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Original article

Characterization of gut bacterial flora of *Apis mellifera* from north-west Pakistan

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ABSTRACT

Gut microbiota has been recognized to play a beneficial role in honey bees (*Apis mellifera*). Present study was designed to characterize the gut bacterial flora of honey bees in north-west Pakistan. Total 150 aerobic and facultative anaerobic bacteria from guts of 45 worker bees were characterized using biochemical assays and 16S rDNA sequencing followed by bioinformatics analysis. The gut isolates were classified into three bacterial phyla of Firmicutes (60%), Proteobacteria (26%) and Actinobacteria (14%). Most of the isolates belonged to genera and families of *Staphylococcus*, *Bacillus*, *Enterococcus*, *Ochrobactrum*, *Sphingomonas*, *Ralstonia*, *Enterobacteriaceae*, *Corynebacterium* and *Micrococcineae*. Many of these bacteria were tolerant to acidic environments and fermented sugars, hence considered beneficial gut inhabitants and involved the maintenance of a healthy microbiota. However, several opportunistic commensals that proliferate in the hive environment including members *Staphylococcus haemolyticus* group and *Sphingomonas paucimobilis* were also identified. This is the first report on bee gut microbiota from north-west Pakistan geographically situated at the crossroads of Indian subcontinent and central Asia.

1. Introduction

As a pollinator, honey bee has a prominent role in sustainable agriculture in addition to production of honey and other natural products (Klein et al., 2007; Potts et al., 2010). Compared to other bee species, honey bees have been reported to increase the yield in animal pollinated crops which account for 35% of the global food production (Genersch, 2010; Klein et al., 2007). Hence, research related to physiology and pathology of honey bees in particular *Apis mellifera* has attracted a lot of attention (Muli et al., 2014).

Numerous causes of severe honey bee colony losses have been proposed, including pesticides toxicity (Desneux et al., 2007), poor nutrition (Brodschneider and Crailsheim, 2010) and genetic diversity (Mattila and Seeley, 2007). A high load of parasites and microbial pathogens, especially bacteria strongly connected with the disappearing of bee population (Core et al., 2012; Di Prisco et al., 2013; Olofsson and Vásquez, 2008). Therefore, characterization of bee gut microbiome can provide valuable insight about of parasites and bacterial pathogens.

Bacteriological analysis along with molecular techniques based on 16S rRNA sequences precisely characterize insects gut bacterial flora (Prabhakar et al., 2013; Ahn et al., 2012). The composition of bacterial assemblage in the digestive tract of honey bee *A. mellifera* is relatively simple compared with other gut-associated communities (Babendreier et al., 2007; Cox-Foster et al., 2007; Engel and Moran, 2013). A distinctive set of bacteria including Firmicutes, Actinobacteria, α- and γ-proteobacteria found in the honey bee alimentary canal has been assessed by using Sanger as well as next generation sequencing techniques (Engel et al., 2012; Evans and Schwarz, 2011; Jeyaprakash et al., 2003; Li et al., 2012; Martinson et al., 2011).

Pakistan is located at the north-western frontier of the distribution range of the honey bees *A. cerana*, *A. dorsata and A. florae*.

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However, due to the low honey yields of eastern honey bees, commercial beekeepers in Pakistan use western honey bee *A. mellifera* since late 1980s (Waghchoure-Camphor and Martin, 2008). In this study, we characterized the aerobic and facultative anaerobic bacteria isolated from alimentary canal of *A. mellifera* from honey producing areas in north-west Pakistan.

2. Material and methods

2.1. Sample collection and dissection of the bees

In order to study the cultivable honey bee gut bacteria, 45 worker honey bees (*A. mellifera*) were collected from the bee farms located in cruciferous vegetation in districts of Kohat, Karak and Bannu in north west Pakistan. After collection, live bees were transported to the laboratory in the small cages containing sugar powder followed by storage at −20 °C until processing. Before dissection, whole bees were washed in 95% ethanol and complete alimentary canals of bees were aseptically dissected by clipping the stinger with sterile forceps. The dissected guts were macerated with sterile dissection scissors in 0.8% NaCl solution and immediately stored at −20 °C.

2.2. Culturing of bacteria

From the preserved bee gut samples, different dilutions (i.e. 1/10, 1/100 and 1/1000) were made and 100 µl aliquots of the diluted sample were inoculated in LB agar plates and incubated for 24–48 h at 37 °C. The separated colonies in master plates were sub-cultured in LB agar plates and incubated at 37 °C and the morphology of isolated colonies in subculture plates was noted.

2.3. Biochemical tests

Various biochemical tests were performed including Coagulase test, Oxidase test, Urease test, Lactose fermentation test, and hydrogen sulfite production test etc. for the identification of bacterial isolates with the help of Bergey's Manual and API 20 NE identification system for non-fastidious, non-enteric Gram negative rods (Biomerieux, France).

2.4. Colony PCR and DNA sequencing

Isolated bacterial colony was subjected to amplification of the 16S rDNA gene according to Khan et al. (2014). The forward primer 5′-GGCTCAGAACGAACGCTGGCGGC-3′ and the reverse primer 5′-CCCACTGCTGCCTCCCGTAGGAGT-3′ were used. These primers are highly specific for conserved regions of bacterial 16S ribosomal DNA. PCR product (10 µl) was subjected to electrophoresis and 40 µl was purified with PCR clean up kit (Invitrogen Inc. USA). The DNA estimation was carried by using Qubit dsDNA Hs assay kit with Qubit 2.0 Fluorometer (Invitrogen, USA). The PCR products were sequenced using Big Dye Terminator kit and Genetic Analyzer ABI 377 (Applied Biosystems Inc., USA).

2.5. Sequence analysis

The resultant sequencing data was analyzed by Sequence Scanner v1.0 (Applied Biosystems Inc., USA). The 16S rDNA sequence of each bacterial isolate was compared using BLAST (Camacho et al., 2008) against '16S ribosomal RNA sequences (Bacteria and Archaea) database' (a subdivision of GenBank) and Ribosomal Database Project (Cole et al., 2014).

3. Results and discussion

Honey production is a profitable small enterprise in Khyber Pakhtoonkhwa province in north-west of Pakistan. Currently ~7000 beekeepers are involved in bee business in Pakistan with a total of 300,000 colonies of *A. mellifera* which produce ~7500 metric tons of honey each year (Waghchoure-Camphor and Martin, 2008). Due to importance of gut bacteria in the development, nutrition and immunity of honey bees, we carried out an analysis of gut bacteria in worker bees in different apiaries in north-west Pakistan.

We isolated 150 aerobic and facultative anaerobic bacteria from guts of 45 worker *A. mellifera* collected from different apiaries located in cruciferous vegetation in honey producing districts of Khyber Pakhtoonkhwa (i.e. Kohat, Karak and Banuu) in north-west Pakistan. Based on colony morphology and other bacteriological characteristics, 100 bacterial isolates were subjected to 16S ribosomal DNA (rDNA) amplification followed by sequencing. Consequently, 77 sequences of 16S rDNA were obtained and analyzed by BLAST (Camacho et al., 2008) and Ribosomal Database Project (Cole et al., 2014). In general agreement with previous studies (Martinson et al., 2011; Jeyaprakash et al., 2003; Li et al., 2012; Ahn et al., 2012; Prabhakar et al., 2013), these sequence analyses approaches classified isolated gut bacteria into three phyla i.e. Firmicutes (60%), Proteobacteria (26%) and Actinobacteria (14%) (Fig. 1). Among Firmicutes, most of the isolates belonged to genera *Staphylococcus*, *Bacillus* and *Enterococcus* (Fig. 2). Members of family Enterobacteriaceae and following genera belonging to α-, β- and γ-Proteobacteria were also found i.e. *Ochrobactrum*, *Sphingomonas*, and *Ralstonia* (Fig. 2). Several actinobacterial isolates were classified in suborders of Corynebacterium and Micrococcineae. Hence culture based method adopted during this study revealed important members of "core" bacterial community present in alimentary canals of honey bees present in apiaries located in north-west of Pakistan (Fig. 3).

The phylogenetic tree of the partial 16S rDNA gene sequences of the bacterial isolates from the gut of honey bees showed relatedness among the bacteria when aligned with reference strains in GenBank (Fig. 4), which revealed that the bacterial population in the gut of honey bee foragers in North West Pakistan was diverse, including the phyla Firmicutes, Actinobacteria, and alpha-, beta-, and gamma-proteobacteria. The richness of these bacterial assemblages suggests their ecological importance. For instance, the abundance of one representative of *Staphylococcus* was estimated at 29% of the total microbial gut samples analyzed. Additionally, most of the 16S rDNA gene sequences were found very comparable

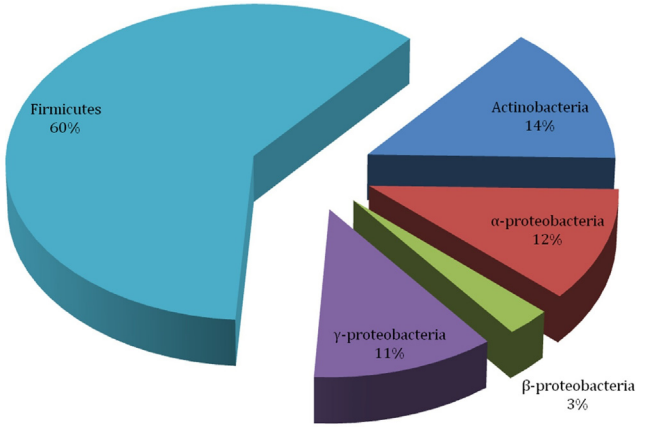

Fig. 1. Phyla-wise classification of honeybee gut bacteria obtained during the present study.

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