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RESEARCH ARTICLE

Molecular Diversity of Bacterial Isolated from Cozy and Cold Spring Water in Mosul / Iraq

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Abstract

The study involved determining the phenotypic and genotypic characterizations of the bacterial isolates from cozy springing water. Three bacterial species were elite supported on morphological bacterial, physiological and biochemical tests. In addition to molecular diagnosis using 16srRNAsequences. The cells were Rod_cocci, non-motile and their colonies appeared pigment red, yellow-orange and white, the range of their growth was at ph 4-8, temperature range 10-40 and the range of growth in NaCl 2-15%. All isolates were aerobic, positive for catalase, oxidase, nitrate redaction and negative methyl red, also produced acid from fermentation of (arabinose, fructose, maltose and trehalose), while our isolates exhibited variable results formation of acid from sugares such as glucose, galactose, mannose, sorbitole, lactose and sucrose). Also the isolates were resistant for the foiiowing antibiotics amoxicillin, carbencillin, erythromycin, streptomycin and tetracycline but with various resistance to gentamycin and novobiocin. The 16srRNA sequences were performed by the ABI 3130XL gene analysis in Biology Bolumu 21280_Diyarbakir/Turkey. The isolated strains were mainly *Serratia nematodiphila*, *Staphylococcus saprophyticus* and Bacillus *vietnamensis*. Bioinformatic analysis alignment was done by Clustal W program mega 5 and phylogenetic tree analysis was constructed and drawn by Nieghbour_ joining program.

Keywords: *Phylogenetic tree, Spring water, Bioinformatic.*

Introduction

The long success and vast diversity of microbes reflect the variety of habitats found on earth and water. In addition to differences in temperature, pressure, salinity, pH, and nutrient availability, Variation in redox potential is also extremely important in determining microbial activity, The Bacteria are ubiquitous and highly divers they can survival in all kinds of inhospitable environments [1, 2].

Research Studies in the last twenty years have discovered that 99% of the bacteria available in our environment are still unknown or unnoted in laboratory cultivation and accordingly remain vague as far as their ecological functions are concerned moreover, They remain unexploited for biotechnological applications, Recent molecular phylogenetic analyses which are based on a small subunit 16rRNA sequencing have shown that the microbic diversity of present microbial communities is way bigger than the previously assumed methods which were based on standard cultivation and isolation [3, 4].

Although several studies centered on the identification and variety of prokaryotes of the domain Bacteria have been conducted, increasing attention is currently paid to the genetic diversity and ecological significance of phylogenetic prokaryotes of the eubacterium and archaea [5, 6]. Currently, generally accepted that life originated between 3.5 and 4 billion years past within the aquatic environment, at the start as self-replicating molecules. Potential disadvantages of aquatic environments include osmotic differences between the organism and also the encompassing aquatic medium and a high degree of physical disturbance several aquatic systems [7, 8].

In order to highlight the important bacterial species present in cozy spring waters, and because there are no prior studies at the molecular level, as well as the lack of interest in aquatic springs, their contents and their biological composition as natural environments, this first study was planned locally to attract researchers' attention to

further studies on environments of springs and Iraqi environments, Therefore, the aims of this study are isolation of bacteria from cozy spring water, classification of the isolates and determination their phylogenetic trees.

Materials and Methods

Study of Site and Samples Collection

- The springs near the fortress of Bashtabia stem under the wall of the Nineveh wall on the right side of the city of Mosul in the northern province of Nineveh on the bank of the right Tigris River. Fig.1.
- Hammam Alalil lies west of the Tigris River, 20 km south of the city of Mosul is characterized by high sulfur content. Fig.2.

In situ samples were collected during the collection time and included water temperature ranging from 20-40 C^{O}. The PH recorded 7-8 indicating that it is a base function.

Samples were collected from October to December 2017 from four different locations, using sterile plastic containers, containing a transporter medium broth; samples were transferred to the laboratory for bacteriological tests.

Fig.1: GPS map showing springs near the fortress of Bashtabia

Fig.2: GPS map showing location of Hammam Al-Alel

Isolation and Identification

Water samples were serial diluted and 1ml from 10^{-3} dilution sample was plated on Luria Bertani (LB) and pepton yeast extract (PYE) agar by pour plate technique. The plates were incubated aerobically condition at 37C^{O} ±2 for 7- 10 days Selected water isolates were grown on LB and PYE agar media Colonies were enumerated and hand-picked colonies variant in shap, size and colouring were selected and sub cultured to get pure culture, Then it preserved using (LB

and PYE) broth containing 15% glycerol at -70C°.

The shape and colours of colonies were examined under microscope then isolates were biochemically analyzed for the activities of oxidase, catalase, voges-proskauar test, methyl red, gelatin and starch hydrolysis, motility, in dole test and citrate utilization, the isolates according to Bergys manual [9,10].

Determination of Optimal Growth Condition

The optimal growth condition with reference to NaCl, PH and temperature were determined : the isolates were grown in LB and PYE medi with different PH values (4,6,8 and 10), and incubation was carried out at temperatures 10,15,20,25,35,40 and 50) C°. The ability to growth in different concentrations of (2, 4, 6, 8, 10 and 15) %w/v NaCl.[11, 12].

Antimicrobial Susceptibility Testing

After twenty four h incubation at 37±1°C, the cells were centrifuged and far from the medium with saline. The matter suspension in saline with McFarland density of 0.5 was fastidiously unfold on Petri plates containing the quality growth medium.Once the suspension was absorbed by the agar. When the suspension was absorbed by the agar, applying antibiotics of µg/disc Amoxicillin 30, Carbenicillin 25, Gentamicin 30, Erythromycin15, Streptomycin 25, Novobiocin 30, Tetracyclin 10, piperacillin 30. Were purchased from Oxoid [13, 14].

DNA Isolation and PCR

DNA isolation was done by using genomic DNA purification Kit (Promega, USA) depending on the manufacture instructions. 16srDNA genes of the bacterial were isolates amplified by PCR with specific primer sequences that used (27F 5′-AGAGTTTGATCMTGGCTCAG, 1492R 5′-TACCTTGTTACGACTT) primers consist of forward and reverse respectively.

PCR was performed with a 50µl 1X buffer reaction mixture containing 1,5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 pmol primers and added 1.5 U Taq polymerase used as recommended by manufacturer. A PCR interaction component was carrid out in appendroff PCR Thermo cycler (Germany). The reaction consists of an initial denaturation step 94˚C, 5 minutes, followed by 35 cycles of denaturation 94˚C, 35 seconds, annealing 58˚C, 1.35 min and elongation 72˚C, 1.35 min. A final elongation step 72˚C, 10 minutes has been followed by cooling to 4˚C. PCR products were analyzed by 1.5 % w/v agarose gel electrophoresis in 1X TAE buffer with ethidium bromide 0.5µg/ml.

DNA Sequnce Analysis

The device ABI 3130XL Genetic analysis in (BIOLOGY BOLUMU 21280-DIYARBAKIR/ TURKEY) was used for DNA analysing and sequencing. Sequencing homology search was conducted with previously published bacterial 16srRNA sequences in the NCBI data bases using basic local alignment search tool (Blast) program wich is available at the national center biotechnology information

Construction of the Phylogenetic Tree

The phylogenetic analysis based on the scoring index the most similar sequences of other representative bacterial 16srRNA regoin was constructed. The clustal W program Mega5 program was used. Aphylogenetic tree UPGMA method and compared with known sequences and drawn by Neighbour-joining program (NJ).

Results and Discussion

Isolation and Identification

All isolates were subjected to the cultural, microscopial, biochemical, physiological examinations and molecular study for identification to the strains level Table1. The Colonies appeared red and yellow-orange and white with entire margin Fig (3), (4) and (5) on both media (PYE and LB), Microscopic examination and gram stain showed negative and positive rods and cocci bacteria, single or chains., all of them were motile and all bacteria were Gram positive except Serratia was Gram negative according to [15, 16].

Biochemical tests identify three genus Bacillus, *Staphylococcus* and *Serratia*. The percentages of these three genus were (48%, 28% and 22%) respectively. They are catalase and oxidase positive and reduce nitrate to nitrite while negative for gelatin hydrolyse except *Bacillus* were positive in latter test. All isolates except *Serratia* were positive for indole test, negative for methyl red, positive for voges-proskauar and negative for citrate utilization, positive growth on manitol salt agar.

Although many isolates produced enzymes such as *Serratia* and *Staphylococcus* produced protease, while all isolates except *Saphylococcus* did not produce lipease and lecethinase enzyme according to [17, 18].

Figure.3: Staphylococcus Saprophyticus on PYX agar

Figure: 4: Bacillus vietnamensis on PYX agar

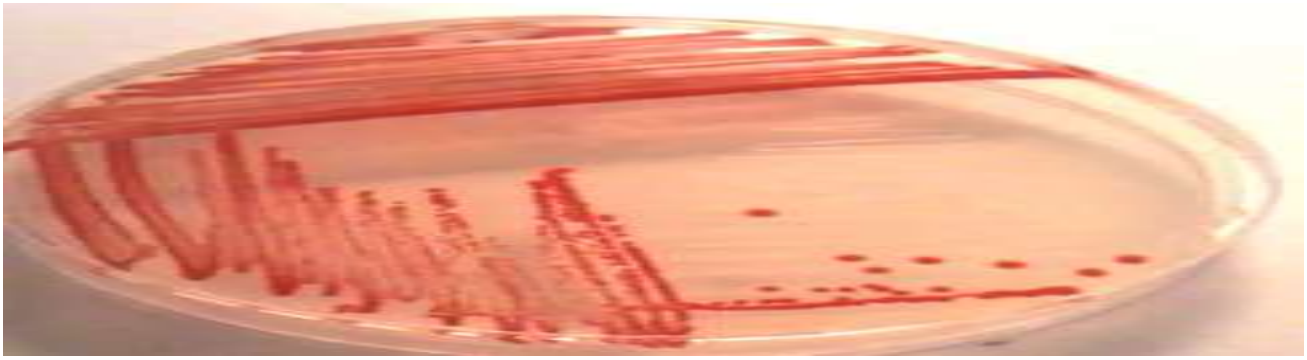


Figure: 5: Serratia nematodiphila on nutrient agar

Determination of Optimal Growth Condition

The results showed that the bacterial isolates were able to multiply at the pH values above 4. They were suitable also growth at PH 7.5 were optimal range was between7-8. The isolated bacteria stopped the activity and the effectiveness of their enzymes and the stability of the proteins and could not grow when the acid function was less than 4 because the high acidity reduces the activity of the proteins and affects the transfer of materials inside [19].

The current study showed that the optimum temperature for the species under study ranged from 37 to 40 C°. The enzyme activity increases in this temperature and increases the duration of the logarithmic phase. Too much temperature leads to the enzymes that are essential to cell life and leads to the reduction of the logarithmic phase, and may affect the fat of the cell membrane of the bacterial cells. Also, the low temperature will cause the protein to become inactive.

The results showed that the minimum temperature at which the isolates grew was 10 C° and the maximum temperature was 50 C° [20], the results showed the bacterial isolates could grow at the concentration of sodium chloride between 2-15 %. The best growth of these bacteria was between 7-8% and the lowest concentration in which

isolates grew 2% while the highest concentration was 15%. The different concentrations of sodium chloride Changes in the appearance of bacterial species where these bacteria are found in the form of regular cells in the ideal concentrations of sodium chloride, but appear in heterogeneous forms when growing with saline concentrations is not ideal for sodium chloride, where the shape of the bacillus turns into the ball and the later decomposition [21, 22].

Antimicrobial Susceptibility Testing

Antibiotic sensitivity was examined out by Kirby-bauer disc diffusion method following national committee for clinical laboratory standard [23] Muller hinton agar oxid wase used as the culture medium the followig antibiotics were applied µg/disc Amoxicillin 30, Carbenicillin 25, Gentamicin 30, Erythromycin15, Streptomycin 25, Novobiocin 30, Tetracyclin 10, piperacillin 30. The antibiotics were purchased from Bioamalyse Company /Turkey The result was recorded as sensitive and resistance after 24h of incubation at 37±2 C°. The zone diameters around all discs were interpreted by using the recommendation of the NCCL [24, 25] Table 2.

Table 1: Phenotypic characteristics of the strains isolated from sulfur springing water:

Phenon	**Bacteria**		
species	*Serratia*(10)	*Staphylococcus*(13)	*Bacillus* (22)
Gram stain	-	+	+
Catalase	+	+	+
Oxidase	+	+	+
Nitrate reduction	+	+	w
Gelatin	-	-	+
Indole	_	+	w
Methyl red	-	-	-
Voges – Proskauar	+	+	+
Citrate	+	w	-
Manitol	-	+	+
protease	+	+	-
Lipease	-	+	w
Lecithinase	-	+	-
Starch	-	+	-
Carbohydrate Fermentation			
Arabinose	+	+	+
Fructose	+	+	+
Maltose	+	+	+
Trehalose	+	+	+
Glucose	+	-	+
Galactose	+	-	+
Mannose	-	w	+
Sorbitol	-	+	+
Sucrose	+	-	-
Lactose	+	w	-

+ = positive, - = negative, w = weak

Table 2: Resistance patterns of Strains to antibiotics

Antibiotic	**Bacteria**		
	Serratia	*Staphylococcus*	*Bacillus*
Amoxicillin	R	R	R
Carbenicillin	R	R	R
Erythromycin	R	R	R
Gentamicin	R	S	S
Novbiocin	I	R	R

Piperacillin	R	R	I
Streptomycin	R	R	R
Tetracyclin	R	R	R

R: resistant, I: intermediate, S: sensitive

Molecular Study

Phylogenetic Analysis

A PCR amplification of 16srRNA gene in (Biology Bolumu21280_Diyar Bakir / Turkey) PCR product size at 1500 bp compare with the 100 bp ladder when analyzed through 1.5 % agarose gel electrophoresis as in the Fig 7.

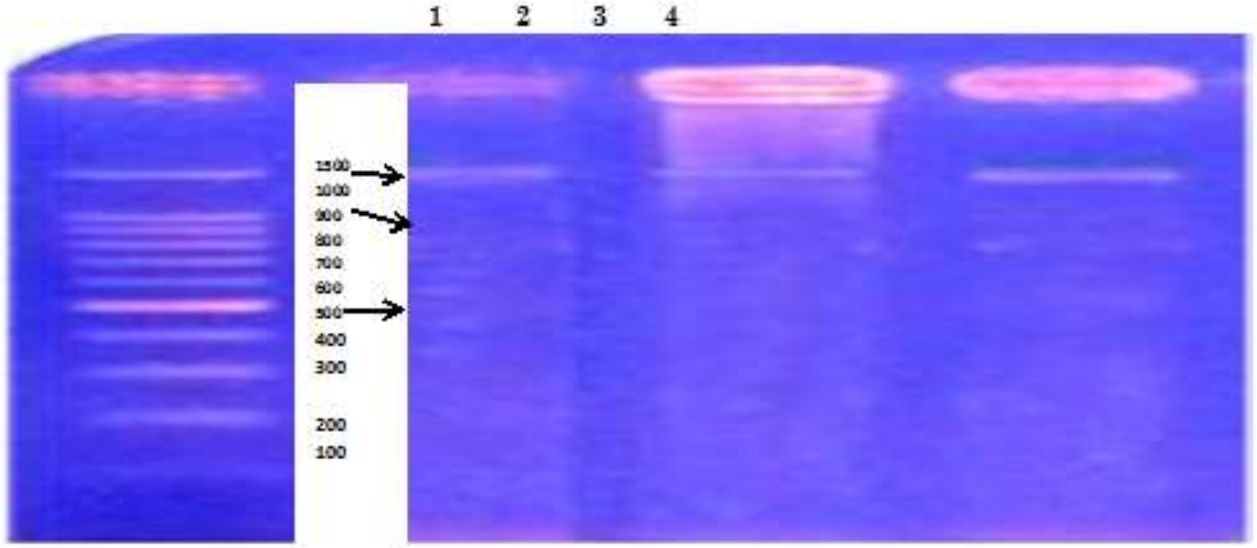


Figure 6: Agarose gel electrophoresis of 16srRNA gene 1500 bp bands using universal primers 27F& 1492R **Lane 1: DNA ladder 100bp, lane 2: *Serratia nematodiphila*, lane 3: *Staphylococcus saprophyticus* lane 4: *Bacillus vietnamensis***

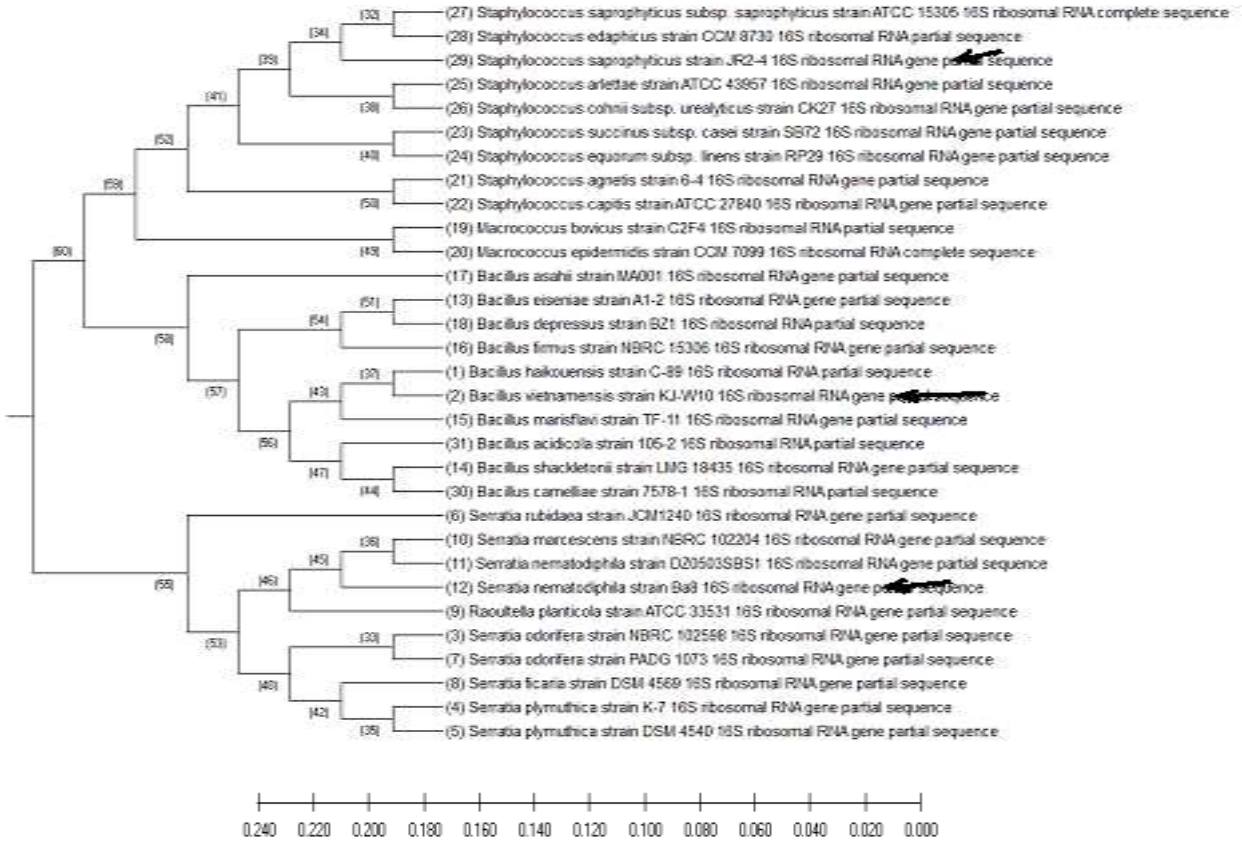
The 16srRNA gene sequence obtained was analyzed by the NCBI genbank BLAST analyses on partial 16srRNA gene sequences of each isolate were performed with the genbankBLAST (http//www.ncbi.nlm.nih.gov) search tool. *Staphylococcus saprophyticus* JR2-4*, Bacillus vietnamensis* ks- w10 *and Serratia nematodiphila* Ba8 isolates exhibited 90 -98% similarity with the sequnces in the NCBI genbank database Fig: 8. A phylogenitic tree was constructed based on comparison of the 16s rRNA sequences of reference strains showing high similarities by using MEGA 5 phylogenetic tree was constructed using the UPGMA method. The strain grouped into three major clusters A, B&C.

Within each of clusters A, B&C strained shared identical 16s rRNA gene sequence compared with the sequencing of the *Bacillus*, *Staphylococcus* and *Serratia* respectively taken from NCBI. From the results, it can be seen that all Bacillus isolates cultured clustered together (the appear side of the tree) including Bacillus *vietnamensis* ks- w10. B.V. KS-W10 appears to be closely related to B.h.c. 89 and B. m. TF-11.

Also, the strains of Staphylococcus species were clustered to gether (the middle side of the tree) including S.s JR-2-4 that was closley related to S.e. ccm 8730 and S.sap. ATCC 15305 strains. However, S.s JR-24 belongs to different species according to the results. Finally, *Serratia nematodiphila* Ba8 was closly related to S.nemDZ0503SBS1 and S.nem NBRC 102204(the lower sid of the tree). In general, *Bacillus* and *Staphylococcus* species were close to gether according to their 16srRNA sequencing as both genera are Gram positive while *Serratia* species were Grame Negative. The obtained results give the power of 16srRNA for identification and classification of bacterial strains isolated from water samples including spring water. Figure 7: phylogenetic tree of Staphylococcus, *Bacillus* and *Serratia*. Depending on 16srRNA by Clustal W. The evolutionary history was inferred using the UPGMA method [26].

The optimal tree with the sum of branch length = 0.61618642 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

The evolutionary distances were computed using the Maximum Composite Likelihood method [27].And is in the units of the number of base substitutions per site. This analysis involved 31 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1583 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5 [28].



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