratory of Fukuoka University for the purpose of this study, eggs from the Nagasaki colony were hatched in cool boiled water and newly hatched larvae were raised at a density of 100 per plastic tray (27 × 36 × 6 cm) filled with 2.5 L of sterilised and dechlorinated water. They were fed daily with a mixture of powdered mouse pellet diet (CLEA Japan, Inc., Tokyo, Japan) and dry yeast (1:1) (Tanabe Seiyaku Co. Ltd., Osaka, Japan). Pupae were transferred into sterilised plastic dishes, and emerging adults placed in cages (20 x 20 x 30 cm) were given access to a 3% sucrose solution. Three-day-old females were blood-fed on immobilised mice. Eggs were dried under rearing laboratory conditions (27 ± 2.0°C, 70 ± 10% RH and 12:12 h photoperiod) and were kept at room temperature. For aseptic production of adults for the experi- ments, all instruments used for rearing were sterilised and wiped frequently with isopropanol prior to use. 100 newly hatched larvae were reared in covered plastic trays. Sterilised food was supplied to larvae at 2 g per day. The pupae were transferred to sterilised plastic dishes filled with sterilised and dechlorinated tap water. Emerging adults were placed in cages without food prior to experimental use.

Construction of GFP-expressing *E. coli*

The plasmid vector pGFPuv (Clontech Laboratories, Inc., Mountain View, CA) was transformed into *E. coli* DH5 (Takara Bio Inc. Shiga, Japan) by electroporation using a Gene Pulser (Bio-Rad Laboratories, Inc., Hercules, CA). The transformants were cultured in Luria-Bertani rich nutrient (LB) medium overnight at 37°C. All reagents for bacterial culture were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Feeding *A. albopictus* with GFP-expressing *E. coli*-infected meal

Recombinant *E. coli* carrying the plasmid pGFPuv were grown in LB medium supplemented with isopropyl -D-1-thiogalactopyranoside (IPTG) at 37°C until the optical density at 660 nm (OD_{660}) reached 4.0. Aliquots of 5 mL of the bacterial cultures were centrifuged at 5000 rpm for 10 min at 4°C. The resulting pellets suspended in 5 ml of sterilised 3% sucrose solution served as the experimental meal. A total of 50 three-day old *A. albopictus* females were transferred into the cages and allowed free access to the infected meal. Adults were further sampled and feeding status was confirmed under a dissecting microscope based on expansion of the abdomen. Fed individuals were maintained without food in sterilised glass test tubes until dissection.

Dissection of crop and midgut

Dissection was performed under laminar air flow to avoid potential contamination. The fed mosquitoes were divided into two groups: those in the first group were dissected immediately after feeding (0 h), while those in the second group were dissected 24 h after the infected meal. Following the method reported by Pumpuni et al. (1996), individuals were anaesthetised with CO_2 and wiped with

70% ethanol before dissection under a binocular microscope (Nikon SMZ- 1B; Nikon, Tokyo, Japan). Only successful dissections, defined as leaving the crop and midgut intact, were considered in the analyses. Body carcasses containing an undamaged crop and midgut were irradiated with UV (366 nm) on a UV transilluminator (Funakoshi Co. Ltd., Tokyo, Japan) and photographed. A similar group of mosquitoes were used to confirm the presence of bacteria at different time's post-ingestion of the infected meal. A group of fed individuals were dissected at 1, 3, 5, 7 and 24 h. Those dissected at t = 24 h will have had longer to interact with the meal than their counterparts dissected at t=1 h.

The crop and midgut were then placed in 1.5 mL plastic tubes with 200 l of physiological saline solution and homogenised with an additional 800 l of the same solution. The homogenates were diluted 1×, 10×, and 100× and aliquots of 100 l from each dilution were spread on LB agar plates and incubated at 37°C overnight.

Data collection and analysis

Photographs of carcasses showing the intact crop and midgut were taken with a digital camera fixed to a binocular microscope with a UV lamp (Model UVL/Blak-Ray UV Lamp, long wavelength UV 366 nm; UVP, San Gabriel, CA) as the light source. At least 4 body carcasses were photographed at 0 and 24 h post-feeding, and if visualised by UV were considered present if observed in all photos. Bacteria were scored as present if consistent images were obtained in all photos. The homogenates were diluted as described above and three replicates of each dilution were spread on Luria- Bertani medium (LB). To estimate the number of GFP-expressing *E. coli* present, colonies that showed luminescence under UV illumination were counted 48 h post-feeding. For each homogenate, the mean value of the number of bacteria was calculated and expressed as colony forming units per ml (cfu/mL). The midgut homogenates were treated in a similar manner. This procedure revealed the number of bacteria for both the crop and migdut throughout the time course of the experiment. The mean bacterial numbers were calculated and expressed as the mean ± SE (standard error). The proportion of bacteria disappearing from each organ at each time point was calculated using the formula: mean number of bacteria at a given time point/mean number of bacteria at 1 h post-feeding × 100. Statistical analyses were performed using Analysis of Variance and Tukey's HSD multiple comparisons with the SYSTAT®11 software package (SYSTAT®11 DATA, 2004).

RESULTS

Analysis of organ photographs

Visual analysis of photographs of the carcasses of *A. albopictus* females just after feeding (0 h) with the infected meal indicated the presence of the green fluorescent marker in the crop and midgut in most cases (Figure 1A). In contrast, no luminescence of GFP was observed in the midgut or crop on photographs from those dissected 24 h post-feeding (Figure 1B). In some cases, the Malpighian tubules also showed fluorescent labelling (Figure 1A).

Bacterial density

The time post-feeding, but not the organ type, significantly affected the number of bacteria (Table 1).

The bacterial population present in the midgut showed a shorter period of persistence than that in the crop (Figure 2). In both organs, the size of the bacterial population tended to decrease over time. In the midgut, the number of bacteria differed significantly between 1, 3, and 5 h ($F = 123.23$, $df = 4$, $p < 0.001$), while a pairwise comparison probability matrix did not reveal any differences between population sizes at 5, 7 and 24 h. In the crop, the bacterial popula-tion size decreased significantly between 1 and 3 h post-feeding. Thereafter, there was a steady decrease of that population about ten times less cfu/ml are found after 7 hours than at after 3 hours ($F = 9.30$, $df = 4$, $p < 0.001$). The bacterial population size was similar between the two organs at 1 ($F = 0.38$, $df = 1$, $p = 0.540$) and 3 h ($F = 0.28$, $df = 1$, $p = 0.601$) post-feeding. However, at 5 ($F = 9.92$, $df = 1$, $p = 0.004$), 7 H ($F = 12.81$, $DF = 1$, $p = 0.001$), AND 24 H ($F = 4.19$, $DF = 1$, $p = 0.05$) after ingesting the infected meal, the size of the bacterial population in the midgut was significantly smaller than that in the crop. The poor persis-tence of transgenic *E. coli* in the midgut may be related to the noxious contents of physiological activity in the lumen.

The data shown in Table 2 indicate variable patterns of bacterial disappearance from the crop and the midgut. Both organs showed a marked decrease in size of the *E. coli* population between 1 and 3 h post-ingestion. Further, the sizes of the bacterial populations decreased slowly, and at 5 h post-feeding both the crop and the midgut still harboured bacteria. At 7 h post-feeding, the crop still har-boured a residual bacterial population, whereas this was completely absent in the midgut.

DISCUSSION

Preliminary trials on the transformation of *E. coli* using *E. coli* DH5 and the expression plasmid vector pGFPuv produced viable bacteria characterised by the presence of green fluorescence under UV illumination. These bacteria were maintained at -20°C and sub-colonies still showing luminescence after months. We assume that the fluorescence actually measures the GFP. Such sub-colonies were mixed with sucrose solution to prepare the experimental meal for *A. albopictus.*

Most females showed fluorescence in the midgut and crop following meal intake, and the marker had disappeared completely from the epithelium in both organs after 24 h. There have been few studies of time- related bacterial persistence in mosquitoes. St. John et al. (1930) reported that ingested *Staphylococcus aureus* , *Bacillus prodigiosus* , *Bacillus leprae* and *Cytoryctes variolae* mixed with blood remained in the *A. aegypti* midgut for more than 24 h. In a related study, Pumpuni et al. (1996) fed a population of *A. stephensi* a 1% sucrose solution containing *Xanthomonas maltophilia*, *Serratia marcescens* or *Pseudomonas aeruginosa*. They reported retention of the two first bacteria in the midgut after 24 h in 75% of cases, whereas only 19% showed persistence of

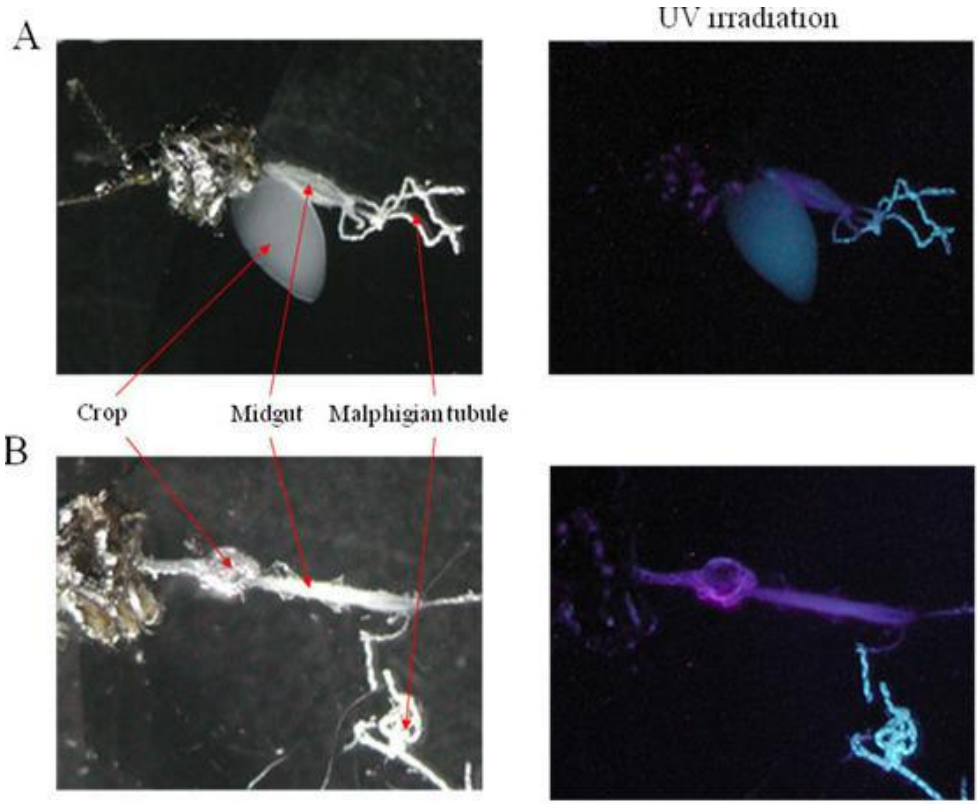

Figure 1. Carcasses of *A. albopictus* females showing the presence of the green fluorescent marker in the crop and midgut just after (A) feeding with the sugared meal contaminated with the transgenic *E. coli* and its absence 24 h later (B).

Table 1. Analysis of Variance of the effects of organ type and time on bacterial population size of GFP *E. coli* within the digestive tract of *A. albopictus*

Source variables	df	F-*ratio*	P
Organ	1	0.20	0.65
Time post-feeding (TPF)	1	40.81	0.000
Organ × TPF	1	11.67	0.74

Crop and Midgut; 0 h and 24 h; x=Interaction.

Pseudomonas aeruginosa. These reports suggested that the period of bacterial retention in the midgut is dependent on the species of bacteria and/or mosquito, but this may not be the sole reason for disappearance of bacteria from the insect alimentary canal.

The results obtained using the homogenates in the present study indicated that the period of persistence of ingested bacteria was much shorter in the midgut, in which the number of bacteria disappeared almost completely after 5 h post-ingestion of the infected meal. In the crop, however, although the size of the bacterial population decreased in the initial 3 h after meal ingestion, it remained at a relatively constant level thereafter. This difference in retention between the crop and midgut may be related to the difference in physiological roles of the epithelium between the two organs. As in any epithelial tissue, the physiological roles are derived from cell function and the degree at which they vary depends largely on the number and proportion of different cell types. Indeed, an epithelium with a limited number of cells will tend to have reduced physiological roles as compared to another with a larger cell number. The crop epithelium is composed of unspecialised cells, which therefore have reduced potential to impact the ingested meal. Although this epithelial tissue has been reported as a site of partial hydrolysis of disaccharides, it is important to realise that this action was due to a salivary enzyme (Clements, 1992). In contrast, the midgut contains rege- nerative, endocrine, and columnar cells, which account for much of its epithelium. The functions of this organ are well established: it is the principal organ of digestion and thus the site of synthesis of digestive enzymes, including trypsin, proteinases, peptidases, esterases, and glucosidases. In addition to its digestive function, the midgut is the site of nutrient absorption, hormonal synthesis (Clements, 1992), infection processes and defensive responses (Wang et al., 2001). Although morphologically different, the midgut and the crop share a similar physiological function, that is, storage of sugar-containing

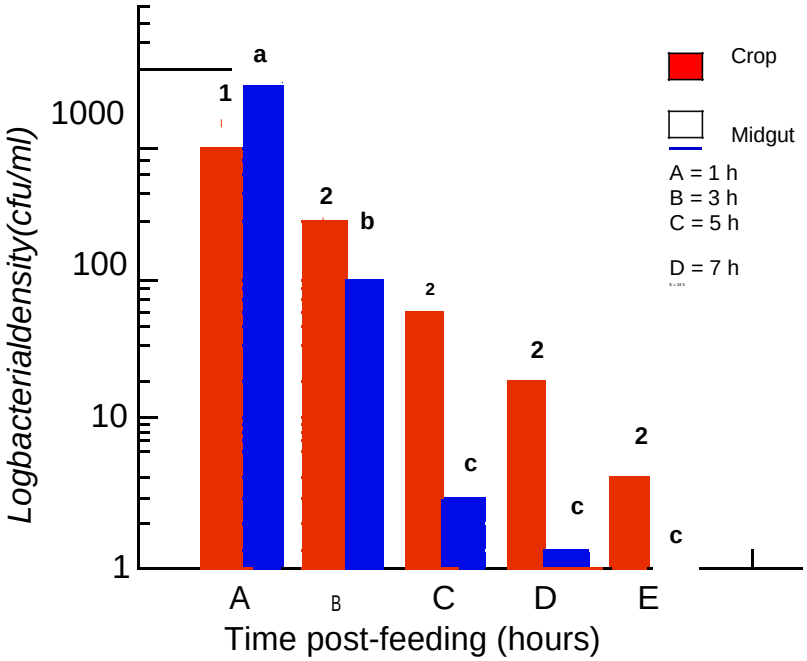

Figure 2. *E. coli* populations in homogenates of the crop and midgut of *A. albopictus* females fed the sugared meal contaminated with transgenic *E. coli* and allowed to interact with the meal at different time points. The vertical axis of this figure shows the mean bacteria density on a $\log_{10}$-based scale.

Table 2. Proportions of *E. coli* cells that disappeared from the crop and midgut of *A. albopictus*. The meal was taken only once, and that the fate of the bacteria inside the crop and midgut was examined at different time points after feeding. By ANOVA, bars of the color and with the same letter do not show a significant difference ($P < 0.05$).

	Mean ±SE bacterial cells	% disappearance			
	at 1 hour post-feeding	3 h	5 h	7 h	24 h
Crop	1389.5 ± 390.83	81.2	97.8	99.1	99.75
Midgut	1692.2 ± 1 69.64	87.6	99.96	99.99	100

meals. These two organs have been shown to interact during digestion processes in *A. aegypti*, a species closely related and ecologically similar to *A. albopictus* studied here (Hawley, 1988). Sugar solu- tion ingested by this mosquito has been detected in the midgut 30 min after filling the crop (Jones and Brandt, 1981). The difference in bacterial persistence between the crop and midgut seen in the present study was likely due to discrepancies in their potential actions on the ingested meal. In addition to its suggested hydrolytic role, the crop is also known to release its contents in small amounts into the midgut (Clements, 1992). Here, ingested meals and any associated micro-organism may be exposed to lumen conditions, such as pH, digestive enzymes, ionic strength, ROS, and the immune system (Valley-Gely et al., 2008), which may alter the bacterial population in the midgut.

In addition to the severe midgut conditions, the bacterial population associated with an ingested meal may be affected by post-midgut physiological processes as food residue undigested in the midgut moves to the hindgut and is excreted (Clements, 1992; Chapman, 1998). These phenomena may have occurred in the present study, but no experiments were performed to determine whether bacteria were present in the excreta of the mosquitoes. Therefore, further studies should include monitoring of the post-midgut bacterial populations.

The results of the present study demonstrated that the crop and midgut differ fundamentally in their interactions with sugar-containing meals, despite their similar status as storage organs. Another interesting result of the present study was the ease of detection of bacterial infection, which was made possible by the use of a fluorescent marker.

There are, however, three possible sources of error in our approach. First, we did not control for loss of plasmid by the *E. coli*, without a selective preasure the *E. coli* might have lost the gfp-expressing plasmid and still undetected in the mosquitoes after 24 h. Secondly, the bacteria culture used for the experimental meal was grown to OD_{660}. In reference to the OD_{600} used in a previous study (Riehle et al., 2007), one may consider that our OD is overgrown and may result in many dead and ruptured bacteria. The cell material from these dead bacteria might trigger immune defences in the mosquito that would not happen in the intact bacteria cells intact. This in turn might have lead to a faster clearence of the bacteria from the midgut. Finally, after spreading the diluted homogenates on LB plates, no deliberate efforts were performed to count the colonies that did not show any luminescence. According to Riehle et al. (2007), the number of fluorescent colonies on each plate represents one half of the total *E. coli* population for an individual mosquito midgut.

Further studies of bacteria-*Aedes* interactions may facilitate the use of such microorganisms to control disease transmission by arthropod vectors. Therefore, further studies involving one or prefereably several bacteria isolated from *A. albopictus* or other *Aedes* mosquitoes, if a known insect pathogen, are required.

ACKNOWLEDGMENTS

The authors thank Dr. Takagi (Department of Vector Ecology and Environment, Nagasaki University, Japan), and the staff of the Department of Microbiology, Faculty of Pharmaceutical Sciences, Fukuoka University, Japan.

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