International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 16s,2025
https://theaspd.com/index.php

Detection Of Aph And Tlr C Genes In Local Isolate Of Streptomyces Fradiae

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Abstract
Background: Streptomyces is a genus of Actinobacteria, which are Gram-positive bacteria that generate spores and have a filamentous structure. Streptomyces fradiae belongs to the Actinomycetota species. Various variants of S. fradiae have been identified as producers of the antibiotics neomycin, tylosin, and fosfomycin. ***Aim of the study:*** *Isolation and identification of Streptomyces fradiae and detection of aph and tlrC genes in the isolates.* ***Methods:*** *A total of fifty soil samples were taken between December 2023 and January 2024. Specimens were gathered from several locations inside the city of Baghdad. After sample dilution it put on ISP2 agar for Streptomyces isolation. Several biochemical tests were done for the isolates. Aph and tlrC genes were detected by PCR.* ***Results*** *Thirty Streptomyces isolate were obtained depended on morphological, cultural and biochemical characteristic. The colonies under suspicion were cultivated on ISP2 agar and chosen based on their color (gray, creamy, or white) and colony diameter (ranging from 1 to 10 mm). Additionally, their morphology was considered, with the colonies initially having a smooth surface and later becoming powdery, soft, and granular as they formed aerial mycelium. One isolate only was contain the aph and tlrC genes among thirteen Streptomyces isolates.* ***Conclusion:*** *Actinomycetes were observed in culturing the diluted soil sample (10^9) for 7-10 days on ISP2 agar. The PCR amplification exhibited that the aph and tlrC genes was appeared in the one isolated and purified Streptomyces fradiae that associated with resistance.*
Keywords: *Streptomyces fradiae, aph gene, tlr C gene, PCR*

INTRODUCTION

The Streptomyces genus comprises spore-forming, filamentous, and Gram-positive bacteria belonging to the Actinobacteria phylum (1,2). These bacteria can be found in diverse ecosystems, including extreme and underexplored habitats, both terrestrial and marine locations, as well as in symbiotic relationships, endophytes, and mangroves. A total of 850 species of Streptomyces have been examined (3). Streptomyces are widely distributed bacteria found on land, known for their strong metabolic powers and adaptable behaviour. These bacteria can use new growth and dispersion behaviours as they compete for environmental niches. In addition, they utilise their varied metabolic capabilities for a range of purposes, including optimising food absorption, impeding phage replication, and suppressing the growth of bacteria and fungi. They are increasingly discovered to exist in symbiosis with plants and insects, often providing protective advantages to their host by producing antimicrobial chemicals that inhibit pathogens (4,5).Approximately 109 colony-forming units (CFU) of bacteria, with 107 CFU exclusively belonging to Actinobacteria, are estimated to be present in a gramme of soil (6). Streptomyces species undergo an intricate process of multicellular growth, starting with the emergence of hyphae from spores and ending with the creation of septa that enclose a chain of spores, each containing a single nucleus. Additionally, they possess a wide network of branching material and above-ground fungal threads, accompanied by complex physical features. Streptomyces bacteria have genomes that are linear and of intermediate size, often ranging from 8 to 10 megabases (Mb). Additionally, they possess a substantial G + C% composition, surpassing 70%, which is regarded as exceptional among the bacterial community. A distinguishing feature of Streptomyces is the existence of several biosynthetic gene clusters (BGCs) on every chromosome. These clusters are accountable for generating a diverse array of bioactive substances with uses in the fields of medicine .The initial categorization of Streptomyces species primarily depended on morphological findings. Consistent and dependable criteria in classifications include morphological traits such as spore colour, spore chain morphology, melanoid pigment production, spore wall ornamentation, and the utilisation pattern of nine distinct sugars as a carbon source in physiological tests (12,13). The International Streptomyces Project (ISP) has released accurate descriptions of type strains for 458 Streptomyces species, utilising standardised criteria to establish species classification. Subsequently, other fundamental characteristics such as physiological and biochemical qualities, chemotaxonomy, and DNA–

DNA hybridization (DDH) of total chromosomal DNA were utilised for categorization (14). Streptomyces fradiae belongs to the Actinomycetota species. Various variants of S. fradiae have been identified as producers of the antibiotics neomycin, tylosin, and fosfomycin (15,16). Aminoglycoside phosphotransferases (APHs) and macrolide phosphotransferases (MPHs) are key factors contributing to the lack of success in therapy. The genes responsible for encoding APHs have been the subject of study since the 1970s. The genes were discovered on plasmids and mobile genetic elements of clinical strains of both Gram-positive and Gram-negative bacteria, as well as actinobacteria like Streptomyces, which are known for producing aminoglycoside antibiotics. Previous studies have demonstrated that APH enzymes play a role in preserving innate resistance to aminoglycoside antibiotics in many microorganisms, such as soil bacteria (17). The gene aph, derived from the neomycin-producing bacterium Streptomyces fradiae, encodes an enzyme called APH. This enzyme phosphorylates neomycin, rendering it inactive as an antibiotic.

tlrC is a protein belonging to the ABC-F subfamily, which is discovered in Streptomyces fradiae. It provides resistance to mycinamicin, tylosin, and lincosamides. tlrC is located within the tylosin biosynthetic cluster and serves as a mechanism employed by S. fradiae to safeguard itself against self-destruction during the production of this macrolide (19). The Streptomyces fradiae strain that produces tylosin (Ty) contains a minimum of three genes, tlrA, tlrB, and tlrC, which confer resistance to Ty (TyR) (20). The objective of the study was to isolate and identify Streptomyces fradiae and to detect the presence of aph and tlrC genes in the isolated strains.

METHODS

Study design

A grand total of fifty soil samples were collected during the period spanning from December 2023 to January 2024. Specimens were collected from several sites inside the city of Baghdad. Separate areas were employed to isolate Streptomyces spp. at the various location. The specimens were obtained from a 10-15 cm depth by excavating approximately 3 cm of the surface soil. The specimens were enclosed in plastic bags, tightly sealed, and stored in a refrigerator. The soil samples underwent incubation at a temperature of 70°C for a period of 2 hours to eradicate any other bacteria present. Subsequently, a screening procedure was employed to selectively isolate Streptomyces.

Isolation and Identification of Streptomyces spp. from Soil Samples

A stock suspension was made by combining 1 gramme of desiccated soil samples with 99 millilitres of sterile distilled water. The specimens were stirred vigorously in a mechanical device at a rate of 120 rotations per minute for a period of 30 minutes at the surrounding temperature. Serial dilutions ranging from 10-1 to 10-9 were created by mixing the stock suspension with different amounts of solvent. The mixtures were then left undisturbed for a duration of 10 minutes. After agitation, 0.1 ml of each dilution was extracted using a pipette and deposited onto Yeast extract-malt extract agar (ISP2) supplemented with Tetracycline at a concentration of 50 µg/L and Nystatin at a concentration of 50 µg/L. To achieve equal distribution, the suspension was evenly spread throughout the surface of the media using a sterile swab. The plates that underwent treatment with an antimicrobial agent were placed in a controlled environment at a temperature of 28°C for a duration of 7 to 10 days to facilitate their growth and development. Actinomycete colonies were selected for characterization based on their cultural features, which encompass being small, white, pinpoint, rough, chalky, and exhibiting a distinct zone of inhibition surrounding them. The probable colonies were identified using Gram's stain, as well as by observing the colour of their aerial and substrate mycelium, pigment production, and pigment colour. Specific biochemical tests are also employed for the purpose of identification. The colonies were transferred from the initial screening stage (mixed culture) to separate agar plates and subjected to incubation at a temperature of 28±1°C for a period of 7 days in order to get a pure culture of actinomycetes species on ISP2 agar. This method was repeated several times. The aseptic culture was kept at a temperature of 4°C for further examination (21).

Molecular study

Molecular detection of aph &tlrC genes

Molecular detection by Polymerase Chain Reaction (PCR) amplification of aph and tlrC gene, primers were showed in (Table 1,2).

Table(1): Primer pair for the aph gene in Streptomyces fradiae

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	CCTTCGTCTACCAGCTCACC	Plus	20	104	123	59.83	60.00
Reverse primer	AAGGCCAGCTTCTCCTTGTC	Minus	20	809	790	59.96	55.00
Product length	706						

Table(2): Primer pair for the tlrC gene in Streptomyces fradiae

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	AGCAGCAGTACGACGAGTG	Plus	19	724	742	59.79	57.89
Reverse primer	CTCCAACTCCTCCACCAGC	Minus	19	1457	1439	59.70	63.16
Product length	734						

DNA Extraction

Bacterial genomic DNA was extracted from bacterial isolates by using (ABIOpureTM Total DNA Bacteria Kit) and done according to company instructions

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to verify the existence of amplification after PCR amplification. The success of PCR relied entirely on the specific requirements of the isolated DNA. The PCR products were loaded without any intermediary steps. For the PCR product, a volume of 5µl was directly injected into the well. An electrical power supply was activated at a voltage of 100 volts and a current of 1 milliampere for a duration of 60 minutes. The DNA migrates from the cathode to the anode, which has a positive charge. The bands stained with Ethidium bromide in the gel were observed using a Gel imaging equipment.

RESULTS

Out of the 50 soil samples analyzed, 35 (86.6%) were found to potentially contain Streptomyces. Among these samples, 33 (80%) isolates were successfully isolated, each exhibiting distinct morphological traits. To obtain a pure isolation, the probable Actinomycetes colonies were carefully moved to ISP2 agar medium. The colourful aerial and substrate mycelium, dry, rough or smooth texture, and uneven or regular edge were then used to characterise the isolate. The colony, in general, had a convex shape. The majority of solitary colonies exhibit earthy aromas, as described by (22).

Figure (1) displays little powdery colonies ranging from white to grey, which are likely to represent isolates of Actinomycetes. This picture displays a solitary Actinomycete colony within a collection of mixed colonies. The figure (2) clearly shows a single colony of Actinomycetes isolate. Colonies that come from sources other than Actinomycetes in the culture can be linked to the presence of their spores in the soil or their ability to withstand heat treatment. The colonies under suspicion were cultivated on ISP2 agar and chosen based on their color (gray, creamy, or white) and colony diameter (ranging from 1 to 10 mm). Additionally, their morphology was considered, with the colonies initially having a smooth surface and later becoming powdery, soft, and granular as they formed aerial mycelium. Similar findings were reported by (23,24)

Figure (1): Actinomycetes 's powdery colonies

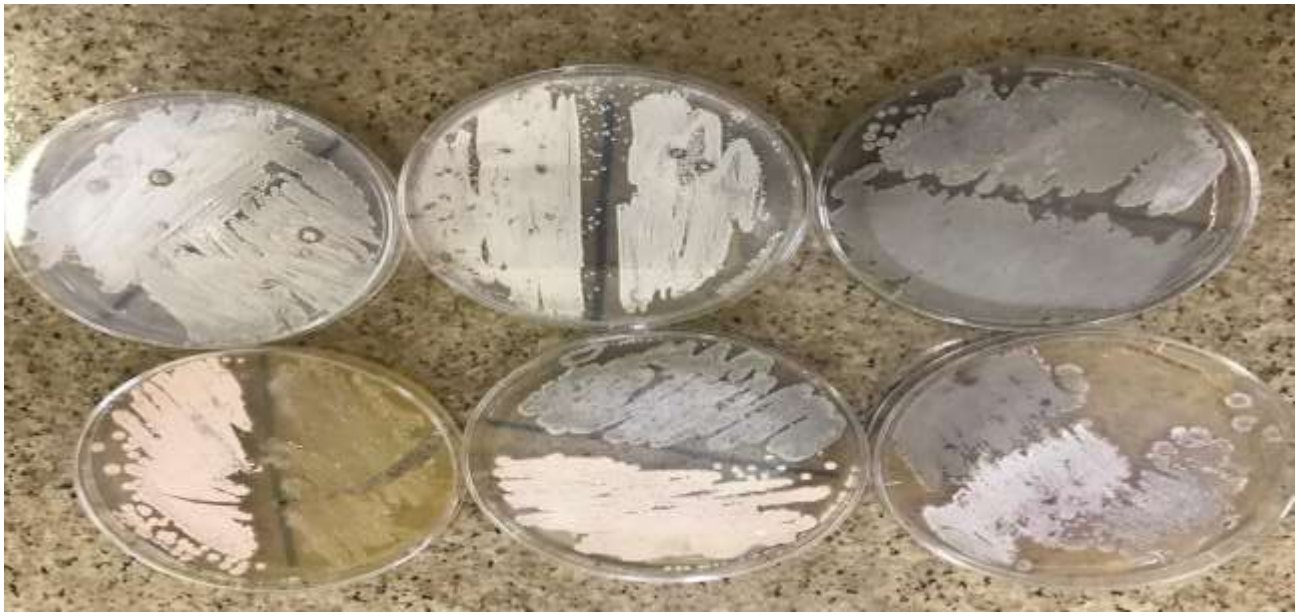


Figure (2): Single colonies of Streptomyces fradiae on ISP2 agar

The biochemical findings of **Streptomyces fradiae** are presented in table (3-2). Streptomyces possess the capacity to synthesize enzymes such as catalase. Simmon's citrate usage was confirmed, indicating a positive result, but indole synthesis was not detected, indicating a negative result. The use of sugar was investigated by cultivating Streptomyces in media containing Dextrose, starch, or Glycerol as carbon sources. The analysis was conducted using a biochemical test (25).

Table (3): Biochemical results of Streptomyces fradiae

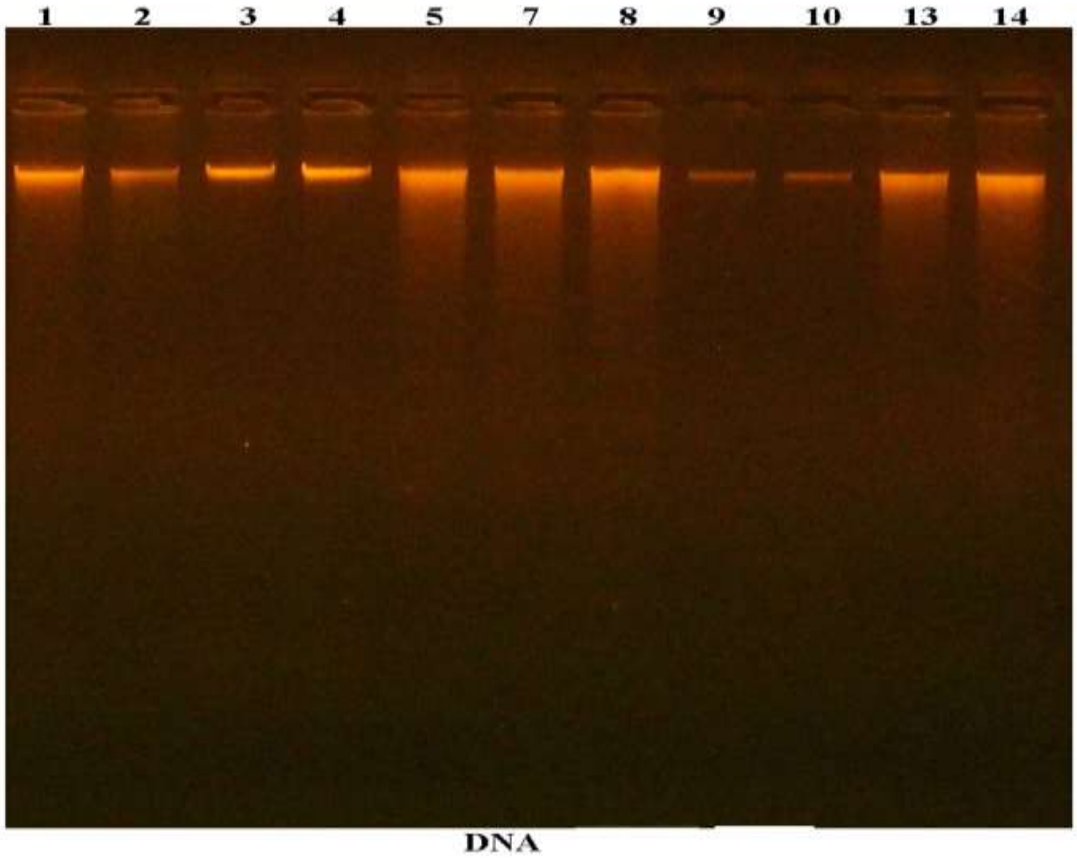
No	Test	Reaction	Result
1.	Melanin	Black to brown	Negative

2.	Catalase	Bubbles	Positive
3.	Citrate Utilization	Deep blue color	Positive
4.	Indole production	No color zone	Negative
5.	Sugar utilization	Growth	Positive
6.	Hydrogen sulfide production	No reaction	Negative
7.	Nitrate reduction	Red color	Positive
8.	Oxidase production	No reaction	Negative
9.	Casein hydrolysis	Clear transparent zone	Positive
10.	Melanine reaction	No reaction	Negative
11.	Starch hydrolysis	Clear halo	Positive

Molecular Assay

Extraction of genomic DNA

Whole genome DNA from overnight cultures of 13 isolates of Streptomyces fradiae were extracted efficiently by ABIOpure TM Total DNA extraction kit, following the procedure described previously in chapter two. The concentration and purity of the isolated DNA were assessed using Nanodrop. Moreover, Figure (3) demonstrates the presence of an unique band of extracted DNA, indicating the efficacy of the DNA extraction method employed.



Figure(3):Agarose gel imaging of the DNA extracted for the bacterial isolates

Detection of aph (The aminoglycoside phosphotransferase) gene in Streptomyces fradiae The results showed that 1 out of the 13 isolates (7.7%) have aph gene (The aminoglycoside phosphotransferase). The aph-F and aph-R primers are used in detection of aph gene. The fragment size was determined to be 706 base pairs. as shown in figure (4).

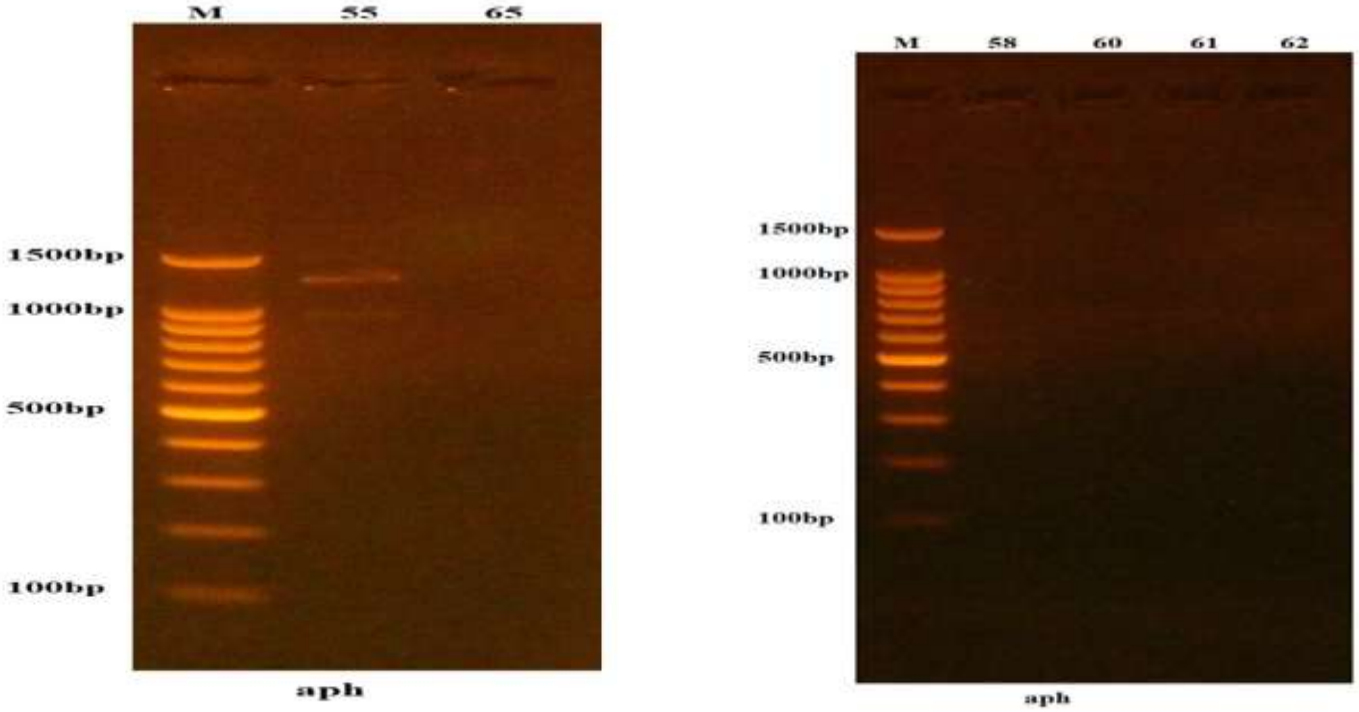


Figure (4): PCR product the band size 706 bp. The product was electrophoresis on 1.5% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100).

Detection of tlrC (tylosin resistance) gene in Streptomyces fradiae

The results showed that 1 out of the 13 isolates (7.7%) have tlrC gene (Tylosine resistance C). The tlrC-F and tlrC-R primers are used in detection of tlrC gene. The fragment size was determined to be base pairs. as shown in figure (5).

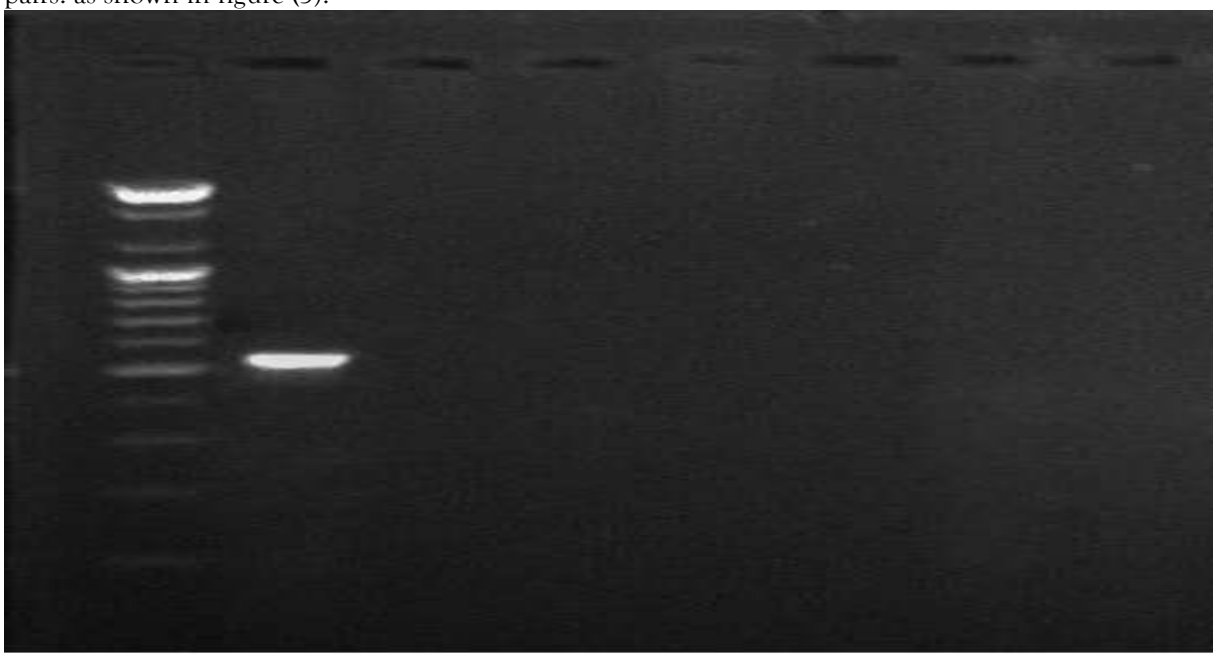


Figure (5): PCR product the band size 734 bp. The product was electrophoresis on 1.5% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100).

DISCUSSION

The classification of the Streptomyces isolates was conducted by evaluating the variations in their microscopic traits and colony morphology, such as the existence of aerial and substrate mycelium, the existence of soluble pigment, and the organisation of spore chains. Multiple Streptomyces fradiae isolates had the capacity to produce a coloured material that spread into the surrounding medium, matching the colour of the aerial mycelium (26). A total of 154 Streptomyces samples were obtained from 46 soil samples collected from various places in Nineveh (27). In the investigation conducted by Al-Rubaye et al. (28), it was found that around 88% of the soil samples, which is equivalent to 44 samples, were suspected to contain Streptomyces. A total of 42 Streptomyces spp. (84%) have been isolated, each exhibiting distinct morphological characteristics. The aminoglycoside phosphotransferase gene (aph) from the neomycin producer Streptomyces fradiae encodes an enzyme (APH) that phosphorylates, and thereby inactivates, the antibiotic neomycin (18).Aminoglycosides, a broad category of medically significant antibiotics, are mostly synthesised by actinomycetes, particularly Streptomyces. These antibiotics are classified as secondary metabolites derived from different species of Streptomyces. S. fradiae possesses the regulatory gene for aminoglycoside phosphotransferase (aph). (29). These genes can be transmitted to them from strains that produce antibiotics (30). The investigation conducted by Risan et al. (24) shown that out of the four Streptomyces isolates analysed, one isolate (25%) tested positive for the presence of the aph gene, whereas the remaining three isolates (75%) lacked this gene. These findings align with the results obtained in the present study.
Streptomyces fradiae is a bacterium that synthesises tylosin, which is an antibiotic commonly employed in veterinary medicine. Research on the biosynthetic genes of tylosin-producing strains has shown that the genes responsible for biosynthesis and self-resistance are located near together in the genome(31). S. fradiae has been documented to harbour four tylosin resistance genes, namely tlrA, tlrB, tlrC, and tlrD. The tlrC gene is situated in the terminal region of the tylosin biosynthetic cluster and encodes for an ATP-binding protein that plays a role in the expulsion of tylosin (32).

CONCLUSION

Actinomycetes were observed in culturing the diluted soil sample (10^{-9}) for 7-10 days on ISP2 agar. The PCR amplification exhibited that the aph and tlrC genes was appeared in the one isolated and purified Streptomyces fradiae that associated with resistance.

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