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Bacterial groups associated with *Nyssomyia neivai* (Diptera: Psychodidae) sandflies

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Phlebotomine sandflies are hematophagous insects involved in the transmission of leishmaniasis. The association of sandflies and bacteria is still an incipient field although there are evidences of interaction amongst the sandfly host-*Leishmania* parasite-gut microbiota[1]. Most of the knowledge of microbiota present in sandflies of the New World was carried out with *Lutzomyia longipalpis* (Lutz and Neiva), vector of American visceral leishmaniasis, using standard bacteriological methods[2] or molecular techniques[3]. The molecular approach used to identify bacteria from *L. longipalpis* in four regions of Brazil and Colombia resulted in 19 distinct bacterial phylotypes[3]. The authors pointed out the presence of bacterial plant pathogens, as for instance *Ralstonia* spp and *Erwinia billingiae*, and raised the question whether sandflies may act as vectors of plant pathogens[3]. Considering that 99% of the micro-organisms on earth do not grow in culture medium[4], this is a preliminary report combining molecular biology and genetics in an attempt to evaluate the bacteria associated with the sandfly species *Nyssomyia neivai* (Pinto) (=*Lutzomyia neivai*) considered to be a vector of American cutaneous leishmaniasis in South America.

Sandflies were collected from São Paulo state, southeast Brazil (21° 35′ 13′ S and 48° 04′ 15′ W) area where 99.9% of sandflies were previously identified as *N. neivai* [5]. In all, 10 females were surface cleansed using ethanol 70%, washed in autoclaved saline and stored in tubes with ethanol 70% following a modified methodology[3]. Total DNA extraction was done following the procedure of de Paiva *et al*[6] with some modifications. Sandfly females were washed with distilled water, following the addition of 20 µl of lysis solution (Tris 50 mM, NaCl 50 mM, EDTA 10 mM, Triton X-100 to 1%, 10 mM DTT); insects were crushed with the help of a plastic pestle followed by three cycles of liquid nitrogen freeze-thawing. The material was heated to 60°C for one hour, with subsequent addition of proteinase K (180 µg/ml final concentration), extra 80 µl of lysis buffer and Triton X-100 to 10% final. The samples were further incubated for three hours at 60°C, and after clarification by centrifugation for 10 min at 12,000 rpm. The supernatant was transferred to another tube and phenol extracted once. Total DNA samples were dialysed against TE (10 mM Tris-HCl pH 8, 1 mM EDTA) prior to storage at –20°C. The presence of bacteria into the DNA extracts obtained from the insects was evaluated by 16S-PCR diagnosis using the universal primers EUB r1387 (5′-GCCCGGGAACGTTCACCG), and EUBf933 (5′-CACAAGCGGTGGAGCATGTGG). The reaction composition was: 1 µl of DNA sample (from stock, or dilutions 1:10, 1:100, 1:200 or 1:400; diluted DNA samples were used to overcome inhibition effects of Taq DNA polymerase by possible contaminants carried over from the insect lysis), 1 U of Taq DNA polymerase, 1 µM of each primer, 2 mM $MgCl_2$, and 200 µM of dNTP. The polymerase chain reaction (PCR) cycle was: initial denaturation at 92°C/3 min, followed by 30 cycles of 92°C/30 sec, 50°C/1 min, and 70°C/1 min. PCR products were ligated into pCR2.1 TOPO-TA vector (Invitrogen), transformed in *E. coli* DH10B, and a total of 22 clones were evaluated by DNA sequencing (Macrogen Inc., Korea).

Using the pipeline generation system EGene, the sequences were filtered by quality using phred values, masked against primers EUB r1387 and EUB f933 and vector sequences (pCR2.1 TOPO-TA), trimming and selected by size (>100 bp). The high quality 16S sequences obtained were identified using the ribosomal database project (*http://rdp.cme.msu.edu/*).

According to the present study three bacterial phyla were identified: Verrucomicrobia (10%), Bacteroidetes (40%) and Proteobacteria (50%). Phylum Verrucomicrobia presented 100% of genus *Opitutus* species (one sequence). Within the phylum Bacteroidetes 25% were

Hydrotalea species (one sequence) and 75% unclassified *Chitinophagaceae* species (three sequences). The phylum Proteobacteria presented 40% of *Gammaproteobacteria* species (100% of unclassified – three sequences) and 60% of *Betaproteobacteria* with 33.3% belonging to genus *Pelomonas* species (one sequence) and 66.7% to genus *Schlegelella* species (two sequences) (Fig. 1).

Despite high number of sandfly species described in South America (470 estimated species), the studies related to bacteria associated to these insects are concentrated in *L. longipalpis*. The present study is a preliminary report using molecular methodology to search bacteria associated with the sandfly species *N. neivai*. Our results showed that some unclassified bacteria and genera have not been reported in other studies of sandflies.

Verrucomicrobia is a group of bacteria which encompasses few cultivated species, and one genus, *Opitutus* identified in *N. neivai*. The genus *Opitutus* was described from rice paddy soil on the basis of comparative analysis of the 16S rRNA genes[7]. Within the phylum Bacterioidetes the genus *Hydrotalea* and the unclassified *Chitinophagaceae* were identified. *Hydrotalea* spp have been isolated from water samples taken at different locations in southern Sweden[8].

Betaproteobacteria and *Gammaproteobacteria* (phylum Proteobacteria) were found in high densities in the sandfly *L. longipalpis*[3]. However, *Pelomonas* and *Schlegelella* genera (*Betaproteobacteria*) found in the present study have not been reported in previous sandfly searches. All sequences belonging to *Gammaproteobacteria* were unclassified.

The genus *Pelomonas* means a monad isolated from mud although other species were also isolated from

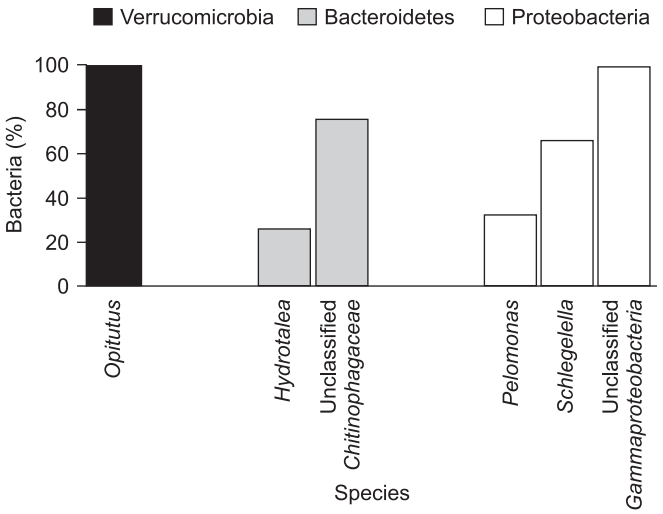

Fig. 1: Relative percentages of bacterial phyla associated with the sequences found on *Nyssomyia neivai* sandflies.

different water sources as for instance industrial and haemodialysis water. The genus *Schlegelella* was proposed based on type species (*Schlegelella thermodepolymerans*) isolated from an activated sludge and considered thermophilic[9].

All the groups of bacteria found in the current study were previously related to water or soil isolates. These findings are consistent with habitats of sandflies, which have their immature development in soil and organic material as the main food sources for larvae[10]. It has been shown that there is bacterial transtadial transmission in sandflies, thereby emerged adults can harbour bacteria from the larvae phase[11]. Sap of plants can also be another source of bacterial contamination for sandfly adults[11].

In accordance with the previous studies on sandflies, our results showed a predominance of gram-negative rods. There are reports about the possible negative role of gram-negative bacteria on the development of parasites in the mosquitoes' gut[12]. A recent study has shown that *Serratia marcescens* a gram-negative bacteria affects the innate immune pathways of the vector *L. longipalpis* and also presents negative effects on the parasite *Leishmania*[1].

Further studies must be performed with a higher number of sandflies to improve the basic knowledge about the bacteria community associated to *N. neivai* or other sandflies species and the possible relation with the protozoa parasites. Moreover, one possible attempt to solve the difficulties of maintaining colonies of sandflies in laboratory, including *N. neivai*, may be the search for symbiotic bacteria that are present in natural diet during larval instars and are not provided in laboratory conditions[13].

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REFERENCES

1. Diaz-Albiter H, Sant'Anna MR, Genta FA, Dillon RJ. Reactive oxygen species-mediated immunity against *Leishmania mexicana* and *Serratia marcescens* in the sand phlebotomine fly *Lutzomyia longipalpis*. *J Biol Chem* 2012; *287:* 23995–4003.
2. Gouveia C, Asensi MD, Zahner V, Rangel EF, Oliveria SM. Study on the bacterial midgut microbiota associated to different Brazilian populations of *Lutzomyia longipalpis* (Lutz and Neiva) (Diptera: Psychodidae). *Neotrop Entomol* 2008; *37:* 597–601.
3. Sant'Anna MRV, Darby AC, Brazil RP, Montoya-Lerma J, Dillon VM, Bates PA, *et al*. Investigation of the bacterial communities associated with females of *Lutzomyia* sandfly species from South America. *Plos One* 2012; *7*(8): e42531.

4. Sleator RD, Shortall C, Hill C. Metagenomics. *Lett Appl Microbiol* 2008; *47:* 361–6.
5. Pinto MC, Barbieri K, Silva MC, Graminha MA, Casanova C, Andrade AJ, *et al*. Octenol as attractant to *Nyssomyia neivai* (Diptera: Psychodidae: Phlebotominae) in the field. *J Med Entomol* 2011; *48:* 39–44.
6. de Paiva BR, Secundino NF, Pimenta PF, Galati EA, Andrade Jr F, Malafronte Rdos S. Standardization of conditions for PCR detection of *Leishmania* spp. DNA in sandflies (Diptera: Psychodidae). *Cad Saude Publica* 2007; *23:* 87–94.
7. Durham AM, Kashiwabara AY, Matunaga F, Ahagon P, Rainone F, Varuzza L, *et al*. EGene: A configurable pipeline generation system for automated sequence analysis. *Bioinformatics* 2005; *21*(12): 2812–3.
8. Kampfer P, Lodders N, Falsen E. *Hydrotalea flava* gen. nov., sp. nov., a new member of the phylum Bacteroidetes and allocation of the genera Chitinophaga, Sediminibacterium, Lacibacter, Flavihumibacter, Flavisolibacter, Niabella, Niastella, Segetibacter, Parasegetibacter, Terrimonas, Ferruginibacter, Filimonas and Hydrotalea to the family Chitinophagaceae fam. nov. *Int J Syst Evol Microbiol* 2011; *61:* 518–23.
9. Elbanna K, Lutke-Eversloh T, Van Trappen S, Mergaert J, Swings J, Steinbüchel A. Schlegelella thermodepolymerans gen. nov., sp. nov., a novel thermophilic bacterium that degrades poly (3-hydroxybutyrate-co-3-mercaptopropionate). *Int J Syst Evol Microbiol* 2003; *53:* 1165–8.
10. Killick-Kendrick R. The biology and control of phlebotomine sandflies. *Clin Dermatol* 1999; *17:* 279–89.
11. Volf P, Kiewegova A, Nemec A. Bacterial colonisation in the gut of *Phlebotomus dubosqui* (Diptera: Psychodidae): Transtadial passage and the role of female diet. *Folia Parasitol (Praha)* 2002; *49:* 73–7.
12. Pumpuni CB, Beier MS, Nataro JP, Guers LD, Davis JR. *Plasmodium falciparum*: Inhibition of sporogonic development in *Anopheles stephensi* by gram-negative bacteria. *Exp Parasitol* 1993; *77:* 195–9.
13. Andrade Filho JD, Galati EA, Falcao AL. Biology of the first generation of a laboratory colony of *Nyssomyia intermedia* (Lutz and Neiva 1912) and *Nyssomyia neivai* (Pinto 1926) (Diptera: Psychodidae). *Mem Inst Oswaldo Cruz* 2004; *99:* 597–601.

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