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Research Article

Biotechnological Application of Production β-Lactamase Inhibitory Protein (BLIP) By Actinomycetes Isolates from Al-Khurmah Governorate

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Abstract

Many pathogenic bacteria secrete β-lactamase enzymes as a mechanism of defense against β-lactam antibiotics. Sixty-nine un-repeated actinomycetes isolates were isolated from different localities in Al-Khurmah governorate, Saudi Arabia kingdom. Actinomycetes isolates were screened for producing β-lactamase inhibitory effect against amoxicillin –resistant bacteria. There were eleven isolates (15.94 %) which had β-lactamase inhibitory protein (BLIP) effect against amoxicillin –resistant *Staphylococcus aureus*, *pseudomonas aeruginosa* and *Klebsiella pneumonia*. The KH-3201-144 isolate has been considered the most potent, this was identified by biochemical, chemotaxonomic, morphological and physiological properties consistent with classification in the genus *Streptomyces*, with the nearest species being *Streptomyces rimosus.* Furthermore, a phylogenetic analysis of the 16S rDNA gene sequence and ribosomal database project consistent with conventional taxonomy confirmed that strain KH-3201-144 was most similar to *Streptomyces rimosus* (96%). The highest amount of β-lactamase inhibitory protein was precipitated at 40% of saturated ammonium sulphate. The purification was carried out by using both diethyl-aminoethyl-cellulose G-25 and sephadex G-200 column chromatography, respectively. The β-lactamase inhibitory protein was separated at 40 KDa. The minimum inhibition concentrations "MICs" of the purified β-lactamase inhibitory protein (BLIP) effect against amoxicillin –resistant *Staphylococcus aureus*, *pseudomonas aeruginosa* and *Klebsiella pneumonia* were also determined.

Keywords: Actinomycetes, Conventional taxonomy, Phylogenetic analysis and β-lactamase inhibitor

Introduction

Beta-lactam antibiotics are a broad class of antibiotics that contains a β-lactam ring in their molecular structures have been critical in the fight against infections caused by bacteria and other microbes **(Livermore, 2006).** However, bacteria can become resistant to antibiotics **(Arias & Murray, 2009).** The most common and important mechanism of resistance in Gram-positive and Gram-negative bacteria is production of β-lactamases. β-lactamases enzymes are able to inactivate a particular class of antibiotics, called the β-lactams (**Tiersma, 2013**). β-lactamase inhibitory proteins (BLIPs), including BLIP and BLIP-II, are inhibitors of class A β-lactamase are produced from the soil *Streptomyces* (**Gretes, *et al.*, 2009**). *Streptomyces* spp. are known for their complex life cycle and their production of a large variety of biologically active secondary metabolites, degradative enzymes and enzyme inhibitors (**Wendy *et al.*, 2001**). **Tiersma, 2013** reported that to limit the effectiveness of β-lactamases, β-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) are used in combination with β-lactams for the treatment of infections by β-lactamase-producing bacteria (**Manickam and Alfa 2008**). Clavulanic acid, sulbactam and tazobactam are all irreversible 'suicide inhibitors' which can permanently inactivate the β-lactamase, this may be more effective than reversible inhibition (**Drawz and Bonomo 2010)**. These inhibitors greatly improve the effectiveness of their partner β- lactams (amoxicillin, ampicillin, piperacillin, and ticarcillin) in the treatment of severe infections (**Drawz and Bonomo 2010**). The clavulanic

acid is a natural inhibitor of β-lactamases that was first isolated from *S. clavuligerus* **(Neto *et al.*, 2005)**. Other natural clavulanic acid producers include *S. jumonjinensis*, *S. katsurahamanus*, *Streptomyces* sp. FERM-P 2804 **(Challis and Hopwood, 2003)** and *Streptomyces* sp. NRC-35 (**Awad, *et al.*, 2009 and Lewis 2013**). In this study, a *Streptomyces* strain KH-3201-144, that produces β-lactamase inhibitors was isolated from soil sample collected from Al-Khurmah governorate, KSA, characterized and identified phenotypically and phylogenetically. The β-lactamase inhibitory protein was purified by using both diethyl-aminoethyl-cellulose G-25 and sephadex G-200 column chromatography.

Materials and Methods

Actinomycetes Isolation and β-lactamase Inhibitors Detection

Sixty-nine actinomycetes isolates were isolated from different localities in Al-Khurmah governorate, Saudi Arabia kingdom. Strain KH-3201-144 was isolated on three different media (Starch-nitrate agar medium, ISP-3 and ISP-4) using the serial dilution plate technique has been described by **(Johnson *et al.*, 1972)**, and were incubated at 30°C for 7 days **(Awad, *et al.*, 2009).** The isolates were primarily tested for their abilities as β-lactamase inhibitors through the specific synergistic bioassay using *Staph. aureus*, *K. pneumoniae* and *Pseudomonas aeruginosa* capable of resistanting 31.25 µg/ml of amoxicillin was used as a test strains **(Romero *et al.*, 1984).**

Test organisms

The test strains amoxicillin –resistant *Staphylococcus aureus*, *pseudomonas aeruginosa* and *Klebsiella pneumonia* was collection, National Research Centre, Dokki-Giza, Egypt.

Conventional Taxonomy

The cultural, morphological, physiological and biochemical characteristics of strain KH-3201-144 were assessed following the guidelines adopted by the International *Streptomyces* Project (ISP) **(Shrilling and Gottlieb, 1966).** The diaminopimelic acid (LL-DAP) isomers in the cell wall were analyzed as described by **(Lechevalier and Lechevalier, 1980).** The media composition and the cultivation conditions were implemented as described by **(Shrilling and Gottlieb, 1966).** Colors characteristics were assessed on the scale developed by **(Kenneth and Deane, 1955).**

DNA Isolation and Manipulation

The preparation of total genomic DNA was conducted in accordance with the methods described by **(Sambrook *et al.*, 1989).**

Amplification and Sequencing of the 16S rDNA Gene

PCR amplification of the 16S rDNA gene of the local actinomycete strain was conducted using two primers, StrepF; 5.-ACGTGTGCAGCCCAAGACA-3. and Strep R; 5.ACAAGCCCTGGAAACGGGGT-3., in accordance with the method described by **(Edwards *et al.*, 1989).** The PCR reaction mixture was then analyzed via agarose gel electrophoresis, and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The 16S rDNA gene was sequenced on both strands via the dideoxy chain termination method, as described by **(Sanger *et al.*, 1977).**

Sequence Similarities and Phylogenetic Analysis

The BLAST program (www.ncbi.nlm.nih. gov/blst) was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluating using BioEdit software **(Hall, 1999)**.

Preparation of Cell Free Extract

The starch nitrate broth medium was inoculated by most potent actinomycete isolate and then incubated at 30°C for seven days on shaking incubator at 200 rpm. After incubation period, the filtrate was centrifuged at 10,000 rpm for 10 min for precipitation of the mycelia.

Purification of β-Lactamase Inhibitory Protein Ion-Exchange Chromatography

Only 100 g of DEAE-Cellulose was placed in 1 L conical flask and then washed with distilled water followed by using 1 N HCl and water till the pH of the suspension was about 6.5. It was then washed several times with 0.5 M NaOH until no more color was

removed after the late alkaline wash the resin was rinsed with distilled water until it was free from alkalinity. The washed resin was then suspended in about three volume of the phosphate buffer pH 7.5. At this stage, the supernatant fluid was almost clear indicating the removal of the fine particles; this precipitate was used for column packing **(Dale and Smith, 1971).**

Gel Filtration

Ten gram of sephadex G-200 were dissolved in 400 ml phosphate buffer pH 7.5, boiled in water bath for 6 h, then cold to 50°C and packed in column (2 × 50 cm). Gel filtration was done basically according to **(Andrews, 1969).** Active fractions were pooled and dialyzed from ion-exchange column were applied to a sephadex G- 200 column which was pre-equilibrated with phosphate buffer pH 7.5, at a flow rate of 5 ml/ 25 min.

Separation of β-lactamase Inhibitor by SDS-PAGE

The β-lactamase inhibitory protein was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, which carried out with 10% polyacrylamide gel as described by **(Blackshear 1984)**, in the presence of 0.1% sodium dodecyl sulphate at pH 8.8 and 1 mM –dithiothreitol. Gel and gel buffers were prepared as described by **(See and Jackowski 1990).** Gel was stained for showing the formed bands clearly by Coomassie Blue dye as described by **(Fairbanks *et al.*, 1971).**

β-Lactamase inhibition assay

The MIC of β-lactamase inhibitory protein produced by KH-3201-144 for Susceptibility of amoxicillin-resistant bacteria have been described by **(Marciano *et al.*, 2008).**

Results

Screening of β-lactamase Inhibitory Protein Activity Produced by Actinomycete Isolates

The results of the primary screening showed a noticeable β-lactamase inhibitory activity against amoxicillin –resistant bacteria. The obtained data emphasized that, out of sixty-nine actinomycete isolates obtained from different locations. There were eleven isolates (15.94 %) which have an inhibitory effect against *Staphylococcus aureus*, *pseudomonas aeruginosa* and *K. pneumoniae*. Nevertheless, KH-3201-144 isolate was considered most potent where the highest activity of β-lactamase inhibitory protein (diameter of inhibition zone = 40.0 mm, 36.0 mm and 37.0 mm respectively) (Table 1).

Identification of the Most Potent Actinomycete Isolate:

Morphological Characteristics

The vegetative mycelia grew abundantly on both synthetic and complex media. The aerial mycelia grew abundantly on Starch- nitrate agar medium Oat-meal agar medium (ISP-3) and Inorganic salts-starch agar medium (ISP-4). The Spore chains were spiral, and had a smooth surface (Plate 1). Neither both sclerotic granules and sporangia nor flagellated spores were observed (Table 2).

Cell Wall Hydrolysate:

The cell wall hydrolysate contains LL-diaminopimelic acid (LL-DAP) and sugar pattern not detected.

Physiological and Biochemical Characteristics

The actinomycete isolate KH-3201-144 could hydrolyzes starch, protein, lipid and lecithin, whereas pectin hydrolysis and catalase test are negative, melanin pigment is negative, degradation of esculin & xanthin was positive, nitrate reduction, citrate utilization, urea and KCN utilization were positive, whereas H_2S production is negative (Table 2). The isolate KH-3201-144 utilizes mannose, mannitol, glucose, fructose, *meso*-inositol, galactose, maltose, lactose, starch, raffinose, arabinose, phenylalanine, valine, arginine, tyrosine, lysine and histidine, but do not utilize xylose, L-rhamnose, sucrose and cycteine. Growth was detected in presence of up to (7%) NaCl. The isolate KH-3201-144 utilizes sodium azid (0.01%), phenol (0.01%); but do not utilize in thallous acetate (0.001). Good growth could be detected within a temperature range of 30 to 45 ºC. Good growth could be detected within a pH value range of 6 to 9. Moreover, the actinomycete isolate KH-3201-144 are active against *Bacillus subtilis*, NCTC 1040*; Micrococcus luteus*, ATCC 9341*; Saccharomyces*

cerevisiae ATCC 9763 and *Aspergillus niger* IMI 31276 (Table 2).

Color and Culture Characteristics

The actinomycete isolate shows the aerial mycelium is white; substrate mycelium is light yellowish brown, and the diffusible pigment moderate yellowish brown for ISP-2, 6 & 7 (Table 3).

Taxonomy of Actinomycete Isolate

This was performed basically according to the recommended international Key's viz. **(Buchanan and Gibsons, 1974; Williams, 1989; and Hensyl, 1994) and Numerical taxonomy of *Streptomyces* species program (PIB WIN).** On the basis of the previously collected data and in view of the comparative study of the recorded properties of actinomycete isolate in relation to the closest reference strain, viz. *Streptomyces rimosus* it could be stated that the actinomycetes isolate KH-3201-144 is suggestive of being likely belonging to *Streptomyces rimosus*, KH-3201-144 (ID Score 0.99094).

Molecular phylogeny of the selected isolate

The 16_S rDNA sequence of the local isolate was compared to the sequences of *Streptomyces* spp. In order to determine the relatedness of the local isolate to these *Streptomyces* strains. The phylogenetic tree (as displayed by the Tree View program) revealed that the locally isolated strain is closely related to *Streptomyces* sp., the most potent strain evidenced an 96% similarity with *Streptomyces rimosus* (Fig. 1).

Precipitation and Purification of β-lactamase Inhibitory Protein (BLIP)

The protein inhibitor was precipitated by saturated ammonium sulfate with a wide range of concentrations (10 to 90%), it was precipitated only at three fractions (30-50%), but the highest activity was presented at 40% (Table 4). The active fractions of saturated ammonium sulfate (30-40-50 %) were pooled and exposed to ion (anion) exchange column chromatography using diethylaminoethyl cellulose G-25 (DEAE-cellulose). Elution was done by phosphate buffer at pH 7.5 and active fractions were dialyzed overnight against the same buffer. Also, active fractions were pooled and exposed to gel filtration column chromatography using sephadex G-200 and so dialyzed. Purified β-lactamase inhibitory protein was separated electrically by gel protein electrophoresis through one band at 40 KDa (Figure 2).

MIC of β-lactamase Inhibitory Protein

The MIC of β-lactamase inhibitory protein produced by *Streptomyces rimosus*, KH-3201-144 for Susceptibility of amoxicillin-resistant bacteria. The results showed that the minimum inhibitory concentration (MIC) of the compound against *Staphylococcus aureus*, was 93.75 µg/ml and 125 µg/ml with *K. pneumoniae* and *Ps. aeruginosa*.

Discussion

This study was begun with isolation of sixty-nine un-repeated actinomycetes isolates were isolated from different localities in Al-Khurmah governorate, Saudi Arabia kingdom. It is worth mentioning that, eleven out of sixty-nine actinomycete isolates were obtained showed noticeable inhibitory activity against *Staph. aureus*, *K. pneumoniae* and *Pseudomonas aeruginosa* that was resistant to amoxicillin. The actinomycete isolate KH-3201-144 was chosen in particular due to its high metabolic ability for production of β-lactamase inhibitory protein as previously reported **(Hozzein *et al*., 2008 and Dhananjeyan *et al*., 2010).** Identification process had been performed **(Williams, 1989; Hensyl, 1994 and Holt *et al*., 2000).** The morphological characteristics and microscopic examination emphasized that the spore chain is spiral. Spore mass is white, while spore surface is smooth, substrate mycelium is light yellow-brown and diffusible pigment moderate yellow-brown. The results of physiological, biochemical characteristics (Table 2) and cell wall hydrolysate of actinomycete isolate, exhibited that the cell wall containing LL-diaminopimelic acid (DAP). These results emphasized that the actinomycetes isolate related to a group of *Streptomyces* as previously studied **(Reddy *et al*., 2011; Afifi *et al*., 2012 and Muharram *et al*., 2013).** The phylogenetic tree (diagram) revealed that the local isolate is closely related *Streptomyces rimosus*, similarity matrix is 96% as identified strain of *Streptomyces plicatus* (strain 101) by **(Kang *et al*., 2000; Zamanian *et al*., 2005 and Atta *et al*., 2013).** *Streptoverticillium* sp. and two *Streptomyces* sp. by **(Raja *et al*. 2010)**. In view of all the previously recorded data, the identification of actinomycete

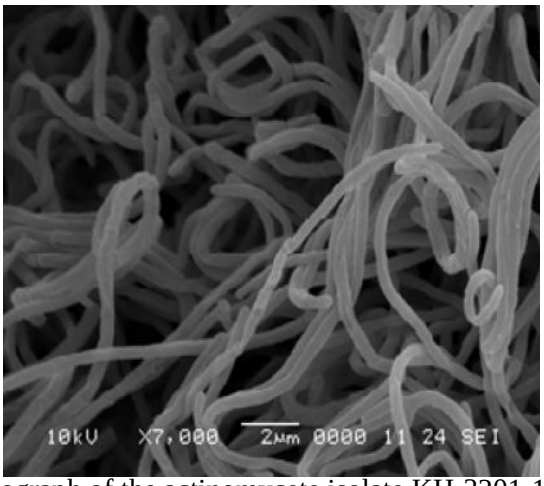

Plate 1. Scanning electron micrograph of the actinomycete isolate KH-3201-144 growing on starch nitrate agar medium showing spore chain Spiral shape and spore surfaces smooth (X7,000).

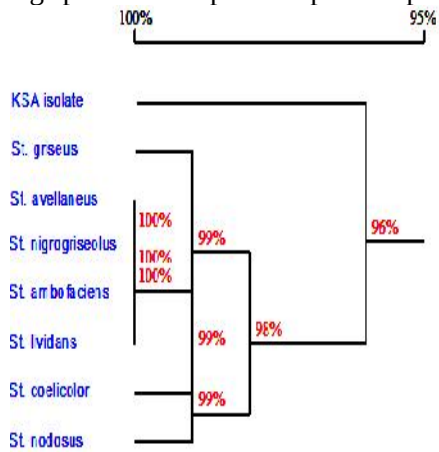

Fig. 1. The phylogenetic position of the local *Streptomyces* sp. strain among neighboring species. The phylogenetic tree was based on the multiple alignments options of 16_S rDNA sequences.

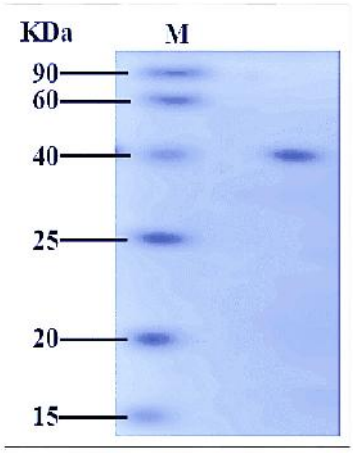

Fig. 2. SDS-PAGE of beta lactamase inhibitory protein.

Table 1. Screening of β-lactamase inhibitory protein activity produced by actinomycete isolates against amoxicillin resistant bacteria

Number of active isolates	**Mean values of inhibition zone (mm) against**		
	Staphylococcus aureus	***Pseudomonas aeruginosa***	***Klebsiella pneumonia***
KH-3201-12	20.0	14.0	15.0
KH-3201-19	15.0	24.0	25.0
KH-3201-34	20.0	19.0	20.0
KH-3201-47	20.0	14.0	15.0
KH-3201-78	35.0	29.0	30.0
KH-3201-98	20.0	14.0	15.0
KH-3201-121	18.0	16.0	16.0
KH-3201-144	**40**.0	**36**.0	**37**.0
KH-3201-155	20.0	14.0	15.0
KH-3201-170	20.0	19.0	20.0
KH-3201-179	35.0	29.0	30.0

isolate KH-3201-144 was suggestive of being belonging to *Streptomyces rimosus*, KH-3201-144, which can produce a β-lactamase inhibitory protein (BLIP) as previously reported **(Ghadin *et al*., 2008 and Ubukata *et al*., 2007).** The β- lactamase inhibitory protein was precipitated by ammonium sulphate (40%), precipitated inhibitor was dissolved in 50 ml of 0.2 M phosphate buffer at pH 7.5. The enzyme inhibitor solution (50 ml) was subjected to dialysis in cellophane tubing for 18 h at 4°C. Similar results were obtained by **(Abdulkhair, 2012).** The purification process was done through two steps. The first step was carried out by using DEAE– Cellulose (diethyl-aminoethyl cellulose G-25) which considered anion exchange column chromatography. The elution was exerted by phosphate buffer at pH 7.5. Sodium chloride was supplemented through this step by certain gradient (0.1 to 0.2 M) to activate the particles of cellulose. One peak illustrated due to presence of one type of β-lactamase inhibitory protein. The second step was carried out by using sephadex G-200 column chromatography. Also one peak illustrated due to presence of purified inhibitory protein. Similar results were obtained by **(Spencer *et al.,* 2005 and Abdulkhair, 2012).** A purified protein was dialyzed through overnight against phosphate buffer at pH 7.5. A purified protein was separated by polyacrylamide gel electrophoresis (10%) in the presence of sodium dodecyl sulphate (SDS). Protein was localized by staining with coomassie blue. The active protein was homogenous in disc gel electrophoresis and gave only one band of protein at 40 KDa. Similar results were recorded by **(Brown & Palzkill 2010 and Awad & El-Shahed, 2013).** The MIC of β-lactamase inhibitory protein produced by *Streptomyces rimosus*, KH-3201-144 for Susceptibility of amoxicillin-resistant bacteria, Staphylococcus *aureus*, was 93.75 µg / ml and 125 µg/ml with *K. pneumoniae* and *Ps. aeruginosa*. Similar investigations and results were attained by **((Chen, *et al.,* 2003; Parag *et al.,* 2003; Parag *et al.,* 2006 and Awad *et al.,* 2009).**

Table 2. The morphological, physiological and biochemical characteristics of the actinomycete isolate KH-3201-144

Characteristic	Result	Characteristic	Result
Morphological characteristics:		Mannitol	+++
Spore chains	Spiral	L- Arabinose	+
Spore mass	white	*meso*-Inositol	+++
Spore surface	smooth	Lactose	+
Color of substrate mycelium	Light yellowish brown	Maltose	+
Motility	Non-motile	D-fructose	++
Cell wall hydrolysate		Utilization of amino acids:	
Diaminopimelic acid (DAP)	LL-DAP	L-Cycteine	-
Sugar Pattern	Not-detected	L-Valine	+
Physiological and biochemical properties:		L-Histidine	++
Hydrolysis of:-		L-Phenylalanine	++
Starch	+	L-Arginine	+
Protein	+	L-Tyrosine & L-Lysine	+
Lipid	+	Growth inhibitors	
Pectin	-	Sodium azide (0.01)	+
Lecithin	+	Phenol (0.1)	+
Catalase test	-	Thallous acetate (0.001)	-
Production of melanin pigment on:		Growth at different temperatures (˚C):	
Peptone yeast- extract iron agar	-	20	-
Tyrosine agar medium	-	25	±
Tryptone– yeast extract broth	-	30-45	+
Degradation of**:**		50	-
Xanthin	+	Growth at different pH values:	
Esculin	+	4	-
H_2S Production	-	5-9	+
Nitrate reduction	+	10	-
Citrate utilization	+	Growth at different concentration of NaCl (%)	
Urea test	+	1-7	+
KCN test	+	10	-
Utilization ofcarbon sources		Antagonistic Effect:	
Xylose	-	*Bacillus subtilis*	+
Mannose	+	*Micrococcus luteus*	+
Glucose	++	*Saccharomyces cerevisiae*	+
Galactose	+	*Aspergillusniger*	+
Sucrose	-		
L-Rhamnose	-		
Raffinose	+		
Starch	+++		

+=Positive, - = Negative and ± = doubtful results, ++ = good growth.

Table 3. Cultural characteristics of the actinomycete isolate KH-3201-144.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
1- Starch-nitrate agar medium	Good	263 - white white	76.1.y Br light yellowish brown	77 m-y Br moderate yellowish brown
2- Yeast extract - Malt extract agar medium (ISP-2)	Moderate	263 - white white	76.1.y Br light yellowish brown	77 m-y Br moderate yellowish brown
3- Oat-meal agar medium (ISP-3)	Good	263 - white white	76.1.y Br light yellowish brown	-
4- Inorganic salts-starch agar medium (ISP-4)	Good	263 - white white	76.1.y Br light yellowish brown	-
5- Glycerol-Asparagine agar medium (ISP-5)	Moderate	93-y-Gray yellowish gray	76.1.y Br light yellowish brown	-
6- Melanin test: Tryptone-yeast extract broth (ISP-1)	No growth	-	-	-
b- Peptone yeast extract-iron agar medium (ISP-6)	Good	263 - white white	76.1.y Br light yellowish brown	77 m-y Br moderate yellowish brown
c- Tyrosine agar (ISP-7)	Good	263 - white white	76.1.y Br light yellowish brown	77 m-y Br moderate yellowish brown

The color of the organism under investigation was consulted using the ISCC-NBS color - Name charts II illustrated with centroid color.

Table 4. Precipitation of β-lactamase inhibitory protein (BLIP) by different concentrations of saturated ammonium sulphate

Ammonium sulphate concentration %	*Total activity of BLIP (u)	Total protein content (mg)	Specific activity (u/mg)
Control	200	180	1.2
10	0	3.0	0.0
20	0	5.0	0.0
30	170	6.5	25.0
40	185	7.5	26.0
50	180	7.0	24.0
60	0	5.5	0.0
70	0	5.0	0.0
80	0	4.5	0.0
90	0	4.0	0.0

BLIP: defined as the amount of protein which gives 1 mm of inhibition zone in the presence of amoxicillin as the substrate of β-lactamase enzyme.

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