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ISOLATION AND IDENTIFICATION OF COMMON PATHOGENIC BACTERIA FROM GUT OF HONEY BEE (APIS MELLIFERA)

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Abstract

The study focused on isolating and identifying the species of bacteria that might have possible relations with the pathogenesis in the digestive tract of the honey bees (Apis mellifera) and determining their susceptibility patterns of antibiotics in the Sanghr district. To attain the objectives, a cross sectional type of study was used. Forty pooled samples of live adult honey bee were taken. The frequency and percentages of the occurrence of bacterial pathogens were described using summary of descriptive statistics. Pearson Chi-square test was used in order to analyze the association between the proportion of different bacteria in the gut and the variables used in the study. P less than =0.05 was held significant. Our research conducted 40 samples, 30 on each side were positive and 10 were negative. Out of 30 positive isolate 28 were targeted organism's bacterium and the rest 2 were gram positive bacteria. There were a total of 8 (28.57%), 7 (25%) and 13 (46.42%) bee samples that showed the presence of K. pneumonia, E. coli and Serratia marcescenin, respectively. The isolated bacterial species exhibited a unique impact in a different research area. The analyzed hives contained 18 (60%) and 12 (40%) of the bacterial species present in traditional and contemporary hives, respectively. The study examined the influence of study region and hive type as potential risk factors; however, no statistically significant differences were observed in the number of identified bacterial species (P > 0.05). Six commonly used antibiotics were employed against the bacterial isolates. These medications were evaluated against the isolates utilizing the disc diffusion technique. The tested medications included amoxicillin, ampicillin, tetracycline, vancomycin, gentamicin, and kanamycin. The susceptibility pattern tests revealed that all identified bacterial species exhibited high resistance to ampicillin, amoxicillin, tetracycline, and vancomycin, while demonstrating significant sensitivity to gentamicin and kanamycin. In this study, the pathogenic bacterial species were identified using the gram stain, morphology, and biochemical test. It will need careful observation and experimentation to determine the best method for removing pathogenic bacteria from honey bee guts

INTRODUCTION

Apis mellifera Honey bees are social insects and are common crop pollinators of crops globally or food production (Hristov, Shumkova, Palova, & Neov, 2020). They have a core microbiota of eight dominant phylotypes in their guts and this comprises 95 percent of all microbes in their guts (Shapira, 2016). Honeybees have a complex intestinal symbiont structure and nine sets of highly conserved bacteria are present. The species which are found in the distal portion of hindgut include *parasaccharibacter apium*, *Acetobacter*, as well as *Gluconobacter* species (Motta & Moran, 2024).

Honey bees are flying insects within the genus Apis under the largest family Apidae of order Hymenoptera (Requier et al., 2023). In Bangladesh, only one genus of honey bee Apis is being found which includes four species: Apis mellifera, Apis dorsata, Apis cerana, and Apis florea (Akter, Kibria, Sultana, Ahmed, & Begum, 2022). For the past few decades, their number has been declined throughout the world alarmingly (Ollerton, 2017).

Various types of microbes are associated with the animal kingdom and some of them are beneficial and some only cause nuisance and diseases (De Beer, Sole, Pirk, & Weldon, 2023). The honey bees gut bacteria can be a pathogenic and or symbiotic. The presence of pathogenic bacteria has been significantly affected honey bee overall health while the symbiotic association with some microbes are obligatory for their survival (Sonmez Oskay, Uygur, Oskay, & Arda, 2025). The microbiota associated with the honey bee is mainly composed of yeasts, gram positive bacteria (such as Lactobacillus rigidus apis, L. constellatus, Bacillus spp., Streptococcus and Clostridium), and gram-negative or gram variable bacteria (Achromobacter, Citrobacter, Enterobacter, Erwinia, Escherichia coli, Flavobacterium, Klebsiella, Proteus and Pseudomonas) (Akter et al., 2022). Some gram-positive bacteria in honey bee gut i.e., Lactobacillus and Streptococcus are considered to be beneficial bacterial species which are immensely used as the important probiotics in the dairy products (Akter et al., 2022).

Apicultural economic development strongly relies on the health status of honey bee colonies. Honey bees face many diseases and consequently rely on a diverse set of individual and group-level defenses to prevent disease. One route by which honeybees and other insects might combat disease is through the shielding effects of their microbial symbiont (Pattabhiramaiah, Reddy, & Brueckner, 2012).

Honey bees can serve as a model One Health organism to investigate the interactions between environmental change and AMR due to their inseparable symbiosis with the determinants of environmental health (Chowdhury et al., 2023). For example, environmental pollutants in water, soil, and air can negatively impact honey bee and hive health through leaching into pollen and honey foodstuffs (Smart, Pettis, Rice, Browning, & Spivak, 2016). Moreover, warming temperatures and other climatic factors related to climate change can increase the prevalence and spread of honey bee diseases and decrease the efficacy of antimicrobials in treating pests and pathogens (Neto et al., 2017). Drug efficacy is further challenged by years of liberal antibiotic use contributing to an increase in multidrug-resistant microorganisms. Apiaries globally are reporting greater colony losses than ever before (Pires & Maués, 2020). It is generally believed that complex interactions between multiple environmental, pathogenic, and climatic factors are responsible for the majority of these losses, which have come to be referred to under the umbrella term of "colony collapse disorder". Interdisciplinary research into these interactions is therefore highly beneficial and inherently relevant to honey bee health (Meixner, 2010).

The intestinal floras of most organisms play a crucial role in nutrient assimilation and immune function. So far, most studies on honeybee microflora have focused on disease causing microorganisms (de Jongh et al., 2022), while much less emphasis has been given to non-pathogenic microorganisms and their potential benefit for individual bees or whole colonies. However, there is growing awareness of the importance of the composition of the intestinal micro-flora for health and growth of honeybees (Mustar & Ibrahim, 2022).

Microbial diversity is associated with the existence o f a diverse array of bacteria in honey bees (Kumpitsch, Koskinen, Schöpf, & Moissl-Eichinger, 2019) While the majority of these bacteria are benign, a small

number of them are diseases that can infect both adult bees and their young. Some pathogens are more detrimental than others, and their infections have the potential to lead to colony collapse (Yañez et al., 2020). Individuals around have discussed various potential causes of colony collapse disorder or the decline of bee populations. Numerous studies have been conducted in recent years about bee health; yet, the issue persists. There is no data on the isolation and identification of several harmful bacterial illnesses in honeybees, and no study has been conducted in this field. Therefore, this study will aim to close this information gap by identifying the types of dangerous bacteria that honey bees' stomachs contain. Additionally, it will provide the necessary health information for the bees. It also needs a research study that can be utilized as a starting point for future research that will look at harmful bacteria in the guts of honey bees in the US and throughout the world.

- Identifying and isolating specific harmful bacteria, including *E. coli*, *Serratia spp*, and *Klebsiella pneumonia*, that inhabit the gastrointestinal tracts of honey bees.
- Examine the local beehive species to gain insights into the spread of infectious diseases.
- The objective is to determine the antimicrobial susceptibility testing profile of the target organism.

Review of Literature

Pakistan is located at the north-western frontier of the distribution range of the honey bees *A. cerana*, *A. dorsata* and *A. florae*. However,due to the low honey yields of eastern honey bees, commercial beekeepers in Pakistan use western honey bee *A. mellifera* since late 1980s (Qadir et al., 2021). 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.

A.mellifera colonies were introduced to subcontinent by Sir Louis Dane during 1908, but these colonies soon died due to excessive rains and lack of expertise. During that time, a Punjab Beekeepers Association was founded by Mr. Brooks (Shimla High School) and Mr. Carson, Assistant Professor Entomology at Lyallpur (now Faisalabad). In 1910, A. mellifera colonies were shifted to Lahore (Pakistan) for rearing and acclimatization but no satisfactory results were obtained. In 1927, Jagjit Singh who was working at Faisalabad imported the honey bees from Italy but not succeeded. In 1930, Mian Afzal Hussain (Entomologist) started to rear A. mellifera first time in Murree. In 1934, A. mellifera colonies were brought to Entomology Department, Faisalabad Agriculture College that initiated their rearing first time in plain areas and started experiments. It was found at that time if an old queen bee would be replaced by a new one, then the bee colonies would become stronger and tolerant to hot summer. In 1940, A. mellifera colonies acclimatized in local environmental conditions that proved to be the start of a potential beekeeping industry in the country. Beekeeping started in Haripur (1940) and many research stations were established in Peshawar (1948), Chattar, Sialkot, Lahore, Rawalpindi, and Hassanabdal (Khan, 2020).

Like ants, bees also belong to hymenoptera which are eusocial insects that survive in colonies with queen and thousands of worker bees which can forage large distances for collecting nectars and pollens and return to the hive. Hence their contact with various environments acts as a vector of diverse bacterial flora. More than fifty bacterial species from 31 genera have been found associated with different ants including *Escherichia*, *Staphylococcus*, Enterobacter, *Pseudomonas*, *Bacillus*, *Streptococcus*, and *Klebsiella* (Pesquero, Carneiro, & Pires, 2012).

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 (Diriba, Abraha, & Jemal, 2022). 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. Beekeepers usually feed sugar solution during starvation but quality and diversity of sugar sources can affect number of bees. 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, Steele, Leonard, Motta, & Moran, 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. It is suggested that

these pathogenic bacteria in bee gut are transferred from foraging sites or sugar supplements through contaminated water (Anjum et al., 2021).

3. Material and Methods

3.1. Study Area

The study area of District Sanghar in the south eastern province of Sindh in Pakistan has been chosen because of multiple socio-economic and ecological importance attached to the area. It is geographically found between 26 °N latitude and 68 °E longitude even having the urban and rural landscapes. The area is highly agricultural and the largest proportion of the people are involved in crop and livestock production. These climatic regimes (arid to semi-arid) of Sanghar are characterized by hot summers and mild winters, this aspect has lent to the sentiments of human livelihoods and diseases endemic to the region. The demographic profile of the district is mixed and there are many ethnic and cultural groups comprising the communities living in this district.

3.2. Study Design

The study was done targeting worker honeybee in Sanghar and took place between January 2024 and June 2024, where a cross sectional kind of study was carried out to retrieve samples of honeybee worker. The major bacteria were identified in gut of honey bee by using the honey bee colonies which are handled by use of traditional and modern method of beekeeping. After that isolation and identification of bee diseases that causes bacterial pathogens were done. These were *Serratia marcescenes*, *Klebsiella pneumomia* and *E. coli* as the target pathogens.

3.3. Study Population

The study population in this study is the bee colonies maintained in both traditional and modern hives in Hyderabad district.

3.4. Sampling Methods and Sample Size Determination

To investigate the honey bee gut bacteria that can be cultivated, Purposive sampling methods was adopted to select sites, because of the availability of the honey bee colony and honey bee colonies that are reared with the help of traditional and modern methods of bee keeping in the district of Sanghar. In 20 sites, there is a total numbers of bee hives of 1050. This is a bee hives of 148 bee hives contained in three sites which were sampled and a procedure called simple random sampling technique was used to sample three sites and bee colonies within each of the sites and, 40 beehives included getting a total of 200 bees by taking 5 bees each from each hives of three localities. The number of sampling divided by Sanghar was as follows: Jhol-n = 13; Panjo Mori: n = 13 and Kandri: n = 14 totaling to 200 heads of the individual bees. The live bees were stored in -20 ^{0} C until processed after being put in the small tubes that contained sugar powder and transported to Microbiology laboratory in University of Sindh, Pakistan.

3.5. Laboratory Works

3.5.1. Sample collection

We employed sterile scissors and donned protective attire to collect samples of honeybees from the colony. We categorized the samples according to the types of hives and the locations from where they were sourced. The caught bees were placed in clean tubes with perforated covers to facilitate respiration. They received little portions of sugar cake till their arrival to the Microbiology laboratory at the University of Sindh in Pakistan.

3. 5. 2. Sample Processing

In order to perform aseptic dissection, complete alimentary canal of bees was dissected by clipping the stinger using a pair of sterile forceps and subsequently whole bee's bodies were placed in 95 percent ethanol before dissection. The sterile dissection scissors were used to macerate the dissected gut in 0.8 % NaCl and immediately stored at -20 ^{0} C unless it was processed immediately as required to culture bacteria.

3. 5. 3. Culturing and Identification of Isolates

To detect the bacteria in the gut of the honey bee, plates containing nutrient agar, Eosin Methylene Blue agar and MacConkey agar were incubated at temperature of 37 o C in 24 h. The strain of the identified bacterial isolates was determined by culture performances of colonies such as shape and colours, morphology and biochemical properties as outlined by Quin (2017).(Quinn, Brainard, & Szendrei, 2017). Following the isolation of a pure culture from the Nutrient Agar gel slide, a series of tests were

conducted, including the Gram stain, Catalase test, Oxidase test, Triple Sugar Iron test, Indole test, Methyl Red Voges-Proskauer test, Urease test, and Citrate test (refer to Table 1 and Annex 2). The appearance of smoother, smaller, fusion-pink, flat MacConkey agar colonies separated *E. coli*. *Klebsiella pneumonia* was identified by its large, mucoid, light pink MacConkey agar colonies. Red colonies on nutritional agar were also found in *Serratia marcescens*.

Table 1. Biochemical Characteristics of Identified bacterial species

Biochemical Tests		Organism		
		E.coli	***k.pneumonea***	***S.marcescens***
Oxidase		-	-	-
Catalase		+	+	+
Triple Sugar Iron	**Slant**	Acid	Acid	Alkaline
	Butt	Acid	Acid	Acid
	Gas	Gas(+), H2s(-)	Gas(+), H2s(-)	Gas(+), H2s(-)
Indole		+	-	-
Citrate		-	+	+
MR		+	+	-
VP		-	+	+
Urease		-	+	+

3.6. Antimicrobial Susceptibility Test

To determine the susceptibility of *E. Coli, Klebsiella*, and *Serratia marcescens* isolates to common antibiotic disks and their likelihood of becoming ill, we employed disk diffusion methods. The zone of inhibition on Mueller Hinton Agar was read by the National Committee for Clinical Laboratory Standards Institute (CLSI, 2020). The research utilized six antimicrobial disks: Gentamicin (GEN 10 µg), Amoxicillin (AMX 30 µg), Ampicillin (Amp 10 µg), Tetracycline (TTC 30 µg), Kanamycin (KA 15 µg), and Vancomycin (VA 30 µg). We analyzed the zones of inhibition measured in millimeters to determine resistance or susceptibility.

3.7. Methods of Data Analysis

The information regarding the pathogenic bacteria present in the gut of honey bee was entered in to Microsoft excel spread sheet and these Excel sheets were analyzed using SPSS version 20.0 software. Descriptive statistics of occurrence of bacterial pathogens (in the form of frequency and percentages) were given. Pearson Chi-square test was used to analyze the proportion of different bacteria present in the gut in terms of its association with the variables of the study. The p-value was regarded as significant less than 0.05.

4. RESULT

4.1. Prevalence of Bacterial Isolates of Bee and Risk Factor

Out of 40 sample analyzed 30 were positive to targeted organism. Through this, 28 of them were identified down to the species level and the other two isolates were broadly described as gram positive bacteria. As it specifies below in (table 2). The general distribution of the frequency of the species of bacteria discovered in the research location was *Escherichia coli* (17.5%), *Serratia marcescens* (32.5%) and *Klebsiella pneumonia* (20%). These revealed that the isolates were highest for Serratia marcescens in turn which was followed by *Klebsiella pneumonia* and fewest of all was the occurrence of *Escherichia coli*. Frequency of the common species of bacteria isolated on the basis of sites are 9 (69.2%), 9 (69.2%), 10 (71.4%) Jhol, Panjo Mori and Kandri respectively.

Table2. Bacteria Isolated from gut contents of adult bee

Organism	Number Isolated	%
Escherichia coli	7	25%
Serratia marcescens	13	46.42%
klebsiella pneumonia	8	28.57%
Total	28	100%

Based on the locations, the distribution of bacterial species was as follows: Jhol had 9 (69.2%), Panjo mori also had 9 (69.2%), and Kandri had 10 (71.4%) (see Table 3). The quantity of bees containing *S. marcescens, Escherichia coli*, and *Klebsiella pneumoniae* in their stomachs, as well as the proportion of those bees, exhibited variability. The overall frequency of bacterial species found in the studied sites was *Escherichia coli* 15.4%, 15.4%, and 21.4% in Jhol, Panjo mori, and Kandri, respectively; *Serratia marcescens* 30.8%, 30.8%, and 35.7% in Jhol, Panjo Mori, and Kandri, respectively; and *Klebsiella pneumonia* 23.1%, 23.1%, and 14.3% in Jhol, Panjo Mori, and Kandri, respectively.

Table 3. The frequency of bacterial species isolated from the selected areas in the study district.

District Sanghar	Organism			Total	%
	E.coli	***Klebsiella pneumonia***	***Serratia marcescens***		
Jhol	2(15.4%)	3(23.1%)	4(30.8%)	9	69.2%
Panjo mori	2(15.4%)	3(23.1%)	4(30.8%)	9	69.2%
Kandri	3(21.4%)	2(14.3%)	5(35.7%)	10	71.4%
Total	7	8	13	28	100

The diversity of bacterial types identified in hives of both categories was variable (Table 4). The p-value for all examined data exceeded 0.05. The results indicated no impact on bee hives associated with a limited diversity of bacterial species.

Table 4. Isolation by bacterial species across hives.

	E.coli			***S.marcescens***			***Klebsiella***		
Hive Types	**Examined**	**+ve**	**Chi² (p-value**	**examined**	**+ve**	**Chi²(p-value)**	**Examined**	**+ve**	**Chi² (p-value)**
Traditional	26	6(23.1%)	1.600(.208)	26	6(23.1%	3.007(.155)	26	5(19.2%	.027(1.00)
Modern	14	1(7.1%)		14	7(50%)		14	3(21.4%	
Total	40			40			40		

A total of 18 types of bacteria were identified in traditional hives, representing 60%, while 12 types of bacteria were found in contemporary hives, accounting for 40%. The SPSS analysis indicated that No statistically significant difference in positivity rates between hive types ($p > 0.05$) (Table 5).

Table 5. ***Comparison of Positive Cases Between Traditional and Modern Hives with Chi-Square Test***

Hive Type	Number Examined	Positive Cases	Negative Cases	Total (%) Positive	Expected Positive	Chi-Square Value
Traditional	26	18	8	60%	19.5	
Modern	14	12	2	40%	10.5	
Total	40	30	10	100%	-	**$\chi^2 = 1.318$**

P-value = 0.251 (df = 1)

Table 6 indicates that *E. coli* exhibited complete resistance to four medications while demonstrating full susceptibility to two others. *Serratia marcescens* and *Klebsiella pneumonia* demonstrated sensitivity to gentamicin and kanamycin, while exhibiting resistance to vancomycin, amoxicillin, ampicillin, and tetracycline. The data presented in Table 6 illustrates the antimicrobial susceptibility testing results for the isolated organism.

Table 6. ***Antimicrobial Susceptibility Test Pattern of Isolated Organism***

Drug	**Organism**					
	Escherichia coli (n=7)		Serratia marcescens (n=13)		Klebsiella pneumonia (n=8)	
	S	R	S	R	S	R
Ampicillin	0	7 (100%)	0	7 (100%)	0	7 (100%)
Amoxicillin	0	7 (100%)	0	7 (100%)	0	7 (100%)
Tetracyclin	0	7 (100%)	0	7 (100%)	0	7 (100%)
Vancomicin	0	7 (100%)	0	7 (100%)	0	7 (100%)
Kanamicin	7 (100%)	0	7 (100%)	0	7 (100%)	0
Gentamicin	7 (100%)	0	7 (100%)	0	7 (100%)	0

S= susceptible, R= resistant

5. Discussion

Out of the 40 sample studied, 30 samples tested positive to targeted organism. The Sanghar bacterial species isolation frequency was 9 (69.2), 9 (69.2), 10 (71.4) and frequency of Jhol, Panjo Mori and Kandri respectively. The gut microbiomes of the bees of the Riyadh (36.4%) as well as those found in bees of Al-Baha (45.5%) were less complex than the current observation (Khalid et al. 2017). This variation in the gut microbial community could be based on disparities in the gut physiological conditions, existence of passing germs within the pollination setting, the maturity of the honey bees, and/or the time of year or dominant attributes of environments that are influenced by such geographical positions.

Out of the 40 sample, 28 were found positive for particular bacterial species. Among the positive sampled gotten; the percentages of the bacterial species isolate varied and 13(32.5) were Serratia marcesens; that make higher numbers of occurrence in this study area followed by Klebsiella pneumonia that counts 8 (20) of the total of isolated species. The level of occurrence of E. coli in this study area was 7(17.5) percent which signifies low figures in an occurrence.

This current study has found out that the identified and separated species of bacteria were sampled out of the two forms of bee hives. Out of 40 samples 26 were gathered through the traditional kinds of hives and the rest of the sample belonged to the bee hives that were managed through the modern one. The general percentages of incidences were 73.1 percent and 78.6 percent respectively on traditional and modern. Out of the three bacterial species isolated and identified, *E. coli* presence in the traditional and modern hives were 6 (23.1%) and 1(7.1) respectively. In *Klebsiella pneumonia* 5 (19.2%), 3 (21.4) of traditional and modern hive respectively. Likewise, in the Species of *Serratia marcescens* 6 (23.1%) and 7 (50) there was a traditional and modern hive respectively occurrence. The percentage prevalence of bacterial species in this study region showed that the prevalence of the bacteria species was higher in traditional hives than the isolations of the modern hives except *Serratia marcescens* which showed the opposite of other two bacteria.

6. Conclusion & Recommendations

The current results of this study told that 28 incidental pathogenic beings of bacteria revealed from the of honey bee, *Apis mellifera* in Sanghar district. Based on study, the bacteria strains of dominant pathogenic digestive guts included *Klebsiella pneumonea*, *Serratia*

marcenscens and *Escherichia coli* and they were prevalent in digestive guts of the adult honeybees of the three areas that were to be studied. Also, the current result indicated that antimicrobial susceptibility pattern of Amoxacilin, ampicillin, tetracyclin, vancomicin, gentamicin and kanamacin were performed on the isolated organism.

- In accordance with the acquired results and conclusion, the following recommendation are forwarded
- Honey bee is also important insects so now we should focus on bee health
- More research and even additional experiment will be necessary to make accurate decision on isolation, determinate pathogenic bacteria in the digestive tracts of the honey bee and their vulnerability tendency to antibiotics.
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7. Limitations

The study titled *"Isolation and Identification of Common Pathogenic Bacteria from Gut of Honey Bee"* presents valuable insights but is not without limitations. First, the study does not cover the whole of Sindh as it is geographically restricted to one of its districts only; it could not reveal the whole macrobiotic diversity of the rest of the ecological regions of the Sindh region or any other environmental site in Pakistan. Secondly, the sample size and collection duration may have been too small to consider seasonal or colony-specific differences of the gut microbiota. Third, the bacteria might have been identified only to those that could be cultured, and non-culturable or fastidious organisms that can only be identified with molecular methods such as 16s rRNA sequencing may be missed. In addition, the environmental variables where the pesticide exposure or the flower origins were not determined, and this would also have altered the bacterial makeup. These restrictions present the sense of a broader, multi-seasonal research that includes the methods of molecular work to understand better the state of honey bee health in the gut.

8. 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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