GENE EXPRESSION of *bla*$_{OXA-51-like}$ and *bla*$_{OXA-23}$ in RESPONSE to β-Lactam ANTIBIOTIC IN CLINICALLY ISOLATED *ACINETOBACTER BAUMANNII* AND *ACINETOBACTER LOWFFII* FROM URINE SAMPLES

H. H. Al-Haideri
Lecturer
Dept. of Bio., Coll. of Sci. for Women, University of Baghdad, Baghdad, Iraq
hhmalhaideri@gmail.com

ABSTRACT

In this study, two isolates *A. lowffii*, and *A. baumannii* were obtained clinically, and genomically identified by 16S rRNA assessment with the accession number (MH685113.1 and MH685112.1) for *A. lowffii* and *A. baumannii* respectively. The sensitivity profile of the isolates was variable, and *A. baumannii* was the most resistant strain towards a wide range of antibiotics, and it did not show any growth defect in the presence of β-lactam antibiotic, in comparison with *A. lowffii*. We identified that the *bla*$_{OXA-23}$ gene was responsible for imipenem resistance in *A. baumannii*, whereas, *bla*$_{OXA-51-like}$ was moderately confer resistance towards *A. lowffii* which lack of *bla*$_{OXA-23}$. This was determined when the isolates were subjected to qRT-PCR. We identified that the *bla*$_{OXA-23}$ gene was increased about 1-fold in the presence of imipenem, whereas, *bla*$_{OXA-51-like}$ did not increased in comparison to the control. Bioinformatic analyses revealed that *bla*$_{OXA-23}$ is located in the cytoplasm, and *bla*$_{OXA-like\ 51}$ is located in the periplasm. Our hypothesis suggests that *bla*$_{OXA-23}$ gene have a major contribution in the outbreak of multidrug resistance *Acinetobacter* species.

Key words: *Acinetobacter* spp., imipenem, qRT-PCR

التعبير الجيني لـ bla$_{OXA-51-like}$ و bla$_{OXA-23}$ كأستجابة للمضادات الحيوية الحاوية على β-Lactam في بكتريا الراكدة البومانية *Acinetobacter baumannii* والراكدة اللوفية *Acinetobacter lowffii* المعزولة سريرياً

هالة هيثم محمد علي
مدرس
قسم علوم الحياة ، كلية العلوم للبنات ، جامعة بغداد ، بغداد ، العراق

المستخلص

تم الحصول في هذه الدراسة على اثنين من عزلات بكتريا *A. lowffii* و *A. baumannii* سريريا من عينات الادرار، وتم تحديدها جينيا بواسطة rRNA 16S مع رقم الانضمام (MH685113.1 و MH685112.1) لـ *A. lowffii* و *A. baumannii* على التوالي. كان مستوى الحساسية للعزلات متغايرًا ، وكانت بكتريا الراكدة البومانية *A. baumannii* أكثر السلالات المقاومةً لمجموعة واسعة من المضادات الحيوية، ولم يظهر أي خلل في نمو البكتريا مع وجود المضاد الحيوي الحاوي على β-lactam ، بالمقارنة مع بكتريا *A. lowffii*. تم تحديد ان جين *bla*$_{OXA-23}$ كان مسؤولا عن مقاومة المضاد الحيوي الاميبينيم imipenem في بكتريا *A. baumannii* ، في حين أن جين *bla*$_{OXA-51-like}$ كان يعطي مقاومة متوسطة في بكتريا *A. lwoffii* التي تفتقر إلى جين *bla*$_{OXA-23}$. وتم تحديد ذلك عند اخضاع العزلات لل qRT-PCR ، وحدد أن التعبير الجيني لجين *bla*$_{OXA-23}$ زاد بحوالي ضعف واحد في وجود مضاد imipenem ، في حين أن جين *bla*$_{OXA-51-like}$ لم يزداد بالمقارنة مع السيطرة. وكشفت التحليلات المعلوماتية الحيوية أن جين *bla*$_{OXA-23}$ موجود في السايتوبلازم، بينما يقع جين *bla*$_{OXA-51-like}$ في الجدار الخلوي (الجبلة المحيطة) . نتائجنا اظهرت ان الجين *bla*$_{OXA-23}$ في أنواع بكتريا *Acinetobacter* لديه مساهمة كبيرة في تفشي المقاومة للعديد من للأدوية .

الكلمات المفتاحية: بكتريا الراكدة، الاميبينيم، qRT-PCR

***Received:18/12/2018, Accepted:24/4/2019**

INTRODUCTION

Acinetobacter spp. is a non fastidious Gram-negative bacteria, belongs to gamma protobacteria, nonfermenting, stringency aerobic and non motile. It was known to be positive to catalyse and negative to oxidase, in addition to the high GC contents (29). The genus *Acinetobacter* comprises of 31 well known published species that predominantly exist in nature and mainly associated with nosocomial infection, urinary tract infection, skin and other soft tissues, and in bloodstream (13) The identification and characterization of *Acinetobacter spp* by molecular technique like 16S-23S rRNA gene spacer region was more accurate and reliable, in addition to the method based on *rpoB* gene sequence (5, 28,30). *A. lowffii*, amongst the *Acinetobacter* spp that generally isolated from blood, and may associated with bacteremia, which resulted from skin contamination (44). Moreover, *A. baumannii* is another species which is mainly associated with nosocomial infection and important opportunistic pathogen acquired infection, especially in intensive care unit (54). Interestingly, *A. lowffii* is considered to be normal flora of some parts in the human bodies like skin, oropharynx and perineum of non infectious human (26). Recently, resistance of pathogens to multiple classes of antibiotics is of great concern to the scientists. *Acinetobacter* species has been known for their ingenuin resistance to antibiotics, and also for their ability to acquire genes encoding determinants, and other mechanisms that involved β-lactamases production and aminoglycoside-modifying enzymes (2). Moreover, the presence of multidrug-resistance (MDR) *Acinetobacter spp*. in long-term care facilities and in acute care hospitals has been reported (36). The overcome and persistence of MDR species is patient care unit is due to the ability of patient to get the infection, or to the presence of patients that already colonized or infected with these organisms, and also lack of infection control procedures (2). Nevertheless, *Acinetobacter spp*. possesses several mechanisms that mediate antibiotic resistance. One of the most potential ways is the antimicrobial inactivating enzymes, which includes a wide spectrum of β-lactamases that degrade and confer resistance to penicillins, cephalosporins and carbapenems, however, cephalosporins can be repelled by chromosomally encoded gene *AmpC* (55). According to amino acid sequence consensus, these β-lactamases are classified into four major molecular classes; A, B, C and D, where A, C and D are inactivate the β-lactam ring via active catalytically serine residue, like *AmpC* (59). On the other hand, zinc is required for catalyst the activity of β-lactamase Class B, which is a metallo-enzyme and exhibits different mechanism of resistance (32). Amongst these enzymes, OXA-51 was found to be involved in carabapenem resistance, when it was characterized form two isolates of *A. baumannii* clones in Argentina (5). OXA-23 was firstly mentioned in 1995, and belongs to β-lactamase Class D or Carabpenem-hydrolyzing class D β-lactamase (CHDL) that confers resistance towards imipenem and carbapenem. Because it was reported mainly in *A. bumannii*, it was formally named as ARI-1 (59,60). Moreover, antibiotics can be repelled by efflux pump system, which are able to remove a wide range of antibiotics out of the bacterial cell (2). Furthermore, antibiotic resistance genes can be acquired either from other organisms or from mutant construct which leads to develop the resistance phenotype over time in *Acinetobacter spp.* In a comparative genomic study of an epidemic and MDR *Acinetobacter* strains isolated in France, revealed that 45 resistance gene-containing resistance island, were acquired from *Pseudomonas*, *Salmonella* and *Escherichia* genera (17). In this study, we demonstrate that the expression of blaOXA-like 51 and blaOXA-23 is variable in response to imipenem addition in *A. baumannii* and in *A. lowffii*. In addition, the ability of these isolates to survive under different concentrations of β-lactam drug was examined. We also determined the localization pattern of these enzymes in *Acinetobacter* isolates.

MATERIALS AND METHODS

Bacterial Isolates Media and Growth Conditions

The bacteria were clinically isolated from urine and identified as *A. lowffii, A. baumanni* by normal Vitek 2 system (bioMerieux). The isolates were routinely subcultured either on

Mackonkey agar and on nutrient agar (NA) plates at 37 ℃ every 24 hr and under aerobic conditions. For liquid cultures, the isolates were inoculated in Mueller Hinton (MH) broth and incubated aerobically at 37℃ for 24 hr with gentle shaking. Bacterial growth curve was performed either under normal conditions, or with the presence of antibiotic. In both cases, bacterial growth was monitored by measurement of OD_{600} against the relevant media as a control (blank). The growth was observed every hour started from $OD_{600}\approx0.1$ to approximately $OD6_{00}\approx1.5$.

DNA Manipulation and Isolation, 16S rRNA Sequencing, PCR

Genomic DNA from isolates was isolated using Wizard Genomic DNA purification Kit (Promega), and according to manufacturer's instructions. In order to confirm the identification of *Acinetobacter* species, 16S rRNA sequencing was required. The amplification and sequencing of 16S rRNA was performed according to (33), with some modification. The PCR amplification reaction of interested genes was carried out in a 25µl reaction containing 12.5µl of Green Master Mix, 1ul of 10 pmol/µl of forward and/ or reverse primer (Table 1), 2 µl of genomic DNA template. The volume was completed to 25µl by adding nuclease-free water. PCR reactions were set up as detailed in manufacturers' guideline with a modification as necessary. PCR products were resolved on 1.5 % agarose gel, and visualized by a UV transilluminator and the image was captured by digital camera (Canon, US).

qRT-PCR Analyses

Acinetobacter cultures were grown in triplicate in MH broth under normal aerobic conditions for 24 hr. The growth was adjusted to an OD_{600} nm of 0.1 before 20 µg of imipenem was added. The growth was monitored till mid phase (OD_{600} nm of 0.5) and the RNA was extracted directly from the cultures using the SV total RNA isolation system (Promega, US) according to the manufacturer instructions. Similar was done with cultures without imipenem as a control. Primers used in RNA extraction are listed in (Table 1).

Table1. nucleotides used in this study

Primers	Primer sequence 5`-3`
27-FWD	AGA GTTTGATCMTGGCTCAG
blaOXA-like51 FWD	TAATGCTTTGATCGGCCTTG
blaOXA-like51 R	TGGATTGCACTTCATCTTGG
blaOXA-23 FWD	GATCGGATTGGAGAACCAGA
blaOXA-23 R	ATTTCTGACCGCATTTCCAT
blaOXA-51 RT-FWD	GAAGTGAAGCGTGTTGGTTATG
blaOXA-51 RT-R	GCCTCTTGCTGAGGAGTAAT
blaOXA-23 RT-FWD	CTTTCTGCAGTCCCAGTCTATC
blaOXA-23 RT-R	GCTGTCCAATTTCAGCATTACC

Antibiotic Sensitivity Assay

The susceptibility assay of *Acinetobacer* isolates was conducted by disc diffusion test (Kirby-Bauer), according to (1). The antibiotics used in this study were selected according to their target and mode of action. The sensitivity test was repeated three independent times.

Preparation of Periplasm by Osmotic Shock Procedure

The periplasm was isolated according to Nossal *et al*, (1966) (40) procedure, with some modifications. *Acinetobacter* broth cultures were aerobically grown in normal MH broth and MH broth containing 20 µg imipene. The cells were harvested and resuspended in 10 ml STE buffer, and then incubated with gentle shaking for 30 min at room temperature. The cells were pelleted and then resuspended in 5 ml ice-cold 10 mM Tris-HCl pH 8.0, followed by incubation with gentle shaking at (2000 xg , 2 hr, 4℃). Finally, periplasm preparation solution was obtained by centrifugation at (15,000 xg, 25 min, 4 ℃) and then either stored at -20 ℃, or mixed with 1X SDS loading dye and electrophoresed on 12 % SDS-PAGE.

Isolation of Cytoplasm

Acinetobacter broth cultures were aerobically grown in 100 ml MH broth for 20 hr at 37 ℃ with gentle shaking. The cells were harvested by centrifugation at (10,000 xg, 10 min, 4 ℃) and resuspended in 10ml PBS (1X) buffer. Cell suspensions were subjected to sonication (6 x 15 s pulses at a frequency of 16 µ amplitude in a Soniprep 150 ultrasonic

disintegrator, Q Sonica), and centrifuged (10.000 xg, 15 min, 4 ℃) to remove cell debris. The supernatant was either stored at -20 ℃ for further used, or mixed with 1X SDS loading dye and electrophoresed on 12 % SDS-PAGE.

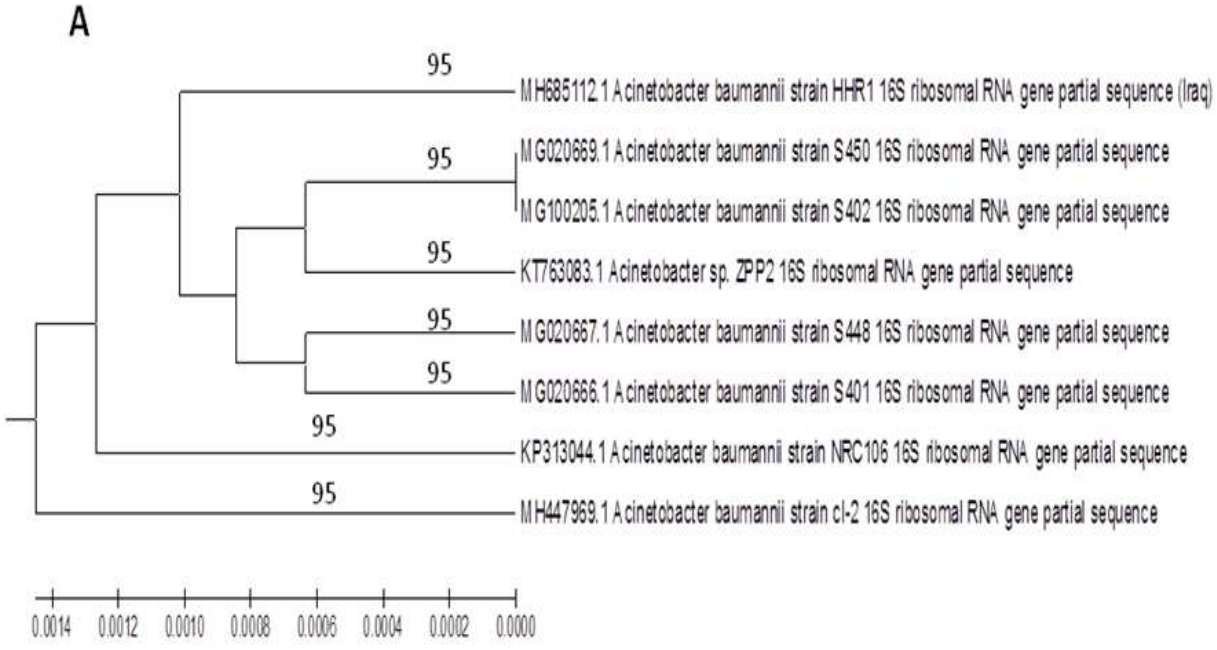
blaOXA-23 structure modelling

A predicted structure model of blaOXA-23 was built in Swiss-Model server (https://swissmodel.expasy.org) using blaOXA-225 as a template to compare between both structures, and under the SMTL ID 4x55.1.A. The amino acid sequence of blaOXA-23 in our isolate was analysed by Swiss-Model Server, and the structure was superimposed with the relative template. The PDB entry 4x55.1.A is available in: https://swissmodel.expasy.org/interactive/8nCF4W/

RESULTS AND DISCUSSION

Dendogram of *Acinetobacter* isolates

Since the 16S rRNA gene was developed as a new standard for identifying bacteria, a wide range of bacteria has been determined by 5S, 16S and 23S rRNA gene sequences (20,56). The comparison of 16S rRNA gene is done among all bacteria and archeobacteria, and within eucaryotes by 18S rRNA gene (10). In general, the 16S rRNA is universal in bacteria, large, about 1,550 bp in length, with variable and conserved regions, and has enough interspecific polymorphisms of 16S rRNA to confer distinguishing measurements. The conserved region at the beginning and at the end of 16S rRNA gene is almost used as a template for primers, whereas, a comparative taxonomy is provided by a variable region sequence (10). The clinically isolated *A.baumannii* HHR1 possesses 16S rRNA gene with 794 bp, which is then used to generate the dendrogram of phylogenetic tree (Figure S1). *A. lowffii* HHR2 is shown to have 1354 bp 16S rRNA gene (Figure S2). The sequence alignment of entire gene of HHR1 is showed 95 % similarity with the relative isolates available in the NCBI (Figure 1 A), while, the degree level of similarity of *A. lowffii* HHR2 with the corresponding isolates is about 94 % (Figure 1 B), as it was showed by tree relationship. Using of 16S rRNA gene to distinguish between species was recorded in many studies, where *Niesseria meningitides* could be tracked by 16S rRNA (50,3) The variability in the length of 16S rRNA initial gene in both isolates is depend on the sequenced region, and in most cases, initial base pairs provide a comparable data in clinical isolates (25), whereas, it was reported that describing a new species required sequencing of entire 16S rRNA gene (48,50).



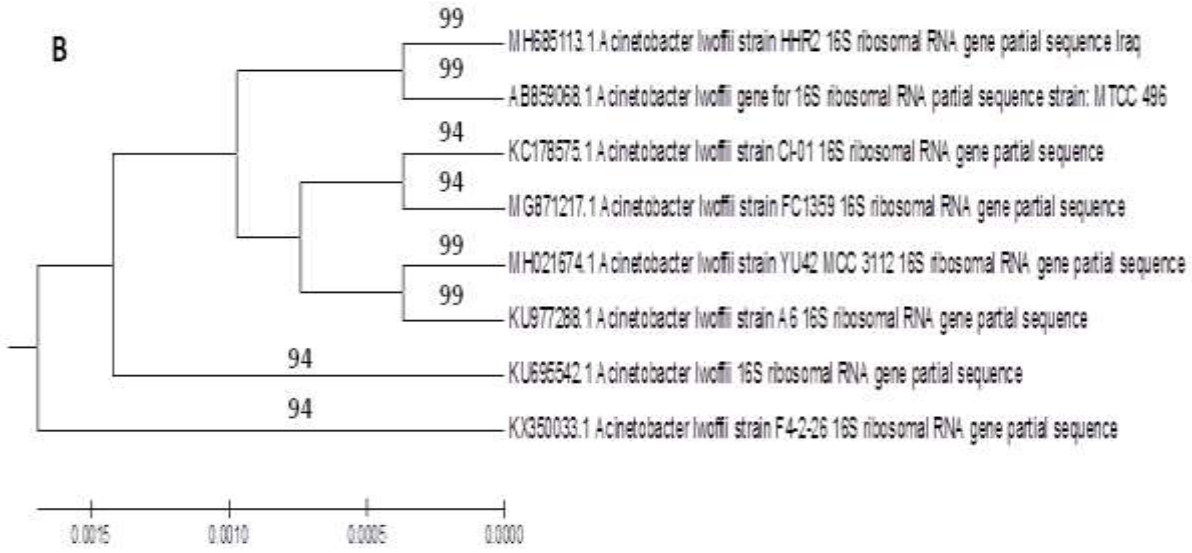


Figure 1. Phylogenetic relationship constructed for *A.baumannii* and *A. lowffii*. (A): the neighbour-joining algorithm showed the overall degree of gene similarity of *A. baumannii* between isolates depicted with species classification. (B): the tree revealed the position of *A. lowffii* between the relative isolates coincided within the genus *Acinetobacter*

Identification of *bla*$_{\text{OXA-51-like}}$ and *bla*$_{\text{OXA-23}}$ in *A. baumannii* HHR1 and *A. lowffii* HHR2

The genetic map of *A. baumannii* showed that it possesses a robust protection enzyme clusters associated with carbapenems, which so called OXA-type carbapenemases. OXA-23, OXA-24, OXA-58, and OXA-51 among these groups, which might be either chromosomally or plasmid encoded genes, and they are defined according to the basis of sequence comparison (22,53), and most notably that transferable imipenem resistance can be conferred by OXA-23 (22). To assess the existence of OXA-51 and OXA-23 genes in isolates, *bla*OXA-51 and *bla*OXA-23 were PCR amplified using blaOXA51-FWD/blaOXA51-R, blaOXA23-FWD/blaOXA23-R and blaOXA361-FWD/blaOXA361-R primers respectively (Figure 2 A, B) (Table 1), and genomic DNA of *A. lowffii* and *A. baumannii* were used as a template. The PCR reactions were used in DNA electrophoresis and the DNA products were electrophoresed and visualized under the UV light (Figure 2 C). Interestingly, the results showed that the *bla*$_{\text{OXA-like 51}}$ (353 bp) was found in *A. lowffii* and *A. baumannii*, whereas, the *bla*$_{\text{OXA-23}}$ (501 bp) was found only in *A. baumannii* (Figure 3C). The *bla*$_{\text{OXA-51-like}}$ is may be a sole carbapenem resistance in *A. lowffii* and *A baumannii* only if IS*Aba1* is located upstream *bla*$_{\text{OXA-51-like}}$, as the former (IS*Aba1)* provides promoter for this gene (Figure 2B) (58). Unlike, *bla*$_{\text{OXA-23}}$ is found only in *A. baumannii*, and may be chromosomally located and flanked by one or two copies of IS*Aba1* (Figure 2A) (11, 37).

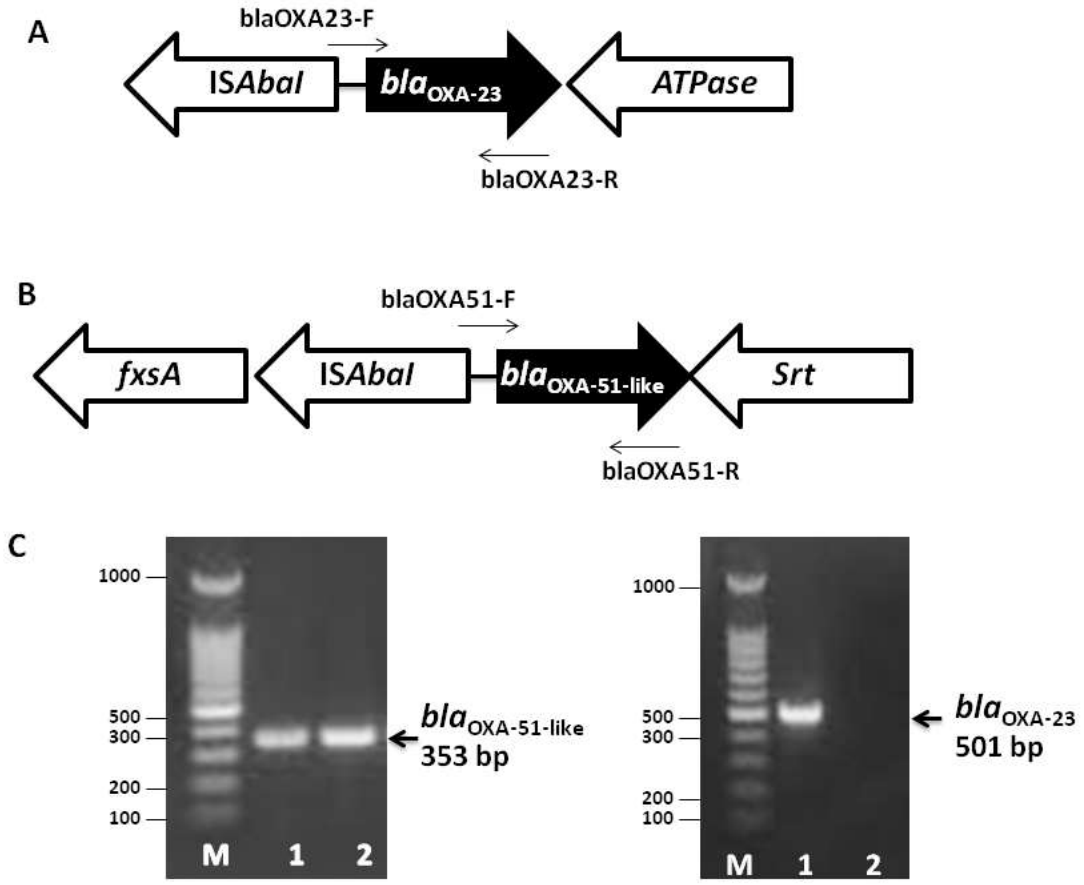


Figure 2. Identification and gene organization map of blaOXA genes in *A. baumannii* HHR1 and *A. lowffii* HHR2. *(*A); gene organization and amplification of bla_{OXA-23} using the specific primer pairs in *A. baumannii,* (B); $bla_{OXA-51-like}$ amplified by PCR from the genomic DNA of *A. baumannii* and *A. lowffii,* (C); DNA agarose gel of *bla*OXA variant genes by PCR amplification. Lane1; PCR amplification product of $bla_{OXA-51-like}$ (353 bp) in *Acinetobacter spp* and lane 2; bla_{OXA-23} (501 bp), Lane M; HypperLadder™ I Molecular Marker (Biolone). The amplified genes were subjected into 2 % agarose gel and electrophoresed before visualized by UV light source.

Antibiotic sensitivity phenotype of *Acinetobacter* isolates

In order to investigate the antibiotic susceptibility pattern of *A. lowffii* HHR1 and *A. baumannii* HHR2, antibiotics were selected according to their target and mode of action; gentamycine (aminoglycoside), imipenem (β-lactum), ciprofloxacime (floroquinolone), Azithromycine (macrolids). In addition, tetracycline, doxycycline and rifampicine were also used in this study. The result showed that the drug resistance pattern of *Acinetobacter* isolates is highly variable among the species, thus reflect the variation in the mechanism that mediate drug resistance. In (Figure 3), it was clearly obvious that *A. baumannii* was significantly resistant to the most of antibiotics, compared with *A. lowffii* which exhibits a remarkable inhibition toward these antibiotics. In addition, *A. baumannii* was completely resistant to trimethoprim, aminoglycoside, floroquinolone, samacyclin and doxycline, and slightly sensitive to tetracycline and macrolide, whereas, *A. lowffii* was susceptible to these drugs. However, both strains were resistant to rifampicin. Moreover, *A. lowffii* exhibits a moderate resistance toward floroqunolone, samacycline, tetracycline and macrolide. The β-lactam antibiotic (imipenem) was able to inhibit the growth of *A. lowffii* compared to *A. baumannii*, where the former showed a significant inhibition (85 mm), and also slightly attenuate the viability of *A. baumannii*. The majority of *Acinetobacter* spp. is considered as a multi-drug resistance which due to their ability to acquire genes encoding resistance determinants, as well as up-

regulation of efflux pump compartments and enzyme-target modification (18). The susceptibility of *Acinetobacter* species towards floroquinolone (ciprofloxacime) was variable, where *A. baumannii* exert a complete resistance. Like other Gram-negative bacteria, *Acenitobacter* acquired resistance to floroquinolones by point mutations in DNA gyrase or DNA topoisomerase IV. The amino acid substitution of *gyr*A (Ser83 to Leu 83) and *par*C (Ser80) have been reported to decrease the binding with the drug (53). Mutation occurs firstly in *gyr*A and accompanied by mutation in *par*C, and thus suggests that *gyr*A is the target of floroqunolone (6). Aminoglycosides (gentamycine) were also exhibit a fluctuated effect on *A. lowffii* compared to *A. baumannii*. The resistance mechanism of *A. baumannii* to gentamycine is performed by efflux pump, which play an important role to extrude these compounds out of the cells (13, 31, 49). Among different antibiotics, imipenem is the most effective antibiotics against *A. lowffii*, whereas, *A.baumannii* was slightly inhibited. This is due to the ability of *A. baumannii* to produce class D β-lactamases like $bla_{\text{OXA-51}}$, $bla_{OXA\text{-}58}$ and $bla_{\text{OXA-24}}$ (33).The gene encoding oxacillinases (OXA) have been reported in several *Acinetobacter* species, where $bla_{\text{OXA-51}}$ like gene is the naturally occurring gene in *A. baumannii* and contributes in resistance mechanism. Noteworthy that the serine carbapenemase ($bla_{\text{OXA-23}}$) was firstly identified in *A. baumannii* isolated from blood culture in Eidenbergh (16).

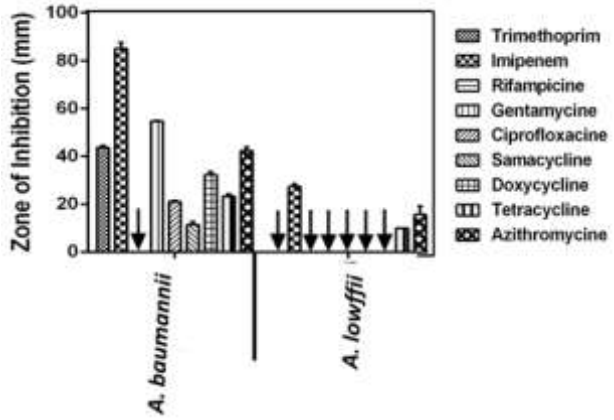


Figure 3. Antibiotic resistance profile of *Acinetobacter* isolates

Overnight cultures of *A. lowffii* HHR2 and *A. baumannii* HHR1were diluted up to 10^{-5} cfu/ml. A 100 µl was spread out on MH agar plates by a sterile cotton swab, and prepared antibiotic discs were placed on the plates with equal distance. The plates were incubated aerobically at 37 ℃ for 24 hr, and the zone of inhibition was calculated. The black arrow means that there is no inhibition zone. Error bars represent the standard deviation from the mean of three independent biological replicates.

Effect of β-lactam imipenem on the Viability of *Acinetobacter* isolates

Among all examined antibiotics, *Acinetobacter* isolates exhibit variable response to imipenem, so imipenem was selected to determine its effect on the viability of *Acinetobacter* isolates, the growth of all strains was monitored in the presence of different concentrations of imipenem (Figure 4), and the results showed that *A. lowffii* HHR2 was strongly inhibited by imipenem , particularly at 30 µg ml^{-1} after 9 hr incubation (Figure 4B). In contrast, *A. baumannii* HHR1 was able to survive under different concentrations ranged from 10-30 µg ml^{-1} (Figure 4C). However, it is worth noted that the growth of both isolates were comparable and reached to the same OD_{600} after 20 hrs incubation (Figure 4A). Till to date, there is no evidence that carbapenem resistance profile in *A. lowffii* is acquired by OXA-23 or OXA-51, however, OXA-51 is a natural occurring CHDL and OXA-143 is intrinsic to *A. lowffii* (16). Carbapenem/Imipenem resistance can be acquired by several mechanisms, such as decrease the affinity of pencilline-binding proteins to carbapenem/ imipenem, decrease the permeability of the outer membrane, repel of β-lactam drugs, as well as β-lactamases activity. Although, most of carbapenem/imipenem resistance in bacteria is attributed to class B β-lactamases, the combination of over-expression of β-lactamase especially AmpC β-lactamase, and reduced permeability, will overcome the resistance to carbapenems (61). To some extent, many researchers stated that OXA-51 was characterized from two clones of imipenem – resistance *A. baumannii* in Argentina (4). High level resistance pattern toward imipenem and meropenem was also observed in nosocomial *A. baumannii* isolates harbouring OXA-51

with MIC >> 8 mg ml^{-1} (21,39). Moreover, the south East clone in the United Kingdom and T clones from hospital in Midland region are associated with only *bla*$_{OXA-51}$ and carbapenem resistance. In contrast, two clones (OXA-23-1, OXA-23-2) have been detected in hospitals in the United Kingdom, were show resistant pattern toward imipenem and meropenem, and both possess *bla*$_{OXA-51-like}$ along with *bla*$_{OXA-23}$ (4). Similarly, (60) reported that the clones of *A. baumannii* isolated from United Kingdom hospital are resistance to carbapenem only if ISA*ba1* is located adjacent to *bla*$_{OXA-51-like}$, whereas, others with no ISA*ba1* are susceptible. In the mean time, the clones producing *bla*$_{OXA-23}$ and having ISA*ba1* were consistently resistance. In another study also confirm that the insertion sequences ISA*bA1* and ISA*bA3* provide strong promoter to *bla*$_{OXA-23}$ and *bla*$_{OXA-58}$ genes respectively, when it is located to the upstream region for both genes and increase the production of the genes, thereby increase the resistance to carbapenems (20). A question is raised up whether the resistance to imipenem is due to *bla*$_{OXA-51-like}$ or *bla*$_{OXA-23}$ or both, by taking the above findings stated that *A. baumannii* HHR 1 is more resistance to imipenem than that of *A. lowffii* HHR2 where the later have only OXA-51 carbapenemase. To unequally evacuate the mechanism mediated resistance to imipenem, (50) revealed that OXA-50, OXA-51 and OXA-60, as a part of OXA-type carbapenamases, are naturally occurring enzymes in *A, baumannii*, *Psuedomonas aeruginosa* and *Riktsia pickettii*, however, some *A. baumannii* strains lack *bla*$_{OXA-51}$ family genes, and (22,9) clearly showed the ability of OXA-51 to resist imipenem if linkage with ISA*ba1*. Furthermore, (27) documented that strains harboring *bla*$_{OXA-23}$ exhibit imipenem MICs one to two dilution higher than that of those not carrying *bla*$_{OXA-23.}$ In *A. lowffii*, the genomic DNA clearly revealed that it producing *bla*$_{OXA-51-like}$ gene but it was so susceptible to imipenem, even the gene is flanked by ISA*ba1*, and *A. baumannii* harbouring both genes with ISA*ba1*. Our results conclude that the presence of *bla*$_{OXA-51-like}$ gene in all isolates did not mainly confer resistance to imipenem, whereas, *bla*$_{OXA-23}$ like gene in *A. baumannii* clearly refers to the major contribution to imipenem resistance.

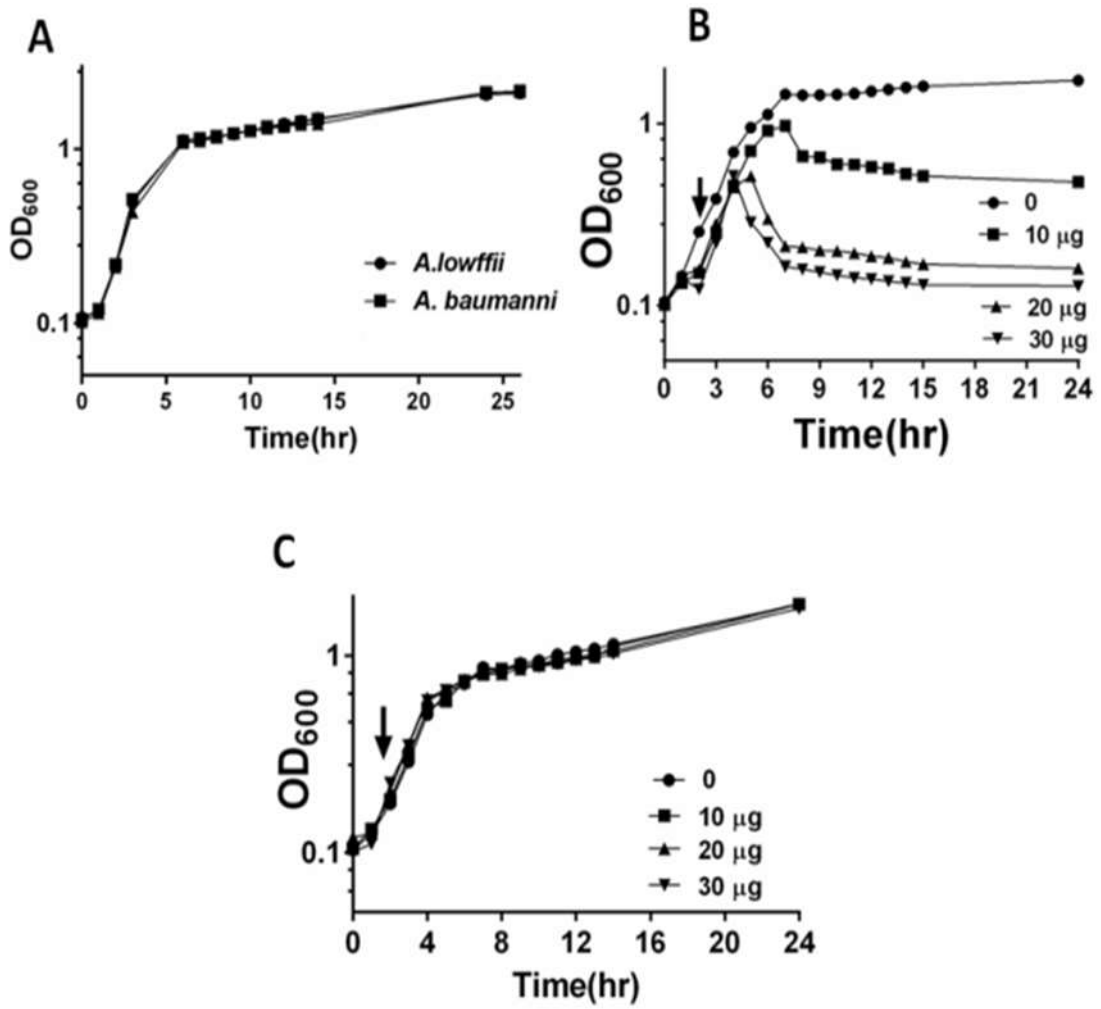


Figure 4. Effect of imipenem on *Acinetobacter* isolates growth curve. (A): Growth curve of *A. lowffii* HHR2and *A. baumannii* HHR1 under normal conditions, (B) growth of *A. lowffii* HHR2 in the presence of imipenem and (C) *A. baumannii* HHR1 growth supplied with imipenem. In both cases, cultures was prepared and serial concentrations of imipenem were added to each culture (represented by black arrow in B and C), otherwise the culture was left without imipenem (A). In all cases, the growth was monitored every hour until the growth reached to the late stationary phase. The experiments were repeated three times and the data represent one of the three independent biological replicates.

Gene-Expression analyses of *bla*$_{\text{OXA-23}}$ in *A. baumannii* HHR1 *and A. lowffii* HHR2

Hence, the imipenem was able to significantly reduce the viability of *A. lowffii* HHR2, and with no effect on the *A. baumannii* HHR1 growth, qRT-PCR was performed to evaluate the expression of *bla*$_{\text{OXA-51-like}}$ and *bla*$_{\text{OXA-23}}$ genes in response to imipenem, however, up regulated of β-lactamases is not critically compromised the increase/ decrease the level of carbapenem resistance to its substrate. RNA was extracted from *A. lowffii* HHR2 and *A. baumannii* HHR1during mid-log growth under two conditions (-/+ imipenem). The primers were used to amplify both *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-51-like}}$ in the presence of 20 µg impenem (Figure 5A, B). The expression of *bla*$_{\text{OXA-51-like}}$ with imipenem is shown relative or less to the expression levels normalized to 1.0-fold under normal condition in both strains (Figure 5 C, D),whereas, the expression of *bla*$_{\text{OXA-23}}$ was increased to 1.0-fold compared to the control (Figure 5C). These results confirmed the vital role of *bla*$_{\text{OXA-23}}$ in B-lactam antibiotics. The identification of *Acinetobacter* species that particularly isolated from patients is vital and essential. Molecular identification methods such as DNA_DNA hybridization, 16S rRNA gene sequence were widely used as an efficient way to identify

bacteria (34). The nosocomial infection is attributed to *A. baumannii* which develop a robust system to resists wide spectrum of antibiotics worldwide, especially carabpenem and emipenem (19, 38). Generally, $bla_{\text{OXA-51}}$ like gene is weakly expressed, unless, it may up regulated by insertion sequence IS*Aba1*that supply a promoter sequence to increase the copy gene number by change the location from chromosome to a plasmid (7,58). It was reported that OXA-51-like β-lactamse could be deleterious to *A. baumannii* and attenuate the resistance pattern, whereas, the OXA-51-type enzymes forms a large group of CHDLs with significant role in protection (50). Unlike, the plasmid-born $bla_{\text{OXA-23}}$, $bla_{\text{OXA-58}}$ genes are highly up-regulated and associated with IS*Aba1*, IS*Aba2*, IS*Aba3* or IS*18*, and thus lead to high resistance capacity to carbapenems (43). According to the expression pattern of OXA-23, we hypothesized that imipenem is act as a signal to promote the expression of $bla_{\text{OXA-23}}$ by IS*Aba1*copy in *A. baumannii*, but it does not be a good inducer for OXA-51 neither in *A. baumannii* nor in *A. lowffii.*

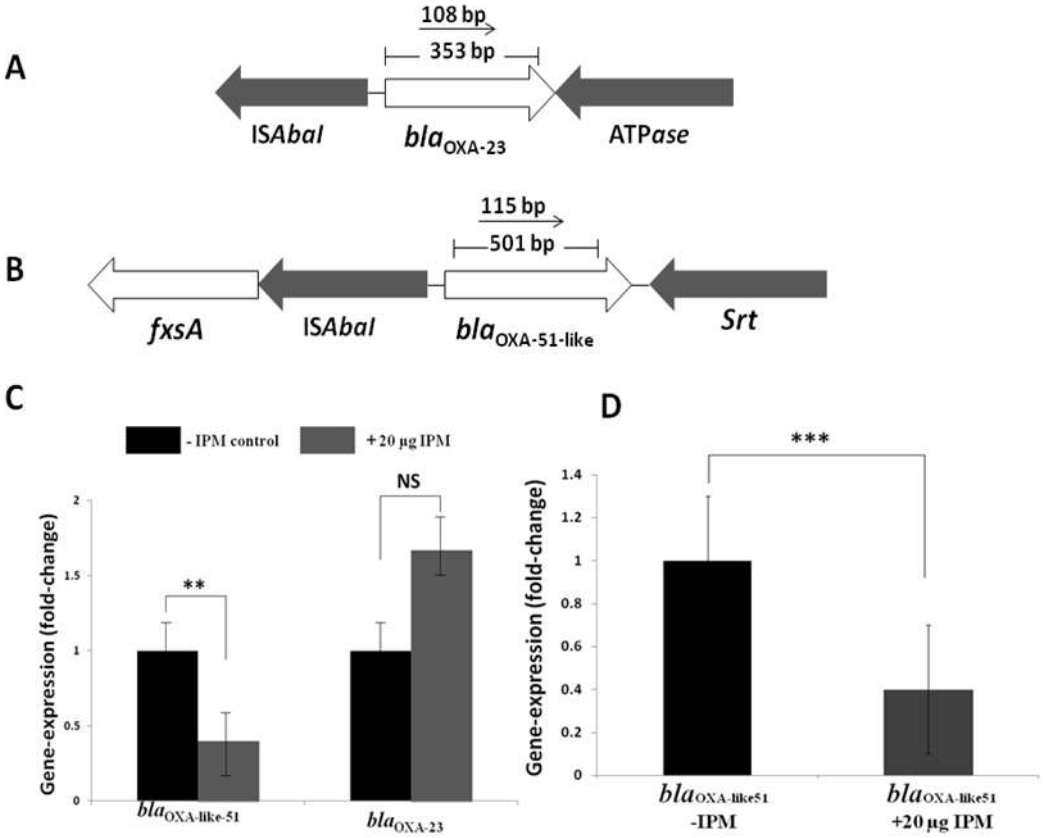


Figure 5. qRT-PCR analyses of $bla_{\text{OXA-like-51}}$ and $bla_{\text{OXA-23}}$ genes and gene organization. (A and B); Gene organization map and neighbourhood of *A. baumannii*HHR1 and *A. lowffii* HHR2 chromosome containing β-lactam encoding genes $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-like-51}}$. The black arrows above each gene refer to the entire PCR products resulted from RT-PCR primers. (C). qRT-PCR analysis of gene expression of $bla_{\text{OXA-like-51}}$ and $bla_{\text{OXA-23}}$ using blaOXA-51 RT-F, blaOXA-51 RT-R, blaOXA-23 RT-FWD and blaOXA-23 RT-R for both respectively in the absence of imipenem (- IPM) and in the presence of 20 µg of imipenem (+20 µg IPM). The data shown are the means and SD from three qRT-PCR reactions, each from three independent cultures grown at both conditions. Statistical significance was determined by Student *t*-test (P<0.01; NS, not significant). The statistical analysis was done by GraphPad Prism.**

Bioinformatic analysis of the sequences for Prediction of cellular localization of blaOXA-51-like and blaOXA-23 in *A. lowffii* HHR2 and *A. baumannii* HHR1 The amino acid sequence of blaOXA-23 and blaOXA-51-like, suggested that they might be located in the cytoplasm and periplasm via Sec system (9) respectively. blaOXA-23 is predicted to have signal peptidase I cleavage site between A_{25} and N_{26} (Figure 7A) (SignalP 4.0) (41). blaOXA-like-51 is predicted to be located in the cytoplasm and have no signal sequence (Figure 6B).

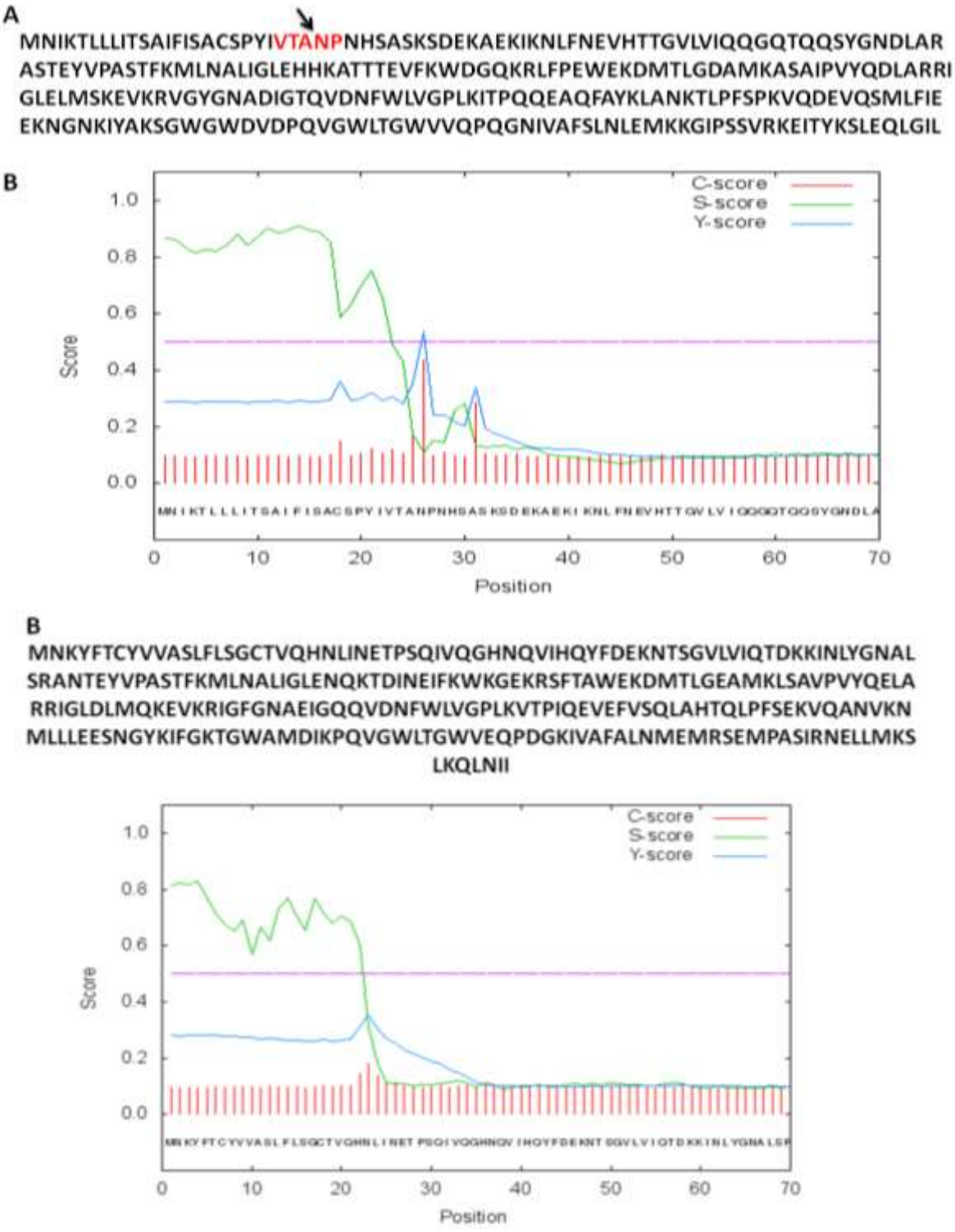


Figure 6. Bioinformatic analysis and prediction of blaOXA protein sequences. (A); the amino acid sequence and the peptidase signal sequence of blaOXA-23. The amino acid sequence shown in red is the predicted signal sequence that will be cleavage by signal peptidase I during translocation across the periplasm. The black arrow refers to cleavage site between A and N. The black sequence is referring to the mature protein start at asparagine residue after cleavage site. (B); the amino acid sequence of blaOXA-like-51. The black sequence is the predicted mature protein. There is no SP according to (SignalP v 4.0).

Localization of blaOXA proteins in the cell envelope of *Acinetobacter* isolates

In order to unequivocally determine the localization of blaOXA proteins, *Acinetobacter* isolates were grown under aerobic conditions with 20 µg imipenem and fractionated into periplasm and cytoplasm fractions, similar assay was performed without imipenem as a control. All fractions were subjected into 12 % SDS-PAGE. The SDS-PAGE (Figure 7) of the cytoplasm fractions showed that blaOXA-51 is located in the cytoplasm and the molecular weight is around 30 kDa in *A. lowffii* HHR2 and *A. baumannii* HHR2 (Figure 7A, C), and it did not show any expression pattern according to imipenem addition. This is clearly indicated that blaOXA-like 51 have no potential role in B-lactam resistance. On the other hand, blaOXA-23 protein is shown to be located in the periplasm with the molecular weight around 30 kDa in *A. baumannii* HHR1 and it is absence in the PM fraction of *A. lowffii* HHR2 (Figure 7B, D). Most notably that blaOXA-23 is slightly exhibits a pattern of expression when *A. baumannii* HHR1 was grown under imipenem stress (Figure 7 D). These results are clearly revealed that blaOXA-like51 is located in the cytoplasm and blaOXA-23 is a periplasmic protein with putative signal sequence, and have an indispensable role in β-lactam drug resistance. These findings are consistence with qRT-PCR results.

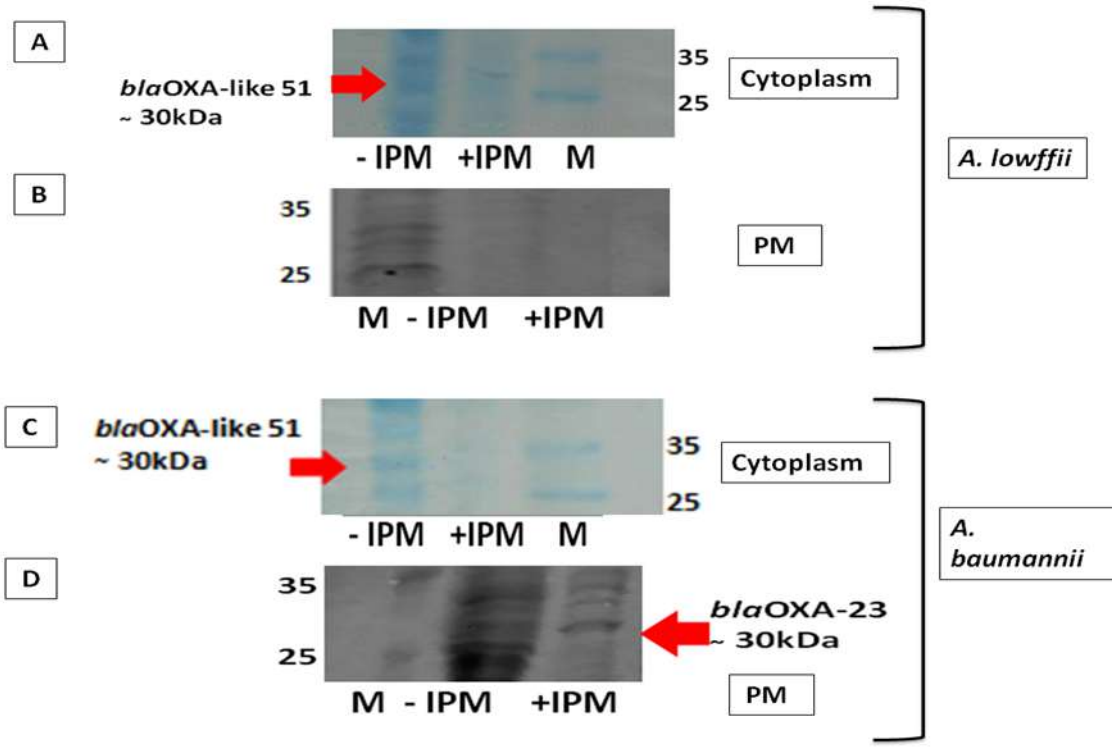


Figure 7. SDS-PAGE of cytoplasm and PM fractions of *Acinetobacter* isolates.

Acinetobacter isolates were grown in MH broth media under aerobic conditions to an OD_{600} of 0.3, and 20 µg of imipenem was added to the strains. The strains were incubated for 24 hr before being fractionated into cytoplasm and periplasm which was prepared by osmotic shock. Control is represented by fractions without imipenem. The fractions were mixed with 1X protein loading dye, and subjected into 12 % SDS-PAGE. (A); Cytoplasm fraction of *A, lowffii*, the red arrow represents the blaOXA-like 51, (B); PM fraction of *A. lowffii*, (C); the cytoplasm of *A. baumannii*, the blaOXA-like 51 is represented by thick red arrow, and (D); is refer to the PM of *A. baumannii* , and the 30 kDa bla OXA-23 is indicated by thick red arrow. Lane-IPM is refers to the PM or cytoplasm fractions in *A. baumannii*; and *A. lowffii* with no addition of imipenem (as a control). Lane +IPM; represents the fractions with imipenem addition. Lane M is the PageRuler™ Plus Unstained Rec. Protein Ladder (Promega).~ 20 µg of protein was loaded into each well.

Structural modelling of blaOXA-23 protein in *A. baumannii* HHR1

The predicted structure of blaOXA-23 was modelled by Swiss-Model Server and superimposed with blaOXA225 according to (35), with some differences. The structure of blaOXA-23 in our isolate is seems to be similar to blaOXA-225 with identity of 99.21, whereas, only 77% similarity with blaOXA-24 has been found. The surface structure of blaOXA-23 protein is composed of uncharged residues and that why this protein does not purified properly by ion exchange (57). The 3D crystal structure of blaOXA-23 (Figure 8 A right) revealed that it composed of 6 β-strands running antiparalel at N-terminal, and wrapped by 7 α-helices near the centre, connected with many turns and short β-strand with long turn at the C-terminal. The differences between blaOXA-225 and blaOXA-23 are between (Lys 82 and Asp 62) in the α-helix 1, and between (Pro225 and Ser205) in the loop at the centre of the active site (Figure 8 B). It was noted that OXA-23 has a long loop connecting β5 and β6, composed of A220, D222 and I225, and thus provides a large loop area contributes to the binding affinity to carbapenem. Unlike OXA-23, OXA-24 has G224 homologues to D222, and G218 and V223 which homologues for A220 and I225 respectively in OXA-23 (24). In our study, OXA-23 has A220, D222 and P225 in the loop connecting β5 and β6. The presence of proline in the loop makes a kink that might have a significant role in the binding affinity not only for imipenem, but also for other drugs. Instead, OXA-225 has a Ser205 which may due to alter the position of the loop near the active site. For specificity and activity of blaOXA-23 to binds and hydrolyse imipenem, the amino acid arrangement of both protein seems to be identical and both have as so called the bridge above the active site (Figure 8 A left). The bridge of blaOXA-23 is distinctive and distinguishes it from that of blaOXA-48, which has a wide open active site (12). The bridge of F110 and Met 221 in blaOXA-23 mediates a tight imipenem binding site, and play an important role in resistance mechanism. Note worthy that blaOXA-24 and blaOXA-40 have an identical bridge across the active site, this suggests that all enzymes use the same mechanism with strong enzyme-substrate binding (47,48).

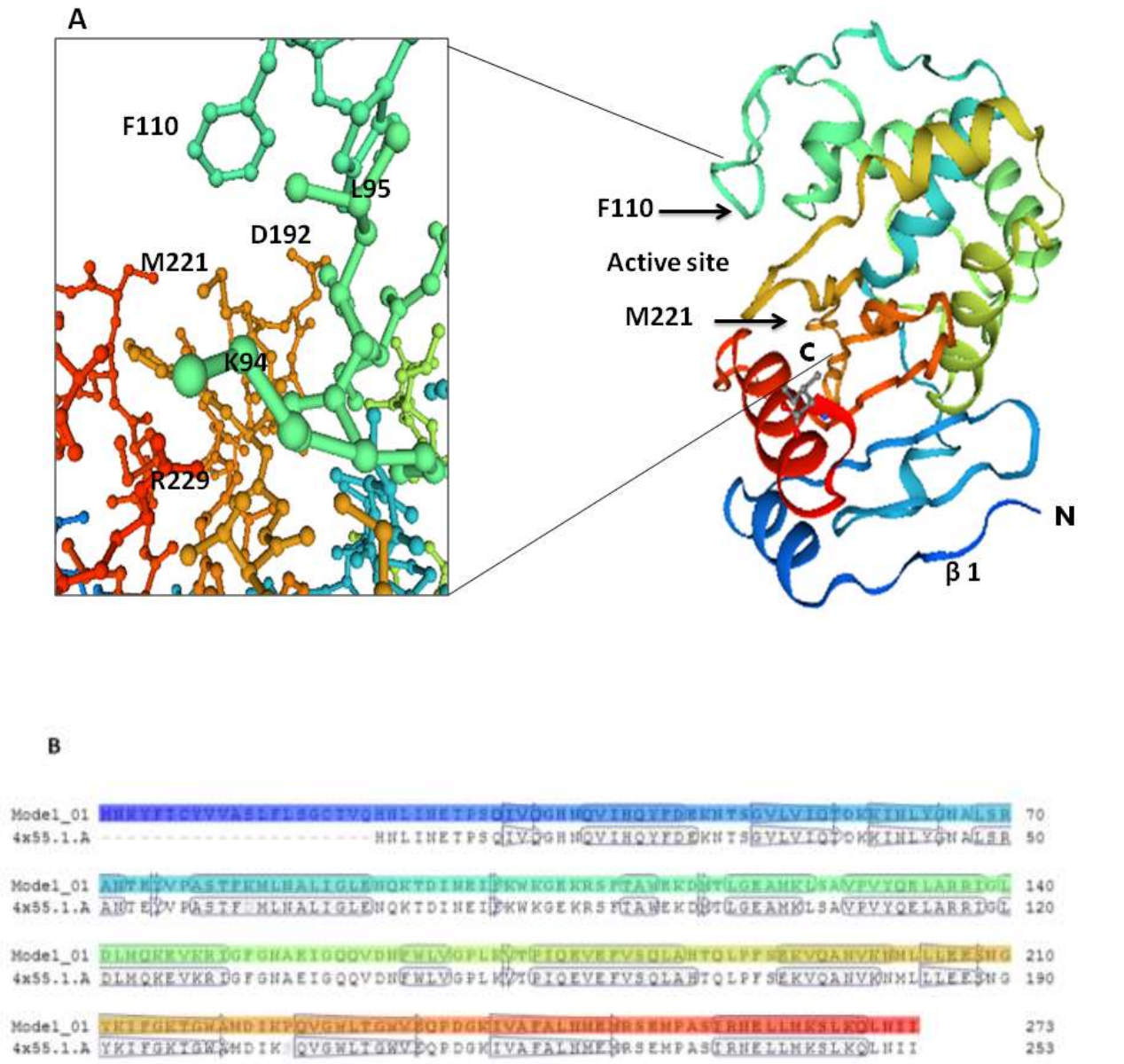


Figure 8. *A. baumannii* HHR1 OXA-23 structure modelling and sequence comparison. (A): Swiss model of blaOXA-23 in *A. baumannii* HHR1 superimposed on the determined structure of blaOXA225 (PDB: 4x55.1.A, [56]). The region of the active site is shown by black arrows, and enlarged with relevant OXA-23 of *A. baumannii* by box region on the left. The ball stick refers to the bridge across the active site of F110 and M221. D192, K94 and R229 are predicted to involve in affinity of imipenem binding site. (B): Sequence alignment of OXA-23 (Model-01) and the relevant OXA-225. The different colour is relevant to the rainbow structure, and β-strand is indicated by blank arrow, and helices by blank box. The figure is adopted by Swiss-Model Server.

In this study, *A. lowffii* HHR2 and *A. baumannii* HHR1 were previously isolated and identified by 16S rRNA, and OXA-51 and OXA-23 genes were PCR screened. The efficiency of these genes to resist imipenem was determined by different aspects. Here, we found that OXA-23 has a major contribution in drug resistance than OXA-51, and the former could be over-expressed by IS elements. This expression is enhanced or up-regulated in the presence of several concentrations of imipenem, where OXA-23 was increased by 1-fold, whereas, up-regulation of OXA-51 was not detected in *A. baumannii*. Another approach confirms that *A. lowffii* is completely inhibited after 24 hr, as OXA-23 is missing. Interestingly, ribbon structure model of OXA-23 showed it has a distinctive bridge across the

active site to facilitate the binding of enzymes with the substrate. The fractionation of both isolates showed that OXA-23 is located in the periplasm and OXA-51 in the cytoplasm, and thus clearly ruled out the significant contribution of OXA-23 in imipenem resistance.

Acknowledgment

I would like to thank Hiba A. Saleh for her enthusiastic efforts to provide us with clinical specimens, and Dr. Safaa A. A. Al-Qaysi for his valuable support throughout my work

REFERENCES

1. Arciola, C. R., D. Campoccia, Y. H. An, L. Baldassam, V. Pirini, M. E. Donati, F. Pegreddi and L. Montanaro 2006. Prevalence and antibiotic resistance of 15 minor Staphylococcal species colonizing orthopaedic implants. Int J Artif Organs 29, 395-401

2. Bonomo, A. R. and D. Szabo 2006. Mechanisms of multidrug resistance in *Acinetobacter* species and *Pseudomonas aeroginosa*. Cid Oxford J 43, S49-S56

3. Bosshard, P. P., S. Ables, R. Zbinden, E. C. Bottger and M. Altwegg 2003. Ribosomal DNA sequencing for identification of aerobic gram-positive rods in the clinical laboratory (an 18-month evaluation). J Clin Microbiol 41, 4134-4140

4. Brown, S., H. K. Young and S. G. B. Amyes 2005. Characterization of OXA-51 a novel class D carbapenemase found in genetically unrelated clinical strains of *Acinetobacter baumannii* from Argentina. Clin Microbiol 11, 4825-4829

5. Brown, S. and S. Amyes 2006. OXA β-lactamase in *Acinetobacter*: the story so far. J Antimicrob Chemother 57, 1-3. doi:10.1093/jac/dki425

6. Charrier, C. A. M. Salisbury, V. J. Savage, E. Moyo, H. Forward, N. Ooi, J. Cheung, R. Metzger, D. Mc Garry, R. Walker, I.R Cooper, A.J. Ratcliffe , N.R. Stokes. 2016. In vitro biological evaluation of novel broad-spectrum isothiazolone inhibitors of bacterial typeII topoisomerases. J Antimicrob Chemother 71, 2831-9

7. Chen, T. L. , Y. T. Kuo, P. R. Hsueh, F. Y. Chang, L. K. Siu, W. C. Ko and C. P. Fung 2010. Emergence and distribution of plasmids bearing the blaOXA-51-like gene with an upstream ISAba1 in carbapenem-resisteant *Acinetobacter baumannii* isolates in Taiwan. Antimicrob Agents. Chemother 54, 4575-4581.

8. Chen, Y. J. Gao, H. Zhang, and C. Ying. 2017. Spread of the blaOXA-23 containing Tn2008 in carbapenem-resistant *Acinetobacter baumannii* isolates grouped in CC92 from China. Fron Microbiol 8, 1-6. doi.org/10.3389/fmicb.2017.00163

9. Chiu, H. Y. H. Liu, Y. C. Wang, Y. T. Lee, S. C. Kuo, T. L. Chen, and et al., 2016. In Vitro activity of SecA inhibitors in combination with carbapenems against carbapenem-hydrolysing class D β-lactamase-producing *Acinetobacter baumannii*. J Antimicrob Chemother 71, 3441-3448. 10.1093/jac/dkw331

10. Clarridge, J. E. 2004. Impact of 16S rRNA Gene Sequence analysis for Identification of bacteria on clinical microbiology and infectious diseases, Clin Microbiol Rev 17, 840-862. DOI: 10.1128/CMR.17.4.840–862.2004

11. Corvec, S., L. Poirel, T. Naas, H. Drugeon and P. Nordmann 2007. Genetics and expression of the carbapenems-hydrolysing oxacillinase gene *bla*$_{\text{OXA-23}}$ in *Acinetobacter baumannii*. Antimicrob Agents Chemother 51, 1530-3. DOI: 10.1128/AAC.01132-06

12. Deng, M. M. H. Zhu, J. J, Li, S. Bi, Z. K. Sheng, F. S. Hu, J. J. Zhang, W. Chen, X. W. Xue, J. F. Sheng, and L. J. Li. 2014. Molecular epidemiology and mechanisms of tigecycline resistance in clinical isolates of *Acinetobacter baumannii* from a Chinese university hospital. Antimicrob Agents Chemother 58, 297-303

13. Dijkshoorn, L., A. Nemec, and H. Seifert 2007. An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. Nat Rev Microbiol 5,939–951

14. Docquier, J. D., V. Calderone, F. DeLuca, M. Benven, F. Giuliani, L. Bellucci, A. Tafi, P. Nordmann, M. Botta, G. M. Rossolini, S. Mangani 2003. Crystal structure of the OXA-48β-lactamase reveales mechanistic diversity among class D carbapenemases. *Chem Biol* 16, 540-547

15. Donald, H. M., W. Scaife, S. G. Amyes and H. K. Yung 2000. Sequence analysis of ARI-1, a novel OXA β-lactamase, responsible for imipenem resistance in *Acinetobacter*

baumannii 6B92. Antimicrob Agents Chemother 44,196-199

16. Figueiredo, S., H. Seifert, P. Mugnier, D. Benhamou and P. Nordmann 2010. OXA-134, a naturally occurring carbapenem-hydrolysing class D beta-lactamase from *Acinetobacter lowffii*. Antimicrob Agents Chemother 45, 5372-5375. http://dx.doi.org/10.1128/AAC.00629-10

17. Fournier, P. E., D. Vallenet, V. Barbe, S. Audic, H. Oqata, L. Poirel, H. Richet, C. Robert, S. Mangenot, C. Abergel, P. Nordmann, J. Weissenbach, D. Raoult and J. M. Claverie 2006. Comparative genomics of multidrug resistance in Acinetobacter baumannii. PLoS Genet 2:e7.

18. Gordon, N. C. and D. W. Wareham. 2010. Multidrug-resistant *Acinetobacter baumannii* mechanisms of virulence and resistance. Int J Antimicrob Agents 35, 219-26.

19. Hanlon, G. W. 2005. The emergence of multidrug resistant *Acinetobacter* species: a major concern in the hospital setting. Litt.Appl Microbiol 41, 375-378

20. Harmsen, D. and H. Karch 2004. 16S rDNA for diagnosing pathogens: a living tree. ASM News 70, 19-24

21. Heritier, C., A. Dubouix, L. Poirel, N. Marty and P. Nordmann 2005. A nosocomial outbreak of *Acinitobacter baumannii* isolates expressing the carbapenem hydrolysing oxacillinase OXA-58. J Antimicrob Chemother 55, 115-118

22. Heritier, C., L. Poirel, T. Lambert and P. Nordman 2005. Contribution of acquired carbapenem- hydrolysing oxacillinase to carbapenem resistance in *Acinetobacter baumannii*. Antmicrob Agents Chemother 49, 3198-3202. DOI: 10.1128/AAC.49.8.3198-3202.2005

23. Jiang, M., Z. Zhang, and S. Zhao. 2014. Epidemiological characteristics and drug resistance analysis of multidrug-resistant *Acinetobacter baumannii* in a china hospital at a certain time. Pol J Microbiol 63, 275-281

24. Kaitany, K. J., N. V. Klinger, C. M. June, M. E. Ramey, R. A. Bonomo, R. A. Powers and D. A. Leonard 2013. Structure of the class D carbapenemases OXA-23 and OXA-146: mechanistic basis of activity against carbapenems, extended-spectrum cephalosporins, and Aztreomnam. Antimicrob Agents Chemother 47, 4848-4855. doi:10.1128/AAC.00762-13

25. Kattar, M. M., J. F. Chavez, A. P. Limaye, S. L. Rassoulian-Barret, S. L. Yarfitz, L. C. Carlson, Y. Houze, S. Swanzy, B. I. Wood and B. T. Cookson 2001. Application of 16S rRNA gene sequencing to identify *Bordetella hinzii* as the causative agent of fatal septicaemia. J Clin Microbiol 38, 789-794

26. Ku, S. C., P. R. Hsueh, P. C. Yang and K. T. Luh 2000. Clinical and Microbiological characteristics off bacteremia caused by *Acinetobacter lowffii, Eur.* J Clin Microbial Infect Dis 19, 501-5.

27. Kubo, Y. M. Komatsu, E. Tanimoto, K. Sugimoto, S. Tanaka, S. Migita, Y. Kondo, S. Yano, K. Maehara, R. Murata, A. Nakamura, T, Fujita, and Y. Kawata. 2015. Spread of OXA-23 producing *Acinetobacter baumannii* ST2 and ST246 in a hospital in Japan. J Medical Microbiol 64, 739-744. Doi:10.1099/jmm.0.000077

28. La Scola, B. V. A. Gundi, Khamis and D. Raoult 2006. Sequencing of the rpoB gene and flanking spacers for molecular identification of *Acinetobacter* species. J Clin Microbiol 44, 827–32.

29. Lee, C.R. J. H. Lee, K. S. park, B. C. Jeong, and S. H. Lee. 2015. Quantitative proteomic view associated with resistance to clinically important antibiotics in Gram-positive bacteria: a systematic review. Front Microbiol 6, 828.

30. Lin, M. F. and C.Y. Lan. 2014. Antimicrobial resistance in *Acinetobacter baumannii*: From bench to bedside. World J Clin Cases 16, 787-814.

31. Lee, C. J. H. Lee, M. Park, K. S. Park, I. K. Bae, Y. B. Kim, C. J. Cha, B. C. Jeong, and S. H. Lee. 2017. Biology of *Acinetobacter baumannii*: Pathology, Antibiotic resistance, Mechanism, and Prospective treatment options. Front Cell Infect Microbiol 7, 55. doi: [10.3389/fcimb.2017.00055

32. Majiduddin, F. K., I. C. Materon and T. G. Paizkill 2002. Molecular analysis of β-lactamase structure and function. Int J Med Microbiol 292, 127-37.

33. Mayilraj, S., P. Saha, S. Korpole and S. H. Saini 2006. Ornithinimicrobium kibberense sp. nov.isolated from the Himalayas, India. Int J Syst Evol Microbiol 56,1657–1661

34. Misbah, S., S. AbuBakar, H. Hassan, Y. A. Hanifah and M. Y. Yousof 2004. Antibiotic susceptibility and REP-PCR fingerprints of *Acinetobacter spp*. isolated from a hospital at ten years apart. J Hosp Infect 58,254-61

35. Mitchell, J. M., J. R. Clasman, C. M. June, K. J. Kaitany, J. R. LaFleur, M. A. Taracila, N. V. Klinger, R. A. Bonomo, T. Wymore, A. Szarecka, R. A. Powest and D. A. Leonard 2015. Structural Basis of Activity against Aztreonam and Extended Spectrum Cephalosporins for Two Carbapenem-Hydrolyzing Class D β-Lactamases from *Acinetobacter baumannii*. Biochemistry 54, 1976–1987. DOI: 10.1021/bi501547k

36. Mody, L., S. F. Bradley, L. J. Strausbaugh and R. R. Muder 2001. Prevalence of ceftriaxone and ceftazidime-resistant gram-negative bacteria in longterm-care facilities. Infect Control Hosp Epidemiol 22, 193–4

37. Mugnier, P. D., L. Poirel, T. Naas and P. Nordmann 2010. Worldwide dissemination of the *bla*$_{\text{OXA-23}}$ carbapenemase gene of *Acinetobacter baumannii*. Emerging Infec Dis 1,35-40

38. Murray, C. K. and D. R. Hospenthal 2005. Treatment of multidrug resistant *Acinetobacter*. Curr Opin Infect Dis 18, 502-506

39. Naas, T., M. Levy, C. Hirschauer, H. Marchandin and P. Nordmann 2005. Outbreak of carbapenem resistant *Acinetobacter baumannii* producing the carbapenemase OXA-23 in a tertiary care hospital of Papeete, French Polynsia. J Clin Microbiol 43, 4826-4829

40. Nossal, N. G. and L. A. Heppel 1966. The Release of Enzymes by Osmotic Shock from *Escherichia coli* in Exponential phase. *The J Biol Chemist* 241, 3055-3062

41. Petersen, T. N., S. Brunak, G. van Heijnee and H. Nielsen 2011. Discriminating signal peptides from transmembrane regions. *Nature Method* 8, 785-786

42.Poirel, L. T. Naas, and P. Nordmann 2010. Diversity, Epidemiology, and genetics of class D-β-lactamases.. Antimicrob Agents Chemother 54, 24-38. doi:10.1128/AAC.01512-08 .

43. Poirel, L., E. Lebessi, C. Heritier, A. Patsoura, F. Foustoukou and P. Nordmann 2006. Nosocomial spread of OXA-58-positive carbapenem-resistanct *Acinetobacter baumannii* isolates in a pediatric hospital in Greece. Clin Microbiol Infect 12, 1138-1141

44. Regalado, N. G., G. Martin and S. J. Antony 2009. *Acinetobacter lwoffii*: bacteremia associated with acute gastroenteritis. Travel Med Infect. Dis 7,316–317

45. Sacchi, C. T., A. M. Whitney, M. W. Reeves, L. Mayer and T. Popovic 2002. Sequence diversity of *Neisseria meningitideis* 16S rNA genes and use of 16S rRNA sequencing as a molecular subtyping tool. J Clin Microbiol 40, 4520-4527

46. Sacchi, C. T., A. M. Whitney, L. W. Mayer, R. Morey, A. Steigerwalt, A. Boras, R. S. Weyant and T. Popvic 2002. Sequencing of 16S rRNA gene: a rapid tool for identification of *Bacillus anthracis*. Emerg Infect Dis 8, 1117-1123

47. Santillana, E., A. Beceiro, G. Bou and A. Romero 2007. Crystal structure of the carbapenemase OXA-24 reveals insights into the mechanism of carbapenem hydrolysis. Proc Natl. Acad Sci USA 104,5354–5359

48. Schneider, K. D., C. J. Ortega, N. A. Renck, R. A. Bonomo, R. A. Powers and D. A. Leonard 2011. Structures of the class D carbapenemase OXA-24 from *Acinetobacter baumannii* in complex with doripenem. J Mol Biol 406,583–594

49. Shrestha, S. T. Tada, B. Shrestha, T. Kirikae, H. Ohara, B.P. Rijal, B. M. Pokhrel, J.B. Sherchand, and J. Nepal. 2016. Emergence of Aminoglycoside resistance due to armA methylase in multi-drug resistant *Acinetobacter baumannii* isolates in a university hospital in Nepal. J Nepal Health Res Counc 14, 72-76

50. Smith, C. A., N. T. Antunes, N. K. Stewart, H. Frase, M. Toth, K. A. Kantardjieff and S. Vakulenko 2015. Structural Basis for enhancement of carbapenemase activity in the OXA-52 family of class D β-lactamase. ACS Chem Biol 21, 1791-1796. . doi:10.1021/acschembio.5b00090

51. Souha, S. H. Kani, R. Tayyar, M. Shehab, B. El-Hafi, S. Sari, H. Rasheed, K. A. B. Kissoyan, Z. A. Kanafani, R.H. Wakim, N. K. Zahereddine, G. F. Araj, G. Dbaibo, and G. M. Matar. 2018. Increase of *bla*$_{\text{OXA-23-like}}$ in *Acinetobacter baumannii* at a tertiary care

center in Lebanon between 2007 and 2013. J Infect Dev Ctries 12, 2S-4S. doi:10.3855/jdc:10100.

52. Song, Y., C. liu, D. R. Molitoris, T. J. Tomzynski, P. A. Lawsen, M. D. Collins and S. M. Finegold 2003. *Clostridium boltae* sp. Nov., isolated from human sources. Syst Appl Microbiol 26, 84-89

53. Spence, R. D. and K. J. Towner 2003. Frequencies and mechanisms of resistance to moxifloxacin in nosocomial isolates of *Acinetobacter baumannii*. J Antimicrob Chemother 52,687–690

54. Sunenshine, R. H., M. O. Wright and L. N. L. Maragakis 2007. Multidrug-resistant Acinetobacter infection mortality rate and length of hospitalization. Emerg Infect Dis 13, 97–103

55. Thomson, J. M. and R. A. Bonomo 2005. The threat of antibiotic resistance in gram-negative pathogenic bacteria: beta-lactams in peril! Curr Opin Microbiol 8, 518–24

56. Tortoli, E. 2003. Impact of genotype studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin Microbiol Rev 16, 231-237

57. Torol, S. and M. Kasap 2012. Purification and characterization of OXA-23 from *Acinetobacter baumannii*. J Enzym InhibMed Chem 28,836-842. DOI: 10.3109/14756366.2012.689296

58. Turton, J. F., M. E. Ward, N. Woodford, M. E. Kaufmann, R. Pike, D. M. Livermore and T. L. Pitt 2006. The Role of ISAba1 in expression f OXA carbapenemase genes in *Acinetobacter baumannii*. FEMS Microbiol Letter 258, 72-77. doi:10.1111/j.1574-6968.2006.00195.x7

59. Walther- Rasmussen, J. and N. Hoiby 2006. OXA-type carbapenemases. J Antimicrob Chemother 57, 373-383. doi:10.1093/jac/dki482

60. Wu, W. Y. He, J. Lu, Y. Lu, J. Wu, and Y. Liu. 2015. Transition of blaOXA-58-like to blaOXA-23-like in *Acinetobacter baumannii* clinical isolates in southern China: An 8-year study. PLOS ONE 4, 1-11. Doi:10.1371/journal.pone.0137174

61. Zarrilli, R. S. Pournaras, M. Giannouli, and A. Tsakris. 2013. Global evolution of multidrug-resistant *Acinetobacter baumannii* clonal lineages. Int J Antimicrob. Agents 41, 11-19. doi: 10.1016/j.ijantimicag.2012.09.008