

Isolation and characterization of bacterial community in the gut of the *Androctonus crassicuda*, *Hottentota schach* and *Mesobuthus epeus* scorpions

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Scorpions (order: Scorpiones) are widely distributed predatory arachnids and highly resistant to environmental conditions. Some factors such as microorganisms of internal cavity of scorpions may affect their biology. In addition, microbial communities of gut effect on immunity system and nutrition in arthropoda. However, few data are available for the microbiota diversity of scorpion gut. Thus, the present study aimed to evaluate the microbial diversity of scorpions related to the Buthidae family which is considered as the most important scorpions from south-west of Iran by chemical test and sequence analysis of 16S rRNA gene. To this aim, both gram positive and negative bacteria were detected from the gut of *Androctonus crassicuda*, *Hottentota schach* and *Mesobuthus epeus*. The *staphylococcus saprophyticus*, *Bacillus cereus*, *Bacillus firmus*, *Corynebacterium variabilis*, *Pseudomonas putida*, *Pseudomonas oryzihabitans*, *Enterobacter aerogenes*, *Pantoea agglomerans*, *Serratia aureilytica*, *staphylococcus gallinarum* were isolated from the gut of scorpion. The isolated bacteria may have symbiotic or pathogenic relationships with scorpions. The present study was first considered the gut of buthidae scorpion of Iran and can provide a reference for future studies on the digestive system microbiota from other scorpions by determining the role of these bacteria in the biology of scorpion.

Key words: *scorpion, gut, bacteria, 16s rRNA.*

INTRODUCTION

Discovered by paleontologists, scorpions appeared as one of the oldest arthropods on earth with very little morphologic transformation from the beginning of their life until now (Mullen *et al.*, 2009). Regardless of dangerous circumstances which threat human safety in district that poisonous scorpions are distributed, nowadays beside production of anti-venom of these arthropods are intensively considered for new treatment approaches for diverse diseases as new drugs (Hadley *et al.*, 1974; Polis *et al.*, 1990). These arachnids are very proficient and effectual predators which tolerate environmental pressures such as temperature changes, drought, and starvation (Hadley *et al.*, 1974). Scorpions are carnivorous creatures and involve an extremely efficient digestive system. Therefore, the behavior, physiology, ecology, and life history can enhanced the biologists' interest to promote

their knowledge. Further, the diversification and evolutionary success of scorpion symbiosis is very a sophisticated phenomenon in nature and symbionts are in a tight biologic contrast with these toxic arthropods (Ashida *et al.*, 2012; Mullen *et al.*, 2009).

The compatibility and evolutionary of arthropods depend on the relationship with microbial communities. Bacterial in arthropods can affect their metabolism; upgrade nutrient-poor diets, fermentation (Desai, 2012), reproduction, and behavior, as well as producing the components of aggregation pheromone (Kohler, 2012). Furthermore, longevity and immune responses protect the host from other pathogen and increase the tolerance to abiotic challenges such as heat stress (Yun *et al.*, 2014; Engle *et al.*, 2013).

For instance, bacteria are routinely present but little is known about the kind of bacteria available in gut of scorpions. Accordingly, there is not enough information about the effect of such organisms on biology and health of scorpion. Obviously, the kind of bacteria in the gut of arthropods is related to many factors such as species, diet, geographic situation, and stage of the arthropod stage (Schloss *et al.*, 2006; Corby *et al.*, 2007; Engel *et al.*, 2013; Werren *et al.*, 2008). In addition, there is a lack of data about the inhabitants of digestive system of scorpions.

The nature and humid and hot climate of Khuzestan province, Iran, which is located in the south-west part of the country provide a very extraordinary and unique condition for the living condition of scorpions (Navidpour, 2015) both from the density or diversity of the species in which about 95% of scorpion species of Iran are living in this zone (Jalali *et al.*, 2014; Navidpour *et al.*, 2008). Thus, the present study aimed to evaluate the bacteria in the gut of scorpion in Khuzestan province to establish some insights for future studies on the possible contributions of microorganisms on the different aspects of scorpion biology and physiology.

MATERIAL AND METHODS

Scorpions

The scorpions were gathered from different zones of Khuzestan province and transferred separately to the Reference Laboratory of Razi Institute. Then, the species of the scorpions were determined by the morphologic criteria suggested by Navidpour *et al.*, 2008. Accordingly, a total of 21 scorpion specimens representing 3 species *Androctonus crassicuda* (n=7), *Hottentota schach* (n=5) and *Mesobuthus epeus* (n=9) were selected for subsequent evaluation. In addition, scorpions were prepared for dissection by surface sterilization. To this aim, each scorpion was washed by hypochlorite 5% and sterile distilled water. Further, the legs, tail, and pinchers from individual scorpion were removed, and discarded under laminar hood. The gut of scorpion was transferred precisely and aseptically into sterile 1.5mL microtubes containing sterile PBS. This suspension was used for isolating the bacteria by culture dependent approach, biochemical test, and culture-independent method.

Culture-dependent method and biochemical test

The microbial isolation protocol was followed by adding 1 ml of gut suspension in 5-7 ml in Reasoner's 2 Agar (R2A) and TSB and placed in the incubator at 26°C until detecting visible growth. Then, the pure colonies were determined by gram staining, and accordingly colonial morphologies were described. Catalase and oxidase Biochemical tests, and Triple Sugar Iron (TSI), (SIM), Citrate, Urease, Methyl Red Vogues Proskauer (MRVP), Phenol Red with 1% glucose, Phenol Red with 1% lactose, Phenol Red with 1% sucrose, Phenol Red with 1% maltose, lysine decarboxylase media were utilized for initial determination and culturing, as well (Garrity, 2004).

16S rRNA Gene Amplification and Sequencing

Scorpion gut suspension was considered for Polymerase chain reaction. Then, the bacterial genomic DNA was extracted by using DNA extraction (Cinnapure™ IRAN) kit according to manufacture illustrations. In addition, the general target of the bacteria 16S rRNA sequence was examined for amplification by PCR. Thus, 5'- AGAGTTTGGATCCTGGCTCAG-3' and 5'- GGTTACCTTGTTACGACTT -3' were used as forward and reverse primers, respectively (Yaraz *et al.*, 2014). Accordingly, the amplification of 16S rRNA was conducted in 50 mL of the reaction volume including 5 µl of DNA template, 1.5 mM of MgCl₂, 200 mM of each dNTP, 12.5 pmol of each oligonucleotide primer, and 1.25 U of Taq DNA polymerase. The cycling protocol included an initial hot start (95°C for 5 min), 40 PCR cycles of 95°C for 60 s, 55°C for 45 s, 72°C for 60 s and final extension of 72°C for 5 min. Finally, PCR products were analyzed by electrophoresis in 1 % agarose gel. DNA was sequenced by Biosystems DNA Analyzers Sequencing (BIONEER_ Korea) and the sequence analysis data was blasted in NCBI database for identifying isolates. Then these sequences were compared with the available 16S rRNA gene sequences in the GenBank database.

RESULTS

Culture-dependent method and biochemical test

Table 1 indicates the result of the total viable bacterial count of the scorpion samples expressed in colony forming unit per gram (CFU g⁻¹).

Staphylococcus saprophyticus, *Bacillus cereus*, *Bacillus firmus*, *Enterobacter aerogenes*, *Pseudomonas oryzihabitans*, and *staphylococcus gallinarum*, were considered as the bacteria species found by the culture-dependent method in gut of *Mesobuthus epeus*. *Bacillus firmus* and *Enterobacter aerogenes* were detected in *Hottentota schach* and *Staphylococcus saprophyticus*, *Bacillus cereus*, *Bacillus firmus*, *Enterobacter aerogenes* isolated in *Androctonus crassicauda*. In the next procedure, the genus *Enterobacter aerogenes* and *Bacillus firmus* were isolated in all species in the gut of scorpion and *Pseudomonas oryzihabitans* and accordingly *staphylococcus gallinarum* was detected in *Mesobuthus epeus* by using the culture-dependent method and biochemical test.

TABLE 1. Frequency of bacterial agents isolated by Culture dependent and biochemical test from gut of scorpion

Isolated bacteria	<i>Androctonus crassicauda</i>	<i>Hottentota schach</i>	<i>Mesobuthus epeus</i>
<i>staphylococcus saprophyticus</i>	57%	-	44%
<i>staphylococcus gallinarum</i>	-	-	55%
<i>Bacillus cereus</i>	100%	-	33%
<i>Bacillus firmus</i>	42%	40%	44%
<i>Enterobacter aerogenes</i>	71%	80%	88%
<i>Pseudomonas oryzihabitans</i>	-	-	33%

Sequence Analysis of 16S rRNA Gene

The gut bacteria of scorpion were evaluated by using a combination of 16S rRNA analysis of PCR amplification. Then, each sequence of 16SrRNA gene was compared with the GenBank database by using the BLAST tool (available on <http://www.ncbi.nlm.nih.gov/blast/>).

Staphylococcus saprophyticus, *Bacillus cereus*, *Bacillus firmus*, *Enterobacter aerogenes*, *Pseudomonas putida*, *Pantoe aagglomerans*, *Serratia aureilytica* were detected gut of *Androctonus crassicuda*.

In addition, *staphylococcus saprophyticus* *Bacillus firmus*, *Enterobacter aerogenes*, *Pseudomonas putida*, *Serratia aureilytica*, *Corynebacterium variabili* were isolated in gut of *Hottentota schach* while genera bacteria detected in the gut of *Mesobuthus epeus* included *Staphylococcus saprophyticus*, *staphylococcus gallinarum* *Bacillus cereus*, *Bacillus firmus*, *Enterobacter aerogenes*, *Pseudomonas oryzihabitans*, *Pseudomonas putida*, *Pantoe aagglomerans*, *Serratia aureilytica*, and *Corynebacterium variabili* *Mesobuthus epeus*.

Finally, *pseudomonas putida*, *Pantoe aagglomerans*, *Serratia aureilytica* and *Corynebacterium variabili* were isolated from this method (Table 2).

TABLE 2. Bacterial identified by culture-independent analysis of scorpion guts based on 16SrRNA gene sequence analysis

Scorpion	Bacterial division	Accession No.	%Sequence similarity
<i>Androctonus crassicuda</i>			
<i>Mesobuthus epeus</i>	<i>Staphylococcus saprophyticus</i>	(KJ958203.1)	(98%)
<i>Mesobuthus epeus</i>	<i>Staphylococcus gallinarum</i>	(AF325886)	(97%)
<i>Androctonus crassicuda</i>			
<i>Mesobuthus epeus</i>	<i>Bacillus cereus</i>	(AF363440)	(96%)
<i>Androctonus crassicuda</i>			
<i>Hottentota schach</i>	<i>Bacillus firmus</i>	(JN990980)	(94%)
<i>Mesobuthus epeus</i>			
<i>Androctonus crassicuda</i>			
<i>Hottentota schach</i>	<i>Enterobacter aerogenes</i>	(KX254413.1)	(97%)
<i>Mesobuthus epeus</i>			
<i>Mesobuthus epeus</i>	<i>Pseudomonas oryzihabitans</i>	(KC139422.1)	(95%)
<i>Androctonus crassicuda</i>			
<i>Hottentota schach</i>	<i>Pseudomonas putida</i>	(KT67824.1)	(97%)
<i>Mesobuthus epeus</i>			
<i>Androctonus crassicuda</i>			
<i>Mesobuthus epeus</i>	<i>Pantoe aagglomerans</i>	(KT075214.1)	(96%)
<i>Androctonus crassicuda</i>			
<i>Hottentota schach</i>	<i>Serratia aureilytica</i>	(HQ2427341.1)	(96%)
<i>Mesobuthus epeus</i>			
<i>Hottentota schach</i>			
<i>Mesobuthus epeus</i>	<i>Corynebacterium variabilis</i>	(KU663670.1)	(100%)

DISCUSSION

Regarding the significance of scorpions as dangerous arthropods which are very tolerant to the numerous environmental conditions (Mullen *et al.*, 2009), the study of different aspects of their relation criteria with other organisms seems crucial for understanding their complicated biology and physiology (Engel *et al.*, 2013) since the gut bacteria communities in arthropods contribute to nutrition, immune system, growth, and development of life stage (Engel *et al.*, 2013). Further, determining this microorganism in the gut of scorpion started to elucidate the composition and functional properties of bacteria in scorpion.

The presented study was conducted on 3 important distributed species including *Androctonus crassicauda*, *Hottentota schach*, and *Mesobuthus eupeus*. In this study, two different detection pathways including culture dependent and 16S rRNA gene amplification and sequencing were used to screen for bacteria in scorpion midguts.

Staphylococcus saprophyticus, *Bacillus cereus*, *Bacillus firmus*, *Corynebacterium variabilis*, *Pseudomonas putida*, *Pseudomonas oryzae*, *Enterobacter aerogenes*, *Pantoea agglomerans*, *Serratia aureilytica*, *staphylococcus gallinarum* were detected in the gut of scorpion. The bacterial diversity in three species of scorpion does not seem to be the same. Each microbiota of scorpion species gut has a differential bacterial species composition, although some bacterial species were similar in all scorpions. *Enterobacter aerogenes*, *Bacillus firmus*, *Pseudomonas putida* and *Serratia aureilytica* were isolated in all species of scorpion and *Pseudomonas oryzae* and *staphylococcus gallinarum* were detected in *Mesobuthus eupeus*.

The genera *Serratia aureilytica* identified in this study had already been described for some Arthropoda species. Thus, it may have some competitive advantages over the other bacteria such as *Anopheles sp* (Favia *et al.*, 2007) *Aedes aegypti* (Gusmo *et al.*, 2010) and *schistocera gergaria* (Dillon, 2002). This bacteria may play a role in host nutrition, chitin degradation, and the production of the components in aggregation pheromone (Iverson *et al.*, 1984, Dong *et al.*, 2009).

The proposed role of *Enterobacter sp*, *Pseudomonas sp.*, *Pantoea sp.* in *Manduca sexta* and Gypsy moth are unknown but may increase susceptibility to toxin by affecting midgut epithelial (Broderick, 2004; Mason, 2011).

Both gram-negative and gram-positive bacteria were isolated in this study. In one study conducted on the gut of scorpion, gram-negatives were dominant, compared to gram-positives (Wong *et al.*, 2007). The present study was different due to the hole of digestive system in the arthropod. Further, the survival of some bacteria is multifactorial. In host habitat, the competition for nutrient supply and competence of bacteria to produce colloidal particles which allow for changing the charge of this surface in order to attach the bacteria firmly are important issues for surviving (Vanthournout *et al.*, 2015; Gaio *et al.*, 2011; Corby *et al.*, 2007).

Bacteria are available in *Androctonus crassicauda*, *Hottentota schach*, and *Mesobuthus eupeus* in mutual, commensal, and/or pathogenic relationships. However, it is not clear whether the isolated bacteria in this work are in symbiosis relation with scorpions or not. If they are, microorganisms may be key contributors for adaptation scorpions successfully in many different environmental conditions (Engel *et al.*, 2013). Bacterial symbionts may influence the ecology and evolution of their host because of the existence of a diverse relationship between microbes and the host (Engel *et al.*, 2013). Host trophic membrane integrity maintenance is considered as one of the roles in gut microbiotas among physiological processes. In spite of commensal or even mutualistic relation aspects of bacteria, different pathogenic features of microorganisms which are not the symbionts of scorpions should be considered (Yan *et al.*, 2014; Polis, 1990). In addition, the microbiota richness in the gut of arthropods may be influenced by host diet, habitat, and developmental stage (Engel *et al.*, 2013).

It appears that scorpions have strength resistance to many bacteria. This criterion is relies on their innate immune defense based upon oxidation reactions followed by classic phagocytosis, as well as the effects of existed defending in hemolymph (Ehret-Sabatier., 1996; Rodríguez de la Vega., 2004). Further, symbionts may act as habitat rivals or play a role in detoxification of harmful bacteria (Undheim *et al.*, 2015). Furthermore, scorpions may be parasitized with mites, nematodes, phoridae flies, pathogenic Rickettsia, and other bacteria which often induce the white marks (Polis *et al.*, 1990; Lourenco *et al.*, 2001).

Finally, some bacteria were isolated from this deleterious environment for microorganisms, and the role of these bacteria is still under investigation. Many studies are necessary to understand the interaction of midgut microbiota and their role in scorpion physiology.

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