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Identification of cultivable bacteria from natural honey of different botanical origin

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Abstract: We assessed cultivable bacterial community of nectar honey samples of different botanical origin produced in Pakistan. Inoculation of different dilutions of honey in specific media resulted in several bacterial isolates which were characterized by bacteriological and molecular biological techniques including 16S rDNA amplification, sequencing and bioinformatics. The results showed the presence of low number of colony forming units (CFU) of *Staphylococcus aureus, Micrococcus luteus, Streptococcus sp., Corynebacterium sp. Klebsiella pneumonia, Escherichia coli, Salmonella sp.* and *Proteus sp.* in honey samples tested. According to our knowledge, this is the first report on honey bacteriology from honey.

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INTRODUCTION

High osmolarity, acidity and the presence of hydrogen peroxide in natural honey make the growth of microorganism difficult[1,2]. Honey contains fewer microorganisms than other neutral-foods. Microorganisms present in honey are those that can withstand the concentrated sugar, acidity and other anti microbial components of honey. Conventional microbiology and PCR based studies reported several species of cultivable and non-cultivable bacteria, yeasts and filamentous fungi associated with honeys of different geographical and botanical origin[3,4]. Several orders of bacteria including *Sphingomonadales, Burkholderiales, Pseudomonadales, Enterobacteriales, Actinomycetales*, and *Bifidobacteriales* have been found in honey[4].

There are two sources of microbial contamination in honey. The primary sources of microbial contamination are pollen, the digestive-tracts of honey bees, dirt, dust, air, earth and nectar. Primary sources are very difficult to control. Secondary sources of microbial contamination in honey are post-harvest sources and include human, equipment, containers, wind and dust[5,6]. These microbes could be placed in three categories: (1) microorganisms that are commonly found in honey (certain strains of yeast and spore-forming bacteria); (2) microorganisms that indicate sanitary or commercial quality (coliforms or yeasts); and (3) microorganisms that infer certain conditions (e.g. germination and growth in a non heat-treated food product)[7].

The microorganisms present in honey include bacteria, yeast and moulds. Members of genera *Bacillus, Clostridium* and *Micrococcus* are common in air and dust and they can easily enter into honey. Most bacteria and other microbes are present in dormant state. Spore-forming bacteria can survive in honey for a long period of time. The presence of *Clostridium botulinum* spores in honey has been reported extensively[8]. Numerous honey samples have tested positive for *Clostridium botulinum* spores and toxins. Infant botulism is an infection that results in a blockade of voluntary motor and autonomic functions. Several studies have shown that the ingestion of honey is linked with infant botulism. Therefore, Center for Disease Control and Prevention (CDC) in United States recommended that honey not be given to infants younger than 12 months old[8].

Coliforms total bacteria counts have assessed in order report adulteration in honey[9]. Honey naturally contains different osmo-tolerant yeast which can cause undesirable fermentation. Osmo-tolerant yeasts can particularly grow in honey with high moisture content. The low moulds counts reported by Piana et al[10] suggest that molds may survive but do not tend to grown in honey. Although some data available about microbiology of honey, further studies are required to shed light on microorganisms present in honey samples produced in different geographical regions by different honey bee species. We carried out a pilot study to find out culturable bacteria present in honey samples produced in northern Pakistan.

MATERIAL AND METHODS

Collection of samples

Commercially available honey samples produced by *Apis mellifera* bees in honey farms located in different districts of Khyber Pakhtoonkhwa, Pakistan were purchased and transported to the laboratory for analysis.

Isolation of bacteria

The honey samples were 2-fold serially diluted up to 10^{-6} dilution in sterile distilled water. An aliquot (0.1 ml) from 10^{-4} and 10^{-6} dilutions was

transferred to the pre-incubated nutrient agar plates and spread over the surface using sterile spreader. The plates were incubated at 37 °C for 24 hours and colonial characteristics of microorganisms were observed.

Identification and characterization of bacteria

Following assays were carried out for identification of bacteria. Gram staining, Spore staining, Motility test, Catalase test, Coagulase test, Oxidase test, Oxygen requirement test and IMVIC test (Indole test, Methyl Red test, Voges-proskauer test, Citrate test).

PCR amplification of bacterial 16S ribosomal DNA

The PCR of selected isolates was carried out for the amplification of the 16S rDNA gene. For this purpose, bacterial colony was picked and directly subjected to the PCR amplification[11]. Two specific primers for the 16S rDNA were used as forward primer Y1 (5'-GGC TCA GAA CGA ACG CT GG CG GC-3') and reverse primer Y2 (5'- CCC ACT GCT GCC TCC CGT AGG AGT-3'). The PCR reaction mix (25 µl) was made by adding 12.5 µl PCR master Mix, 2.5 µl each primer, 7.5 µl Ultra pure nuclease free water and an determined number of the bacteria from a single colony with the help of sterile pipette tip was added. PCR amplification was carried by using PCR Master Cycler (BioRad Inc., USA). The PCR program was set as; Pre-denaturation step at 94°C for 4 minutes, followed by Initial denaturation at 94°C for 40 seconds, annealing temperature at 65°C for 1 min and extension temperature 72°C for 1 min for the 30 cycles, final denaturation step at 94 °C for 30 sec and final extension step at 72°C For 4 minutes were also given for the amplification of uncompleted fragments. Aliquots of the reaction mixture (10 µl) were analyzed in 1% agarose gels, and detected by Ethidium Bromide staining. Amplified PCR products were purified with PCR clean up kit (Promega Inc., USA) following the manufacturer's instructions. The DNA estimation of purified DNA products was carried by double beam UV spectrophotometer.

DNA sequencing and sequence analysis

The purified PCR products were processed for sequencing using primers used for amplification and DTCS kit (Beckman Coulter Inc, USA). The DNA sequencing was carried out by Genetic Analyzer CEQ8000 (Beckman Coulter Inc., USA). Analysis of sequence was carried out through BLAST (Basic Local Alignment Search Tool)[12], using the NCBI GenBank database. The discrepancies within the sequences were corrected by using Chromas and Applied Biosystems Software.

RESULTS AND DISCUSSION

Here we report cultivable bacterial species found in different honey samples produced in Pakistan. On the basis of cultural, biochemical and morphological characteristics, *Staphylococcus aureus, Micrococcus luteus, Streptococcus pyogenes, Streptococcus faecalis, Streptococcus epidermidis, Corynebacterium sp. Klebsiella pneumonia, Escherichia coli, Salmonella sp.* and *Proteus sp* were identified as members of bacterial community present in honey samples tested (Tables 1 and 2). This list includes food borne pathogens in honey samples. Honey can be expected to contain a small number and a limited variety of microorganisms because the intrinsic properties of honey such as low pH and high sugar content prevent the growth of microorganism[7]. Few microorganisms that are present in honey are those which can withstand the high concentration of sugar, acidity and other antimicrobial characteristic of honey. Microorganisms can be introduced in honey during the process of biosynthesis of honey by the bees (primary source) as well as after it has been harvested (secondary source). Secondary source of contamination can be controlled by standard sanitation and good manufacturing practices. In the study, five gram positive bacteria including *Staphylococcus aureus, Corynebacterium sp., Streptococcus pyogenes, Streptococcus faecalis, Streptococcus epidermidis* and *Micrococcus luteus* and four gram negative bacteria including *Klebsiella pneumoniae, Escherichia coli, Salmonella sp*. and *Proteus sp.* were isolated from different commercially available honey samples available (Table 1). These isolates were identified by both conventional and molecular biology techniques (Table 2). Olaitan et al.[5] reported presence of several bacteria (*Enterobacter, Escherichia coli, Klebsiella, Micrococcus* and *Pseudomonas*), yeast (*Saccharomyces, Schizosaccharomyces, Trichosporium* and *Nematospora*) and mould (*Aspergillus*).

There are several reports in the literature about the presence of spores of *Clostridium botulism* in honey[13-15]. We could not find *C. botulism* during this study because procedure for aerobic bacterial culture was adopted. In the present study, microorganism isolated from the honey samples were first identified using conventional biochemical tests and then confirmed through colony PCR using primers specific for 16S rDNA followed DNA sequence analysis. In Conclusion, different species of bacteria that can survive in honey are typically found in honey at low numbers. Microbial survival may be influenced by the type of honey. The microbial count of microbial population in honey can be decreased by following appropriate sanitation guidelines.

Table 1: Characteristics of bacterial isolates from honey samples produced in Pakistan.

Bacterial isolates	Colonial morphology	Microscopic characteristics		
		Gram reaction	Shape	Arrangement
Staphylococcus aureus	Golden yellow colonies, Circular, pinpointed, convex	Positive	Cocci	Clusters pairs
Micrococcus luteus	Yellow colonies, non-diffusible pigment, circular, convex, opaque, smooth	Positive	Cocci	Tetrads, clusters pairs
Streptococcus pyogenes	Yellowish white, pinpointed, circular.	Positive	Cocci	Chains Pairs
Streptococcus faecalis	Pinpointed, white, smooth, round, opaque colonies.	Positive	Cocci	Clusters
Streptococcus epidermidis	White, pinpointed, circular, opaque, colonies	Positive	Rods	Palisade
Corynebacterium Sp.	White to gray, Circular, raised, mucoid colonies.	Positive	Rods	Palisade
Klebsiella pneumonia	Round, smooth, convex, shiny, opaque, white colonies	Negative	Rods	Singly, pairs short chains
Escherichia Coli	Colorless to yellowish-white circular, smooth colonies	Negative	Short Rods	Singly pairs
Salmonella sp.	White to Transparent shiny, circular, medium sized colonies.	Negative	Rods	Pairs chains
Proteus sp.	Cream colored, medium to large, circular, shiny, swarming colonies	Negative	Rods	Short chains, pairs singly

REFERENCES

1. Aurongzeb M and Azim MK. Antimicrobial properties of natural honey: a review of literature. *Pak. J. Biochem. Mol. Biol.* 2011; 44: 118-124.
2. Whadan HA. Causes of the antimicrobial activity of honey. *Infection*, 1998; 26: 26-31.
3. Sinacori M, Francesca N, Alfonzo A, Cruciata M, Sannino C, Settanni L and Moschetti G. Cultivable microorganisms associated with honeys of different geographical and botanical origin. *Food Microbiol.*, 2014; 38: 284-294.
4. Olivieri C, Marota I, Rollo F and Luciani S. Tracking plant, fungal, and bacterial DNA in honey specimens. *J. Forensic Sci*., 2012; 57: 222-227.
5. Olaitan PB, Adeleke OE and Ola IO. Honey: a reservoir for microotganisms and and inhibitory agent for microbes. *Afr. Health Sci*., 2007; 7: 159-165.
6. Kim SA, Oh SW, Lee YM, Imm JY, Hwang IG, Kang DH and Rhee MS. Microbial contamination of food products consumed by infants and babies in Korea. *Lett. Appl. Microbiol*., 2011; 53: 532-538.
7. Snowdon JA, Cliver DO. Microorganisms in honey. *Int. J. Food Microbiol*., 1996; 31: 1-26.
8. Tanzi MG, Gabay MP. Association between honey consumption and infant botulism. *Pharmacotherapy*, 2002; 22: 1479-83.
9. Lawal RA, Lawal AK and Adekalu JB. Physico-chemical studies on adulteration of honey in Nigeria. *Pak. J. Biol. Sci*., 2009; 12: 1080-1084.
10. Piana, ML, Poda, G, Cesaroni, D, Cuetti, L, Bucci, MA and Gotti, P. Research on microbial characteristics of honey samples of Udine province. *Riv. Sot. Ital. Sci. Aliment*., 1991; 20: 293-301.
11. Khan IA, Khan A, Asif H, Jiskani MM, Mhlbach HP and Azim MK. Isolation and 16S rDNA sequence analysis of bacteria from dieback affected mango orchards in southern Pakistan. *Pak. J. Bot*., 2014; 46: 1431-1435.
12. Altschul SF, Gish W, Miller W, Meyers EW and Lipman DJ. Basic local alignment search tool. *J. Mol. Biol.*, 1990; 215: 403-410.
13. Aureli P, Franciosa G and Fenicia L. Infant botulism and honey in Europe: a commentary. *Pediatr. Infect. Dis. J.*, 2002; 21: 866-868.
14. Grant KA, McLauchlin J, Amar C. Infant botulism: advice on avoiding feeding honey to babies and other possible risk factors. *Community Pract*., 2013; 86: 44-46.
15. Abdulla CO, Ayubi A, Zulfiquer F, Santhanam G, Ahmed MA and Deeb J. Infant botulism following honey ingestion. *BMJ Case Rep*., 2012; 7. doi: 10.1136/bcr.11.2011.5153.

Table 2: Biochemical assay based characterization of bacterial isolates from honey samples.

Bacterial isolates	*Staphylococcus aureus*	*Micrococcus luteus*	*Streptococcus pyogenes*	*Streptococcus faecalis*	*Streptococcus epidermidis*	*Corynebacterium sp.*	*Klebsiella pneumonia*	*Escherichia coli*	*Salmonella sp.*	*Proteus sp.*
Catalase	+	+	-	-	-	+	+	+	-	+
Oxidase	-	+	-	-	-	-	-	-	-	-
Coagulase	+	-	-	-	-					
Motility	-	-	-	-	-	-	-	+	+	+
Indole	-	-	-	-	-	-	-	+	-	+
Ethyl red	+	-	-	-	-	-	-	+	+	+
VP test	+	-	-	-	-	-	-	+	+	+
Citrate test	+	-	-	+	-	-	+	-	+	-
Growth on MacConkey	-	-	-	+	-	-	+	+	+	+
Heamolysis on blood agar	β	γ	β	γ	γ	γ	γ	α	γ	α
Oxygen requirement	F.anaerobe	O.aerobe	F.anaerobe	F.anaerobe	Aerobe	F.anaerobe	F.anaerobe	F.anaerobe	F.anaerobe	F.anaerobe
Lactose fermentation	+	-	-	+	+	-	+	+	-	-
Sucrose fermentation	+	-	-	+	+	-	+	+	-	-
Glucose fermentation	+	-	-	+	+	-	+	+	-	-