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Dissemination and Genetic Structure of Carbapenemase Encoding Genes (*bla*$_{OXA-23}$ and *bla*$_{OXA-24}$) in *Acinetobacter baumannii* from Southern Texas

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Abstract

Acinetobacter baumannii **is one of the most important human pathogens causing a variety of nosocomial infections. Carbapenem antibiotics have been primarily used to treat the** ***A. baumannii*** **infections. However, carbapenem resistant** ***A. baumannii*** **producing carbapenemases causes serious treatment problems worldwide. Outbreaks of carbapenem resistant isolates have reported in some area of the United States, but their dissemination and genetic structure of the carbapenemase encoding genes are currently little known. To understand outbreaks, dissemination, and genetic structure of the carbapenemase encoding genes in Southern Texas, 32 clinical isolates collected from Austin and Houston, TX were characterized. Twenty-eight of 32 isolates were resistant to all tested** ***β*****-lactam antibiotics including carbapenem (imipenem and meropenem). Three of them carried** ***bla***$_{OXA-23}$ **as a part of Tn2008 integrated into a known plasmid (pACICU2) and all others carried** ***bla***$_{OXA-24}$ **flanked by XerC/XerD-like recombinase binding sites that were adjoined by DNA sequences originated from multiple plasmids. Genotype analysis revealed that the 25 isolates carrying** ***bla***$_{OXA-24}$ **were all identical genotypes same as a representative isolate carrying** ***bla***$_{OXA-24}$ **from Chicago, IL but the 3 isolates carrying** ***bla***$_{OXA-23}$ **was a distinct genotype as compared with isolates carrying** ***bla***$_{OXA-23}$ **from Chicago, IL and Washington, D.C. Each of the** ***bla***$_{OXA-23}$ **and** ***bla***$_{OXA-24}$ **was transferred to carbapenem susceptible** ***A. baumannii*** **and** ***E. coli*** **with similar minimal inhibitory concentration (MIC) of carbapenem as that of their parental isolates but significantly lower levels of MIC in** ***E. coli*****. Overall results suggest that a unique strain carrying** ***bla***$_{OXA-23}$ **and a similar strain carrying** ***bla***$_{OXA-24}$ **as seen in other geographic areas are currently disseminated in Southern Texas.**

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Keywords

***Acinetobacter baumannii*, Dissemination and Genetic Structure of Carbapenemase-Encoding Genes**

1. Introduction

Acinetobacter baumannii is a gram-negative human pathogen characterizing strictly aerobic, non-pigmented, non-motile, non-fermenting, oxidase-negative, and catalase-positive coccobacillus [2]. A major clinical impact of *A.* baumannii is hospital-acquired infections including ventilator-associated pneumonia, skin and soft-tissue infections, wound infections, urinary-tract infections, secondary meningitis, and bloodstream infections [2] [3]. The treatment of this pathogen is very difficult due to its innate resistance to a number of commonly used antibiotics and an enormous capability to acquire resistance mechanisms to almost all commercially available antibiotics [4].

For the last decade *A. baumannii* has acquired resistance mechanisms to most of commercially available antibiotics. Carbapenem has been used to treat these multidrug resistant strains. However, carbapenem resistant clinical isolates of *A. baumannii* have been emerged and rapidly spread worldwide with serious treatment problem [5]. Carbapenems (imipenem and meropenem) are broad-spectrum β-lactam antibiotics and the most important antimicrobial agents for treatment of multidrug resistant gram-negative pathogens including *A. baumannii* [6]. Carbapenem resistant isolates of *A. baumannii* are usually resistant to all classes of β-lactams and also resistant to most of other classes of antibiotics [6]. Carbapenem resistance in *A. baumannii* can be mediated by one or multiple mechanisms including enzymatic inactivation, active efflux, decreased membrane permeability, and modification of target sites. The most common carbapenem resistant mechanism is enzymatic inactivation by carbapenem-hydrolyzing β-lactamases (or carbapenemases) in clinical isolates of *A. baumannii* [2].

Three Ambler classes of β-lactamase have been identified in *A. baumannii*. Ambler Class B metallo-β-lactamases (VIM-, IMP-, and SIM-types) are mostly associated with class 1 integrons and conferring resistance to all β-lactams including carbapenems with exception of monobactams [2]. Ambler Class A *Klebsiella pneumonia* carbapenemases (KPC) was also identified in *A. baumannii* from Puerto Rico [7]. Ambler Class D β-lactamases (or oxacillinases) are the most prevalent in *A. baumannii*, which includes an intrinsic chromosomal OXA-51-type and three acquired types (OXA-23-, OXA-24-, and OXA-58-types) located in either chromosome or plasmids [2]. The intrinsic chromosomal OXA-51-type is required by insertion of an insertion sequence element (IS*Aba*1) into upstream of this gene to confer significant carbapenem resistance. The OXA-23- and -58-types are also associated with insertion elements (e.g., IS*Aba*1, IS*Aba*2, IS*Aba*3, IS*Aba*4, or IS18), but OXA-24-type (or OXA-40-type) is not associated with any insertion element to confer carbapenem resistance [2] [8].

Outbreaks of carbapenem resistant *A. baumannii* have been increasingly reported and the carbapenem resistant strains rapidly spread among different cities of worldwide [9]. In the United States, outbreaks of carbapenem resistant *A. baumannii* were first reported from New York City in 1994 [10]. Study showed that carbapenem resistance of the isolates from New York City was caused by OXA-51-type carbapenemase encoding genes that were activated by insertion sequence (IS*Aba*1) [11]. Similar outbreaks of carbapenem resistant *A. baumannii* isolates associated with OXA-23-type and OXA-24-type were reported in Pittsburgh, PA and Chicago, IL [12] [13]. Emergence of carbapenem resistant *A. baumannii* isolates associated with OXA-24-type and OXA-58-type were also reported in Houston, TX as a part of SENTRY antimicrobial surveillance program [14]. Carbapenem resistant *A. baumannii* isolates infected in U.S. military personnel combatting operations in Iraq, which were associated with OXA-23-type and OXA-58-type, were reported from the Walter Reed Army Medical Center, Washington, D.C. [15].

In this report, 32 clinical isolates of *A. baumannii* collected from Southern Texas (Austin and Houston) were characterized to understand outbreaks and dissemination of carbapenem resistant *A. baumannii* and genetic structure surrounding the carbapenemase encoding genes. Results revealed that carbapenem resistant *A. baumannii* isolates carried genes encoding OXA-23 (3 isolates) and OXA-24 (25 isolates). Genotype of the isolates carrying OXA-24 and genetic structure surrounding the $bla_{\text{OXA-24}}$ were all identical and same as the isolate carrying $bla_{\text{OXA-24}}$ from Chicago, IL. However, genotype of the isolates carrying OXA-23 and genetic structure surrounding the $bla_{\text{OXA-23}}$ were distinct as compared with the isolates carrying $bla_{\text{OXA-23}}$ from Chicago, IL and Washington, D.C.

2. Materials and Methods

2.1. Clinical Isolates and Culture Conditions

Thirty-two clinical isolates of *A. baumannii* were collected from Southern Texas (24 isolates from Clinical Bacteriology Laboratory Services Section, Texas Department of State Health Services, Austin, TX; 8 isolates from Pathology Department of Baylor College of Medicine, Houston, TX). All isolates were collected from individual patients and from August 2010 to July 2013. Two carbapenem resistant *A. baumannii* collected from Chicago, IL (C1: H26, OXA-23 positive; C2: AC1204, OXA-24 positive) and one carbapenem resistant *A. baumannii* AB0057 collected from Washington, D.C. (OXA-23 positive; a complete genome sequence strain) were obtained from Lolans *et al.* [12] and Adams *et al.* [16], respectively. *A. baumannii* ATCC 19606 was also included as a reference strain in this study. To ensure identity of *A. baumannii*, *gyrB* multiplex PCR was performed for each of the isolates as described [17] and their identities were confirmed by DNA sequence determination (data not shown). The clinical isolates were routinely grown in Luria-Bertani (LB) broth or LB agar plates.

2.2. Antibiotic Susceptibility Testing

All antibiotics used in this study were purchased from Sigma (Sigma, St. Louis, MO) except for meropenem (U S Pharmacopeia, Rockville, MD). Antibiotic susceptibility was determined by minimal inhibitory concentrations (MICs) using the broth dilution method as described [18]. Each antibiotic was added to divalent cation-adjusted Mueller-Hinton (MH) (Oxoid, Ogdensburg, New York) broth (pH 7.0) to achieve serial two-fold dilutions between 0.25 and up to 128 μg/mL using sterile 17 × 100 mm snapped-cap Falcon culture tubes (1 mL/tube; Fisher Scientific). Fresh overnight cultures of each isolate were diluted in saline to an optical density at 600 nm of 0.1 to 0.12 (approximately 1×10^8 viable cells per mL). A portion of the adjusted cell suspension (3 μL for ~10^5 cells) was inoculated to each MH broth containing antibiotics as indicated. The cell cultures were then incubated overnight (16 to 18 hrs) at 37˚C without shaking. MIC was defined as the lowest concentration that completely inhibited growth of the inoculums. MIC values were confirmed by three independent experiments. Antibiotic resistant breakpoints were used as suggested by Clinical and Laboratory Standards Institute (CLSI) cited by Peleg *et al.* [2].

2.3. Detection of Genes Encoding Carbapenemases and an Insertion Sequence Element (IS*Aba*1)

Presence of genes encoding carbapenemases known as Ambler Class A *β*-lactamase (bla_{KPC2}), Ambler Class B metallo *β*-lactamase (bla_{IMP}, bla_{GIM}, bla_{SIM}, bla_{SPM}, and bla_{VIM}), and Ambler Class D *β*-lactamase or oxacillinase ($bla_{OXA\text{-}23\text{-}type}$, $bla_{OXA\text{-}24\text{-}type}$, $bla_{OXA\text{-}51\text{-}type}$, and $bla_{OXA\text{-}58\text{-}type}$) was examined by multiplex PCR methods as described [1] [19]. The presence of an insertion sequence element (IS*Aba*1) and its location in the upstream of $bla_{OXA\text{-}51\text{-}type}$ was also examined by PCR methods as described [8]. All PCR amplified fragments from clinical isolates were used to determine DNA sequences of both strands from commercial DNA sequencing services (GENEWIZ, Inc., South Plainfield, NJ).

2.4. Gene Cloning Experiment

Genomic DNA from *A. baumannii* isolates A21 ($bla_{OXA\text{-}23}$ positive) and H1 ($bla_{OXA\text{-}24}$ positive) was extracted by Qiagen DNeasy kit (Valencia, CA). The genomic DNA was completely digested by BamHI or EcoRI and ligated to pGH240 (a *bla* gene of pBluescriptSK+ [Stratagene, La Jolla] was destroyed by inserting a chloramphenicol resistant gene cassette [20] into ScaI site) digested by BamHI or EcoRI, respectively. *E. coli* DH5*α* was then transformed with the ligation mixture by a standard chemical transformation method. Transformed *E. coli* DH5*α* carrying $bla_{OXA\text{-}23}$ or $bla_{OXA\text{-}24}$ was selected on LB agar plates containing chloramphenicol (15 μg/mL) and Ampicillin (100 μg/mL). Insert DNA fragments from the recombinant plasmids were confirmed by BamHI or EcoRI digestion and used to determine DNA sequences of both strands from commercial DNA sequencing services (GENEWIZ, Inc., South Plainfield, NJ).

2.5. Construction of Rifampicin Resistant *A. baumannii* ATCC 19606 and Transfer of Carbapenem Resistance

Carbapenem susceptible *A. baumannii* ATCC 19606 grown in LB broth was concentrated at 10^{10} cells and

spread on LB agar plates containing 50 μg/mL of rifampicin for 24 hours and one of colonies grown on the agar plates was used to grow in LB broth containing 50 μg/mL of rifampicin for 24 hours. The cells grown in LB broth containing 50 μg/mL of rifampicin were spread on agar plates containing 100 μg/mL of rifampicin. One of the colonies grown on the agar plates containing 100 μg/mL of rifampicin was subcultured on LB agar plates without any antibiotic and passaged three times on the same LB agar plates. The cells on the third passage was used to confirm stability of rifampicin resistance (MIC > 100 μg/mL) and used for a recipient strain. Two carbapenem resistant clinical isolates (A21 for OXA-23 positiveand H1 for OXA-24 positive) were confirmed as rifampicin susceptible (MIC 0.5 μg/mL) and used as donor strains for conjugative transfer of carbapenem resistance. Mating conditions for the donor and recipient strains were essentially identical as described previously [21] except for an extended-mating period (16 to 18 hours). The mating mixtures were spread on LB agar plates containing meropenem (5 μg/mL) or carbenicillin (100 μg/mL) and rifampicin (100 μg/mL). Individual transconjugants (3 colonies) were used for phenotype analysis and genomic DNA extraction.

2.6. Transformation of *A. baumannii*

Electroporation for transformation of *A. baumannii* ATCC 19606 was performed as described [22]. Briefly, electrocompetent cells were prepared by washing the cells (OD_{600} of 0.8) 3 times with 10% (v/v) glycerol and concentrating the cells at 2×10^{10} cells per mL of 10% (v/v) glycerol. Plasmid DNA (up to 3 μg in 40 μL) extracted by Qiagen kit (Valencia, CA) mixed with the electrocompetent cells and placed in a pre-chilled sterile electroporation cuvette (2 mm electrode gap, Bio-Rad) following immediately pulse by using a Bio-Rad Gene Pulser (2.5 kV, 200 Ω, and 25 μF). The mixture was incubated at 37˚C for 1 hr with 1 mL of LB broth and spread the cells on agar plate containing carbenicillin (100 μg/mL) or meropenem (5 μg/mL). Natural transformation of carbapenem susceptible *A. baumannii* ATCC 19606 was also alternatively performed as described [23]. Briefly, 100 ng of plasmid DNA was added in a mixture containing 50 μL of fresh LB broth and 50 μL of culture in a stationary phase, followed by incubation at 37˚C for 1 hour and plating on LB agar plates containing carbenicillin (100 μg/mL) or meropenem (5 μg/mL). As a control for the natural competency of *A. baumannii* ATCC 19606, kanamycin resistant plasmid (pJHCMW1) was used for the natural transformation by selection on LB agar plates containing kanamycin (25 μg/mL) [23] [24].

2.7. GenBank Accession Numbers

BamHI DNA fragment carrying bla_{OXA-23} (4183-bp) from clinical isolate of *A. baumannii* A21 and EcoRI DNA fragment carryingbla_{OXA-24} (6285-bp) from clinical isolate of *A. baumannii* H1 were deposited in GenBank accession numbers JN207493 and JN207494, respectively.

3. Results

3.1. Antibiotic Susceptibility of Clinical Isolates of *A. baumannii*

MIC levels to multiple antibiotics were determined for the 32 clinical isolates collected from Austin and Houston, TX including 3 representative carbapenem resistant clinical isolates from Chicago, IL, Washington, D.C., and the reference *A. baumannii* ATCC 19606. Results revealed that 28 isolates (87%) were resistant to all tested *β*-lactams including carbapenem (imipenem and meropenem). Four isolates were resistant to polymyxin B (MIC ≥ 8 μg/mL) and 3 of them were resistance to all tested antibiotics including polymyxin B (**Table 1**). The 3 isolates from Chicago, IL and Washington, D.C. were also resistant to all tested antibiotics except for polymyxin B. The reference strain ATCC 19606 was susceptible to all tested antibiotics (**Table 1**).

3.2. Detection of Carbapenemase Encoding Genes

Multiplex PCR was performed to detect the carbapenemase encoding genes as described [1] [19]. Results revealed that a gene encoding OXA-51-type was detected in all 36 isolates. A gene encoding OXA-23-type was detected in 5 of the 36 isolates (3 from Austin, TX, 2 positive controls from Chicago, IL and Washington, D.C.). A gene encoding OXA-24-type was detected in 26 of the 36 isolates (17 from Austin, TX, 8 from Houston, TX, and a positive control from Chicago, IL) (**Table 2**). None of the other carbapenemase encoding gene was detected from any isolate in this study.

Table 1. Antibiotic susceptibility of clinical isolates of *A. baumannii*.

Isolate[a]	MICs (μg/mL)[b]								
	ATM	CAR	CAZ	IMP	MEM	CIP	GEN	TCN	PXB
ATCC19606	16	64	16	0.5	0.5	1	2	1	1
AB0057	>256	>256	>256	16	32	32	64	128	0.5
C1 (H26)	64	>256	64	128	64	32	>256	64	2
C2 (1204)	64	>256	>256	>256	128	64	>256	16	2
A1	128	>256	64	0.5	1	16	8	16	2
A2	64	>256	>256	32	32	32	4	8	4
A3	64	>256	>256	64	64	128	>256	16	4
A4	128	>256	>256	32	64	128	128	8	2
A5	>256	>256	64	16	64	16	128	8	2
A6	128	>256	64	64	64	128	>256	>256	8
A7	>256	>256	>256	64	64	32	128	8	0.5
A8	64	>256	>256	64	64	64	128	16	2
A9	64	>256	>256	64	64	16	128	4	0.5
A10	64	>256	>256	64	64	64	64	8	2
A11	128	>256	>256	16	64	64	>256	8	2
A12	128	>256	>256	64	64	64	>256	8	2
A13	>256	>256	>256	64	64	32	64	16	0.5
A14	64	>256	>256	64	64	32	16	16	0.5
A15	>256	>256	>256	64	32	64	>256	>256	4
A16	>256	>256	128	64	32	64	>256	16	4
A17	>256	>256	>256	64	64	64	>256	16	16
A18	>256	>256	>256	1	0.5	128	>256	>256	4
A19	>256	>256	>256	0.5	0.5	32	>256	>256	4
A20	>256	>256	>256	0.5	0.5	64	>256	>256	1
A21	>256	>256	>256	64	64	128	>256	>256	2
A22	>256	>256	>256	64	64	64	>256	16	2
A23	>256	>256	>256	32	64	16	128	>256	2
A24	>256	>256	>256	32	64	64	>256	128	2
H1	>256	>256	>256	64	64	128	>256	0.5	8
H2	128	>256	128	64	64	128	64	0.5	2
H3	128	>256	128	64	32	128	64	2	2
H4	64	>256	>256	64	32	128	128	16	4
H5	64	>256	>256	64	32	128	>256	0.5	4
H6	>256	>256	>256	64	32	128	64	4	2
H7	128	>256	>256	64	64	128	>256	16	16
H8	128	>256	>256	64	64	128	128	2	2

[a]The isolate AB0057 was obtained from Washington, D.C. [16]; isolates (C1 and C2) were obtained from Chicago, IL [12]; isolates of A1 to A24 were collected from Austin, TX; isolates of H1 to H8 were collected from Houston, TX. [b]MIC measurements were repeated three times with identical results. Note: aztreonam, ATM; carbenicillin, CAR; ceftazidime, CAZ; ciprofloxacin, CIP; gentamicin, GEN; imipenem, IMP; meropenem, MEM; polymyxin B, PXB; tetracycline, TCN.

Table 2. Detection of carbapenemase-encoding genes in clinical isolates of *A. baumannii*.

Isolate	PCR amplification[a] for:		
	$bla_{\text{OXA-23-type}}$	$bla_{\text{OXA-24-type}}$	$bla_{\text{OXA-51-type}}$
ATCC19606	-	-	+
AB0057	+	-	+
C1	+	-	+
C2	-	+	+
A1	-	-	+
A2	-	+	+
A3	-	+	+
A4	-	+	+
A5	-	+	+
A6	+	-	+
A7	-	+	+
A8	-	+	+
A9	-	+	+
A10	-	+	+
A11	-	+	+
A12	-	+	+
A13	-	+	+
A14	-	+	+
A15	+	-	+
A16	-	+	+
A17	-	+	+
A18	-	-	+
A19	-	-	+
A20	-	-	+
A21	+	-	+
A22	-	+	+
A23	-	+	+
A24	-	+	+
H1	-	+	+
H2	-	+	+
H3	-	+	+
H4	-	+	+
H5	-	+	+
H6	-	+	+
H7	-	+	+
H8	-	+	+

[a]PCR primer pairs used for $bla_{\text{OXA-23-type}}$ was 5'-GATCGGATTGGA GAACCAGA-3'/5'-ATTTCTGACCGCATTTCCAT-3', for $bla_{\text{OXA-24-type}}$ was 5'-GGTTAGTTGGCCCCCTTAAA-3'/5'-AGTTGAGCGAAAAGGGGATT-3' and for $bla_{\text{OXA-51-type}}$ as described [1].

Presence of the insertion sequence element (IS*Aba*1) at upstream of the gene encoding OXA-51-type requires for production of significant carbapenem resistance in *A. baumannii* [8]. To understand involvement of the OXA-51-type in carbapenem resistance, presence andlocation, if any, of the IS*Aba*1 was examined from the isolates as described [8]. Results revealed that the IS*Aba*1 was detected in all isolates except for 8 isolates from Houston, TX. However, none of them was preceded to the gene encoding OXA-51-type.

3.3. Genotype Analysis of Clinical Isolates Carrying Carbapenemase Encoding genes

To understand dissemination of carbapenemase encoding genes among the clinical isolates, two PCR-based genotype methods (REP-PCR and RAPD-PCR) proved as similar discrimination power as a pulse field gel electrophoresis (PFGE) method [25] were used. REP-PCR profiles for the 3 isolates carrying OXA-23-type from Austin, TX (A6, A15, A21) showed an identical pattern (**Figure 1**, lane 3) which was different from that of isolate carrying OXA-23-type from Washington, D.C. or from Chicago, IL (**Figure 1**, lane 1 and 2). REP-PCR profiles for all isolates carrying OXA-24-type from Austin and Houston, TX and Chicago, IL showed an identical pattern (**Figure 1**, lanes 4 and 5); interestingly, this genotype pattern was very similar as that of the isolate carrying OXA-23-type from Chicago, IL (**Figure 1**, lane 2).

RAPD-PCR profiles for the 3 isolates carrying OXA-23-type from Austin, TX showed an identical pattern (**Figure 1**, lane 8) which was different from that of isolate carrying OXA-23-type from Washington, D.C. or from Chicago, IL (**Figure 1**, lanes 6 and 7). As shown in REP-PCR profiles, RAPD-PCR profiles for all isolates carrying OXA-24-type from Austin and Houston, TX and Chicago, IL showed an identical pattern (**Figure 1**, lanes 9 and 10). The two genotype methods produced repeatedly consistent results, suggesting that a unique isolate carrying OXA-23-type is currently propagated in Austin, TX but the isolate carrying OXA-24-type in Austin and Houston, TX may be clonally disseminated from isolates carrying OXA-24-type in Chicago, IL or *vice versa*.

3.4. Genetic Structure Surrounding the Carbapenemase Encoding Genes

The genes encoding carbapenemase of OXA-23-type and OXA-24-type were cloned from the clinical isolates A21 and H1, respectively. BamHI fragment (4183-bp) from total DNA of the clinical isolate A21 conferred resistance to ampicillin (100 μg/mL) in *E. coli* DH5*α* and the insert DNA was used for DNA sequencing analysis. As shown in **Figure 2(a)**, the gene encoding OXA-23 was a part of Tn2008 that was integrated into a plasmid

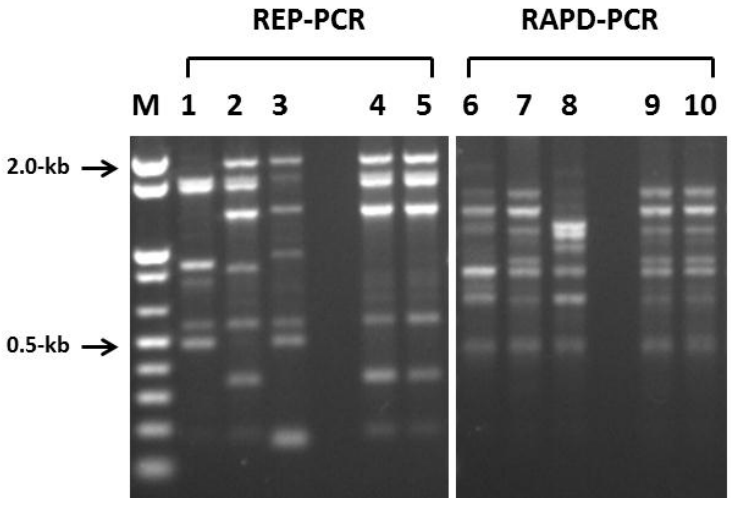

Figure 1. Genotype analysis of clinical isolates of *A. baumannii*. Genotype analysis of clinical isolates was performed by REP-PCR and RAPD-PCR as described [25]. Lanes 1, 2, 3 and 6, 7, 8 for isolates carrying bla_{OXA-23}; lanes 4, 5 and 9, 10 for isolates carrying bla_{OXA-24}. Lanes 1 and 6: AB0057 (Washington, D.C.), lanes 2 and 7: isolate C1 (Chicago, IL), lanes 3 and 8: isolate A6 (Austin, TX), lanes 4 and 9: isolate C2 (Chicago, IL), lanes 5 and 10: isolate H1 (Houston, TX). Three isolates carrying bla_{OXA-23} from Austin, TX (A6, A15, A21) were an identical genotype pattern (same as lane 3 or lane 8). All isolates carrying bla_{OXA-24} from Austin and Houston, TX were an identical genotype pattern (same as lane 5 or lane 10).

pACICU2 (GenBank accession number CP000865) and deduced amino acid sequence of the gene was 100% identical as previously reported OXA-23 (GenBank accession number EU594641) [26]. A newly designed forward PCR primer for upstream 500-bp from the start codon of OXA-23 (5'-GTATCTGAATTT CCACGTTT-3') and a reverse primer for downstream 500-bp from the stop codon of OXA-23 (5'-AAGTGTACTTGATGGCTCAT-3') were used to amplify a full length of $bla_{\text{OXA-23}}$ from the other two OXA-23 positive isolates (A6 and A15). DNA sequence analyses of the PCR fragments containing $bla_{\text{OXA-23}}$ from the two isolates were 100% identical as that from the isolate A21.

EcoRI fragment (6285-bp) from total DNA of the clinical isolate H1 conferred resistance to ampicillin (100 μg/mL) in *E. coli* DH5*α* and the insert DNA was used for DNA sequencing analysis. As shown in **Figure 2(b)**, the gene encoding OXA-24 was flanked by XerC/XerD-like recombinases binding sites and surrounded by DNA sequences originated from multiple plasmids. Deduced amino acid sequence of the gene was 100% identical as previously reported OXA-24 (GenBank accession number GU199040) [13]. A newly designed forward PCR primer for upstream 500-bp from the start codon of OXA-24 (5'-AGGTGTGCATCATACTTCTA-3') and a reverse primer for downstream 500-bp from the stop codon of OXA-24 (5'-TAAAGAATCAGATCGATTTT-3') were used to amplify a full length of $bla_{\text{OXA-24}}$ from all the other OXA-24 positive isolates (24 isolates). DNA sequence analysis showed that all PCR fragments were 100% identical as that from the isolates H1.

3.5. Gene Transfer for Carbapenem Resistance

Gene transfer experiment was performed by bacterial conjugation to test whether the carbapenem resistance of the clinical isolates was transferrable to a carbapenem susceptible *A. baumannii*. Each of carbapenem resistant but rifampicin susceptible clinical isolates (OXA-23 for the isolate A21, OXA-24 for the isolate H1; **Table 1**) was mated to the rifampicin resistant but carbapenem susceptible strain of *A. baumannii* ATCC 19606-rif-R.

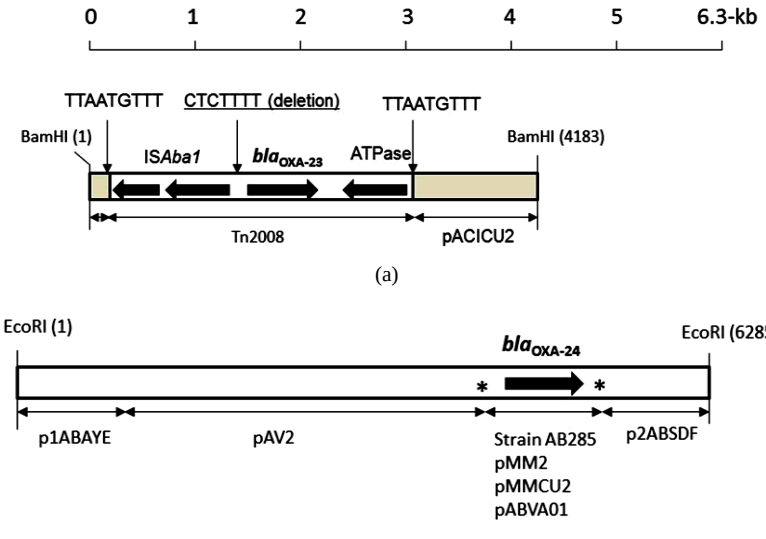

Figure 2. Genetic structure surrounding $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-24}}$ from clinical isolates of *A. baumannii* from Austin and Houston, TX. (A) BamHI fragment cloned from *A. baumannii* A21 (4183-bp; GenBank accession number JN207493). The gene $bla_{\text{OXA-23}}$ was a part of Tn2008 that was integrated into plasmid pACICU2 (shaded region; GenBank accession number CP000865) with the direct repeat target site duplications (TTAATGTTT) and 7-bp deletion (CTCTTTT) in the promoter region of $bla_{\text{OXA-23}}$ as compared to that of $bla_{\text{OXA-23}}$ from *A. baumannii* AB0057 (GenBank accession number CP001182). (B)EcoRI fragment cloned from *A. baumannii* H1 (6285-bp; GenBank accession number JN207494). The gene $bla_{\text{OXA-24}}$ was flanked by XerC/XerD-like recombinase binding sites (*for 5': ATTTCGTATAAcgcccaTTATGTTAAAT; *for 3': AATTAACATAAtacaccTTATACGAAAT) with adjoining DNA sequences originated from p1ABAYE (GenBank accession number CU459137), pAV2 (GenBank accession number DQ278486), and p2ABSDF (GenBank accession number CU468232). The $bla_{\text{OXA-24}}$ was 100% identical as that of *A. baumannii* AB285 (GenBank accession number GU199040) and 99% identical as that of plasmid pMM2 (GenBank accession number GQ377752), pMMCU2 (GenBank accession number GQ476987), and pABVA01 (GenBank accession number FM210331).

Results revealed that countless transconjugants from each conjugation mating mixture grew on LB agar plates containing carbenicillin (100 μg/mL) or meropenem (5 μg/mL) and rifampicin (100 μg/mL) suggesting carbapenem resistance can be transferrable and may be mediated by a plasmid-specific gene.

To test whether the plasmid-specific gene is associated with carbapenem resistance plasmid DNA extracted from the clinical isolates (A21 and H1) was used for transformation of the carbapenem susceptible ATCC 19606 with selection on LB agar plates containing carbenicillin (100 μg/mL) or meropenem (5 μg/mL). Results revealed that each plasmid DNA from the carbapenem resistant clinical isolates produced countless transformants on the agar plates. Presence of the genes encoding OXA-23 and OXA-24 in the transconjugants and transformants (3 colonies for each) was confirmed by PCR methods as done above.

3.6. Expression of Carbapenemase Encoding Genes in *A. baumannii* and *E. coli*

To understand roles of the carbapenemase encoding genes in *β*-lactam susceptibility the transconjugants of rifampicin resistant *A. baumannii* ATCC 19606 and the transformants of *E. coli* DH5*α* carrying $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-}}$24 were used to measure *β*-lactam susceptibility. As shown in **Table 3**, MIC levels to carbapenem (imipenem and meropenem) and penicillin (carbenicillin) were significantly increased (up to 64-fold) in both *A. baumannii* ATCC 19606 and *E. coli* DH5*α* for both $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-24}}$. However, MIC levels to monobactam (aztreonam) and cephalosporin (ceftazidime) were slightly increased or no difference in both *A. baumannii* ATCC 19606 and *E. coli* DH5*α*for both $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-24}}$. These results suggest that the transconjugants of *A. baumannii* ATCC 19606 are similar MIC levels for both $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-24}}$ as that of their clinical isolates while the transformants of *E. coli* DH5*α* are significantly lower MIC levels for both genes than that of their clinical isolates.

4. Discussion

Outbreaks of carbapenem associated multidrug resistant *A. baumannii* may be the most problematic issue to treat the infected patients since the only viable therapeutic option for this infection is polymyxins [9], but polymyxins cause neuro- and nephro-toxicity [27] and polymyxin selection pressure can readily develop polymyxin resistant *A. baumannii* [28] [29]. In this study, most of the isolates showed carbapenem associated multidrug resistance, which was similar as other areas of the United States and worldwide [9] [11] [30], and more sadly some of them conferred intermediate levels of polymyxin resistance. Outbreaks of polymyxin associated carbapenem resistant *A. baumannii* should alert in this area.

Carbapenem resistance of the isolates in Southern Texas was associated with $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-24}}$. The carbapenem resistant isolates reported previously in Houston, TX carried $bla_{\text{OXA-24}}$ and $bla_{\text{OXA-58}}$ [14]. However, the $bla_{\text{OXA-58}}$ was not detected in this study; instead, $bla_{\text{OXA-23}}$ was newly detected in the isolates from Austin, TX. In the United States, all known OXA-type carbapenemase encoding genes are currently associated with carbapenem resistance in clinical isolates. Carbapenem resistant isolates associated with OXA-23-type, OXA-

Table 3. Expression of genes encoding OXA-23 and OXA-24 in *A. baumannii* ATCC 19606 and *E. coli* DH5*α*.

Strains	MIC (μg/mL)				
	ATM	CAR	CAZ	IMP	MEM
A. baumannii ATCC 19606-rif-R	64	64	16	0.5	0.5
A. baumannii ATCC 19606-rif-R/pA21 (OXA-23 positive)	128	>256	32	32	32
A. baumannii ATCC 19606-rif-R/pH1 (OXA-24 positive)	128	>256	64	32	32
E. coli DH5*α*	0.03	8	0.25	0.12	0.015
E. coli DH5*α*/pBA41 ($bla_{\text{OXA-23}}$)	0.03	>256	0.5	0.5	0.06
E. coli DH5*α*/pBA49 ($bla_{\text{OXA-24}}$)	0.06	>256	0.5	1	0.5

pA21 is a plasmid transferred from clinical isolate A21. pH1 is a plasmid transferred from clinical isolate H1. Note: aztreonam, ATM; carbenicillin, CAR; ceftazidime, CAZ; imipenem, IMP; meropenem, MEM.

24-type, and OXA-58-type were reported in northeast and northwest area [12] [15] [26] [31]. The carbapenem resistant isolates from New York City harbored genes encoding OXA-51-type which were fused by IS*Aba*1 providing promoter function for the genes [11]. OXA-51-type carbapenemase is intrinsic to all *A. baumannii* and usually not associated with carbapenem resistance unless authentic expression of the carbapenemase [2].

All isolates carrying $bla_{\text{OXA-24}}$ including the representative isolate carrying $bla_{\text{OXA-24}}$ from Chicago, IL were identical genotype. The isolate carrying $bla_{\text{OXA-24}}$ (or $bla_{\text{OXA-40}}$) from Chicago, IL was one of predominantly (97%) circulating isolates [12]. Therefore, this result suggests that a similar genotype strain carrying $bla_{\text{OXA-24}}$ is currently disseminating in Chicago, IL and Southern Texas. Interestingly, this genotype isolate carrying $bla_{\text{OXA-24}}$ was very similar genotype as that of the isolate carrying $bla_{\text{OXA-23}}$ from Chicago, IL, suggesting the similar genotype isolate may carry two different types of carbapenemase encoding genes in Chicago, IL. It is consistent to the note that a limited number of genotypic clusters of carbapenem associated multidrug resistant isolates of *A. baumannii* are successfully spread among hospitals of different cities and countries [9]. However, genotype of the isolates carrying $bla_{\text{OXA-23}}$ in Southern Texas was unmatched to any of the isolates from Chicago, IL and Washington, D.C., indicating that a distinct genotype strain was currently propagated in Southern Texas. The $bla_{\text{OXA-23}}$ in Southern Texas was carried on Tn2008 while the same genes from Washington, D.C. and Chicago, IL were carried on Tn2006 and others. This observation suggests that the different vehicles for the carbapenemase encoding genes may be delivered by different genotype strains. The $bla_{\text{OXA-23}}$ carried on Tn2008 was reported in clinical isolates from Pittsburgh, PA [26]; thus, it is interesting to test whether the isolate carrying $bla_{\text{OXA-23}}$ from Pittsburgh, PA has a similar genotype as the isolate carrying $bla_{\text{OXA-23}}$ from Southern Texas.

The genetic structures surrounding the genes of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-24}}$ were reported in clinical isolates from Pittsburgh, PA [13] [26]. In this study, genetic structure surrounding the $bla_{\text{OXA-23}}$ was essentially identical as that from Pittsburgh, PA, characterizing that the gene was carried on Tn2008 integrated into plasmid pACICU2 [26] [32]. However, genetic structure surrounding the $bla_{\text{OXA-24}}$ from Southern Texas was different from that of Pittsburgh, PA. The $bla_{\text{OXA-24}}$ from Southern Texas, which was flanked by XerC/XerD-like recombinase binding site, was surrounded by *A. venetianus* plasmid pAV2 [33] and *A. baumannii* plasmids p1ABAYE and p2ABSDF [34] while the $bla_{\text{OXA-24}}$ from Pittsburgh, PA was surrounded by *A. venetianus* plasmid pAV2 [13] [33]. XerC/XerD-like DNA sequences are recognized by recombinase and adjoining by different DNA modules in different *Acinetobacter* plasmids [35], suggesting that XerC/XerD-mediated recombination events may direct multiple genetic motifs from multiple plasmids to be surrounded by a specific gene (or locus) as shown in this case of $bla_{\text{OXA-24}}$ from Southern Texas.

The genes of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-24}}$ have been identified in chromosome and/or plasmid [2]. Two copies of $bla_{\text{OXA-23}}$ were also identified in both chromosome and plasmid in a single isolate of *A. baumannii* [36]. Theoretically, even more than two copies of genes for $bla_{\text{OXA-23}}$ or $bla_{\text{OXA-24}}$ would be also possible since the vehicles for the genes are usually large sizes of conjugative plasmids that can be integrated (or excised) into (or out) chromosome and, if the genes are carried on transposons, they would be also transposed to other incompatibility group of plasmids in a single strain. In this report, we confirmed that both $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-24}}$ from the clinical isolates were transferrable to the carbapenem susceptible *A. baumannii* ATCC 19606, indicating that at least one copy of each gene was carried on a plasmid DNA, which was also supported by the genetic structure for the genes that were surrounded by DNA sequences from plasmids as mentioned above. Both genes of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-24}}$ on the plasmid DNA were expressed in the recipient *A. baumannii* at the similar MIC levels as their parental isolates. However, expression of the same genes cloned into a commercial plasmid vector in *E. coli* DH5α was significantly lower MIC levels than that of their parental clinical isolates of *A. baumannii*. Similar results were also observed in other studies [13] [37], suggesting that expression of the $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-24}}$ may be hindered at the level of transcription and/or translation in *E. coli*.

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