RESEARCH ARTICLE

Bacterial screening of historic site of Qarah caves, biosphere analysis

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Microbial activities play major roles in both the building foundations of historical sites and ecotourism health. The Qarah Caves, located in the historical site of Qarah Mountain in Al-Ahsa Oasis, a UNESCO world heritage site, have experienced an increase in the daily tourist number, consequently increasing human activity inside the caves. In the current study, ten spots in the caves were screened to identify bacterial communities, and two unknown samples were subjected to phenotypic and molecular identification. We used small subunit ribosomal RNA (16S rRNA) gene DNA data to identify bacteria from ten sites. The phenotypic analysis of the unknown samples identified rod-shaped Gram-negative bacteria under a microscope. The molecular analysis indicated that the unknown bacterial samples were *Salmonella enterica* and *Kluyvera intermedia*. The study emphasized the importance of managing procedures for historical sites to maintain a better status and prevent disruption. Healthy ecotourism requires taking care during caving and after by washing hands. Some cave microbes are pathogenic such as *Salmonella enterica* while others carry bioremediation potentials such as *Kluyvera intermedia*.

Key Words: Cave microbiology, Bacteria screening, Bacterial isolates, Ecotourism, Public health.

INTRODUCTION

Microbial biodiversity is an important factor in maintaining a healthy environment in caves and historical sites. However, the geophysical nature of caves may remain relatively constant for thousands of years until anthropogenic activities are encountered. The anthropogenic activities with the greatest impact on cave ecosystems are unrestrained eco-tourism and unrestrained mining [1-3]. The Qarah (or Al-Qarah or Al-Garah) Caves are located in the historical site of Qarah Mountain in Al-Ahsa Oasis in the Eastern province of Saudi Arabia, has been added to The United Nations Educational, Scientific and Cultural Organization (UNESCO) world heritage site in 2018. Qarah Mountain is 246 ft high, with a greatest rise of 225 m above sea level. The Qarah Caves have a highly distinctive shape as a result of subaerial weathering and include 28 tall linear passageways that are 1.5 km (0.93 mi) in length [4,5]. The Qarah caves passageways evolved as result of environmental and climate changes during and after the Plio-Pleistocene period [6].

The bacterial communities that contribute to landscape degradation mostly have a "potential pathogenic character" [7]. Understanding how microbial communities affect landscapes and historical sites might provide insight into this understudied ecology leading to better cave management and preservation and a healthier ecotourism. In the current study, soil samples were collected from 10 spots in the caves, and DNA sequencing was performed to identify bacterial communities. The aim of this study was to explore the dominated microbial community in the Qarah Caves and to contemplate their foreseen benefits and anticipated threats for the biosphere and ecotourism.

MATERIALS AND METHODS

Site sampling

During August of 2019 (summer, the maximum temperature was 20°C inside the caves), soil samples were collected from 10 spots from the historical site of the Qarah Caves, Al-Hassa, Saudi Arabia (25.417450, 49.666120) (Figure 1). Soil samples were diluted in 9 mL of serial sterile deionized water to reach a concentration of 10-5 for pure bacterial colony isolation. The soil suspension was spread on an agar plate for subsequent study of the characteristics of pure cultures and Gram staining. The incubation was at 37°C for 48 h to get full microbial growth. The bacterial colonies' characteristics were compared with those of known organisms from the literature. Bacterial isolations were carried out by employing direct bacterial dilution techniques, under which one gram of soil sample was placed in 9 mL of sterile distilled water within nutrient agar medium [8,9].

Phenotypic identification

Gram staining: Following Coico, 2006 method, a small bacterial colony was picked with an inoculation needle and mixed over glass slide with water to create a smear [10]. The smear dried naturally with air and heat fixed. The dried smear was stained with the Gram pigment then a drop of oil was added to the slide to observe the bacteria under 100X magnification.

Molecular Identification

Colony PCR: Housekeeping small subunit ribosomal RNA (16S rRNA) gene primers (16s F: AGAGTTTGATCCTGGCTCAG and 16s - R:GGTTACCTTGTTACGACTT) were used for the molecular identification of bacterial colonies from two dominant colonies; they were picked from an agar plate after growing for 48 h at 37°C and named Qarah-FD1 and Qarah-FD2. The PCR reaction tube contained 50 µl of solution, comprising 33 µl of Nuclease free water, 2.0 µl of each Primer (10 µM) (Forward, Reverse), and 13 µl of the PCR master mix (2.5 U/µl) (Promega, USA). The PCR program consisted of 10 min of the initial denaturation at 94°C followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1.5 min at 72°C. The final extension was for 10 min at 72°C. The amplification was carried out in a BioRad Thermocycler.

Sequencing

Following effective visualization of amplified DNA by the 1% agarose gel electrophoresis technique, the forward and reverse of the PCR amplified 16s rDNA regions were sequenced. The sequence was performed using DNA sequence-3037xl DNA analyzer from Applied Biosystems using a BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems). Sequenced information were adjusted and dendrograms were produced utilizing Sequence investigation programming form 5.2 from Applied Biosystems.

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Phylogenetic analysis

The two colonies' sequences were compared to the non-redundant NCBI database using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi_) [11]. The expected value and e values were noted for the most similar sequences (98%-99%) and aligned using MUSCLE. There were 17 similar sequences for Qarah-FD1 and 20 similar sequences for Qarah-FD2, with unknown bacterial sequences (Appendix: Table 1). The multiple-alignment file was obtained and used to create a Phylogram using MEGAX software [12]. The evolutionary tree was inferred using the Neighbor-Joining method [13]. The bootstrap consensus tree utilized from 500 replicates representing the evolutionary history of the analyzed taxa [14]. In less than 50% of bootstrap replicates, corresponding branches reproduced and replicates were removed. The Maximum Composite Likelihood computed according to evolutionary distances and shown of base substitutions per site. This analysis involved 37 nucleotide sequences [15]. The codon positions included were the three first Noncoding. All uncertain positions were removed for each sequence pair (pairwise deletion option). There were a total of 1616 positions in the final dataset. Evolutionary analyses were conducted in MEGAX [16].

Figure 1) The Qarah (or Al-Qarah or Al-Garah) Caves are located in a small mountain in Al-Ahsa located in the East of Saudi Arabia; Qarah Mountain is 75 m (246 ft) high with 12 passage.

RESULTS

Gram Staining

The colonies from Qarah-FD1 and Qarah-FD2 were observed as rod-shaped Gram-negative bacteria under a microscope (Figure 2: a, b).

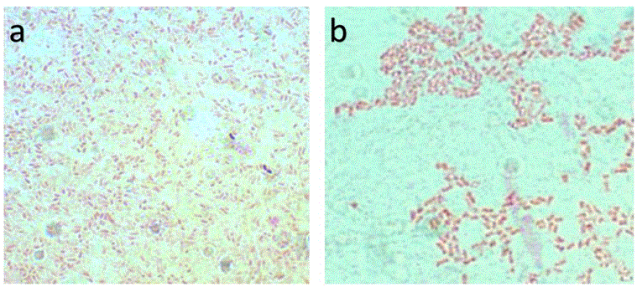

Figure 2) (a) Qarah-FD1 and (b) Qarah-FD2. Gram-staining pictures of colonies from the agar plates of samples Qarah-FD1 and Qarah-FD2 were observed under oil immersed 100x magnification microscope.

Sequencing and analysis of the PCR amplicon from 16s primers

The sequencing and bioinformatics analysis results of different PCR products are given below, considering contigs of forward and backward amplicons for each of Qarah-FD1 and Qarah-FD2.

>Qara-FD1

GCACACGAGAAGCTCTTGCTCTCCGTGGTGGACGGAGTAGGCG
GACGGGTGAGTAATAGTCTGGGAAACTGCCTG
ATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAA
CGTCCCAAGACCAAAGAGGGGGACCTTCGG
GCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGT
GGGGTAACGGCTCACCTAGGCGACAATCCCT
AGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACG
GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAAT
GGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGC
CTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGG
TTAATAACCCCGGCAATTGACGTTACCCGCAAAAAAAACACCGG
CTAACTCCGTGTCAGCGGCCGCGGTAATATACAAGGTGTGCACG
TTTATCTGAATTATTGTGCGTAAAGCGCGTGCAGGCG
GTCTTTTAATTCTGGATTTGAAATCCCCGGGCTCAAAATCCCCG
GGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTGGAGTC
TTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTA
GAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACA
AAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAG
GTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATC
GACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTA
GATGCAACGCGAAGAACCTTACCTCGTCTTGACATCCACAGAAC
TTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTG
CTGCATGGCTGTTGTCAGCTCGTGTTGTGAAATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTCTCCTTTGTTGCCAGCGGTTAG
GCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAG
GTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTA
CACACGTGCTCCAATGGCGCATACAAAGAGAAGCGACCTCGCG
AGAGCAAGCGGACCTCATAAACTGCGTTGTAGTCCGGATTGGAG
TCTGCAATTGGACTCCATGAAGTCGGAATCGTTAGTAATCGTAGA
TCACAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCG
CCCGTCACACCCATGGGAGTGTGGTT
GCAACAAAGAAAGCTAGGTAGCTTTA

>Qara-FD2

CTCAGCTAGAGCTTTGCTCTCTTGGTGACAAGCGGCGGACAGGT
GAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTG
GAAACGGTAGCTAATACCGCATAACGTCTCAAGACCAAAGAGGG
GGACCTTCTGGCCTCTTGCCATCACATGTGCCCAGATGGGATTA
TCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACAATCCCTAGC
TGGTCTGAGAGGATGACCACCCACACTGGAACTGAGACACGGTC
CACACTCCTACGGGAGGCAGCACTGGGGAATATTGCACAATGG
GCGCAAGCCTGATGCACCCATGCCGCGTGTATGAAAAAAGCCT
TCGGGTTGTAAAGTACTTTCACCGAGGAGGAAGGCGTTAAGGTT
AATAACCTTGGCGATTGACGTTACCCGCAGAAGAAACACCGGCT
AACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGT
TAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTC
AAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCTTTT
TAAACAGGCATGCTATAGTCTTGTATAGGGGGGTATAATTTCCAC
GTGTAGCGGTGTAATGCGACGTACCCGCAAAAGAAGCCCCGGT
AACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGT
TAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTC
AAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTC
GAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAG
GTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGG
CGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTT

CCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCG
CAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACC
TACTCTTGACATCCAGAGAACTTTGCAGAGATGCTTTGGTGCCTT
CGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGT
GTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTA
TCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTG
CCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCA
TGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCATATA
CAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGT
ATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGT
CGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACG
TTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGG
GTTGCAAAAAGAAAGTAGGTAGCT

Molecular identification

The evolutionary relationships of taxa showed nine clusters of DNA sequences with unknown bacterial sequences clustered in two of them. The DNA sequence of Qarah-FD1 matches best with *Salmonella enterica* (NR_074910.1 and NR_074799.1). Qarah-FD2 matches best with *Kluyvera intermedia* (NR_112007.1) (Figure 3). Table 1, show the Blast sequences best match results.

Table 1) Unknown bacterial sequences of Qara-FD1 and Qara-FD2 BLAST search result best matched from NCBI website (heightened rows are the best match that showed evolutionary relationships according to phylogenetic analysis).

Qara-FD1 BLAST search result Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Enterobacter cloacae subsp. dissolvens strain LMG 2683 16S ribosomal RNA, partial sequence	2180	2180	0.99	0	0.9502	NR_044978.1
Enterobacter cloacae strain DSM 30054 16S ribosomal RNA, partial sequence	2172	2172	0.99	0	0.9495	NR_117679.1
Enterobacter cloacae strain NBRC 13535 16S ribosomal RNA, partial sequence	2172	2172	0.99	0	0.9495	NR_113615.1
Enterobacter cloacae subsp. dissolvens strain ATCC 23373 16S ribosomal RNA, partial sequence	2172	2172	0.99	0	0.9496	NR_118011.1
Enterobacter cloacae strain 279-56 16S ribosomal RNA, partial sequence	2172	2172	0.99	0	0.9495	NR_028912.1
Enterobacter cloacae strain ATCC 13047 16S ribosomal RNA, complete sequence	2156	2156	0.99	0	0.9475	NR_102794.2
Pantoea agglomerans strain JCM1236 16S ribosomal RNA, partial sequence	2121	2121	0.99	0	0.9431	NR_111998.1
Salmonella enterica subsp. enterica strain LT2 16S ribosomal RNA, partial sequence	2115	2115	0.97	0	0.9477	NR_074910.1
Kosakonia oryzendophytica strain REICA_082 16S ribosomal RNA, partial sequence	2115	2115	0.99	0	0.9424	NR_125586.1
Enterobacter tabaci strain YIM Hb-3 16S ribosomal RNA, partial sequence	2111	2111	0.97	0	0.9488	NR_146667.2
Leclercia adecarboxylata strain CIP 82.92 16S ribosomal RNA, partial sequence	2109	2109	0.99	0	0.9417	NR_104933.1
Leclercia adecarboxylata ATCC 23216=NBRC 102595 strain LMG 2803 16S ribosomal RNA, partial sequence	2108	2108	0.99	0	0.941	NR_117405.1
Leclercia adecarboxylata strain NBRC 102595 16S ribosomal RNA, partial sequence	2106	2106	0.99	0	0.941	NR_114154.1
Enterobacter ludwigii strain EN-119 16S ribosomal RNA, partial sequence	2104	2104	0.99	0	0.941	NR_042349.1
Klebsiella pneumoniae strain ATCC 13883 16S ribosomal RNA, partial sequence	2104	2104	0.99	0	0.941	NR_114506.1
Klebsiella pneumoniae strain JCM1662 16S ribosomal RNA, partial sequence	2100	2100	0.99	0	0.9403	NR_112009.1
Salmonella enterica subsp. enterica strain Ty2 16S ribosomal RNA, partial sequence	2098	2098	0.97	0	0.9455	NR_074799.1
Qara-FD2 BLAST search result	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Description						
Klebsiella pneumoniae subsp. rhinoscleromatis ATCC 13884 16S ribosomal RNA, partial sequence	1731	2676	0.99	0	0.9907	NR_114507.1
Klebsiella pneumoniae subsp. ozaenae strain ATCC 11296 16S ribosomal RNA, partial sequence	1731	2681	0.99	0	0.9907	NR_041750.1

Klebsiella aerogenes KCTC 2190 16S ribosomal RNA, complete sequence	1720	2692	0.99	0	0.9886	NR_102493.2
Klebsiella aerogenes strain ATCC 13048 16S ribosomal RNA, partial sequence	1720	2692	0.99	0	0.9886	NR_118556.1
Klebsiella aerogenes strain JCM 1235 16S ribosomal RNA, partial sequence	1720	2692	0.99	0	0.9886	NR_024643.1
Klebsiella aerogenes strain NBRC 13534 16S ribosomal RNA, partial sequence	1716	2689	0.99	0	0.9876	NR_113614.1
Kluyvera cryocrescens strain NBRC 102467 16S ribosomal RNA, partial sequence	1714	2644	0.99	0	0.9876	NR_114108.1
Kluyvera cryocrescens strain 12993 16S ribosomal RNA, partial sequence	1714	2642	0.99	0	0.9876	NR_028803.1
Klebsiella aerogenes strain NCTC10006 16S ribosomal RNA, partial sequence	1712	2685	0.99	0	0.9865	NR_114737.1
Klebsiella quasipneumoniae subsp. similipneumoniae strain 07A044 16S ribosomal RNA, partial sequence	1709	2657	0.99	0	0.9865	NR_134063.1
Kluyvera intermedia strain 256 16S ribosomal RNA, partial sequence	1703	2609	0.99	0	0.9855	NR_028802.1
Klebsiella pneumoniae subsp. rhinoscleromatis strain R-70 16S ribosomal RNA gene, partial sequence	1703	2648	0.99	0	0.9855	NR_037084.1
Citrobacter murliniae strain CDC 2970-59 16S ribosomal RNA, partial sequence	1703	2631	0.99	0	0.9855	NR_028688.1
Citrobacter braakii strain 167 16S ribosomal RNA, partial sequence	1703	2615	0.99	0	0.9855	NR_028687.1
Klebsiella quasipneumoniae subsp. quasipneumoniae strain 01A030 16S ribosomal RNA, partial sequence	1701	2639	0.99	0	0.9845	NR_134062.1
Enterobacter ludwigii strain EN-119 16S ribosomal RNA, partial sequence	1698	2631	0.99	0	0.9845	NR_042349.1
Klebsiella pneumoniae strain ATCC 13883 16S ribosomal RNA, partial sequence	1698	2631	0.99	0	0.9845	NR_114506.1
Citrobacter freundii ATCC 8090 = MTCC 1658 16S ribosomal RNA, partial sequence	1698	2626	0.99	0	0.9845	NR_028894.1
Serratia liquefaciens strain JCM1245 16S ribosomal RNA, partial sequence	1698	2642	0.99	0	0.9845	NR_112008.1
Kluyvera intermedia strain JCM1238 16S ribosomal RNA, partial sequence	1698	2604	0.99	0	0.9845	NR_112007.1

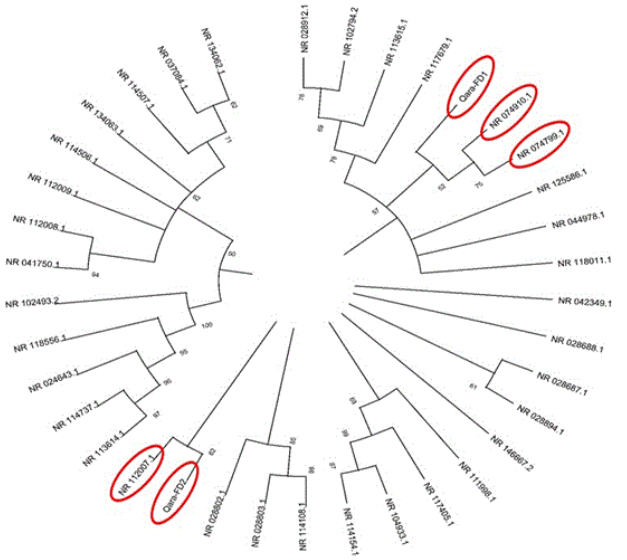

Figure 3) Evolutionary relationships of taxa: The evolutionary history was induced utilizing the Neighbor-Joining method. The bootstrap consensus tree induced from 500 replicates was taken to represent the evolutionary history of the examined taxa. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were removed. The evolutionary distances were computed using the Maximum Composite Likelihood method. The analysis performed for 37 nucleotide sequences. Codon positions included were the three first Noncoding regions. All uncertain positions were removed for each sequence pair (pairwise deletion option). There were a total of 1616 positions in the final dataset. Evolutionary analyses were conducted in MEGAX.

DISCUSSION

Historical sites are full of microbial communities, and visitors to these sites increase the diversity and quantity of such communities. In addition, microbial communities might change over time in response to biological and geochemical changes [17]. The bacterial communities of caves have been reported to contain contamination at some sites [1].

Recent studies on microbial species ecosystems in caves have led to improvements in cave conservation practices. The identification of infrequent cave biota makes it crucial to implement practices to limit human contamination and conserve caves as microbial habitats [18]. A taxonomic composition analysis of the Manao-Pee cave soil community (a subterranean limestone cave in western Thailand) identified two dominant bacterial groups, in which 51.2% were of the Gram-positive class Actinobacteria and 24.4% were of the Gram-negative class Gammaproteobacteria [19].

Caves that are repeatedly visited by human beings have already been reported as reservoirs of pathogenic microorganisms [20].

In New Mexico, a four million years old cave was accounted to contain profoundly antibiotic resistant bacteria, in which of them several strains were resistant to 14 known antibiotics [21]. Many studies have reported the isolation of pathogenic bacteria, for example, *Micrococcus luteus* was isolated from the Meghalaya caves in India, *Staphylococcus sciuri* was isolated from a Chinese cave, and *Micrococcus varians* was isolated from a cave in Poland. However, various human-being-oriented bacteria (*Staphylococcus epidermis*, *Stomatococcus mucilaginosus*, *Staphylococcus salivarius*, *Staphylococcus sciuri*, and *Micrococcus varians*) were also isolated from the Kotumsar cave, which directly suggests that these caves have been subjected to high anthropogenic pressure and consequently, their ecosystems have been disturbed [7,22-24].

In the current study, two infrequent cave bacteria were isolated, namely, *Salmonella enterica* and *Kluyvera intermedia*. According to the World Health Organization (WHO), *Salmonella enterica* is estimated to affect over 17 million people worldwide each year. *Salmonella enterica* a known human pathogen, are able to colonize plants and soil and are transferred to humans via direct interactions [25]. In addition, *Salmonella enterica* is known as foodborne pathogen that can survive for long periods and transfer to and persist in food [26]. *Salmonella serovars* are resilient microorganisms that can survive different stress factors such as changes in temperature, acidity and osmotic pressure beyond their normal growth range [2]. Meanwhile, *Kluyvera intermedia* was identified as one of the biocorrosion factors for oil pipelines in Iran, and some Kluyvera spp. can act as aerobic arsenate-reducing bacteria (aARB) and might be used in arsenic transformation [20,27,29]. They have also been identified as lead-resistant bacteria [30]. In addition, *Kluyvera intermedia* can transform sulfamethoxazole (SMX) and reduce the toxicity of this antibiotic in water [31,32]; thus, they can be used in bioremediation procedures for soil or water.

CONCLUSION

The morphological and molecular identification of samples identified two pathogenic bacteria, *Salmonella enterica* and *Kluyvera intermedia*, in the historical site of the Qarah Caves. This study suggests that management practices should be improved, e.g., limiting the number of daily visitors to the caves and taking precautions for tourists before and after caving, to limit pathogenic microbe exposure. It is also advisable for visitors to wash their hands before and after visiting the Qarah Caves and to prevent food or beverage consumption during caving. Better cave management practices will save the cave environment from disruption and promote healthy ecotourism. On the other hand, the bioremediation potential of *Kluyvera intermedia* from the Qarah Caves should be explored further.

Significance for public health

The study emphasized the importance of managing procedures for historical sites to maintain a better status and prevent disruption. Healthy ecotourism requires taking care during caving and after by washing hands. Some cave microbes are pathogenic such as *Salmonella enterica* while others carry bioremediation potentials such as *Kluyvera intermedia*.

AUTHOR CONTRIBUTIONS

The study was conducted solely by the author Faten Dhawi.

COMPETING INTERESTS

The author declares no competing financial and non-financial interests.

DATA AVAILABILITY

Data that are supplementary to the manuscript will be available by request.

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