

Occurrence and molecular characterization of metallo- β -lactamases (MBLs) among *Acinetobacter baumannii* isolates from cancer patients

Husam F. qouzah¹,
Feras Hawari²,
Luay F. Abu-Qatouseh³,
Asem A. Shehabi¹

- 1 Department of Pathology-Microbiology, School of Medicine, The Jordan University, Amman, Jordan
- 2 King Husain Cancer Center (KHCC), Amman, Jordan
- 3 Faculty of Pharmacy, Department of Biomedical Sciences, University of Petra, Amman, Jordan.

Abstract

Background: During the last decade, the prevalence of carbapenem-resistant infection associated with multidrug resistant (MDR) *Acinetobacter baumannii* in patients has been continuously increasing. This prospective study aimed to determine the occurrence and molecular characterization of metallo- β -lactamases (MBLs) and carbapenem hydrolyzing oxacillinases among *A. baumannii* isolates from cancer patients over a period of 6-month.

Methods: Antimicrobial susceptibility profile of 70 randomly collected *A. baumannii* isolates was first determined using disc diffusion test, and second, the MICs of 45 representative multidrug resistant (MDR) isolates were tested to most clinically used drugs in treatment of their infections using E-test. PCR assays were used to detect the common four types of class D carbapenem hydrolyzing oxacillinases, two types of class A carbapenemases, four types of class B metallo- β -lactamases, and prevalence of Class 1 Integron among MDR isolates.

Results: All 70 isolates were MDR, including 100% resistance to meropenem, aztreonam, piperacillin/tazobactam and 99% to carbapenem. All isolates carried *bla*OXA-23 and *bla*OXA-51, but none carried a *bla*OXA-24 like or *bla*OXA-58. The isolates were also positive for NDM-1, VIM, GES, KPC and SPM at the rates of 29%, 20%, 29%, 19%, 7% and 2%, respectively. Class 1 Integron was positive in 82% of *A. baumannii* isolates.

The clonal relationship of 42 MDR *A. baumannii* isolates using ERIC-PCR and constructed dendrogram showed 3 major genotype clusters

Contact information:

Asem A. Shehabi.

Address: Department of Pathology-Microbiology, School of Medicine, The Jordan University, Queen Rania str. 100, Amman, Jordan.

 ashehabi@ju.edu.jo

of genetically related isolates. These include 4 genotype groups, each composed of 2 isolates with 100% similarity of DNA bands.

Conclusion: This study demonstrates that *A. baumannii* colonize frequently cancer patients in association with antibiotic treatment. The organism is mostly carrying wide spectrum of antibiotic resistance genetic factors, especially many types of ESBLs and MBLs and Class 1 Integron. This fact should be considered when therapy is selected for treatment of patients infected with MDR *A. baumannii*.

Keywords

Acinetobacter baumannii, ESBLs, MBLs, Class 1 Integron, Jordanian cancer patients.

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Introduction

In recent years worldwide including Jordan, most clinical *A. baumannii* isolates have developed resistant to most clinically used drugs, and caused increased morbidity and mortality among hospitalized patients in intensive care units (ICUs) [1-4].

Acinetobacter spp. develop multidrug resistance through mechanism of mutation in penicillin-binding proteins (PBPs), alterations in outer membrane proteins (OMPs) and increased activity of efflux pumps [5]. However, resistance to β -lactams appears to be primarily caused by production of β -lactamases which include extended-spectrum- β -lactamases (ESBLs), oxacillinases, and metallo- β -lactamases [6].

A. baumannii acts first often as colonizer rather than a pathogen because of its wide distribution and colonizing capability [1, 7], especially in immune-compromised and among patients with prolonged hospital stay [8]. The spectrum of infections caused by this pathogen include ventilator-associated pneumonia (VAP) which is developed after prolonged hospitalization or mechanical ventilation and prior use of antibiotics [4, 9-10].

Many factors contribute to the ability of *A. baumannii* to cause infections in hospitalized patients. First, *A. baumannii* has the capacity to survive for long periods in various body parts of patients and their environment. Secondly, the genomic makeup of this organism allows it to acquire resistance to many antibiotics within a short period [11]. However, the rates of antibiotic resistance vary from region to region and among hospitals [12]. Almost all studies indicate that MDR *A. baumannii* infections are difficult to treat, spread quickly among hospitalized patients and can be associated with high mortality due to blood sepsis or ventilator associated pneumonia [2, 4, 12-14].

This study describes the occurrence and the mechanism of antibiotic resistance of *A. baumannii* isolated from cancer patients.

Patients and Methods

Collection of *A. baumannii* isolates

This prospective study included a total of 70 *A. baumannii* isolates, were recovered from clinical specimens of patients who were investigated for

presence of *A. baumannii* during hospitalization or investigation as outpatients at a Cancer Center in Amman, Jordan, over a period of 6-months (2016) as shown in the **Table 2**. Approval was obtained from the Institution Ethical Review Board (IERB) at the King Hussein Cancer Center (KHCC), Amman, Jordan, for conducting and publishing this study.

Identification of *A. baumannii* isolates

All isolates were first identified using BioMerieux *VITEK 2* Automated Microbiology System (France). Later all collected isolates were sub-cultured on Blood and MacConkey agar plates and incubated at 37°C for 24 hr. Pure growth was again confirmed as *A. baumannii* according to the following characteristics; negative oxidase test, negative lactose and glucose fermentation in Kligler iron tubes, and later by presence of OXA-51 gene using PCR. Five fresh colonies were inoculated in brain-heart infusion agar plus 15% glycerol and kept frozen at -70 °C until used for further investigation.

Antibiotic susceptibility tests

The susceptibility of *A. baumannii* isolates to antibiotics was determined using the disc diffusion method according to the guidelines of CLSI 2016 [15]. The minimum inhibitory concentrations (MICs) of isolates were determined by the E-test (ABBioMérieux, France) for imipenem, amikacin and colistin. Interpretations of the MICs in the E-test were done according to the guidelines of CLSI 2016 [15].

Genomic DNA Extraction from *A. baumannii* Isolates

Genomic DNA was extracted according to the manufacturer's instructions of Wizard Genomic DNA purification kit (Promega, USA).

Plasmid DNA extraction from *A. baumannii* isolates

The bacterial plasmid was extracted using the EZ-10 Spin Column Plasmid DNA Minipreps Bio Basic kit (Canada) according to manufacturer's protocol.

Detection of genes encoding *bla*OXA carbapenemases, metallo-beta-lactamases, and class-1 integron in *A. baumannii* using PCR

All primer target genes of *bla*OXA carbapenemases, metallo-beta-Lactamases, their sequences, product sizes, annealing temperatures were used according to references as shown in **Table 1**. β -lactamases genes (OXA-58, OXA-51, OXA-24, OXA-23 [16], *IMP-2*, *VIM-2*, *SPM*, *NDM*, [17], *GES* [18], *NDM-1*(19)KPC [20] and class-1 integron [21].

The following control strains were used in the experiment, which were kindly donated by Prof. Monzer Hamza, Laboratoire Microbiologie Sante et Environnement (LMSE), Ecole Doctorale des Sciences et de Technologie, Faculte de Sante Publique, Universite Libanaise, Tripoli, Lebanon): *A. baumannii* (OXA-51 positive), *A. baumannii* (OXA-23 positive), *A. baumannii* (*IMP-2* positive), *K. pneumonia* (*bla*KPC positive; ATCC BAA-1705), *K. pneumonia* (*bla*NDM-1 positive; ATCC BAA-2146), and *P. aeruginosa* (ATCC 2 7853) was used in susceptibility test.

PCR tests were carried out in 25 μ l reaction with 2.5 μ l of extracted DNA, 20 pmol of each primer (Alpha DNA, Montreal, Canada), 12.5 μ l GoTaq® Green master mix (Promega, USA). The volume was made up to 25 μ l using nuclease free water. DNA concentrations of each sample was evaluated using Nanodrop 2000c (Thermo scientific, USA). The PCR amplification assays for the target genes were performed using programmable PCR-Thermocycler (Bioerxp cycler, China). Control tubes containing master mix without template DNA were included in each run as negative control. Tubes were held at 4°C when the cycles were ended. The amplified products and the PCR DNA marker were separated via electrophoresis on 2% agarose gels containing 15% Red safe™ stain (5 μ l), for 40-50 min at 120 volts, and then visualized using Gel documentation system including: UV camera, monitor and printer (UVP, USA).

Table 1. Primers of blaOXA - carbapenemases, Metallo-beta-Lactamases ,Class-1 integrons, ERIC.

Gene targets	Primer Sequence (5'→3')	Annealing Temp.oC	Product size (bp)	Reference
<i>bla</i> _{OXA-51}	F→TAATGCTTTGATCGGCCTTG R→TGGATTGCACTTCATCTTGG	60	353	16
<i>bla</i> _{OXA-23}	F→GATCGGATTGGAGAACCAGA R→ATTCTGACCGCATT TCCAT	57	501	
<i>bla</i> _{OXA-24}	F→GGTTAGTTGGCCCCCTTAAA R→AGTTGAGCGAAAAGGGGATT	57	246	
<i>bla</i> _{OXA-58-}	F→AAGTATTGGGGCTTGTGCTG R→CCCCTCTGCGCTCTACATAC	57	599	
<i>IMP</i>	F→GGA ATA GAG TGG CTT AAY TCTC R→GGT TTA AYA AAA CAA CCA CC	52	232	17
<i>VIM</i>	F→GAT GGT GTT TGG TCG CAT A R→CGA ATG CGC AGC ACC AG	52	390	
<i>SPM</i>	F→AAA ATC TGG GTA CGC AAA CG R→5'- ACA TTA TCC GCT GGA ACA GG	52	271	
<i>NDM</i>	F→GGT TTG GCG ATC TGG TTT TC R→CGG AAT GGC TCA TCA CGATC	52	621	
<i>GES</i>	F→ATG CGC TTC ATT CAC GCA C R→CTA TTT GTC CGT GCT CAG GA	56	863	18
<i>NDM-1</i>	F→ATT AGC CGC TGC ATT GAT R→CAT GTC GAG ATA GGA AGT G	58	154	19
<i>KPC</i>	F- GATACCACGTTCCGTCTGG R- GCAGGTTCCGGTTTTGTCTC	52	246	20
<i>int1</i> <i>int1</i>	F→ACATGTGATGGCGACGCACGA R→ATTCTGTCTGGCTGGCGA	55	600	21
<i>ERIC-1</i> <i>ERIC-2</i>	ATGTAAGCTCCTGGGGATTCAACAAG TAAGTGACTGGGGTGAGCG	52	Multiple Bands	22

Enterobacterial repetitive consensus (ERIC)-PCR

This test was performed in 50 µl volumes containing 10 ng of genomic DNA from *A. baumannii* clinical isolates, 4 mM MgCl₂, 50 pM each of primer ERIC-1 and 2 as described by Jeong et al. [22].

Statistical analysis

Statistical analysis was done using SPSS version 20, chi square test and t-test were used to analyzed categorical variables and to measure the significance of the association between the study variables. If less than 5 a fisher exact test was used. P-value of ≤ 0.05 was considered significant \.

Results

Table 2 Shows the sources of 70 *A. baumannii* isolates within the period of study (March 2016 to October 2016). The majority of samples were collected from nasal swabs (30%) and perianal swabs (21%). **Table 3** shows the demographic characteristics of cancer patients with positive *A. baumannii* isolates. The majority of patients (56%) were over 50 years old and were in-patient (90%) rather than out-patient (10%). Most of the patients (68%) have received medication prior to collection their specimens. The results of antimicrobial susceptibility using disc diffusion method are presented in **Table 4** *A. baumannii* isolates were 100% resistant

Table 2. Sources, number and percent of collected samples from patients.

Source	Samples*	
	No	%
Nasal swab (NS)	21	30
Perianal swab (PS)	15	21
TRAP	8	11
Wound swab (WS)	8	11
Blood	4	6
Urine	5	7
High vaginal Swab (HVS)	3	4
Sputum	2	3
Broncho alveolar lavage (BAL)	2	3
Throat	1	1.5
Pleural fluid	1	1.5
Pleural fluid	1	1.5

*: Only one sample was included from each patient.

to meropenem, piperacillin/tazobactam, aztreonam and ceftazidime, 99% resistant to imipenem, and ciprofloxacin, 76%, 77% resistant to amikacin and gentamycin, respectively. The results of MICs for the three commonly used drugs amikacin, colistin and imipenem against *A. baumannii* isolates are shown in **Table 4**.

A total of 45 *A. baumannii* isolates were 100% positive to OXA23 like and OXA51, and all of these were negative to OXA58 like and OXA24 genes. **Table 5** shows the distribution of other potential genes of *VIM*, *GES*, *NDM-1*, *NDM*, *IMP α* , *KPC*, and *SPM* were found in 64%, 42%, 29, 20%, 7%, 7%, 2%, respectively, while integron-1 was presented in 83% of the isolates.

The genetic relationship of 42 MDR *A. baumannii* isolates from hospitalized patients as shown in the dendrogram indicated 3 major genotype clusters of genetically related isolates (**Figure 1**). These include

Table 3. Demographic characteristics of 70 cancer patients with positive *A. baumannii* isolates.

Patients characteristics	Hospitalized patients		Out-patients		Total		P-Value
	No	%	No	%	No	%	
Skills							
Males	38	54	1	1	39	55	0.026**
Females	25	36	6	9	31	45	
Total	63	90	7	10	70	100	
Age /year							
Less than 14	2	3	0	0	2	3	0.601
15-50	26	37	2	3	28	40	
More than 50	34	49	5	7	39	56	
Total	63	90	7	10	70	100	
Prior use of antibiotic							
No antibiotic	18	26	4	6	22	32	0.033**
Treatment with antibiotics*	45	64	3	4	48	68	
Total	63	90	7	10	70	100	

*: Mostly amikacin and colistin. **: Significant

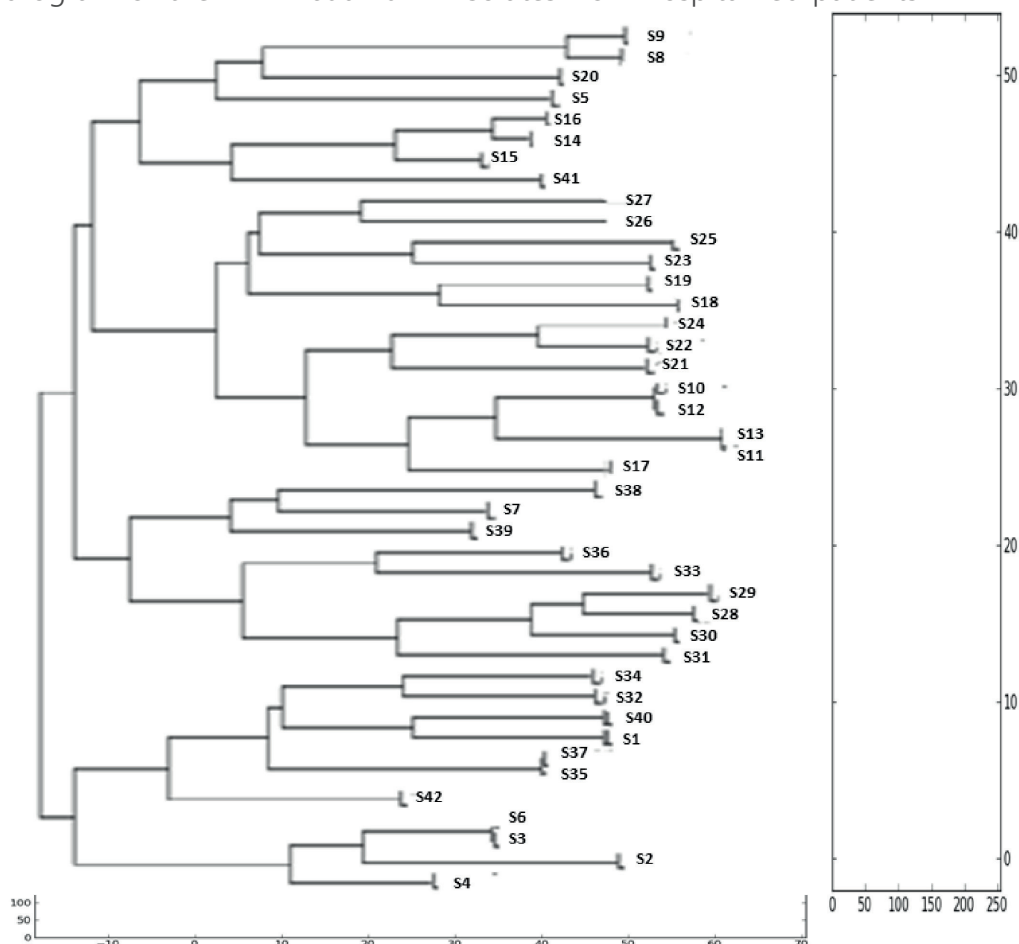
Table 4. Antimicrobial resistance patterns of 70 *A. baumannii* isolates using disc diffusion and E-tests for MICs.

Antimicrobial agent	resistant isolates		MIC ₅₀	MIC ₉₀	MIC-range
	No	%	mg/L	mg/L	mg/L
Meropenem (Mem)	70	100	-	-	-
aztreonam (Atm)	70	100	-	-	-
Piperacillin/Tazobactam (Ptz)	70	100	-	-	-
Ceftazidime (Caz)	70	100	-	-	-
Ciprofloxacin (Cip)	69	99	-	-	-
Imipenem (IMI)	69	99	15.6	28.5	0.38-32
Gentamicin (GM)	54	77	-	-	-
Amikacin (Ak)	53	76	98.3	177	1-256
Colistin	-	-	0.26	0.47	0.019-2

Table 5. Distribution of bla OXA-carbapenemases, MBLs and Integron-1 genes among 45 *A. baumannii* isolates.

Gene name	Positive isolates	
	No	%
OXA-23	45	100
OXA-24	0	
OXA-51	45	100
OXA-58	0	
VIM	29	64
GES	19	42
NDM-1	13	29
NDM	9	20
IMP	3	7
KPC	3	7
SPM	1	2
Integron-1	37	82

Figure 1: Dendrogram of the 42 *A. baumannii* isolates from hospitalized patients.



4 genotype groups, each composed of 2 isolates with 100% similarity of DNA bands.

The identity of class-1 integron PCR products of 5 *A. baumannii* isolates were confirmed by sending them to Macrogen Inc. (Seoul, Korea) for sequencing and the results were analyzed using alignment search tool of Blast. (<http://www.ncbi.nlm.nih.gov/BLAST>) and the multiple sequence alignment program, CLUSTALW (<http://www.ebi.ac.uk/clustalw>).

Discussion

This study has demonstrated that many body sites of cancer patients are frequently colonized with *A. baumannii*, especially their nares and perianal site. Invasive blood infection was detected among few percentage (6%) of hospitalized cancer patients during the 6-month study period (**Table 2**). The rest of the isolates can be considered colonizers and might become potential pathogens for patients under certain condition, especially in association with the use of invasive procedures [23-24]. There is no significance difference in general demographic characteristics between male and female patients or their ages, whereas treatment with antibiotics showed increased significantly the chance of ($P=0.033$) colonization with *A. baumannii*. Additionally, hospitalized patients were at increased risk for colonization with AB (90%; $P=0.026$) when compared to outpatients (10%) with *A. baumannii* (**Table 3**). Recent studies from Jordan and other countries reported that *A. baumannii* frequently colonizes hospitalized patients, causing sporadic invasive infection and nosocomial outbreaks, especially among critically ill patients and in ICUs [2, 25-27].

This study demonstrates that the majority of *A. baumannii* species isolates were resistant in the range between 76% to 100% to commonly useful antimicrobial agents in the treatment of gram-negative infections, including infections due to *A. baumannii* (**Table 4**). Previous studies which has been performed over the last few years at the Jordan

University Hospital and KHCC in Amman, have also found that the majority of *A. baumannii* isolates from patients and hospital environment sources was also multidrug resistant [2, 3, 28].

The ability to acquire resistance to a broad range of antimicrobial agents within a short period and during treatment is commonly observed in *A. baumannii* [4, 29]. High mortality rates among critically ill patients infected with MDR or extensively drug-resistant (XDR) *A. baumannii* strains (resistant to all antibiotics except colistin and tigecycline, have been recently described in many regions [30-32].

This study shows extremely high rate of carbapenems-resistance (99-100%) among *A. baumannii* isolates. The prevalence of imipenem-resistance among *A. baumannii* isolates was 99%. This rate is very high compared to resistance rates of clinical isolates reported few years ago from Jordan and neighboring countries which ranged between 90% to 64% [3, 31, 32, 33]. A previous Jordanian study has also indicated that majority of *A. baumannii* isolates from environmental and clinical sources was multidrug resistant, except for colistin and tigecycline similar to the case in other countries such as Lebanon [3, 32].

Carbapenems are frequently used as the drugs of choice in treatment of infections caused by MDR Gram-negative bacteria including *A. baumannii*. Increased resistance to carbapenems in *A. baumannii* has raised special concerns during the last decade, especially since it is associated mostly with the production of acquired carbapenemases belonging to either carbapenem-hydrolyzing OXA-type class D β -lactamases or class B metallo- β -lactamases [32, 35-36]. The results of this study demonstrate that all representative examined 45 MDR *A. baumannii* isolates were 100% positive for genes of *blaOXA-51*, and *blaOXA-23*, but none of the isolates was positive to *OXA-24* or *OXA-58*. A recent Jordanian study carried at the Jordan University Hospital also showed that all examined *A. baumannii* isolates harbored a *blaOXA-51* gene, 58% has a *blaOXA-*

23gene, and 38.8% has a *blaOXA-24* gene [3]. It is well known that *blaOXA-51* gene, is an enzyme that naturally exists in *A. baumannii* and has very weak carbapenem hydrolyzing activity [37]. While the presence of plasmid-borne *blaOXA-23* and *blaOXA-58* genes have been shown to contribute significantly to carbapenem resistance in *A. baumannii* worldwide [38]. However, the distribution of carbapenem-hydrolyzing oxacillinases varies among regions and hospitals [3, 32-35, 38]. A study used multivariate analysis demonstrated that the risk factors for acquisition of MDR *A. baumannii* were not related specifically to presence of cancer, but rather due to exposure of patients to health care procedures, especially dialysis, antibiotic treatment and length of stay in intensive care unities [4, 7].

The present study also demonstrates that all *A. baumannii* isolates were susceptible to colistin. This result is similar to most recent studies from neighboring countries which has reported high susceptibility rates of clinical *A. baumannii* isolates to colistin [2-3, 25, 32, 35]. However, with an increase of using colistin to treat carbapenem-resistant *A. baumannii* infections, colistin resistance could be later emerged [39].

This study revealed that 37 of the 45 (82%) MDR *A. baumannii* isolates harbored class-1 Integrations (Table 5). Previous studies from Jordan has demonstrated that class 1- integrations is frequently found in clinical and environmental isolates of Gram-negative bacteria such as *A. baumannii* and *E. coli* and are often associated with their MDR strains [28, 40]. The presence of the integrase gene in the majority of our *A. baumannii* clinical isolates in association with other resistance markers provided a strong evidence that these isolates have the potential for acquisition more antimicrobial resistance genes in future. It has been often observed that MDR *A. baumannii* strains involved in hospital outbreaks have carried class 1 integrations [41-42].

The ERIC-PCR analysis as shown by dendrogram demonstrated that few *A. baumannii* isolates might

have been circulated among cancer patients (Figure 1). Further testing of these isolates using multilocus sequence typing (MLST) or DNA sequencing could confirm this result.

In conclusion, the results of this study demonstrate that body colonization with *A. baumannii* increased in association with antibiotic treatment of cancer patients. The majority of *A. baumannii* are carrying a wide spectrum of genetic resistance factors, especially many important types of *blaOXA*-carbapenemases which will limit using valuable drugs in treatment of *A. baumannii* invasive infection.

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Conflicts of interest

There is no conflict of interests

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