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Microbiological analysis, antimicrobial activity, and heavy-metals content of Jordanian Ma'in hot-springs water

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ABSTRACT

Ma'in hot springs are known as sites of balneotherapy. However, little is known about their microbiology and chemistry. In this study, we aim at evaluating the antimicrobial activity of Ma'in hot-springs water (MHSW), studying its microbiology, and determining its physicochemical properties including the heavy metals content. Therefore, water samples were collected from Ma'in hot springs and tested for antimicrobial activity using agar diffusion method. Water was then cultivated on nutrient agar to isolate and identify the dominant bacteria by chemical and molecular methods. The identified strains were tested by cross streak method to evaluate their antimicrobial activity against different clinical and standard strains. Finally, water samples were chemically analyzed and the heavy-metals content was assessed. Results revealed that MHSW was not active against any of the clinical isolates. Nevertheless, MHSW was found to be active against five standard bacterial strains, namely, *Staphylococcus epidermidis* ATCC 12228 (inhibition zone: 20 mm), *Staphylococcus aureus* ATCC 29213 (inhibition zone: 19 mm), *Micrococcus luteus* ATCC 9341 (inhibition zone: 15.3 mm), and *Bacillus cereus* ATCC 11778 (inhibition zone: 12.3 mm). After cultivation of MHSW, five bacterial isolates were obtained and identified based on 16S rRNA gene analysis as new strains of *Anoxybacillus flavithermus* (identity percentage ranges between 96–99%). Physicochemical analysis revealed that the *in situ* temperature was 59 °C, pH was 7.8, salinity was 1.6 ppt, and dissolved oxygen was 3.8 mg l^{-1}. In respect to heavy-metals content in MHSW, the following metals were present in the order: Cr (0.571 ppm) > Mn(0.169 ppm) > Fe (0.124 ppm) > Zn (0.095) > Cu(0.070 ppm) > Ni(0.058 ppm) > Cd (0.023 ppm) > Pb (0 ppm). Cd, Cr, Ni and Mn were found to be higher than permissible levels set by international organizations and countries. This study highlights new chemical and microbiological data about Ma'in hot springs.

Introduction

Jordan is known to contain several hot springs along the country. The mean temperature of these springs ranges between 45 °C to 80 °C [1]. Most springs are frequently visited by tourists for the purpose of balneotherapy. Among the well-known hot springs in Jordan is Ma'in hot springs which are found in a touristic place that contains a series of hot springs and waterfalls located about 58 km south from Amman, the capital of Jordan. This touristic place is about 150 m below sea level and the temperature of its water usually fall in the range of 42–63 °C.

Ma'in hot springs are known as a site of balneotherapy which involves the use of natural mineral water, gases, or peloids for therapy [2]. It was proposed that Ma'in hot springs can be used to heal diseases like ankylosis, arthritis, muscle contractions, influenza, respiratory system troubles, rheumatism, central circulation troubles and skin diseases [3]. Despite this medical importance of Ma'in hot springs, little is known about the biological activity and the chemical composition of their waters. For instance, some microbiological studies have already indicated that Ma'in springs are populated with a number of thermophilic bacteria. Consequently,

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several bacterial isolates were isolated from Ma'in hot-springs water (MHSW) and described. Most of the isolates from the MHSW were found to belong to the genus *Bacillus* [4–7]. Lately, we have isolated and identified two new thermophilic bacteria, namely, *Geobacillus pallidus* and *Anoxybacillus flavithermus* from MHSW [1]. In more recent study by other researchers, antimicrobial activity of some isolated thermophiles (aerobic thermophilic strains of the genus *Bacillus*) from Ma'in hot springs were also screened [8].

Literature survey clearly shows that hot springs from other countries were already explored and studied [9–11]. It was also demonstrated that thermophilic bacterial strains from such hot springs can produce several valuable products like thermostable enzymes, antibiotics, and anti-cancer substances [12,13].

Along the same line, literature survey indicates that no study was done to evaluate the antimicrobial activity of MHSW even though very few studies were carried out in other countries. Studying the antimicrobial activity of MHSW is important to answer the question about the curing effect of MHSW used for treatment of certain diseases, especially the skin infections. As mentioned before, some studies have been already done evaluate the antimicrobial activity of the water of certain hot springs in other parts of the world like the acidic water of Kusatsu hot-spring in Japan which was found to possess an antimicrobial activity against *Staphylococcus aureus* strains isolated from patients of atopic dermatitis [14,15]. The potential antibacterial activity of Kusatsu hot-spring's water was linked to ions like iodide and manganese in addition to acidic conditions (pH 2.0–3.0) [15].

In the current study, we aim at examining the antimicrobial activity of MHSW against different clinical and standard Gram-positive, Gram-negative bacteria and yeasts. We also aim at isolating and identifying the dominant bacteria in MHSW and examine their antibiotic-production potential. Finally, we aim at determining the physicochemical properties of the MHSW including the heavy-metals content.

Materials and methods

Sampling of MHSW

MHSW samples were collected from Ma'in hot springs located in Ma'in, Jordan (N 35-36-36, E 31-36-40) during April, 2014. The *in situ* temperature of water was 59 °C and the *in situ* pH was 7.8. Water was collected in sterile glass bottles and transported to the laboratory for further analysis.

Microbiological analysis

Enrichment and isolation of bacteria

MHSW water sample (10 ml) was transferred to nutrient broth (90 ml). The broth was incubated for at least 12 h in dark with shaking (100 rpm) at 40 °C. A portion of 0.1 ml from the enrichment culture was transferred to nutrient agar and allowed for growth at 37 °C for 24 h or more. Separated colonies were then obtained by streaking on nutrient agar for several times. A glycerol stock (30%, glycerol: broth culture) from each pure culture was subsequently prepared and stored at −80 °C.

Molecular identification of bacterial isolates

Genomic DNA was extracted from isolates as previously described previously [16]. Briefly, cells were pelleted at 7000 rpm, and then lysed, and finally protein was precipitated. The DNA was precipitated and 16S rRNA gene sequence amplification and sequencing were done by Macrogen Inc., Seoul, Korea as previously described [16]. The 16S rRNA gene sequence was analyzed by Basic Local Alignment Search Tool (blastn) (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi).

Phylogenetic analysis was carried out by maximum likelihood method based on the Tamura-Nei model [17] and a phylogenetic tree was constructed by the software Molecular Evolutionary Genetics Analysis (MEGA6) [18].

Antibiotic-production by the isolated bacteria

The ability of antibiotic production by the isolated bacteria was carried out by cross-streak method [19]. The test strains were *S. aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Micrococcus luteus* ATCC 9341, and *Bacillus cereus* ATCC 11778.

Antimicrobial activity testing of MHSW

Test microorganisms

Two types of test organisms were used in this part of the study: clinical and standard microorganisms. In respect to the clinical microorganisms, different bacterial and fungal isolates were obtained from diverse clinical sources such as: bloodstream infections, urinary tract infections, cerebrospinal fluid infections, and lower respiratory tract infections. Isolates were collected from the diagnostic clinical microbiology laboratories of King Abdullah University Hospital, Jordan. These clinical isolates were collected aseptically, and obtained from patients who admitted in King Abdullah University Hospital and suffering from many distinct bacterial infections. All clinical bacterial and fungal isolates were identified by standard bacteriological methods (bacterial species identification schemes were used). In the current study, definitive identification was based on bacterial cell morphology, Gram stain, and biochemical tests.

Clinical isolates in the present study which were used include eight Gram-negative bacterial strains: *Serratia marcescens*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Proteus vulgaris*, *Haemophilus infuenzae*, *Pseudomonas aeroginosa*; and four bacterial strains of Gram-positive cocci: *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecalis*, and *S. aureus*, and one yeast, *Candida albicans*.

In respect to standard microorganisms, they include *C. albicans* ATCC 10231, *S. epidermidis* ATCC 12228, *S. aureus* ATCC 29213, *M. luteus* ATCC 9341, *S. marcescens* ATCC 27117, *E. faecalis* ATCC 29212, *K. pneumoniae* ATCC 10031, *Pseudomonas aeruginosa* ATCC 27853, *E. aerogenes* ATCC 13048, *E. coli* ATCC 25922, *B. cereus* ATCC 11778, and *Salmonella typhi* ATCC 6539.

Antimicrobial activity assay

Antibacterial activity test was done by the agar well diffusion method [20]. Water samples were first filter sterilized through 0.2 μm pore size-nitrocellulose membranes and used. The test bacteria and yeast were freshly prepared and a fresh inoculum was transferred to Mueller-Hinton agar plates. Equidistant wells (6 mm diameter) on were done in the inoculated Mueller-Hinton agar plates. The wells in the Mueller-Hinton agar plates were filled with 50 μl of filter-sterilized hot spring water. The culture plates were kept to stand for few minutes to allow pre-diffusion and then incubated at 37 °C overnight or more. Inhibition zones around the wells were then measured in millimeters. The positive control for these experiments was ampicillin (10 mg ml^{-1}), whereas the negative control was sterile distilled water. All experiments were done in triplicates.

Physicochemical analysis and heavy-metals measurement

Temperature (°C) and pH were determined by a portable pH-meter (model HD 2105.1), whereas DO (mg l^{-1}) was measured by a portable oximeter (Pro 20, YSI, USA). The determination of salinity (PSU) was done by the titration method according to Strickland and Parsons [21].

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