Identification of the Midgut Microbiota of An. stephensi and An. maculipennis for Their Application as a Paratransgenic Tool against Malaria

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Abstract

The midgut microbiota associated with Anopheles stephensi and Anopheles maculipennis (Diptera: Culicidae) was investigated for development of a paratransgenesis-based approach to control malaria transmission in Eastern Mediterranean Region (EMR). Here, we present the results of a polymerase chain reaction (PCR) and biochemical-based approaches to identify the female adult and larvae mosquito microbiota of these two major malaria vectors, originated from South Eastern and North of Iran. Plating the mosquito midgut contents from lab-reared and field-collected Anopheles spp., was used for microbiota isolation. The Gram-negative and Gram-positive bacterial colonies were identified by Gram staining and specific mediums. Selected colonies were identified by differential biochemical tests and 16S rRNA gene sequence analysis. A number of 10 An. stephensi and 32 An. maculipennis adult mosquitoes and 15 An. stephensi and 7 An. maculipennis larvae were analyzed and 13 sequences of 16S rRNA gene bacterial species were retrieved, that were categorized in 3 classes and 8 families. The majority of the identified bacteria were belonged to the γ-proteobacteria class, including Pseudomonas sp. and Aeromonas sp. and the others were some closely related to those found in other vector mosquitoes, including Pantoaea, Acinetobacter, Brevundimonas, Bacillus, Sphingomonas, Lysinibacillus and Rahnella. The 16S rRNA sequences in the current study aligned with the reference strains available in GenBank were used for construction of the phylogenetic tree that revealed the relatedness among the bacteria identified. The presented data strongly encourage further investigations, to verify the potential role of the detected bacteria for the malaria control in Iran and neighboring countries.

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Introduction

Malaria, tuberculosis and AIDS are the most important infectious diseases in the world, while malaria has been considered as the major parasitic and vector-borne disease in Iran and also the main health problem in South-Eastern parts of the country. Across the Kerman, Hormozgan and Sistan and Baluchestan provinces, around 10,000 cases of malaria infections have been reported in 2008 and 4.1 million people are considered to be at risk of infection (Centers for Disease Control and prevention (CDC), Iran, unpublished data).

Since the discovery that mosquitoes are the vectors of malaria parasite by Ronald Ross [1,2], vector control has become an important part of malaria control programs. Several strategies have been designed and put into place to reduce the mosquito population, and several others are currently being investigated as possible solutions for rendering the vector less competent to transmit malaria. These strategies include environmental management, insecticide treatments and molecular entomology approaches [3]. One of the newly developed approaches that has been proposed as an anti-Plasmodium effector delivery strategy is paratransgenesis, which is the genetic modification of symbiotic microorganisms to deliver anti-pathogenic products and thus reduce vector competence[4].

Paratransgenesis studies were started by Durvasula et al. [5] to break the transmission of American trypanosomiasis (Trypanosoma cruzi) by Rhodnius prolixus, which, in turn, fostered attempts for controlling other diseases like malaria. In early 1960s, a few studies were carried out on midgut microbiota of laboratory-bred species of Culex [6,7,8]. Further, the presence of oxidase-positive bacteria from the midgut of anopheline mosquitoes [9] and also, the successful colonization of Serratia marcescens in laboratory-bred An. stephensi were reported [10]. A study of wild Aedes triseriatus, Cx. pipiens, and Psorophora columbiae using routine laboratory bacteriologic techniques indicated the presence of S. marcescens, Klebsiella ozonae, Pseudomonas aeruginosa, and Enterobacter agglomerans [11]. Moreover, the examination of the Cx. quinquefasciatus larvae midgut indicated the presence of bacteria represented by Bacillus spp., Staphylococcus spp. and Pseudomonas spp., while Aspergillus and Streptomyces spp. represented the fungal and actinomycete inhabitants, respectively. Chao and Wistreich [7], Vasanthi and Hoti [12], Jadin et al. [9] found Pseudomonas sp. in the midgut of mosquitoes from the Democratic Republic of the Congo. Straif
et al. [13] identified 20 different genera of midgut bacteria from An. gambiae sensu lato and An. funestus mosquitoes caught in Kenya and Mali. They identified Pantoea agglomerans (synonym Enterobacter agglomerans) as the most frequently isolated bacterium, apart from Escherichia coli. Gonzalez-Ceron et al. [14] isolated Enterobacter amnigenus, Enterobacter cloacae, Enterobacter sp., Serratia marcescens, and Serratia sp. from An. albimanus mosquitoes caught in southern Mexico.

Pidiyar et al. [15] reported the isolation and taxonomic characterization of some species, Aeromonas culicicola, from the midgut of Cx. quinquefasciatus and two strains of A. culicicola from Aedes aegypti, indicating that different mosquito species in the same environment may harbor common representatives of the microbiota. Pidiyar et al. [16] studied the midgut microbiota of wild Cx. quinquefasciatus and Lindh et al. [17] reported 16 bacteria species from 14 genera in An. gambiae sensu lato and An. funestus.

In this report, we studied the midgut microbiota of Iranian An. stephensi and An. maculipennis mosquitoes by using biochemical identification and molecular techniques with the aim to identify suitable candidates to develop an effective strategy for malaria control in Iran and perhaps Eastern Mediterranean region.

Results and Discussion

Here, we have presented the results of culture-dependent biochemical tests and polymerase chain reaction (PCR)-based approach to identify midgut bacteria associated with An. stephensi and An. maculipennis, two major malaria vectors in Iran.

Plating the mosquito midgut contents from lab-reared and field-collected An. stephensi and An. maculipennis (Female adult/larvae) and also the water of their breeding sites was used for isolation of culturable microflora. At first, prepared samples were cultured on blood Agar medium and next, different morphological colonies (based on the colony size, shape, color and margin) were cultured on Blood Agar (for isolation of Gram-positive bacteria) and MacCON-KEY media (for isolation of Gram-negative bacteria) in parallel.

The Gram-negative bacteria colonies were identified by culture-dependent biochemical tests such as EMViC, oxidase and etc. Also, the Gram-positive bacteria colonies were studied by Gram staining (for morphology characteristics) and supplemental tests such as Bacitracine sensitivity, catalase, coagulase, Novobiocine sensitivity, Optochine and etc. They were further selected on the basis of colony characteristics too. The selected colonies were identified by the analysis of the 16S rRNA gene sequence.

From 10 An. stephensi and 32 An. maculipennis adult mosquitoes and 15 An. stephensi and 7 An. maculipennis larvae analyzed, we retrieved 13 sequences of 16S rRNA gene bacterial species in 3 classes and 8 families.

The majority of 16S rRNA sequences detected are from bacteria belonging to the Gammaproteobacteria class (Table 1). The Pseudomonas sp. and Aeromonas sp. are species detected in most of the

| NO. | Strain | Nearest species | Ac. no. of identified bacteria | Ac. no. of the closest species in gene bank | Class | Family | Product size(bp) |
|-----|--------|----------------|-----------------------------|------------------------------------------|-------|--------|-----------------|
| 1   | Ast1L  | Pantoea agglomerans | 99 | AY849936.1 | Gammaproteobacteria | Enterobacteriaceae | 1500 |
| 2   | Ast2L  | Pantoea stewartii | 98 | AF373198.1 | Gammaproteobacteria | Enterobacteriaceae | 911  |
| 3   | Lst1L  | Bacillus pumilus | 99 | GQ152134.1 | Bacillales | Bacillaceae | 1507 |
| 4   | Lst2L  | Sphingomonas paucimobilis | 98 | D16144.1 | Alphaproteobacteria | Sphingomonadaceae | 1447 |
| 5   | Lst3F  | Brevundimonas aurantiaca | 99 | AJ227787.1 | Alphaproteobacteria | Caulobacteraceae | 1419 |
| 6   | Lst4F  | Brevundimonas aurantiaca | 99 | AJ227787.1 | Alphaproteobacteria | Caulobacteraceae | 1419 |
| 7   | Lst5F  | Brevundimonas aurantiaca | 99 | AJ227787.1 | Alphaproteobacteria | Caulobacteraceae | 1419 |
| 8   | Lst6F  | Lysinibacillus sphaericus | 99 | CP000817 | Bacillales | Planococcaceae | 1510 |
| 9   | Lst7F  | Rahhnella aquatilis | 98 | U90757.1 | Gammaproteobacteria | Enterobacteriaceae | 1501 |
| 10  | Lma1F  | Aeromonas bivakum | 99 | DQ504429.1 | Gammaproteobacteria | Aeromonadaceae | 951  |
| 11  | Lma2F  | Pseudomonas mendocina | 100 | DQ178224.1 | Gammaproteobacteria | Pseudomonadaceae | 1495 |
| 12  | Lma3F  | Lysinibacillus sphaericus | 99 | CP000817.1 | Bacillales | Planococcaceae | 1510 |
| 13  | Lma4F  | Aeromonas punctata | 95 | GQ259885.2 | Gammaproteobacteria | Aeromonadaceae | 1446 |
| 14  | RW1    | Pseudomonas otitidis | 99 | AY953147.1 | Gammaproteobacteria | Pseudomonadaceae | 1495 |
| 15  | RW2    | Pseudomonas otitidis | 99 | AY953147 | Gammaproteobacteria | Pseudomonadaceae | 1496 |
| 16  | TW1    | Acinetobacter calcoaceticus | 99 | AJ888983.1 | Gammaproteobacteria | Moraxellaceae | 1508 |
| 17  | TW2    | Aeromonas hydrophila | 99 | GQ184148.1 | Gammaproteobacteria | Aeromonadaceae | 1506 |
| 18  | TW3    | Bacillus pumilus | 99 | FJ68456.1 | Bacillales | Bacillaceae | 1507 |
| 19  | TW4    | Rahhnella aquatilis | 98 | U90757.1 | Gammaproteobacteria | Enterobacteriaceae | 1500 |
| 20  | TW5    | Rahhnella aquatilis | 98 | U90757.1 | Gammaproteobacteria | Enterobacteriaceae | 1500 |

Ast (Adult sample of An. stephensi), Lst (Larvae of An. stephensi), Lma (Larvae of An. maculipennis), RW (Rasht Breeding Sites), TW (Talesh Breeding Sites). L and F are lab-reared and field-caught specimens, respectively. 
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Table 2. Molecular identification of bacteria species based on breeding sites, adults and larvae of mosquitoes.

| Source of sample | Identified bacteria species |
|------------------|-----------------------------|
| Breeding sites   | Talesh                      |
|                  | Rahnelia aquatilis          |
|                  | Aeromonas hydrophila        |
|                  | Bacillus pumilus            |
|                  | Acinetobacter calcoaceticus |
| Rasht            | Pseudomonas stewartii       |
| Larvae sample    | An. maculipennis            |
|                  | Pseudomonas mendocina       |
|                  | Aeromonas punctata          |
|                  | Aeromonas bivalvum          |
|                  | Lysinibacillus sphaericus   |
| An. stephensi    | Bacillus pumilus            |
|                  | Brevundomonas aurantiaca    |
|                  | Lysinibacillus sphaericus   |
|                  | Sphingomonas paucimobilis   |
| Adult sample     | An. stephensi               |
|                  | Pantoena agglomerans        |
|                  | Pantoena stewartii          |

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Table 3. Molecular identification of bacteria species based on common bacterial species in each source.

| Bacteria species | Pan. agg | Pan. ste | Bac. pum | Sp. pau | Bre. aur | Lys. sph | Rah. aqu | Aer. biv | Pse. men | Aer. pum | Pse. oti | Aci. cal | Aer. hyd |
|------------------|----------|----------|----------|---------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| Breeding sites   | Talesh   |          |          |         |          |          |          |          |          |          |          |          |          |
|                  |          |          |          |         |          |          |          |          |          |          |          |          |          |
|                  |          |          |          |         |          |          |          |          |          |          |          |          |          |
| Rasht            |          |          |          |         |          |          |          |          |          |          |          |          |          |
| Larvae sample    | An. maculipennis |          |          |         |          |          |          |          |          |          |          |          |          |
|                  |          |          |          |         |          |          |          |          |          |          |          |          |          |
|                  |          |          |          |         |          |          |          |          |          |          |          |          |          |
| Adult sample     | An. stephensi |          |          |         |          |          |          |          |          |          |          |          |          |

[1](Pantoena agglomerans), 2(Pantoena stewartii), 3(Bacillus pumilus), 4(Sphingomonas paucimobilis), 5(Brevundimonas aurantiaca), 6(Lysinibacillus sphaericus), 7(Rahnella aquatilis), 8(Aeromonas bivalvum), 9(Pseudomonas mendocina), 10(Aeromonas punctata), 11(Pseudomonas stewartii), 12(Acinetobacter calcoaceticus), 13(Aeromonas hydrophila).

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Bacteria Flora Identification in *Anopheles* sp.
same PCR conditions. Some of the identified bacteria (Pseudomonas, Aerornonas, Bacillus) in breeding sites were also detected in larvae (molecular identification) and adults (biochemical identification) (Pseudomonas), but according to low number of identified samples in adult it should be examined further. Although we had enough adult mosquitoes, from 32 examined blood-fed and non blood-fed adult An. maculipennis we could not find any bacteria whereas in all An. stephensi mosquitoes, the bacteria detection was successful.

In the current study, specimens were identified by biochemical and culture-dependent pathways. Moreover, in spite of using two different pathways, including culture-independent and culture dependent in some recent papers, we did not use culture-independent pathway because by using culture independent pathway (PCR based methods), the identified genera could not be retrieved by the culture-dependent method [17]. One explanation might be the remnants of the midgut cells or human blood interfere in PCR reaction. Another explanation could be the competition of DNAs from different bacteria that led to the successful amplification of ones with the higher abundance [17].

So far, several bacteria have been isolated from anophelines, from both laboratory colonies and wild populations and at least one bacterium, Enterobacter agglomerans, has been identified as an excellent candidate for paratransgenesis [13,21]. Inhibition of malaria parasite development has been shown in Yoshida et al. [22] study where E. coli was fed to An. stephensi. Using a single-chain antibody against Plasmodium berghei ookinete, Pbs21 linked to the lytic peptide Shiva-1, resulted in 95.6% transmission blockage. Furthermore, in a recent study, the expression of two anti-Plasmodium effector molecules (SM1 and phospholipase-A2 (PLA2)) on the surface of E. coli, partially inhibited the P. berghei development, showed the potential role of this strategy for malaria control programs [21,23].

The data presented from the current study revealed a great diversity of the midgut microbiota in An. stephensi and An. maculipennis and presented potentially new species. This justifies further search and isolation of useful bacteria for paratransgenesis.

All of the bacterial isolates in this study will be further evaluated for their suitability as paratransgenic tools. The first step will be the sustainability study in mosquito midguts after reintroduction and the second step is the assessment of the immune response that induced by the bacteria. The survival of a reintroduced bacterium will depend on the tolerance level to immune response that mounted by the mosquito and putative antagonistic effects of other midgut bacteria. Several studies have shown that Gram-negative midgut bacteria can suppress Plasmodium parasites development [14,24,25], possibly by acting as elicitors of the mosquito immune response that affecting on Plasmodium development [25]. Hence, an ideal bacterium for paratransgenics would be one that elicits a potent immune response that suppresses other bacteria and Plasmodium parasites but does not affect on own survival. Therefore, genetic modification of this bacterium by introducing a gene that expresses an antiparasitic molecule, could achieve the clearance of Plasmodium parasites from the mosquito midgut. From this point of view, it is promising that several isolates detected in the current study are Gram-negative, proteobacteria, and these are suitable elements for genetic modification [17].

Further, recently a great deal of attention has been addressed to bacteria of the genus Asaia, which have been proposed as the best potential candidates for malaria control [26,27,28]. In the original screening, we could not identify Asaia that this is most likely due to the fact that it grows in very peculiar conditions [26]. Also, bacterial identification by molecular analysis can be strongly biased by the kind of primers that are used in ribosomal gene amplification. However, by using Asaia specific primers, we could not detect its presence in any of the analyzed samples.

On the other hand, Girimotch et al [29] identified an Enterobacterium isolated from wild mosquito populations in Zambia that renders the mosquito resistant to infection with the human malaria parasite Plasmodium falciparum by interfering with parasite development before invasion of the midgut epithelium.

These means that despite the use of identified bacteria in paratransgenic strategy, it is also possible to raise the anti-Plasmodium mechanism through mediating a mosquito-independent interaction of small populations of replicating bacteria with the malaria parasite, which is reported to be largely caused by bacterial generation of reactive oxygen species.

Accordingly, the detection of 13 sequences of 16S rRNA gene bacterial species (categorized in 8 families) in two main malaria vector species, An. maculipennis from Palearctic region and An. stephensi from western extend of Oriental region in current study, strongly encourage further investigations, to verify the potential role of the detected bacteria in malaria control in Iran and neighboring countries.

Materials and Methods

Ethics Statement

All projects, prior to the approval by Pasteur institute of Iran (PII) and Ministry Of Health (MOH), should had been reviewed and cleared by the ethical committee of PII and hence, in mosquito collection from private residences, all residents provided their oral informed consent to have their residences used in the study by coordination through “Local Manager of Malaria Control Program” of Public Health Center in the study areas. Briefly, the PI for the project, N. Dinparast Djadid (NDD), presented the different sections of project to the local staff, especially the field collection section. One to two weeks prior to field collection, the “Local Manager of Malaria Control Program” of Public Health Center, had a visit from the study areas and in a meeting with residents, asked their permission for mosquito collection in the defined date and time. As it is a routine procedure for other activities of malaria control program in the region, the residents accepted to cooperate in the project, which followed by the presence of the research team in the agreed date and time within the study areas and performing the mosquito collection. This has been documented in “Weekly Activity Report Book” of the Malaria Control Program in the study areas, signed by the PI (NDD), the manager of malaria control program and a representative from the study areas.

Collection of mosquito species and isolation of bacterial flora

Adult and larvae mosquito specimens of An. stephensi and An. maculipennis were collected by total catch and hand catch in human and animal shelters and also their breeding sites from provinces of Sistan and Baluchestan in South West of Iran and Guilan in northern Iran (Fig. 2). Specimens species were identified by using the morphological key of Iranian Anophelines [30] to distinguish...
An. stephensi and An. maculipennis adults from other Anopheles species. The details of the origin and the number of specimens that were sequenced and the detected bacteria have been presented in Table 1.

Living mosquitoes were anesthetized by putting them on ice and dissection was done under sterile conditions after surface sterilization with 70% ethanol. The midgut content was suspended in 50 µl of sterile saline (0.9% NaCl); A 25 µl aliquot of this content was plated onto blood agar base (Merck, Germany) with 7% (v/v) human blood, and 25 µl aliquot of the content was plated onto MacConkey agar base (Merck, Germany) followed by incubation at 37°C for 18–24 hours. The sterility of all reagents was checked during the entire procedure. The morphology of the Gram-positive bacteria was examined by Gram staining and light microscopy. IMVic, oxidase and different types of supplemental analysis were used for classical phenotyping, depending on the bacterial genus.

Water of the breeding sites were collected in 50 ml sterile falcon tubes and transferred to local laboratories for further process. On arrival, the waters centrifuged in 10,000 rpm for 3 minutes and the pellet from each water was plated on blood agar and MacConkey mediums, followed by biochemical and molecular identification of their bacteria, as carried out for the detection of microbiota of the mosquitoes’ midgut.

Genomic DNA extraction, amplification, cloning and sequencing of 16S rRNA genes

The optimum growth of bacteria was gained in LB by shaking at 160 rpm at 37°C and spectrophotometric reading. DNA was extracted by using the phenol- chloroform extraction protocol for Gram-negative and -positive bacteria according to the adapted protocols in Microbiology Department, Pasteur Institute of Iran.

The 1.5 kb of the 16 s rRNA gene was amplified by using 16 s forward (5'-AGT TTG ATC CTG GCT CAG-3') and 16 s reverse (5'-GCT ACC TTG TTA CGA CTT G-3') primers that were designed by Gene Runner, Version 3.05. All PCR reactions were performed in a total volume of 25 µl. The reaction mixture contained 100 ng of each of the specific primers, 0.2 unit of Taq polymerase, 0.1 mM each of dNTPs, 0.001% spermidin, 2.5 µl of 10× reaction buffer and 2 mM MgCl2. The amplification profile was as follows: denaturation at 95°C for one minute, followed by 35 cycles of annealing at 54°C for one minute and extension at 72°C for one minute with 10 minute extra extension time in the last cycle.

Specific forward and reverse primers were designed based on the 16 s rRNA gene of Asaia bacteria Gene Runner, Version 3.05. Asafor (5'-GCC CGT AGG CGG TTT ACA C-3') and Asarev (5'-AGC GTG AGT AAT GAG CCA GGT T-3') [26] were used to amplify a 180 bp fragment. The expected size of PCR products were confirmed by electrophoresis on a 1% agarose gel that was stained with ethidium bromide and the bands were visualized by UV transillumination. Amplified fragments were purified by Core-One™ Gel Elution Kit (Core Bio System Co. Ltd., Seoul, Korea) and PCR products with the expected size were cloned into pDrive TA cloning vectors (QIAGEN). The inserted gene in the plasmids was subjected to sequencing by Millegene Company (Labege, France), using M13 primers [(M13 (21) and M13R (29)].

Data analysis and phylogenetic tree

The sequencing signals of the specimens were double-checked and annotated, followed by comparison with GenBank data. For data analysis, alignment and construction of phylogenetic tree, a series of softwares, including DNASTAR, Lasergene (Version 7.1), Chromas (Version 2.31), Gene Runner (Version 3.05), ClustalX

Figure 2. Map of study area, including Guilan (G), Sistan and Baluchestan (S&B) provinces.

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To determine the phylogenetic relatedness of the strains, the 16S rRNA gene sequence of the mosquito midgut contents were subjected to analysis by using Molecular Evolutionary Genetic Analysis (MEGA4, 2007) software.

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Author Contributions

Conceived and designed the experiments: NDD GF. Performed the experiments: HJ AR. Analyzed the data: HJ AR. Contributed reagents/materials/analysis tools: NDD HJ AR. Wrote the paper: HJ. Supervised the field and laboratory research works and finalized the manuscript: NDD GF. Performed most of the practical in laboratory and drafted the manuscript: HJ. Performed some of the practical, shared the bioinformatics analysis and reviewed the manuscript: AR. Jointly designed the project with NDD, partly advised in practical sections and reviewed the manuscript: GF. Partly cooperated in practical: IR. Advised on practical and reviewed the manuscript: NZ.

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