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Novel approach to overcome the β-lactam resistant bacteria using an actinobacterial inhibitory protein

Nada A. M. Abdelbary[1*]; Rawia F. Gamal[1]; Waleed M. Abdulkhair[2]; Sawsan Y. Elateek[3]

[1]Microbiology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt; [2]Microbiology Department, Egyptian Drug Authority (EDA), Giza, Egypt; [3]Genetics Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt

[*]Corresponding author E-mail: NadaAbdelbary@agr.asu.edu.eg

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Abstract

β-lactam resistance is a serious problem that the hospitals face worldwide; particularly in the developing countries. The widespread of this resistance is attributed to various mechanisms used by the nosocomial bacteria. The aims of this study were to monitor the spread of the β-lactam resistant bacteria in the different provinces of Egypt; to create a biocontrol strategy by producing the β-lactamase inhibitory protein from the *Streptomyces* bacteria, and to knowing its suitability for the human use. Seventy β-lactam resistant bacterial isolates were sampled randomly from several hospital laboratories across ten governorates of Egypt. The isolates were screened against six different antibiotics; mainly Amoxicillin; Amoxicillin-Clavulanate, Penicillin, Ampicillin-Sulbactam, Cefepime, and Piperacillin-Tazobactam at 250 µg/ ml, and their Minimum inhibitory concentration (MIC) was recorded. The Bn67 isolate was the most promising isolate, which was molecularly identified using 16SrRNA partial sequence as *Pseudomonas aeruginosa* (LC710315.1). So in order to overcome this bacterial resistance; eighty actinobacteria were isolated from several soil samples collected from Giza governorate, Egypt, and were screened for their effectiveness against Bn67. The actinobacterial isolate (Stn-01) showed the maximum inhibitory efficacy against Bn67 and was identified using 16SrRNA partial sequence as *Streptomyces katsurahamanus* (LC710314.1). The inhibitor protein (β-LIP-n) was isolated; precipitated, and purified to give 35 kDa with 17 amino acid sequences. The β-LIPn exhibited no cytotoxic potential against the Human Skin Fibroblast (HSF) cell line at 200 µg/ ml. This approach of using the bacterial soil-based inhibitor protein to biocontrol the β-lactam resistant bacteria is considered as novel and as a promising start-up to using the environmental bacteria to overcome this problem of β-lactam resistance.

Keywords: β-lactamase, β-lactam antibiotics, Nosocomial bacteria, Actinobacteria, 16SrRNA gene

1. Introduction

During the 20th century, the human infections have been treated with antibiotics, which are significant anti-infective agents. Since the 1950s, the β-lactam antibiotics are the first-line of chemotherapeutic treatment for the G (+) and G (-) bacteria, and they are clinically considered as significant antimicrobial drugs (Hutchings *et al*., 2019; Walesch *et al*., 2023; Soliman *et al*., 2023). Over the past few decades, the bacterial resistance to the β-lactam antibiotics has significantly increased (Jani *et al*., 2021; Zaatout *et al*., 2021; Mutuku *et al*., 2022; Tan *et al*., 2023).

The β-lactam antibiotics have lost their importance, as they are no longer capable of inhibiting the pathogenic bacteria. The emergence and prevalence of this problem is attributed to the abuse of the antibiotics; especially in the developing countries (Veeraraghavan *et al*., 2018).

The majority of the pathogenic G (-) bacteria; particularly those from the family *Enterobacteriaceae*, have been classified by the World Health Organization (WHO) as critical-priority microorganisms, which are represented by the multidrug resistant (MDR) bacteria that are detected in the healthcare settings (Janda and Abbott, 2021; Dantas Palmeira *et al*., 2022).

The WHO. (2023) reported that antibiotic resistance is one of the top ten worldwide hazards to the public health. Meanwhile, in 2019, the antibiotic resistance was recorded by several bacterial spp., including *Escherichia coli*; *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Streptococcus pneumonia*, which have caused approximately 4.95 million deaths worldwide (Antimicrobial Resistance Collaborators, 2022).

Although there are four main mechanisms of β-lactam resistance; however β-lactamases (EC 3.5.2.6) production is the most regular mechanism through which the antibiotic becomes completely inhibited (Avatsingh *et al*., 2023). Some of the common pathogenic bacteria that have been spotted as the well-known MDR bacteria, include *Acinetobacter* spp.; *Proteus* spp., *E. coli*, *Klebsiella* spp., and *Pseudomonas* spp. (Kumari *et al*., 2007).

A previous study reported by Viana Marques *et al*., (2018) that in order to overcome these β-lactam resistant bacteria, the actinobacteria are well known to produce unique characteristic metabolites, which are used in various fields including the pharmaceutical industry. The *Streptomyces* species are more ubiquitous than any other genera of the actinobacteria, as they produce 90 % of the antibiotics used in the markets. This genus is highly productive of several bioactive compounds including the β-lactamase inhibitors, such as clavulanic acid; sulbactam and tazobactam. These inhibitors significantly reduce the proliferation of the β-lactam resistant bacteria

Furthermore, it is crucial to identify these bacterial isolates using 16SrRNA sequencing, as it helps with the discovery of new bacterial isolates in the diagnostic laboratories; detects bacteria with unique phenotypic characteristics, and the rare types of bacteria. 16SrRNA sequencing also allows the physicians to select and evaluate the antibiotics. Moreover, it can be used for the precise detection of any types of prokaryotes; especially the pathogenic bacteria (Raheem and Shareef, 2021). The objective of this study was to present an effective approach to safely control the β-lactam resistant bacteria; through disarming the β-lactamases using the β-lactamase inhibitor protein obtained from the *Streptomyces katsurahamanus* actinobacteria.

2. Materials and methods

2.1. Sampling of the nosocomial bacteria

Seventy nosocomial bacterial isolates that were resistant to the β-lactam antibiotics at a concentration of 10 µg/ ml were collected within six months from July-Dec, (2019) from several hospitals and laboratories across ten governorates in Egypt, including Giza governorate (27 isolates); Cairo (14), Al-Kalyobia (9), AL-Dakahlia (6), 3 isolates from each of AL-Menoufia, AL-Gharbia, Bani Swaef, and AL-Minya governorates, and a single isolate from AL-Fayoum and Al- Sharkia. These clinical isolates were identified as *E. coli* (21/ 70), *Klebsiella* spp. (11/ 70), *Staphylococcus aureus* (11/ 70), *Pseudomonas* spp. (10/ 70), *Proteus* spp. (6/ 70), *Enterococcus* spp. (4/ 70), *Enterobacter* spp. (2/ 70), *Acinetobacter* spp. (2/ 70), *Proteus mirabilis* (1/ 70), and *Streptococcus pneumonia* (1/ 70). These clinical bacterial isolates were collected from both genders; especially those suffering from severe infections; mainly urogenital tract; wounds, throat, blood, vagina, sputum, prostate, and the ear.

2.2. Preparation of the nosocomial bacterial isolates

The seventy isolates were plated on Soybean Casein Digest (SCD) agar with amoxicillin (250 µg/ ml) and incubated at 37°C for 24 h, to obtain the most resistant bacterial isolates to the β-lactam antibiotics. The obtained β-lactam resistant bacterial isolates were maintained on SCD agar slants amended with amoxicillin (250 µg/ ml) (Andrews, 2001).

2.3. Determination of the minimum inhibitory concentrations (MIC)

The minimum inhibitory concentrations (MIC) of the tested six β-lactam antibiotics; mainly amoxicillin, amoxicillin-clavulanate, penicillin, ampicillin-sulbactam, cefepime, and piperacillin-tazobactam, was determined against the potent β-lactam resistant bacteria using the McFarland assay that were made with two replicates for each concentration (Andrews, 2001), in order to detect the potent resistant bacterial isolate to these tested antibiotics. The McFarland standard solution was prepared; where 0.5 ml of 0.048 M $BaCl_2$ was supplemented with 99.5 ml of 0.18 M H_2SO_4 (1 % v/v) with constant stirring. The mixture was distributed in equal volume screw capped test tubes, which were tightly sealed to prevent the evaporation, and then placed in darkness at room temperature. The resulting turbid standard solution was vigorously agitated using a vortex mixer before use (Leslie *et al*., 1990).

2.4. Identification of the potent β-lactam resistant bacteria

The promising β-lactam resistant bacterial isolate was identified biochemically, according to Cowan and Steel, (1974); Pradhan and Tamang, (2019); Zheng *et al*., (2000). In addition, this isolate was identified molecularly. The 16SrRNA partial gene of the selected β-lactam resistant bacterium was sequenced using MiSeq Sequencer in (Macrogen Inc. Seoul,, Korea); with the universal primers (518F, CCAGCAGCCGCGGTAATACG, 800R TACCAGGGTATCTAATCC) (Zheng *et al*., 2000). The sequence of the isolate was run by Codon Code Aligner program ver. 9.0 (Codon Code Corporation, Dedham, MA, USA), to view the chromatograms and generate contigs. The contigs were checked on BLASTn search against NCBI's GenBank database (https://www.ncbi.nlm.nih.gov/blast), to determine the species and subspecies (Altschul *et al*., 1990), and the E-values were calculated. A CLUSTAL W program was used to align the multiple sequences (Thompson *et al*., 1994), while the pairwise distance was computed. The phylogenetic tree was generated using "MEGA" ver. 5.2.2 (Tamura *et al*., 2011) with the Neighbor-Joining method and 1000 bootstrap (Saitou and Nei, 1987). The sequence of 16SrRNA was compared with those deposited in the GenBank database, to find the closely related species and create the Phylogenetic tree.

2.5. Isolation of the actinobacteria

Eight soil samples were collected at a distance of 20 cm from the soil surface of two locations in Giza Governorate, Egypt; transferred immediately to the microbiology laboratory in sterile polypropylene bags,

and allowed to dry for 10 d at room temperature. The soil extract was prepared and serially diluted up to 10^{-8} with sterile dist. water. The agar surface of the Starch nitrate (SNA) Petri plate was coated individually with one ml of each soil dilution, and incubated at 30°C for 7 d. The developing single colonies were purified on SNA (Abdulkhair, 2012; El-Gammal *et al*., 2013).

2.6. Screening for antibacterial potential of the actinobacteria

Eighty actinobacterial isolates were screened against the selected β-lactamase resistant bacterium for the production of β-lactamase inhibitory protein. Nutrient agar (NA) plates were used; each containing a single antibiotic of the following: cefepime; ampicillin-sulbactam, amoxicillin, amoxicillin-clavulanate, penicillin, and piperacillin-tazobactam (250 µg/ ml), which were seeded individually with 100 µl the potent β-lactam resistant bacterium. Actinobacterial disks (10 mm in diameter) that were cut from 7th d old cultures using a sterile cork borer were placed aseptically and individually onto the NA surface; kept at 4 °C for 1 h to allow for diffusion of the β-lactamase inhibitory protein, and then incubated at 37 °C for 24 h (Waksman, 1961; Abdulkhair, 2012; El-Gammal *et al*., 2013).

2.7. Identification of the promising actinobacterial isolates

The most potent of actinobacterial isolates that demonstrated the highest inhibitory activity against the β-lactam resistant bacterium due to the production of β-lactamase inhibitory protein, were identified using several conventional techniques; mainly cultural; morphological, and biochemical traits (Shirling and Gottlieb, 1966). The morphology of these actinobacteria was investigated using a JEOLISM 541OLV scanning electron microscope (SEM); obtained from the Electron Microscopy unit, Mansoura University, Egypt, to determine the shape of the spore chains, in addition to the shape and texture of these spores (Abdulkhair, 2012). Moreover, identification of these actinobacterial isolates was confirmed molecularly, as described before for the β-lactam resistant bacterial isolate.

2.8. Isolation of the β-lactamase inhibitory protein

2.8.1. Preparation of the actinobacterial cell free extract

The selected actinobacterium was grown on SN broth medium, and shaken at 28°C for 7 d. After incubation, the bacterial broth culture was filtered using Whitman No. 1 filter paper, and the filtrate was centrifuged for 10 min. at 5000 rpm. The β-lactamase inhibitory protein was found in the supernatant (cell free extract), which was collected and salted-out to precipitate this protein (Abdulkhair, 2012; El-Gammal *et al*., 2013).

2.8.2. Salting-out of the β-lactamase inhibitory protein

Saturated ammonium sulfate (10 % - 90 %) was gradually added to the cell free extract containing the β-lactamase inhibitory protein. Each precipitated protein fraction was left standing for 2 h at 4°C, and then centrifuged at 5000 rpm for 20 min. at 4°C. Afterwards, 10 ml of sterile dist. water was used to dissolve the precipitate. The overall amount of the protein (mg/ ml) (Lowry *et al*., 1951); activity, and total activity (U), was determined for each protein fraction (Abdulkhair, 2012; El-Gammal *et al*., 2013).

2.8.3. Purification of the β-lactamase protein

a-Ion exchange column chromatography

Approximately 100 g of Diethylaminoethyl cellulose (DEAE-cellulose) were placed in an Erlenmeyer flask (1 l); washed with dist. water, 1N HCl was added, and finally bringing the pH to 6.5. Afterwards, the DEAE-cellulose was washed several times with 0.5 M NaOH until the color was completely gone, and then washed with dist. water to be alkaline free. DEAE-cellulose was suspended in 3X phosphate buffer (pH 7.5), and then gently poured into a glass column (2.5 × 20 cm) to be tightly packed. Active fractions with progressive salting were pooled and

gently delivered to the chromatographic column (Abdulkhair, 2012).

b- Gel filtration technique

About 10 g of "sephadex" (G-200) was combined with 400 ml of phosphate buffer (pH 7.5); heated for 6 h in a water bath at 60 °C, cooled to 50 °C, and then packed into a 2.5 × 20 cm glass column. The purified protein was concentrated after the active fractions were being combined and dialyzed overnight through a dialysis bag in dist. water (Andrews, 1964).

2.8.4. Separation of the β-lactamase inhibitory protein by SDS-PAGE

The purified β-lactamase inhibitory protein was electrophoresed using Sodium Dodecyl Sulate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE), in reference to Laemmli, (1970). The gel was created between two glass plates (16 × 16 cm), which were spaced apart by a Teflon spacer that was 1 mm thick. Each well of the gel was inoculated with no more than 25 µl of the protein sample. A high molecular weight protein marker with a range of 14.4 - 116 kDa was present during the electrophoresis, which was conducted at roughly 100 V. Analysis of the obtained results followed washing the gel in a stain removal solution, and the gel was then stained with 0.1 % Coomassie Brilliant Blue G-250 (Hames and Richwood, 1985).

2.8.5. Amino acid analysis of the inhibitory protein by HPLC

The amino acids sequence of the purified β-lactamase inhibitory protein was determined using High-performance liquid chromatography (HPLC) technique (Spectra-Physics Analytical, Inc. A0099-600 with spectra focus optical scanning detector and spectra system U.V. 2000 detector and ultra-sphere C Beckman column). Analysis was performed using a gradient of Pico-Tag solvent A and B at 40°C and a flow rate of 1 ml/ min. The Pico-Tag discrete amino acids were detected at the wavelength of 254 nm (Steven *et al*., 1989).

2.9. Cytotoxicity of the β-lactamase protein on the Human Skin Fibroblast (HSF) cell line

2.9.1. Cell culture

The Human Skin Fibroblast is a normal human cell line obtained from Nawah Scientific Inc. (Mokatam, Cairo, Egypt). The cells were kept alive in a humidified 5 % (v/v) CO_2 atmosphere at 37°C in Dulbecco's Modified Eagle Medium (DMEM) medium, which was supplemented with 100 mg/ ml of streptomycin; 100 U/ ml of penicillin, and 10 % of heat-inactivated fetal bovine serum.

2.9.2. Cytotoxicity assay

The cytotoxic effect of the β-lactamase inhibitory protein (β-LIPn) was tested on the Human Skin Fibroblast (HSF) cell line using SulfoRhodamine B assay (SRB), according to Skehan *et al*., (1990); Allam *et al*., (2018). This experiment was conducted at Nawah Scientific Inc. (Mokatam, Cairo, Egypt). Approximately 5×10^3 cells/ 100 µl cell suspension aliquots were inoculated individually into 96-well plates and incubated for 24 h. Another aliquot of 100 µl of a medium containing the inhibitor protein at varied doses (0.02; 0.2, 2.0, 20, and 200 µg/ ml), which were made with two replicates for each concentration, was applied individually to the wells. The cells were fixed for 72 h after the inhibitor protein exposure; by removing the media and adding 150 µl of 10 % TriChloroAcetic acid (TCA), and incubation at 4 °C for 1 h. The cells were washed five times with dist. water after the TCA solution was removed. Aliquots of a 70 µl SRB solution (0.4 % w/v) were added individually, and then incubated for 10 min. in darkness at room temperature. The plates were cleaned with 1 % acetic acid three times before being let to air dry overnight. Then, 150 µl of Tris buffer (10 mM) was added to dissolve the protein-bound SRB stain, and the absorbance was measured at 540 nm using a BMG LABTECH® - FLUOstar Omega microplate reader (Ortenberg, Germany).

The change in morphology of the cells after being treated with the β-lactamase inhibitory protein (β-

LIPn) was investigated. The investigated changes in cell morphology included partial or complete monolayer loss; rounding, shrinkage, and/ or cell granulation.

3. Results

3.1. Sampling of the nosocomial β-lactam resistant bacteria

Seventy clinical isolates were collected from several hospitals and laboratories in the Egyptian's governorates. The most detected frequent infection site was the urine followed by wounds; throat extrusions, blood, vagina, sputum, prostate, and the ear.

The MICs were determined for the six selected bacterial isolates, as shown in Table (1). Results showed that the isolate Bn67 was the potent β-lactam resistant bacterium, as it resisted the effects of the β-lactams at a high concentration of 250 µg/ ml. Meanwhile, cefepime had the highest inhibitory effect on the isolate Bn67; recording MIC of 10^3 µg/ ml, followed by amoxicillin and amoxicillin-clavulanate (12×10^2 µg/ ml), piperacillin-tazobactam (14×10^2 µg/ ml), ampicillin-sulbactam (18×10^2 µg/ ml), while the least effective antibiotic was penicillin recording MIC of 2×10^3 µg/ ml.

3.2. Determination of the MICs

Table 1: Determination of MICs of the six selected bacteria against the six β-lactam antibiotics

Bacterial isolate no.	MIC of the antibiotics (µg/ ml)					
	Cefepime	Ampicillin-Sulbactam	Amoxycillin	Amoxycillin-Clavulanic acid	Penicillin	Piperacillin-Tazobactam
B8	800	1400	1000	1200	1600	1400
B29	800	1600	800	1000	1800	1200
B30	600	1600	800	1000	1800	1200
B61	600	1400	1000	1200	1600	1400
Bn67	1000	1800	1200	1200	2000	1400
B69	400	1200	600	800	1400	1000

Where; two replicates were used for each concentration

3.3. Identification of the β-lactams resistant bacterium

The Bn67 isolate that recorded the highest resistance to the six β-lactams antibiotics, was identified using the morphological; cultural, and physiological characteristics. Bn67 was a rod-shaped Gram (-) bacterium that grew on several selective and differential media; where green, white, pink, and white colonies were observed on Cetrimide Agar; MacConkey Agar, Eosin-Methylene Blue agar, and Selenite Cystine agar medium, respectively. This bacterial isolate was motile, and had a positive reaction to citrate test; however, it reacted negatively

to the methyl red; Voges-Proskauer, indole, and H2S production assays. Only mannitol was fermented by this isolate, but the glucose; fructose, arabinose, inositol, inulin, lactose, maltose, mannose, raffinose, ribose, sorbitol, and sucrose, were not fermented. The isolate was capable of producing catalase; oxidase, nitrate reductase, arginine dihydrolase, and β-lactamase; however, no coagulase; gelatinase, and urease were produced. Therefore, the bacterial isolate Bn67 was identified as *P. aeruginosa*.

Based on its highest resistance to the β-lactam antibiotics; Bn67 isolate was partially sequenced using 16SrRNA universal primers. The contig of the isolate was generated using Codon-Code Aligner program. The contig was (656 bp) and had been submitted to the GenBank and assigned an accession number of LC710315.1. Subsequently, the nearest species and subspecies referenced sequences were imported from the GenBank; added to the query to perform multiple sequence alignments with CLUSTAL W, and computing pairwise distances with the maximum composite likelihood. A phylogenetic tree was generated using the Neighbor-Joining method with 1000 bootstrap values. As demonstrated in Fig. (1), the Bn67 belonged to the Family *Pseudomonadaceae* and was most closely related to members of the Genus *Pseudomonas*. Bn67 belonged to three clusters: cluster one contains *P. aeruginosa* (LC710315.1); cluster two contains *P. aeruginosa* (KF815703.1), (EU170480.1), and (KR349544), and cluster three contains *P. aeruginosa* (MW301141.1) and (EU834943.1); with (99 %) sequence similarity, as demonstrated in Table (2).

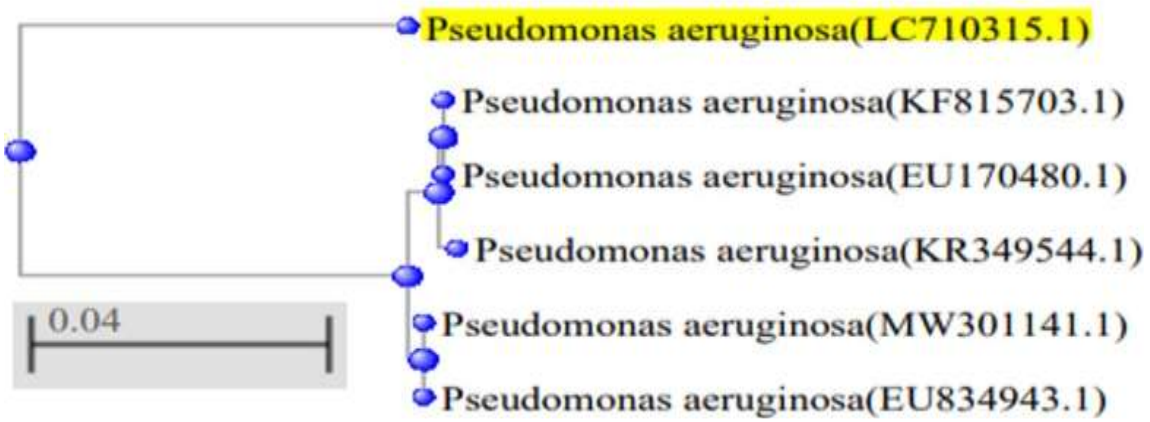

Fig. 1. The phylogenetic tree of *P. aeruginosa* (LC710315.1) (Bn67 isolate)

Table 2: 16 SrRNA-sequences demonstrating significant alignments of *P. aeruginosa* (Bn67) compared with the different *Pseudomonas* strains

Description	Scientific Names	Query_2562 Cover (%)	Identities (%)	Accession numbers
Pseudomonas aeruginosa strain GSN26, 16S ribosomal RNA gene partial sequence	*P. aeruginosa*	99 %	88.16	KF815703.1
Pseudomonas aeruginosa strain L-4, 16S ribosomal RNA gene partial sequence	*P. aeruginosa*	99 %	88.16	EU170480.1
Pseudomonas aeruginosa strain ZSBM1, 16S ribosomal RNA gene partial sequence	*P. aeruginosa*	99 %	87.92	MW301141.1
Pseudomonas aeruginosa strain ISTDF1, 16S ribosomal RNA gene complete sequence	*P. aeruginosa*	99 %	87.92	EU834943.1
Pseudomonas aeruginosa strain VV163, 16S ribosomal RNA gene partial sequence	*P. aeruginosa*	97 %	88.10	KR349544.1

3.4. Isolation and screening of the actinobacteria

Eighty actinobacterial isolates were isolated from the soil onto Starch Nitrate medium, where 25 isolates had white colonies, 35 isolates were grey, 13 isolates were yellow, and 7 isolates were red. The effective inhibition of growth of the β-lactam resistant *P. aeruginosa* by the eighty actinobacterial isolates was tested in the presence of 6 individual antibiotics; mainly cefepime, ampicillin-sulbactam, amoxicillin, amoxicillin-clavulanate, penicillin, and piperacillin-tazobactam at 250 µg/ml. The results showed that only the Stn-01 and St-02 actinobacterial isolates were found to have effective inhibitory clear zone diameters (mm) against *P. aeruginosa* (LC710315.1); however, the Stn-01 isolate had a higher clear zone than St-02, as presented in Table (3).

3.5. Identification of the actinobacteria

The Stn-01 actinobacterial isolate showed white colonies on SN agar medium. The cultural characteristics were determined on seven recommended media. The isolate had enriched growth on Tryptone Yeast Extract Broth; Yeast-Malt Extract, Inorganic Salt Starch, and Peptone Yeast Extract Iron media. However, it displayed moderate growth on Oat-Meal Extract and Tyrosine media, and poor growth on Glycerol Asparagine medium.

Table 3: The inhibitory effects of the actinobacterial (Stn-01, Stn-02) isolates against *P. aeruginosa* (LC710315.1) and the β-lactam antibiotics

Antibiotic	β-lactam bacterial resistant strain	Concentration (µg/ ml)	Clear zone diameter (mm)	
			Stn-01	Stn-02
Cefepime	*P. aeruginosa* (LC710315.1)	250	30	25
Ampicillin-Sulbactam		250	28	20
Amoxicillin		250	25	15
Amoxicillin-Clavulanate		250	25	15
Penicillin		250	30	22
Piperacillin-Tazobactam		250	27	20

On Yeast-Malt Extract; Oat-Meal Extract, Glycerol Asparagine, Peptone Yeast Extract Iron, and Tyrosine media, Stn-01 exhibited white aerial mycelia; however, on Tryptone Yeast Extract broth and Inorganic Salt Starch medium, it presented creamy aerial mycelia. The surface of the substrate mycelia was colorless on Tryptone Yeast Extract, but it was light yellow on all the other media; with the exception of the Tyrosine medium, which was light brown. No diffusible pigment was developed on all the tested media. The results of the cultural characterization allowed for identification of this Stn-01 isolate as *Streptomyces* sp. The isolate lacked the sugar pattern but was motile and had LL-DiAminoPimelic acid (LL-DAP) in its cell wall. Stn-01 may have had generated lecithinase and nitrate reductase, in addition to amylase; protease, cellulase, pectinase, and catalase, but no lipase. In addition, H_2S was produced; although xanthan and esculin were not broken down. Both streptomycin and amoxicillin (50 g/ ml) represented effective treatments for this isolate. Rhamnose; xylose, lactose, and inositol were not fermented by the Stn-01 isolate, although it can ferment glucose; fructose, galactose, sucrose, mannose, mannitol, arabinose, and starch. Fermentation was performed on cysteine; proline, valine, alanine, lysine, leucine, tyrosine, and phenylalanine. This bacterial isolate could withstand sodium chloride solutions with concentrations between 1 % and 10 %. Using SEM, the isolate had a spiral spore chain made up of spores with an ellipsoidal shape and a smooth surface, as illustrated in Fig. (2).

Since Stn-01 isolate was the potent actinobacterium against *P. aeruginosa*; this isolate was partially sequenced using 16SrRNA universal primers. The contig of the isolate was generated using Codon Code Aligner program. The contig was (697 bp) and has been submitted to the GenBank and assigned an accession number (LC710314.1). Subsequently, the nearest species and subspecies referenced sequences were imported from the GenBank; added to the query

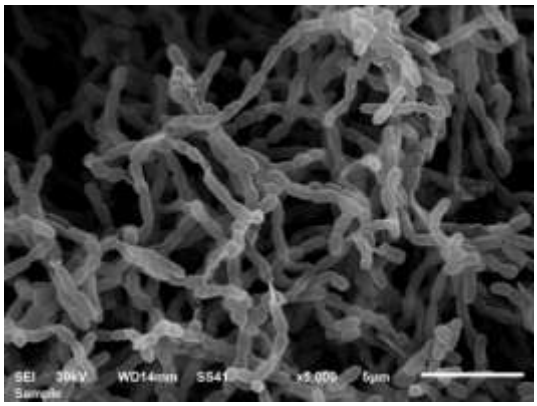

Fig. 2. Scanning electron micrograph (SEM) of *S. katsurahamanus* (LC710314.1) (Isolate Stn-01)

to perform multiple sequence alignments with CLUSTAL W, and computing pairwise distances with the maximum composite likelihood. A phylogenetic tree was generated using the Neighbor-Joining method with 1000 bootstrap values. As shown in Fig. (3), Stn-01 belonged to the family *Streptomycetaceae* and was most closely related to members of the Genus *Streptomyces*. Stn-01 belonged to three clusters: cluster one contains *S. katsurahamanus* (LC710314.1); cluster two contains *S. katsurahamanus* strain (AY999726.1), *S. jumonjinensis* strain (MW548247.1), (DQ026629.1) and (AB184538.1), and cluster three contains *S. jumonjinensis* strain (AB045863.1); with (99 %) sequence similarity as shown Table (4).

3.6. Isolation of the β-lactamase inhibitory protein

The β-lactamase inhibitory protein was precipitated at 40 % and 50 % saturated ammonium sulfate. As shown in Table (5), the activity was 35 mm and 25 mm; the total activity was 180 U and 160 U, and the specific activity was 43.9 U/ mg and 35.5 U/ mg; for the 40 % and 50 % fractions, respectively. The purification fold was 39.1 and 31.6, while the yield ratio was 97.2 and 86.4 for the 40 % and 50 % fraction, respectively.

3.7. Purification of the β-lactamase protein

The fractions containing the β-lactamase inhibitory protein (40 % and 50 %) were pooled and delivered to the ion-exchange column chromatography; where the protein was detected in 7 fractions (19 to 25), the highest activity was 30 mm as shown Fig. (4). Seven protein fractions were pooled and dialyzed overnight with the phosphate buffer (pH 7.2), and then delivered to a gel filtration column chromatography; where the protein was detected in 5 fractions (18 to 22), the highest activity was 30 mm (Fig. 5).

3.8. Partial characterization of the β-lactamase protein

The purified β-lactamase inhibitory protein (β-LIPn) was separated using SDS-PAGE and stained with Coomassie blue dye. The protein was separated as a single band at 35 kDa as shown in Fig. (6). The purified β-lactamase inhibitory protein was analyzed by HPLC to determine the amino acid sequence composition. The protein had different concentrations of 17 amino acids measured in milli absorbance unit (mAU). It was found that Threonine had the highest concentration (80 mAU); followed by lysine (74 mAU), arginine (73 mAU), alanine (70 mAU), glycine (60 mAU), proline (58 mAU), glutamic acid

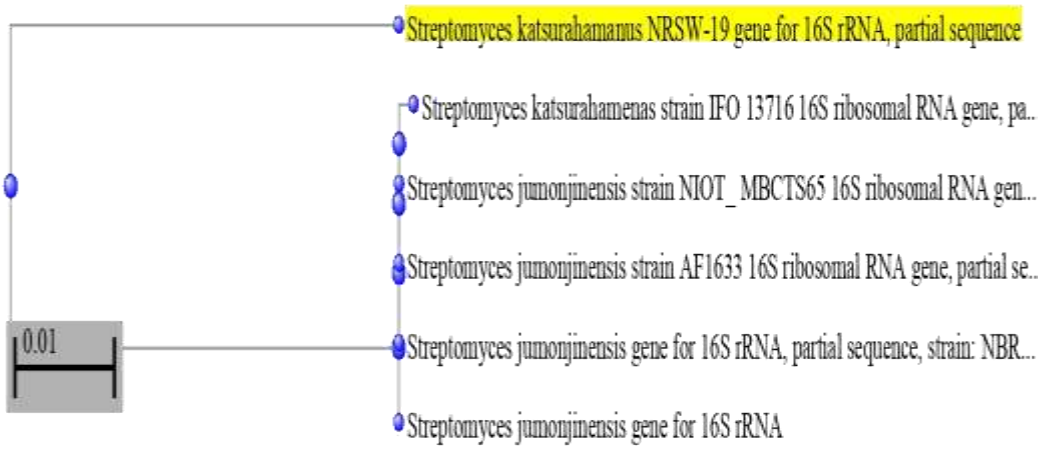

Fig. 3. The phylogenetic tree of *S. katsurahamanus* (LC710314.1) (Isolate Stn-01)

Table 4: 16SrRNA sequences producing significant alignments of *Streptomyces* isolates compared with different *Streptomyces* strains

Description	Scientific names	Query_2562 cover (%)	Identities (%)	Accession numbers
S. katsurahamanus strain IFO 13716, 16SrRNA gene partial sequence	*S. katsurahamanus*	99 %	99.60	AY999726.1
S. jumonjinensis strain AF1633, 16SrRNA gene partial sequence	*S. jumonjinensis*	99 %	90.35	DQ026629.1
S. jumonjinensis strain NIOT_ MBCTS65, 16SrRNA gene partial sequence	*S. jumonjinensis*	99 %	90.35	MW548247.1
S. jumonjinensis strain: NBRC 13869, 16SrRNA gene partial sequence	*S. jumonjinensis*	99 %	90.23	AB184538.1

Table 5: Purification of β-lactamase inhibitory protein produced by *S. katsurahamanus* (LC710314.1)

$(NH_4)_2SO_4$ (%)	Activity (mm)	Total activity (U)	Total protein (mg)	Specific activity (U/ mg)	Purification fold	Yield (%)
Filtrate	40	185	165	1.121	1.0	100
10	0	0	2.4	0	0	0
20	0	0	2.9	0	0	0
30	0	0	3.6	0	0	0
40	35	180	4.1	43.9	39.1	97.2
50	25	160	4.5	35.5	31.6	86.4
60	0	0	4.9	0	0	0
70	0	0	5.1	0	0	0
80	0	0	5.4	0	0	0
90	0	0	5.9	0	0	0

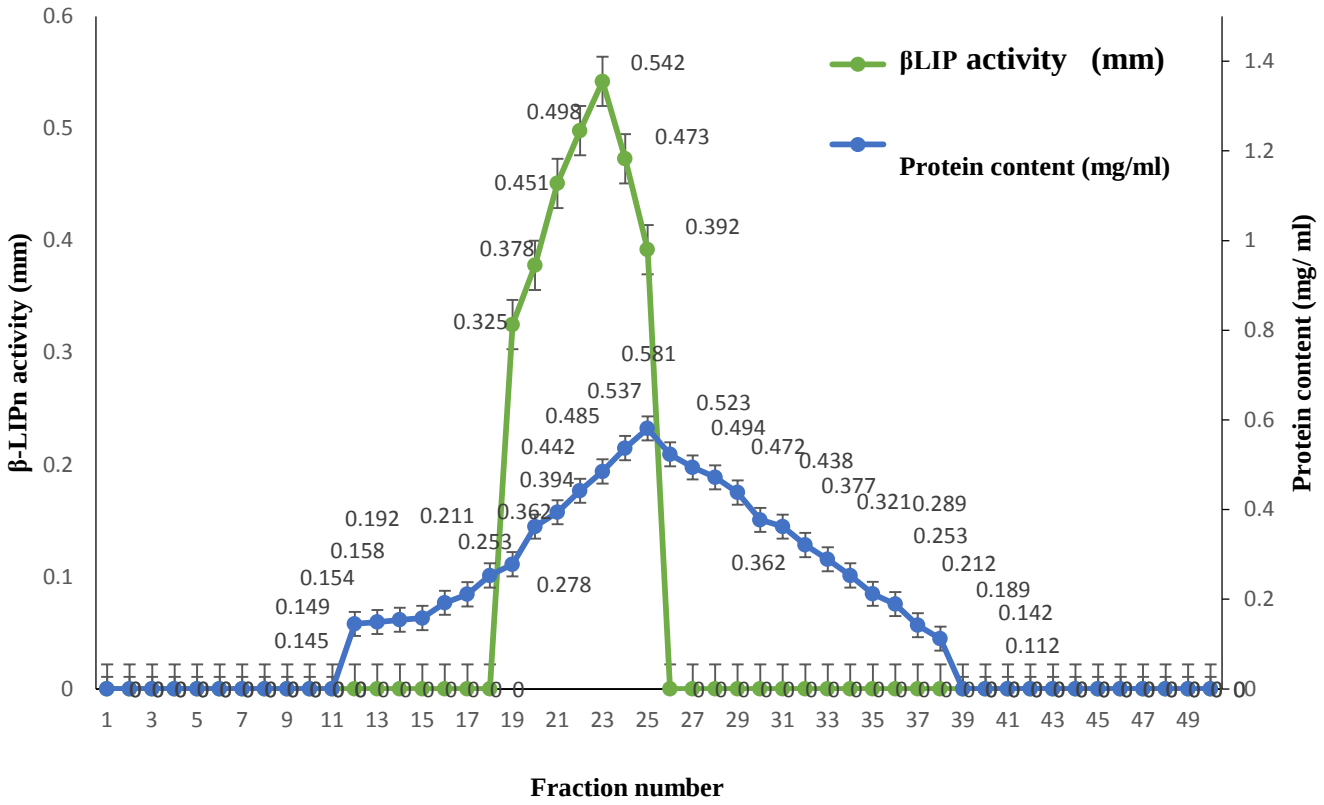

Fig. 4. Purification of the β-lactamase inhibitory protein (β-LIPn) using Ion-exchange column chromatography

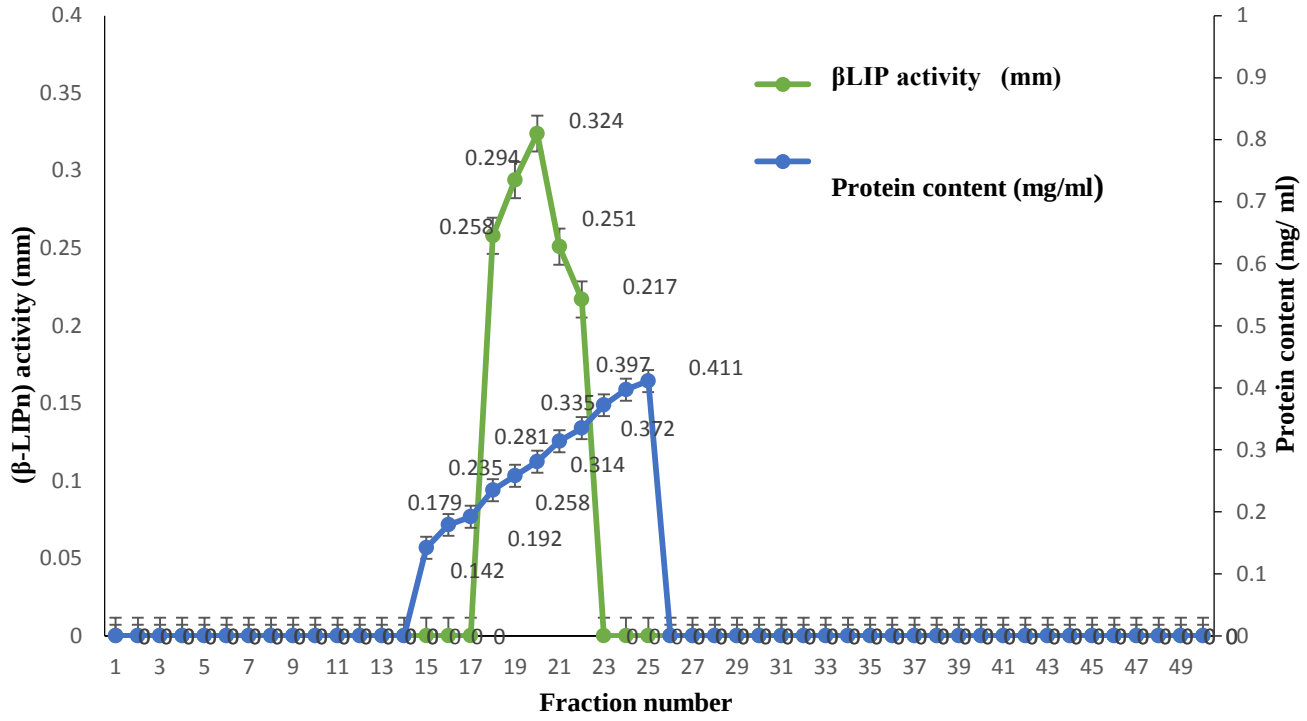

Fig. 5. Purification of the β-lactamase inhibitory protein (β-LIPn) using Gel filtration column chromatography

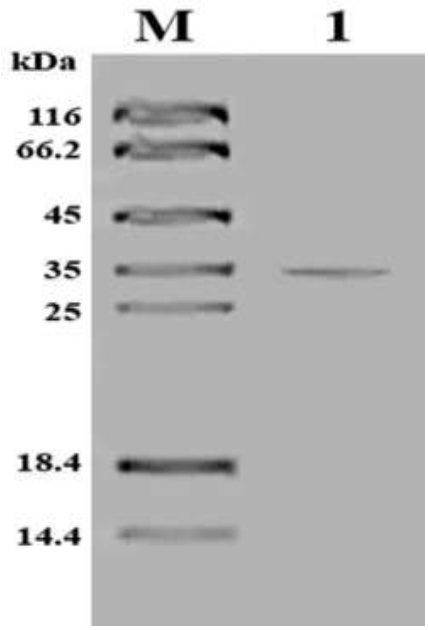

Fig. 6. Sodium Dodecyl Sulate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) of the β-lactamase inhibitory protein (β-LIP-n) demonstrating a single band obtained at 35 kDa

(48 mAU), isoleucine (43 mAU), leucine (42 mAU), serine (35 mAU), valine (33 mAU), methionine (32 mAU), phenylalanine (32 mAU), tyrosine (30 mAU), histidine (29 mAU), cystine (26 mAU), and aspartic acid (24 mAU).

3.9. Cytotoxicity of the β-lactamase inhibitory protein (β-LIPn) against the HSF cell line

In this study, the SRB assay was used to detect the cytotoxic activity of the β-LIPn against the HSF; through treatment with five concentrations of this β-LIPn. According to the results shown in Table (6), the HSF cell line after being exposed to β-LIPn at a concentration of up to 0.20 µg/ ml for 24 h, had maintained a mean viability of 99.0 %, as demonstrated in Fig. (7). At the concentration of 0.02 µg/ ml, the β-LIPn did not show any cytotoxicity against the HSF. Meanwhile, at a concentration of 200 µg/ ml of β-lactamase inhibitory protein (β-LIPn), the cell viability was reduced to 95.4 %; however, the cytotoxicity was non-significant because more than 95 % of the cells remained alive. The recorded IC_{50} was 200 µg/ ml. When the treated HFS cells were stained with SRB and compared with the control, we did not detect any cell decomposition, which proves that the β-lactamase inhibitory protein (β-LIPn) is safe for human use.

4. Discussion

β-lactam resistance in the bacteria spreads in the last few years with no apparent treatment; that is why the infectious diseases became a major challenge in the medical field (Alekshun and Levy, 2007; Lota and Latorre, 2014; Avatsingh *et al*., 2023). Consecutive generations have high resistance to the β-lactams that is observed by many pathogenic bacteria and the *Enterobacteriaceae* in particular, such as *E. coli*; *Enterobacter* spp., *Morganella* spp., *Proteus* spp., *Providentia* spp., *Klebsiella* spp., and *Serratia* spp. This made it easier for these resistant bacteria to attack different locations of the human body, such as the urinary tract; respiratory tract, blood stream, and the wounds (Tham, 2012; WHO. 2017; Avatsingh *et al*., 2023).

Several previous studies reported by Rossi *et al*., (2006); Reinert *et al*., (2007) revealed that 29 countries were highly affected by the β-lactam resistant bacteria, due to the production of Extended Spectrum β-Lactamase enzyme" (ESBL), which hydrolyzes the β-lactam ring of an antibiotic. *K. pneumoniae* and *E. coli* are highly prevalent in the Latin America; the Middle East, Africa, and Asia, while their prevalence has been limited in Europe and the United States (Rossi *et al*., 2006; Reinert *et al*., 2007). The Middle Eastern countries have had the worst outbreak of these ESBL bacteria; since they lack the appropriate hygiene in their hospitals (Al-Agamy *et al*., 2006; Tawfik *et al*., 2011; Storberg, 2014).

A recent study conducted by Guo *et al*., (2022) reported that there are 379 Enterobacterales isolates that have high level of resistance to cefepime (MIC >128 × 10^3 µg/ ml). The authors revealed that 756 of them belong to *P. aeruginosa* and 630 are *A. baumannii* isolates, which are resistance to cefepime; recording MICs ranging from 6 × 10^2 to 128 × 10^3 µg/ ml. Furthermore, this study added that the susceptibility of *P. aeruginosa* to many of the commonly used broad-spectrum β-lactams, *i.e*., Cefepime; ranged from 70 % to 80 %.

Escherichia coli is highly resistant to ampicillin-sulbactam and has sporadic resistance intervals (Jones and Dudley, 1997). An *in vitro* infection model was used to investigate the activity of ampicillin-sulbactam against the TEM-1β-lactamase-producing *E. coli*. Four strains of *E. coli* have been selected to represent a group of the TEM-1 β-lactamase production. *E. coli* ATCC 25922 has been used as a negative control; as it is a non β-lactamase producer, while *E. coli* EC11; TIM2, and GB85 produced different amounts of TEM-1 β-lactamase; as indicated by the MIC for each strain. *E. coli* EC11 has been sensitive to ampicillin-sulbactam; with MICs similar to those for ATCC 25922. *E. coli* TIM2 has an average sensitivity at an

Table 6: Cytotoxicity assay of the β-lactamase inhibitory protein (β-LIPn) produced by *S. katsurahamanus* (LC710314.1) against the Human Skin Fibroblast cell line

Conc. of the (β-LIPn) (µg/ ml)	Mean of Cell viability (%)
Control	100
0.02	100
0.2	99.0758
2	94.1107
20	94.1053
200	95.4164

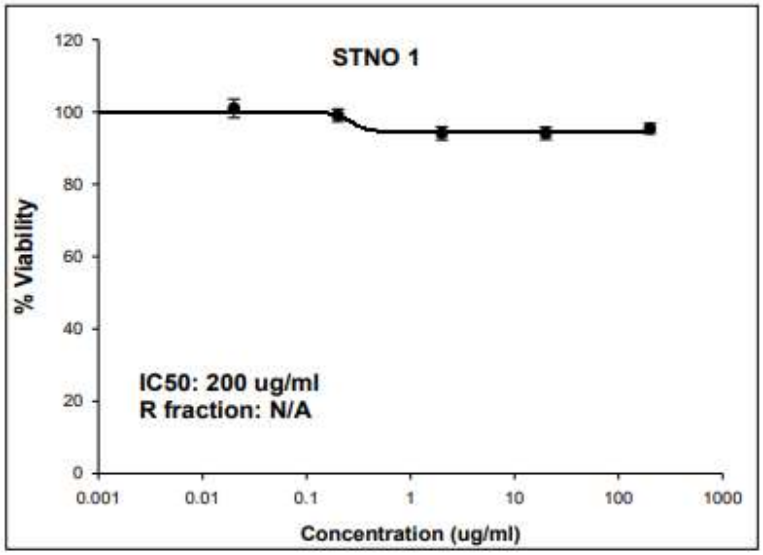

Fig. 7. Viability curve showing the percentage viability (%) of the HFS cells after treatment with the (β-LIPn) protein at the tested concentrations of; 0.02, 0.20, 2.0, 20, and 200 µg/ ml. Where STNO 1: represents the potent actinobacterial isolate

inoculum of 5 × 10^5 cfu/ ml. When tested at the inoculum level of 10^7 cfu/ ml, TIM2 has been classified as resistant to ampicillin-sulbactam. Meanwhile, Ampicillin-sulbactam is not expected to have an activity against the GB85 strain on the basis of its MIC and achievable concentrations (Jones and Dudley, 1997).

The problem of antimicrobial resistance due to excessive antibiotics abuse must be fully controlled to avoid worse implications. The used systematic monitoring systems should determine and take into account that the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria are applied (Díez-Aguilar *et al*., 2015), and higher rates of amoxicillin-clavulanate resistance should be expected for *E. coli* and *Klebsiella* sp. In addition, the empirical and targeted therapy with amoxicillin/ clavulanate is not recommended if the EUCAST is applied (Delgado-Valverde *et al*., 2017).

In this study, piperacillin-tazobactam was found to have the MIC of 10^3 µg/ ml for the B69 isolate; 12 × 10^2 µg/ ml for the isolates B29 and B30, and 14 × 10^2 µg/ ml for the isolates of B8; B61, and Bn67. In accordance, Guo *et al*., (2022) recently revealed that about 630 *A. baumannii* isolates and 756 *P. aeruginosa* isolates were resistant to Piperacillin-tazobactam; with MICs that ranged from 2× 10^3 - 256× 10^3 µg/ ml.

High mortality rates worldwide are usually associated with severe bacterial infections; especially with the Gram-negative bacteria (Esper *et al*., 2006; Vincent *et al*., 2009). Furthermore, changes in the Pharmaco Kinetics (PK) profiles of the used drugs may also occur in the infected patients (Patel *et al*., 2010). Prolonged treatment may be an important factor in raising the mortality rate, because the altered PK of the drugs within the body may lead to adverse health effects and death. Therefore, antibiotic therapy should be prescribed by the physician with prescribed doses for a limited period of time to ensure effective recovery and to avoid development of the antimicrobial resistance (Drusano, 2004). The multi-drug-resistant (MDR) microorganisms affect the morbidity and duration of hospitalization, and may eventually increase the mortality rate. Therefore, effective measures are being sought to prevent transmission of the resistant microorganisms from the infected patients to the others, including prescribing of the appropriate antimicrobial agents in suitable doses and for fitting periods of time (Shlaes *et al*., 1997). The conventional techniques used for identifying the bacterial isolates based on their phenotypic characteristics are widely used; however, it has been observed that comparing the bacterial 16SrRNA gene sequence is the best genetic approach (Clarridge, 2004). Currently, results of the morphological; cultural, and biochemical assays that were used for identification of the bacterial isolate agree with the previous results reported by Al-Daraghi and Abdulkadhim Al-Badrwi, (2020), who similarly isolated *P. aeruginosa* as a nosocomial bacterium. Furthermore, the results of molecular identification of the obtained bacterial isolate that was made by the 16SrRNA sequencing method are in agreement with the previous study reported by Eremwanarue *et al*., (2021), who isolated *P. aeruginosa* as one of the nosocomial bacterial isolates.

In this study, there are eighty Streptomyces isolates that were isolated from soil and were screened against *P. aeruginosa* LC710315.1; where there were two isolates which had an inhibitory effect against this bacterial strain. Nevertheless, one isolate (Stn-01) was considered as the most potent actinobacterium, which produced the β-lactamase inhibitory protein and recorded the highest inhibition zone diameter that ranged from 25-30 mm. According to results of the morphological and cultural characteristics of the International Streptomyces Project (ISP), in addition to the biochemical characteristics and cell morphology that was photographed using SEM, this isolate was identified as *S. katsurahamanus*. These obtained data agree with those of Abdulkhair, (2012), who isolated

Streptomyces spp. from the soil. By comparing the identities (%), the obtained sequences of 16SrRNA genes of the selected actinobacterial isolate (Stn-01) presented 99 % sequence similarity with that of *S. katsurahamanus*, which had been documented in the Genbank under the accession number of LC710314.1. The collected data are consistent with the soil-isolated *Streptomyces* spp. reported by Hamid *et al*., (2020); Avatsingh *et al*., (2023).

The β-lactam inhibitors are generally effective against the plasmid mediated drug resistance, which bind irreversibly to the active site of the lactamase enzyme to prevent hydrolysis of the β-lactams. Several previous studies have shown that the inhibitors are similar to the β-lactam antibiotics, but they do not have any significant antimicrobial effect. Three β-lactam inhibitors; mainly clavulanic acid, sulbactam, and tazobactam that combine with the β-lactam antibiotics, are available for clinical use (Lakshmi *et al*., 2014). The β-lactama inhibitor protein was precipitated by ammonium sulphate (40 % to 50 %). Similar results were obtained by Abdulkhair, (2012); where clavulanic acid was widely used with the β-lactams; mainly penicillin and cephalosporin, to treat the infectious diseases caused by the β-lactam resistant bacteria. Clavulanic acid is produced on a large scale by *S. clavuligerus*, which ferments the organic sludge (AbuSara *et al*., 2019).

Purification of the β-lactamase inhibitory protein (BLIP-I) was carried out through two steps, including ion-exchange column chromatography; where the protein was found in 7 fractions, and a gel filtration column chromatography; where the protein was detected in 5 fractions, and the highest activity was 30 mm, in agreement with the previous results obtained by Abdulkhair, (2012). Similarly, a new β-lactamase inhibitory protein produced by *S. exfoliates* SMF19 has been purified and characterized. Using gel filtration fast protein liquid chromatography, the molecular mass of BLIP-I is 17.5 kDa, while the N-terminal sequence is NH2-Asn-Ser-Gly-Phe-Ser-Ala-Glu-Lys-Tyr-Glu-Gln-Ile-Gln-Phe-Gly (Kang *et al*., 2000).

Results of the cytotoxicity assay of the β-lactamase inhibitory protein on the Human Skin Fibroblast (HSF) proved that the used protein is safe for human use. These data are agreement with those obtained by Allam *et al*., (2018).

Conclusion

Pseudomonas aeruginosa (LC710315.1) was the most potent resistant strain for all the tested β-lactam antibiotics; however, this resistance was overcome by the β-lactamase inhibitory protein that was isolated from *S. katsurahamanus* (LC710314.1). This purified protein was separated at 35 kDa using SDS-PAGE with seventeen amino acids. By testing the cytotoxicity of this inhibitor protein, it was proven to be safe for human use.

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Conflict of interest

There are no competing interests, according to the authors.

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Ethical approval

None applicable.

Authors' Contributions

All authors contributed equally in this study.

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