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Full Length Research Paper

Emergence of extensive drug-resistant *Acinetobacter baumannii* in North of Jordan

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The aim of this study is to investigate the antimicrobial susceptibility profile of *Acinetobacter baumannii* and the contribution of the insertion sequence upstream of ampC β-lactamase on the susceptibility profile of 64 *A. baumannii* clinical isolates collected from a Jordan hospital. A total of 64 consecutive clinical isolates of *A. baumannii* were recovered (between March 2005 and December 2006) at the King Abdullah University Hospital (KAUH). The antimicrobial susceptibility profile against 11 different antibiotics was determined by disk diffusion method. The minimum inhibitory concentration (MIC) of these antibiotics against all isolates was determined using Etest. Polymerase chain reaction (PCR) screening was carried out to identify the presence of ampC gene and its adjacent insertion sequence. *A. baumannii* showed high resistance profile to β-lactam antibiotics (cefotaxime 92.2%, cefuroxime 98.4%, ceftazidime 89.1%) and ciprofloxacin 89.1%. imipenem and meropenem showed increased resistant rates (70.1 and 71.6%, respectively) with nearly half of the isolates being resistant to amikacin (52.5%). All isolates were susceptible to colistin. AmpC gene was detected in all isolates and only β-lactam including carbapenems and β-lactamase inhibitor resistant isolates were found to carry the IS-AmpC gene. Our present study confirms the essential role of the insertion sequence which could represent a gene regulatory system, able to regulate various genes in *A. baumannii*. Finally, inappropriate infection control measures and inaccurate antibiotic usage are highly potential factors that might increase the prevalence and spread of antibiotic resistant *A. baumannii* isolates.

Key words: *Acinetobacter baumannii, Antimicrobial susceptibility, IS-AmpC*

INTRODUCTION

Acinetobacter baumannii a non-glucose fermenting Gram negative bacillus, has emerged in the last three decades as a major etiological agent of hospital-associated infections giving rise to significant morbidity and mortality particularly in immunocompromised patients. Multidrug-resistant *A. baumannii* has become a global enemy to the seriously infected patients who critically rely on antibiotic therapy. It has developed resistance to major classes of antibiotics and carbapenem-resistant isolates have been increased and singly reported worldwide as a cause of nosocomial outbreaks. Therapeutic options have become very limited raising infection control concern worldwide (Perez et al., 2007) Meanwhile, the ability of these bacteria to develop resistance rapidly has raised the suggestion that unless newer therapeutic options are developed we may be closer to the end of the antibiotic era with *A. baumannii* compared to methicillin-resistant *Staphylococcus aureus* (Giamarellou et al., 2008).

A. baumannii is the most common species associated with hospital-associated infections and like the rest of the *Acinetobacter spp*. it has the ability to survive in the hospital environments as well as on dry surfaces and this aid in their easy transmission amongst hospitalized patients resulting in possible hospital outbreaks (Wybo et al., 2007). The selective pressure by the extremely high usage of antibiotics against this bacteria as well as its ability to accept foreign DNA containing antimicrobial resistant determinants could be one of the reasons

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behind *Acinetobacter* incredible resistance to major different antibiotics worldwide (Hawkey et al., 2008).

Different classes of β-lactamases confer various resistant phenotypes to *A. baumannii.* However chromosomal AmpC cephalosporinase has been reported widely to be responsible for a wide range of β-lactams resistance in *A. baumannii.* It has recently sequenced from a multi-resistant *A. baumannii* clinical strain (Corvec et al., 2003). The expression of AmpC cephalosporinase is naturally occurring among *A. baumannii* at basal level and thus does not reduce the efficacy of cephalosporins broad-spectrum antibiotics. However the introduction of the insertion sequence upstream to the β-lactamases AmpC gene confer an over expression of that particular genes by acting as a strong promoter sequence resulting in a high resistant rate to β-lactam antibiotics (Corvec et al., 2003).

In this study we examined the susceptibility profile of *A. baumannii* clinical isolates to eleven major classes of antibiotics including ciprofloxacin (CIP), amikacin (AK), colistin (CL), amoxicillin/calvulinic acid (AMC), ampicillin/sulbactam (SAM), cefotaxime (CTX), cefuroxime (CXM), ceftazidime (CAZ), cefepime (FEP), meropenem (MEM) and imipenem (IMP) and the influence of the presence of AmpC gene and its adjacent sequence on the susceptibility profile of these clinical isolates and comparison the susceptibility pattern among all the major classes of antimicrobial agents β-lactams including carbapenems, aminoglycosides and fluoroquinolones.

MATERIALS AND METHODS

Bacterial isolates

A total of 64 consecutive clinical isolates of *A. baumannii* were recovered (between March 2006 and December 2007) from different specimens including sputum, wound swab, urine, blood and body fluids submitted to the clinical microbiology laboratory of the King Abdullah University Hospital (KAUH) the major hospital in north Jordan. All isolates were immediately identified to the species level by conventional methods and confirmed using the standard Vitek techniques. All of these isolates were suspended in 20% glycerol stock and kept at -70℃ for storage. During the processing of the isolates for the experiments, the isolates were subcultured twice onto nutrient agar plates and incubated at 37°C overnight before they were used. Repeated isolate for the same patient was strictly ignored.

Susceptibility testing

The susceptibility of *A. baumannii* to the selected antimicrobial agents obtained from OXOID, UK including ciprofloxacin (CIP), amikacin (AK), colistin (CL), amoxicillin/calvulinic acid (AMC), ampicillin/sulbactam (SAM), cefotaxime (CTX), cefuroxime (CXM), ceftazidime (CAZ), cefepime (FEP), meropenem (MEM) and imipenem (IMP) was determined using disc diffusion test and the results were interpreted according of the CLSI guidelines (Clinical and Laboratory Standards Institute, 2010)

The *in vitro* minimum inhibitory concentration (MIC) for the previous antibiotics was determined by Etest method (AB BIODISK, Solna, Sweden). Newly prepared plates with Mueller–Hinton medium were inoculated with *A. baumannii* suspensions equivalent to 0.5 McFarland standard, Etest strips of each antibiotic were immediately applied. The plates then were incubated for 24 h at 37℃, (MICs) were read and interpreted according to the manufacturer's instructions. *Pseudomonas aeruginosa* ATCC 25923 was used as a control for susceptibility testing methods and the values were within the accepted limits.

Detection of the insertion sequence of AmpC gene by PCR

Bacterial DNA was extracted from 64 *A. baumannii* isolates according to the instructions provided with the extraction kit from Promega company USA (Promega, USA). PCR amplification tests were performed in Princess Haya Biotechnology Center using Biorad DNA thermal cycler PCR. Forward and reverse primers ACI5 (5' ACTTACTTCAACTCGCGACG 3') and ACI6 (5' TAAACACCACATATGTTCCG 3') were used to be corresponding to the nucleotides 486-505 and 1128- 1148 of the structural AmpC gene, respectively.

The PCR was performed in a final volume of 25 µl containing 10 mM Tris-HCL pH8.3, 2.5 mM MgCl2, 50 mM KCL, 200 of each nucleotides, 0.5 µM of each primer, 2.5U of Taq DNA polymerase (promega, USA) and 2 µL of bacterial DNA extract. Cycling conditions were; an initial denaturation step at 94℃ for 2 min, amplification steps of 94℃ for 30 s, 59℃ for 30 s, 72℃ for 1 min for a total of 30 cycles and a final extension at 72℃ for 7 min. The PCR products were detected on a 1.5% agarose gel. The amplification of 347 bp fragment of the insertion sequence upstream of ampC gene was performed using the forward and reverse primers (5' ACI2 TAGTACTGCTATTTACGGCT) and ACI1 (AACTCATTGAGATGTGTCATA) respectively, PCR was carried out as previously described (Corvec et al., 2003).

RESULTS

A total of 64 *A. baumannii* clinical isolates were collected from KAUH in Jordan within the period from March 2006 to December 2007. The isolates were cultures of: sputum (42%), wound swabs (28%), blood (20%), urine (6%) and body fluids (4%). The results of the susceptibility profile of 64 *A. baumannii* clinical isolates to the examined antibiotics were obtained. The MIC range and the MIC50 and MIC 90 of all isolates as well as the susceptibility profile of each antibiotic are shown in (Table 1). All isolates were showed high resistance profile for the following antibiotics, cefuroxime 98.4%, cefotaxime 92.2%, ceftazidime, cefepime and ciprofloxacine both 89.1%, amoxicillin/calvulinice acid 86%, ampicillin /sulbactam 68.8%. Resistant rates were found to be 70.1 and 71.6% for imipenem and meropenem, respectively and 52.5% for amikacin. Resistant rate for colistin was 0%. Thirty five (65.7%) *A. baumannii* isolates were multidrug-resistant based on disk diffusion method (isolates resistant to at least three different classes of antibiotics were considered as a MDR *A. baumannii*). The agarose gel electrophoresis showed that the chromosomally encoded cephalosporinase AmpC gene was present in all isolates included in this study with the size 663 bp (Figure 1). All β-lactams including

Table 1. Susceptibility profile and Minimum inhibitory concentrations (MICs) of *Acinetobacter baumannii* isolates (*n* = 64) for 11 antibiotics.

Antibiotics	Antibiotic Susceptibility rate (%)			MIC (µg/mL)		
	Susceptible	Intermediate	Resistant	Range	MIC_{50}	MIC_{90}
Cefotaxime	0	7.8	92.2	32- >256	>256	>256
Cefuroxime	0	1.6	98.4	32- >256	>256	>256
Ceftazidime	10.9	0	89.1	1.5- >256	128	>256
Cefepime	10.9	0	89.1	3- 256	128	>256
Meropenem	26.3	3.6	70.1	0.25- >32	32	>32
Imipenem	26.8	1.6	71.6	0.125- >32	32	>32
Ampicillin/sulbactam	26.6	4.7	68.8	0.5- >256	64	>256
Amoxicillin/calvulinice acid	1 1.6	12.5	86	1.5- >256	128	>256
Ciprofloxacin	7.8	3.1	89.1	0.094- >32	>32	>32
Amikacine	40.9	6.6	52.5	0.38- >256	32	>256
Colistin	100	0	0	0.125- 2	0.38	0.5

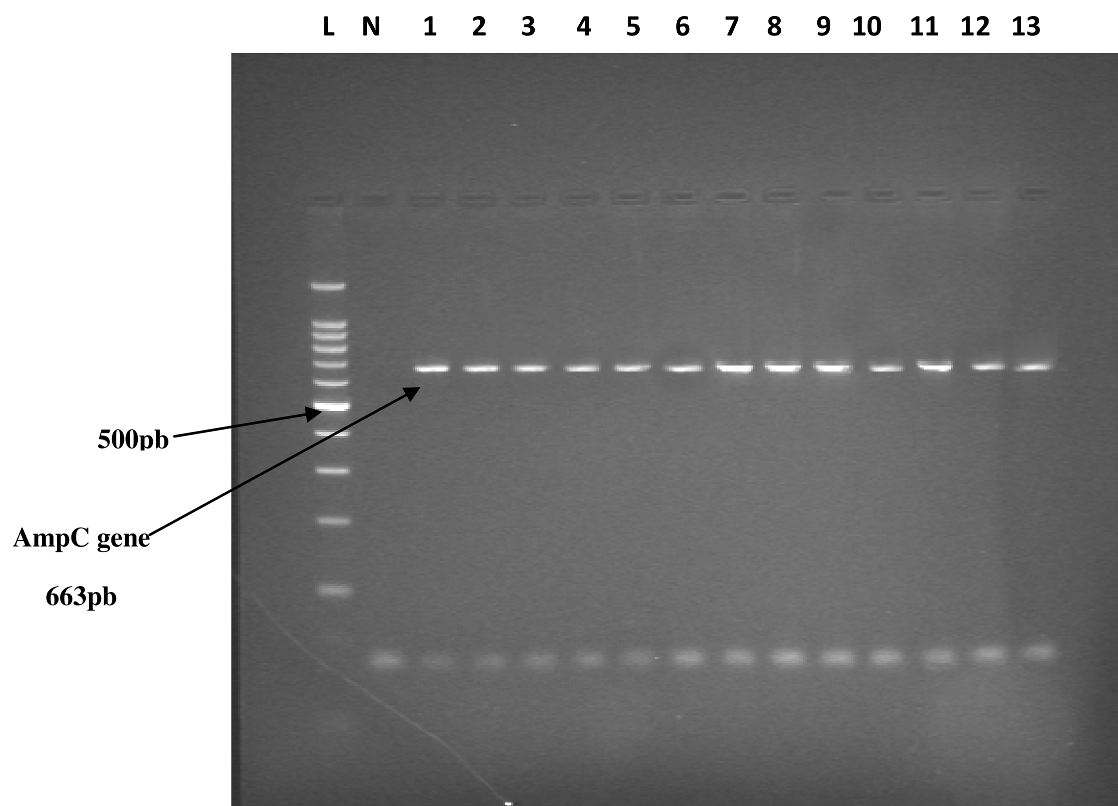

Figure 1. Agarose gel electrophoresis of PCR product for the presence of ampC gene in *A. baumannii* isolates. Lane L represents the 100pb DNA ladder. Lane N represents the negative control. Isolates 1-4 represent the (B-lactams resistant isolates) which showed positive for the presence of *ampC* gene. Isolates 6-13 represent the (B-lactams susceptible isolates) which showed positive for the presence of *ampC* gene.

carbapenems and β-lactamase inhibitor resistant isolates showed positive for the presence of insertion sequence with the size 347 bp compared to those susceptible isolates which were negative for the presence of the insertion sequence (Figure 2).

DISCUSSION

In concurrence with studies worldwide our study also showed that *A. baumannii* has highly increased resistance profile to imipenem and meropenem (Zarrilli et

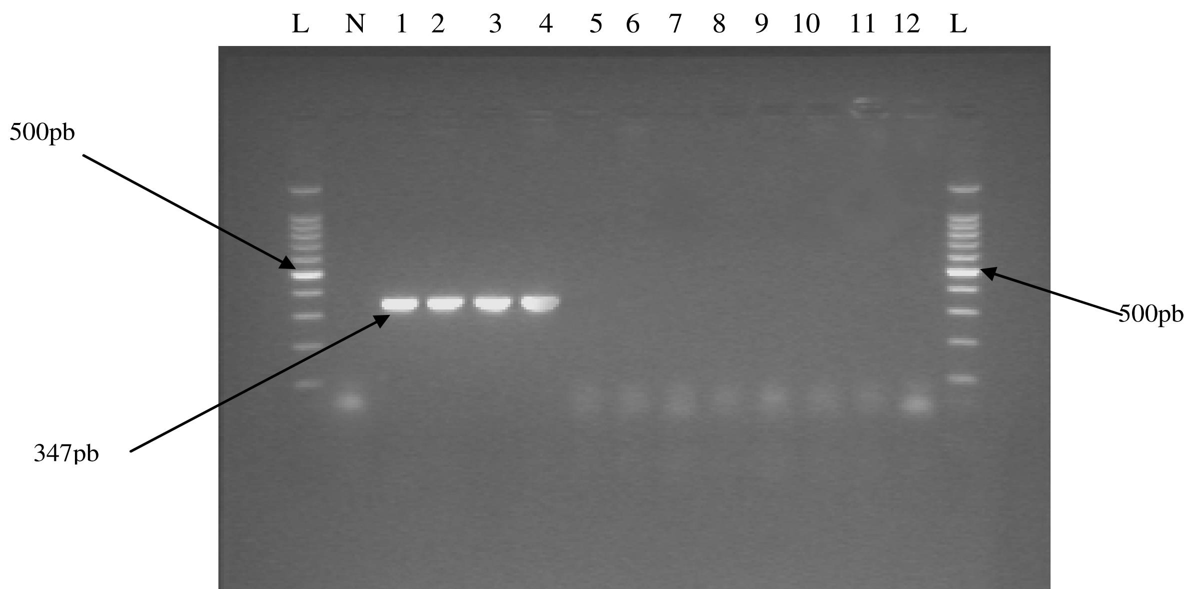

Figure 2. Agarose gel electrophoresis of PCR product for the presence of IS upstream of ampC gene of *A. baumannii*. Lane L represents the 100pb DNA ladder. Lane N represents the negative control. Isolates 1-4 represents the (B-lactams resistant isolates) which showed positive for the presence of the IS- ampC gene. Isolates 5-12 represents the (B-lactams susceptible isolates) which showed negative for the presence of IS ampC gene.

al., 2009). The development of carbepenem resistant Acinetobacter has been a major concern of infection prevention worldwide (Nemec et al., 2008). Imipenem has long been the drug of choice for serious infection caused by multidrug resistant *Acinetobacter* species. The development of imipenem- resistant *A. baumannii* has seriously limited the available antibiotics that can be used against those MDR isolates. It is interesting to note that although geographically connected, studies from Saudi Arabia showed that imipenem resistance was only 13% amongst Acinetobacter isolates compared with our study results that imipenem resistant rate was 70.1% (Hanan et al., 2003). Amongst the factors that may have contributed to the rapid development of resistance are ineffective infection control measures, overuse of antibiotics, inaccurate diagnosis and thus inaccurate use of initial or empirical antibiotics, the ability of the Acinetobacter to accept foreign DNA containing antimicrobial resistant determinants (Hanan et al., 2003; Abu Setteh, 2005). Our isolates showed high susceptibility (100%) to colistin. The limited usage of this antibiotic might be the reason for the high susceptibility profile of *Acinetobacter* to colistin (Sinirtas et al., 2009; Dizbay et al., 2008). The *Acinetobacter* susceptibility profile showed that the antibiotic combination such as ampicillin/sulbactam and amoxicillin/clavulinic acid did not show promising activities against these isolates despite it being a recommended antibiotic treatment for *Acinetobacter* infections (Oliveira et al., 2008). A combination of antibiotic resistant mechanisms could be one of the most likely reasons for a wide range of resistance to different classes of antibiotics by Acinetobacter (Rahal, 2009).

The role of mobile elements in the over expression or reduce expression of bacterial resistant genes have been confirmed by a study from France (Corvec et al., 2003). The researchers found that the insertion sequence upstream of AmpC gene led to overexpression of that particular gene. Meanwhile they also observed that mobile elements seem to be widespread and might represent a gene control system able to regulate different genes in *Acinetobacter* (Corvec et al., 2003; Mak et al., 2009; Mansour et al., 2005). This was in agreement with our findings which showed that the insertion sequence was detected amongst β-lactams, carbapenems and β-lactams / β-lactamase inhibitor resistant isolates. This may indicate the role of the insertion sequence in regulating and overexpressing of AmpC gene and probably other genes that might be existed at same motif which increased the resistance profile of *A. baumannii* to those particular antibiotics including carbapenem.

Conclusion

A. baumannii is developing resistance to antibiotics extremely rapidly particularly to carbapenems. It is becoming a global health concern especially when it develops resistance to the other major classes of antibiotics which makes the therapeutic option very limited. β-lactamase gene expression may be enhanced by the presence of Insertion sequences (ISs) by providing promoters. An adjacent sequence to the AmpC

gene is probably responsible for the overexpression of β - lactamase AmpC gene in combination with other mechanisms that leads to increase of the resistance profile of the isolates that contains the mobile element. Further molecular studies are needed to clarify the bacterial mechanisms of rapid resistant development especially under antibiotic selective pressure.

Antimicrobial stewardship programs and infection prevention and control measures must be strictly adhered to in the efforts of tackling the rising resistance amongst *A. baumannii* to multiple antibiotics. The failure to do so may impact on outbreak containment and the spread of these resistant strains in healthcare settings.

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