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Antibacterial activities of River Sediment-Derived *Streptomyces* spp. against Multi drug resistant *Klebsiella pneumoniae.*

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ABSTRACT

The aim of this study determined by the screening of antibacterial compounds exhibited in *Streptomyces* spp. isolated from Tigris river sediment against Extended specrtrum beta-lactamases (ESBLs) producing *Klebsiella pneumoniae* and Carbapenem and Colistin resistant *Klebsiella pneumoniae* isolates to find a promising *Streptomyces* spp. with a new antibiotic working on these resistant bacteria. Out of 20 *K. pneumoniae*, 6 (30%) isolates showed Extended specrtrum beta-lactamases (ESBLs) producing *K. pneumoniae* and 2 (10%) Carbapenem and Colistin resistant *K. pneumoniae* by using disc diffusion method. The supernatant of 6 *Streptomyces* spp. out of 15 isolates cultured in IPS2 broth showed good bioactive compounds production by well diffusion method which appeared by a very good zone of inhibition around resistant *K. pneumoniae*. DNA sequencing of the highest active *Streptomyces* spp isolate showed that the identities was 96% to *Streptomyces azureus.*

Keywords: River Sediment; *ESBLs;Streptomyces*; Carbapenem and Colistin resistance; *Klebsiella pneumoniae.*

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INTRODUCTION

ESBL-producing organisms may be susceptible to some extended-spectrum Cephalosporins and inhibited by β-lactamase inhibitors such as clavulanic acid [1]. The *Klebsiella* and *E. coli* are the main bacteria that produce ESBLs [2]. Plasmids for ESBL may carry genes encoding resistance to other drugs like Aminoglycosides, Chloramphenicol, Sulfonamides, Trimethoprim, and Tetracycline are multidrug resistance, which are mobile genetic elements and can transmitted *in vivo* among Gram negative species. Therefore, antibiotics used in the treatment of ESBL-producing organisms are very limited [3]. Carbapenems are drug of choice for ESBL-producing organisms, recently Carbapenem-resistance have been reported. However, treatment with such antibiotics has been associated with high failure rates. Reports showed that ESBL-producing organisms resist to the Carbapenems, primarily Ertapenem are inhibited by Clavulanic acid [4, 5, 6] reported that the outbreak of ESBL-producing *K. oxytoca* in an Neonatal intensive care unit spread from *K. oxytoca* to *K. pneumoniae*, *E. coli*, *E. cloacae,* and *C. freundii*.

The National Committee for Clinical Laboratory Standards Laboratory (NCCLS) recommends routine screening for ESBL activity in *K. pneumoniae,* and *K. oxytoca* isolates by determining susceptibility to several Cephalosporins (Cefpodoxime, Cefotaxime, Ceftriaxone, and Ceftazidime) [7]. Confirmatory tests for an ESBLs are detected by increase the sensitivity to Cefotaxime or Ceftazidime in the presence of Clavulanic acid. Carbapenem resistance among the nosocomial pathogen *Klebsiella pneumoniae* is increasing worldwide, increasing morbidity, mortality and healthcare costs. Colistin is the drug of choice for treating infections caused by Carbapenem-resistant *K. pneumoniae* (CR-*KP*); however, its usefulness is threatened by the dissemination of the Colistin resistance gene *mcr-1*. Chromosomally mediated resistance appears to be the most common resistance mechanism [8,9]. Colistin is considered the last resort antibiotic for treating infections caused by Carbapenem-resistant bacteria. However, the emergence of Colistin resistant *K. pneumoniae* (CoR*KP*) emphasizes the urgent need to identify another drug, so we tried to find alternative choices like *Streptomyces* spp. bioactive compounds against the CoR*KP* isolates [10].

MATERIALS AND METHODS

Table (1): The Antibiotic discs used throughout this study to detect Carbapenem resistant *K. peumoniae* (CR-*KP*) and Colistin resistant *Klebsiella peumoniae* (CoR*KP*).

Antibiotics	Discs symbols	Concentration (µg)	Company	Origin
Colistin	CT	10	Bioanalyse	Turkey
Trovafloxacin	TRV	10		
Imipenem	IPM	10		
Meropenem	MEM	10		

Table (2): The Antibiotics discs used throughout this study for detection ESBLs producing *K. pneumoniae* by Double disc synergy method (DDST).

Antibiotics	Discs symbols	Concentration (µg)	Company	Origin
Ceftriaxone	CRO	10	Bioanalyse	Turkey
Cefotaxime	CTX	10		
Ceftazidime	CAZ	10		
Amoxycillin/Clavulanic acid	AMC	10		

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Table (3): Media used in the study for *Streptomyces* characterization and fermentation.

NO	Media name	Composition	Amount	Ref
1.	ISP2 Yeast Extract Malt Extract Agar	Yeast Extract	4 gm	[11]
		Malt Extract	10 gm	
		Dextrose	4 gm	
		Agar	20 gm	
		Distilled water	1 liter	
		pH 7. 4		
2.	ISP4 Inorganic salts starch Agar	Soluble starch	10 gm	[11]
		Casein	0.3 gm	
		K2HP04	2 gm	
		KNO3	2 gm	
		MgS04.7HzO	0.05 gm	
		NaCl	2 g	
		FeSo4.7H2O	0.01 gm	
		CaC03	0.02 gm	
		Trace salts solution	1 ml	
		Agar	20 gm	
		Distilled water	1 liter	
		pH 7. 4		

River sediment samples

About 37 Tigris river sediment samples were collected from Abu Nuwas Zone at Baghdad \ Iraq.

Tested *K. pneumoniae*

The total number (20) isolates of *K. pneumoniae* were used to determine the antimicrobial activity of *Streptomyces* isolates. The *Klebsiella* spp. were obtained from Biotechnology College\ University of Baghdad, which activated by culturing in a Nutrient Broth at 37°C for 24 hours then species confirmation was done by Enterosystem 18R (Liofilchem\Italy).

Disk diffusion method

By using Kirby-Bauer Single disk diffusion method [12], the susceptibility of 20 *K. pneumoniae* isolates was determined against Carbapenem and Colistin discs (table 1). The inocula were prepared by growing the isolates on selecting three to five colonies from the plate and transferred with inoculating loop into 3 ml of normal saline in a test tube. The density of these suspensions was adjusted to 0.5 McFarland standards. The surface of Mueller-Hinton agar plate was evenly inoculated with the organisms using a sterile swab. The swab was dipped into the suspension and pressed against the side of the test tube to remove excess fluid. The wet swab was then used to inoculate the Mueller-Hinton agar, evenly streaked across the surface. The antibiotic discs were applied to the surface of the inoculated agar and the plates were incubated overnight at 37 °C for 18-24hrs. The diameter of zone of inhibition was observed and measured and compared to the chart provided by National Committee for Clinical Laboratory Standard institute [13].

Double disc synergy test

Double disc synergy test [14] was indicated to test the ability of 20 *K. pneumoniae* isolates for ESBLs production by using Cephalosporins (Ceftriaxone, Cefotaxime, Ceftazidime) and Amoxycillin/Clavulanic acid (table 2). The three antibiotics were placed at distances of 14 mm (edge to edge) from the Amoxycillin/Clavulanic acid disc that was placed in the middle of the plate on the surface of Mueller-Hinton agar plate (the inocula were prepared as that in agar disk diffusion method). After 24-h incubation, if an enhanced zone of inhibition between

either of the Cephalosporin antibiotics and the Amoxycillin/Clavulanic acid disc occurred, the test was considered positive. This indicates synergistic activity with Clavulanic acid and the presence of an ESBL.

Isolation and identification *Streptomyces* spp.

One gram of Tigris river sediment samples was used to make suspension, by adding it to 99 ml of distilled water (stock suspension), The samples were shacking in a shaker at 120 rpm for 30 minutes at room temperature. Serial dilutions from 10^{-1} to 10^{-3} were made from the stock suspension and left for 10 minutes. After shaking, 0.1 ml of each dilution was pipetted and put on supplemented starch casein agar (ISP4) with Tetracycline 50 mg/L and Nystatin 50 mg/L and Nystatin 50 mg/L (table 3), then spread by a sterile swab to make a uniform distribution of the suspension on the surface of the media. The inoculated plates were incubated at 28°C for 7 to 14 days. The suspected colonies subcultured in ISP2 media (table 3) as reported by Al-rubaye *et al*., (2018) [15].

Cultural Characterization of *Streptomyces* isolates

The *Streptomyces* isolates grew on ISP2 medium (table 3) were characterized morphologically according to the colony characteristics, Gram's Stain and morphological characterization following the directions given for the International *Streptomyces* project (ISP) [11,16,17,18].

Screening of *Streptomyces* isolates for bioactive compounds production (well plate method)

About Fifteen *Streptomyces* isolates were grown on ISP2 broth (table-3 without agar) and submitted to screening for antimicrobial activity. Shake flask fermentation was carried out by the inoculation of 1.5ml of prepared stock suspension cultures with 80ml of Antibiotic production medium (ISP2 broth), pH 7.5 and incubated at 28°C, 170rpm for 7days in a shaking incubator [18,19,20]. Supernatant were collected and separated from the crude precipitation from each isolate by centrifugation at 10,000 rpm\2 min. The flasks fermentation of the selected *Streptomyces* (figure 3) were subjected to plate well method by using the filtrate. The bioactive compounds were screened by checking the activities of the supernatant filtrate (filtered by 0.45µm) against the ESBLs producing *K. pneumoniae*, CR*KP* and CoR*KP*. After solidification of 20 ml of sterilized muller-Hinton agar, spread 100 microliters of pathogenic activated bacteria (*Klebsiella pneumoniae*) by L shape spreader. Wells (6 mm in diameter) were prepared in each seeded agar plate and each well was filled with 100 microliters of filtered supernatant (0.45µm) and screened via agar well diffusion procedure mentioned previously, the plates incubated at 37 °C and the zone of inhibition was determined after 24 hours overnight [21].

DNA Extraction

Six *Streptomyces* isolate with the highest antibacterial activity were selected and cultured in ISP2 medium (table 3). Cells were harvested by centrifugation (5 min, 4000× *g*), washed [2× 10 mL of 10% (w/v) sucrose] as described by Nikodinovic *et al*., (2003) [22] and Al-rubaye (2016) [23]. Genomic DNA extraction and purification carried out by Bioneer extraction kit. According to manufacturer instructions. The DNA samples measured for their concentration and purity using Microvolume UV Spectrophotometer (ACTGene, USA).

DNA sequencing

The primers *16S rDNA* F: **5' TCACGGAGAGTTTGATCCTG 3'** and *16S rDNA* R: **5' GCGGCTGCTGGCACGTAGTT 3'** were used for identification of *Streptomyces* spp. as used in a previous study [23]. PCR was performed in a 50 µl mixture containing 1× PCR buffer (10 mM Tris–HCl, 1.5 mM MgCl2, 5 mM KCl [pH 9]) (Merck, India), 100 µM (each) deoxynucleoside triphosphates, 1 U of Taq DNA polymerase (Merck, India), 10 pM each of forward and reverse primers, and 100 ng of templet DNA. The program for PCR included an initial denaturation 94°C for 5min, 30-40 cycles of denaturation at 97°C for 30s, annealing at 50°C for 1min, extension at 72°C for 1min and a final extension at 72 °C for 7min. The PCR products were loaded on a 1.5% agarose gel, stained with ethidium bromide (5ng/ml) and bands observed using a gel documentation system (ATTA, Japan). PCR products were sent for sequencing at Bioneer, Korea. The obtained sequence was compared for similarity with sequences resent in the genomic database banks, using the "NCBI Blast" program available at the ncbi.nlm.nih.gov web site and highest watching sequences downloaded.

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RESULTS AND DISCUSSION

Detection ESBLs producing *K. pneumoniae*

The DDST is considered positive when the inhibition zone of any of the antibiotics is larger towards the Clavulanic acid disc (fig 1) or a ghost inhibition zone appears between the central disc and any of the other antibiotics. This is happening because of the ESBL's inhibition by the clavulanic acid. In proximity to the central disc the enzyme's activity is blocked. Thus, the growth inhibition zone appears only towards the Clavulanic acid disc. If resistance to Cephalosporins is not due to ESBL production, the test results negative. Seven isolates had been shown ESBLs activity according to DDST (fig 2), a same result was observed by Zhang *et al*., (2016) [24], they found an increase in the percentage of ESBL producing *K. pneumoniae* isolates (31.8%). The spread of ESBL-producing bacteria is rapidly increasing worldwide, so the continuous monitoring systems and effective infection control measures are essential. The most risk factors for acquisition of ESBL-producing bacteria are using Cephalosporins with an oxyimino side chain and the hospital transfer [25].

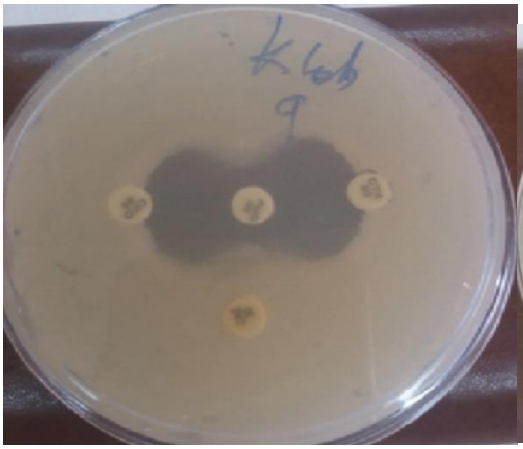

Fig (1): Detection of ESBLs producing *K. pneumoniae* by Double disc synergy method: Cefotaxime disk (CTX); Ceftazidime disk (CAZ) and Ceftriaxone (CRO) disk around Amoxicillin-clavulanate (AMC) disk in center. Note the synergy between the AMC and CRO in the left and the AMC and CTX in the right.

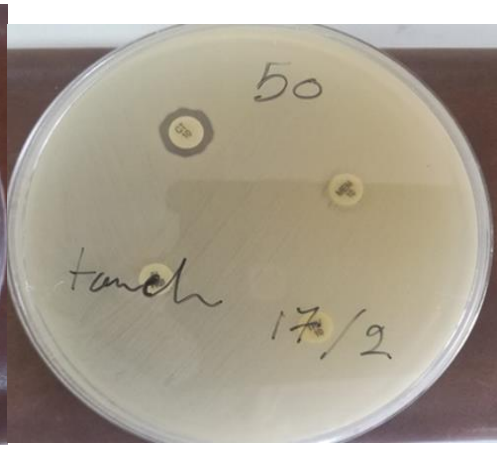

Fig (2): *Klebsiella pneumoniae* resist to Carbapenem and Colistin.

CR*KP* and COR*KP* results

Figure (2) shows *K. pneumoniae* resist to Carbapenem and Colistin, about 2 (10%) isolates showed a Carbapenem and Colistin resistance, which is in disagreement with a study by Sarab and Alrubaye (2019) [26], they showed that the Colistin and Carbapenem resistance about 33.3%, this may be due to small group population of *K. pneumoniae* isolates. This case is very urgent because the drug of choice to Carbapenem resistant *K. pneumoniae* is Colistin, so an alternative drug is very important for treatment this case.

Characterization of *Streptomyces* spp.

Morphological characterization

The *Streptomyces* spp. isolates were identified according to the variability in their colony morphology and microscopic characteristics like the aerial and substrate mycelium, soluble pigment, spore chain arrangement. Some isolates produced diffusible pigment in the surrounding media in accordance with the aerial mycelium color, soluble pigment was also observed in some isolates, figure (3) showed aerial mycelium of *Streptomyces* grown in ISP2 (series established in the Bergey's manual of determinative bacteriology by Buchanan and Gibbons [27]. The Total number of characterized *Streptomyces* were 15 isolates.

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Screening of *Streptomyces* isolates for bioactive compounds production

Fifteen *Streptomyces* in flasks fermentation (fig 4) were subjected to plate well method by using the filtrate. The bioactive compound production screened by observing the activities of the supernatant filtrate (filtered by 0.45µm) against the ESBLs producing *K. pneumoniae,* CR*KP* and COR*KP*. Figure (5) shows a very good zone of inhibition of the supernatant against ESBLs producing *K. pneumoniae,* while figure (6) shows the *Streptomyces* supernatant activity against the CR*KP* and COR*KP.*

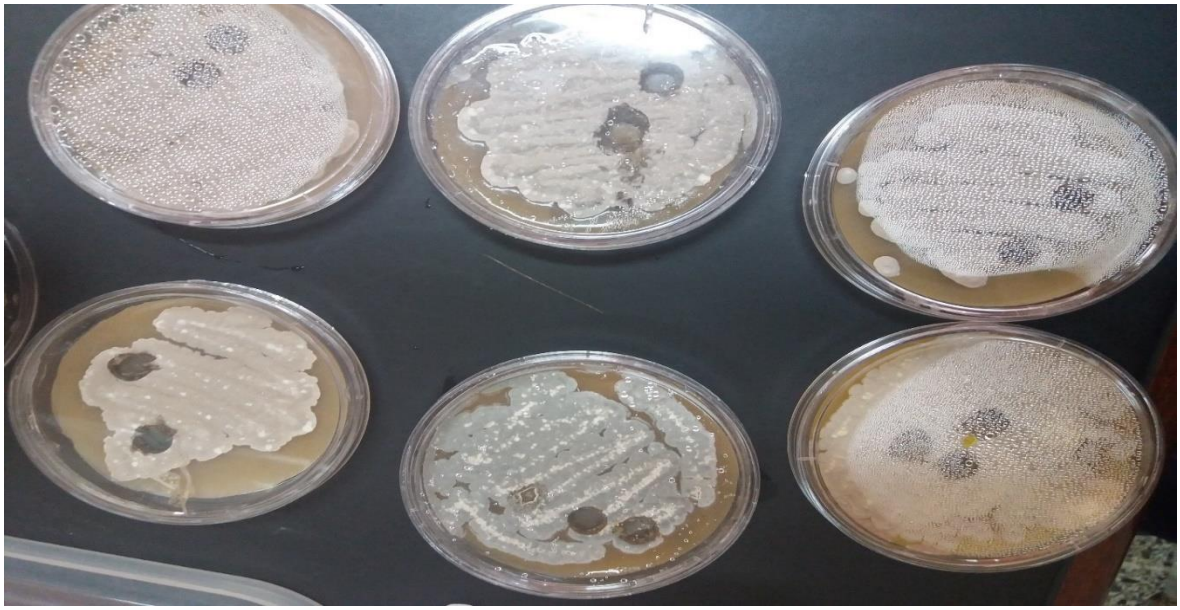

Fig (3): Arial mycelium of *Streptomyces* grown in ISP4.

Fig (4): Shake flask fermentation for most active *Streptomyces* isolates, selected from primary screening, was carried out in flasks containing 80 ml of production medium (ISP2) broth, shaken at 170 rpm for 7 days.

ISSN: 0975-8585

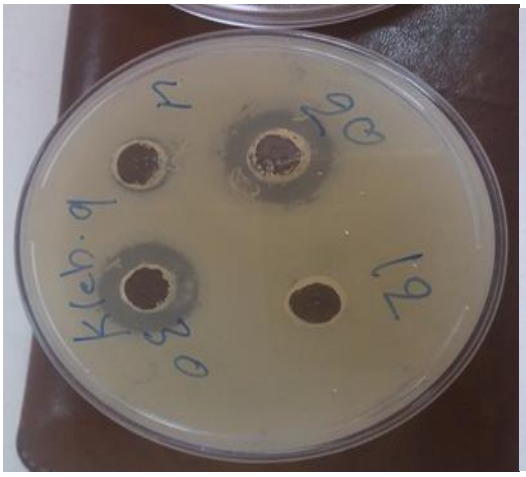

Fig (5): Antibacterial activity of *Streptomyces* supernatant against ESBLs producing *K. pneumoniae*.

Fig (6): Antibacterial activity of *Streptomyces* supernatant against Carbapenem and Colistin resistant *K. pneumoniae*.

Molecular Characterization (PCR technique and DNA sequencing)

According to the cultural characteristics including the aerial and substrate mycelia and spore chains arrangement of the isolates, they are placed under the genus *Streptomyces*. The *Streptomyces* identity was specified as spp. by PCR and sequencing of *16S rDNA* as reported by Kandhasamy and Sun (2012) [28]. About 6 isolates with the highest antibacterial activities against *K. peumonia* were selected for PCR and sequence. By partial amplification of *16S rDNA,* 500-bp were recovered from each of the 6 isolates (fig 7), in addition to sequences identity as shown in figure (8). PCR and *16S DNA* gene partially sequenced in *Streptomyces* species detection was also used by Alrubaye (2016) [23]. The conclusion from this study showed that river derived *Streptomyces* had a good activity against resistant pathogens with interest to ESBLs, Carbapenem and Colistin. The *16S rDNA* sequencing of the highest active *Streptomyces* against ESBLs, Carbapenem and Colistin resistant *K. pneumoniae* was in similarity to *Streptomyces azureus*. Further studies recommends to the genes responsible for antibiotics production in addition to other lifestyle genes, whole genomic sequence for the isolates from Tigris river sediments, optimization of these biproducts, evaluation to treat infection and evaluation the mechanism of action.

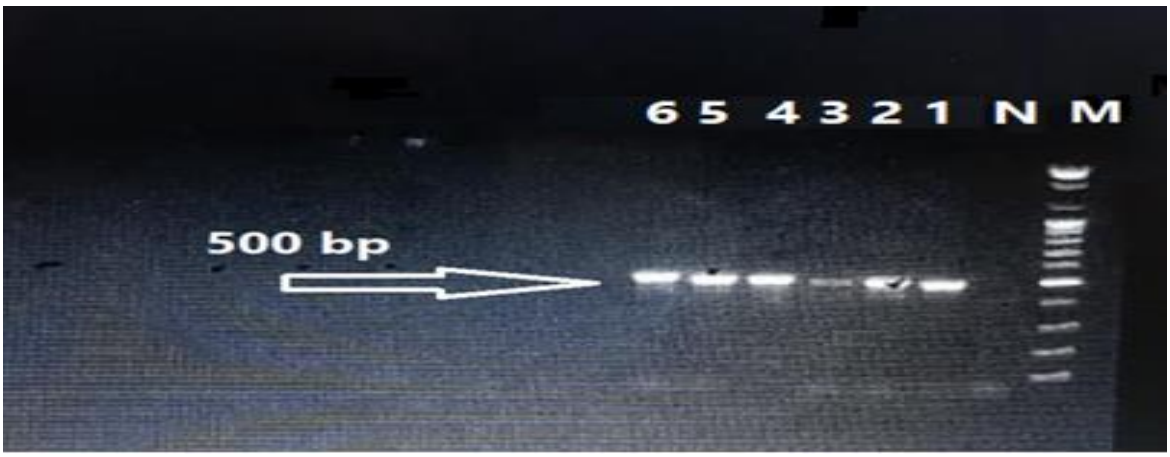

Fig (7): Agarose gel electrophoresis of 500bp specific PCR product for *16S rDNA* using 1.5% agarose gel at 90V for 1hr. in 1x TBE buffer, visualized under transilluminator UV after staining by ethidium bromide. Lane M: 100 bp DNA Marker, lane N: Negative control and Lane (1,2,3,4,5,6): Samples.

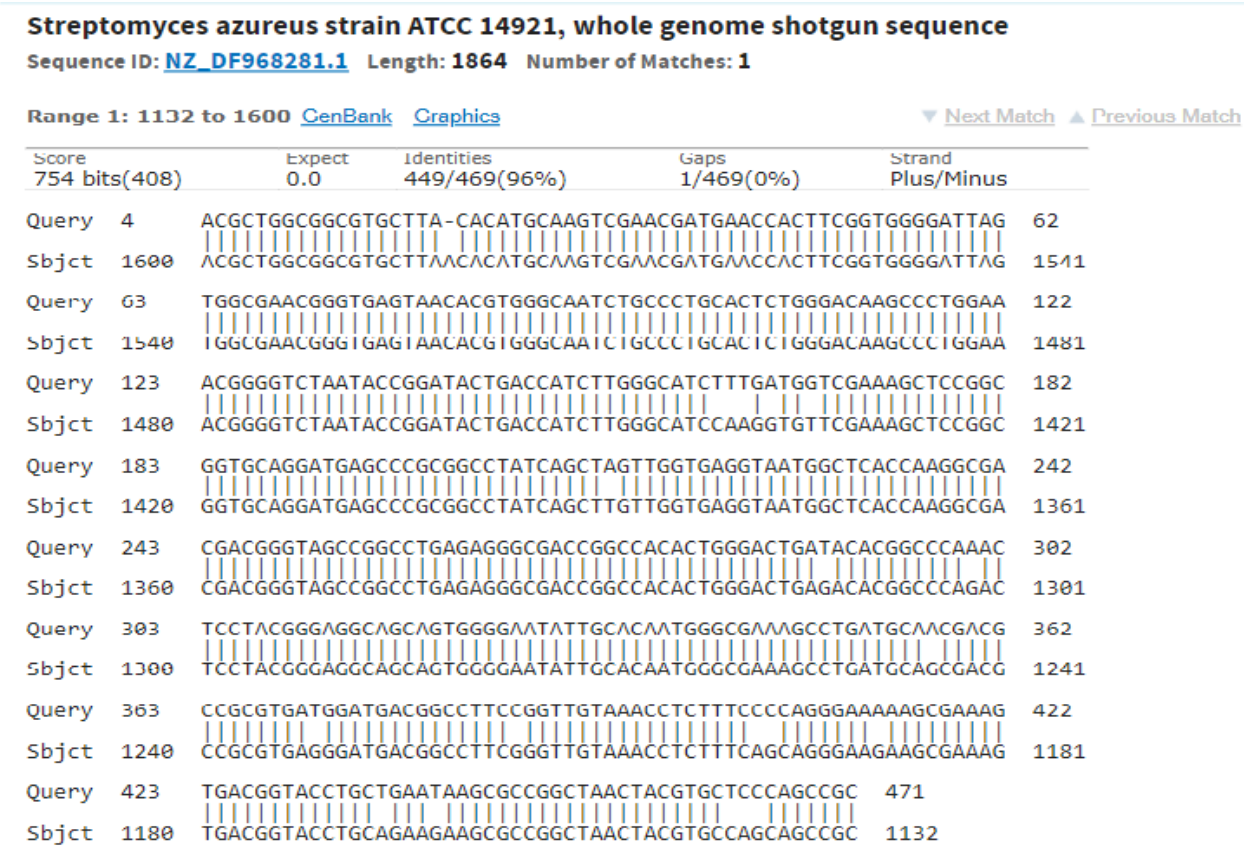

Fig (8): Sequences producing significant alignments.

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