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Isolation and identification normal flora bacteria from different areas of Ninava governorate

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ABSTRACT: In this study, different types of bacteria were isolated and diagnosed from soils collected from different areas of Ninava governorate, new strain of bacteria was discovered, based on biochemical tests and genetic examination (16S rRNA) and the strain was recorded under accession number (MW690182) in the Gene Bank (NCBI). The isolated bacteria were *Bacillus Subtilis* strain that given new name (AHM1976) it was positive for gram stain, motility, hemolytic, beta type.
Keywords: isolation; identification; normal flora bacteria.

Isolamento e identificação de bactérias da flora normal de diferentes áreas da província de Ninava, Iraque

RESUMO: Neste estudo, diferentes tipos de bactérias foram isolados e diagnosticados em solos coletados em diferentes áreas da Província de Ninava, Iraque. Foi descoberta uma nova cepa de bactéria, com base em testes bioquímicos e exame genético (16S rRNA), sendo essa cepa registrada sob o número de acesso (MW690182) no Gene Bank (NCBI). A bactéria isolada foi a cepa *Bacillus Subtilis*, que recebeu novo nome (AHM1976), testando de forma positiva para coloração de gram, motilidade, hemolítica, tipo beta.
Palavras-chave: isolamento; identificação; bactérias da flora normal.

1. INTRODUCTION

Bacteria are among the most widespread forms of life on Earth. One gram of soil contains more than 40 million bacterial cells and nearly a million bacterial cells per millimeters of fresh water (WHITMAN, 1998; RAMBO et al., 2019). Bacteria make up the biomass that exceeds the biomass of plants and animals (KUNDAVVARAM, 2015).

Because of the large number of bacterial species that live on Earth, they have not been fully identified and identified. Therefore, many technological and biological techniques have been developed to identify these different types, such as the VITEK2 device, DNA hybridization, PCR techniques, which are the most popular at the present time, and biochemical tests.

Among these bacteria spread in the soil are Gram-positive Bacillus.subtilis bacteria, which is one of the best types of bacteria that are used for basic research on bacteria because of its unique characteristics such as its response to inappropriate environmental conditions such as lack of food sources, temperatures, pH, osmotic pressure, and the formation of a rich (Biofilm) layer It contains sugar, protein, fatty compounds and nucleic acids (MIROUZE; DUBNAU, 2016). Therefore, bacteria B. subtilis were used in laboratory studies aimed at discovering the basic characteristics of gram-positive bacteria and the formation of spores (EARL et al., 2008).

2. MATERIALS AND METHODS

2.1. Collection of samples

Soil samples were randomly collected from different areas of Nineveh governorates. Soil samples were taken from five areas. Samples are put in sterile polyethylene bags and the required information (sample number, date, weight) was recorded on them and transferred to the laboratory and kept in the refrigerator at a temperature of 4 C° until use.

2.2. Isolation of bacteria

The collected soil samples were mixed homogeneously and passed from a 2 mm sieve to get rid of the unwanted gravel and impurities. The dilution and plate method was used to isolate the bacteria by adding 1 g of soil samples to 9 ml of distilled water (DW) and after homogenization, the dilution series was prepared from 10^{-1} to 10^{-4}, after which the decanting method was used, where 1 ml of each dilution was transferred to a sterile Petri dish and added to it the nutrient medium, MacConkey agar medium and blood agar medium, with three repetitions for each dilution, and the dishes were incubated at a temperature of 37 C° for 24 hours. 48 hours.

2.3. Purification

Depending on the shape, texture and color of the bacterial colonies growing on the petri dishes, the bacterial isolates were purified and with the help of the differential and selective media (MacConkey and Mannitol) as well as the nutrient medium so that this technique was repeated to obtain pure isolates, after obtaining the pure isolates, they are stored formally. Sterilized and safe at a temperature of 4 C°.

2.4. Identify and diagnose bacteria

2.4.1. Phenotypic diagnosis

The phenotypic characteristics of the developing colonies were relied on the nutrient agar media in terms of texture, shape, size, height, color and odor in the diagnosis of bacterial isolates (LOGAN, 2009).

2.4.2. Microscopic diagnosis

A Loop campaign was taken from the developing colonies and spread them in a circular motion on a glass slide after being heat-fixed and stained with a gram stain (COLLEE et al, 1996), depending on the chemical and physical properties of the cell wall and using an oily lens of 100x strength, the shapes of bacteria were identified (SONI, 2013).

2.4.3. Biochemical Indemnification Test

1. The Oxidase Test

A drop of oxidase reagent is placed on a filter paper, after which a portion of the bacterial culture is transferred by a sterile wood stick. We wait about 10 seconds. The positive result is the appearance of a blue color (FORBES et al., 2007).

2. The catalase test

We transfer a Loop campaign from the bacterial colony to a glass slide and sprinkle it with a few drops of catalase reagent (3% hydrogen peroxide). The appearance of bubbles is evidence of a positive result (FORBES et al., 2007).

3. Voges – Proskauer test

After preparing the MR-VP medium in laboratory tubes, these tubes were inoculated with bacterial growth and immunized at 37 C° for 24 hours. After the incubation period, 0.5 ml of VP1, VP2 solution was added, waiting for about 30 minutes, the appearance of the red color evidence of a positive result (ABEDIN, 2015).

4. Motility

The movement medium was inoculated by the stabbing method using the needle with bacterial growth and incubated for a period of 24 hours at a temperature of 37 C°. The appearance of a cloudy area in the movement medium is evidence of the positive of the examination as for the growth of the stabbing area, only evidence of the negative result (FORBES et al., 2007).

5. Citrate consumption test

The medium of consumption of citrate was inoculated with the bacterial inoculum and immunized for 24 hours at 37 C°. The transformation of the medium from green to blue is evidence of positive testing (CAPPUCCINO; SHERMAN, 2010).

6. Mannitol test

The bacterial isolates were cultivated on a medium of mannitol for a period of 24 hours at a temperature of 37 C°. The color of the medium changed from red to yellow, indicating a positive result (fermentation of mannitol sugar), while the remaining red color was considered a negative result (non-fermentation of mannitol sugar) (MUDAWI et al., 2007).

7. Test of indole production

The tubes containing the indole medium were inoculated with bacterial growth and immunized for 24 hours at a temperature of 37 C°, after which 5-6 drops of the Kovac's reagent were added, the appearance of the red ring representing the positive result and its absence representing a negative result (MACFADDEN; JEAN, 2013).

8. The urease test

A slant tube containing urea medium was inoculated with bacterial growth by the planning method and immunized for 48 hours at a temperature of 37 C°. The color of the medium changed from yellow (acid) to pink (alkaline) representing the positive result, and the reason is due to the ability of the bacteria to obtain the production of the enzyme urea, which works on the analysis of urea and the production of ammonia, which increases the alkalinity of the medium and there is evidence in the medium that turns pink in the alkaline conditions (COLLEE et al., 1996).

9. Triple iron sugar test

An oblique tube containing TSI-iron agar medium was inoculated with bacterial growth by planning method and pricking to the depth of the medium and incubated 48 hours at 37 C°. After the end of the incubation period, the color of the medium, gas formation, and hydrogen sulfide production were observed (ATLAS, 1995; COLLEE, 1996).

2.5. Molecular Biology Identification

2.5.1.DNA Extraction

The analysis kit provided by Geneaid was used to extract DNA from bacterial samples.

1. The precipitate of bacteria that were grown in the liquid medium is taken into a 1.5ml Eppendorf tube

2. Add 200 µl to an Eppendorf tube with Lysozyme at a concentration of 0.8 mg / 200 ml with mixing with Vortex.

3. The tube was incubated at 37 C° for 30 minutes and the tube was turned over every 3 minutes during the incubation period.

4. Add 20 µl of Proteinase K and mix Vortex

5. The mixture was incubated at 60 C° for 10 minutes.

6. Add 200 µl of GB buffer, mix with Vortex and incubate at 70 C°.

7. 200 µl of absolute ethanol are added and mixed manually, then the mixture is transferred to the GD tube installed in the collection tube and a (16000g) centrifugation is performed for 30 seconds and the filtrate is removed, then 600 µl of the washing solution are added and centrifuged at the same speed and time before the sediment is removed. Re-centrifuge for 3 minutes to get rid of all washing solution residues.

8. The GD column is transferred to a new 1.5 ml tube and 100 µl of dissolving solution are added, then left for 3 minutes. After that, centrifugation at 16000g for 30 seconds is carried out, then the DNA is kept at -20 ° C until use.

9. Quanti Fluor® (Promega, USA) was used to measure DNA concentration. 199µl quantus stain was mixed with 1µl of DNA sample and left in a dark place for 5 minutes after which the DNA concentration was measured by a quantus ™ fluorometer.

2.5.2. DNA in Gel Electrophoresis

Electrophoresis was performed to separate the extracted DNA mixture and test it according to the following steps: (MISHERA et al., 2009; SAMBROOK; RUEESL, 2006).

1. To prepare the 1% agarose gel, 1 g of agarose powder was dissolved in 100 ml of TBE 1x buffer and 3 µl of red safe dye was added by using a heat source with constant stirring until boiling. Then it was left to cool down to a temperature of (60-50 C°).

2. The tray was prepared for pouring gel, and the two edges of the template were surrounded by adhesive tape and the comb was fixed to create the wells at a distance of 1 cm from the edge of the template. After that, the gel was poured into the template, which is placed completely horizontally, taking into account that the pouring was quietly in order to avoid the formation of bubbles and leave for a period 30 minutes to harden, after which the comb and tape gently lifted from the template and placed in the electrophoresis bath that contains buffer1x TBE so that the agarose gel covered approximately 1 mm because the large amount causes the DNA bundles not to appear in the horseshoe shape or straight and evenly.

3. Migration samples were prepared by mixing 5 µl of DNA sample with 3 µl of loading solution

4. Using a (2000bp) ladder by taking 6 µl of it and mixing it with 2 µl of bromophenol dye solution and taking out 5 µl.

5. The electric migrate was Done under a voltage of 100V for a period of 60 minutes.

6. The gel was examined by UV Trans illumination and photographed by a camera.

2.5.3. Polymerase chain reaction

The DNA concentration in all study samples is adjusted by dilution with TBE buffer solution to obtain the required concentration for PCR reactions and it was (50) nanogram(ng) / µl per sample. As the Master Reaction mixture was prepared for each PCR reaction by mixing the DNA sample and the initiator of each gene with the master-mix components inside a 0.2 ml Eppendorf tube supplied by the English company Biolabs, the reaction volume was fixed to 20 µl with distilled water, the mixture was in the Microfuge device for a period between (3-5) seconds to ensure that the components of the reaction were mixed, then the reaction tubes were inserted into the Thermocycler device for the purpose of performing the amplify reaction using the special program for each reaction, after which the sample was loaded into the wells of pre-prepared agarose gel at a concentration of 1% with the addition of the volumetric guide Ladder DNA prepared by Biolabs in one of the wells, then the samples are migrated by running the Electrophoresis device for a period of (60-70) minutes, after which the gel is photographed using the UV Trans illumination device.

The PCR polymerase technique was used to amplify the DNA encoded for the 16S rRNA gene and using the prefixes shown in Table (1) with the DNA extracted in paragraph (2.5.1), 4 µl (100 ng) of DNA template were added with 1 µl (10 picomole) of each Gene-specific prefix to Master Mix contents.

Table 1. Universal primers.
Tabela 1. Primários universais.

Primer	Sequence	Bp size	Ref.
Forward Reverse	GACCTCGGTTTAGTT CACAGACACACGCTG ACGCTGACCA	1200	Kumar et al. (2016)

The reaction was carried out according to the leaflet supplied with the kit supplied by Geneaid with a volume of 20 µl according to the Table 2. The program shown in Table 3 was followed to amplify the DNA samples with PCR technology.

Table 2. Components and volumes of the polymerization reaction mixture.
Tabela 2. Componentes e volumes da mistura de reação de polimerização.

Reaction components	Volume
Master MIX	5 µl
Primer Forward	1 µl
Primer Reverse	1 µl
DNA	5 µl
dd.H2O	8 µl
Total	20 µl

Table 3. DNA amplification program.
Tabela 3. Programa de amplificação de DNA.

No.	Stage	Temperature (°C)	Time	Cycle number
1.	Initial denaturation	95	6 min.	1
2.	denaturation	95	45 sec.	
3.	Annealing	56	1 min.	35
4.	Extension	72	1 min.	
5.	Final extension	72	5 min.	1

2.5.4. Electrophoresis of samples

The same method was used for DNA detection, where a solution (1500bp) ladder was placed in the first hole of the gel in order to locate the 16S rRNA Gene in the carried DNA. After the migration process was completed, the samples were examined by a (UV Trans illumination) device to view the orange beams that were photographed by the camera.

2.5.5. DNA extraction from agarose gel

The bands resulting from the PCR reaction were extracted from the gel to be purified and sent for a nucleotide sequence sequencing test, based on the analysis kit supplied by Geneaid, according to the steps:

1. The bands are cut from the agarose gel using a sterile scalpel with the removal of the largest amount of gel surrounding the bands.

2. Transfer approximately 300 mg of gel piece to a 1.5 mL Eppendorf tube and add 500 µl of buffer solution DF and mix using a Vortex device.

3. The tube is incubated at a temperature of 55-60 C° for 15 minutes to ensure a complete piece of gel with inverting the tube every 3 minutes during the incubation period. The tube is left to cool down at room temperature.

4. Transfer 800 µl of the sample mixture to a DF column installed in the collection tube, then perform a centrifugation with a force of 16000g for 30 seconds and the filtrate is removed.

5. Re-install the DF column again to the collection tube, add 600 µl of washing solution and leave for one minute.

6. Conduct a centrifuge with the same force of 16000g for 30 seconds and get rid of the filtrate.

7. The previous process is repeated.

8. Conduct a 3-minute centrifugation to ensure that the DF column is dry.

9. The collection tube is discarded and the DF column is transferred to a new 1.5 mL tube.

10- (20-50 µl) of Ellution buffer solution are added to the center of the column.

11- Leave for two minutes to ensure absorption of the dissolving solution.

12- Two-minute centrifugation of 16000g to obtain the dissolved DNA.

2.5.6. Detection of genetic sequences

After the completion of the DNA extraction process and the PCR process, the samples were sent to Macrogen (South Korea), where 20 µl of each sample was placed in an Eppendorf tube and 50 µm of the primers were placed in other Eppendorf tubes to determine the genetic sequence of the bacterial isolates, depending on the Genetic Analyzer 3130 device. Equipped from Japanese Hitachi company.

The gene sequences were matched with data available at the National Center Biotechnology Information (NCBI).

3. RESULTS

3.1. Isolation and identification of bacteria:

The results of Table 4 showed the bacterial growth that was cultured on nutrient agar medium, the growth of bacterial isolate from soil samples, which were collected from different areas of Nineveh governorates, at dilutions from 10^{-1} to 10^{-4}, Studies differ in the possibility of isolating different types of bacteria, as Azeez; Shareef (2012) managed to obtain six bacterial isolates from different regions of Nineveh Governorate and its dependent regions, while (JASIM, 2016). Isolation of 44 bacterial isolates from soils for different regions of Sulaymaniyah Governorate.

Table 4. Phenotypic characteristics of bacterial isolate.
Tabela 4. Características fenotípicas do isolado bacteriano.

	Isolate	*Bacillus subtilis* strain (AHM1976)
Nature of colonies on solid nutrient medium	Shape	Circular
	Colour	Light pink
	Texture	Creamy
	Appearance	Dark
	Hight	Flat
	Edge	Regular
	Gram stain	+
	Cell pool	Single and binary
	Cell shape	Rod

3.2. Biochemical tests

Biochemical tests, which included the tests shown in Table 5 based on (Collee et al., 1996; Macfadden; Jean, 2013), where the test for catalase, oxidase, hemolysis, and culture was performed on McConkey's medium, mannitol medium, motility test, IMVIC group of tests, as well as TSI test (Triple Sugar Iron) and urease test, the growing isolates were diagnosed based on colony shape, color, edge shape, interaction with gram stain and biochemical assays (Brookse et al., 2013) as shown in Tables 4 and 5 and Figures 1 and 2.

3.3. Molecular Diagnostics

The final diagnosis of bacterial isolates was made using DNA Sequencing technology due to the ability of this technique to diagnose genus and species with high accuracy and in less time than traditional methods of diagnosis, where special primers are used to amplify this gene, which contains specialized regions that allow the diagnosis of genus and species. This gene consists of many nucleotides (JENKIS et al., 2012).

16S rRNA technology is considered one of the basic criteria for classifying and identifying bacteria due to the difficulty of genetic mutations in them, and because this gene contains high change regions between bacterial species and provides an independent sequence for each bacterial species. (ABDULLAH; MAHDI,2016).

Table 5. Biochemistry tests for bacterial isolate.
Tabela 5. Testes bioquímicos para isolado bacteriano.

N°	Tests	*Bacillus subtilis* strain (AHM1976)
1	Motility	+
2	Indole	-
3	Methyl red	+
4	Voges-Proskaoer	-
5	TSI	A\A
6	Oxidase	+
7	Catalase	+
8	Urease	-
9	Simmon Citrate	-
10	MacConkey agar	-
11	Mannitol salt agar	+/Non-F
12	Blood hemomysis	β

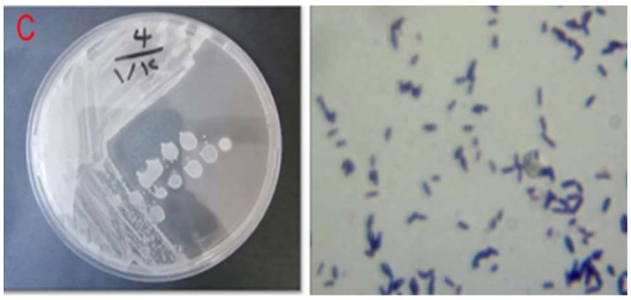

Figure 1. Bacterial cell growth on nutrient agar and its Shaps under microscope.
Figura 1. Crescimento de células bacterianas em ágar nutriente e suas formas ao microscópio.

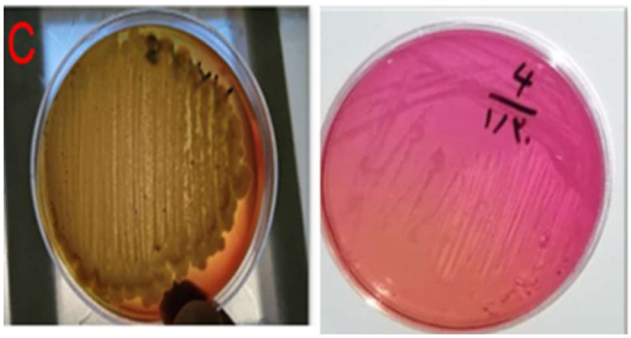

Figure 2. Bacterial cell growth on Blood and MacConkey agar.
Figura 2. Crescimento de células bacterianas em ágar Blood and MacConkey.

3.4. Polymerase chain reaction PCR

The results of electrophoresis on an agarose gel of DNA samples extracted from bacterial isolates using the 16S rRNA gene primer showed that the DNA bundles were about 1200bp as shown in Figure 3.

3.5. Detection of a nucleotide sequence

The results of samples sent to Macrogen Company in North Korea, and in comparison, with the database of the National Center for Biotechnology NCBI, showed the discovery new strain of bacteria, and it is registered on the NCBI website for the first time with new names and given a special number called an Accession number (MW690182).

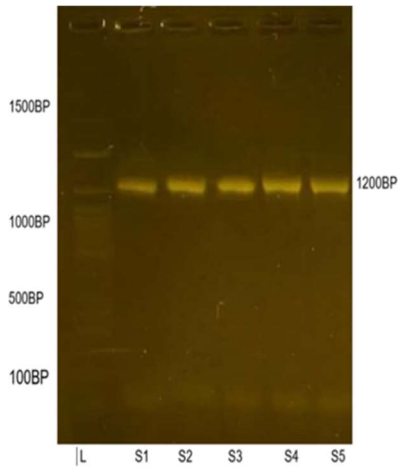

Figure 3. Agarose gel electrophoresis showing the analysis of the PCR product for the 16S rRNA gene of bacterial isolate.
Figura 3. Eletroforese em gel de agarose mostrando a análise do produto de PCR para o gene 16S rRNA do isolado bacteriano.

4. DISCUSSION

Through this research, it became clear to me that the bacteria present in different soils of Nineveh Governorate are exposed to mutations. The reason for this may be due to the excretion of factory waste and war remnants in different environments. It is also clear that the effect of fertilizers and hydrocarbons on the sequence of genes in the natural bacteria of the soil is obvious.

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