Bacterial diversity in the lumen of wild caught leishmaniasis vector *Phlebotomus argentipes* (Psychodidae: Phlebotominae)

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Abstract

Leishmaniasis is a vector-borne disease transmitted through female sand flies which caused by a protozoan parasite belong to genus *Leishmania*. Parasite development happens mostly in the midgut of the sand fly with presence of bacterial community in lumen. In Sri Lanka, *Phlebotomus argentipes* is known to be the vector for leishmaniasis. Several studies have reported the presence of aerobic bacteria in the gut of sand flies which evidence potential approach to control Leishmaniasis transmission through paratransgenic strategy. However, such investigations have not been conducted in Sri Lanka. Sand flies were collected by Cattle baited collections from three selected Medical Officer of Health (MOH) areas (Polpithigama, Maho and Galgamuwa) in Kurunegala district on a monthly basis from August to December 2018. Female sand flies were immobilized on ice and sterilized in 30 µL of 70% w/v ethanol followed by phosphate-buffered saline (PBS) washing. Midgut offive unfed female sand flies were pooled and homogenized with 150 µL of sterile PBS (pH 7.3). The lysate was diluted to 500 µL with PBS and 100 µL of each homogenate was plated onto Brain Heart Infusion (BHI) agar followed by incubation at 28°C up to 2 weeks under aerobic conditions. The experiment procedures were repeated 10 times. Colony separation was done byphenotypical differences. Stab cultures of isolates were sequenced for 16S ribosomal RNA partial gene. A total of 1,969 specimens of sand flies were collected. Morphological identification revealed the presence of only one species, *Phlebotomusargentipes*(n=1,969). The male sand flies are the most represented with 91.4% (n= 1,800), whereas females represent only 8.6% (n=169). A total of 50 randomly selected unfed female *P. argentipes*was examined. The average Colony Forming Unit (CFU) ranged from $8 \times 10^1 - 130 \times 10^2$. A total of 11 bacterial isolates namely; *Serratiamarcescens, Enterobacter*sp, *Staphylococcus saprophyticus, Enterobacter* cloacae, Staphylococcus *sciuri, Stenotrophomonas maltophilia, Pseudomonas aeruginosa, Staphylococcus arlettae, Aeromonascaviae, Staphylococcus warneri, Bacillus megaterium*. Sequences showed 99% - 100% identities to the existing sequences, which was assigned to the same genus and species. *S. marcescens,* shown to have an anti-leishmanial activity. Therefore, further studies are required to explore the potential use of this species for paratransgenesis.

Keywords: Leishmaniasis, sand fly, midgut, bacteria

Introduction

Leishmaniasis is a vector-borne disease transmitted through female sand flies (Psychodidae: Phlebotomine) which caused by a unicellular protozoan parasite belong to genus *Leishmania*. It is considered as a neglected tropical disease in the world accounting 102 countries as disease endemic (Karakus*et al.,* 2017).

In Sri Lanka, *P. argentipes* is known to be the vector for leishmaniasis transmission (Wijerathna *et al.,* 2017). Several studies have recorded the prevalence of bacterial community in the midgut sand fly vectors in other countries. However, such investigation has not been conducted in Sri Lanka. It is important to emphasize that when considering any means of biological control its effectiveness, ecological soundness and sustainability should be determining factors. Most of the available biological control methods are focused on killing the insect vector, while other methods such as the sterile insect technique (SIT), Release of Insects carrying a Dominant Lethal (RIDL) and paratransgenesis are also being tested (Benelli *et al.*, 2016). Of these, the use of paratransgenic sand flies has emerged recently as a promising option for the control of Leishmaniasis transmission (Hurwitz *et al.,* 2011).

In paratransgenic strategies to control insect vectors, symbiotic gut-associated bacteria of insects are transformed to express molecules with anti-parasitic activity (Durvasula, 1999; Beard, 2002). Introduction of these transformed organisms will results in anti-parasitic activity in the gut of sand flies by which means the pathogen's transmission is prevented. Therefore, first-hand understanding of what species of gut bacteria are present in sand flies local to Sri Lanka is essential in order to select a possible species to be transformed. Hence, the proposed study aims to screen the availability of such gut flora that may have an anti-parasitic property in order to evaluate the effectiveness of a paratransgenic strategy as a means to control leishmaniasis in Sri Lanka.

Method

Field collection

Sand flies were collected from three selected Medical Officer of Health (MOH) areas (Polpithigama, Maho and Galgamuwa) in Kurunegala district (Cattle Baited Net Traps (CBNT) were used to collect sand flies on a monthly basis from August to December 2018.

Identification of field-caught sand flies

The field caught sand flies were transferred live to the laboratory at the Department of Parasitology, Faculty of Medicine, University of Kelaniya, Ragama, Sri Lanka. Female sand flies were separated and immobilized on ice. Unfed females were taken for the study. Each sand fly was sterilized with 30 µL of 70% w/v ethanol and rinsed using phosphate-buffered saline (PBS).

Dissection of the mid-gut

The sterilized sand flies were transferred onto a drop of sterilized PBS placed on a sterile microscope slide. The specimens were dissected under a dissecting microscope and the midgut was removed. Genitalia was used to confirm the species identification referring to morphological. The midgut of fivesand flies were pooled to a 1.5 ml sterile microcentrifuge tube containing 150 µL of sterile PBS (pH 7.3) and homogenized using a disposable pestle. The lysate was diluted to 500 µL with PBS. From this stock solution a dilution series of the lysate (10^{0}-10^{-9}) were prepared.

Culturing and isolation of bacteria

About 100 µL of each homogenate was plated onto Brain Heart Infusion (BHI) agar Petri dishes. The BHI was picked as a non-selective medium to promote the growth of a diversity of microbes including nutritionally fastidious bacteria. Plates from the isolation were incubated at 28°C for up to 2 weeks under aerobic conditions. In order to assess microbial growth, the total number of total colony-forming units (CFUs) were counted. The whole experiment procedures were repeated 10 times.

Biochemical characterization of bacterial isolates

A record of the phenotypically different colonies was used to determine the diversity of bacteria in the mid but of each sand fly. Each bacterium was isolated by subculture from these primary plates. All of the isolates were differentiated by Gram staining, biochemical tests and morphological characterization.

Identification of isolated bacteria by DNA sequencing

Stab cultures were prepared from isolated single colonies of bacteria and those samples were send to Macrogen, South Korea (Macrogen Inc., 1001, 254 Beotkkot-ro, Geumcheon-gu, Seoul, Republic of Korea) for 16S ribosomal RNA partial gene sequencing with 27F (5' AGAGTTTGATCCTGGCTCAG 3) and 1492R (5' TACGGCTACCTTGTTACGACTT 3') universal primers. Sequencing results were analysed by BioEdit sequence alignment editor v7.0.9 software. The database search for homologous sequences was performed by Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI, USA). Sequences were deposited on NCBI GenBank to obtain accession numbers. Phylogenetic analyses were conducted according to neighbour-joining method (Saitou and Nei, 1987) in MEGA7 (Kumar *et al.,* 2016).

Results and Discussion

Entomological investigation

A total of 1,969 specimens of sand flies were collected. Morphological identification revealed the presence of only one species, *Phlebotomus argentipes* which is reported to be the vector for cutaneous leishmaniasis in Sri Lanka. The male sand flies are the most represented with 91.4% (n= 1,800), whereas females represent only 8.6% (n=169) of the entire collection in 3 localities (Table 1). The highest sand fly abundance was reported from the Polpithigama collection site with an 84.7% (n=1,668) of the entire collection followed by Galgamuwa (11.1%, n=219) and Maho (4.2%, n=82) sites.

Table 1. Abundance of sand flies among different localities in Kurunegala District

Collection site	Male	Female	Total
Maho	75	7	82
Galgamuwa	186	33	219
Polpithigama	1,539	129	1,668
Total	**1,800**	**169**	**1,969**

Biochemical characterization of mid-gut bacteria

In the present study, a total of 50 randomly selected unfed female *P. argentipes*was examined. The average Colony Forming Unit (CFU) ranged from $8 \times 10^1 - 130 \times 10^2$. There were more than 25 bacterial colonies with different morphological characters were isolated from sand flies (Figure 1).

Figure 1. Some mid-gut isolated bacterial colonies grown on Brain Heart Infu

The isolated organisms were first subjected to biochemical tests to identify up to genus level (Table 2). Only three strains were identified as gram positive and majority of them were "Rod in shaped. All strains indicated aerobic growth and almost all strains except PaKu-7, PaKu-8 and PaKu-13 showed the ability to grow under anoxic condition also. The catalase test was positive for all strains and only PaKu-7, PaKu-8 and PaKu-13 denoted oxidation ability. Acid production was observed from bacteria defined as PaKu-7 and PaKu-8.

Table 2. Characterization of bacteria based on morphology and biochemical investigations

Isolate No.	Gram stain	Shape	Motility	Aerobic growth	Anaerobic growth	Oxidase	Catalase	Acid Production	Oxidative/ Fermentative
PaKu-1	-	R	+	+	+	-	+	+	F
PaKu-2	-	R	+	+	+	-	+	+	F
PaKu-3	+	S	-	+	+	-	+	+	F
PaKu-4	-	R	D	+	+	-	+	+	F
PaKu-5	-	R	D	+	+	-	+	+	F
PaKu-6	+	S	-	+	+	-	+	+	F
PaKu-7	-	R	+	+	-	+	+	-	-
PaKu-8	-	R	+	+	-	+	+	-	-
PaKu-9	+	S	-	+	+	-	+	+	F
PaKu-10	-	R	+	+	+	+	+	+	F
PaKu-11	-	R	+	+	+	+	+	+	F
PaKu-12	+	S	-	+	+	-	+	+	F
PaKu-13	+	R	+	+	-		+	+	-

Abbreviations; R: Rod, S: Spirillum, +: Positive, -: Negative, F: Fermentative, O: Oxidative

Molecular characterization and diversity for midgut bacteria

A total of 11 bacterial isolates were identified by comparing 16S rRNA partial sequences with those present in NCBI gene bank. Among them *Staphylococcus* (30.7%) and *Enterobacter* (23%) were the most prominent genera. Sequences showed 99% - 100% identities to the existing sequences, which was assigned to the same genus and species (Table 3).

On the basis of phylogenetic tree in Figure 2, two main clusters were identified and *Bacillus* species were clustered with *Staphylococcus* species with the basis of 16S rRNA partial gene sequences. Therefore, those two genera have a close relationship than others.

Table 3. Molecular identification of isolated bacteria from mid gut of Sand flies

Bacterial Isolates	Genus/species identification	Similarity %	Accession no.	Phylum
PaKu1	*Serratia marcescens*	99%	MK841543	Proteobacteria
PaKu2	*Enterobacter* sp.	100%	MK841544	Proteobacteria
PaKu3	*Staphylococcus saprophyticus*	100%	MK841545	Firmicutes
PaKu4	*Enterobacter cloacae*	99%	MK841569	Proteobacteria
PaKu5	*Enterobacter cloacae*	100%	MK841570	Proteobacteria
PaKu6	*Staphylococcus sciuri*	100%	MK841316	Firmicutes
PaKu7	*Stenotrophomonas maltophilia*	100%	MK841317	Proteobacteria
PaKu8	*Pseudomonas aeruginosa*	100%	MK841321	Proteobacteria
PaKu9	*Staphylococcus arlettae*	100%	MK841329	Firmicutes
PaKu10	*Aeromonas caviae*	100%	MK841331	Proteobacteria
PaKu11	*Aeromonas caviae*	100%	MK841333	Proteobacteria
PaKu12	*Staphylococcus warneri*	99%	MK841411	Firmicutes
PaKu13	*Bacillus megaterium*	100%	MK841412	Proteobacteria

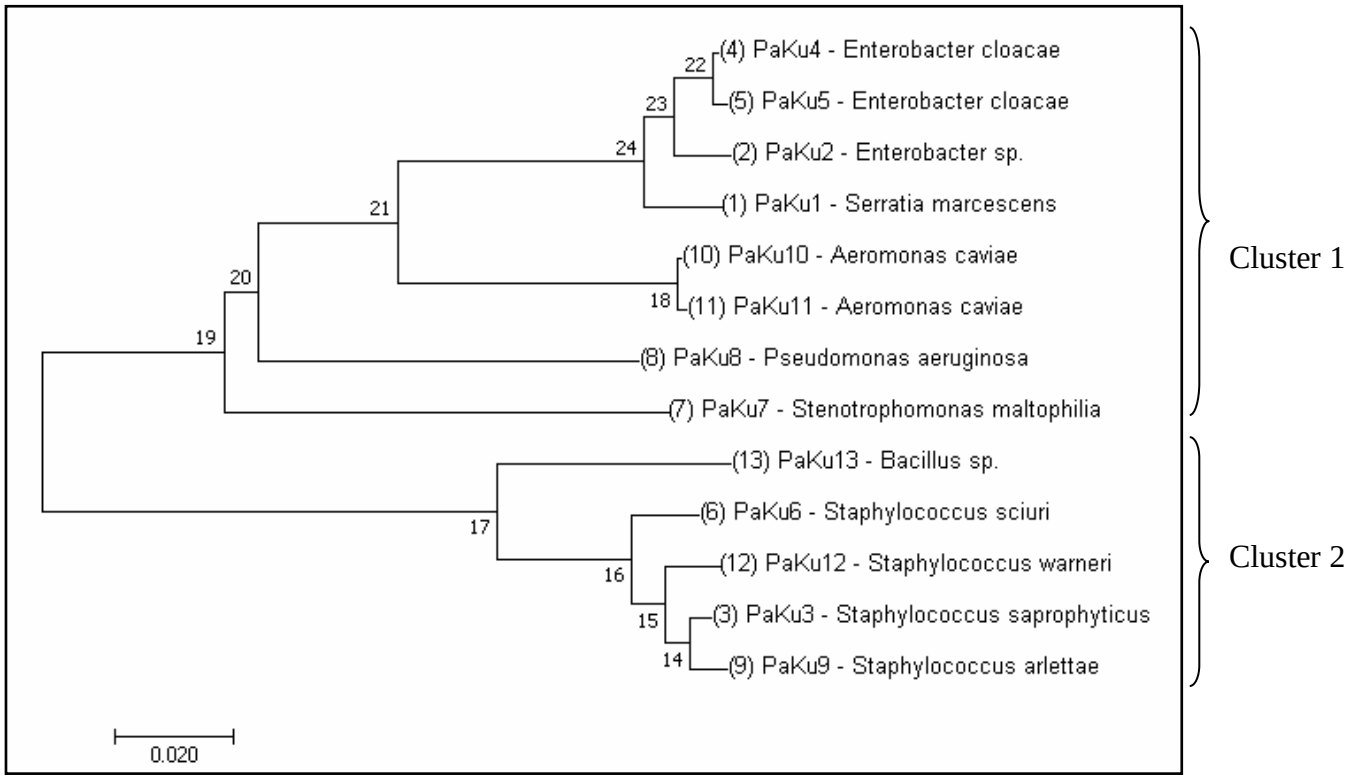

Figure 2. Phylogenetic tree constructed for partial 16S rRNA gene of isolates from midgut of sand flies

Among the species reported during the study, certain variants of *S. marcescens,* shown to have an anti-leishmanial activity (Moraes *et al.,* 2008). However, this experiment was conducted under *in-vitro* conditions using *L. chagasi*. Therefore, further studies are required to gain a full insight on the potential use of this bacterium in the control of *Leishmania* parasites through paratransgenesis.

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