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RESEARCH ARTICLE

Antimicrobial Resistance Patterns and Metallobeta-lactamases Producing of Imipenem Resistant *Pseudomonas aeruginosa* Isolated from Clinical Samples of Iraqi Patients

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Abstract

In this study, a total of 160 clinical samples (72 burned skin, 25 wound, 18 sputum, 24 urine and 21 ear samples) were collected from five hospitals in Baghdad during the period December 2017 - April 2018. The samples were cultured on MacConkey agar and blood agar plates to isolate *Pseudomonas aeruginosa* in order to estimate its prevalence rate, which was 65% (104/160) of the total clinical samples. It was found that 37.5% (39/104) of *P. aeruginosa* isolates were resistant to imipenem (IRPA). A phenotypic detection of beta-lactamases showed that all IRPA isolates did not produce extended spectrum beta-lactamases, while 89.7% (35/39) of these isolates produced metallo-betalactamases (MBLs). Antibiotic susceptibility test against 11 antibiotics using the disk diffusion method showed that all metallo-betalactamases producing IRPA isolates were sensitive to polymyxin B, while resistance rates were 100% to both cefotaxime and ceftazidime, 94.3% to both netilmicin and amikacin, 91.4% to each of meropenem, gentamicin and tobramycin, 88.6% to cefepime, 82.9% to aztreonam and 77.1% to piperacillin/tazobactam. The resistance of metallo-betalactamase producing IRPA isolates for antibiotics according to MIC values was 100% to both tigecycline and cefazolin, 91.4% to meropenem, 85.7% to tobramycin, 80% to both cefepime and gentamicin, 71.4% to each ceftazidime, amikacin and levofloxacin, and 51.4% to ciprofloxacin.

Keywords: *Pseudomonas aeruginosa; Metallobeta-lactamases; and Antimicrobial Resistance.*

Introduction

Pseudomonas aeruginosa (Family: Pseudomonadaceae) is aerobic, gram-negative rods, ubiquitous environmental organism, nonlactose-fermenting, pyocyanin pigment producer [1].It is an opportunistic pathogen that causes a wide variety of human infections; including severe burn and wound infection, urinary tract infections, otitis media, and respiratory tract infection (pneumonia) and other infections [2].

P. aeruginosa nosocomial isolates are often resistant for different types of antibiotics and the resultant infections are difficult to be treated because of emergence of multidrug resistant (MDR) strains [3].Carbapenems are very important antibiotics used for the treatment of MDR *P. aeruginosa* infections [4] and imipenem is the first line of defense to treat *P. aeruginosa* infection [5]. However, prevalence of carbapenems-resistant *P. aeruginosa* has increased worldwide [6]. Metallobeta-lactamases (MBLs) are important clinical problems of concern to all societies in the world, because they are nearly able to hydrolyze and inactive all beta-lactam drug [7]. The first MBL from *Bacillus cereus* was reported in 1966 [8] and *P. aeruginosa* producing MBLs was first reported from Japan in 1991 and then spread to other countries of the world [9].

Carbapenems resistant *P. aeruginosa* has different mechanisms, which either carbapenemase production or loss of outer membrane porin (OPrD) and resistance-nodulation cell division (RND) and efflux pump system [10] and over production Ampc [4]. Production of MBLs is major defense in these bacteria against beta-lactam drug [11]. There are different MBL genes among carbapenems-resistant *P. aeruginosa* including *VIM*, *IMP*, *GIM*, *SPM*, *NDM* and *FIM*. These genes are carried by specific genetic elements including integrons, transposon, plasmids and chromosome

[12].The present study aimed to evaluate the prevalence rate of imipenem resistant *P. aeruginosa* in clinical specimens, their susceptibility to different antibiotic groups and their ability to produce metallo-beta-lactamases.

Material and Methods

Sample Collection

A total of 160 clinical samples were collected from burns, wounds, sputum, urinary tract infections and otitis media from five hospitals in Baghdad during the period December 2017 - April 2018. The samples were distributed as the following: 72 burn, 25 wound, 18 sputum, 24 urine and 21 ear samples.

Sample Processing

For urine, mid-stream samples were collected from patients using sterile container. The samples were inoculated on MacConkey agar and blood agar plates by streaking without flaming loop, and then they were incubated aerobically at 37°C for 24 hours [13]. In the case of burns, wounds, and ears (middle ear) swabs were collected and cultured similarly. Sputum samples were collected from patients using clean sterile containers, and then they were streaked on MacConkey agar and blood agar plates. The plates were incubated aerobically at 37°C for 24 hours.

Identification of Bacterial Isolates

The bacterial isolates were first identified according to cultural characteristics**.** Non lactose-fermenting colonies of growing bacterial isolates on MacConkey agar, ß-hemolysis colonies on blood agar, green color colonies on cetrimide agar and produce green-blue pigment on nutrient agar were observed. After that, Gram stained smears of young bacterial isolates were examined under oil immersion lens to observe cell morphology, cell arrangement and reaction with Gram-stain. Finally, **s**everal biochemical testes were used to identify the *P. aer*uginosa isolates. They included oxidase, catalase, IMViC tests, urease, glucose fermentation and H_2S production [14].

Imipenem Resistance P. aeruginosa (IRPA)

Screening for IRPA isolates among the total *P. aeruginosa* isolates was done primarily by Kirby Bauer disk diffusion method using imipenem disk (10µg), and then the isolates were further confirmed by the minimum inhibitory concentration (MIC) test.

Molecular Identification

Identification of IRPA isolates was confirmed by detection of specific *16SrDNA* gene using polymerase chain reaction (PCR) technique. Genomic DNA of IRPA isolates was extracted using a DNA extraction kit (Gene aid, Thailand) according to the manufacture instructions, and its purity and concentration was checked by a Nanodrop. The isolated genomic DNA was used as template to amplify product size of 956bp of 16S rDNA gene by using two primers: forward 5'-GGGGGATCTTCGGACCTCA-3' and reverse 5'-TCCTTAGAGTGCCCACCCG-3' [15].

Extended Spectrum Beta-lactamases (ESBLs) Production

All IRPA isolates (39) were tested phenotypically for ESBLs production by the double-disk synergy (DDS) test. The inoculum was adjusted to 0.5 tube McFarland standard. The swab was dipped into the bacterial isolate suspension and pressed against internal side of tube to remove excess fluid. Then, swab was cultured on the Muller-Hinton agar by evenly streaking across the surface. The antibiotic amoxicillin/clavulanic acid disk (20/10µg) was placed at the center of the Muller-Hinton agar plate, and then disks of ceftazidime, cefotaxime, aztreonam, and piperacillin were placed around the central amoxicillin/clavulanic acid disk at distances of 22-25 mm (center to center) from a disk containing amoxicillin/clavulanate. The plates were incubated at 37ºC aerobically for 18-24 hrs. The test was considered positive if the inhibition growth zone of any of the antibiotics was larger towards the amoxicillin/clavulanate disk [16].

Metallo - Betalactamases (MBLs) Production

All IRPA isolates (39) were tested phenotypically for MBLs production by the imipenem-EDTA combined-disk test. EDTA solution (0.5M) was prepared by dissolving 18.61 g of disodium EDTA in 100 ml of distilled water, the pH was adjusted to 8.0 using NaOH solution, and then the solution was sterilized by autoclaving. Test isolates were adjusted to the 0.5 tube McFarland standard and then were inoculated to Mueller Hinton agar plates. Two imipenem disks (10µg) were placed on the surface of

Mueller Hinton agar plates, and 10µl of 0.5M EDTA solution was added to one imipenem disk only.

Then the plates were incubated at 37ºC aerobically for 18-24 hours. The inhibition growth zones around the imipenem disk plus EDTA and imipenem disk without EDTA were compared. An increase of 7 mm or more in inhibition growth zone diameter around the imipenem-EDTA disk compared to the IMP disk was considered a positive test [17].

Antibiotic Sensitivity Test

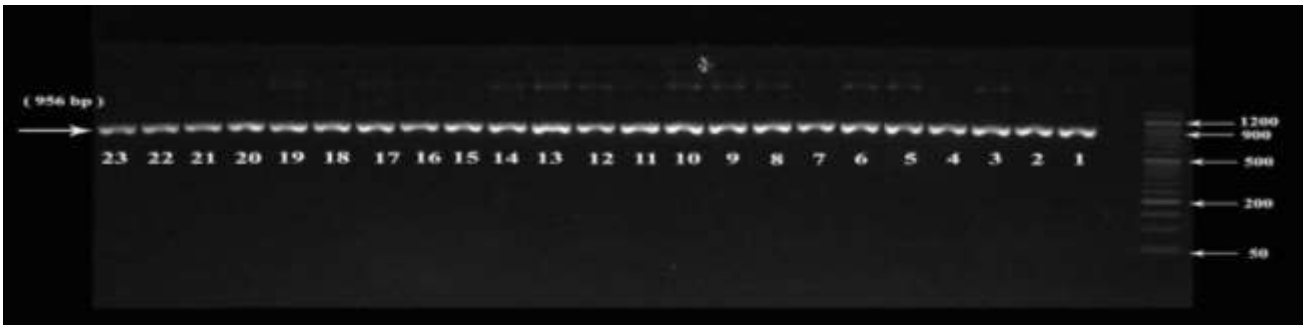
The sensitivity of MBLs producing isolates (35) against 11 different antibiotics was done by Kirby-Bauer disk diffusion method [18]. The used antibiotics were meropenem (10 µg), aztreonam (30µg), cefotaxime, (3oµg), ceftazidime (30µg), cefepime (30µg), amikacin (10µg), tobramycin (10µg), gentamicin (10µg), netilmicin (30µg), piperacillin/tazobactam (100/10µg) and polymyxin B (300µg). The inoculum of bacterial isolates suspensions was adjusted to 0.5 tube McFarland turbidity and cultured evenly on the surface of Muller-Hinton agar plates with sterile cotton swabs. Antibiotic disks were applied to the surface of inoculating plates and incubated aerobically at 37ºC for 24 hours. The growth inhibition zone diameters around the antibiotic disks were measured and compared to standard criteria in Clinical Laboratory Standards Institute [19].

Minimum Inhibitory Concentration (MIC) Test

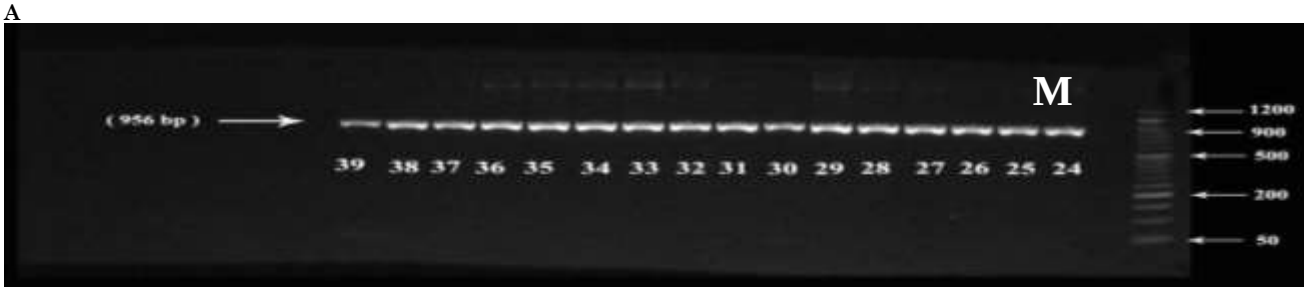
The MIC of MBLs producing isolates (35) against 10 antibiotics was detected by using a commercial kit (BioMerieux, France) as manufacture instructions. These antibiotics were cefazolin, ceftazidime, cefepime, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, levofloxacin and tigecycline.

Result and Discussion

Depending on cultural and morphological properties, microscopy and conventional biochemical testes, 104 (65%) isolates were identified as *P. aeruginosa* out of the 160 clinical samples. Depending on antibiotic sensitivity test and minimum inhibitory concentration (MIC), 37.5% (39/104) isolates were identified as imipenem resistant IRPA, and the MIC values of IRPA isolates were ≥ 16 µg/ml. All bacterial isolates were Gram-negative rods, non-spore forming, non-lactose fermenters, produced green-blue pigment on nutrient agar, green colored colonies on cetrimide agar, oxidase (+), catalase (+), Simmon`s citrate (+), methyl red (-), urease (+/-), indole (-), and Voges-Proskauer (-). Identification of IRPA isolates (39) was further confirmed by presence of specific 16*SrDNA* gene (956 bp). The results of polymerase chain reaction (PCR) technique showed that all isolates contain this gene which confirmed that all isolates belong to the species *P. aeruginosa*, as shown in Figure (1: A, B).



A



B

Figure (1A, and B): Gel-Electrophoresis of PCR products of 16SrDNA gene (956 bp) of IRPA isolates, Lane M: Ladder 50-1200 bp, Lane 1-39: positive IRPA isolates

The *16SrRNA* gene is used by most researchers to identify different clinical isolates of *P.* aeruginosa [20]. Marhoon *et al.* also used *16S rDNA* gene in the detection and differentiation of *P. aeruginosa* from *Pseudomonas* species and other pathogen genera from clinical and environmental samples [21].

Prevalence of IRPA

The results in table (1) shows that the prevalence rate of *P. aeruginosa* was 65% (104/160) of total clinical samples, and 39 (37.5%) isolates were resistant to imipenem (IRPA) out of 104 isolates. The IRPA distributed as 49.1% burned skin, 18.8% wounds, 27.3% sputum, 38.5% urine and 11.1% ear isolates (Table 1).

Table 1: Source and number (%) of clinical *P. aeruginosa* isolates

Isolation Source	No. of Total samples	*P. aeruginosa* isolates		IRPA Isolates	
		No.	%*	No.	%*
Skin burns	72	55	76.4	27	49.1
Wounds	25	16	64	3	18.8
Sputum	18	11	61.1	3	27.3
Urines	24	13	54.2	5	38.5
Ear swabs	21	9	42.9	1	11.1
Total	160	104	65	39	37.5

*The percentage is calculated according to isolate number of each source

In Iran, Neyestanaki *et al.* mentioned that rate isolate of IRPA from skin burns was 46.7% [22], and such finding is in agreement with the current study. In India, Qureshi and Bhatnagar reported that rate isolate of clinical IRPA accounted for 45.8% of investigated cases [23]. In Iraq, Khorsheed and Zain Al Abdeen reported that rate isolate of IRPA from burned skin accounted for 93% [24]; a finding that is not supported by the results of present study.

Beta-lactamases Tests

The results of phenotypic betalactamases detection showed that all IRPA isolates (39) did not produce extended spectrum beta-lactamases (ESBLs) by using double-disk synergy test (DDST), while 89.7% (35/39) of these isolates produced metallo-betalactamases (MBLs) by the IMP-EDTA combined disk test. The MBL producing *P. aeruginosa* were distributed as 96.3% burned skin, 100% wound, 100% sputum, and 60% urine isolates (Table 2 and Figure 2). We believe that most bacteria that produce MBLs do not produce ESBLs. Al-charrakh revealed that rate of IRPA metallo-betalactamase producing isolates account for 66.7% [25].

Ellappan *et al.* found that 37.2% of the CARPA isolates were phenotypically positive for MBL producing [10], while Fallah *et al.* showed that 57.9% was metallo-beta-lactamase producers [26].

Table 2: Number and percentage (%) IRPA MBLs producing isolates

Source of isolates	No. of isolates	MBLs producers	
		No. of isolates	*%
Skin burns	27	26	96.3
Wounds	3	3	100
Sputum	3	3	100
Urine	5	3	60
Ear	1	-	-
Total	39	35	89.7

*The percentage is calculated according to isolates number of each source

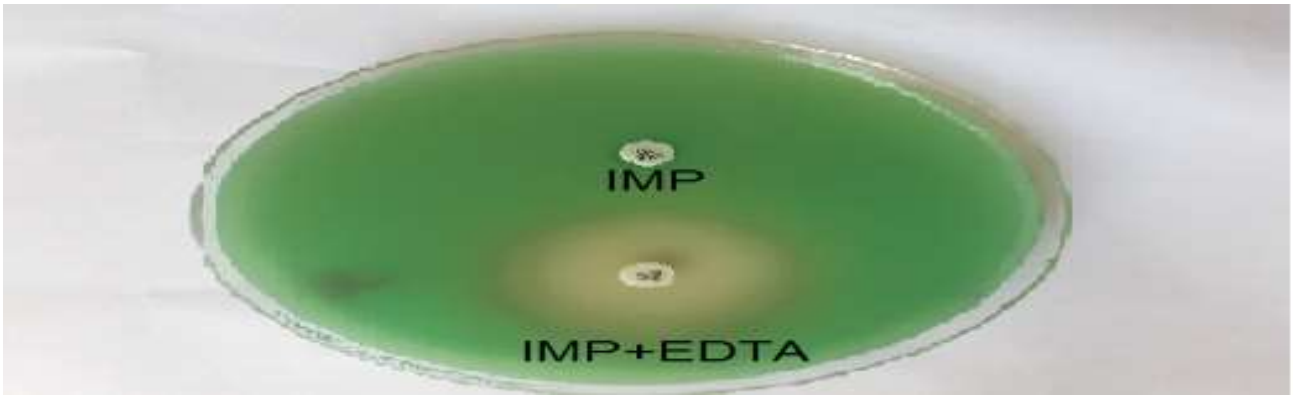


Figure 2: MBL-producing IRPA isolate by Imipenem-EDTA combined-disc test

Antibiotic Susceptibility Test

The percentage of antibiotic resistance in MBL producing IRPA isolates (35) are shown in table 3. Based on these data, all isolates were sensitive to polymyxin B2. This is in agreement with Sood who mentioned that MBL producing *P. aeruginosa* isolates were 100% sensitive to polymyxin B [27]. The result of antibiotic susceptibility also showed that MBL producing isolates were 100% resistant for cefotaxime and ceftazidime, 94.3% for netilmicin and amikacin, 91.4% for meropenem, gentamicin and tobramycin, 88.6% for cefepime, 82.9% for aztreonam and 77.1% for piperacillin/tazobactam. These findings are not in agreement with Abaza *et al*., who reported that the resistance rates of MBL producing *P. aeruginosa* isolates were 60% ceftazidime, 70% cefepime, 66% meropenem, 73.3% aztreonam, 66.6% gentamicin, 86.7% amikacin, and 13.3% polymyxin B [28]. Acharya *et al*. mentioned that the rates of resistance in MBL producing *P. aeruginosa* isolates were 100% for ceftazidime, cefepime and meropenem, 91.7 gentamicin, 83.4% Amikacin, and 37.5% aztreonam [29]. While Zafer *et al*. revealed that the rates of resistance in MBL producing *P. aeruginosa* isolates were 100% resistant to meropenem, 93.9% ceftazidime, 81.8% amikacin, 84.8% gentamicin and 6% polymyxin B [30].

Table 3: Percentage of antimicrobial susceptibility of MBLs producing IRPA isolates

Antibiotics (µg/disc)	MBLs producing IRPA isolates (n=35)	
	Resistant (%)	Sensitive (%)
Piperacillin / Tazobactam (10/100)	27(77.1)	8 (22.9)
Cefotaxime (30)	35(100)	0.0 (0.0)
Cefepime (30)	31 (88.6)	4 (11.4)
Ceftazidime(30)	35 (100)	0.0 (0.0)
Aztreonam (30)	29 (82.9)	6 (17.1)
Meropenem(10)	32 (91.4)	3 (8.6)
Polymyxin B(300)	0.0 (0.0)	35 (100)
Amikacin (30)	33 (94.3)	2 (5.7)
Netilmicin(30)	33 (94.3)	2 (5.7)
Gentamicin (10)	32 (91.4)	3 (8.6)
Tobramycin(10)	32 (91.4)	3 (8.6)

Minimum Inhibitory Concentration (MIC) Test

MIC is the lowest antibiotic concentration that prevents growth of microorganism, and it is used to confirm the results obtained by disk diffusion method. The current study used MIC test to detect the susceptibility of MBL producing isolates toward six tested and four untested antibiotics to give a real picture for susceptibility of this microorganism to these antibiotics. The MIC of 10 antibiotics against IRPA isolates produced MBLs were done by using a commercial kit. The results of MIC values in table 4 showed high resistance rates of the antibiotics toward IRPA isolates produced MBLs, and they were 100% tigecycline and cefazolin with MICs values ≥64 and ≥8 µg/ml respectively, 91.4% meropenem with MICs values ≥16 µg/ml, 85.7% tobramycin with MICs values ≥16 µg/ml, 80% gentamicin and cefepime MICs values ≥ 16 µg/ml, 71.4% amikacin, levofloxacin and ceftazidime with MICs values ≥64 , ≥ 8 and 32 - ≥ 64 µg/ml respectively, and 51.4% ciprofloxacin with MICs values ≥4 µg/ml.

Table 4: The minimum inhibitory concentration (MIC) values of antibiotics against IRPA isolates producing MBLs

Antibiotics	MBLs producing IRPA isolates by Vitek2 system (n=35)					
	Resistant (R)		Intermediate (I)		Sensitive (S)	
	No. (%)	MIC µg/ml	No. (%)	MIC µg/ml	No .(%)	MIC µg/ml
Cefazolin	35(100)	≥64	-	-	-	-
Ceftazidime	25(71.4)	32-≥64	5(14.3)	>16	5(14.3)	4
Cefepime	28(80)	≥64	2(5.7)	8	5(14.3)	2-4
Meropenem	32(91.4)	≥16	3(8.6)	4	-	-
Amikacin	25(71.4)	≥64	4(11.4)	23	7(20)	≤2
Gentamicin	28(80)	≥16	1(2.9)	8	5(14.3)	≤1
Tobramycin	30(85.7)	≥16	-	-	5(14.3)	≤1
Ciprofloxacin	18(51.4)	≥4	7(20)	2	11(31.4)	0.5≤0.25
Levofloxacin	25(71.4)	≥8	5(14.3)	0.25-4	(14.3) 5	0.25-2
Tigecycline	35(100)	≥8	-	-	-	-

Franco *et al*. mentioned that the rates of resistance in MBL producing *P. aeruginosa* isolates were 100% to imipenem, meropenem, cefepime, gentamicin and tobramycin [31]. Rajabnia *et al*. reported that all MBL-producing *P. aeruginosa* strains were 100%

resistant to cefepime, ceftazidime, cefotaxime and imipenem [32]. Akhi *et al.* found that MBL producing *P. aeruginosa* isolates were 100% resistant to imipenem, meropenem, ceftazidime, cefepime, 95.45% to ciprofloxacin and 9.09% to amikacin [4]. In Table 5, IRPA metallo-betalactamases producing isolates showed six antibiotypes (six different patterns of resistance to 10 different antibiotics). The commonest antibiotype was antibiotype 1 (24 isolates) with resistance to all tested antibiotics except polymyxin B, antibiotype 2 (5 isolates) with susceptibility to both polymyxin B and piperacillin /tazobactam, antibiotypes 3 (2 isolates) with susceptibility to polymyxin B and aztreonam, antibiotypes 4 (2 isolates) with resistance to cefotaxime and ceftazidime, antibiotype 5 (1 isolate) with resistance to netilmicin, amikacin and ceftazidime, antibiotype 6 (1 isolate) with resistance to polymyxin B, piperacillin /tazobactam and aztreonam, cefepime.

Table 5: Resistance profiles of IRPA producing MBLs isolates (antibiotypes)

Antibiotype	NET	CN	PB	PTZ	ATM	CPM	CTX	MEM	AK	TOB	CAZ	No. of isolates (%)
Antibiotype1	R	R	S	R	R	R	R	R	R	R	R	24(68.6%)
Antibiotype2	R	R	S	S	R	R	R	R	R	R	R	5(14.3%)
Antibiotype3	R	R	S	R	S	R	R	R	R	R	R	2(5.7%)
Antibiotype4	S	S	S	S	S	S	R	S	S	S	R	2(5.7%)
Antibiotype5	R	S	S	S	S	S	R	S	R	S	R	1(2.9%)
Antibiotype6	R	R	S	S	S	S	R	R	R	R	R	1(2.9%)

R: Resistant, S: Sensitive, NET: Netilmicin, CN: Gentamicin, PB: Polymyxin B, PTZ: Piperacillin/Tazobactam, ATM: Aztreonam, CPM: Cefepime, CTX: Cefotaxime, MEM: Meropenem, AK: Amikacin. TOB: Tobramycin, CAZ: Ceftazidime

Conclusion

Prevalence rate of IRPA among clinical *P. aeruginosa* isolates was high (37.5% %). Metallo-b-lactamases producing among IRPA was detected in 89.7% (53/39) of IRPA isolates. All metallo-betalactamase producing IRPA isolates had multidrug resistance wit high resistance to all tested antibiotics except polymyxin B, which were sensitive.

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