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MOLECULAR AND BIOCHEMICAL DIAGNOSIS OF LOCAL ISOLATES OF *STREPTOMYCES* SPECIES FROM SOILS OF IRAQ

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ABSTRACT

In this study, (154) local isolates of *Streptomyces* has obtained from soil samples collected from different locations in some regions of Iraq (Nineveh, Erbil, Duhok). The isolates showed bioactivity against some negative and positive species of bacteria, gram stain and species of fungus. The *Streptomyces* isolates are microscopically and morphologically diagnosed. The results Polymerase chain reaction specific-PCR a 8 bands of DNA gene elected from bacteria local isolates with one volume rang between (900-1000) base pair. These bands are kept the used DNA ladder volume. The nitrogenic base sequences determined the Polymerase chain reaction products of DNA Samples elected from 6 local isolates. The results of analysis shows, using DNA Blast NCBI, the species are from the following: *S. luteus, S. thermocarboxydus, S. griseorubens, S. albogriseolus* and *S. deccanesis* are the same genus of biochemically and microscopically diagnosed *Streptomyces*.

***Keywords*:** Sequencing analysis, specific PCR. *Streptomyces*.

Introduction

The actinomycetes are thread like, Eukaryotic, single-celled, Gram-positive bacteria. They grow as branched threads over the media which contains them known as the hyphae. They either elongate to form the mycelium or segregate to form bacillus or cocci cells (Chater, 2016). The colonies might be Leathery, Chalky, waxy, or mucus-like cells. Sometimes it is embedded inside the media they are normally aerial and substrate, or substrate hyphae (Al-Obaidi, 2012). They are shown in different colors, as they produce multiple pigmentations that affect the color of the colony or the color of the media surrounding it.

One of the geniuses of Actinomycetes is *Streptomyces*, which is considered as the most medical antibiotic-producing genus -that is used in aiding the immune system- of all the Actinomycetes genus, they are mostly grey series, but few are red, white, and green, while the yellow is rare، and the blue are the rarest of them all (Al-Obaidi, 2012).

The distinctive odor of the soil is due to the scented products produced by the *Streptomyces* like Isobutanol or Geosmin (Ismael *et al*., 2019). *Streptomyces* are known for their ability to produce active metabolic products, the data had shown that about 80% of the metabolic products in nature are produced by this genus (Bull & Stach, 2007).

Along with antibiotic-producing, the *Streptomyces* can also: decompose complex materials, play an important role in materialization, and helps in maintaining the balance of the soil (Kim *et al*., 2006). Plus, it can produce nucleotide antibiotics that have a role in fighting some types of cancer like blood cancer (leukemia), one of the most important types of these nucleotide antibiotics is sangivamycin (Ahmad *et al*., 2017).

The aim of this research is the isolation and identification of *Streptomyces* that inhibit the growth of parasitic, antibiotic-resistant bacteria, and fungi, which are isolated from the soil in Nineveh, Duhok, and Erbil and to diagnose them by the microscopic and morphologic experiments, also to determine their genetic sequences using the PCR test based on the 16s rRNA.

Materials and Methods

Collecting of Samples

Forty Six soil samples were taken from different locations in farms from Nineveh from depths of (5-15) cm, after washing away 3 cm from the surface, the samples were treated with calcium carbonate ($CaCO_3$) with a ratio of (1:10), then dried out by (40-45) degrees Centigrade for 4 days. Then the samples were put in bags with polyethylene and they were shut tightly, then they were placed in the refrigerator until the time of use. 1gm was put in tubes with 15ml of distilled water and then they were mixed thoroughly, a series of dilutions were made until the 6th dilution was reached, 1ml was taken from the last dilution and placed in a sterilized petri dish, the culture medium (starch-casein medium) was pured on it (which were cooled to 45 degrees centigrade) this was done three times for each sample. Plates with (10-35) colonies were chosen, and the number of solitary colonies was used to be re-cultured in the same medium to get pure culture. (Ceylan *et al*., 2008; Al-Obaidi, 2012; Vercesi *et al*., 1992)

Culture Medias:

Starch-casein medium

It was prepared by mixing: 10gm starch, 0.3 casein, 2gm KNO_3, 2gm NaCl, 0.02gm $CaCo_3$, 2gm KH_2PO_4, 0.05gm $MgSo_4.7H_2O$, 0.01gm $FeSO_4.7H_2O$, 18gm Agar, in 1 liter of distilled water, with a Ph of (7.2), sterilized with autoclave (Kuster & Williams, 1965) this medium was used in isolation.

Glycerol yeast extract-agar medium

Was prepared by mixing: 5gm glycerol, 2gm yeast, 0.1gm K_2HPO_4, 25gm pipton, 15gm agar, in 1 liter of distilled water, the mixture was sterilized in the autoclave at 121 degrees centigrade and under 5 pounds/inch square for 15 minutes. Nystatin 50 micrograms/ml was added to the medium after it was cooled to 40-degree centigrade (Oskay *et al*., 2004).

This exact sterilization method was used to sterilize the medias used in this research- these media were used in isolation.

Glycerol, asparagine agar medium

Was prepared by mixing: 1gm asparagine, 10gm glycerol, 1gm K_2HPO4, 20gm agar, 1ml of trace salt solution, 0.64gm $CuSo_4.5H_2O$, 0.11gm $FeSo_4.7H_2O$, 0.79gm $MnCl_2.4H_2O$, 0.15gm $ZnSo_4.7H_2O$, in 1 liter of distilled water with (7.4) Ph, and all were sterilized in the autoclave. (Williams& Cross, 1971) this medium was used in isolation.

Nutrient agar medium

Was prepared by the instructions of (oxiod) the supplier company, by melting 23gm of the nutrient agar in 1 liter of distilled water, and a Ph of 7.2 and all were sterilized in the autoclave. This medium was used to measure the optic capacity.

Trypton-yeast extract glucose agar medium

Was prepared by mixing: 10gm glucose, 3gm yeast extract, 5gm tryptone, 1gm KH_2PO_4, 20gm agar, in 1 liter of distilled water, and all were sterilized in the autoclave (Wu & Chen,1995). This medium was used in the diagnosis.

Starch mineral salts medium

Was prepared by mixing: 10gm starch, 2gm $(NH_4)_2SO_4$, 2gm $CaCO_3$, 1gm KH_2PO_4, 1gm NaCl, 20gm agar in one liter of distilled water, with a Ph of (7.0), and all were sterilized in the autoclave (Williams *et al*.,1983). This medium was used in the isolation.

Czapic Dox Agar_Dox agar medium

Was prepared by mixing: 30gm sucrose, 3gm $NaNO_3$, 1gm K_2HPO_4, 0.5gm $MgSO_4$, 0.5gm KCl, 0.01gm $FeSO_4$, 15gm agar, in 1 liter of distilled water, with a Ph of (7.3) and all were sterilized in the autoclave. (Lennette *et al*., 1983) this medium were used in the isolation

Antibiotics Production Medium

The augmented antibiotics production medium was prepared by mixing: 0.8gm NaCl, 1gm NH_4Cl, 0.1gm KH_2PO_4, 0.2gm $MgSO4.7H_2O$, 0.1gm $CaCl_2$, 10gm glucose, 3gm yeast extract, in 1 liter of distilled water and a Ph of (7.3), all were sterilized in the autoclave (Ilic *et al*., 2005).

Isolation

Identification of *Streptomyces*

The isolates were identified as *Streptomyces* depending on the morphology and color of the colonies, the starch mineral agar medium, the shape of the aerial and medial twig, and the arrangement of Spores using slide culture technique (Williams& Cross, 1971).

Test Bacteria

Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, E. coil, Klebsiella pneumoniae, Candida albicans, Fusarium solani, Rhizoctonia solani and Alternaria alternata which brought from department of biology/college of education/ Mosul University for identify the antagonistic activity of isolated *Streptomyces* isolates.

Testing the antagonistic activity of isolates

Agar discs diffusion method is used to test the ability of *Streptomyces* isolates to produce antibiotics (Egorov, 1985).

Diagnosis of the selected *Streptomyces*

The selected *Streptomyces* were diagnosed to the level of species based on the diagnostic experiments (Oskay *et al*., 2004; Williams *et al*., 1983; Holt *et al*., 1994; Pridham & Gottlieb, 1948)

Preparation of inoculation

The media of inoculation were prepared from the same components of the production Media. They were cultured in 250ml flasks, 50ml in each one, and they were sterilized in the autoclave, inoculation occurred by transporting a lop of the *Streptomyces* grown on the (Glycerol asparagine agar) to the inoculum. Then the flask was put in a shaker incubator at (28±1) degree centigrade, at 140 round/second for 3 days.

Cultural Conditions

Antibiotic production media was prepared in a 250ml flask, 50ml in each one, the flasks were shut tight and sterilized in the autoclave, they were left to cool all then inoculated with the inoculum prepared from the 3 days old selected isolation at the ratio 2% (v/v). The flasks were put in the shaker incubator at (28±1) degrees centigrade with 240 round/minute for 7 days.

Purification and acquisition of DNA from *Streptomyces*

Geneaid's analysis kit was used to obtain the DNA from the *Streptomyces* samples

PCR Reactions

The DNA concentration in all the samples used in this study was tuned using the TE solution to get the best concentration to carry on the PCR reactions, and it was (50 nanogram per microliter). The master reaction for each PCR reaction was produced by mixing the DNA sample with the specific primer of each gene, with the appender pre-mix inside a (0.2ml) Eppendorf tube provided by the British company (biolaps). The size of the reaction was fixed to 20 microliters by distilled water, the reaction then were placed in a microfuge for (3-5) seconds to mix the components of the reaction. Then the tubes were put inside a thermal cycler to achieve the polymerss reactions by the specific program for each reaction, the samples then were placed inside wells of 2% agarose gel for 60-70 minutes to undergo

electrophoresis alongside the ladder from biolps, the gel was filmed by Gel documentation.

The molecular diagnosis of *Streptomyces* based on the 16SrRNA

The amplification area were spotted by adding 4 microliters (100 nanograms) of the DNA template &1 microliter (10 picompl) from each gene primer to the master mix

Primer	Sequence
Forward	AAGCCCTGGAAACGGGGT
REVERS	CGTGTGCAGCCCAAGACA

After that the reaction tubes were inserted in the thermal cycler to achieve the polymers reaction by specific programs as shown in the table:

Table 1 : Illustrate apparatus program of thermocycler device

NO.	Stage	Temperature	Time	Cycle number
1	Initial denaturation	95	6 min	1
2	Denaturation	95	1-30 min	35
3	Annealing	58	1-30 min	
4	Extension	72	2 min	
5	Final extension	72	5 min	1

DNA Sequencing analysis

DNA sequencing is considered the main and most important technique to find out the mutations and variations of SNP in DNA samples, usually, the results from PCR reactions are used to select the sequence of the amplified parts of the DNA used to determine and study the mutations, in the last years the results from DNA sequencing are highly precise in determining mutations (Smith *et al*., 2014; Cheng *et al*., 2012).

On the other hand, if the results from the PCR reaction had more than one band; they are then purified and the wanted peace of the DNA is isolated from the gel, but if the reaction had only one band it is going to be the main band and it would be used to determine the sequences. (Figure 1) (DePristo *et al*., 2011)

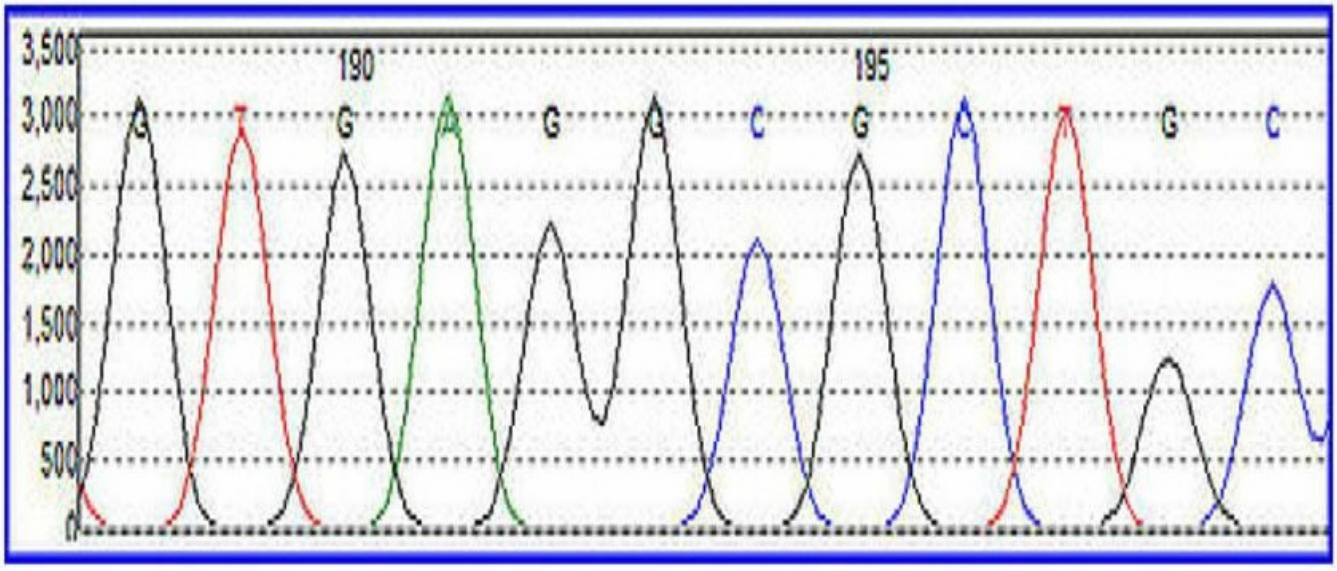

Fig. 1 : Tracking of DNA Sequences

Determining the nucleotides sequences of the amplified segment using DNA sequencing technique

The genetic sequences of the samples used in the study are identified, as the results from the PCR reaction for the samples mentioned earlier, along with the primers have been read with the 3130 Genetic Analyzer device, manufactured by the Japanese company "Hitachi". The genetic sequences have been compared with those in the National Center Biotechnology Information NCBI, the results were analyzed by BLAST program.

Results and Discussion

One hundred fifty four *Streptomyces* samples were isolated from 46 soil samples from different locations in Nineveh. The samples were chosen based upon their chalky appearance of the colonies grown in the media, and the earthy smell (wet soil) they produce. (Carpenter, 1977; Ford & Alexander, 1977).

The treatment of the soil with $CaCO_3$ 1:10 and applying temperature (40-45) degree centigrade, a great effect on the

flourishing of the bacteria in the primary isolation as drying the soil seize the growth of the vegetative bacteria, and the addition of $CaCO_3$ increase the Ph inhibiting the growth of fungi, and so the thread-like bacteria (*Streptomyces*) grows. (Nahar *et al*., 1997)

Diagnosis

The samples of *Streptomyces* were identified using the slide culturing technique as it's considered as one of the best ways (on the genus level) to show substrate and aerial hyphae which are the special features that differentiate the thread-like bacteria from each other. (Holt *et al*., 1994)

The substrate hyphae are known to be heavily branched, unsegmented, and have no spores. While the aerial hyphae showed as darker threads, thicker, and less branched than the substrate hyphae (figure 2). The aerial hyphae have a long chain of spores surrounded by the sporophore, it takes various chaps depending on the arrangement of these spores, it's either erect (rectus), or having a spiral shape (spiral), erict with a curved end (retinaculum-a precum), or erect with waves (rectus-flexibilis) (Saadoun *et al*., 1999).

The isolates showed multiple colors when they were cultured on different types of media, they were incapable of forming melanin and some other pigments, the gray-colored were the most appearing colonies figure (1), these results are similar to those of Saadoun *et al*., (1999)

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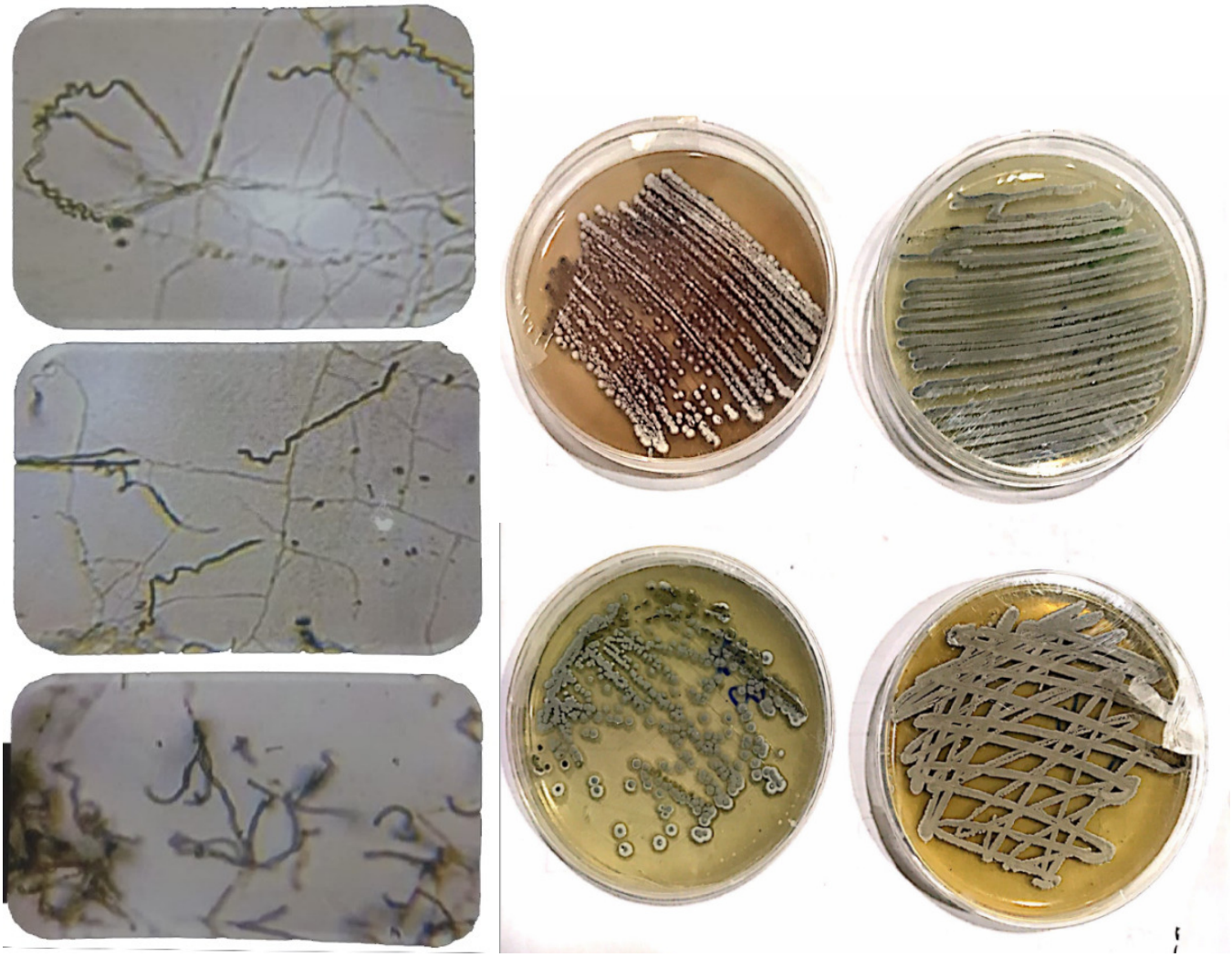

A **B**

Fig. 2: **A :** Shapes of various spore chains of Isolates of *Streptomyces spp*
B:Aerial and Substrate Mycelia in *Streptomyces spp.*

The diagnosis by using biochemical tests

After confirming the ability of selected isolates to inhibit test's organisms (bacteria, fungi), they diagnosed to species evel based on (Atta *et al*., 2011a; Atta *et al*., 2011b; Arifuzzaman *et al*., 2010), it shows that they belong to : *S.luteus, S. thermocarboxydus, S. griseorubens, S. albogriseolus* and *S. deccanesis*

The results obtained from the comparison of *Streptomyces* were as follows:

Table 2 : Diagnostic tests of *S. luteus*

Test	**Result**
Gram stain	+
The acid fast stain	-
Aerial mycelia color	Gray - White
Substrate mycelia color	Yellow
The shape of Spores' chains	Straight
Spore formation on substrate mycelia	**-**
Melanin pigment on a medium Tyrosine – agar	-
Add another strains	**-**

Enzyme tests	**Result**
Tests	
Catalase	+
Oxidase	**-**
Gelatin analysis	**-**
Starch analysis	+
Casein hydrolysis	+
Ureolysis	+
nitrate reduction	+
H_2S production	+
indole test // indole output	**-**

Consumption of carbon sources	**Result**
Tests	
Glucose	+
Fructose	+
Lactose	+
Galactose	+
Arabinose	+
Cellulose	**-**
Starch	+
Mannitol	+
Sucrose	+

The ability to growth in different concentrations of NaCl	**Result**
Tests	
NaCl % (1.5)	+
NaCl % (3)	+
NaCl % (5)	+
NaCl % (7)	+
NaCl % (15)	-
NaCl % (20)	-
Heat tolerance of (45) Celsius	-
The ability to shape of growth in the liquid medium (Nutrient broth)	Shape of small balls spread in the medium

The ability to inhibit growth	**Result**
Tests	
Staphylococcus aureus	+
Proteus vulgaris	-
Pseudomonas aeruginosa	+
E. coil	+
Klebsiella pneumoniae	+
Candida albicans	+
Fusarium solani	+
Rhizoctonia solani	+
Alternaria alternate	+

Table 3 : Diagnostic tests of *S. thermocarboxydus*

Test	**Result**
Gram stain	+
The acid fast stain	-
Aerial mycelia color	Gray, Black
Substrate mycelia color	Brown
The shape of Spores' chains	Straight with swastikas.
Spore formation on substrate mycelia	**-**
Melanin pigment on a medium Tyrosine – agar	**-**
Add another strains	**-**

Enzyme tests	**Result**
Tests	
Catalase	+
Oxidase	+
Gelatin analysis	±
Starch analysis	+
Casein hydrolysis	+
Ureolysis	+
nitrate reduction	+
H_2S production	+
indole test // indole output	-

Consumption of carbon sources	**Result**
Tests	
Glucose	+
Fructose	+
Lactose	+
Galactose	+
Arabinose	+
Cellulose	+
Starch	**-**
Mannitol	+
Sucrose	+

The ability to growth in different concentrations of NaCl	**Result**
Teat	
NaCl % (1.5)	+
NaCl % (3)	+
NaCl % (5)	+
NaCl % (7)	+
NaCl % (15)	-
NaCl % (20)	-
Heat tolerance of (45) Celsius	+
The ability to shape of growth in the liquid medium (Nutrient broth)	Shape of small balls spread in the medium

The ability to inhibit growth	**Result**
Tests	
Staphylococcus aureus	+
Proteus vulgaris	+
Pseudomonas aeruginosa	-
E. coil	-
Klebsiella pneumoniae	-
Candida albicans	-
Fusarium solani	-
Rhizoctonia solani	+
Alternaria alternate	-

Table 4 : Diagnostic tests of *S. griseorubens*

Test	Result
Gram stain	+
The acid fast stain	-
Aerial mycelia color	Gray - White
Substrate mycelia color	dark **blue** - **Nilotic**
The shape of Spores' chains	Straight with swastikas.
Spore formation on substrate mycelia	**-**
Melanin pigment on a medium Tyrosine – agar	+
Add another strains	**-**

Enzyme tests	**Result**
Tests	
Catalase	+
Oxidase	+
Gelatin analysis	**-**
Starch analysis	+
Casein hydrolysis	+
Ureolysis	-
nitrate reduction	-
H_2S production	+
indole test // indole output	+

Consumption of carbon sources	**Result**
Tests	
Glucose	+
Fructose	+
Lactose	-
Galactose	+
Arabinose	-
Cellulose	**-**
Starch	+
Mannitol	+
Sucrose	+

The ability to growth in different concentrations of NaCl	**Result**
Tests	
NaCl % (1.5)	+
NaCl % (3)	+
NaCl % (5)	+
NaCl % (7)	+
NaCl % (15)	-
NaCl % (20)	-
Heat tolerance (45) Celezia	-
The shape of growth in the liquid medium Nutrient broth	In the form of small balls scattered in the middle.

The ability to inhibit growth	**Result**
Tests	
Staphylococcus aureus	-
Proteus vulgaris	-
Pseudomonas aeruginosa	-
E. coil	-
Klebsiella pneumoniae	-
Candida albicans	-
Fusarium solani	-
Rhizoctonia solani	-
Alternaria alternate	-

Table 5 : Diagnostic tests of *S. albidoflavus* Molecular and biochemical diagnosis of local isolates of *Streptomyces* species from soils of Iraq

Test	Result
Gram stain	+
The acid fast stain	-
Aerial mycelia color	Gray – green
Substrate mycelia color	Dull brown
The shape of Spores' chains	Rectus - Flexibilis
Spore formation on substrate mycelia	-
Melanin pigment on a medium Tyrosine – agar	-
Add another strains	+

Enzyme tests	Result
Tests	
Catalase	+
Oxidase	-
Gelatin analysis	+
Starch analysis	+
Casein hydrolysis	+
Ureolysis	+
nitrate reduction	-
H_2S production	-
indole test // indole output	-

Consumption of carbon sources	Result
Tests	
Glucose	+
Fructose	+
Lactose	+
Galactose	∓
Arabinose	+
Cellulose	+
Starch	+
Mannitol	+
Sucrose	±

The ability to growth in different concentrations of NaCl	Result
Tests	
NaCl % (1.5)	+
NaCl % (3)	+
NaCl % (5)	+
NaCl % (7)	-
NaCl % (15)	-
NaCl % (20)	-
Heat tolerance (45) Celezia	-
The shape of growth in the liquid medium Nutrient broth	In the form of small balls scattered in the middle.

The ability to inhibit growth	Result
Test	
Staphylococcus aureus	+
Proteus vulgaris	+
Pseudomonas aeruginosa	+
E. coil	+
Klebsiella pneumoniae	+
Candida albicans	+
Fusarium solani	+
Rhizoctonia solani	+
Alternaria alternate	+

Table 6 : Diagnostic Tests of *S. deccanesis*

Test	Result
Gram stain	+
The acid fast stain	-
Aerial mycelia color	White – pink
Substrate mycelia color	Honey
The shape of Spores' chains	Straight
Spore formation on substrate mycelia	**-**
Melanin pigment on a medium Tyrosine - agar	+
Add another strains	-

Enzyme tests	Result
Result	
Catalase	+
Oxidase	**-**
Gelatin analysis	+
Starch analysis	+
Casein hydrolysis	+
Ureolysis	-
nitrate reduction	+
H_2S production	+
indole test // indole output	**-**

Consumption of carbon sources	Result
Tests	
Glucose	+
Fructose	+
Lactose	+
Galactose	+
Arabinose	+
Cellulose	+
Starch	+
Mannitol	+
Sucrose	+

The ability to growth in different concentrations of NaCl	Result
Tests	
NaCl % (1.5)	+
NaCl % (3)	+
NaCl % (5)	+
NaCl % (7)	+
NaCl % (15)	-
NaCl % (20)	-
Heat tolerance (45) Celezia	-
The shape of growth in the liquid medium Nutrient broth	In the form of small balls scattered in the middle.

The ability to inhibit growth	Result
Tests	
Staphylococcus aureus	+
Proteus vulgaris	+
Pseudomonas aeruginosa	-
E. coil	+
Klebsiella pneumoniae	+
Candida albicans	-
Fusarium solani	-
Rhizoctonia solani	-
Alternaria alternate	+

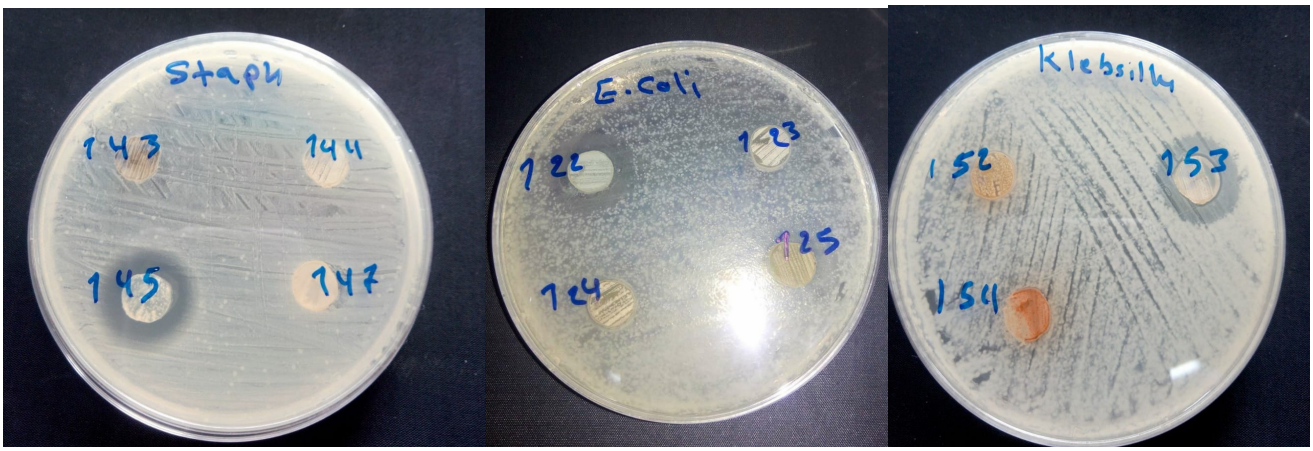

Fig. 3: Effect of antibiotics on isolates

Genomic DNA polymers reaction

The specified DNA's reaction has done on the purified DNA obtained from the organisms taken from local samples according to Geneaid protocol, using the forward primer: (AAG CCC TGG AAA CGG GGT)
And the revers: (CGT GTG CAG CCC AAG ACA) (Maleki *et al*.,2013).

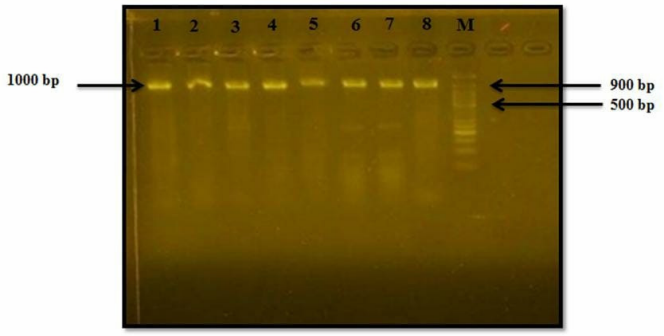

Fig. 4: The results from the specific polymers reaction of the local bacterial samples based on primer 16s rRNA
* (8-1) *Streptomyces* isolates
*(M) Marker examining and analyzing the sequence of nitrogenous bases resulted from specific PCR

In figure (4) it can noted 8 bands of purified DNA from the samples are of the same length (500-1000) base pairs obtained from the DNA specified polymers reaction of the *Streptomyces*.

It's been found that they have the same length, the appearance of these bands is a proof that there are mutual sequences of nitrogenous bases in the genomic DNA of these isolates, that can join with the primer and carry on the reaction creating new DNA bands of the same length. These results are similar to those of (Carpenter, 1977)

When getting a positive result by using the polymers reaction technique in the identification of the bacterial local samples, the length of the DNA resulted was 1000 nitrogenous base pairs and so on. (Table 2)

Table 2 : Show analyzing the sequence of nitrogenous bases resulted from specific PCR.

Sample	Diagnosis
1	*Streptomyces* luteus strain TRM 45540 (T) VINDHYACHAL 16S ribosomal RNA gene , Sequence ID : MN134076.1 Length : 1175Number of Matches : 1
2	*Streptomyces* thermocarboxydus strain XY190 16S ribosomal RNA gene , partial sequence . Sequence ID : MH432689.1 Length : 1509Number of Matches : 1
3	*Streptomyces* griseorubens strain bdu15 16S ribosomal RNA gene , partial sequence . Sequence ID : MG198770.1 Length : 1580Number of Matches : 1
4	*Streptomyces* albogriseolus strain LBX - 2 chromosome , complete genome . Sequence ID : CP042594.1 Length : 7210477Number of Matches : 3
5	*Streptomyces* deccanensis strain QY - 3 16S ribosomal RNA gene . partial sequence . Sequence ID : MT355909.1 Length : 1491 Number of Matches : 1
6	*Streptomyces* aureofaciens strain IMET 43577 16S ribosomal RNA gene , partial sequence . Sequence ID : AY289116.1 Length : 1509Number of Matches : 1

The table shows the species of *Streptomyces* isolated locally, and their level of similarity with the sequences of *Streptomyces* in the Gene bank.

The purpose behind using the Sequencing technique is to find out the genus of the organisms tested, and to identify the similarity between nitrogenous bases sequences of the DNA obtained from the specific PCR, from local samples.

The results from the specified polymers reaction of the purified DNA of the local samples were sent to the NCBI, to be analyzed with DNA BLAST, to find out their similarity with the sequences present in the gene bank.

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