Bacteria of Phlebotominae Sand Flies Collected in Western Iran

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ABSTRACT

Microorganisms particularly bacteria presenting in insects such as *Phlebotominae* may play an important role in the epidemiology of human infectious disease. Nowadays, because of vector implications, the routine methods of controlling and spraying have no more beneficial effects on vectors and reservoirs. Little knows about the prevalence and diversity of sand fly bacteria. The main objective of this study was to determine the presence of bacteria of phlebotominae sand flies collected in Hamadan, west of Iran. This information is important in order to development of vector control strategies.

The microbial flora of *Phlebotomus papatasi* and *P. sergenti* the main vector of *Cutaneous Leishmaniasis* in the old world, were investigated.

We characterized 8 bacteria, including 5 Gram-negative bacteria: *Acinetobacter lwoffii, Pseudomonas aeruginosa, Enterobacter cloacae, Edvardsiela sp*. and *Proteus mirabilis* and *Gram-positive bacteria: Bacillus subtilis, Staphylococcus saprophyticus* and *Micrococcus luteus*.

Our study provides some data on the microbiota diversity of field-collected sand flies for the first time in Hamadan. Our results indicate that there is a range of variation of aerobic bacteria inhabiting sand fly, which possibly reflect the ecological condition of the habitat where the fly breeds. Microbiota is increasingly regarded as an important factor for modulating vector competence in insect vectors. So, mirobiota can be effects on the biology of *phlebotominae* and their roles in the sandfly-*Leishmania* interaction.

Further experiments are required to clearly delineate the vectorial role of sand flies. Because it is probable that in the future, factors such as environmental changes, migration and urbanization can ease the transmission of *leishmaniasis* in this area.

Key words: Bacteria, Iran, *Leishmaniasis*, *Phlebotmine* sand Flies.

INTRODUCTION

Phlebotominae sand flies (Diptera: Psychodidae) are important vectors of *leishmaniasis*, Carrion's disease or *bartonellosis*, and a variety of arboviral diseases [1-3]. Not only are novel viruses currently being discovered in sand flies, but also different reservoirs are being identified for pathogens and parasites of human diseases, transmitted by sand flies. The distribution areas of sand flies and the diseases they transmit are also expanding. New viral diseases of humans transmitted by sand flies are being reported as well [4-7].

The disease can present in three main ways as: *cutaneous*, *mucocutaneous*, or *visceral leishmaniasis* [8]. *Cutaneous leishmaniasis* is more prevalent throughout the world and causes disfiguration and other associated complications. Anthroponotic *Cutaneous leishmaniasis* and Zoonotic *cutaneous leishmaniasis* caused by *L. tropica and L. major*, respectively, are widely distributed in Turkey, Egypt, Israel, Iran, Saudi Arabia and the northern part of India, where mainly *P. sergenti* and *P. papatasi* have been incriminated as the vectors [9].

The disease is endemic in many rural districts in 17 out of 31 provinces of Iran [10]. In Hamadan Zahirnia and *et al.* carried out an epidemiological survey in Hamadan that indicated the occurrence of about 210 cases *of cutaneous leishmaniasis* in this province. According to their study 99% of the patients had a history of traveling to stay at endemic areas [11].

One of the most important factors in transmission of leishmaniasis is the presence of sandflies harboring leishmanial infection [12].

Adult sand flies usually remain close to their larval development sites [13]. The sites where larval

developments take place are usually a mixture of animal feces and mud which are found in both wild and anthropized biotopes [14]. Larvae feed on the decomposing organic materials in these sites and the adults can therefore acquire a part of their microflora during their larval development. Furthermore, male and female sand flies feed daily on natural sugars, especially nectars or sap secretions and drink water from plants [15]. These sugars are **the main source of carbohydrates for adults.** Additionally, females require a blood-meal to complement their diet, during the maturation of their eggs and completion of the gonotrophic cycle [16]. During these feeding events, they can also acquire various microorganisms including bacteria (e.g. *Bartonella bacilliformis*), fungi, Phleboviruses or other trypanosomatidae and co-colonization by human pathogenic and non pathogenic species of Leishmania [17-18].

In addition such bacteria may interfere with the development of medically important pathogens. For example, high midgut counts of Gram-negative bacteria are well known for significantly reduce oocyst numbers in plasmodium-infected mosquitoes [19]. Clearly, a complex interaction exists between Leishmania and midgut microbiota that has an effect on the development of mature infections [20].

Based on few previous studies it is believed that the microorganisms existing naturally in wild and laboratory-reared insects might have an important role as determinants of parasite survival and development in insect hosts. Some bacteria have attracted attention because they induce a number of intriguing abnormalities in the host's reproductive system [21]. Also, intracellular microorganisms affect the biology of their invertebrate hosts in many ways, ranging from mutualistic effects to the establishment of reproductive isolation and speciation [22-23]. A very preliminary study on *P. papatasi* from Morocco identified just two bacteria [24].There is also a small report on the distribution of bacteria from *P. papatasi* collected in Egypt [25]. Adler and Theodor suggested as early as 1929 that the presence of microbes in sand flies might interfere with *Leishmania* infection [26]. Later, Schlein *et al.* saw a reduction of infection rate of *L.major* in *P. papatasi* under the influence of bacteria [27]. Notwithstanding these studies, there are a few reports available on the micro flora of *P. papatasi* [24, 28].

A high prevalence of microbial infection in the digestive tract of wild-caught *P. papatasi* females was suggested to have a negative effect on Leishmania transmission in endemic areas [29].

There is a new vector borne disease control method called paratransgenesis that leads to decrease pathogen transmission by an insect vector (30). So, microbes particularly bacteria presenting in insects may have an important role in the epidemiology of human infectious disease [31].

Sand flies bacterial flora has been investigated on the isolated or via culture of bacterial gut content and were identified by the use of classical bacteriology, cloning [29-30].

Little is known about the prevalence and diversity of sand fly microflora colonizing. This information is important for development of vector control strategies [32]. It is now widely recognized that symbiotic microorganisms of arthropods play a crucial role in the ecology and evolution of their hosts.

The aim of the present study was to isolate, identify and examine the prevalence of bacteria in *P. papatasi* and *P. sergenti* in Hamadan through a culture dependent methodology. Results of this study may lead to identify appropriate candidate/s for paratransgenesis approach. Further experiments are required to clearly delineate the vectorial role (passive or active) of sand flies. This information is important for the development of new strategies for possible vector control.

MATERIALS AND METHODs

This is a descriptive cross-sectional study. In The city of Hamadan (33°59'-35°48' N and 47°34'-49°36' E) the average minimum and maximum monthly temperatures in January and June are -2.7 °C and 25.6 °C, respectively. The average relative humidity ranges are from 25% in July to 71% in January.

Sand flies were collected weekly, in 5 sites (north, south, east, west and center) of Hamadan , using 30 sticky traps (castor oil coated white paper 20×32 cm) from the beginning (May) till the end of the active season (October) [33]. Traps were installed at 18:00 pm and run until 07:00 am the following morning. Then traps containing insects were collected and transported to the laboratory in Hamadan. Isolation of sand fly guts was conducted in a sterile environment under a microbiological lab hood on a sterile glass slide. Before dissection, individual flies were surface sterilized for 2 min in 70% ethanol. The gut from each sand fly was micro-dissected and homogenized in test tubes (34). In each case, a fixed volume of samples (0.01 ml each) was cultured on non-selective and selective bacterial growth media containing blood Agar, Mac Conkey Agar and EMB at 37°C for 24-48 hours. Using inoculating loop different colonies subculture to aerobic and enriched anaerobic blood agar plates to purify the colonies and later pure isolates used for further identification procedures. For species identification, sand flies were mounted in Puri's medium and identified after 24 h, using the keys of Mesghali *et al.*, Nadim and Smart

[35-37]. The specimens were identified based on morphological characters of the head and the abdominal terminalia using rest of sand flies body for Bacterial Identification under sterile conditions.

Bacterial Identification

The initial identification of bacterial species was based on the colony characteristics (involving colony size, shape, color, margin, opacity, elevation and consistency) and the morphology of isolates based on Gram's staining procedure.

Pure cultures for each microbe were used for further identification procedure. All of the isolates were differentiated by standard gram staining and morphotypes. Mac Conkeys Agar is a special selective medium for gram negative bacteria [28, 38-39].

Finally, the API identification kit (API 20E, BioMerieux) was used for final identification of Gram-negative bacteria. The identification of Gram-positive bacteria was performed using the API Staph, API 20 Strep and API50CH B following the manufacturer's recommendations.

The colonies with different phenotype were sub cultured sequentially to obtain a single colony of the microbes. The best growing colonies and the most characteristic ones were picked up by a sterile loop and subjected to purification in the same isolation medium. Agar streak method was used for the purification process. A well separated colony from each isolate was picked up on nutrient agar slopes and incubated at 35°C for 24 h. Purity was checked by microscopic examination of the isolate using Gram stain. All cultures were maintained under aerobic conditions.

Gram-positive isolates that was not able to grow on the Mac Conkeys Agar medium, tested with manual laboratory examinations such as oxidase, catalase, coagulase, novobiocin susceptibility tests and mannitol medium. Bacteria were isolated after 24-48 h. Gram stain: Jensen's modified method was applied using crystal violet as a basic dye and safranine as counter stain [40]. Sterilization efficiency was controlled during the whole procedure.

Total colony counts were recorded for each sample and the average for every sample was calculated.

Statistical analysis: Data were organized through Excel 2007, which was also used in the descriptive statistics (SPSS-16 software). Graph Pad Instat software was used for statistical analysis.

RESULTS

The microbial flora of *Phlebotomus papatasi* and *P. sergenti* the main vector of *Cutaneous Leishmaniasis* in the old world was investigated.

The present study revealed that *P. papatasi* and *P. sergenti* female and male harbored both Gram–negative and Gram–positive bacteria in their body.

Among the 200 processed sand flies, only 4 of them (3 males and 1 female) were negative for bacteria. Eight bacterial strains were isolated from the processed *Phlebotominae* sand flies. The bacterial isolates corresponded to eight bacterial taxa: *Acinetobacter lwoffii* (19.5%), *Pseudomonas aeruginosa* (21.5%), *Enterobacter cloacae* (18%), *Edvardsiela sp.* (11%) and *Proteus mirabilis* (10.5%)) Gram-negative bacteria (80.5%)) as well as *Bacillus subtilis* (12%), *Staphylococcus saprophyticus* (4.5%) and *Micrococcus luteus* (3%) (Gram-positive bacteria (19.5%)) (Figure 1.).

Also, our observation showed that the majority of the bacterial strains isolated were Gram-negative bacteria with 80.5 percent. The most frequently isolated bacteria species of *P. papatasi* and *P. sergenti* in the present study were *Pseudomonas aeruginosan* and *Acinetobacter lwofii*, 21.5 and 19.5 percent respectively.

In addition, in our study where flies collected from the same region harbored almost the same kinds of bacteria.

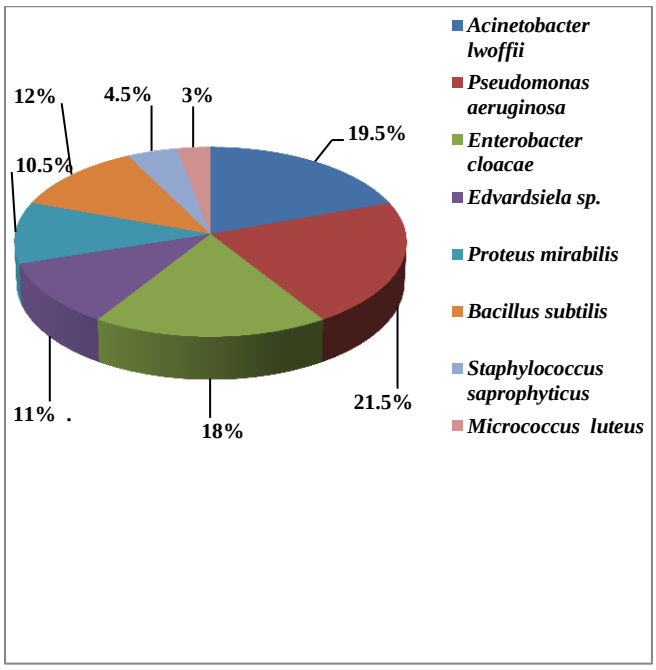

Fig.1: The percentage of each bacteria in sand flies

DISCUSSION

The presence of bacteria in insects can strongly influence their hosts' biology. Information on the biological interactions between bacteria of sand flies and Leishmania parasites they transmit is somewhat limited. However, differences in susceptibility to

leishmanial infection based on geographical distribution of sandflies were studied by many authors [41-42].

It is important to consider the microorganisms in vector insects. The present study revealed that P. papatasi and P. sergenti female and male harbored both Gram–negative and Gram–positive bacteria in their body.

In this survey, 200 insects were screened, and 8 bacterial species were isolated and identified. These species were*: Acinetobacter lwoffii, Pseudomonas aeruginosa, Enterobacter cloacae, Edvardsiela sp.* and *Proteus mirabilis*) Gram-negative bacteria) as well as *Bacillus subtilis*, *Staphylococcus saprophyticus* and *Micrococcus luteus* (Gram-positive bacteria). Hassan and *et al.* isolated 4 species of Gram–negative bacteria namely; *Acinetobacter calcoacetius, Staphylococcus aureus, Staphylococcus haemolyticus* and *Neisseria mucosa* of *P. papatasi* collected from *North Sinai* and *P. langeroni* collected from El Agamy [43].

The most frequently isolated bacteria species of *P. papatasi* and *P. sergenti* in the present study were *Acinetobacter lwofii* and *Pseudomonas aeruginosan*. But in one study in Egypt *B. thuringiensis* was the most frequently isolated bacteria species of *P. papatasi* [38]. Dillon *et al.* reported that the predominant bacteria species in the *P. papatasi* caught of Sinai, Egypt were *Enterobacter cloacae, E. sakazaki* and *Aeromonas sobria* (order *Enterobacteriaceae*) [25].

Although we used a nonselective medium to promote growth in a wide range of bacteria, due to not using specific media and culture conditions (e.g. an aerobic condition) the medium generally favored in growth of gram negative bacteria. Similarly, almost all of the studies analyzing the sand fly for bacterial communities have also relied on culture dependent techniques in their analysis where gram negative bacteria constituted the majority of their findings [28, 44].

In addition, several studies have reported a higher prevalence of Gram negative bacteria than Gram-positive ones in different vector insects [31, 45]. This is in agreement with our observation that the majority of the bacterial strains isolated in the present study were Gram-negative bacteria.

In addition, a correlation between the type of microbial flora detected and the area inhabited by the sand fly has been showed by Hillesland and *et al.*, where flies collected from the same region harbored almost the same kinds of bacteria [44]. Mukhopadhyay and *et al.* carried out a survey to study the abundance of different natural flora of *P. papatasi* in different habitats of Tunisia, Turkey, India and Egypt. They found variation in the species and abundance of flora in sand flies collected from different habitats [28]. These results fit with our study. Therefore, it was suggested that flora diversity more or less is a reflection of the environment where the sand fly resides.

Bacillus subtilis is a Gram-positive bactria which found in the present study. Mukhopadhyay and *et al.* succeeded in introducing of *B. subtilis* as candidate species for paratransgenesis due to the ability of this bacterium in induction of sand fly oviposition behavior and the real function of this as symbiont and not merely as an environmental contaminant [28].

Also Proteus species are found in many animals including insects [46-47]. Interestingly, in maggots, the bacteria *P. mirabilis* secrete antibacterial toxins that kill other microbes but do not harm the maggots. *Proteus mirabilis* is also highly resistant to the action of antimicrobial peptides, such as polymyxin. It referred to the active anti-bacteria constituents as "mirabilicide" [46]. The presence of *P. mirabilis* in other studies showed that this microorganism was considered beneficial [46-47]. However, these data have to be confirmed in the future by further studies carried out on more specimens.

Lack of Wolbachia isolate in the present study might be due to the isolation and characterization methodology that we have used.

Further studies examining bacteria species in sand flies are needed to reveal the relationship between bacteria and *phlebotominae* hosts.

Whether or not, the resident microbiota as a micro ecological factor can regulate the prevalence of sand flies with transmissible infections need to be investigated. Also, more investigation needs to find the most effective bacteria which can be used as bio-agent for combating *Leishmania* parasites.

In nature, despite the probable well balanced associations between some bacteria and sand flies, there could be natural selective pressure involving some species of bacteria, *Leishmania* and their vectors.

A study showed that the importance of considering the host microbiota as an "extended immune phenotype" in addition to the host immune system itself provide a unique perspective to understanding insects in health and disease (48). Other study showed that the capacity of bacteria to decrease viral and parasitic infections in mosquito and tsetse fly vectors by activating their immune responses or directly inhibiting pathogen development (49). This could be happen in the sand fly and may lead to a reduction in *Leishmania* infection within the sand fly host. Alder and Theodor (50) were the first to suggest that the presence of other microorganisms might prevent the development of *Leishmania spp*. in the

sand fly. Dillon and *et al*. showed that the *Leishmania* parasites often grow poorly in competition with bacteria in *P. papatasi*, probably because of their relatively slow generation time [25].
There are urgent needs for new strategies to control major human parasitic diseases; it might include engineering transgenic insects to reduce parasite transmission. It is hoped that these bacteria may be able to be used as a system to decrease vector-borne-diseases and to reduce the transmission of diseases in the future.

CONCLUSION

Bacteria are increasingly seen as an important factor for modulating vector competence in insect vectors so the presence of the bacteria in *P. papatasi* and *P. sergenti* were discussed. However, a depth research on the interactions between sand fly and their bacteria and *Leishmania* are required.

ETHICAL ISSUES

We hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct and the presented paper is all our own work and was produced without the use of other than the stated resources.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORS'CONTRIBUTIONS

A..Salehzadeh, S.Rafatbakhsh, R.Yousefi-mashouf and M najafimosleh designed the experiments.
S. Rafatbakhsh collected the sand flies.
A. Salehzadeh, M. khedri and S. Rafatbakhsh indentified the sand flies.
S. Rafatbakhsh and Z.Karimitabar, carried out the bacteriological experiments.
S. Rafatbakhsh did the analysis of results and prepared the preliminary version of manuscript. All mentioned authors read the manuscript, made their comments and approved the final version of manuscript.

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