Susceptibility of Male Testis Pathogenic Bacteria-a Probable Cause of Primary Infertility among Men in Egypt to Antibacterial Activity of *Streptomyces minutiscleroticus*, Strain Al-AK-6

Mohamed Helal El-Sayed[1], Zeinab Khaled Abd El-Aziz[2], Aziza Mansour Aly[3], Wael Refaat Hablus[4] and Eman Abdullah Elhusseiny[3]

[1]Botany and Microbiology Department, Faculty of Science (Boys), Al-Azhar University, Cairo, Egypt.
[2]Botany and Microbiology Department, Faculty of Science (Girls), Al-Azhar University, Cairo, Egypt.
[3]Obstetrics and Gynecology Department, International Islamic Center for Population Studies and Research (IICPSR), Al-Azhar University, Cairo, Egypt.
[4]Clinical Pathology Department, Faculty of Medicine (Boys), Al-Azhar University, Cairo, Egypt.
m_helal2007rm@yahoo.com

Abstract: Infertility has been known to cause serious social and emotional problems worldwide, especially in developing countries like Egypt and there is no certainty as to the probable cause hence this research work. In this study a total of 100 samples, comprising 50 Seminal fluidand 50 testicular biopsy samples were collected from one hundred infertile men patients, attending the fertility clinic at the International Islamic Center for Population Studies and Research (IICPSR), Al-Azhar University, Cairo, Egypt. A variety of six pathogenic bacterial species; *Enterobacter cloacae* Es-1, *Escherichia coli* Es-2, *Staphylococcus haemolyticus* Es-3, *Staphylococcus aureus* Es-4, *Bacillus cereus* Es-5 and *Kocuriarhizophila* Es-6 were isolated. The obtained bacterial species were subjected for antibacterial activity of different actinomycete cultures isolated from different localities at Egypt, it was found that an actinomycete culture Al-AK-6 isolated from a water sample collected from Abo Keer city, Alexandria governorate, Egyptwas found to be the most active against the isolated bacterial pathogens. Identification of this isolate was performed according to spore morphology and cell wall chemo-type, which suggested that this strain is a streptomycete. Further cultural, physiological characteristics and phylogenetic analysis of 16S rRNA gene indicated that this strain is identical to *Streptomyces minutiscleroticus*(accession number JX905302.1) and then designated *Streptomycesminutiscleroticus*, strain Al-AK-6. In its culture supernatant, this organism could produce one major bioactive compound belonging to macrolide antibiotics groupexhibited strong antibacterial activity against the isolated testis-pathogens.

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I. Introduction

Infertility affecting couples around the World is both a medical as well as social problem particularly in Egypt. The psychological problem associated with cases of primary infertility cannot be emphasized. Childlessness as infertility is generally known in our community is caused by a variety of factors; male and female alike**[1].** In about 60% of married couples, 90% would achieve pregnancy in 12 months and 95% would be able to achieve pregnancy in 18 months to 24 months **[1].**

Male urogenital tract infections are one of the most important causes of bacterospermiaand male infertility worldwide. Genital tract infection and inflammation have been associated with 8-35% of male infertility cases **[2,3,4].** Male accessory sex gland infection is a major risk factor in infertility **[5].**

The significant of pathophysiology of bacteriospermia has been discussed in recent years. Some possible pathomechanisms of the development of infertility linked with infection are considered: direct effect on sperm function (motility, morphology, etc.), deterioration of spermatogenesis, auto-immune processes induced by inflammation and dysfunction of accessory sex glands. Hence, a microbial investigation of male partners in infertile couple can be useful to detect the male urogenital tract infection, especially asymptomatic infections **[2,6].**

The demand for new antibiotics continues to grow due to the rapidly emerging of multiple antibiotic resistant pathogens causing life threatening infection. Although, considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of antibacterial compounds,

nature still remains the richest and the most versatile source of new antibiotics **[7,8,9].**

Throughout the ages, natural products have been the most consistently successful sources of useful compounds that have found many applications in the fields of medicine, pharmacy and agriculture. Microbial natural products have been the source of most antibiotics in current use in the treatment of various infectious diseases**[10].**

Marine microbes represent a potential source for commercially important bioactive compounds **[11].** Among marine microorganisms, actinomycetes have gained special importance as the most potent source of antibiotics and other bioactive secondary metabolites **[12].**

Among the genera of marine actinobacteria, the genus *Streptomyces* is represented in nature by the largest number of species and varieties, which differ greatly in their morphology, physiology, and biochemical activities. An evaluation is made on the present state of research on marine *Streptomyces* and its perspectives. The highlights include the production of metabolites such as antibiotics, anticancer compounds, enzyme inhibitors and pigments by marine *Streptomyces* and their applications as single cell protein and as probiotics in aquaculture **[13].** The present study was conducted to screen the ability of different actinomycete isolates for antibacterial activity against the isolated testis bacterial pathogens complaining infertility of men attending the fertility clinic at the International Islamic Center for Population Studies and Research (IICPSR), Al-Azhar University, Cairo, Egypt.

2. Subjects and Methods

2.1. Isolation and identification of bacterial isolates complaining male infertility

In this study a total of 100 samples, comprising 50 Seminal fluid and 50 testicular biopsy samples were collected from one hundred infertile men patients (age: 22-49y), attending the fertility clinic at the International Islamic Center for Population Studies and Research (IICPSR), Al-Azhar University, Cairo, Egypt during January 2011 to May 2012. The semen samples were collected from patients who have had 3-7 days of sexual abstinence from intercourse, using the masturbation method. Upon collection, samples were transferred to the laboratory in a near as possible to body temperature by placing the container inside a plastic bag. To collect testicular biopsy samples a transverse scrotal incision is made with the opening of the tunica vaginalis until there was full exposure of the testis. The biopsy material was inserted into tubes containing Ham F 10 (HTF) medium and transferred to the laboratory for sperm search and isolation. The collected samples were cultured in a septic condition using blood agar, chocolate agar and macConkey agar media at 37°C for 24 hours. The grown cultures were subcultured on trypticase soy agar, sabouraud dextrose agar and nutrient agar media**[14].** The obtained isolates were phenotypically characterized by a Biolog identification system (Biolog, Hayward, Calif.) **[15,16].**

2.2. Studying antibacterial activity of actinomycete cultures against the obtained bacterial isolates infecting male testis

2.2.1. Isolation of actinomycetes

Fifty five different type's samples included; soil, water and sediments were collected from four different localities at Egypt; Alexandria (Abo Keer region), MarsaMatrouh (Matrouh city) and Suez ("Kilo 60" from Cairo). The isolation and enumeration of actinomycete coloniesfrom the different collected samples was carried out onstarch nitrate agar medium (g/L): Soluble starch 20.0; $NaNO_3$2.0;K_2HPO_4 (anhydrous) 1.0; KCl 0.5; $MgSO_4.7H_2O$ 0.5; $CaCO_3.2H_2O$ 2.0; Agar 15. The medium was adjusted to the initial pH 7.0 prior to sterilization using 0.1 N NaOH or 0.1 N HClsolution**[17].**The obtained actinomycete cultures were purified using the dilution plate technique described by Williams and Davis**[18].**

2.2.2. Screening for antibacterial activity of the isolated actinomycetes

Testing antibacterial activity of the isolated actinomycete cultures against the isolated male testis bacteria were performed by diffusion plate methods**[19,20]**, based on the observation of inhibition zone of bacterial growth on agar media.

2.3. Taxonomic characterization of the most active actinomycete isolate, Al-AK-6

2.3.1. Conventional taxonomy

The characterization of isolated actinomycete, Al-AK-6 followed the guidelines adopted by International *Streptomyces* Project **[21].** The cultural characteristics were studied according to the guidelines established by the ISP **[21]**,colours were assessed on the scale adopted by Kornerup and Wanscher**[22]**. Micro-morphological studies were carried out using light and scanning electron microscope (JEOL JSM 5300, JEOL Technics Ltd., Japan) **[23]** according tothe method of Tresner and Davies **[24].** Diaminopimelic acid isomers in the cell-wall and whole cell sugar pattern were analyzed using the method of Becker *et al.***[25].**The physiological and biochemical characteristics; melanin pigment production, utilization of carbon and nitrogen sources, enzymatic activities and other physiological characters were also studied **[21,26,27].**

2.3.2. Molecular and phylogenetic identification

The nucleotide sequence of partial 16S rRNA gene of the local actinomycete strain Al-AK-6 was performed through inoculation of Al-AK-6 spores on

50 ml of starch nitrate broth and the culture was incubated at 200 rpm and 28°C for 72 hours. The total genomic DNA was extracted according to the method of Sambrook *et al.***[28]**. The 16S rRNA of the strain was amplified by PCR using a GeneAMP PCR System 9700 from PE Applied Biosystems (Perkin Elmer, Ohio, USA). The following primers were used: F27, 5'-AGAGTTTGATCMTGGCTCAG-3'and R1492 5'-TACGGYTACCTTGTTACGACTT-3'using Biolegio BV software (Biolegio, Nijmegen, Netherlands) **[29]**, PCR mixture conditions were performed according to El-Naggar**[30]**.The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and were detected using a gel documentation system, (Alpha-Imager 2200, CA, USA). The PCR products were sequenced using gene analysis unit in genetics laboratories of the Holding Company for Biological Products and Vaccines (VACSERA), El-Dokki, Giza, Egypt using an ABI PRISM 377 DNA sequencer and ABI PRISM BigDye Terminator Cycle Sequencing (Perkin Elmer, Ohio, U.S.). BLAST (www.ncbi.nlm.gov) was used to assess the DNA similarities. A multiple sequence alignment and molecular phylogenetic analyses were performed using BioEdit software **[31]**. The phylogenetic tree was constructed using the TreeView program **[32]**.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of testis-bacterial pathogens complaining of male infertility

In the present study thirty bacterial isolates symbolized (E1-E30) were obtained from the collected clinical specimens and phenotypically characterized to the species level by the Biolog identification system (Biolog, Hayward, Calif.). The results of identification revealed that, the obtained isolates are represented by six different bacterial species with different percentages; *Enterobacter cloacae*, *Es-1*26.66%;*Escherichia coli*, *Es-2* 20.0%;*Staphylococcus haemolyticus*, *Es-3* 16.66%;*Staphylococcus aureus*, *Es-4* 16.66%; *Bacillus cereus*, *Es-*513.33% and *Kocuriarhizophila*, *Es-6* 6.66%. Data of the obtained bacterial isolates are recorded in table (1) and illustrated graphically in figure (1). Momoh*et al.***[33]** reported, a variety of bacterial strains were isolated in which *Staphylococcus aureus* having a prevalence of 38.7% from high vaginal swab and endocervial swabs respectively, and a prevalence of 75% among the bacterial strains from semen.

Table (1): The obtained bacterial isolates in relation to their number and percent.

No.	Microorganism	Number	%
1	*Enterobacter cloacae, Es-1*	8	26.66
2	*Escherichia coli, Es-2*	6	20.0
3	*Staphylococcus haemolyticus, Es-3*	5	16.66
4	*Staphylococcus aureus, Es-4*	5	16.66
5	*Bacillus cereus, Es-5*	4	13.33
6	*Kocuriarhizophila, Es-6*	2	6.66
Total		**30**	**100%**

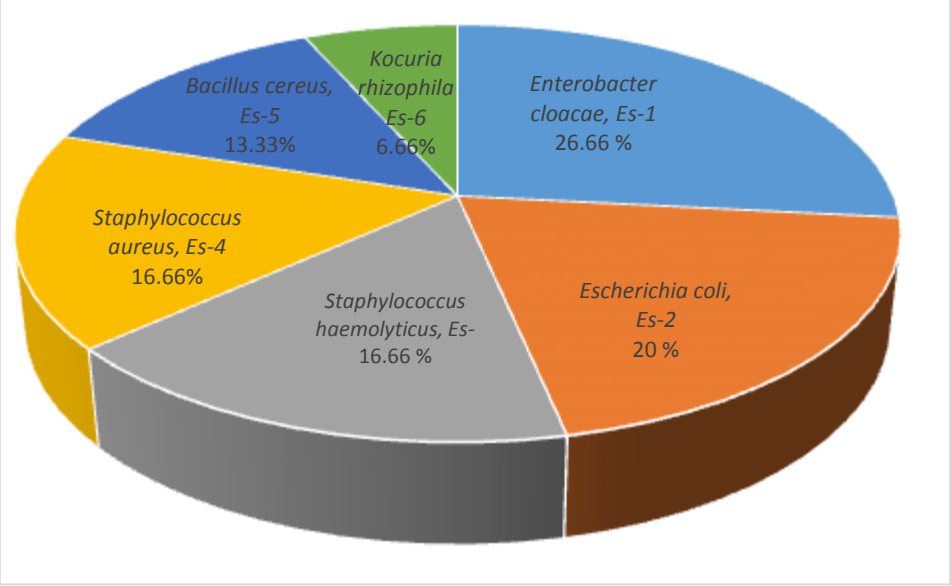


Figure (1): Percent of the obtained bacterial isolates.

3.2. Studying the antibacterial activity of the isolated actinomycetes against testis-bacterial pathogens

Thirty five actinomycete cultures were isolated from different soil and water samples collected from different localities at Egypt, these isolates were screened for their antibacterial activity against the isolated testis-bacterial pathogens. It was found that, twenty five isolates (71.42%) exhibited various degrees of activities against all tested bacteria, on the other hand, ten isolates (28.57%) failed to exhibit antibacterial activity against the all tested bacteria.

The results recorded in table (2) revealed that, actinomycete isolates; Al-AK–6, Mar.M-17, Mar.M-23, Mar.M-24 and SK60-30 were selected as the highest antibacterial producing isolates and Al-AK–6 was the most active one thus, it was selected for further studies concerning its identification. The percent of antibacterial activity of the tested isolates is higher than those described by many authors studying the activities of actinomycetes**[34,35].**

Table (2): The selected actinomycete isolates having the highest antibacterial activity.

Isolate no.	Mean diameter of inhibition zone (mm) of the tested bacterial strains					
	Enterobacter cloacae, Es-1	*Escherichia coli, Es-2*	*Staphylococcus haemolyticus, Es-3*	*Staphylococcus aureus, Es-4*	*Bacillus cereus, Es-5*	*Kocuriarhizophila, Es-6*
Al-AK–6	**27.0**	**29.0**	**18.0**	**20.0**	**18.5**	**16.5**
Mar.M-17	19.5	18.0	15.5	17.4	23.4	15.5
Mar.M-23	20.0	22.0	15.8	17.00	15.0	14.5
Mar.M-24	26.1	24.0	16.0	20.0	21.0	0.0
SK60-30	20.0	22.0	15.8	17.00	15.0	14.5

3.3. Taxonomic characterization of actinomycete isolate, Al-AK-6

3.3.1. Conventional Taxonomy

The cultural characteristics of actinomycete isolate, Al-AK-6 grown on different ISP media (Table 3) exhibited that, the aerial hyphae of the strain was yellowish gray therefore, it was assigned to gray color series. Diffusible pigments are ranged from slight yellow to strong greenish yellow on oat meal agar medium (ISP-3). Also the organism was found to produce brownish orange melanin pigment on tryrptone yeast extract broth (ISP-1)

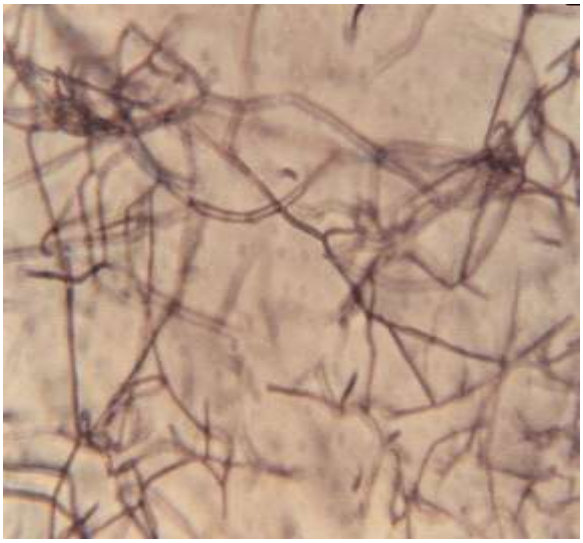
Micro-morphological characteristics of actinomycete isolate, Al-AK-6grown on inorganic salts-starch agar (ISP-4) under light microscopy (x600) figure (2a)exhibited rectiflexibleshaped mycelium and special morphological characteristics were seen under scanning electron microscope (x4500): minute sclerotic granules (small sclerotia) are produced on the agar surface or vegetative mycelium figure (2b).

Whole cell hydrolysate of this strain contained LL-diaminopimelic acid (LL-DAP) and glycine indicating that, the strain has a chemo-type I cell wall but no characteristic sugars could be detected. Cell-wall composition analysis is one of the main methods that can be employed to identify the chemotaxonomic characteristics of *Streptomyces*; the presence of LL-DAP in the cell wall also signifies that this strain is *Streptomyces* **[36].** The physiological and biochemical properties; carbon and nitrogen sources utilization;enzymatic activities, tolerance to NaCl; growth pH;growth temperature; growth inhibitors and resistance to antibiotics were presented in table 4.

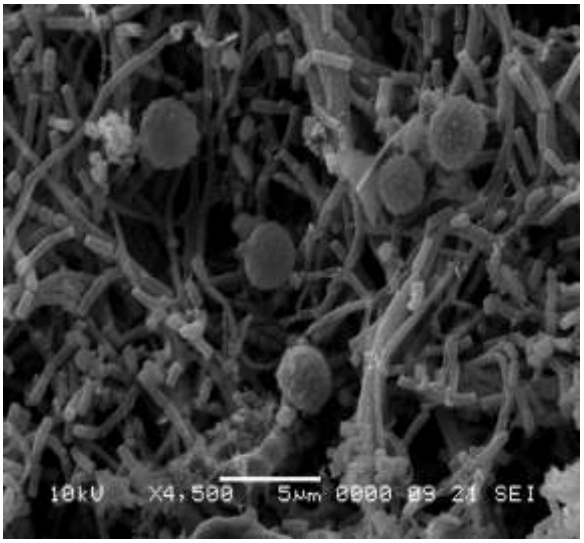
Table (3): Cultural characteristics of actinomycete isolateAl-AK-6 grown on different ISP-media.

Medium	Growth	Substrate mycelium	Aerial mycelium	Diffusible pigments
Tryptone yeast extract broth (ISP–1)	Good	White (ISCC-NBS 263)	Pale yellow (ISCC-NBS 89)	Brownish orange (ISCC-NBS 74)
Yeast -malt extract agar (ISP–2)	Weak	Yellowish white (ISCC-NBS 92)	Grayish yellowish (ISCC-NBS 90)	None
Oatmeal agar (ISP–3)	Good	White (ISCC-NBS 263)	Yellowish gray (ISCC-NBS 93)	Pale greenish yellow (ISCC-NBS 104)
Inorganic-trace salt- starch agar (ISP–4)	Good	White (ISCC-NBS 263)	Yellowish gray (ISCC-NBS 93)	None
Glycerol asparagine agar (ISP–5)	Mode- rate	Moderate gray (ISCC-NBS 264)	Yellowish gray (ISCC-NBS 93)	None
Peptone yeast extract iron agar (ISP–6)	No growth	-	-	-

Tyrosine agar (ISP–7)	No growth	-	-	-



A



B

Figure (2): A**;**Phase-contrast micrograph of actinomycete Al-AK–6 showing rectiflexibleshaped mycelium (x600) **B;** Scanning electron microscopy (SEM) showing rectiflexible mycelium with the appearance of minute sclerotia (x4500).

Table (4): Physiological and biochemical characteristics of actinomycete isolateAl-AK-6

Character	Results	Character	Results
• **Melanin pigment:**		8. Gelatin liquefaction	+
Tryptone-yeast extract broth	Brownish orange	9. Nitrate reduction	+
Peptone-yeast extract iron agar	–[a]	10. H_2S production	–
Tyrosine agar	–	• **Tolerance to NaCl concentrations:**	
• **Carbon sources utilization:**		1.0 - 6.0 %	+++
1. Starch	+[b]	7.0 %	++
2. Maltose	+	8.0 %	Wg[e]
3. D–Glucose	+	9.0 %	–
4. L–Arabinose	+	• **Growth temperature ^oC:**	
5. D–XyLose	+	10.0 – 25.0 ^oC	–
6. Lactose	+	30.0 – 40.0 ^oC	+++
7. Mannitol	+	45.0 ^oC	+
8. Sucrose	–	50.0 ^oC	–
9. D–Galactose	–	• **Growth pH:**	
10. L–Rhamnose	–	4.0 - 6.0	–
• **Nitrogen sources utilization:**		7.0	+++
1. L-asparagine	+++[c]	8.0	++
2. Leucine	+++	9.0	–
3. L-tryptophan	+++	• **Tolerance to growth inhibitors:**	
4. L-glutamic acid	+++	Sodium azide (0.01%)	+
5. L-arginine	+++	Sodium azide (0.02%)	–
6. L-histidine	++ [d]	Phenol (0.1%)	+
7. L-serine	++	Crystal violet (0.0001%)	–
8. Lysine	++	Thallus acetate (0.001%)	+
9. L-methionine	+	• **Resistance to antibiotics:**	
10. L-tyrosine	–	Erythromycin (15 µgm)	–
• **Enzymatic activities:**		Penicillin (25 µg/ml)	–
1. Protease	–	Ciprofloxacine (30 µgm)	–
2. Lecithinase	+	Tetracycline (15 µgm)	–
3. Lipase	+	Bacitracin (50 µgm)	–
4. Pectinase	–	Chloramphinicol (30µgm)	–
5. Catalase	+	Norfloxacin (30 µgm)	+

6. Urease	–	Rifampicin (50 µg/ml)	+
7. Xanthine degradation	–		

a(-) = Negative or no growth, b(+) = Positive or moderate growth, c(+++) = Abundant (Very good growth), d(++) = Good growth, e(wg) = Weak growth.

3.3.2. 16S rRNAgene sequencing and phylogenetic analysis

To confirm the identification of the isolated strain Al-AK-6, 16S rRNAgene sequence of thelocal isolate was compared to sequences of 10 *Streptomyces* spp. Experimental analysis of the PCR amplification was studied through the agarose gel electrophoresis (Fig. 3a) exhibited specific 16S rRNA band. The phylogenetic tree (Fig. 3b) showed that the locally isolated strain is closely related to *Streptomyces minutiscleroticus*. Multiple sequence alignment was done between the sequences of the 16S rRNA genes of *Streptomycesminutiscleroticus* and other nine *Streptomyces* spp and the local isolate. Computer assisted DNA similarly searches against bacterial database revealed that 16S rRNA sequence was 91% identical to *Streptomyces minutiscleroticus* MMA1 accession number JX905302.1.

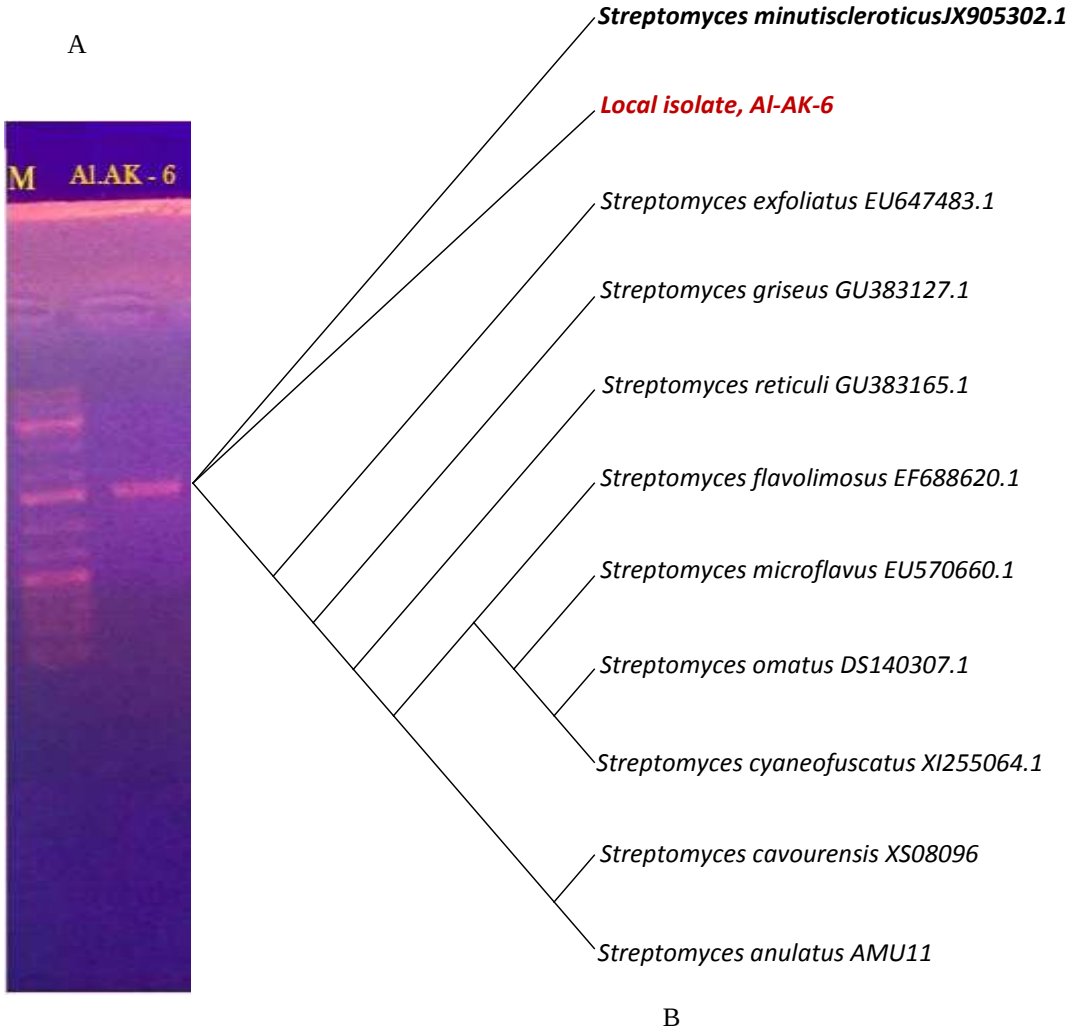


Figure (3): A; Amplified fragment of 16S rRNA gene, (M): Marker DNA. B; Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequence of *Streptomyces minutiscleroticus*, Al-AK-6 and phylogenetically related member of this genus.

The morphological, physiological and phylogenetic characteristics of actinomycete isolate, Al-AK–6 suggested that, this isolate have high similarity with the reference strain *Streptomyces minutiscleroticus***[37,38]** the comparative study of identification properties of the two strains are recorded in table (5).

At the turn of the century, a shift driven by new technologies started in search of exploitable biology. This shift is exemplified by the extent of biodiversity now revealed and recognized by biologically informative and data-rich methods functioning at the molecular scale. Such methods are often employed for characterizing organisms and defining taxon-property relationships through high-throughput screening and the PCR and DNA sequencing**[39].** In this study, the phylogenetic analysis coupled with a conventional method related to Al-AK-6 indicated that the most closely-related strain is *Streptomyces minutiscleroticus*MMA1 (accession number JX905302.1) therefore, *Streptomyces minutiscleroticus* strain Al-AK-6 is proposed as its name. The use of genotypic and phenotypic techniques gives a better resolution in species-level identification**[40].**

Table (5): A comparative study of identification properties of the local isolate, Al-AK-6 in relation to the reference strain *Streptomyces minutiscleroticus***[37,38].**

Characteristics	**Local isolate Al-AK-6**	***Streptomyces minutiscleroticus* [37,38].**
1. Morphological characteristics:		
Spore mass	Gray	Gray
Spore chain	Rectiflexibles	Rectiflexibles, spirals
Spore surface	Smooth	Smooth
Diffusible pigment	+[a]	+
Sclerotia formation	+	+
2. Chemotaxonomic characteristics:		
DAP	LL-DAP	LL-DAP
Sugar pattern	ND[b]	ND
3. Physiological characteristics:		
• **Melanin pigment production**		
Tryptone-yeast extract broth	Brownish orange	–
Peptone-yeast extract iron agar	–[a]	–
Tyrosine agar	–	–
• **Utilization of carbon sources:**		
D-Glucose	+[b]	+
L-arabinose	+	+
D-xylose	+	+
Meso-inositol	+	+
D-mannitol	+	+
D-fructose	+	+
Rhamnose	+	+
Sucrose	–	–
Raffinose	–	–
• **Growth at 45 °C:**	–	–
• **Growth at 6 %NaCl:**	+	+
• **Resistance to antibiotics:**		
Rifampicin (50 µg/ml)	+	+
Penicillin (25 µg/ml	–	+

a(+) = Positive, b(ND) = Not detected, c(-) = Negative.

3.4. Antibacterial activity of *Streptomyces minutiscleroticus*, Al-AK-6.

The actinomycete *Streptomyces minutiscleroticus*, Al-AK-6 was allowed to grow on fermentation nutrient medium under optimum environmental and nutritional conditions. In its culture supernatant, this organism could produce one major compound strongly inhibits the growth of the isolated testis bacterial pathogens complaining of male infertility. The isolated compound was found to be belonging to macrolide group**[41,42]**as a derivative of cirramycin A **[43]**.

1. Conclusion

The study has shown the prevalent microbial cause of infertility in our environment. More importantly, it highlights the fact that these organisms are common. These isolates may later be involved in cross-transmission as the couple engaged in

intercourse. We therefore suggest therapeutic management strategies of couples with primary infertility to include natural, effective products and couples should be managed together to avoid cross transmission. In conclusion, it is believed that a rich source of new drug candidates can be potentially obtained from marine organisms or their metabolites. This preliminary screening of marine actinomycetes for new antibacterial antibiotic revealed their potential to yield potent bioactive compounds for drug discovery programs.

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Corresponding Author:
Mohamed Helal El-Sayed, Botany and Microbiology Department, Faculty of Science (Boys), Al-Azhar University, Cairo, Egypt.
E-mail: m_helal2007rm@yahoo.com

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