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Study on the Bacterial Midgut Microbiota Associated to Different Brazilian Populations of *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae)

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Estudo sobre as Bactérias da Microbiota Intestinal de Diferentes Populações Brasileiras de *Lutzomyia Longipalpis* (Lutz & Neiva) (Diptera: Psychodidae)

RESUMO - A flora bacteriana encontrada no intestino de populações brasileiras de *Lutzomyia longipalpis* (Lutz & Neiva) provenientes de duas áreas endêmicas para leishmaniose visceral (Jacobina, BA e São Luís, MA) e de uma área não endêmica (Gruta da Lapinha, MG) é descrita. Cinco grupos de 35 fêmeas de cada população foram separados, no total de 175 fêmeas analisadas por área de coleta. A identificação das espécies foi baseada em métodos moleculares e em métodos bacteriológicos tradicionais. As bactérias encontradas foram classificadas como pertencentes ao grupo das não-Enterobacteriaceae, como *Acinetobacter*, *Burkholderia*, *Flavimonas*, *Pseudomonas*, ou ao grupo das Enterobacteriaceae, como *Stenotrophomonas*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Serratia*, *Pantoea*, *Morganella* e *Weeksella*. *Stenotrophomonas* esteve presente nas três populações analisadas. Além disso, *Serratia* spp., grupo bem documentado como contaminante laboratorial de insetos, foi detectado apenas na população de Jacobina. O impacto da colonização do intestino de insetos por bactérias no desenvolvimento e transmissão de patógenos é discutido.

PALAVRAS-CHAVE: Flebotomíneo, leishmaniose visceral, microrganismo, microbiota intestinal

ABSTRACT - The bacterial community associated with the midgut of three Brazilian *Lutzomyia longipalpis* (Lutz & Neiva) populations, two from endemic areas for visceral leishmaniasis (Jacobina, Bahia State and São Luís, Maranhão State) and one from a non-endemic area (Lapinha Cave, Minas Gerais State), was identified. Five groups, 35 females each, from each population were separated; a total of 175 females per collecting area were analyzed. The species identification was based on molecular and traditional bacteriological methods. All bacteria were either affiliated to non-Enterobacteriaceae, such as *Acinetobacter*, *Burkholderia*, *Flavimonas*, *Pseudomonas* and *Stenotrophomonas*, or and to Enterobacteriaceae, such as *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Serratia*, *Pantoea*, *Morganella* and *Weeksella*. *Stenotrophomonas* was found to be associated with all three populations studied. In addition, *Serratia* spp., which are well documented as laboratory contaminant of insects, were detected only in the Jacobina population. We also discuss the impact of the colonization of insect gut by bacteria on the development and transmission of pathogens.

KEY WORDS: Sand fly, visceral leishmaniasis, microorganism, gut microbiota

There are many species among Diptera, in which males and females feed on natural sugars, especially nectar, sap and aphid secretions. These sugars are the main source of carbohydrates for adults and complement the females diet, which also need blood feeding (Schlein 1986, Schlein & Warburg 1986, Samie *et al.* 1990). In this context, considering their food sources, these insects harbour microorganisms as

a natural flora that can influence their life cycles (Brooks 1964, Alves 1986).

In nature, sand flies – dipterous associate to the transmission of leishmaniasis – feed on different kinds of sugars, so having the chance to acquire contaminating microorganisms, such as bacteria and fungi, that can eliminate the leishmaniotic infection or even kill the sand fly (Killick-

Kendrick & Killick-Kendrick 1987, Schlein & Yuval 1987, Cameron *et al.* 1995).

Previous studies revealed that the introduction of transitory bacteria during the ingestion of sugars can exert a constant selective pressure on the development of *Leishmania* in the gut of its natural vector (Adler & Theodor 1927, Schlein *et al.* 1985, Pumpuni *et al.* 1996). It was suggested that the presence of a specific bacteria in the gut of an insect may affect the success a pathogen is transmitted by a vector (Pumpuni *et al.* 1996). Despite the apparent importance of the bacterial microbiota, there are few reports on the presence of bacteria in sand flies (Warburg 1991) and the midgut microbiota of Brazilian populations is unknown.

Some reports show a predominance of Gram negative rods in the midgut of wild-caught sand flies (Oliveira *et al.* 2000). For this reason we decided to investigate the bacterial microbiota present in the midgut of natural populations of *L. longipalpis* from different regions of Brazil (endemic and non endemic areas for visceral leishmaniasis). It is an important step to understand the microbial structure of populations of *L. longipalpis*, and allows identifying the organisms that might play a role in the insect life cycle and, possibly, in the pathogen transmission cycle.

Material and Methods

Field sampling. Sand flies were collected in a non-endemic area, Lapinha Cave (Minas Gerais State), and in two transmission areas of visceral leishmaniasis, Jacobina (State of Bahia) and São Luís (State of Maranhão), where *L. longipalpis* occurs in high density. The captures were performed from 2001 to 2004 using CDC light traps (Sudia & Chamberlain 1962), during a 12h interval from 6 p.m. to 6 a.m. In Jacobina and São Luís, traps were installed in the peridomicile in domestic animal shelters (hen houses and corrals), while inside the Lapinha Cave they were left about 50-80 cm from the ground level.

Specimen preparation. Sand flies were transported alive in a nylon cage to the laboratory, where selected females were transferred to plaster lined pots humidified with sterilized distilled water, in which insects remained in absolute abstinence of food until their processing. Five groups, 35 females each, from each population were separated; a total of 175 females per collecting area were analyzed. Females were killed at low temperature, surface-sterilized with sodium hypochlorite (5%), washed thrice with sterile distilled water and dissected in sterile PBS (phosphate buffered saline) under a hood for extracting the digestive tract and the Malpighian tubules.

Bacterial cultures and biochemical identification. The digestive tracts were put in a simple broth for enrichment. Afterwards the broth was spread on different culture media (DIFCO). The primary characterization of the microorganisms was based on the general aspects of colonies, Gram staining and selective biochemistry tests according to Holt *et al.*

(1994). Bacteria were identified at species level according to the Crystal Enteric Fermenter/Nonfermenter (E/NF) Identification System (Becton Dickinson Inc., Cockeysville, Md.). This system contains modified conventional and unconventional biochemical and enzymatic tests and is designed to identify members of the family Enterobacteriaceae and common strains of clinical significance and glucose non-fermenting Gram negative bacilli. Details on the procedure for isolation and identification of bacteria can be found in Oliveira *et al.* (2000).

DNA extraction, purification and quantification. Template DNA for PCR screening was prepared by processing 1 ml of culture grown for 18h at 37°C or 30°C, using the GenomicPrep cell and tissue DNA kit (Amersham Pharmacia Biotech Inc.). DNA concentration of each bacterial strain was measured in a GeneQuant DNA Spectrophotometer (Amersham-Pharmacia).

PCR amplification of 16S rDNA partial sequences. Twenty-five nanograms of DNA were used for PCR reaction. Ultrapure water (Gibco) was used in all negative control reactions (without DNA) and in the preparation of PCR mixtures. Reaction mixtures for amplification of sequences encoding 16S rDNA contained 2 µl of template DNA (10 ng), 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 0.1 mM of each deoxynucleoside triphosphate, 2.5 mM MgCl₂, 0.5 pmol of each primer, and 0.5 U of *Taq* DNA polymerase (Invitrogen). The broadly conserved Eubacterial 16S rDNA gene primers were p515f (GCCAGCAGCCGCGGTAA; *Escherichia coli* position 515-533) and p806r (GGACTACCAGGGTATCTAAT; *E. coli* position 806-787). The PCR amplification conditions consisted of 12 min at 95°C followed by 30 cycles of 95°C for 15s, 55°C for 20s and 72°C for 20s, and a single cycle of 72°C for 30 min on a 9600 Thermocycler (Perkin-Elmer/Applied Biosystems). Amplicons were analyzed by separating 12 µl of the PCR reaction on 2.0% (wt/vol) agarose (Sigma type V) gels at 3.2 V.cm⁻¹ in TBE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]), followed by ethidium bromide staining and examination under UV light. Negative controls (without DNA) were included in all amplifications.

Sequence analysis. Amplicons were purified using Wizard PCR Preps DNA Purification System (Promega) and subjected to direct bidirectional sequencing with the original primers (p515f and p806r) a Big Dye Terminator kit. Putative identifications of the 16S rDNA fragments (~240 nucleotides, excluding the primer sequences) were made by using the BLAST search algorithm. The entry having the highest homology to the submitted sequence was chosen as a reference identity for individual bands.

Results

Based on the sequence similarity to the existing GenBank database entries and traditional methods, bacterial identification revealed a complex flora with up to 13 genera (Murray *et al.* 2003) (Table 1). From the three *L. longipalpis*

populations studied 19 bacteria species could be isolated and characterized (Table 1). *Stenotrophomonas maltophilia* was the only species common to all populations of *L. longipalpis* analyzed, while *Enterobacter gergoviae*, *Enterobacter cloacae* and *E. coli* were isolated from specimens collected in Jacobina and Lapinha Cave. *Pantoea agglomerans* was the only species isolated from São Luís and Lapinha Cave populations, as *Acinetobacter baumannii* in *L. longipalpis* from Jacobina and São Luís.

The most diverse bacterial flora was observed in the population from Jacobina, with 12 isolated species, with the predominance of *E. cloacae* (32%), followed by *Serratia marcescens* (25%). In the population from São Luís, considering the five identified bacterial species, *P. agglomerans* was predominant (40%), followed by *Weeksella virosa* (26%) and *Flavimonas oryzihabitans* (16%). Nine bacterial species were identified in *L. longipalpis* females from Lapinha Cave, with the predominance of *S. maltophilia* (37%) followed by *E. cloacae* (25%) (Table 1).

Discussion

Many insects harbor microorganisms in symbiotic association that are essential for fecundity and viability to the host and can play a role on the establishment of parasites in the gut (Pumpuni *et al.* 1993). Others are stable

residents or transients and are lost during digestion or insect moulting (Endris *et al.* 1982, Gonzalez-Ceron *et al.* 2003). The origin and ecological association of the midgut bacteria and the insect host is difficult to define. In this study we identified the microbiota of different Brazilian populations of *L. longipalpis*. However a more in depth research on the interactions between insects and their midgut bacteria is required.

S. marcescens, *P. aeruginosa* and *P. agglomerans*, commonly associated with the midgut of the analyzed sand flies, were the most frequent bacteria in three mosquitoes, *Aedes triseriatus* (Say), *Culex pipiens* (L.) and *Psorophora columbiae* (Dyar & Knab) (De Maio *et al.* 1996). *K. ozaenae*, also common to these mosquitoes, was not found associated to the three sand fly populations analyzed in here, but was found associated in previous screenings of *L. longipalpis* from Lapinha Cave by Oliveira *et al.* (2000). *S. marcescens* was also isolated in lab-colonies of *Phlebotomus duboscqi* (Volf *et al.* 2002). The ubiquitous *P. agglomerans* is considered the Gram negative bacterium more regularly associate to ground and plants (DeMaio *et al.* 1996). Interestingly, this bacterium is responsible for the production of a toxic phenol in *Schistocerca gregaria* (Forsk.) that acts on other species of bacteria and fungi, being part of a defense mechanism to the host (Dillon & Charnley 1995). *Serratia* and *Klebsiella* were pointed out as pathogens of mosquitoes (Jadin 1967, Seitz *et al.* 1987, Pumpuni *et al.* 1993, Gonzalez-Ceron *et*

Table 1. Percent of bacteria species identified in *L. longipalpis* populations from Jacobina (BA), São Luís (MA) and Lapinha Cave (MG), Brazil.

Bacterial species	<i>L. longipalpis</i> populations		
	Jacobina	São Luís	Lapinha Cave
<i>Acinetobacter baumannii</i>	2	13	---
<i>Burkholderia cepacia</i>	5	---	---
<i>Citrobacter freundii</i>	---	---	7
<i>Enterobacter aerogenes</i>	---	---	2
<i>Enterobacter amnigenus</i>	2	---	---
<i>Enterobacter cloacae</i>	32	---	25
<i>Enterobacter gergoviae</i>	2	---	6
<i>Enterobacter taylorae</i>	---	---	4
<i>Escherichia coli</i>	2	---	3
<i>Flavimonas oryzihabitans</i>	---	16	---
<i>Klebsiella oxytoca</i>	---	---	14
<i>Morganella morganii</i>	6	---	---
<i>Pantoea agglomerans</i>	---	40	2
<i>Pseudomonas aeruginosa</i>	2	---	---
<i>Pseudomonas putida</i>	5	---	---
<i>Serratia liquefaciens</i>	5	---	---
<i>Serratia marcescens</i>	25	---	---
<i>Stenotrophomonas maltophilia</i>	12	5	37
<i>Weeksella virosa</i>	---	26	---
Total	100	100	100

al. 2003).

Reports on gut microbiota of wild sand flies belonging to *Phlebotomus* showed the presence of *S. marcescens*, *A. baumannii* and *P. aeruginosa* (Dillon et al. 1996, Warburg 1991), which were also associated with *L. longipalpis*.

Bacteria from environmental sources (water, ground and debris), such as *Acinetobacter*, *Serratia*, *Pseudomonas*, *Stenotrophomonas*, *Flavimonas* and *Enterobacter*, found to be associated with *L. longipalpis*, were also reported to be associated with the gut of several other insects (Oliveira et al. 1998, Campbell et al. 2004, La Scola & Raoult 2004, Zayed & Bream 2004), suggesting to be part of their natural or transient flora.

Studies demonstrated that *E. cloacae* is the most common bacteria found in the gut of insects of medical importance or not, including sand flies (Rossiter et al. 1983, Tanada & Kaya 1993, Dillon et al. 1996). In the midgut of mosquitoes collected from the field or maintained in insectary, *Enterobacter* has no influence in the insect's survival. However, other bacteria can be pathogenic and lead to a high adult mortality, such as some species of *Serratia* and *Klebsiella* (Jadin 1967; Seitz et al. 1987; Pumpuni et al. 1993, 1996).

The wide geographic distribution and occurrence of *S. maltophilia* in the environment, including vegetal material, could explain its occurrence in all populations of *L. longipalpis* analyzed.

Among the nine identified bacterial species from *L. longipalpis* collected from Lapinha Cave, only *E. cloacae* and *S. maltophilia* were previously reported by Oliveira et al. (2000), suggesting both as residents of this sand fly population. They probably do not influence the establishment of the parasites in the gut as they were isolated from areas with leishmaniasis transmission.

The other bacteria species could be part of the transitory flora, which differ according to the food source exploited. Dillon et al. (1996) suggested that the fluctuation of the bacterial species in the gut of *P. papatasi* increased after blood feeding. Ingestion of the Gram negative bacteria *E. coli* H243 and *S. marcescens* by blood feeding blocked the development of the *Plasmodium falciparum* oocysts (Pumpuni et al. 1993, 1996) in *Anopheles stephensi* (Liston). The presence of high concentration of *S. marcescens* (Azambuja et al. 2004) and *Pseudomonas fluorescens* (Mercado & Colon-Whitt 1982) in *in vitro* experiments induced *Trypanosoma cruzi* lysis. It can be suggested that the parasitic development in the insect vector is controlled by the presence and abundance of these microorganisms.

Representatives of the Gram negative bacteria isolated in the present study, *S. marcescens*, *P. aeruginosa*, *Klebsiella* spp. and *Enterobacter* spp., were able to produce several microbial factors, including prodigiosina (Azambuja et al. 2005). *S. marcescens* and *P. aeruginosa* synthesize others antiparasitic molecules, such as cytotoxic metalloproteases, haemolysins, antibiotics and haemagglutinins (Azambuja et al. 2005).

It is proven that *Enterobacter amnigenus* has the ability to re-colonize and persist in the gut of mosquitoes (Luxanani et al. 1994), being used as a host to express the *Bacillus* sp. toxic genes in *Aedes aegypti* (L.), *Culex quinquefasciatus* (Say)

and *Anopheles dirus* (Peyton & Harrison) to control their larval development (Khampang et al. 1999, Tanapongpipat et al. 2003).

Promastigotes of leishmania in culture grow with difficulty when competing with bacteria. In the same way, the bacteria interfere with the development of promastigotes in the digestive tract of sand flies competing for nutrients and reducing the pH (Dillon et al. 1996). In nature, despite the probable well-balanced associations between some bacteria and sand flies, there should be natural selective pressure involving some species of bacteria, leishmania and their vectors, acting directly over the prevalence of infection by *Leishmania* spp.

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