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

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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\textsubscript{OXA-23} gene was responsible for imipenem resistance in A. baumannii, whereas, bla\textsubscript{OXA-51-like} was moderately confer resistance towards A. lowffii which lack of bla\textsubscript{OXA-23}. This was determined when the isolates were subjected to qRT-PCR. We identified that the bla\textsubscript{OXA-23} gene was increased about 1-fold in the presence of imipenem, whereas, bla\textsubscript{OXA-51-like} did not increased in comparison to the control. Bioinformatic analyses revealed that bla\textsubscript{OXA-23} is located in the cytoplasm, and bla\textsubscript{OXA-like 51} is located in the periplasm. Our hypothesis suggests that bla\textsubscript{OXA-23} gene have a major contribution in the outbreak of multidrug resistance Acinetobacter species.

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

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A. baumannii and A. lowffiiths, the sensitivity patterns were variable. Also, 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\textsubscript{OXA-23} gene was responsible for imipenem resistance in A. baumannii, whereas, bla\textsubscript{OXA-51-like} was moderately confer resistance towards A. lowffii which lack of bla\textsubscript{OXA-23}. This was determined when the isolates were subjected to qRT-PCR. We identified that the bla\textsubscript{OXA-23} gene was increased about 1-fold in the presence of imipenem, whereas, bla\textsubscript{OXA-51-like} did not increased in comparison to the control. Bioinformatic analyses revealed that bla\textsubscript{OXA-23} is located in the cytoplasm, and bla\textsubscript{OXA-like 51} is located in the periplasm. Our hypothesis suggests that bla\textsubscript{OXA-23} gene have a major contribution in the outbreak of multidrug resistance Acinetobacter species.

الكلمات المفتاحية: Acinetobacter spp., imipenem, qRT-PCR

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INTRODUCTION

Acinetobacter spp. is a non fastidious Gram-negative bacteria, belongs to gamma-proteobacteria, 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. baumannii, 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. baumannii by normal Vitek 2 system (bioMerieux). The isolates were routinely subcultured either on
Mackonkey agar and on nutrient agar (NA) plates at 37 °C every 24 hr and under aerobic conditions. For liquid cultures, the isolates were inoculated in Mueller Hinton (MH) broth and incubated aerobically at 37°C 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 against the relevant media as a control (blank). The growth was observed every hour started from OD ≈0.1 to approximately OD ≈1.5.

**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 nm of 0.1 before 20 µg of imipenem was added. The growth was monitored till mid phase (OD 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 GTTGTACMTGGCTCAG |
| blaOXA-like51 FWD | TAAATGCCTTGATCGGCCTTG |
| blaOXA-like51 R | TGGATTGCACTTCATCTTGG |
| blaOXA-23 FWD | GATCGGATTTGGAGAACCAGA |
| 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 *Acinetobacter* 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 imipenem. 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°C). Finally, periplasm preparation solution was obtained by centrifugation at (15,000 xg, 25 min, 4 °C) and then either stored at -20°C, 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°C with gentle shaking. The cells were harvested by centrifugation at (10,000 xg, 10 min, 4 °C) 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 °C) to remove cell debris. The supernatant was either stored at -20 °C 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**\textsubscript{OXA-51-like} and **bla**\textsubscript{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 carbapenemases, 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**\textsubscript{OXA-51} and **bla**\textsubscript{OXA-23} were PCR amplified using **bla**\textsubscript{OXA51-FWD/**bla**\textsubscript{OXA51-R}, **bla**\textsubscript{OXA23-FWD/**bla**\textsubscript{OXA23-R} and **bla**\textsubscript{OXA361-FWD/**bla**\textsubscript{OXA361-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**\textsubscript{OXA-like 51} (535 bp) was found in *A. lowffii* and *A. baumannii*, whereas, the **bla**\textsubscript{OXA-23} (501 bp) was found only in *A. baumannii* (Figure 3C). The **bla**\textsubscript{OXA-51-like} is may be a sole carbapenem resistance in *A. lowffii* and *A. baumannii* only if IS\textsubscript{Aba1} is located upstream **bla**\textsubscript{OXA-51-like}, as the former (IS\textsubscript{Aba1}) provides promoter for this gene (Figure 2B) (58). Unlike, **bla**\textsubscript{OXA-23} is found only in *A. baumannii*, and may be chromosomally located and flanked by one or two copies of IS\textsubscript{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; gentamycin (aminoglycoside), imipenem (β-lactum), ciprofloxacin (floroquinolone), Azithromycin (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 floroquinoone, 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 fluoroquinolone (ciprofloxacin) was variable, where A. baumannii exert a complete resistance. Like other Gram-negative bacteria, Acinetobacter acquired resistance to fluoroquinolones by point mutations in DNA gyrase or DNA topoisomerase IV. The amino acid substitution of gyrA (Ser83 to Leu 83) and parC (Ser80) have been reported to decrease the binding with the drug (53). Mutation occurs firstly in gyrA and accompanied by mutation in parC, and thus suggests that gyrA is the target of fluoroquinolone (6). Aminoglycosides (gentamycin) were also exhibit a fluctuated effect on A. lowffii compared to A. baumannii. The resistance mechanism of A. baumannii to gentamycin 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 blaOXA-51, blaOXA-58 and blaOXA-24 (33). The gene encoding oxacillinases (OXA) have been reported in several Acinetobacter species, where blaOXA-51 like gene is the naturally occurring gene in A. baumannii and contributes in resistance mechanism. Noteworthy that the serine carbapenemase (blaOXA-23) was firstly identified in A. baumannii isolated from blood culture in Eidenbergh (16).

![Figure 3. Antibiotic resistance profile of Acinetobacter isolates](image)

Overnight cultures of A. lowffii HHR2 and A. baumannii HHR1 were 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 °C 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 ISAba1 is located adjacent to bla(OXA-51)-like, whereas, others with no ISAba1 are susceptible. In the mean time, the clones producing bla(OXA-23) and having ISAba1 were consistently resistance. In another study also confirm that the insertion sequences ISAba1 and ISAba3 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 picketii, 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 ISAba1. 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 ISAba1, and A. baumannii harbouring both genes with ISAba1. 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. lowfi* HHR2 and *A. baumannii* HHR1 under normal conditions, (B) growth of *A. lowfi* 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*OXA*-23 in *A. baumannii* HHR1 and *A. lowfi* HHR2**

Hence, the imipenem was able to significantly reduce the viability of *A. lowfi* HHR2, and with no effect on the *A. baumannii* HHR1 growth. qRT-PCR was performed to evaluate the expression of bla*OXA*-51-like and bla*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. lowfi* HHR2 and *A. baumannii* HHR1 during mid-log growth under two conditions (+/− imipenem). The primers were used to amplify both bla*OXA*-23 and bla*OXA*-51-like in the presence of 20 µg imipenem (Figure 5A, B). The expression of bla*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*OXA*-23 was increased to 1.0-fold compared to the control (Figure 5C). These results confirmed the vital role of bla*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 resist wide spectrum of antibiotics worldwide, especially carbapenem and imipenem (19, 38). Generally, *bla*<sub>OXA-51</sub> like gene is weakly expressed, unless, it may up regulated by insertion sequence IS<sub>Aba1</sub> 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*<sub>-51</sub>-like β-lactamase 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*<sub>OXA-23</sub>, *bla*<sub>OXA-58</sub> genes are highly up-regulated and associated with IS<sub>Aba1</sub>, IS<sub>Aba2</sub>, IS<sub>Aba3</sub> or IS18, 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*<sub>OXA-23</sub> by IS<sub>Aba1</sub> copy in *A. baumannii*, but it does not be a good inducer for *OXA*-51 neither in *A. baumannii* nor in *A. lowffi*.

Figure 5. qRT-PCR analyses of *bla*<sub>OXA-like-51</sub> and *bla*<sub>OXA-23</sub> genes and gene organization. (A and B); Gene organization map and neighbourhood of *A. baumannii* HHR1 and *A. lowffi* HHR2 chromosome containing β-lactam encoding genes *bla*<sub>OXA-23</sub> and *bla*<sub>OXA-like-51</sub>. 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*<sub>OXA-like-51</sub> and *bla*<sub>OXA-23</sub> using *bla*<sub>OXA-51</sub> RT-F, *bla*<sub>OXA-51</sub> RT-R, *bla*<sub>OXA-23</sub> RT-FWD and *bla*<sub>OXA-23</sub> 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 *bla*<sub>OXA-51-like</sub> and *bla*<sub>OXA-23</sub> in *A. lowffi* HHR2 and *A. baumannii* HHR1 The amino acid sequence of *bla*<sub>OXA-23</sub> and *bla*<sub>OXA-51-like</sub>, suggested that they might be located in the cytoplasm and periplasm via Sec system (9) respectively. *bla*<sub>OXA-23</sub> is predicted to have signal peptidase I cleavage site between A<sub>25</sub> and N<sub>26</sub> (Figure 7A) (SignalP 4.0) (41). *bla*<sub>OXA-like-51</sub> 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 β-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 7D). 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 (1), 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 (27). The 3D crystal structure of blaOXA-23 (Figure 8 A right) revealed that it composed of 6 β-strands running antiparallel 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.

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