ISSN 0975 -8542

Journal of Global Pharma Technology

Available Online at www.jgpt.co.in

RESEARCH ARTICLE

Molecular Analysis of Genetic Elements Responsible for XDR in Highly Successful Pathogen *Acinetobacter Baumannii* Isolated from Clinical Samples of Iraqi Patients

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Abstract

Objective(s): *A. baumannii* infections have gained attention due to the high number of infected soldiers serving in Iraq and Afghanistan. Current study focus on the phenotypic and genotypic detection of antibiotic resistant genes among Iraqi *A. baumannii* isolates to classify them into appropriate antibiotic resistant level.Material and methods: Antibiotic resistant genes were screened among 24 *A. baumannii* isolates collected from Baghdad and Al- Najaf hospitals using different phenotypic methods. Genotypic detection was done using PCR. Results: All isolates showed positive results to *recA* gene at 425bp. Antimicrobial susceptibility testing revealed 100% resistance towards Ceftazidime, Amoxicillin-clavulanic acid, Ticarcillin-clavulanic acid, and Cefepime. Also, 50% isolates were resistant to Colistin sulphate, while 66.7% isolates were resistant to tigecycline. The MIC test for polymyxin B revealed that 83.3% isolates were resistant to >32µg/ml. Results showed 16.7% of *A. baumannii* isolates was MDR, 70.8% was XDR, and 12.5% of isolates was PDR. Also, 87.5% of isolates were carbapenemase producers; while 33.3% were ESBLs producers. PCR studies showed among 22 antibiotic resistant genes tested, there was 100% and 70.8% for *aac(6)-Ib, ant(4)IIb-,* and *aac*(3)-I, respectively. Also, 13% of the isolates were harbored *mexX* gene. Results showed 50% had *rmtD* followed by *rmtA* and *rmtF* 45.83%. Some of the isolates encode *ParC2* and *GyrA2* (75% and 91.67%, respectively). The *OXA 23* and *OXA 51* were detected among 29.17% and 75%, respectively. Conclusion: Current study found most *A. baumannii* isolates were MDR, XDR, and even PDR pathogens among wound infected patients in Baghdad and Al-Najaf hospitals.

Keywords: *ESBLs, Modified Hodge test, carbapenemase, Pan drug resistant (PDR), multiple resistant (MDR), extremely resistant (XDR).*

Introduction

A. baumannii is a well-known pathogen that causes a variety of diseases such as pneumonia, bacteraemia, urinary tract infections, wound infections and meningitis (1,2), and also it is commonly associated with skin colonization of hospitalized patients, leading to serious infections (3, 4). The ability of this bacterium to survive for long periods on inanimate surfaces and its extensive drug resistance make it a successful microorganism that is able to cause outbreaks (5, 6). *A. baumannii* infections have also gained attention due to the high number of soldiers serving in Iraq and Afghanistan that were infected with this bacterium. Molecular genetic studies of *A. baumannii* clinical strains have often been limited because they are usually resistant to most antibiotics (7, 8). MDR and XDR *A. baumannii* has been increasing worldwide as a result of the combination of two main factors which are high level of genomic plasticity and mutation of endogenous genes (9). Often, MDR pathogens could be resistant towards 3-5 antibiotic classes such as antipseudomonal

cephalosporins, antipseudomonal carbapenems, ß-lactam–ß-lactamase inhibitor, fluoroquinolones, and aminoglycosides, while XDR pathogens are susceptibility to only 2 or less antibiotic classes (10). The alteration of genes are associated with antimicrobial resistance, such as overexpression of the chromosomally encoded ADC ß-lactamase (AmpC) and the OXA-51-like ß-lactamase (9), acquisition of plasmids, transposons, and integrons harboring different antibiotic resistance genes (11), production of aminoglycoside-modifying enzymes, ESBLs, and carbapenemases, in addition to target-site alteration such as changes in outer membrane proteins, penicillin binding proteins, over expression of efflux systems, and topoisomerases (12,13).

There are currently very few reports on the clinical outcome of patients suffering from infection caused by PDR *A. baumannii*, leading to high mortality rate (14). Pan drug resistant PDR pathogens have no options for treatment as they diminished susceptibility to all classes, including polymyxins (10). Iraqi bacter isolates are extremely difficult to treat and pose considerable infection control issues (15); therefore, we focus in our study on the phenotypic and genotypic detection of antibiotic resistant genes among Iraqi *A. baumannii* isolates in order to classify them into multi-, extremely-, or pan drug pathogens.

Material and Methods

Collection and diagnosis of Bacterial isolates: Twenty four isolates of *A. baumannii* were obtained from patients admitted to several teaching hospitals in Baghdad and AL- Najaf during a period between March and September 2015. All isolates were collected from wounds infections. Bacterial diagnosis was performed depending on rapid morphological tests followed by identification using complementary Vitek 2 compact automated system (Biomeriux, USA), and the probability was 99% *Acinetobacter baumannii.*

Genotyping Detection for Isolates: The primers were designed using Geneious Software/ primer 3. Bacteria were diagnosed on the genetic level using amplified housekeeping gene *rec A*. The amplified size was 425 bp from the origin gene sequence F-CCTGAATCTTCYGGTAAAAC; R-GTTTCTGGGCTGCCAAACATTAC.

Total DNA was extracted using Genomic DNA Extraction Kit (Wizards, Promega, USA) following manufacture's protocol for Gram – negative bacteria from overnight bacterial growth. The extract was used as a DNA template for the PCR process. The mixture of PCR is composed from 12.5 of GoTaq®Green Master Mix (2x), 5 µl template DNA, 1.5 µl primers (for each) final concentration (0.6pmol/µl), and nuclease free water up to 25 µl (4.5 µl). Uniplex PCR was stored at -20 ºC, then nucleotides sequence was carried out at NICEM Company, USA. Results of sequencing were analyzed using genious software 7.01R compareid with the NCBI standered strain.

Antimicrobial Sensitivity Tests: Resistance tested for the isolates towards 22 antibiotic agents related to ten categories including penecillins, *β*- Lactam/ *β*-Lactamase, cephalosporines, aminoglycosides, carbapenem, tetracyclines, sulfa drug, Glycylcycline, polypeptide, and quinolones groups. Susceptibility was determined based on the interpretative criteria recommended by the Clinical and Laboratory Standards Institute (CLSI) (16). The antibiotics were: from ß- Lactams, Piperacillin (100µg), Mezlocillin (75µg), Ticarcillin (75µg), Cefotaxime (30µg), Ceftriaxone (30µg), Ceftazidime (30µg), Cefepime (30µg), Amoxicillin/clavulanic acid (20/10µg), Ticarcillin-clavulanic acid (75/10µg), Ampicillin-sulbactam (10/10 µg), Imipenem (10µg), and Meropenem (10µg). Aminoglycoside group: Gentamycin (10µg), Tobramycin (10µg), and Amikacin (10µg).

The quinolone group includes Levofloxacin (5µg) and Gatifloxacin (5µg). Other antibiotics include sulfa group: Trimethoprime-Sulphamethoxazole (1.25/23.75µg), and Tetracyclines: Tetracycline (30µg) and Doxycycline (30µg). Glycylcycline: Tigecycline (15µg). Polypeptide colistin sulphate (25µg) and Polymyxin B was also used however, to achieve the minimum inhibitory concentration (break point 2µg/ml) (17). *E.*

coli HB101 used as the negative control strain. Isolates classified into MDR, XDR, and PDR according to the following criteria revealed by Magiorakos *et al.* for *A. baumannii*: **MDR:** non-susceptible to ≥1 agent in ≥3 antimicrobial categories; and its therapeutic option is carbapenems and polymyxins (E and B). **XDR:** non-susceptible to ≥1 agent in ≥ 8 of the 10 antimicrobial categories but still otherwise sensitive to polymyxins (E and B) and tigecycline. **PDR:** non-susceptible to all antimicrobial agents, and there is no therapeutic options have been revealed yet (18). It should be noted that even the isolate could resist ≥1 agents in one category; it would be considered resistant isolate to that category (18).

Indirect three dimensional agar diffusion method to detect ESBLs: The detection of ESBLs production was performed using modified indirect three dimensional method in which Muller Hinton Agar (MHA) plates were seeded with a lawn of a standard strain *E.coli*HB101 adjusted to Mac Farland (0.5) standard .A slit was performed inside the MHA plate using a sharp scalpel emerged with the test isolate. The following discs were placed 2mm away towards the performed line. They were amoxicillin/clavulanic acid (20/10µg), ceftazidime (30 µg), ceftriaxone (30 µg), and cefexime (5µg). The plate was incubated at 37ºC for16-18 hours. The distortion of inhibition zone around the antibiotic indicates ESBLs production (19).

Modified Hodge test (MHT): Modified Hodge test (MHT) was performed according to Bonnin *et al.* for detection of carbapenemase production (20). Briefly, 5 ml of brain heart infusion broth culture for *Escherichia coli* HB101 equal to 0.5 McFarled was prepared then 100µl from 1:10 dilution was streaked as lawn on to a Mueller Hinton agar plate. Imipenem susceptibility disk (10µg/disc) was placed in the center of the test area. The test isolate was streaked in a straight line from the edge of the disk to the edge of the plate. The plate was incubated overnight at 37º C for 24 hours. After 24 hours, MHT positive test (carbapenemase producer) showed a clover leaf-like indentation of the *Escherichia coli* 25922 growing along the test organism growth streak within the disk diffusion zone. MHT negative test represent by normal growth for the standard strain or without the formation of clover leaf-like shape.

Detection of antibiotic resistant genes using PCR amplification technique: All the twenty four isolates were subjected to molecular screening study using PCR amplification technique to detect XDR profile. Different primers (table 1) were used. PCR mixture was composed from 5µl template DNA which was extracted using Genomic DNA Extraction Kit (Wizards, Promega, USA), 12.5µl of GoTaq®Green Master Mix, 1.5µl from forward and reverse primers (final concentration 0.6pmol\ µl).

The volume was completed to 25µl with nuclease free water. PCR was run under the following conditions: primary denaturation step 95°Cfor 5 min; 30 -40 repeated cycles (according to gene listed in table 2) of 94°C for 30sec , 43- 62 °C annealing temperature for 60 sec and 72°C for 1 min then final extension step at 72 °C for 6 min. PCR products were electrophoresed in 1% agarose gel and visualized under UV light (21).

Table 1: Primers used for detection of antibiotic resistant genes

Primer	Sequences (5'_3') F	Sequences (5'_3') R	Size product	T m	Ref.
RecA	F- CCTGAATCTTCYGGTAAAAC	R- GTTTCTGGGCTGCCAAACATTAC	425	54	Bartual, *et al.*(3)
MexX	F- TGA AGG CGG CCC TGG ACA TCA GC	R- GAT CTG CTC GAC GCG GGT CAG CG	326	62	Lianes *et al.* (22)
MexY	F- CCGCTACAACGGCTATCCCT	R- AGCGGGATCGACCAGCTTTC	250	59	Xavier, *et al.* (23)
aac(3)-I	F-AGCCCGCATGGATTTGA	R- GGCATACGGGAAGAAGT	227	43	Ndegwa *et al.* (24)
aac(6)-	F- TTG CGA TGC TCT ATG AGT GGC TA	R- CTC GAA TGC CTG GCG TGT TT	482	56	Haldorsen

Ib-					*et al.* (25)
ant(4')Ib-	FGACGACGACAAGGATATGGAATTGCCCAATATTATT	RGGAACAAGACCCGTTCAATTCAATTCATCAAGTTT	364	57	Haldorsen *et al.*(25)
aph(3') VI	F-TAT CTC GGC GGC GGT CGA GT	R- CAC GCG GGG AAA CGC GAG AA	800	55	Vaziri *et al.* (26)
bla$_{AmpC}$	F-ATGCAACAACGACAATCCATC	R-GTTGGGGTAGTTGCGATTGG	1150	58	Oliveira *et al.* (27)
IMP-1	F-CTACGCCAGCAGAGTCTTTG	R-AACCAGTTTTGCCTTACCAT	500	55	Ramazanzadeh *et. al.*(28)
VIM	F-GTTTGGTCGCATATCGCAAC	R-AATGCGCAGCACCAGGATAG	382	57	Mendes *et al.*(29)
rmt A	F-CTA GCG TCC ATC CTT TCC TC	R-TTG CTT CCA TGC CCT TGC C	635	58	Vaziri *et. al..(26)*
rmtB	F-CCC AAA CAG ACC GTA GAG GC	R-CTC AAA CTC GGC GGG CAA GC	584	584	Lee *et al.* (30)
rmtC	F-CGA AGA AGT AAC AGC CAA AG	R-ATC CCA ACA TCT CTC CCA CT	711	53	Doi *et al.*(31)
rmtD	F-TCAAAAAGGAAAAGGACGTG	R-CGATGCGACGATCCATTC	500	52	Tijet *et al.* (32)
rmtF	F-GCGATACAGAAAACCGAAGG	R-GGCAGGAGCTTCATCAGAA	453	51	Morovat *et. al.*(33)
OXA 51	F 5'-TAATGCTTTGATCGGCCTTG-3'	R 5'-TGGATTGCACTTCATCTTGG-3'	353	53	Morovat *et. al.*(33)
OXA58	F 5'-AAGTATTGGGGCTTGTGCTG-3	R 5'-CCCCTTGCGCTCTACATAC-3	599	53	Morovat *et. al.*(33)
OXA23	F 5'-GATCGGATTGGAGAACCAGA-3	R 5'-ATTTCTGACCGCATTTCCAT-3	501	53	Morovat *et. al.*(33)
OXA24	F 5'-GGTTAGTTGGCCCCCTTAAA-3'	R 5'-AGTTGAGCGAAAAGGGGATT-3	246	53	Morovat *et. al.*, 2009
gyr A1	F-GGCATTTATTCGTCACCGCC	R-TTCCGGGAACTCGGTTAACG	886	57.2	This study
gyr A2	F-CGACAGCTTCTGGTACGGTT	R-ACTGCACCAACTAGCTCACC	499	57.2	This study
parC1	F-CGCTTCGGCCTGTATCTCAT	R-GTTACCGTATGCGAGCGGTA	536	57	This study
par C2	F-GTTACCGTATGCGAGCGGTA	R-TGATTTCACCTGAGGACGGC	314	57	This study

Results

Molecular Diagnosis of local *A. Baumannii* Isolates

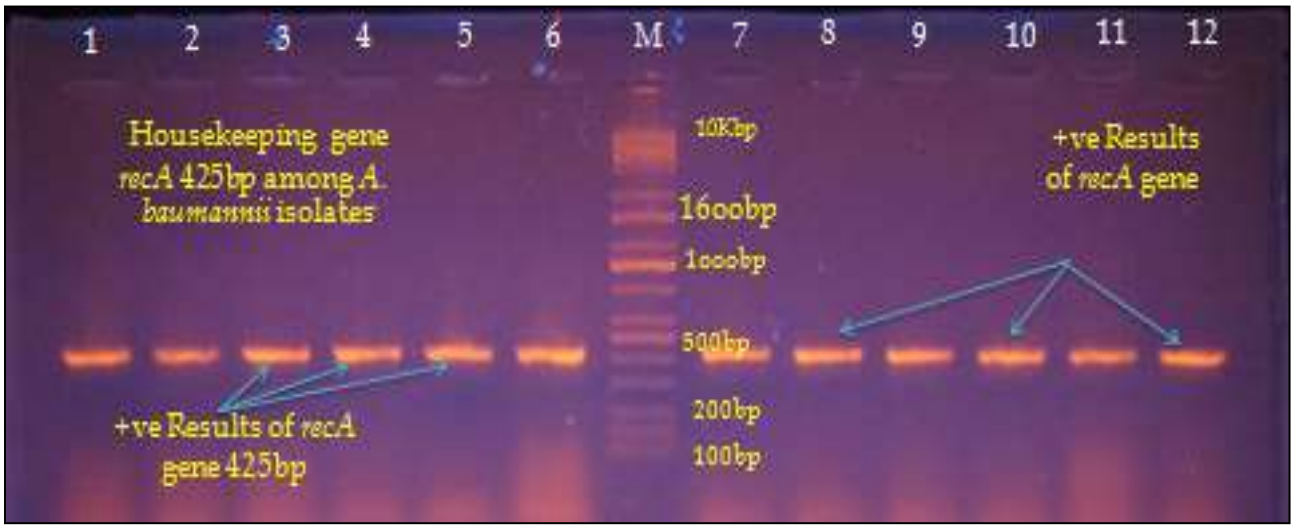


Figure 1 A: Agarose gel electrophoresis (1% agarose, 5 V/cm for 120min) for *recA* gene (amplified size 425bp) of *A. baumannii* obtained from wound infections as compared with (100pb) DNA ladder lane (M).

Results exhibited that 24/24 (100%) isolates were *A. baumannii* using housekeeping gene *recA* with amplified size of 425bp. Figure (1A) illustrated shine bands of positive isolates compared with DNA ladder of (100pb). The amplified DNA segment for *recA* gene was analyzed by Genoius Software as illustarted in figure (1B). It was clear that amplified DNA segment compared to the standered strain *A. baumannii* NBRC: LC014650 (NCBI) and the refference source. Pariwise identity was (98%) which represent the percentage of residues that are identical in the alignment.

Some differences were recognized between the local isolate and the recorded NCBI strain as clear with gaps in the upper green identity line. Data obtained from sequence were submitted to gene bank National center for Biotechnology information (NCBI) and recorded under the accession number (KX244958).

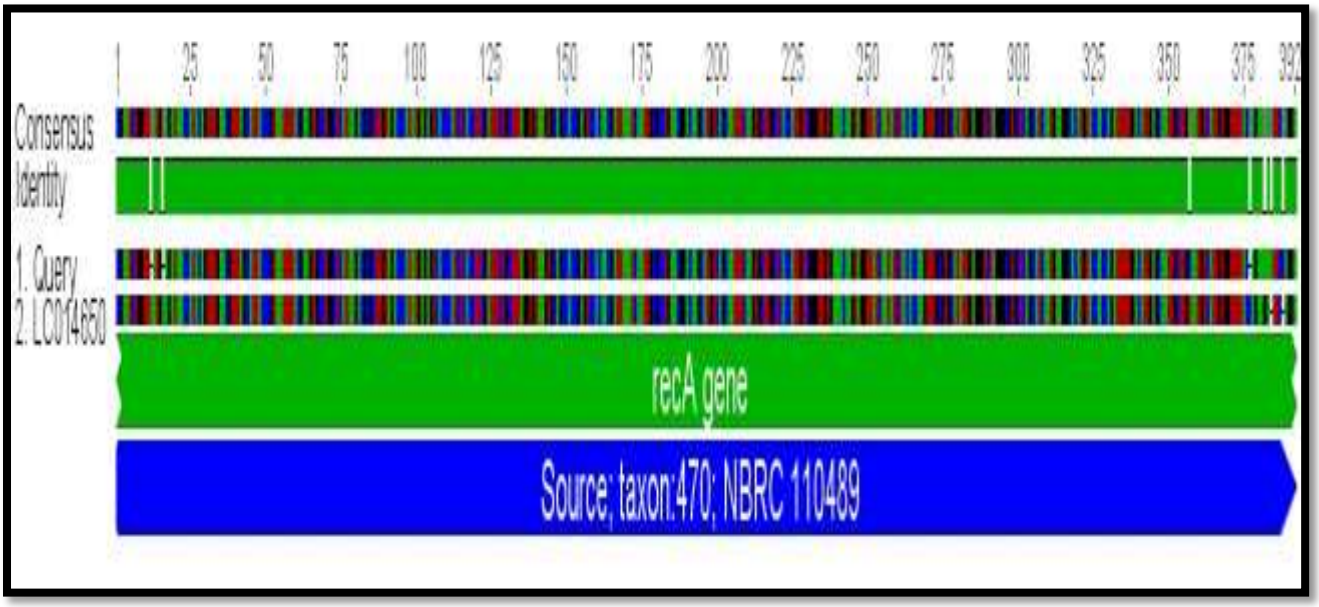


Figure 1 B: Pariwise identity and nucleotide sequence for *recA* gene (amplified size 425bp) as compared with the standered strain *A. baumannii* NBRC: LC014650 (NCBI).

Phenotypic Detection of Antibiotic Resistant Genes

Antimicrobial susceptibility Test: Rates of full resistance for the 24 local isolates were as follows (figure3): first, penicillin group such as 83.3% for each ticarcillin and mezocillin, 66.7% for piperacillin ß-lactams such as 100% for each ticarcillin-clavulante and amoxicillin/clavulanic acid. Second, cephalosporins group such as 91.7% for cefotaxime and 100% for each ceftazidime, ceftriaxone, and cefepime.

Third, resistant profile to carbapenem group was 91.7% for Imipenem and 41.6% for Meropenem. Forth, there were resistant isolates among *A. baumannii* towards aminoglycoside group including gentamycin, amikacin, and tobramycin (95.8%, 62.5%, 62.5%, respectively). Also, there was 37.5% resistance for tetracycline and 16.7% for doxycycline.

In addition, results showed 91.7% resistance to gatifloxacin and 83.3% to levofloxacin. Also, trimethoprime-sulfamethoxazole was non-effective among (87.5%) of *A. baumannii* isolates. It was found that 50% were resistant to colistin sulphate, while 66.7% isolates were tigecycline resistant.

Finally, minimum inhibitory concentration test (MIC) of Polymyxin B revealed that 83.3% isolates were resistance to 32 µg/ml (break point ≥2 µg/ml), and the only *A. baumannii* W13, W16, W17, and W19 were sensitive to polymyxin B, and they showed no growth at 2 µg/ml of this antibiotic. The number of resisted antimicrobial agents by each isolate presented in figure (4). Results also demonstrated that 4/24 (16.7%) of *A. baumannii* were classified as MDR. There was also 17/24(70.8%) of *A. baumannii* isolates were considered as XDR. Pan drug resistant (PDR) was only detected in 3/24(12.5%) of isolates (figure5A and B).

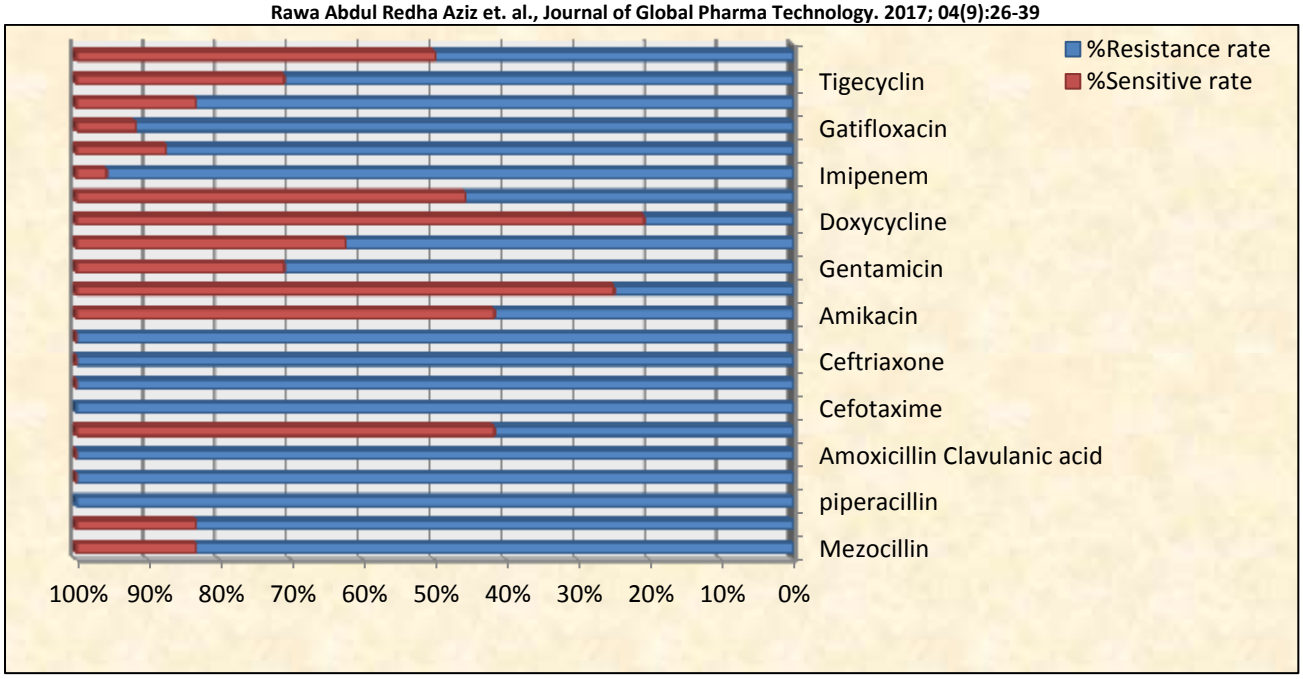


Figure 3: The percentages of resistance and sensitivity rates among 24 *A. baumannii* collected from wound infections towards 22 antimicrobial agents tested.

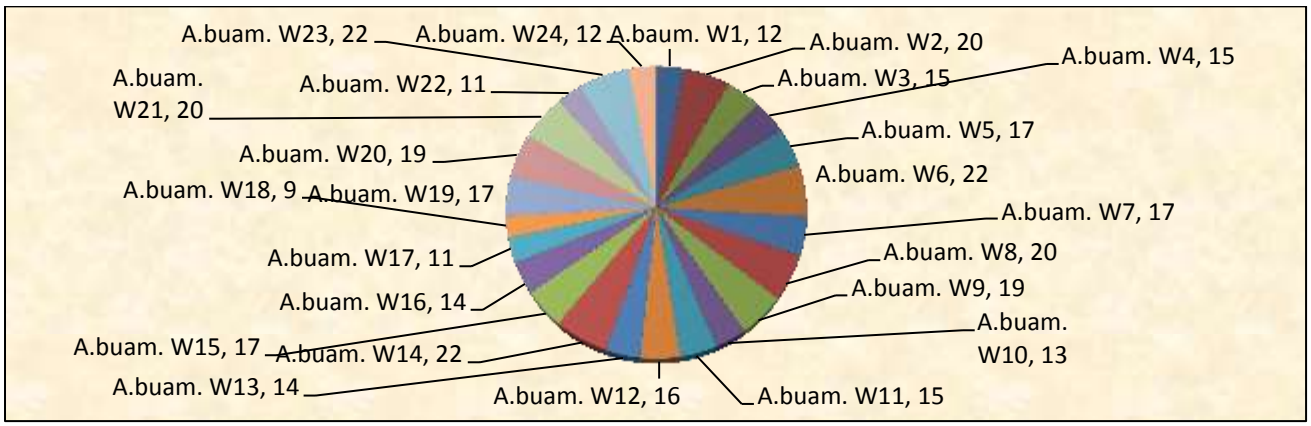
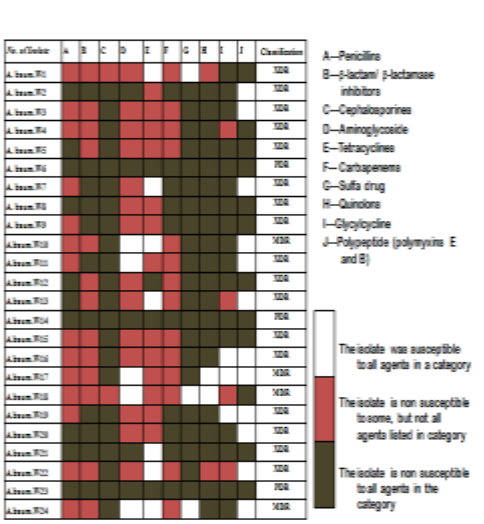
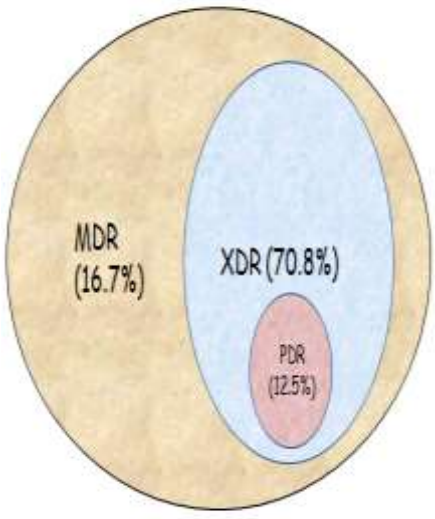


Figure 4: The number of resisted antimicrobial agents for each A. baumannii isolates. Results obtained by Kirby Bauer disk diffusion method in current study.



(A)



(B)

Figure (5): A) Classification of 24 *A. baumannii* isolates into three antibiotic resistance level (MDR, XDR, and PDR) by calculating their antimicrobial susceptibility patterns toward ten antibiotic categories tested in current study according to criteria revealed by Magiorakos *et al.* (2012); B) The percentages of MDR, XDR, and PDR levels among 24 *A. baumannii* isolates collected from wound infections this in study.

Figure 6: Carbapenemase production by *A. baumannii* W14 on MH agar that had a lawn of standard strain HB101 and an imipenem disc (10µg/disc) in the center of the plate.

Modified Hodge's Test: Results showed that 87.3% of *A. baumannii* isolates were positive for carbapenemase production using modified Hodge's test. Figure (6) represented the clover leaf like shape that positive isolate showed on Muller Hinton agar containing lawn of standard strain HB101.

Results demonstrated that highly carbapenem resistant rate detected among 24 *A. baumannii* isolates in antimicrobial test was mediated by carbapenemase production.

Indirect Three Dimensional Agar Diffusion Method: Results conducted via this test showed inhibition zone around the antibiotic disc passed the line of tested isolate which indicates ESBLs production among only 8/24 (33.3%) of *A. baumannii* isolates. Of those eight ESBLs producer isolates, 8/8 (100%) were resistant to each of ceftazidime (CAZ) and cefepime (CFM), 7/8 (87.5%) isolates were resistant to amoxicillin/ clavulanic acid (AMC), and 6/8 (75%) isolates were ceftriaxone (CRO) resistant.

Detection of Antibiotic Resistant Genes using PCR Amplification Technique

Resistance to β-Lactam Antibiotics

PCR analysis showed that no sign was detected for ß-lactamase genes including (*bla*$_{SHV}$, *bla*$_{IMP-1}$, *bla*$_{VIM}$, *bla* $_{AMP-C}$, and *bla* $_{CMY}$) among tested isolates, However, there was 7/24 (29.2%) of *A. baumannii* isolates could harbor *blaOXA23* gene at amplified size of 501bp and 18/24 (75%) of *A. baumannii* isolates had *blaOXA51* gene at amplified size of 353bp (figure7), and none of *A. bauamnnii* isolates had *blaOXA24 and blaOXA58.*

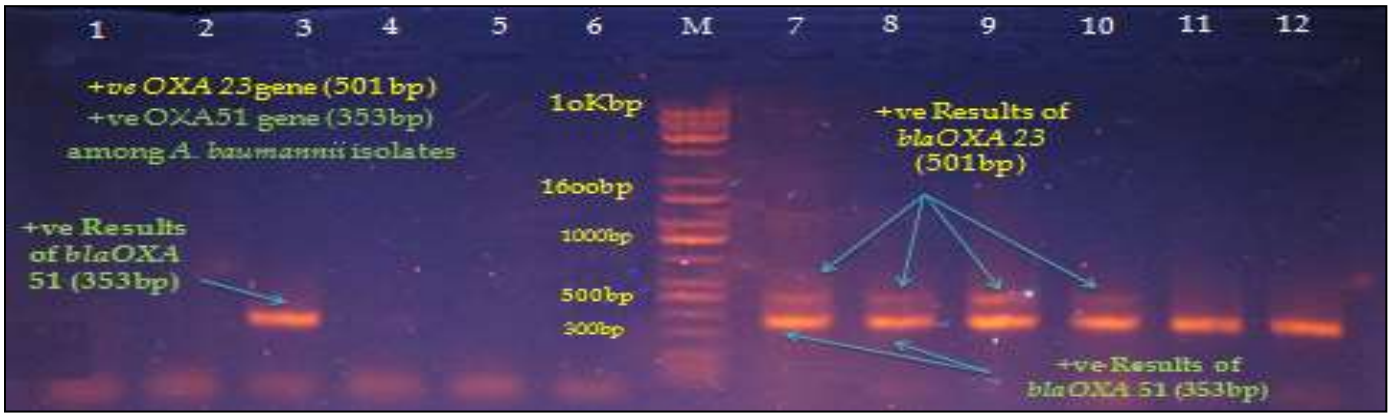


Figure (7): Agarose gel electrophoreses (1% agarose, 50 V/cm for 120min) for PCR multiplex *blaOXA*23 at amplified size of 501bp and *blaOXA*51 at amplified size of 353bp. Lanes 3, 7, 8, 9, 10, 11, and 12 were positive results with 353bp *blaOXA51*gene. Lanes 7, 8, 9, and 10 were positive results with 501bp *OXA23* compared with 100bp DNA ladder lane M. Lanes 1, 2, 4, 5, and 6 showed negative results.

Resistance to Aminoglycoside

Aminoglycoside Modifying Enzymes (AMEs): Four AMEs genes included in PCR study were present singly or in combination among isolates.

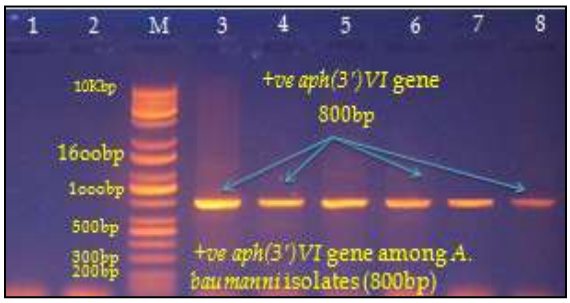
Results exhibited 24/24 (100%) of *A. baumannii* isolates could harbor *aac(6)-Ib* gene at amplified size of 482bp (figure 8 A) and *ant(4')IIb* gene at amplified size of

364bp (figure 8B). Gene *aac(3)-I* was also detected; however among 17/24 (70.8%) of *A. baumannii* isolates at amplified size of 227bp (figure 8C). It was also found that 11/24(45.8%) of the isolates had amikacin resistance gene *aph*(3)-VI when amplified at 800bp (figure 8D).

Efflux Pump Genes: MexXY pump which is related to resistance-nodulation-division (RND) family was detected among 24 *A. baumannii* isolates in current study. PCR data showed that none of *A. baumannii* isolates had the effflux pump *mexY* gene; however they could exhibit *mexX* gene among only 3/24(13%) of isolates at amplified size of (326bp) (figure8E).

16s Ribosomal Methylation Enzymes: *A. baumannii* isolates had 16s ribosomal methylation enzymes related to different genes tested in current study singly or in combination in each isolate. PCR study showed that there was 12/24 (50%) of *A. baumannii* had *rmtD* presented in clear band of 500bp on 1% agarose gel (figure 8F).

There was also 11/24 (45.8%) of the isolates harbored *rmtA* and *rmtF* at amplified size of 635bp (figure 8G) and 453bp (figure 8H), respectively. Gene *rmtB* was also distributed among 10/24 (41.7%) of *A. baumannii* isolates when amplified its size at 584pb (figure 8I). Last gene tested *(rmtC)* of this group was found among only 7/24 (29.2%) isolates when amplified at 711bp (figure 3-9J).



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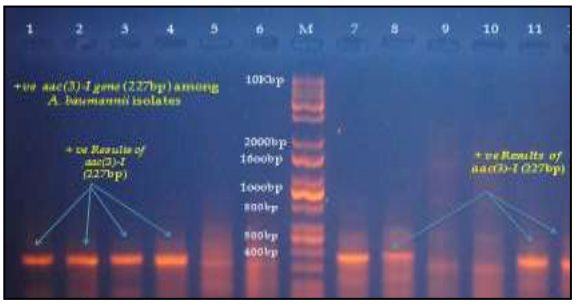

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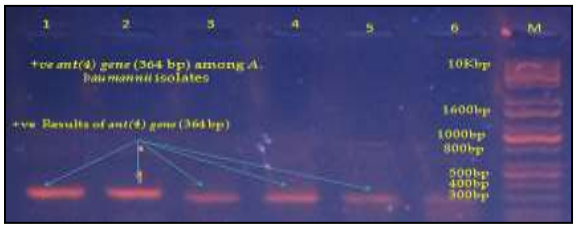

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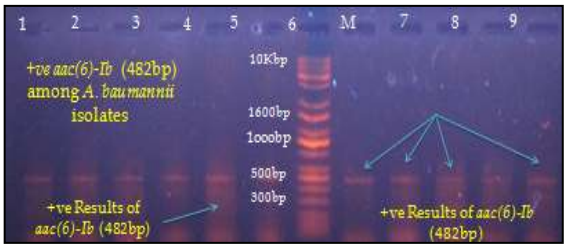

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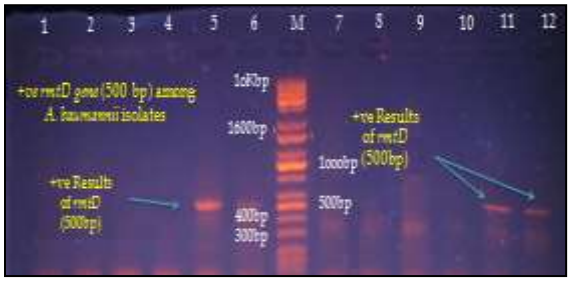

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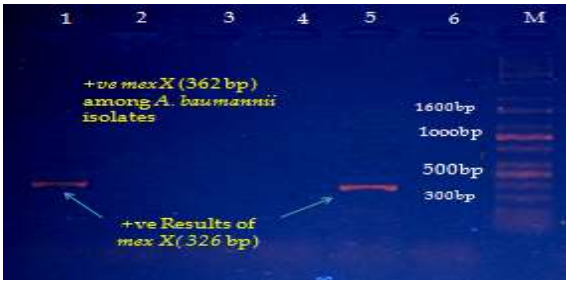

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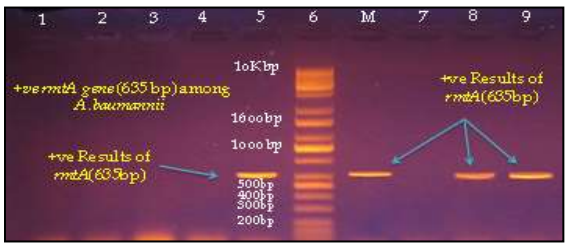

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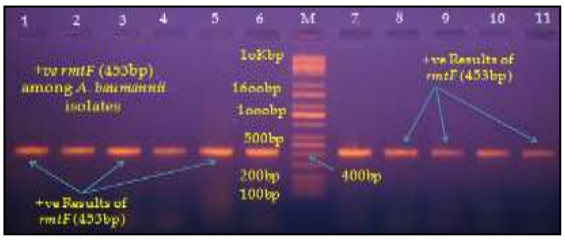

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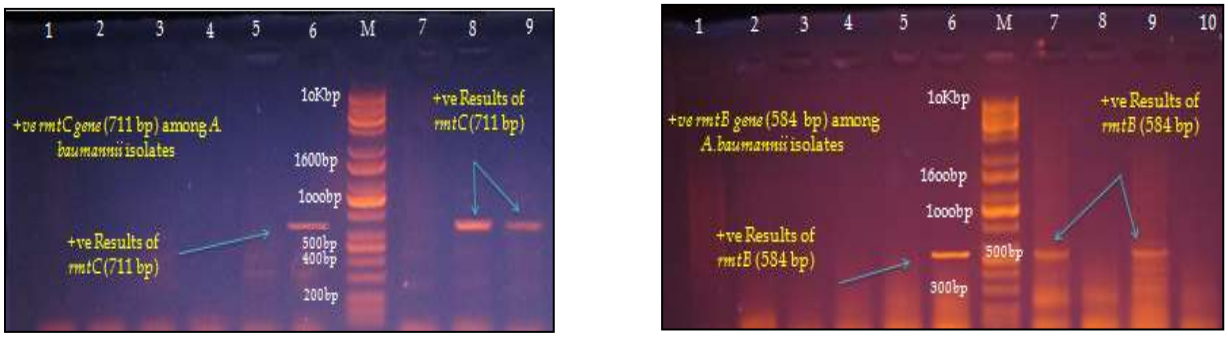

I **J**

Figure (8): Agarose gel electrophoresis (1% agarose, 5V/cm, for 120min) for positive results of aminoglycoside resistant genes related to different resistant mechanisms among 24 *A. baumannii* obtained from wound infection samples compared with DNA ladder (lane M). A) *aac(6)-Ib* gene (482 bp), B) *ant(4')IIb* gene (364bp), C) *acc(3)-I* gene (227bp), D) *aph(3)-VI* (800bp), E) *mex X* gene (326bp), F) *rmtD* gene (500 bp), G) *rmtA* gene (635bp), H) *rmtF* gene (453bp), I) *rmtB* gene (584 bp), and J) *rmtC* gene (711bp). Results obtained by PCR technique in current study.

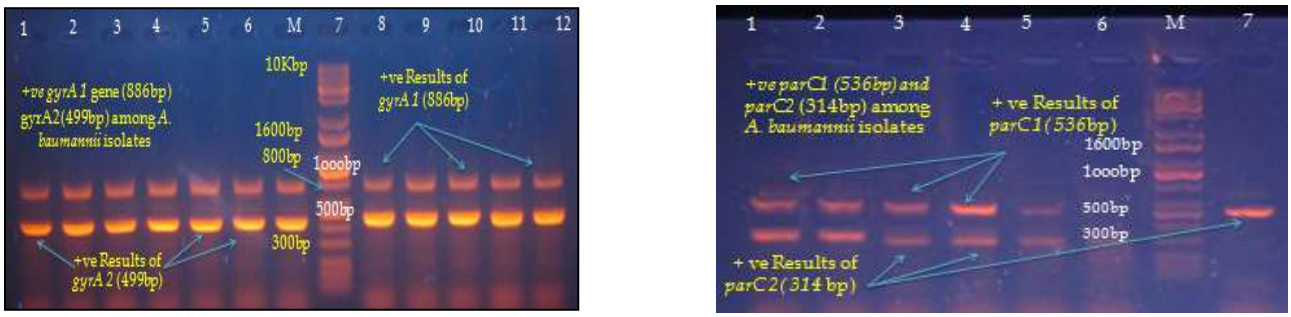

Figure 9: Agarose gel electrophoreses (1% agarose, 50 V/cm for 120 min) for quinolone resistant genes among *A. baumannii* isolates. A) Multiplex PCR of two options of *gyrA* gene 1 (amplified at 886bp) and 2 (amplified at 499bp). B) Multiplex PCR of two options of *parC* gene 1 (amplified at 536bp) and *parC2* (amplified at 314 bp). Positive results were showed by clear shiny bands compared with DNA ladder (100pb) lane M. Results obtained by PCR technique in current study.

Resistance to Quinolones: Two options of primer design *gyrA* (1 and 2) and *parC* (1 and 2) were used, and they gave identical results. The *gyrA* 1 gene was shown by 22/24 (91.7%) of *A. baumannii* isolates at amplified size of 886bp, and same prevalence rate was showed for *gyrA2* gene among isolates; however at amplified size of 499bp (figure 9A). Both *parC* 1 and 2 genes showed identical prevalence rate among 18/24 (75%) of *A. baumannii* isolates when amplified size at 536bp and 314bp, respectively (figure 9B).

It was noted that *A. baumannii* isolates had a range of 6-12 out of 22 antibiotic resistant genes tested in current study. It was interesting to find that *A. baumannii* isolates that was classified as PDR in previous section harbored the highest number of antibiotic resistant genes. Pan drug resistant *A. baumannii* W14 had 12/22 genes followed by 11/22 genes acquired by PDR *A. baumannii* W6 and PDR W23. The lowest number of antibiotic resistant genes (6/22) was detected in *A. baumannii* W17 that was classified as MDR isolate (figure 10).

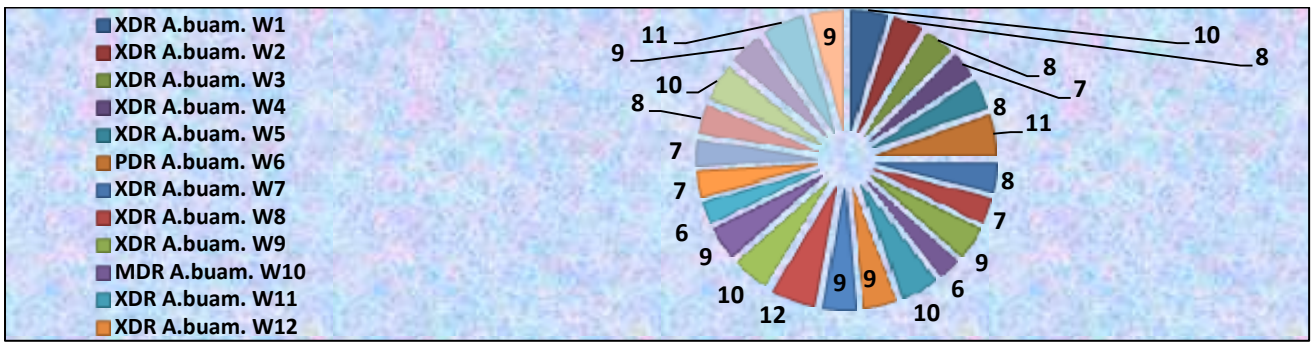

Figure 10: The number of antibiotic resistant genes that each *A. baumannii* isolate could harbor out of 22 total genes tested by PCR technique in current study.

Discussion

In current study, *A. baumannii* isolates were identified using *recA* gene via PCR technique as it was developed as an alternative ways for accurate identification and classification of bacterial species; especially when amplifying bacterial housekeeping genes (34; 35). It was clear from the antimicrobial sensitivity test that each isolate of the 24 *A. baumannii* obtained from wound infection could resist a range of 10-22 antimicrobial agents, resulting in resistance pattern towards wide range of antibiotic categories. It was interesting that *A. bauamnnii* W6, W14 and W23 could resist all antimicrobial agents. Consequently, it was crucial to classify *A. baumannii* isolates into three categories including (MDR, XDR, and PDR) in order to give a complete picture of antibiotic resistant level for each tested isolate.

Classification was done according to the criteria revealed by Magiorakos *et al.* for *A. baumannii* (18), and as the therapeutic options were mentioned in this criteria, it should be taken into account the relatedness among the number of resistant antimicrobial categories, resistance to carbapenems, resistance to polymyxins (E and B), and tigecycline resistance among tested *A. baumannii* isolates as showen in figure (5A).

It was found that *A. baumannii* W10 was resistant to 7/10 antimicrobial categories, but still was considered as MDR with possibility of being XDR as it was resistant to carbapenems and polymyxins (B and E) but sensitive to tigecyclins. Also, there was possibility for some XDR isolates to be PDR since they could be resistant to polymxins (E and B) and tigecycline as in cases of *A. bauamnnii* W5, 8, 9, 12, and15. Results were similar to Park *et al.* who reported that *A. baumannii* isolates collected from patients in ICUs of Samsung Medical Center in Seoul, South Korea were resistant to all tested antimicrobial drugs, including polymyxin B and colistin (36).

Current study also showed that 87.3% of *A. baumannii* isolates were carbapenemase producers via Modified Hodge Test; explaining the reason behind high resistant rate towards imipenem and meropenem in antimicrobial sensitivity test. However, only *A. baumannii* W1, 4, 24 were imipenem resistant isolates but not carbapenemase producers. This might be explained by other antibiotic resistant mechanisms those isolates could have including efflux pump mechanism associated with carbapenem resistance in *A. baumannii* or may refer to smaller number and size of porins contributing to the intrinsic outer membrane impermeability (37).

Results of current study were supported by Nordmann *et al.*(38) and Pateran *et al.* (39). Unlikely, Iraqi study reported that 33.3% of *A. baumannii* isolates recovered from AL-Najaf hospitals were confirmed as carbapenemase producers (40). AL-Harmoosh and Jarallah however, revealed that 60% of *A. baumannii* isolated from Hilla hospitals in Iraq were carbapenemase producing isolates when tested by MHT (41).

In addition, results of ESBLs detection test supportted high resistance rates among ESBLs producer isolates towards amoxicillin/ clavulanic acid and third and fourth generation cephalosporins in antimicrobial sensitivity test compared to non-ESBL producer isolates that may had different resistance mechanisms toward such antibiotics. Similar work were reported in literature by Biswas *et al.* (42) and Goel *et al.*(43). It was found only 12% of *A. baumannii* isolates showed ESBL production along with high level resistance towards cefoxitin and cefotaxime among isolates collected from wound infection and skin/ soft tissue infections (44).

Genotypic detection of antibiotic resistant genes in this study showed that the resistance to carbapenems in *A. baumannii* isolates detected in phenotypic method was mediated mainly by class D OXA-type enzymes and less often by metallo ß-lactamase (MBLs). This finding was also supported by modified Hodge test explained earlier.

These results were similar to Villegas *et al.*(45) and Antunes *et al.*(46). It was revealed that OXA-23 enzyme is frequently detected among *A. baumannii* worldwide (47). In Saudi Arabia, UAE, and Bahrain, the detection of *blaOXA-23* in *Acinetobacter* was more common. In Saudi Arabia, there

was incidence of 50% of *A. baumannii* isolates from Al-Riyadh harbored *OXA23* gene (48). Iraqi study showed that *A. baumannii* isolated from military and civilian personnel injured in the Iraq/Kuwait region during Operations in Iraq harbored 97% *blaOXA-51* and 11% *blaOXA- 23* genes (49). It was found 33% *A. baumannii* isolates had *blaOXA-23* genes positive in Al-Najaf hospitals (40).

Also, it was revealed that 40% *A. baumannii* isolated from Hilla hospitals in Iraq had *blaOXA-23* genes (41).

PCR analysis further demonstrated that aminoglycoside resistant rates exhibited among the 24 *A. baumannii* isolates in phenotypic test was mediated by acquiring aminoglycoside modifying enzymes that could turn aminoglycoside ineffective and conferred high level resistance to all clinically useful aminoglycosides. Similar observation were reported by Lin *et al.*(50) and Wong *et al.* (51).

Besides, Iranian study revealed that the amikacin resistance was due to the prevalence of *aph(3')-VIa* gene among *A. baumannii* isolates which was 45% (52). It also revealed that the resistance rate among *A. bauamnnii* isolates towards gentamicin, amikacin, kanamycin, and tobramycin was due to having *aph(3')-VIa*, *aac(3')-Ia*, and *aac(3')IIa* (53).

In current study, it was also found that PDR *A. baumannii* W6, 14, and 23 could harbor *mexX* gene that ensure resistance profile towards aminoglycosides.

There was increasing evidence that the role of efflux pumps in antibiotic resistance in bacteria was significant (54). It was also studied that inactivation of RND type pump in *A. baumannii* would decrease resistance towards aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and macrolides (1).

Current study results further ensured third resistance mechanism of 16sRNA methylation enzymes among 24 *A. baumannii* isolates against aminoglycosides; especially those genes were found singly or in combination in each isolate. It was noteworthy to reveal that PDR *A. baumannii* isolates could harbor all 16sRNA methylation genes, ensuring the highly resistant pattern that those isolates had to such antibiotic category. It has been revealed that those enzymes confer high level resistance by inhibiting aminoglycosides access to their site of action (55). Similar results were shown by Upadhyay *et al.*(56).

Also, AL-Jubori *et al.* revealed that the resistant rates of *rmtB* and *rmtD* were 10 and 5%, respectively among *A. baumannii* isolated from Baghdad hospitals (57). It was also reported that Gram-negative bacteria become highly resistant to all clinically important aminoglycosides due to having 16S rRNA methylase enzymes singly or in combination (58).

Finally, PCR analysis in this study demonstrated that *A. baumannii* could resist fourth generation quinolons including (gatifloxacin and levofloxacin) during antimicrobial sensitivity test due to acquiring mutations in *gyrA* and *parC* genes, essential as target sites for quinolons antibiotics. These structural topoisomerase changes would then reduce their affinity to fluoroquinolones as a part of developing resistance mechanisms against this type of antibiotics. Similar interpretation was revealed by Minarini and Darini (59).

Quinolones have been prescribed as empirical therapy against most hospital and community infections due to disease severity; therefore, resistance can cause a problem in clinical medicine as revealed in some countries (60).

Conclusion

Results obtained from PCR studies explained above for three key antibiotic categories along with phenotypic detection for antibiotic resistant genes ensure that *A. baumannii* isolates were being MDR, XDR, and even PDR pathogens among wound infected patients in Baghdad and Al-Najaf hospitals. This ability of *A. baumannii* to develop broad spectrum resistance toward wide range of antibiotic categories would result in significant clinical challenge when eliminating such pathogen.

Acknowledgment

Special thanks to Prof. Sawsan Sajid Al-Jubori for here guidance and support.

Abbreviation

MDR: Multidrug resistant pathogen

XDR: Extremely drug resistant pathogen

PDR: Pan drug resistant pathogen

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