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MICROBIAL DIVERSITY AND PHYLOGENETIC STUDIES OF SOME MICROBES OBTAINED FROM UNEXPLORED CAVES OF SAUDI ARABIA

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ABSTRACT

The microbial diversity within cave ecosystems is largely unknown. This study aimed to studying the microbial communities of the three caves viz Mossy, Hotel and Reda caves which are located approximately 200 km of Riyadh region, between Riyadh and Al Kharj road, Saudi Arabia. These caves have an intricate cave system which developed in the calcareous sandstone and clay. Morphological interpretation revealed that Mossy cave was different from Hotel and Reda caves where it is tall and have a linear cave passage and narrow canyons. Various field studies revealed that many bacteria inhibited in caves and obtained their energy from degradation of inorganic substances, thus they directly interact with the surfaces where they live on. In present study, soil and wall dust samples were collected and cultured on Nutrient agar, starch nitrate agar and PDA plates. During study various Gram positive, Gram negative bacteria, actinomycetes and fungi were isolated from the cave soil and wall. The isolated bacteria were characterized and identified by using culture-dependent methods (morphological and physiological methods). Identifications of the most occurring bacterial genera were confirmed by using 16S rRNA gene amplicon sequencing which revealed the presence of 11 broad taxonomic species of bacteria. Among these, Proteobacteria were dominant in all caves and this was followed by Actinobacteria, Firmicutes and Bacteroidetes. Majority of the true bacterial isolates belong to the genera *Alcaligenes*, *Bacillus*, *Brevibacterium*, *Klebsiella*, *Planomicrobium*, *Shigella*, *Pseudomonas* and *Staphylococcus.* During study, percentage of resistance to some metallic cations, heavy metals and antibiotics were also determined. Result of study revealed that *Bacillus cereus*, *Klebsiella pneumoniae*

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and *Pseudomonas earuginosa* showed the highest resistant percentage to the tested heavy metals while *Pseudomonas earuginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus* were the highest resistant to the tested antibiotics. In conclusion, present study provide beneficial information about microbial diversity, taxonomic and their resistance to some heavy metals and antimicrobial agents in three uncharacterized caves, located in Alsoman region, north east of Riyadh.

1 Introduction

All imaginable environment including unexplored caves must be examined for new microorganisms (Barton & Luiszer, 2005). Saudi Arabia is a country with rarest wild caves with interesting and popular geologic and geomorphic features. Its caves have great exploration potential with unsurpassed beauty that provides unending curiosity to a caver. Jabal Al Qarah Cave is located between Dammam and Al-Hofuf, Saudi Arabia and its cool protected passages and considered a gathering place for visitation (Hotzl et al., 1978, Hussain et al., 2006). Harrat Khaybar lava caves, Dahl Rumahah, Kahf Al Shuwaymis, and Umm Jirsan Lava-Tube are some of the oldest caves which explored for their ecology and diversity in Saudi Arabia (Pint, 2009). Very few geological studies have been carried out about the caves located in Saudi Arabia (Forti et al., 2003; Al-Shanti et al., 2003). Microorganisms interact directly with the geology of our planet and their activities have provided the oil, oxygen and the essential nutrients such as carbon, nitrogen and phosphorus. Cave microbiology deals with microorganisms that are found in caves and have the ability to consume inorganic material for energy and survive under extreme conditions (Madigan et al., 2000, Shivaji et al., 2004). Extreme conditions included physical and chemical environmental limits, high temperatures, acid conditions, low light and growth under salt stress (Hathway, 2010). Generally, microorganisms from caves have been considered extreme microbes, because of their ability to grow at low nutrient accessibility, low or high temperature and moderately high humidity, under minimum light conditions of caves (Hathway, 2010). Attempts must be carried out to understand the difficulties associated with microbial activities in cave environments. The significant roles of cavers are the identification of new cave biota and conserving the microbial habitats of caves. Traditional methods such as growing microorganisms in Petri plates, Gram staining, type of respiration and mode of motion are generally used for bacterial identification but unfortunately if the number of bacterial species are higher than 5,000/gram, it is difficult to identify by above said technique (Amann et al., 1966). Thus, recently methods based on genetic sequences (16S rRNA gene) were used to identify and determine the diversity of the isolated bacteria (Al'Abri, 2011; Amasha, 2012, Almalki, 2012). Very little information is available about the diversity and importance of cave microbes, thus further researches on caves microbiology are needed. Moreover, the biodiversity of bacteria in Saudi Arabian caves are not received scientific attentions (Pint, 2006) and available published literature about the geology of the Saudi Arabian caves and their biological composition are in scarcity (Forti et al., 2003, Al-Shanti et al., 2003). There are several caves that have not been explored yet for their geomorphology and microbial diversity. This study aimed at studying and determining bacterial diversity of three caves viz. Mossy, Hotel and Reda caves near Riyadh, Saudi Arabia.

2 Material and methods

2.1 Study area:

Three easily accessible caves in the vicinity of Riyadh named, Mossy Cave: 26°27’33.2″N, 47°14’03.3″E, Hotel cave: 26º 28' 14.2 47º 14' 23.8″E″, and Reda cave: 26°27’10.5″N, 47°15’1.9″E were visited with the help of a local cave explorer (Dr. Mahmoud Ahmed Shanti, Saudi geological Survey). All three caves are located in Somman area and the visitation was low because these were not opened for visitors. During the study period, the average temperature ranged from 11˚C (January) to 43˚C (July and August). Representative photos were taken for each cave and all samples were photo-documented at the collection area. Elevation of the cave (m) at the entrance, temperature, humidity and RH were determined during January 2017. Relative humidity is approximately the ratio of the actual to the saturation vapour pressure and is calculated from the following equation

RH = (Actual vapor pressure) / (Saturation vapor pressure) × 100%

2.2 Sample collocation

From each cave, ten soil samples were collected from surface soils and cave walls (microbial mats) from the entire selected cave by following protocol of Amasha (2012). All the collected samples were taken in sterile containers with a sterilized spoon and brought to the Laboratory of Microbiology, University of King Abdulaziz, Jeddah, Saudi Arabia for further analysis. All soil samples were spread on a clean paper sheet until dries.

2.3 Soil analysis

Analysis of chromium (Cr), cadmium (Cd), cobalt (Co), copper (Cu) and iron (Fe) in each soil sample was carried out. All

samples were homogenized; particle size, distribution and organic carbon (Org. C) content were determined using the loss-of-ignition method (Donkin 1991) and sieving method (Laker & Dupreez, 1982), respectively. The pH value was measured in soil extract (1:2 w/v) by the method of Sonneveld & Van Den Ende (1971). Soil sample digestion was carried by 10 N of mixture of ultrapure nitric acid (HNO_3) and HCl. Metal detection was determined using Agilent 7500 Capillary Electrophoresis series Inductively Coupled Plasma Mass Spectrometer. Geo-accumulation index was calculated from the relation (Muller, 1969; Loska et al., 2004; Ji et al., 2008):

I geo=log2 Cn/ 1.5 Bn (du Preez et al., 2016)

C_n: measured concentration of n (element), B_n: measured geochemical background value.

2.4 Microorganism's isolation and total microbial count

One gram of each sample was suspended in 10 ml sterile distilled water and serial dilution carried out. From the suitable dilution, bacteria, actinomycetes and fungi were isolated on nutrient agar, starch nitrate agar (Pridham et al., 1957) and PDA, respectively. After 2 days of incubation at 37°C for bacteria and 7 days of incubation at 25°C for actinomycetes and fungi, colonies were counted, selected and streaked on new agar plates until pure colonies were obtained. Total bacterial, fugal and actinomycetes counts for different collected samples were determined using agar plate count methods. All pure colonies were preserved on agar slants of the same medium at 4°C until used.

2.5 Bacterial identification

2.5.1 Morphological and physiological characteristics

Isolated bacteria were identified on the basis of various morphological characteristics such as Gram stain, cell shape, colony characteristics and growth on different nutrient media in addition to physiological characters like production of catalase, oxidase, gelatinase, chitinase and amylase (Plotnikova et al., 2010). For Gram negative bacteria, biochemical reactions were carried out using API20E system (Bio Mérieux S.A, France).

2.5.2 Molecular identification

16SrRNA sequencing was used to identify bacteria from caves sample. Genomic DNA was extracted from isolated and purified bacterial isolates and then PCR was used with the appropriate primers to produce large quantities of the 16SrRNA gene (Al'Abri, 2011). Phylogenetic grouping was performed using a multiplex PCR-based assay as described by Clermont et al. (2000). During molecular identification, the name and sequence of the used premiers have been given in Table 1. 16S rRNA gene was amplified using 10 p mol/µl of each forward 785F (5′- GGA TTA GAT ACC CTG GTA -3′) and reverse 907R (5′- CCG TCA ATT CMT TTR AGT TT-3'). DNA extraction, PCR and sequencing were carried out at Macrogen, Geumcheon-gu, Seoul 08511, Republic of Korea.

Table 1 Premiers name and sequences used in this study.

Name of Premiers	Premiers sequences
785F5'	(GGA TTA GAT ACC CTG GTA)3
907R5'	(CCG TCA ATT CMT TTR AGT TT) 3'
27F 5'	(AGA GTT TGA TCM TGG CTC AG) 3
'1492R 5	' (TAC GGY TAC CTT GTT ACG ACT T) 3'

2.5.3 Metal tolerance and antibiotic resistance patterns

All the obtained bacterial isolates were screened for resistance to heavy metals (1300 µg/ml), Cu, Cd and Cr using agar dilution method. After 24 hr of incubation, the plates were examined for bacterial growth (Washington & Sutter, 1980). Antibiotic resistance of the differed bacterial isolates were determined by standard disc diffusion method (Finegold & Martin, 1982) on Mueiler-Hinton agar (Lennette et al., 1974). Antibiotic paper discs (bioMerieux, Charbonnieres-les-Bains, France) containing Ampicillin (10 pg); Streptomycin (10 pg); Tetracycline (30 pg) and Neomycin (30 pg) were put on the surface of the inoculated agar and all plates were incubated at 30°C for 2 days. Zone of inhibitions were measured as described in the instructions (bioMerieux).

3 Results ad Discussion

Caves are found in many types of rocks and are formed by different geological processes. Caves of Somman are formed by dissolving calcareous rocks and slow moving of the groundwater from the passages and cavities (Moore & Sullivan, 1978). In present study, three caves of the Somman were visited, characterized and microbial diversity of these three was determined. A part of the map of Saudi Arabia showing the three studied caves, north east of Riyadh city was represented in Figure 1. The caves under study named Mossy, Hotel and Reda caves. The three studied caves have interesting pattern, among these first two caves have horizontal and easy to walk while the third one viz, Mossy cave has deep pit, with 4 m long, and need ropes and a ladder to go inside (Figure 2). Similarly, Amasha (2012) studied, characterized and isolated various bacterial strains from Ghar Al Hibashi cave which is located 300 km southeast of Makkah, Saudi Arabia (21°10′ N, 42°10′ E). Environmental

Figure 1 A part of the map of Saudi Arabia showing studied caves, north east of Riyadh city

Figure 2 The three studied caves, A: Mosse cave, B: hotel cave, and C: Reda cave, Riyadh, Saudi Arabia

characteristics of the three studied caves and mat colours were determined (Table 2). During study, obtained mat colours (Table 1) were gray, tan, yellow, brown and white. Similarly, tan, white, and yellow colours were reported by Lavoie et al (2017). At the bingeing, dry and wet bulb temperatures (°C) and Barometric pressure (mbar) were measured in January and used to calculate percentage of relative humidity RH). During study, cave air temperatures ranged from 29 to 33ºC, while RH values ranged between 41% and 53%. Similarly, lower temperature and higher relative humidity was recorded for Torgac cave, New Mexico by Forbes (1998). The contents of Cd, Cr, Co, Cu and Fe have been assayed in the cave soils. This area is affected by microorganism growth, thus soil organic carbon content was varied from 0.23 - 0.70 %. The highest value of organic carbon was reported from the soil collected from Mosse cave. Soil contamination was assessed on the basis of heavy metal concentrations (Table 2) and

geo-accumulation index (I geo) as in Figure 3. The results revealed elevated contents of I geo for Cd and Cr. The contents of Cu, Fe, and Co were similar to the levels in the Earth's crust (Table 3).

Caves are like underground chambers that contained extremophilic microorganisms which interact directly with the geology of the place and carried different microbial activities, ranged from the obvious slimy growth to the more subtle deposition of calcite or alteration of the rock surface. In caves the microbial activities were shown as surface dots, unusual coloration, biofilm formation and corrosion residues (Barton, 2006). In this study, total counts of bacteria, actinomycetes and fungi were determined for cave walls and soils. Generally, the cave soil contained more microorganisms compared to cave walls and the counts of bacteria were significantly higher, this was followed by fungal counts and actinomycete counts. The highest bacterial counts were recorded from Mosse cave wall and soil. Further, Mosse wall contained 68% bacteria, 09% actinomycetes and 25% fungi while this percentage was 60.5%, 5.5% and 34% for Mosse cave soil. Actinobacteria were found in all cave samples in the range of 4-10% for cave walls and 5.5-8 % for cave surface soils. The highest actinomycete counts were recorded from soil of Hotel and Reda caves while in case of wall actinomyctes percentage, highest was reported from the wall of Reda cave (10%) and this was followed by the wall percentage of Mosse cave (9%). In case of fungal counts, highest fungal count was recorded for soil of Reda (37%) and Mosse (34%) caves (Table 4). The commonest bacterial isolates obtained on nutrient agar were selected, purified and identified using morphological, physiological and biochemical characterisation. Identifications were carried out according to Krieg & Holt (1984), Sneath et al. (1986), Brenner et al. (2005), and Krieg (2015). Further, isolated microorganism identified and characterized by using 16S rRNA gene, which is very accurate method for bacterial phylogeny and taxonomy (Michael & Abbott, 2007). Total eleven bacterial species viz., *Alcaligenes faecalis, Bacillus cereus, Bacillus nealsonii, Brevibacterium* sp., *Klebsiella pneumoniae, Planomicrobium okeanokoites, Shigella flexneri, Pseudomonas earuginosa, Staphylococcus aureus, Staphylococcus epidermidis* and *Staphylococcus equorum* have been

Table 2 Environmental characteristics of the three studied caves and mat colours

Cave name	No. of entrances	Elevation (m)	Distance of sample from entrance (m)	pH	RH (%)	Mat colours
Mosse	1	4.0	5.0	Nd	53	Gray and tan
Hotel	1	2.11	3.3	7.9	41	Yellow, and tan
Reda	2	1.8	2.9	Nd	47	Brown and white

Nd: Not determined, RH: Relative humidity

Table 3 Total carbon and heavy metal concentrations (µg/g; dry weight), Cadmium, Chromium, Cobalt, Copper and Iron

Cave name	Soil organic carbon content (%)	Cadmium (Cd)	Chromium (Cr)	Cobalt (Co)	Copper (Cu)	Iron (Fe)
Mosse	0.70	0.36	61.9	129	57	1600
Hotel	0.23	0.25	93.3	155	55	1700
Reda	0.60	0.32	62.4	121	75	1200

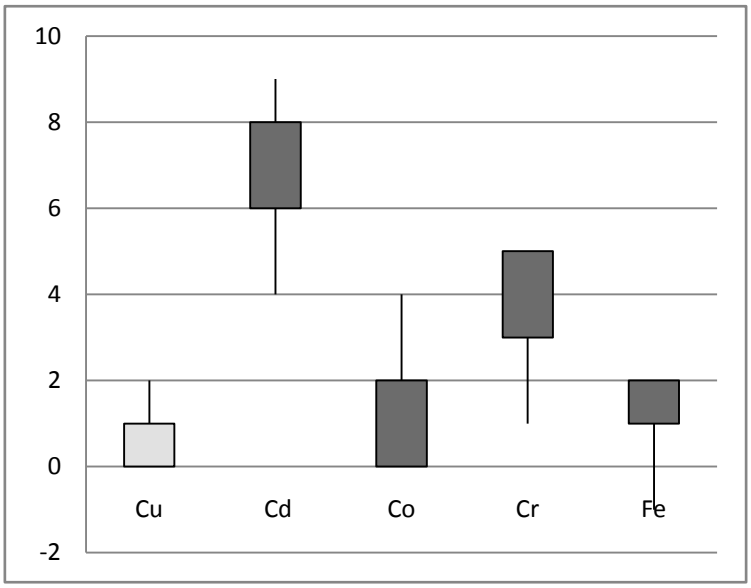

Figure 3 Geoaccumulation index (I geo) for trace metals in the study area I geo < 0: Uncontaminated, 2 < I geo < 3: Mod.–heavy, 4 < Igeo < 5: Heavy–extreme, 5 < I geo : Extreme

Table 4 Total counts (CFU/g) of Bacteria, Actinomycetes and Fungi on agar media and the percentage of occurrence (%) for cave walls and soils.

Microorganisms	Studied Cave					
	Mosse cave		Hotel cave		Reda cave	
	Wall	Surface soil	Wall	Surface soil	Wall	Surface soil
Bacteria	$1.11x10^4$ (68%)	$1.73x10^4$ (60.5%)	$1.00x10^4$ 34%)	$1.33x10^4$ (59%)	$1.03x10^4$ (53%)	$1.43x10^4$ (55%)
Actinomycetes	$1.04\ x10^3$ (9 %)	$1.59x10^3$ (5.5%)	$1.33\ x10^3$ (4%)	$1.99x10^3$ (8%)	$1.99\ x10^3$ (10%)	$1.96\ x10^3$ (8 %)
Fungi	$0.37\ x10^4$ (25%)	$0.98\ x10^4$ (34%)	$0.78\ x10^4$ (26%)	$0.77x10^4$ (33%)	$0.70\ x10^4$ (37%)	$0.94\ x10^4$ (37%)

Table 5 The isolated and identified bacteria, accession numbers and their % of resistance to heavy metals

Isolated bacteria	Accession number	Gram reaction	% of Resistance to heavy metal (1300 µg/ml)		
			Cu	Cd	Cr
Alcaligenes faecalis (n=17)	NR_113606.1	Negative	69	59	49
Bacillus cereus (n=29)	NR_074540.1	Positive	99	77	75
Bacillus nealsonii (n=14)	NR_044546.1	Positive	56	69	65
Brevibacterium (n= 9)	NR_115063.1	Positive	60	59	56
Klebsiella pneumoniae (n=22)	NR_117683.1	Negative	55	77	47
Planomicrobium okeanokoites (n=14)	NR_113593.1	Positive	49	41	60
Shigella flexneri (n=17)	NR_026331.1	Negative	64	33	31
Pseudomonas earuginosa (n=33)	CP012001.1	Negative	94	55	69
Staphylococcus aureus (n=21)	CP011526.1	Positive	69	29	60
Staphylococcus epidermidis (n=32)	LN681574.1	Positive	79	41	29
Staphylococcus equorum (n=16)	NR_041926.1	Positive	89	39	48

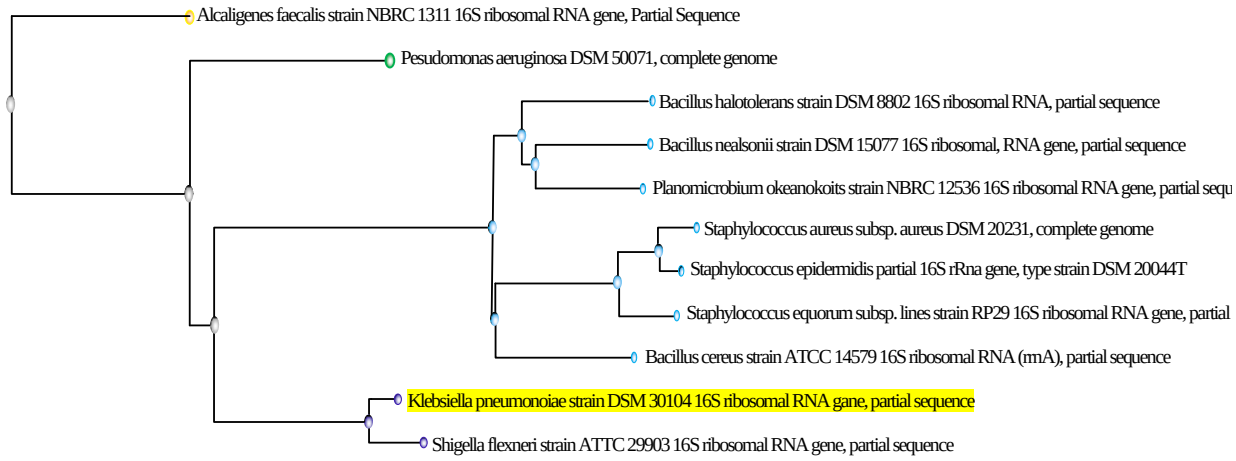

Figure 4 Phylogenetic tree based on 16S rDNA sequence comparisons of 11 identified bacterial isolates, obtained from the tree tested caves, using neighbor joining tree method, maximum sequence difference =0.002.

identified (Table 5, Figure 4). Findings of present study are in agreement with the findings of Amasha (2012) who used 16S rRNA gene for determining the bacterial diversity of Ghar Al Hibashi cave of Makkah, result of this study suggest the presence of *Bacillus* species in higher quantity. Parker Cave, Kentucky received sulphurous water and molecular phylogenetic analysis of the microbial mat revealed the presence of *Thiothrix* spp., *Thiomicrospira denitrificans* and *Thiobacillus baregensis* (Angert

et al., 1998). Moreover, Oliveira et al. (2017) examined five sites of Ozark region caves and used 16S rRNA gene-based metagenomic analysis for bacterial diversity. They reported a variation in bacterial composition, species abundance and diversity among the five caves studied and the lowest richness was found for Sand town cave. Lavoie et al. (2017) studied lava cave microbial diversity of subsurface mats and surface soils using 16S rDNA. They noticed the same genera for both surface soils and cave microbial mats and actinobacteria dominated in all cave samples. These results are in agreement with the findings of present study. Microbial diversity of caves was evaluated and further work on cave environments and their microbes are needed (Al'Abri, 2011; Almalki, 2012; Lavoie et al., 2017).

Chemical reactions that cause mineral dissolution and precipitation were regulated by microorganisms which also affect contaminant remediation (Thomas & Ward, 1992; Engel & Randall, 2011; Lian et al., 2011). The percentages of resistance in all bacterial isolates against metallic cations or heavy metals such as Cu, Cd and Cr were determined (Table 5). The most resistant isolate was *Bacillus cereus* where 99% were resistant to Cu, 77% to Cd and 75% to Cr. Moreover, 94 and 69% of *Pseudomonas earuginosa* isolate were resistant to Cu and Cr, respectively while 77% of the isolates of *Klebsiella pneumoniae* were resistant to Cd (Figure 5). From the contaminated area, two bacterial isolates, *Gemella* sp. and *Micrococcus* sp., which were identified based on morphological, cultural, physiological and biochemical characteristics, showed resistance against Lead, chromium and cadmium (Marzan et al., 2017). Although the tested cave bacteria have not exposed to anthropogenic antibiotics before, isolates of *Pseudomonas earuginosa* were the most resistance to all tested antibiotics; percentages of resistance were 91, 64, 54 and 43 % for Ampicillin, Streptomycin, Tetracycline and Neomycin respectively. Percentages of resistances in *Klebsiella pneumoniae* isolate were 81 and 55 % for Ampicillin and Streptomycin while the resistance of *Staphylococcus aureus* isolate was 63 and 44 % for Tetracycline and Neomycin, respectively (Table 6). Studying the resistance of bacterial isolates that are not exposed to antibiotics before is very interesting. The decrease in annual detection rates of *Staphylococcus aureus* and their increases in cases of *P. aeruginosa* and *Klebsiella pneumoniae* were recorded by Dou et al. (2017). Various factors are responsible for the bacterial resistance, among these some common are target modification, drug influx and efflux control, and presence of inactivation enzymes which are found due to

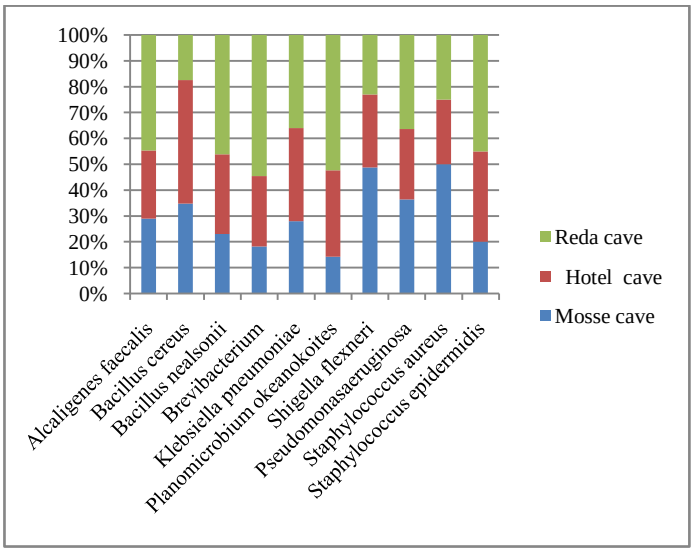

Figure 5 Percentage of occurrence of the isolated and identified bacteria in the three studied caves

Table 6 Percentage of resistance of the isolated bacteria from caves to different antibiotics

Tested bacteria	% of resistance to antibiotics			
	Ampicillin (10 pg)	Streptomycin (10 pg)	Tetracycline (30 pg)	Neomycin (30 pg)
Alcaligenes faecalis (n=17)	67	56	33	12
Bacillus cereus (n=29)	33	33	49	35
Bacillus nealsonii (n=14)	49	31	51	29
Brevibacterium (n= 9)	77	19	41	33
Klebsiella pneumoniae (n=22)	81	55	33	28
Planomicrobium okeanokoites (n=14)	66	54	30	33
Shigella flexneri (n=17)	32	39	39	28
Pseudomonas earuginosa (n=33)	91	64	54	43
Staphylococcus aureus (n=21)	70	51	63	44
Staphylococcus epidermidis (n=32)	46	29	51	34
Staphylococcus equorum (n=16)	17	46	28	22

evolution and natural selection. Non-pathogenic bacteria may act as resistant gene reservoirs which may be transferred to the harmful bacteria (Allen et al., 2009, Donato et al., 2010). The outstanding ecosystem, Lechuguilla cave, was found over 4 million years and is a best biosystem to study bacterial resistant. From Lechuguilla cave, 93 bacterial strains, live under limited nutrient environment, were isolated and surveyed for antibiotic susceptibility and most of these isolates were considered as multidrug resistant. Antibiotic resistant bacteria are common in cave environments (Bhullar et al., 2012). Bacterial resistance patterns to many antibiotics were different and more studies of cave microbiota including extensive cultivation and metagenomic analysis are needed.

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Conflict of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise

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