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Honey bee gut an unexpected niche of human pathogen

Syed Ishtiaq Anjum^{a,*}, Fahad Aldakheel^b, Ayesha Haleem Shah^c, Sanaullah Khan^d, Amjad Ullah^a, Riaz Hussain^a, Hikmatullah Khan^a, Mohammad Javed Ansari^e, Ahmed Hossam Mahmoud^f, Osama B. Mohammed^f

^a Department of Zoology, Kohat University of Science and Technology, Kohat 26000, Khyber Pakhtunkhwa, Pakistan

^b Department of Clinical Laboratory Medicine, College of Applied Medical Sciences, King Saud University, Riyadh 11564, Saudi Arabia

^c Institute of Biological Sciences, Gomal University, Dera Ismail Khan, Khyber Pakhtunkhwa, Pakistan

^d Department of Zoology, University of Peshawar, Khyber Pakhtunkhwa, Pakistan

^e Department of Botany, Hindu College Moradabad (MJP Rohilkhand University Bareilly), 244001, India

^f Department of Zoology, College of Science, King Saud University. P.O. Box 2455, Riyadh 11451, Saudi Arabia

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ABSTRACT

The gut microbiota of honey bees (*Apis mellifera*) can be symbiotic or pathogenic and therefore, important for bee survival and honey production. To study gut cultivable bacteria of honey bees, 30 honey bee samples were collected from district Kohat of Khyber Pakhtunkhwa Province as there is no information about the diversity of bee gut microbiota from Pakistan. Complete digestive system of the worker bee was dissected and processed for bacterial isolation. A total of 219 bacteria were obtained and characterized by bacterialogical parameters. The human pathogenic bacterial isolates were identified and confirmed on 16S ribosomal DNA sequencing. Combined microbiological practices revealed the presence of following bacterial genera: *Bacillus, Enterococcus, Escherichia, Micrococcus, Morganella, Ochrobactrum, Pseudomonas, Salmonella, Shigella, Sphingomonas and Staphylococcus.* Two pathogenic bacteria terized. This work suggested that forager *Apis mellifera* gut acts as reservoir and potential vector of bacterial pathogens.

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1. Introduction

A wide range of pathogens as viruses, bacteria and parasites may affect honey bee colony, some are more harmful that leads to colony collapse (Potts et al., 2010; Evans and Schwarz, 2011). The honey bee populations are going to decline in various parts of the world and these could be due to adverse effect of multiple honey bee pathogens. Further, increasing prevalence of parasites and pathogens are among the most significant threats to managed bee colonies. (O'Neal et al., 2018; Cox-Foster et al., 2007). Like two known bacteria (*Melissococcus plutonius* and Paenibacillus *larvae*) that effect honey bees brood not adults and causes significant

* Corresponding author.

E-mail address: ishtiaq@kust.edu.pk (S.I. Anjum).

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losses to beekeepers around the world. Many aspects of their transmissions, virulence and adult host mortality are poorly documented and still remain obscure (Genersch, 2010).

Contaminated water not only acts a reservoir of pathogenic bacteria (Khalil et al., 1994) but access to polluted water affect the health of insects especially honey bee (Staveley et al., 2014). The usage of reclaimed water sugar solution as a drinking water had negative impacts on the average deaths of the honeybee colonies. Reclaimed water also alters the shape of mid gut of bees (Hananeh, et al., 2014). Water resources are polluted having various animal and plants pathogenic bacteria. Several pathogens, in feces of animals are transported to plants by non-potable irrigation water, fertilizer and insects (Tyler and Triplett, 2008). As honey bee spread plant bacterial pathogens including Erwinia amylovora (fire blight pathogen) and Pseudomonas syringae while pollinating various plants (Pattemore et al., 2014)

A range of techniques has been used so far to characterize the gut bacterial flora of honey bee like culture dependent method and phenotypic screenings. Although biochemical characterization is very helpful in bacterial identification but Sanger-based and next

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generation DNA sequencing techniques are useful in identification of distinctive set of bacteria present in honey bees gut (Li et al., 2012; Cox-Foster et al., 2007; Engel et al., 2012). So this study aimed to investigate the bacterial communities from the digestive tract of managed honey bee workers, captured in North West districts of Khyber Pakhtunkhwa Pakistan, using bacteriological and molecular techniques. These findings will improve current knowledge on the composition and structure of bee gut microbiota and provide the framework for understanding their contribution to honey bee health and potential application in disease control.

2. Material and methods

2.1. Study area

Kohat is 2545 square kilometers area, located at 33°35′13 N 71°26′29E in Khyber-Pakhtunkhwa province of Pakistan. River Indus is present on the east (Zone 1) where cruciferous vegetation attracts beekeepers to manage farms. On the west (Zone 2) a rich agricultural land irrigated by Tanda dam while on the north and the south (Zone 3) rocky dry hills and slopes with large patches of open croplands provide a center of attraction for migratory beekeeping practices.

2.2. Sample collection and dissection of the bees

In order to study the cultivable honey bee gut bacteria, 30 unhealthy worker honey bees (bees were unable to fly and crawl on the ground in front of the hive.) were collected from honey bee farms distributed in cruciferous vegetation (Zone 1–3) in district Kohat Khyber Pakhtunkhwa Pakistan. After collection, live bees were transported to the laboratory of Entomology/Bee lab Department of Zoology Kohat University of Science and Technology Kohat, Khyber Pakhtunkhwa Pakistan in the small cages containing sugar powder followed by storage at -4 °C until processing. Before dissection, whole bees were washed in 95% ethanol in conical tubes. The complete digestive systems of bees were aseptically dissected by clipping the stinger with sterile forceps and carefully pulled the whole gut. The dissected guts were macerated with sterile dissection scissors in 0.8% NaCl solution and immediately stored at -80 °C. (Anjum et al; 2018. Ellegaard and Engel, 2019).

2.3. Culturing and identification of bacteria

From the preserved bee gut samples, different dilutions (i.e. 1/10, 1/100 and 1/1000) were made and 100 μ l aliquots of the diluted sample were inoculated in LB Agar plates 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. The isolated colonies were characterized by various bacteriological techniques like colony morphology, gram staining followed by various biochemical tests with the help of Bergey's Manual and API 20 by procedure mentioned elsewhere (Iqbal et al., 2014; Hussain et al., 2013). Further, identification of selected pathogenic bacteria was achieved by 16s rDNA analysis.

2.4. Colony PCR and DNA sequencing

Single isolated bacterial colony was subjected to amplification of the 16S rDNA gene using thermal cycler (BioRed USA) according to Khan et al. (2014). PCR product (10 μ l) was analyzed after electrophoresis and 40 μ l was purified with PCR clean up kit (Invitrogen Inc. USA). The DNA estimation was carried by using Qubit dsDNA Hs assay Kit USA with Qubit 2.0 Fluorometer (Invitrogen, Life Technology USA). The pure quantified PCR products were sequenced using either forward or reverse primers and Big Dye Terminator Kit (Applied Biosystems Inc., USA) by Genetic Analyzer (ABI 377 Applied Biosystems Inc., USA).

2.5. Sequence analysis

The 16S rDNA sequence of *Salmonella spp* and *Shigella spp* were compared through BLAST with known 16S rRNA gene sequences in the GenBank and analysed using sequence scanner v1.0 (Applied Biosystems) and Chromas Lite 2.1.1 (Technelysium Pty Ltd) software packages mentioned elsewhere (Gantner et al., 2011; Martínez-Hernández et al., 2013).

3. Results discussions

A total of 219 bacterial isolates from gut of 30 worker honevbees were obtained and the presence of 11 genera were confidently identified (Fig. 1). 23% Salmonella and 18% Shigella were characterized up to species level by using bacteriological parameters combined with 16s rDNA gene sequencing. BLAST searching revealed that 87% of the obtained sequences were identical to Salmonella enterica and 84% of Shigella sonnei (Table 1). Generally, gut bacterial diversity of honey bee collected from three zones were almost similar but the Enterococcus spp in zone 1 and zone 2 and Pseudomonas spp in zone 3 were prevalent (Fig. 2). Five bacterial genera, Bacilli, Staphylococci, Escherichia, Pseudomonas and Micrococci (Fig. 1) appeared recurrently and may contribute the major flora of bee gut. Colonies PCR of the selected pathogenic bacterial isolates were amplified, except few colonies of Salmonella and Shigella (Fig. 3). The isolates KHT03 and KHT30 produced low quality PCR product (Fig. 3) which were not subjected to BLAST analysis.

Indeed, honey bee populations all over the world are at high risk of decline due to unidentified reasons however a high load of parasites and microbial pathogens especially bacteria strongly connected with the disappearing of bee population (Olofsson and Vásquez, 2008; Gilliam, 1997; Di Prisco et al., 2013; Core et al., 2012). Additionally, pathogens are not passive microbes when they enter the arthropod vector but actively influence vector gene expression that can manipulate the local environment (Abraham et al., 2017)

The present study demonstrated 219 bacterial isolates of 11 bacterial genera, identified (Fig. 1) from worker honey bee gut, collected from the different bee yards in Kohat. The worker bee harbored some well-known pathogenic or potentially pathogenic bacteria species, including *Shigella sonnei* and *Salmonella enterica*. These bacteria are known for various diseases such as diarrheal illnesses, nausea, vomiting, and fever in human being (Greeley, 2013; Karlsson et al., 2013; Holman et al., 2014). The Diptera flies as a carrier of pathogenic bacteria like *Salmonella enterica* have previously been reported (Holt et al., 2007), however the presence of the human pathogen in bee gut are quit alarming. Although it has been reported that honey bee spread plant bacterial pathogens including Erwinia amylovora and Pseudomonas syringae while pollinating various plants (Pattemore et al., 2014).).

Enterococcus spp in the guts of *Apis nigrocincta* based on an identity BLAST search of its COI region also revealing the richness of the group in the gut *Apis spp.* (Lombogia et al; 2020). Recently, bacterial isolates (*Enterococcus* spp.) were isolated from Egyptian honey bee's intestinal tract and were expected that honey bee has the potential to be a source of new bacteria. (Elzeini et al; 2020). *Pseudomonas spp. Enterococcus spp.* were also recovered from *ventriculum* of *Apis mellifera ligustica* in Italy (Kačániová et al; 2020). Consequently, prevalence of the *Enterococcus spp Enterococcus spp* in bee guts collected from Zone 1–3 are in accordance of the recent published reports. However, differences and richness may be due



Fig. 1. Diversity of bacteria harboring in the guts of worker bees in Kohat.



Selected samples	Number (%)recovered	No. colonies	No. (%)confirmed	sequence homology	Accession no. (GenBank)
Salmonella spp.	52(23)	40	35 (87)	Salmonella enterica subsp. DSM 9220	NR 044372.1
Shigella spp.	40(18)	33	28 (85)	Shigella sonnei strain CECT 4887	NR 104826.1

*Percent recovery was calculated as (number of isolated colonies from which Salmonella/Shigella were recovered/total number of colonies isolated) \times 100. And Percent confirmation was calculated as (number of colonies confirmed as Salmonella enterica/Shigella sonnei /number of typical colonies picked) \times 100.



Fig. 2. Guts bacterial isolates of honeybee collected from different regiones in Kohat.

100bp
KHT02
KHT03
KHT21
KHT17
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KHT1C
KHT30
KHT42
KHT23

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Fig. 3. Showing bands of 16s rDNA gene amplification, two lanes (KHT02 and KHT23) visualizing *Shigella sonnei* DNA fragments while three lanes (KHT21, KHT17 and KHT1C) separating *Salmonella enterica* DNA fragments, 100 bp DNA ladder (L).

to the type strains, procedures, and condition of cultivation. Similarly, *Pseudomonas spp* were also most abundant bacteria followed by *Bacillus* sp in drinking water of the study areas (Hussain et al; 2013).

The high incidence of bacteria presents in bee's gut is a public health risk, as the synanthropic behavior of bees may conducive to disseminate through a wide variety of routes (Menasria et al., 2014). Worker honey bees forage in different sites where sugar food is prepared, processed, stored and thus it may increase likelihood of the risk of bacterial transmission. The lack of sufficient food is partially a management issue in bee keeping practices (Mattila and Otis, 2007). Beekeepers usually feed sugar solution during starvation but quality and diversity of sugar sources can affect number of bees (Pernal and Currie, 2001). Much like the human gut microbiota, many bee gut bacteria are specific to the bee gut and can be directly transmitted between individuals through social interaction. (Zheng et al., 2018). Apart from bee sociability, the main risk factor of transmission is water as in the study area it is highly contaminated with human pathogenic bacteria (Hussain et al., 2013). It is suggested that these pathogenic bacteria in bee gut are transferred from foraging sites or sugar supplements through contaminated water.

Moreover, it has been reported that sugar feeding like HFCS (high fructose corn syrup and SS (Sucrose syrup) decrease life span of worker bees (Sammataro and Weiss, 2013) and also colony collapse disorder (CCD) is an alarming colony mortality, correlated with the deleterious effects of sugar feeding (Van Engelsdorp et al., 2007; VanEngelsdorp and Meixner, 2010). But it is observed in the current study that beekeepers always use feeder continuously without sterilization for sugar feeding during starvation to honey bees. It is obvious that the feed or the water may be contaminated with pathogens like bacteria and may be ultimately resulted in bee's population declining and also a source of transmission. However, there is a need of research to correlate bacteria present in bee gut to honey bee morbidity and mortality and to evaluate the sugar solutions used for bee feeding, hive tools, feeder and water as source of transmission of these bacteria.

Presence of human pathogenic bacteria in bee gut may be the main cause of declining of bee population. The same were proved while injecting various pathogenic bacteria (*Salmonella enterica, Staphylococcus aureus, Listeria monocytogenes, Pseudomonas aeruginosa* and *Serratia marcescen*) to honey bees *Apis mellifera*. Moreover, the infected model bee with *S. aureus* also act as a vector to other hive mate and 50% of them died within 24 hrs (Ishii et al., 2014). Similar studies were carried out on *Drosophilla melanogestar* infected with P. aeruginosa and same results were obtained (Linder et al., 2008). *Serratia marcescens*, an opportunistic pathogen of many plants and animals, including humans, is a virulent

opportunistic pathogen of honey bees, which could contribute to bee decline (Raymann et al., 2018). This indicates that human pathogenic bacteria may be the main cause of honey bee mortality and bee population declining.

4. Conclusion

It is concluded that honey bee gut is an alternate habitat for human pathogenic bacteria as high load of these bacteria recovered from the alimentary canal of honey bees. Furthermore, *Salmonella* and *Shigella* may play major role in mortality of honey bee in the study area.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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