Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

p-ISSN: 0972-6268
(Print copies up to 2016)
e-ISSN: 2395-3454

Vol. 24 | No. S1 | pp. 285-291 | 2025

Original Research Paper

https://doi.org/10.46488/NEPT.2025.v24iS1.021

Open Access Journal

Bioactivity of Rhizospheric *Acinetobacter baumannii* Siderophore Combined with Antibiotics Against Lower Respiratory Tract Pathogenic Bacteria

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Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 21-05-2024
Revised: 11-06-2024
Accepted: 19-06-2024

Key Words:
Antibacterial activity
Siderophore
Acinetobacter baumannii
Respiratory tract bacteria

ABSTRACT

The study focused on extracting and purifying siderophore produced by *Acinetobacter baumannii* isolated from rhizospheric soil in Baghdad city and evaluating its bioactivity both independently and in combination with selected antibiotics. Bacterial identification was performed using CHROM agar, biochemical, and physiological tests, with confirmation via PCR amplification of the 16S rDNA housekeeping gene. The siderophore was extracted using ethyl acetate after culturing the bacteria in succinate broth and was purified through HPLC, detected at a wavelength of 403 nm. A total of 38 bacterial isolates were obtained from lower respiratory tract infections, including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus*, and *Serratia marcescens*. Antibiotic susceptibility testing with 13 antibiotics showed the highest resistance rates to ampicillin (65.7%) and ceftriaxone (63.1%), while the lowest resistance was observed with amikacin (15.7%). The synergistic activity of the siderophore combined with sub-MIC concentrations of ceftriaxone, ceftazidime, and gentamycin was tested against multidrug-resistant (MDR) isolates. The most significant antibacterial activity was observed with the combination of siderophore and gentamycin against *S. aureus*, whereas a minimal effect was noted on *A. baumannii*. In conclusion, 38 bacterial isolates were successfully identified from lower respiratory tract infections. The combination of siderophore with gentamycin exhibited notable antibacterial activity against *S. aureus* but was ineffective against *A. baumannii*.

INTRODUCTION

Acinetobacter spp. are saprophytic organisms found in soil, water, wastewater, vegetables, and animal and human skin. They resist many antibiotics due to chromosome-mediated genetic elements and can persist on surfaces and medical devices in hospitals for long periods(Asif et al. 2018). Microorganisms and plants produce low molecular weight (500–1000 Daltons) iron chelators called siderophores to enhance iron acquisition from the soil, especially under iron-limited conditions. These siderophores selectively bind iron (III) with high affinity(Lis et al. 2015). Siderophores are high-affinity iron chelator proteins that compete with host cells for iron (Chan & Burrows 2023). Iron acquisition mechanisms are crucial virulence factors for bacterial pathogens, including *A. baumannii*, enabling their survival in hosts. Iron is an essential nutrient for nearly every life on earth(Ilbert &Bonnefoy 2013, Artuso et al. 2023).

Siderophores can be employed as a "Trojan Horse Strategy" in the medical profession, forming a complex with antibiotics and delivering them to the appropriate places, notably in antibiotic-resistant bacteria (Prabhakar 2020, Cheng et al. 2024).

Biofilms are one of the primaries that cause lower respiratory tract infections that may particularly complicate the treatment and are one of the foremost causes of death in developing countries worldwide. Patients with chronic respiratory diseases are at a higher risk of such infections and are one of the main causes of morbidity and mortality rate in this group, besides, respiratory infections can also impact economies because they can lead to increasing in treatment costs (Smith et al. 2019, Perry & Tan 2023).

This study aims to extract and purify siderophores produced from *Acinetobacter baumannii* and investigate the activity of synergism against some antibiotics and its action as an antibacterial agent on pathogenic bacteria.

MATERIALS AND METHODS

Isolation and Identification of *A. baumannii*

The soil samples were taken from the wetland rhizosphere region from the plant to isolate *A. baumannii* isolates. Soil sample culturing on (CHROM agar) in a selective media for *A .baumannii* and then were subjected to numerous cultural, and biochemical tests and vitek2 using PCR. All earlier

Table 1: Primer sequences used in molecular detection of *A. baumannii* isolated from soil.

Gene name	Primer name	Sequence 5'→3'	size	Annealing Temperature	Reference
16s rRNA	F	TTTAAGCGAGGAGGAGG	242bp,	56°C,	(Sepahvand*et al.* 2017)
	R	ATTCTACCATCCTCTCCC			

F = Forward R= Reverse

diagnosed isolates underwent DNA extraction using the ABIO pure TM kit (Alliance Bio, USA). The concentration and purity of the extracted DNA were measured with a Nanodrop. The samples were further confirmed by diagnosing with the housekeeping gene 16S rDNA(Tawfeeqet al.2023) (Table 1). The bacterial isolates were cultured overnight in a nutrient broth medium, then subjected to DNA extraction,

Screening for Siderophore Produced from *A. baumannii* Isolates

Chrome Azurol S (CAS) agar assay was used to detect the siderophore producer from *A. baumannii* isolates. According to the modified method of Srimathi & Suji (2018), the isolates were streaked on CAS agar and incubated at 30ºC for 48 hours. The isolate is considered a siderophore producer when bacterial growth can grow and change the medium blue color to green or yellow, indicating a positive result.

Antibiotic Susceptibility Test (AST)

Antibiotic sensitivity of all lower respiratory tract infection isolates was tested using the agar disc diffusion method (Kirby-Bauer method) against 13 different antibiotics(Ampicillin, Ceftriaxone, Ceftazidime, AmoxillinCalvunic acid, Gentamicin, Ciprofloxacin, Cefotaxime, Nitrofurantion, Norfloxacin, Cefepime, Levolfloxacn, Cefazolin, Amikacin). The results obtained were justified according to (Weinstein & Lewis2020).

Extraction of Siderophore

The Siderophore produced by an isolate of *Acinetobacter baumannii* was precultured at 28ºC for 12 hours in succinate liquid broth and incubated in a rotary shaker for 4 days at 110rpm, then siderophore was extracted by centrifuging at 12000rpm for 10min. The supernatant was acidified with pH 2 and was mixed with ethyl acetate. Using a reparatory funnel, the upper layer was collected which represents the crude siderophore, then dried in a desiccated vacuum at 40°C and then dissolved in 1mL of methanol as explained by (Taher 2016).

Purification of Siderophore

The extracted siderophore was analyzed by HPLC using a C18 reverse-phase column with methanol:water (8:2 v/v) mobile phase. The sample was injected at a flow rate of 1 mL/min at 25°C and detected at 403nm. Preparatory separation used the same mobile phase, and retention times (RT) of peaks with similar heights were analyzed.

Synergistic Effect Between Purified Siderophore with Some Antibiotics

The Minimum Inhibition Concentration (MIC) of three antibiotics—ceftriaxone, ceftazidime, and gentamycin—was determined. Overnight cultures of *Staphylococcus aureus*, *Escherichia coli*, *Acinetobacter baumannii*, *Serratia marcescens*, and *Klebsiella pneumoniae* were inoculated into nutrient broth (N.B) in 96-well microtitration plates. Serial dilutions of the antibiotic solutions were added to each well, with each isolate at approximately 1.5 × 10 CFU/mL. The plates were then incubated at 37°C for 24 hours.

The antibacterial activity of purified siderophore in combination with sub-MIC of antibiotics was also carried out by mixing 50µL of sub-MIC of antibiotics with 50µL of purified siderophore. The agar well diffusion method was applied by spreading each isolate on the surface of Muller Hinton Agar and using a cork borer to make 3 wells and loaded with 100µLof (50µL sub-MIC of antibiotic + 50µL purified siderophore) and second loaded with 100µL of sub-MIC of antibiotic alone and the last was control filled with D.W.

RESULTS

Conventional PCR was employed to detect the housekeeping 16S rRNA gene to identify A. baumannii species. The PCR-amplified fragments were 242 bp, Fig. 1.

The method used CAS agar for detecting the production of siderophore was also carried out as shown in Fig. 2.

Extraction and purification of siderophore from *A. baumannii* were also carried out using HPLC Chromatogram for siderophore purification C-18 column (250mm×4.6mm, 5; flow rate:1 mL/min at µm) 25°C; detection wavelength: 403nm; methanol: water (8:2 v/v).

Figs. 4, 5 and 6 show the Antibiotic Susceptibility Test (AST) indicating the resistance percentage of bacteria against different antibiotics.

Combination Effectiveness of Siderophore with some Antibiotics

The combination of antibiotics with siderophore was

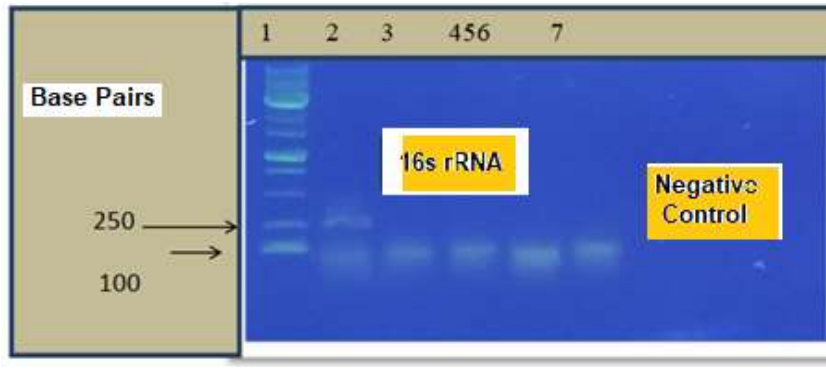

Fig. 1: PCR for 16s rRNA gene (amplified sizes were 242bp).

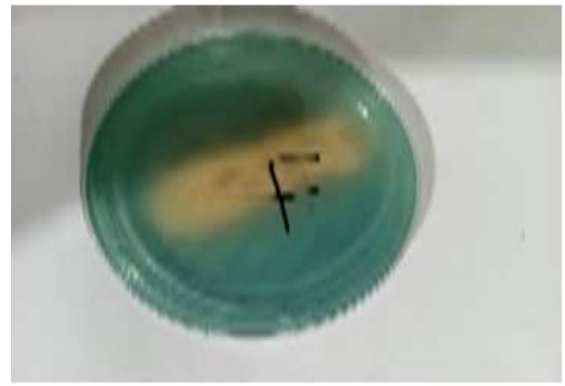

Fig. 2: CAS agar assay for siderophore detection.

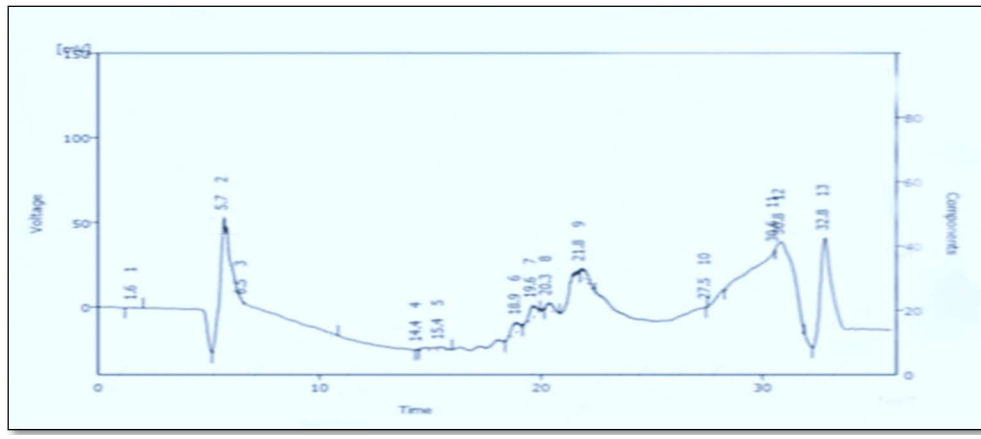

Fig. 3: HPLC Chromatogram flowchart for siderophore purification.

determined against some test bacteria causing lower respiratory tract infections. MIC was determined to 3 antibiotics (Gentamicin, Ceftazidime and Ceftriaxone) and the results were explainable according to clinical laboratory standard institutes (CLSI, 2022) (Tables 2 & 3).

DISCUSSION

Isolation and Identification of *A.baumannii*Isolates

Soil sample culturing on (CHROM agar) selective media for *A. baumannii* isolates were able to grow on CHROM agar and were suspected to be belonging to the genus *Acinetobacter*,

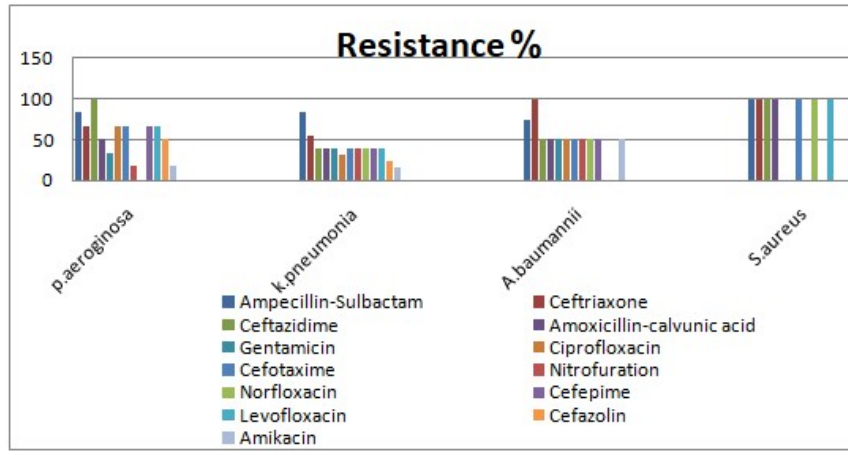

Fig. 4: The resistance percentage of *P. aeruginosa*, *K. pneumonia*, *A. baumannii* and *S. aureus* against different antibiotics.

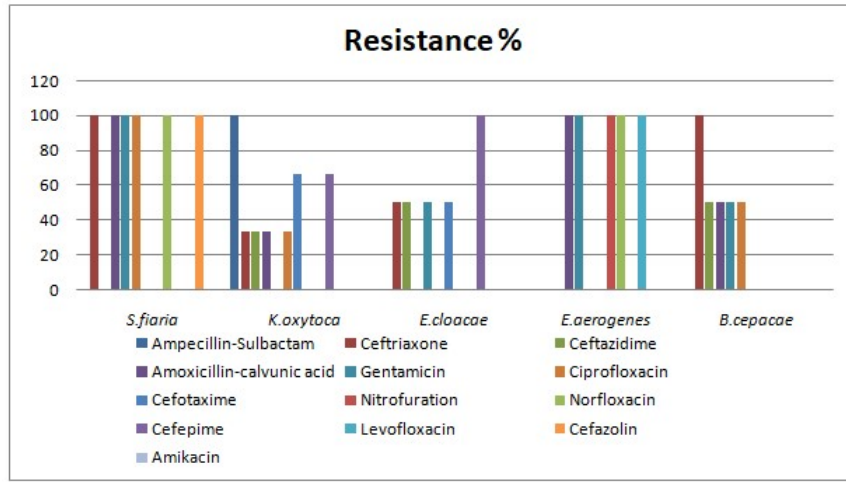

Fig. 5: The resistance percentage of *S. pyogenes*, *E. faecalis*, *E. coli* and *S. marscenes* against different antibiotics.

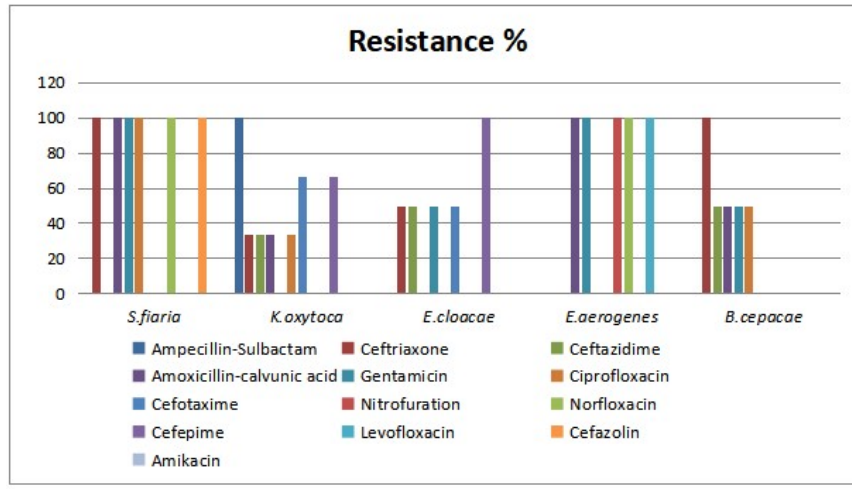

Fig. 6: The resistance percentage of *S. fiaria*, *K.oxytoca*, *E. cloacae*, *E. aerogenes* and *B. cepacian* against different antibiotics.

Table 2: The Value of MIC (µg/mL) for antibiotics against pathogenic bacteria isolates.

Bacterial isolates	MIC value (µg/mL)		
	GN	CAZ	CTX
S. aureus	16	16	64
K. pneumonia	16	32	128
A. baumannii	16	16	128

Table 3: Inhibition zone (mm) of the synergistic effect of antibiotic with siderophore antibacterial against tested bacteria.

Synergistic antibiotic with siderophore	Inhibition zone (mm)		
	S. aureus	*K. pneumoniae*	*A. baumannii*
Gentamicin	25	20	20
Ceftriaxone	28	15	15
Ceftazidime	24	24	18

then subjected to numerous cultural, biochemical tests and vitek2 finely using PCR for confirming the identification. The PCR is a more reliable method for diagnosing A. baumannii in laboratories than Chromogenic media and other methods used, the result appeared only one isolate was diagnosed as *A. baumannii*, which is used in the production of siderophore. The genotypic detection of *A. baumannii* on agarose gel electrophoresis of 16srRNA shows positive results with 242bp bands for 16s rRNA, line 3-6: negative result, line 7: negative control. UV light was used to visualize the DNA bands (Fig. 1).

Detection of Siderophores CAS Agar Medium

By changing the color of colonies into orange or yellow after the incubation period due to the removal of iron Fe from the dye which indicated the + ability of *A. baumannii* isolates for siderophore production. It is a colorimetric method by changing the color of colonies into orange or yellow after the incubation period due to the removal of iron Fe from the dye which indicates the + ability of isolates .*A. baumannii* for siderophore production.

Extraction and Purification of Siderophore from *A. baumannii*

The peaks obtained in retention times RT between 14 to 20 minutes belong to siderophore (Fig. 3). These results agree with Tank et al. (2012). When using the same condition and solvent obtained peaks at RT 14.9, 16.9, and 18.4 min at wavelength 403 using Data. RT allowed dissemination between siderophore and different peptide chains produced by bacterial species.

Antibiotic Susceptibility Test (AST)

The clinical isolates of *P. aeruginosa* were highly confrontation to beta-lactams (Ampicillin, Amoxicillin, Ceftazidime, Ceftriaxone, and Cefepime), this could be related to the hyperproduction of beta-lactamase through the genes of resistance and mutational processes, while the resistance against non-beta lactam antibiotics (Figs. 4, 5 and 6). The results recorded that 33.3% and 16.6% of isolates were resistant to aminoglycoside (gentamycin and amikacin, respectively) this may be due to the inactivation of aminoglycosides by resistant *P. aeruginosa* isolates involving their modification by enzymes that phosphorylate, acetylate, or adenylate these antimicrobials and enzymes are frequent determinants of aminoglycoside resistance in *P. aeruginosa*. Resistance of *Pseudomonas aeruginosa* isolates to cephalosporins was 66.6% (Cefotaxime) this is often associated with stable synthesis of the chromosomal beta-lactamase that robustly hydrolyzes cephalosporins (Barnes et al. 2018).

According to beta-lactam resistance by *A. baumannii* (each of Ampicillin 75% Amoxicillin and Ceftazidime were 50%, ceftriaxone 100%, and Cefepime was 50%), *Acinetobacter* spp. display multidrug confrontation via the creation of β-lactamases, changes in external membrane proteins and penicillin-binding proteins, and increased activity of efflux pumps (Cerqueira et al. 2011).In *K. pneumoniae*, the results show higher resistance for each of Ampicillin 84.6% and Ceftriaxone 53.8%, The production of β-lactamases, particularly extended-spectrum β-lactamases and AmpC β-lactamases, is a major drug resistance mechanism in *K. pneumoniae*, making these isolates resistant to broad-spectrum cephalosporins and β-lactam/β-lactamase inhibitors(Ali 2008).In *A. baumannii*, resistance to aminoglycosides is often due to aminoglycoside-modifying enzymes, with genes for these enzymes commonly located on mobile elements like plasmids and transposes, facilitating their transfer among the *A. baumannii* inhabitants (Lin et al. 2013).

The resistant to Beta-lactam (Amoxicillin, Ampicillin, Ceftazidime, Ceftriaxone, and Cefepime) *Staphylococci* are resistant to beta-lactam antibiotics through two different mechanisms. One is the creation of beta-lactamases, which are hydrolytic enzymes that eliminate beta-lactams. The other is the expression of the beta-lactam antibiotic-resistant penicillin-binding protein 2a. Resistance of *S. aureus* strains to cephalosporins was 100% (Cefotaxime) this is often associated with the widely used of Cephalosporins for the treatment of *staphylococcal* infections. The resistance mechanisms to quinolones in *Staphylococcus aureus* (Levofloxacin, Ciprofloxacin, and Norfloxacin), may be due to mutations in the genes encoding target enzymes, the expression of the efflux pump, fluoroquinolones inhibit

altered enzymes, and cefotaxime has a broader spectrum compared to ampicillin. Cefotaxime is often used alone, offering the benefits of fewer line entries and potentially lower toxicity.

Combination Effectiveness of Siderophore with some Antibiotics

The bacteria are regarded to be sensitive when the MIC value is less than the cut-off value as depicted by Kowalska et al. (2017). The results shown in Table 2 indicate that test isolates were highly resistant as they could grow in concentrations higher than the premium value for certain antibiotics. To determine the synergism effects of siderophore produced from the isolate of *A. baumannii* with sub-MIC of the antibiotics, the results in Table 3 declared the combination effects of siderophore with sub-MIC of gentamycin on *S. aureus* the inhibition zone was 25mm, *K. pneumoniae* and *A. baumannii* the inhibition zone was 20mm, then the combination of Ceftazidime with siderophore on *S. aureus* and *K. pneumoniae* inhibition zone was 24, *A. baumannii* the inhibition zone was 18mm, while the Ceftriaxone with siderophore gave 15 on *K. pneumonia* and *A. baumannii* 25mm. Whereas the inhibition zone gave 28 on *S. aureus*. The capacity of bacteria to acquire antibiotic resistance complicates the treatment of a wide range of bacterial infections. The 'Trojan horse' technique is one method for combating permeability-mediated drug resistance. The Trojan horse concept is the use of a bacterial iron absorption system to enter and kill bacteria after building complexes with antibiotics, as well as facilitating the selective delivery of drugs to antibiotic-resistant bacteria cells (Fan& Fang 2021).

Specific siderophore receptors identify the siderophore-antibiotic combination, which is then actively related across the external membrane. The goal of such an approach is to make it easier for present use or future antibiotics to enter bacterial cells, by increasing their activity or making them active against a wider variety of infections. It has been noticed that siderophore-drug conjugates allow for the development of medicines with enhanced cell transport and lower resistance rates Nguyen et al. (2020) concluded in their research that the combination of siderophore and cephalosporin antibiotics exhibited potent action against various MDR strains of *E. coli*, *K. pneumoniae*, and *Acinetobacter spp*. When coupled with antibiotics, Braun et al. (2009)found that Danomycins and salmycins, natural siderophore–antibiotic conjugates produced by Streptomycin, might decrease protein synthesis in Gram-positive bacteria, particularly staphylococci, and streptococci. Furthermore, various researchers and pharmaceutical companies have produced siderophore-antibiotic hybrids known as cefiderocol, which has advanced to clinical trials in terms of antibacterial activity against Gram-negative species).

CONCLUSIONS

Acinetobacter baumannii, derived from the rhizospheric soil was prepared successfully. The Isolation and purification were carried out via HPLC. Thirty-eight bacterial isolates were obtained successfully from injuries in the lower respiratory tract. The mixed siderophore with gentamycin shows a significant effect on *S. aureus*, while it does not affect *A. baumannii*.

ACKNOWLEDGMENT

The authors would like to thank the head and members of the Biology Department, College of Science, Mustansiriyah University (www.uomustansiriyah.edu.iq) for their support for the present work and special gratitude to every patient and control person.

List of Abbreviations

Abbreviation	Scientific Name
rDNA	Ribosomal Deoxyribonucleic acid
HPLC	High performance liquid chromatography
RT	Retention time
MIC	Minimum inhibitory concentration
MDR	Multi-drug resistance
PCR	Polymerase chain reaction
CAS agar	Chrome azurolsulfonate (CAS) Blue Dye solution
AST	Antibiotic Susceptibility Test
CLSI	Clinical and Laboratory Standards Institute
N.B	Nutrient broth
D.W	Distilled water
GN	Gentamicin
CAZ	Ceftazidime
CTX	Ceftriaxone

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