Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(5): 175-182
Received: 22-07-2017
Accepted: 24-08-2017

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Molecular detection of aminoglycoside phosphotransferase (*aph*) gene responsible for neomycin production from *Streptomyces* spp

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Abstract
The present study was aimed for molecular identification of aminoglycoside phosphotransferase (*aph*) gene from *Streptomyces* spp. Six soil samples were collected randomly from different location of Baghdad city. Nine *Streptomyces* isolates were selected and purified on Glycerol yeast extract agar (GYEA). The isolates were identified as *Streptomyces* spp. based on their morphological, physiological and biochemical characteristics as described in the International *Streptomyces* Project. All *Streptomyces* isolates were screened for their antibacterial activity on malt extract yeast extract agar medium (ISP2) using scross-streak technique. A broad spectrum of antibacterial activity was observed in 44.4% (4 out of 9) (A3, A4, A15 and A17) against all tested pathogens. Four isolates were positive in the PCR technique. Performing PCR reactions using primer pair on DNA from soil isolates produced a single 273 bp band in all tested isolates. The results of *aph* gene detection clarify that one isolate (25%) of *Streptomyces* isolates gave a positive result and carrying *aph* gene while 3 (75%) of *Streptomyces* isolates were lack the gene and no cross reactivity could be observed with any of the A3, A4 and A15 isolates. A17 isolate gave DNA bands 1190 bp in length. The results indicated that the A17 isolate is very close to the species *Streptomyces fradiae* responsible for producing antibiotic neomycin.

Keywords: *aph* gene, antimicrobial activity, *Streptomyces*.

1. Introduction
Actinomycetes, the ubiquitous and abundant soil organisms that give the characteristic earthy smell to soil, are Gram positive bacteria rich in G+C content, its life in the soil as non-motile bacteria (Kieser *et al*., 2000) [23]. *Streptomyces* are Gram-positive, production of pigments, antibiotics and other secondary metabolites is initiated (Kutzner 1986, Williams *et al*. 1989) [27, 7-15-16-50]. *Streptomyces* are known to be producers of many secondary metabolites, which have different biological activities, such as antibacterial, antifungal, antiparasitic, antitumour, and immunosuppressive actions (Sanchez and Damon, 2002, Risan *et al.,* 2017) [43, 36, 40]. The *Streptomyces* are responsible for producing 70% of the world's antibiotics. Much is known about the secondary metabolism of *Streptomyces* due to the variety of useful compounds that can be produced; however, much less is known about other aspects of their biology (Hirsch and McCann-McCormick, 1985) [19]. Secondary metabolites are known to possess antibacterial, antifungal, antioxidant, anti-cancer and anti-inflammatory activities (Ravikumar *et al.,* 2011; Amin *et al.,* 2016; Risan *et al.,* 2016) [37, 2-39, 2-39]. *Streptomyces* species was considered as major producers of bioactive compounds in the biotechnology industry. They are the source of most clinically used antibiotics, as well as of several widely used drugs against common diseases (Bizuye *et al*., 2013) [5]. The biochemistry of replication is similar in all bacteria, but linearity of the *Streptomyces* chromosome brings with it the need for a special replication strategy. Aminoglycosides are mainly produced by actinomycetes including *Streptomyces*, aminoglycosides are a large class of clinically important antibiotics and all are secondary metabolites from various *Streptomyces* sp. *S. fradiae* is carrying the regulatory gene for aminoglycoside phosphotransferase (*aph*). (Thompson and Gray, 1983) [45], Feeding experiment of *S. fradiae* (Nem producer) culture with [6-^{14}C] -glucose or [1-^{14}C] - glucose followed by the isolation and periods degradation of Nem revealed the corresponding labelling pattern in neosamine subunit is without any localisation of C-C bonds (Rinehart *et al.,* 1974) [38]. up - aminoglycoside phosphotransferase gene, which responsible for neomycin production from *S. fradiae* (Janssen *et al.,* 2017) [21]. The present study was aimed for molecular identification of aminoglycoside phosphotransferase (*aph*) gene from *Streptomyces* spp**.**

Materials and Methods

Collection of Soil Samples

Six soil samples were collected randomly from different location of Baghdad city on January 2017. The samples were taken at a depth of 5-10 cm (where most of the microbial activity takes place) from the surface in shaded areas. Soil samples (50 g each soil sample) were collected by using clean, dry and sterile polythene bags. Samples were stored in boxes and transported to the laboratory where they were kept in a refrigerator at 4°C until microbial assays were performed on them (El-Shatoury *et al.,* 2006; Qasim and Risan, 2017) [12, 36, 40].

Preparation of samples for Isolation of *Streptomyces* spp

Each soil sample was air dried at room temperature and sieved through a 2 mm pore size sieve to get rid of large debris. The sieved soil was used for the isolation purpose. Sieved soil samples of 1 g were suspended in 100 ml sterile distilled water and incubated at 28°C with shaking at 180 rpm for 1 h (Oskay *et al.,* 2004) [34]. Mixtures were allowed to settle and then serial dilutions of the soil suspensions were prepared up to 10^{-4} from each dilution**.** One ml of each dilution was spread plated on Glycerol yeast extract agar (GYEA) (2g Yeast extract, 10ml Glycerol, 20 agar and 1 L water) supplemented with tetracycline 50 µg/ml and 50 µg/ml cycloheximide, then incubated at 28°C for 7 days still *Streptomyces* colonies appear. (El-Nakeeb and Lechevalier, 1963; Kuster and Williams, 1964) [11, 26]. Purified *Streptomyces* isolates were sub-cultured on specific media and stored at 4 °C.

Isolation, purification and identification of *Streptomyces* spp

After incubation, plates were examined for the appearance of *Streptomyces* colonies. Colonies of *Streptomyces* were recognized by their microscopic characteristics (optical microscopy, aerial, substrate mycelium colour and Gram's stain). The stock cultures were maintained and transferred on International *Streptomyces* project Medium slants (ISP-2) (4g Yeast extract, 10g Malt extract, 4g Dextrose, 20 agar and 1 L water) to obtain pure colonies used for identification (Nonoh *et al*., 2010) [33]. Once in four months and stored at 4°C and at -20°C in glycerol stock (20%, v/v) for a longer period.

Screening of Antibiotic Producing *Streptomyces* spp.

Primary screening for antimicrobial activities was, according to (Kumar *et al.,* 2012) [25]. by using cross-streak technique, in which the *Streptomyces* isolates were used against four pathogenic bacteria. The *Streptomyces* were streaked as across lines in the center of plates poured with malt extract yeast extract agar medium (ISP2) and inoculated plates were incubated at 28°C for 7 days to secrete antibiotics into the medium. Each streaking was started near the edge of the plates and streaked toward the *Streptomyces* growth line. The positive results were observed by the naked eye. Antimicrobial activity of *Streptomyces* isolates were determined to carry out after the positive results were obtained from the primary screening by Agar- Well Diffusion method (Murray *et al.,* 1995) [32], to test the antibiotic activity of the isolates. Two of them were gram-positive and two were gram-negative bacteria. Gram positive species were *Bacillus cereus* and *Streptococcus* spp. Gram-negative strains were *Pseudomonas aeruginosa* and *Escherichia coli*. The bacteria were maintained in nutrient agar at 4°C and sub-cultured before use. The selected isolates were streaked as parallel line on nutrient agar plates and incubated at 28°C for 5 days. After observing a good ribbon- like growth of the *Streptomyces* sp on the Petri plates, the pathogen was streaked at right angles to the original streak of *Streptomyces* and incubated at 28°C. The inhibition zones in mili meter (mm) were measured after 24 and 48 hours using an antibiotic zone reader. Further, *Streptomyces* isolates were selected for morphological and molecular identification.

Cultural Characterization

The isolates characterized of by morphological studies according to Bergey's Manual of Determinative Bacteriology. The micromorphology of *Streptomyces* isolates were carried out for Gram's straining type, shape by under a light microscope (Vimal *et al.,* 2009) [47], where many colonies with different morphological and cultural characteristics, growth, coloration of aerial and substrate mycelia (Goodfellow, 1989 a, b) [15-16-50]. Morphological features of colonies such as colony pigmentation were used for preliminary classification of the bacterial population.

Biochemical and physiological Characteristics

Biochemical characterization, various biochemical tests were performed for the identification of the potent isolate *Streptomyces* spp. These tests, including Hydrogen sulphide production, Catalase production, Nitrate reduction, Gelatinase, Citrate utilization, Tyrosine degradation, Pectin degradation, Protease production, Urease production, Catalase, Oxidase production, Casein hydrolysis, Indole production, Melanine reaction, Starch, Amylase production, Cellulase and Lipase. Physiological characterization such as, the effect of pH (5-9), temperature (25˚-45˚C) and salinity (NaCl concentrations 2-8%) were also studied. (Lechevalier and Lechevalier 1967; Cowan, 1974; Gordon *et al.,* 1974; Elwan *et al.,* 1977) [28, 6, 17].

Molecular identification of *Streptomyces* spp by amplification of 16S rDNA gene

Extraction of genomic DNA of *Streptomyces* spp.

(According to: FavorPrep Tissue Genomic DNA Extraction Mini Kit)

HINT: Set dry or water baths: one to 37 °C, another to 60 °C and the other to 95 °C.

1. Transfer 1 ml well-grown bacterial culture to a microcentrifuge tube (not provided).
2. Descend the cells by centrifuging at full speed for 2 min and discard supernatant completely.
3. Resuspend the cell pellet in 200 µl lysozyme reaction solution (20 mg/ml lysozyme; 20 mM Tris-HCI, pH 8.0.
4. (Optional) If RNA-free genomic DNA is required, add 4 µl of 100 mg/ml RNase A (not provided). Mix thoroughly by vortexing and incubate at room temperature for 2 min.
5. Add 20 µl Proteinase K to the sample, and then add 200 µl FATG2 Buffer to the sample. Mix thoroughly by pulse-vortexing. Incubate at 60 °C for 30 min and vortex occasionally during incubation.
6. Do a future incubation at 95 °C. for 15 min.
7. Add 200 µl ethanol (96-100%) to the sample mixture. Mix thoroughly by pulse-vortexing.
8. Briefly spin the tube to remove drops from the inside of the lid.
9. Place a FATG Mini Column in a Collection Tube. Transfer the mixture (including any precipitate) carefully to the FATG Mini Column. Centrifuge at full speed (~18,000 x g) for 1 min then place the FATG Mini

Column to a new Collection Tube.

10. Add 400 µl W1 Buffer to the FATG Mini Column. Centrifuge at full speed for 1 min then discard flow-through.
    -Make sure that ethanol has been added into W1 Buffer when first open.
11. Add 750 µl Wash Buffer to the FATG Mini Column. Centrifuge at full speed for 1 min then discard flow-through.
    -Make sure that ethanol has been added into Wash Buffer when first open.
12. Centrifuge at full speed for an additional 3 min to dry the column.
13. Add 100 µl of preheated Elution Buffer or ddH2O (pH 7.5-9.0) to the membrane of the FATG Mini Column. Stand the FATG Mini Column for 3 min.
14. Centrifuge at full speed for 2 min to elute DNA.

Table 1: Primers tested in this study

Primer	Nucleotide sequencet (5' – 3')	Corresponding region
AM48	CCCT(TG)CACTCTGGGACAAGC CCT	nt 130
AM51	ACATTCCACGT(CT)GTC(GC)GT GCC	nt 800
AM52	GGCAC(GC)GC(GA)ACGTGGAAT GT	nt 800 (comp.)

PCR amplifications

PCR amplifications were as follow

Volumes for PCR mixture

- DNA from step 5 µl
- Primer (1) 1 µl F, 1 µl R
- Primer (2), 1 µl F, 1 µl R
- A Volume of D.D.W. of 11 µl was added for a final volumes of 20 µl reaction mixture

Amplifications conditions

The PCR was performed in a thermo cycler under the following conditions according (Volossiouk *et al*., 1995) PCR program was conducted with an initial 5 min denaturation step at 95°C for 1 cycle, followed by repeating cycles of denaturation (94°C for 30 Sec.), annealing (30 Sec. at 55°C), and extension (30 Sec. at 72°C) for 35 cycle, followed by 5 min of final extension step at 72°C, 5 µl of the PCR product is used for analysis by gel electrophoresis and was Product size 275 bp.

Electrophoretic analysis

The PCR amplicons were analysed by electrophoresis in 1.5% (w/v) agarose gel stained with 4 µg of ethidium bromide/ml. After electrophoresis, the PCR products were visualized with UV transilluminator and documented with a Gel Doc 2000 gel system (Bio-Rad, USA). In addition, gels were photographed using a Polaroid camera. Molecular weight analysis of the resulted patterns was performed with the Quantity one software version 4.2.1 (Bio-Rad), as compared to the 250bp DNA ladder.

Molecular identification of *aph* gene for *Streptomyces* isolates -Amplification of (*aph*) gene

To amplification of aminoglycoside phosphotransferase *(aph*) gene using primers in PCR for detection genes were obtained from Bioneer, Korea. Method according to (Edwards *et al.,* 1989; Mehling *et al.,* 1995 and Von Wintzingerode *et al.*, 1997) [10, 29, 49] used 16S rDNA sequencing to determine those regions suitable. Amplified by PCR from *Streptomyces* isolates (A3, A4, A15 and A17). Tow 16S rDNA genes primes using, APH-F (5′-TGAGCCTTGTAAGCGTCCAC-3′) and APH-R (5′-TTCATGCCGTGCTTCTCCAG -3′). The expected product size for these primers is 1000-1190 bp using *(aph*) gene as the template. The PCR amplification was performed using for 30 cycles of (94 °C at 1 min) denaturation step and annealing (53 °C at 1 min), and final extension (72 °C at 10 min). The PCR products were run on a 1% agarose gel and the product bands were cut out and extracted using the Extraction Kit as per manufacturer's instructions and compared with 1-kb ladder DNA ladder.

Table 2. Primer sequences for identification *aph* gene

Gene	Primer	Nucleotides Sequence (5' – 3')		PCR product size Primer
Aph	APH -F APH-R	Forward	GGCTAAAATGA GAATATCACCGG	1000-1190 bp
		Reverse	CTTTAAAAAATC ATACAGCTCGCG	

Results and Discussion

Collection and isolation of *Streptomyces* sp from Soil Samples. In this study 6 samples were collected from soil of different location of Baghdad city. Nine isolates of *Streptomyces* were recovered from soil samples. These isolates have been cultured and purified on Glycerol yeast extract agar (GYEA). Twenty to Thirty-five colonies were found per plate. Maximum number of colonies (3.5×10^5 CFU/gm of soil) were obtained in the soil collected from Baghdad / Utafiyah.

Colonies selected from each plate were five to ten based on colony appearance. Colonies having characteristic features such as powdery appearance with convex, concave or flat surface and color ranging from white, gray to pinkish and yellowish were selected.

Out of the 23 Actinomycetes isolates, only 9 isolates demonstrated cultural characteristics similar to that of genus *Streptomyces*. Nine isolates were selected and purified by pure culture techniques. All isolates were given a number as A1, A 2, A3, A4, A5, A15, A16, A17 and A18 (Table 3).

Nine of the isolates were recovered from soil samples 5 from Al-Jadiria, 2 from Al-Mahmoudiyah and 2 from Utafiyah. All of the isolates were considered as *Streptomyces* depending on their mycelia growth (Branched mycelia) and on their abilities to grow on Glycerol yeast extract agar (GYEA) supplemented with tetracycline 50 µg/ml and 50 µg/ml cycloheximide. Glycerol yeast extract agar medium is specific and sensitive for *Streptomyces* since it contains glycerol, more actinomycetes use as a sole carbon source, cycloheximide reduces fungal growth, whereas tetracycline reduces other bacteria. Colonies size varied, powdery, colour varied from chalky white and grey. Isolation of *Streptomyces* from all soil samples show *Streptomyces* is predominant in these soils. previous studies reported that *Streptomyces* species were the most dominant actinomycetes inhabited, the genus *Streptomyces* species which was the dominant and contributed the largest spectrum of species among 22 (85%) isolates. (Kumar *et al*., 2013; Abdullah *et al.*, 2016) [1]. In study obtain on 27 actinomycetes isolates were identified as *Streptomyces* (16 isolates) (Gurung *et al.,* 2009) [18]

Table 3: Collection site and *Streptomyces* isolates using on Glycerol yeast extract agar medium

Date of collection	Number of samples.	Collection site (Source of soil sample)	Number of actinomycetes Colonies in each gram of soil (CFU/gm of dried weight soil)	Isolates
9/1/2017	2	Baghdad / Al-Jadiria	20×10^5	A1
			2.1×10^5	A2
			2.7×10^5	A3
			2.0×10^5	A4
			2.4×10^5	A5
10/1/2017	2	Baghdad / Al Mahmoudiyah	3.2×10^5	A15
			3.0×10^5	A16
12/1/2017	2	Baghdad / Utafiyah	3.5×10^5	A17
			2.8×10^5	A18

Screening of Antibiotic Producing *Streptomyces* sp.

All *Streptomyces* isolates (A1, A 2, A3, A4, A5, A15, A16, A17 and A18) were screened for their antibacterial activity on malt extract yeast extract agar medium (ISP2) using scross-streak technique. A broad spectrum of antibacterial activity was observed in 44.4% (4 out of 9) (A3, A4, A15 and A17) of the total *Streptomyces* isolates (Table 4).

Table 4: Primary screening of *Streptomyces* isolates using scross-streak technique on malt extract yeast extract agar medium

Isolates	Gram-positive		Gram-negative		Note
	Bacillus cereus	Streptococcus sp.	Pseudomonas aeruginosa	Escherichia coli.	
A1	-ve	-ve	-ve	-ve	Neglected
A2	-ve	-ve	-ve	-ve	Neglected
A3	+ve	+ve	+ve	+ve	Selected
A4	+ve	+ve	+ve	+ve	Selected
A5	-ve	-ve	-ve	-ve	Neglected
A15	+ve	+ve	+ve	+ve	Selected
A16	-ve	-ve	-ve	-ve	Neglected
A17	+ve	+ve	+ve	+ve	Selected
A18	-ve	-ve	-ve	-ve	Neglected

Screening was performed by Agar-Well Diffusion method and growth inhibition zones were measured in millimeters for each of the *Streptomyces* isolates, the results are shown in Table 5. Tested isolates have shown potent *in vitro* antibacterial activities against all tested pathogens. The highest activities were shown by isolate A17 against *Bacillus cereus* (19 mm), *Streptococcus* sp. (15 mm), *Pseudomonas aeruginosa* (17mm), *Escherichia coli* (19 mm). It is also evident in Table 5 that isolates A4 and A15 have shown strong activities against all pathogenic bacteria with inhibition zone diameters ranging between 12 and 15mm. Isolate A3 have shown moderate inhibitory effect against pathogenic bacteria with inhibition zones diameters in the ranging between 6 and 11mm. In a study by Arifuzzaman *et al.,* (2010) [4] showed about 55 actinomycetes were isolated and screened for antibacterial activity, found 20 isolates (36.36%) were active against the test organisms. Denizci *et al.,* (1996) [9] isolation 356 *Streptomyces* sp from the soil and found about 122 isolates were antimicrobial activity.

Table 5: Inhibition zones (mm) by different *Streptomyces* isolates against pathogenic bacteria

Isolates	Zone of inhibition (mm)			
	Bacillus cereus	Streptococcus sp.	Pseudomonas aeruginosa	Escherichia coli.
A3	10	6	11	8
A4	12	12	14	15
A15	14		15	13
A17	19	15	17	19

Cultural and Microscopical Characterization of *Streptomyces* isolates

Four *Streptomyces* isolates that showed great potentialities for antibacterial production were selected and characterized. Characterization was done according to Bergey's Manual of Systematic Bacteriology (Bergy and Holt, 1994; Vimal *et al.,* 2009) [47]. The results of cultural characterization after 5 days of incubation on malt extract yeast extract agar medium (ISP2) are shown in Table 6. The colonies of the tested isolates were round, filamentous and concentric in shape, small, smooth surfaces, aerial mycelium developed and appeared powdery. Most isolates have whitish colonies. Two of the isolates produced white- coloured mycelia, one grey mycelia, one Peach mycelium. Colonies Elevation were flat (A15), convex (A3), raised (A17) or drop like (A4). Colony edges were smooth (A15) filamentous (A3), concave and irregular (A17) and wrinkled (A4). The colonies morphology displayed are typical to those described by Anderson and Wellington (2001) [3] for actinomycetes.

Table 6: Cultural and Microscopical characteristics of *Streptomyces* isolates.

Isolates	Shape	Coloor	Edges	Elevation	Surface	Gram stain	Motility	Coloration of substrate mycelia	Aerial mycelium	Growth
A3	Round	Peach	filamentous	convex	Powdery	+ve	-ve	yellow	Present	Good
A4	Concentric	Grey	wrinkled	drop	Powdery	+ve	-ve	chalky texture	Present	Moderate
A15	Filamentous	White to yellow	smooth	flat	Powdery	+ve	-ve	chalky texture	Present	Good
A17	Round and irregular	White to Gray	concave and irregular	raised	Powdery	+ve	-ve	yellow	Present colour Pink	Good

Microscopical characterization of the tested isolates were filamentous, non motile and Gram positive. Such characters are typical of the actinomycetes (Cross, 1989) [7]. Morphological examination of these isolates, indicates that they belong to the genus *Streptomyces* (Waksman, 1961). Morphological characteristics described by Das *et al.,* (2008) [8]. Vanajakumar *et al.,* (1995) [46] have also reported that white color series of *Streptomyces* they were the dominant forms.

Biochemical and physiological Characteristics

Result of biochemical and physiological testes of *Streptomyces* sp. Show in (Table 7). Ability of *Streptomyces* isolates to produce enzymes was varied. All isolates hydrolyzed starch. All isolates produced Catalase, Gelatinase, Protease, Urease, Catalase, Amylase, Cellulase and Lipase. All *Streptomyces* isolates could produce Nitrate reduction, Tyrosine degradation and Casein hydrolysis. A3, A4 and A15 could not produce Hydrogen sulphide production, Oxidase production, Indole production and Melanine reaction. All *Streptomyces* isolates could Citrate utilization and Pectin degradation except A17. All *Streptomyces* isolates could grow at pH 6-9 and Grow in different temperature 28-30oC. Growth of isolated *Streptomyces* with different NaCl concentration was ranging between 5 to 7. *Streptomyces* is one of the best recognized genera of the whole order of Actinomycetales because of its wide distribution in nature, especially in soils. The biochemical activities of such pure cultures frequently allow genera and species characterization and identification (Gillies and Dods, 1984) [14].

Table 7: Physiological and biochemical characteristics of *Streptomyces* isolates

Isolates	Biochemical characteristics																		Physiological ch.		
	*H2S	Ca.	Ni	Ge	Ci	Ty	Pe	Pr	Ur	Cat	Ox	Cas	In	Me	St	Am	Ce	Li	pH	Te.	NaCl%
A3	-	+	+	+	-	+	-	+	+	+	-	+	-	-	+	+	+	+	8	28°C	6
A4	-	+	+	+	-	+	-	+	+	+	-	+	-	-	+	+	+	+	6	28°C	6
A15	-	+	+	+	-	+	-	+	+	+	-	+	-	-	+	+	+	+	9	30°C	7
A17	-	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	+	+	8	28°C	5

*H2S: Hydrogen sulfide production, Ca: Catalase production, Ni: Nitrate reduction, Ge: Gelatinase, Ci: Citrate utilization, Ty: Tyrosine degradation, Pe: Pectin degradation, Pr: Protease production, Ur: Urease production, Cat: Catalase, Ox: Oxidase production, Cas: Casein hydrolysis, In: Indole production, Me: Melanine reaction, St: Starch, Am: Amylase production, Ce: Cellulase, Li: Lipase, Te: temperature, NaCl concentrations

Molecular Identification of *Streptomyces* isolates

PCR products were separated and the isolation of genomic DNAs was verified with electrophoresis on 1.5% agarose gels (w/v) and run in 1x Tris-borate buffer, pH 8.3. Genomic DNAs were seen as a smear of different molecular weight DNAs under UV light. The ampilified DNA bands were visualised after ethidium bromide staining and photographed under ultraviolet light. The results show the DNA of isolated strains was extracted successfully. The results of PCR amplification which was performed on the DNA extracted from all isolates were confirmed by the electrophoresis analysis.

Three primers were selected for further testing, the forward primer and the reverse primer. The target areas of the primers along the 16S rRNA are bp 260-273. Comparison of PCR and cultivation methods for the detection of *Streptomyces* in soil. Four isolates (A3, A4, A15 and A17) were positive in the PCR T, technique. Performing PCR reactions using primer pair on DNA from soil isolates produced a single 273 bp band in all tested isolates (Fig. 1).

(Kim *et al.,* 1993) [24] showed that advantages of rRNA genes as targets for PCR detection assays are that the genes are essential for the cells, and they are conserved among the prokaryotes. With *Streptomyces* which have a highly unstable genome (Volff and Altenbuchner 1998) [48]. It is important that the rRNA genes are situated in the stable core part of the genome (Bentley *et al.,* 2002) [4]. According to Stackebrandt *et al.,* (1991), the variable regions V2, V6 and V7 are suitable for the discrimination of *Streptomyces* species. Two of the primers developed in this work, StrepB and StrepE partially overlap with published *Streptomyces* - specific primers (Mehling *et al*., 1995) [29]. Thus, the gene fragments amplified with these primers contain sufficient information for diversity studies; however, the 16S rDNA sequence diversity provides no information about the functional diversity of species (Metsä-Ketelä *et al.,* 2002) [30]. According to morphological, physiological and biochemical characteristics and molecular identification, the results indicated that A17 isolate is very close to the species *Streptomyces fradiae*.

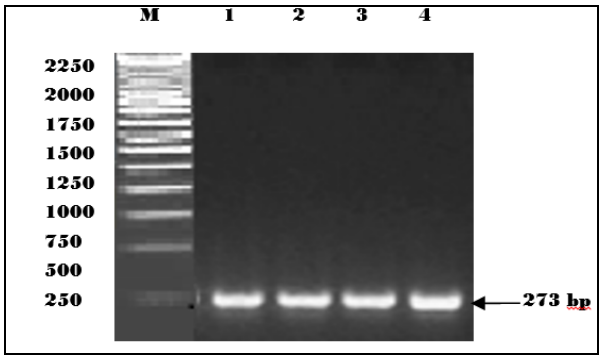

Fig 1: PCR amplification of *Streptomyces* 16S rDNA from soil, using AM48, AM51, AM52 Primers and 1.5% Agarose: Lane M - Molecular weight marker (250 bp), Lanes 1– 4: *Streptomyces* isolates (1: A3; 2: A4; 3: A15; 4: A17), showing positive results (273 bp).

Molecular identification *aph* gene for *Streptomyces* isolates
PCR Detection of *aph* Gene

The amplification of gene *aph* was performed using the APH primers (APH -F and APH -R). The *Streptomyces* isolates then subjected to molecular identification of the *aph* genes by polymerase chain reaction. *Aph* gene was successfully amplified from *Streptomyces* isolates. The results of *aph* gene detection clarify that one isolate (25%) of *Streptomyces* isolates gave a positive result and carrying *aph* gene while 3 (75%) of *Streptomyces* isolates were lack the gene and no cross reactivity could be observed with any of the A3, A4 and A15 isolates. The results showed the amplification of aph gene for four *Streptomyces* isolates were A3, A4, A15 and A17. PCR product 1190bp, this used for identification of *aph* gene for *Streptomyces* isolates and found the A17 isolate gave DNA bands 1190 bp in length, (Fig: 2). Demonstrating that aph gene was present below the detection threshold of the PCR used. This gene should be considered a component of Production of neomycin antibiotic. The results indicated that the A17 isolate is very close to the species *Streptomyces fradiae* responsible for producing antibiotic neomycin. This results were closely related to results of (Thompson and Grayt, 1983; Salauze and Davies, 1991) [45], they found that *aph* gene validly tested of *S. fradiae* isolates harboured the *aph* - aminoglycoside phosphotransferase gene.

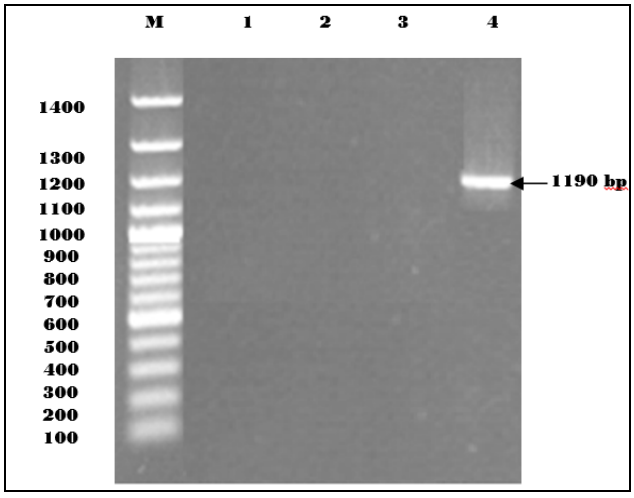

Fig 2: PCR amplification of *aph* gene of *Streptomyces* isolates, Lanes: 100 bp DNA ladder, 1-4 PCR products obtained with the APH PCR primers: 1-3 no Gene: 4, A17: *aph* gene (1190bp)

The sequence differences in the 16S rRNA gene are located in the so-called variable regions V1-V8. The V2 and V3 the regions show the greatest differences between *Streptomyces* and *E. coli*, while the V2 region shows the greatest variability within the *Streptomyces* (Pernodet *et al.*, 1989, Stackebrandt *et al*., 1991) [35]. Sequence analysis of the highly variable V2 region has been applied for *Streptomyces* species identification; however, it was not able to distinguish all of the species (Kataoka *et al*., 1997) [22]. Subba (2006) [44] found that cloned and sequenced an approximately about 42 kb genomic DNA fragment of *S. fradiae* in cosmid (pNEM37) cloned selected from screening. Analysis of the DNA sequence revealed the presence of putative *Nem* gene clusters along with regulatory gene and resistant genes and showed that Aminoglycosides are a large class of clinically important antibiotics and all are secondary metabolites from various *Streptomyces* species. Neomycin is the members of "4, 5-disubstituted 2-deoxystreptamine containing" aminoglycosides antibiotics. These is produced by *S. fradiae* (Inouye *et al.*, 1989) [20]. *Nem* gene cluster as it may be involved in the regulation of neomycin biosynthesis in *S. fradiae*. The genes *neo6* and *neo5* were successfully identified as an aminotransferase with dual function and dehydrogenase in the neomycin biosynthetic gene cluster from *S. fradiae* (Subba, 2006) [44].

References

1. Abdullah QYM, Al-Maqtari MA, Al-Awadhi OA, Abdullah YA. New Records of *Streptomyces* and Non *Streptomyces* Actinomycetes Isolated from Soils Surrounding Sana'a High Mountain, International Journal of Research in Pharmacy and Biosciences, 2016; 3(3):19-31.
2. Amin SM, Risan MH, Abdulmohimin N. Antimicrobial and Antioxidant Activities of Biologically Active Extract from Locally Isolated Actinomycetes in Garmian Area, Journal of Garmian University, 2016; 1(10):625-639.
3. Anderson AS, Wellington EMH. The taxonomy of *Streptomyces* and related genera. International Journal of Systematic and Evolutionary Microbiology, 2001; 51:797-814.
4. Arifuzzaman M, Khatun MR, Rahman H. Isolation and screening of actinomycetes from Sundarbans soil forantibacterial activity, African Journal of Bentley SD, Chater KF, Cerdeño-Tárraga A.-M, Challis GL, Thomson NR, James KD, Complete genome sequence of the model actinomycete Streptomyces coelicolorA3(2). Nature. 2010-2002; 417:141-147.
5. Bizuye A, Moges F, Andualem B. Isolation and screeningof antibiotic producing actinomycetes from soils in Gondartown, North West Ethiopia. Asian Pac.J. Trop. Dis. 2013; 3(5):375–381.
6. Cowan ST. Cowan and Steels Manual for the IdentificationOf Medical Bacteria, second ed. Cambridge, Univ. Press. 1974.
7. Cross T. Growth and Examination of Actinomycetes Some Guidelines. In Bergey's Manual of Systematic Bacteriology. Williams & Wilkins company ,Baltimore. 1989; 4:2340-2343.
8. Das A, Mazumder Y, Dutta BK, Shome BR, Bujarbaruah KM, Sharma G. Clostridium perfringens type A beta-2 toxin in elephant (Elephas maximus indicus) and pygmy hog (*Sussalvanius*) with haemorrhagic enteritis in Assam, India. Afr JMicrobiol Res. 2008; 2(8):196-201.
9. Denizci AA. A study on the detection and production of antibacterial antibiotics from Actinomycetes which

isolated from the soils of Aegean and Eastern Black Sea regions of Turkey. PhD thesis, Ege University, Institute of Science, Izmir, 1996.
10. Edwards U, Rogall T, Bocker H, Emade M, Bottger E. Isolation and direct complete nucleotide determination of entiregenes. Characterization of a gene coding for 16s ribosomal DNA. Nucleic Acid Res. 1989; 17:7843–7853.
11. El-Nakeeb MA, Lechevalier HA. Selective isolation of aerobic actinomycetes, Appl. Microbiol. 1963; 11(2):77-75.
12. El-Shatoury S, Abdulla H, El-Karaaly O, El-Kazzaz W, Dewedar A. Bioactivities of endophytic actinomycetes from selected medicinal plants in the world heritage site of saint Katherine, Egypt. Int J Bot; 2006; 3:307-12.
13. Elwan SH, El-Nagar MR, Ammar MS. Characteristics of Lipase(s) in the growth filtrate dialysate of *Bacillus* stearo thermophilus grown at 55-C using a tributryin- cup plate assay. Bull. Fac. Sci. Riyadh Univ. 1977; 8:105–119.
14. Gillies RR, Dodds TC.. Gillies and Dodds Bacteriology Illustrated 5th edition, Churchill Livingstone. 1984, 122-132.
15. Goodfellow A. Suprageneric classificationof actinomycetes. InBergey's Manual of Systematic Bacteriology, volume IV (Williams, S. T., Sharpe,M. E. and Holt, J. G., Eds) Williams and Wilkins, Baltimore. 1989, 2333-2339.
16. Goodfellow A. Maduromycetes. In Bergey's Manual of Systematic Bacteriology, volume IV (Williams, S. T., Sharpe, M. E. and Holt, J. G, Eds). Williams and Wilkins, Baltimore. 1989, 2509-2510.
17. Gordon RE, Barnett DA, Handerhan JE, Pang CH. Nocardia coeliaca, Nocardia autotrophica and nocardin strain. Int J Syst Evol Microbiol. 1974; 24:54-63.
18. Gurung T, Sherpa C, Agrawal V, Lekhak B. Isolation and characterization of antibacterial actinomycetes from soil samples of Kalapatthar, Mount Everest Region. Nepal Journal of Science and Technology. 2009; 10:173-182.
19. Hirsch CP, McCann-McCormick PA. In A.L. Demain and N.A. Solomon (Eds.), Biology of Industrial Microorganisms, Biotechnology Series. The Benjamin / Cummings Publishing Company Inc., California. 1985; 6:291-314.
20. Inouye S, Watanabe T, Kitasato I. Comparative antimicrobial activities of ribostamycin, gentamicin, ampicillin and lincomycin *in vitro* and *in vivo*. Drugs Under Experimental and Clinical Research, 1989; 15: 465–476.
21. Janssen GR, Judith Ward M, Bibb MJ.Unusual transcriptional and translational features of the aminoglycoside phosphotransferase gene *(aph)* from Streptomyces fradiae. Published by Cold Spring Harbor Laboratory Press. 2017.
22. Kataoka M, Ueda K, Kudo T, Seki T, Yoshida T. Application of the variable region in16S rDNA to create an index for rapid species identifiaction in the genus Streptomyces. FEMS Microbiology Letters 1997; 151:249-255.
23. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA. *Practical Streptomyces genetics.* Norwich, England: The John Innes Foundation 2000.
24. Kim E, Kim H, Hong S, Kang KH, Kho YH, Park YH. Gene organisation and primary structure of a ribosomal RNA gene cluster from Streptomyces griseus subsp. griseus. Gene, 1993; 132:21-31.
25. Kumar P, Preetam RJ, Duraipandiyan V, Ignacimuthu S. Antibacterial activity of some actinomycetes from Tamil Nadu, India. Asian Pac J Trop Biomed. 2012; 2(12):936-943.
26. Kuster E, Williams ST. Selection of mediafor isolation of streptomycetes, Nature, 1964; 202:928-929.
27. Kutzner KJ. The family *Streptomycetaceae*. In: Starr MP, Stolp H, Tr_per HG, Balows A, Schlegel HG (eds) The prokaryotes, A Handbook on Habitats, Isolation, and Identification of Bacteria, vol. 2, Springer-Verlag, New York, 1986; 2028-2090.
28. Lechevalier H, Lechevalier A. Biology of actinomycetes, Ann Rev Microbiol, 1967; 21:71-100.
29. Mehling A, Wehmeier UF, Piepersberg W. Nucleotide sequences of streptomycete 16S ribosomal DNA: towards a specific identification system for streptomycetes using PCR. Microbiology, 1995; 141:2139-2147.
30. Metsä-Ketelä M, Halo L, Munukka E, Hakala J, Mäntsälä P, Ylihonko K. Molecular evolution of aromatic polyketides and comparative sequence analysis of polyketide ketosynthase and 16S ribosomal DNA genes from various Streptomyces species. Applied and Environmental Microbiology, 2002; 68:4472-4479.
31. Mohanraj G, Sekar T. Isolation and screening of actinomycetes from marine sediments for their potential to produce antimicrobials Int. J. LifeSc. Bt and Pharm. Res, 2013; 2(3):115-126.
32. Murray PR, Baroon EJ, Pfaller MA, Tenover FC, Yolke RH. Editors. Manual ofclinical microbiology. 6th ed. Washington DC: American Society for Microbiology, 1995.
33. Nonoh J, Lwande W, Masiga D, Herrmann R, Presnail J, Schepers E, Okech MA, Bagine R, Mungai P, Bernard Nyende A, Boga HI *et al*. Isolation and characterization of *Streptomyces* species with antifungal activity from selected national parks in Kenya. Afri J Microb Res. 2010; 4(9):856-864.
34. Oskay M, TA Usame, Azeri C. Antibacterial activity of some actinomycetesisolated from farmin soils of Turkey, Afr. J. Biotechnol, 2004; 3(9):441-446.
35. Pernodet JL, Boccard F, Alegre MT, agmat J, Guerineau M. Organisation andnucleotide sequence analysis of a ribosomal RNA cluster from Streptomyces ambofaciens. Gene, 1989; 79:33-46.
36. Qasim B, Risan MH. Anti-tumor and Antimicrobial Activity of Antibiotic Produced by Streptomyces spp, World Journal of Pharmaceutical Research, 2017; 6(4):116-128.
37. Ravikumar S, Inbaneson SJ, Uthiraselvam M, Priya SR, Ramu A, Banerjee MB. Diversity of endophytic actinomycetes from Karangkadu mangrove ecosystem and its antibacterial potential against bacterial pathogens. J Pharm Res. 2011; 4:294-6.
38. Rinehart KL, Malik JM, Nystrom RS, Stroshane RM, Truitt ST, Taniguchi M, Rolls JP, Haak WJ, Ruff BA. Biosynthetic incorporation of (1-13C) glucosamine and (6-13C) glucose into neomycin. J. Am. Chem. Soc. 1974; 96:2263-2265.
39. Risan MH, Amin SM, Abdulmohimin N. Production, Partial Purification and Antitumor Properties of Bioactive Compounds from Locally Isolated Actinomycetes (KH14), Iraqi Journal of Biotechnology, 2016; 15(3):51-64.
40. Risan MH, Qasim B, Abdel-jabbar B, Muhsin AH. Identification Active Compounds of Bacteria

Streptomyces Using High-Performance Liquid Chromatography, World Journal of Pharmaceutical and Life Sciences, 2017; 3(6):91-97
41. Salauze D, J Davies. Transcriptional mapping of the promoter of the aminoglycoside acetyltransferase gene (*aacC9*) of neomycin-producing Micromonospora chalcea. Res. Microbiol. 1992; 143:133–138.
42. disubstituted 3 -deoxystreptamine antibiotics J. Antibiot. 44:1432-1443.
43. Sanchez S, Demain AL. Metabolic regulation of fermentation processes. Enzyme and Microbial Technology, 2002; 31:895 - 906.
44. Subba B. Biosynthesis of Ribostamycin and Neomycin: thesis, graduate school, sun moon university, republic of korea, 2006.
45. Thompson CJ, Grayt GS. Nucleotide sequence of a streptomycete aminoglycoside phosphotransferase gene and its relationship to phosphotransferases encoded by resistance plasmids, Proc. Nati. Acad. Sci. 1983; 80:5190-5194.
46. Vanajakumar SN, Natarajan R. Antagonistic properties of actinomycetes isolated from mollusks of the Porto Novo region, South India. In: Thompson M, Sorojini R, Nagabhushanam R, editors. Bioactive compounds from marine organisms. New Delhi: Oxford & IBH Publishing Co. Pvt. Ltd. 1995.
47. Vimal V, Mercy RB, Kannabiran K. Antimicrobial activity of marine actinomycetes, Nocardiopsis sp VITSVK5 (FJ973467). Asian J Med Sci. 2009; 1:57-63.
48. Volff J, Altenbuchner J. Genetic Instability of the Streptomyces chromosome. Molecular Microbiology 1998; 27:239-246.
49. Von Wintzingerode F, Göbel UB, Stackebrandt E. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiology Reviews, 1997; 21:213-229.
50. Williams ST, Goodfellow M, Alderson G. Genus *Streptomyces* Waksmanand Henrici1943, 399AL. In Williams ST, Sharpe ME, Holt JG (ed) Bergey's manual of systematic Bacteriology, vol. 4, Williams & Wilkins, Baltimore, 1989, 2452-2492.