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Research Article

Overexpression of bla_{OXA-58} Gene Driven by IS*Aba3* Is Associated with Imipenem Resistance in a Clinical *Acinetobacter baumannii* Isolate from Vietnam

Anh T. Nguyen,[1,2,3] **Son C. Pham**,[4] **Anh K. Ly**,[4] **Chau V. V. Nguyen**,[5] **Thanh T. Vu**,[3,6] **and Tuan M. Ha**[3,6]

[1]*Research Center for Genetics and Reproductive Health, School of Medicine, Ho Chi Minh City, Vietnam*
[2]*Vietnam National University, Ho Chi Minh City, Vietnam*
[3]*Molecular Biomedical Center, University Medical Center-Campus 2, Ho Chi Minh City, Vietnam*
[4]*Department of Genetics, Faculty of Biology, University of Science, Vietnam National University, Ho Chi Minh City, Vietnam*
[5]*The Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam*
[6]*University Medical Center-Campus 2, Ho Chi Minh City, Vietnam*

Correspondence should be addressed to Tuan M. Ha; hamanhtuan@ump.edu.vn

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The aim of this study was to investigate genetic structures and expression of bla_{OXA-58} gene in five *Acinetobacter baumannii* clinical isolates recovered from two hospitals in southern Vietnam during 2012-2014. *A. baumannii* isolates were identified by automated microbiology systems and confirmed by PCR. All isolates were characterized as multidrug resistant by antimicrobial testing using the disk diffusion method. Four imipenem susceptible and one nonsusceptible isolates (MIC > 32 $\mu g \cdot ml^{-1}$) were identified by E-test. PCR amplification of bla_{OXA-58} gene upstream and downstream sequences revealed the presence of IS*Aba3* at both locations in one multidrug-resistant isolate. Semiquantitation of bla_{OXA-51} and bla_{OXA-58} gene expression was performed by the $2^{-\Delta\Delta Ct}$ method. The bla_{OXA-51} gene expression of five isolates showed little difference, but the isolate bearing IS*Aba3*-bla_{OXA-58}-IS*Aba3* exhibited significantly higher bla_{OXA-58} mRNA level. Higher β-lactamases activity in periplasmic than cytoplasmic fraction was found in most isolates. The isolate overexpressing bla_{OXA-58} gene possessed very high periplasmic enzyme activity. In conclusion, the *A. baumannii* isolate bearing IS*Aba3*-bla_{OXA-58} gene exhibited high resistance to imipenem, corresponding to an overexpression of bla_{OXA-58} gene and very high periplasmic β-lactamase activity.

1. Introduction

Multidrug resistant *A. baumannii* constitutes a serious threat for nosocomial infection control [1]. Carbapenems are currently the antibiotics of choice against multidrug-resistant *Acinetobacter* infections [2], but an increasing rate of resistance to carbapenems was reported worldwide, seriously limiting therapeutic options [3]. Carbapenem-resistant *A. baumannii* has become an alarming health care problem, mainly in developing countries [4]. As a result, carbapenem-resistant *A. baumannii* is classified into the critical priority group according to the urgency of need for new antibiotic treatment and the level of reported antibiotic resistance by the World Health Organization [5].

Multiple mechanisms of carbapenem resistance have been identified in *A. baumannii* including low membrane permeability, mutation in its chromosome genes, overexpression of efflux pumps, and acquisition of mobile resistance genes [6]. However, the production of carbapenemases is considered the principal resistance mechanism [7, 8]. The most frequent ones are carbapenem-hydrolyzing class D β-lactamases (CHDLs) and secondly metalloenzymes (MBL) such as bla_{NDM} [9]. In addition, class A β-lactamases such as bla_{KPC} gene has been recently also detected in *A.*

baumannii [10], presenting a serious threat of expanding resistance spectrum in the bacteria.

Currently, six main groups of CHDLs found in *A. baumannii* include $bla_{\text{OXA-51}}$-like, $bla_{\text{OXA-23}}$-like, $bla_{\text{OXA-24}}$-like, $bla_{\text{OXA-58}}$-like, $bla_{\text{OXA-143}}$-like, and $bla_{\text{OXA-235}}$-like genes [2, 11, 12]. CHDLs exhibit weak carbapenem hydrolysis; however, they can confer resistance mediated by the combination of natural low permeability and IS*Aba* elements located upstream of the gene possibly leading to the gene's overexpression [2]. Overexpression of bla_{OXA} genes usually corresponds to resistance phenotypes [13–15]. Overproduction of oxacillinases, including $bla_{\text{OXA-58}}$ enzyme, results from the presence of insertion sequences such as IS*Aba1*, IS*Aba2*, IS*Aba3*, or IS*18* which provide strong promoters for gene expression [13, 16].

In Vietnam, $bla_{\text{OXA-23}}$ is the most widely disseminated class D-carbapenemase in carbapenem-resistant *Acinetobacter baumannii* while $bla_{\text{OXA-24}}$ is not detected [17]. Even though there is not any information of $bla_{\text{OXA-143}}$ and $bla_{\text{OXA-235}}$ in Vietnam up to now, these genes are believed to emerge in other parts of the world [18, 19]. During 2003-2014, the majority of *A. baumannii* clinical isolates recovered harbored $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-23}}$ genes. The $bla_{\text{OXA-58}}$ gene was only detected in isolates recovered from 2010, after the introduction of imipenem in 2008-2009 into hospitals in Vietnam [17, 20]. The $bla_{\text{OXA-58}}$-positive isolates investigated in the present study probably emerged at the same time. This recent emergence was in contrast with the striking replacement of $bla_{\text{OXA-58}}$ by $bla_{\text{OXA-23}}$ reported in Italy and China for the same period [21, 22]. Furthermore, isolates bearing $bla_{\text{OXA-58}}$-like gene were recovered from different countries during outbreaks and showed remarkable conserved gene sequence [23–25]. The aim of this study was to investigate genetic structures and relative expression of $bla_{\text{OXA-58}}$ gene, which lead to imipenem nonsusceptibility in clinical isolates recovered from two Vietnamese hospitals during 2012-2014.

2. Materials and Methods

2.1. Study Design. The study focused on *A. baumannii* isolates containing $bla_{\text{OXA-58}}$ gene with the purpose of determining imipenem-resistance mechanism related to the gene.

2.2. Bacterial Isolates, Microbial Identification, and Antimicrobial Susceptibility Testing. Five *A. baumannii* isolates were chosen from a total of 252 nonduplicate *Acinetobacter* spp. isolates recovered from patients admitted to hospitals in southern Vietnam during 2012-2014 and were named DN and TN based on their source hospitals [17]. Microbial isolation and identification in source laboratories were performed using the Phoenix System (BD) and the API 20NE system (bioMérieux). Identification of *A. baumannii* isolates was confirmed by PCR amplification and sequencing of 16S-23S intergenic spacer (ITS) regions. The sequences were deposited in GenBank under accession numbers KY659325, KY659326, KY659327, KY659328, and KY659329. Antimicrobial susceptibility testing was performed by the disk diffusion method and interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2014). Tested antimicrobials included ceftazidime, cefotaxime, ceftriaxone, cefpodoxime, cefepime, piperacillin, ampicillin/sulbactam, piperacillin/tazobactam, ticarcillin/clavulanic acid, and meropenem, as well as others not belonging to β-lactams such as amikacin, gentamicin, ankamycin, netilmicin, ciprofloxacin, and levofloxacin. MIC values of imipenem were determined by the *E*-test (bioMérieux); the CLSI-approved breakpoints for imipenem $\geq 8\ \mu g \cdot ml^{-1}$ and $\leq 2\ \mu g \cdot ml^{-1}$ were considered resistant and susceptible, respectively.

2.3. Detection of bla_{OXA}, bla_{NDM}, and bla_{KPC} Genes and Insertion Sequences. Amplification of bla_{OXA} genes including $bla_{\text{OXA-51}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, and $bla_{\text{OXA-58}}$ genes were performed and published in the previous study [17]. bla_{NDM} and bla_{KPC} genes were amplified in this study as previously reported [26]. The presence of IS*Aba1*, IS*Aba2*, IS*Aba3*, IS*Aba4*, and IS18 was detected as previously described [13, 27]. The sequence of all primers is shown in Table 1.

2.4. PCR Mapping of bla_{OXA-58} and bla_{OXA-51} Genes. PCR mapping of bla_{OXA} genes upstream regions was carried out using combinations of insertion sequence-specific forward primers and $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-58}}$ gene-specific reverse primers (Table 1). The presence of IS*Aba3* downstream of $bla_{\text{OXA-58}}$ was determined by a long-range PCR containing 1X PrimeSTAR GXL Buffer, 0.2 mmol dNTPs, 500 nmol OXA-58-F, 500 nmol ISAba3C, and 0.5 U PrimeSTAR GXL DNA polymerase (Takara). PCR products were sent to 1-BASE (https://order.base-asia.com/) for purification and sequencing. The sequences were analysed by BioEdit 7.0.9.0. (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and sequence similarity was assessed using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence of *bla*OXA-58 and its surrounding IS*Aba3* was deposited in GenBank under accession number KY660721.

2.5. Analysis of bla_{OXA-58} and bla_{OXA-51} Gene Expression by Real-Time RT-PCR. The midlog phase of bacterial cultures was treated with $1\ \mu mol \cdot ml^{-1}$ oxacillin for 24 h and was subsequently used for RNA extraction [28]. Treatment with RNAse-free DNAse I (Sigma) was performed at 37°C for 2 h. The concentration and DNase-free quality of RNA samples were spectrophotometrically assessed and confirmed by the amplification of chromosomal $bla_{\text{OXA-51}}$ and 16S rRNA. Fifteen microliters of each RNA sample was reverse-transcribed in a final volume of 20 μl containing random hexamers, MMLV reverse transcriptase (Agilent) at 42° C for 45 min.

Amplification of $bla_{\text{OXA-51}}$, $bla_{\text{OXA-58}}$, and 16S rRNA was performed in a final volume of 25 μl containing 5 μl cDNA, 3 mmol $MgCl_2$, 200 nmol dNTPs, 2 U h-Taq DNA polymerase (Solgent), 300 nmol of OXA-51/58-F/R primers, 150 nmol of OXA-51/58-P probes, 200 nmol of 16S-F/R primers, and 100 nmol of 16S-P probe (IDT). Primer and probe sequences are given in Table 1. Each real-time PCR was performed in triplicate on the Stratagene Mx3005P real-time PCR system (Agilent). The reaction

Table 1: Primers and probes used for PCR amplification and sequencing of antimicrobial resistance genes and related genetic elements.

Primers/probes	Sequence (5′→3′)	Length (bp)	Tm (°C)	Product (bp)	Ref.
OXA-23-F	CACTAGGAGAAGCCATGAAGC	21	55.0	114	Nguyen et al., 2017
OXA-23-R	CAGCATTACCGAAACCAATACG	22	55.0		
OXA-24-F	GCTAAATGCTTTAATCGGGCTAG	24	55.0	141	Nguyen et al., 2017
OXA-24-R	ACTGGAACTGCTGACAATGC	20	55.0		
OXA-51-F	GAAGTGAAGCGTGTTGGTTATG	22	55.0	148	Nguyen et al., 2017
OXA-51-R	GCCTCTTGCTGAGGAGTAAT	20	55.0		
OXA-51-P	FAM-CGACTTGGGTACCGATATCTGCATTGC-BHQ1	27	61.3		This study
OXA-58-F	ATATTTAAGTGGGATGGAAAGCC	23	55.0	110	Nguyen et al., 2017
OXA-58-R	CGTGCCAATTCTTGATATACAGG	23	55.0		
OXA-58-P	FAM-TTTACTTTGGGCGAAGCCATGCAAG-BHQ1	25	60.6		This study
16S-rRNA-F	CCAGTGACAAACTGGAGGAAG	21	55.5	199	This study
16S-rRNA-R	GCTGTGTAGCAACCCTTTGTA	21	55.2		
16S-rRNA-P	HEX-ACGTCAAGTCATCATGGCCCTTACG-BHQ1	25	61.5		
HRF/ISAba1	CACGAATGCAGAAGTTG	17	56.0	520	Segal et al., 2005
HRR/ISAba1	CGACGAATACTATGACAC	18	56.0		
ISAba2A	AATCCGAGATAGAGCGGTTC	20	54.0	1200	Poirel et al., 2006
ISAba2B	TGACACATAACCTAGTGCAC	20	52.1		
ISAba3A	CAATCAAATGTCCAACCTGC	20	52.3	200	Poirel et al., 2006
ISAba3C	AGCAATATCTCGTATACCGC	20	51.8		
ISAba4A	ATTTGAACCCATCTATTGGC	20	50.6	612	Brown et al., 2007
ISAba4B	ACTCTCATATTTTTTCTTGG	20	45.3		
IS18A	CACCCAACTTTCTCAAGATG	20	51.2	925	Poirel et al., 2006
IS18B	ACCAGCCATAACTTCACTCG	20	54.7		
1512F/ITS	GTCGTAACAAGGTAGCCGTA	20	54.1	607	Chang et al., 2005
6R/ITS	GGGTTYCCCCRTTCRGAAAT	20	56.5		
NDM-F	GACCGCCCAGATCCTCAA	18	55.4	52	Yong et al., 2009; CDC 2011
NDM-R	CGCGACCGGCAGGTT	15	57.0		
NDM-P	HEX-TGGATCAAGCAGGAGAT-ZEN/IBFQ	17	48.3		
KPC-F	GGCCGCCGTGCAATAC	16	56.0	61	Garcia et al., 2010; CDC 2011
KPC-R	GCCGCCCAACTCCTTCA	17	56.5		
KPC-P	6FAM-TGATAACGCCGCCGCCAATTTGT-ZEN/IBFQ	23	62.2		

mixture was incubated for 15 min at 95°C, followed by 40 cycles of 10 s at 95°C and 20 s at 60°C. Normalized expression of $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-58}}$ genes was calculated relatively to the 16S rRNA reference gene according to the $2^{-\Delta\Delta Ct}$ method [29].

2.6. Multiple-Locus Variable Number Tandem Repeat Analysis. Multiple-locus variable number tandem repeat analysis (MLVA) as previously described [17, 30, 31] was used to profiling the *A. baumannii* isolates in the study. The method works on eight variable number tandem repeat (VNTR) loci, namely, 3468, 1988, 3002, 845, 2396, 5350, 826, and 2240 to determine relatedness among the *A. baumannii* isolates.

2.7. β-Lactamase Extraction and Quantitation. Isolates were grown on LB medium supplemented with 1 μmol·ml^{-1} oxacillin for 18-24 h at 37° C in a shaking incubator. The supernatants (extracellular fraction) were collected after centrifugation of bacterial cultures and precipitated with absolute ethanol (1 : 4) in 20 min at -20°C [32]. Periplasmic fractions were recovered from cell pellets [33]. Protein concentration was determined by the Bradford method [34].

β-Lactamase activity was determined based on nitrocefin hydrolysis [35, 36]. Briefly, 1-5 μl extracellular and periplasmic fractions obtained from each isolate were incubated with 40 nmol nitrocefin dissolved in 0.1 M phosphate buffer, pH 7.0 in a total volume of 100 μl. Samples were loaded onto microtiter plates, and the absorbance at 482 nm was measured kinetically at room temperature for 2-30 minutes using an ELISA spectrophotometer. The specific β-lactamase activity was calculated and expressed as mU·mg^{-1} of protein based on the quotient of β-lactamase activity (mU·ml^{-1}) and protein concentration (mg·ml^{-1}).

2.8. Statistical Analysis. The analysis of variance (ANOVA) was used to analyse the difference among β-lactamase activity means of isolates. A t-test was used to determine the significant difference of extracellular and periplasmic β-lactamase activity. A p value < 0.05 was considered significant.

3. Results and Discussion

bla_{OXAs} are prevalent in *A. baumannii*. We had previously performed bla_{OXA} identification in *A. baumannii* isolates from three hospitals in Southern Vietnam and found $bla_{\text{OXA-23}}$ was dominant [17]. Even though $bla_{\text{OXA-58}}$ existed with a small number in Vietnamese population, the exact genetic context involving antimicrobial resistance elements remained unknown. Here, we uncovered the imipenem-resistance mechanism of $bla_{\text{OXA-58}}$-positive *A. baumannii* isolates. The overexpression of $bla_{\text{OXA-58}}$ gene has been seen in the isolate with high-resistance phenotype through relative quantification of mRNA of the corresponding gene. The specific possible-intact IS*Aba3* sequence upstream of $bla_{\text{OXA-58}}$ gene could be the key factor for the high expression. In addition, the high β-lactamase activity in the periplasmic space observed in the study could be the outcome of the phenomenon.

3.1. Antimicrobial Susceptibility Testing. All five isolates (DN050, TN078, DN014, TN341, and TN345) were classified as multidrug resistant (MDR) since they were nonsusceptible to at least one agent in three or more antimicrobial categories including aminoglycosides, antipseudomonal carbapenems, antipseudomonal fluoroquinolones, antipseudomonal penicillins and β-lactamase inhibitors, extended-spectrum cephalosporins, folate pathway inhibitors, penicillins and β-lactamase inhibitors, polymyxins, and tetracyclines [37]. In this study, although several antimicrobials were not tested because of their availableness at different times and hospitals, all isolates satisfied the definition to be defined as MDR. Isolate DNA050 was nonsusceptible to all antimicrobials tested. The other four were all susceptible to imipenem (there were three isolates nonsusceptible to meropenem as hospitals reported), but for other antimicrobials, their susceptibility varied. Isolate TN078 and DN014 were nonsusceptible to three categories while isolates TN341 and TN345 were nonsusceptible to five categories (Table 2).

3.2. Isolate Genotyping and Profiling. All isolates were identified as *A. baumannii* based on 16S-23S intergenic spacer (ITS) region sequencing. Based on MLVA profiling, four different MLVA types within the five isolates reflected substantial genetic diversity in the sampled Vietnamese *A. baumannii* isolates, as previously described [17].

No isolate with bla_{KPC} gene was detected, while two isolates contained bla_{NDM} gene (DN050 and TN078). Even though the two isolates were singletons (based on MLVA types from previous study [17]) with different phenotypes, they had close relatedness with just difference in 3/8 loci surveyed and very similar resistance determinants, especially the bla_{NDM} gene. Therefore, the difference in resistance phenotype was mostly because of the distinguished genotype with ISAba3_$bla_{\text{OXA-58}}$ in isolate DN050, compared to isolate TN078. It might be necessary for bla_{NDM} gene located in a specific genetic context to be expressed as one of the important and strong resistance determinants. The mechanism should be explored further.

Regardless of the genetic diversity of the isolates, the $bla_{\text{OXA-58}}$ gene sequence analysis (data not shown) of all isolates was identical with the reported $bla_{\text{OXA-58}}$ gene [38]. This was in agreement with a previous work showing a lack of diversity in this gene, probably due to its recent acquisition by *A. baumannii* from other species [3].

All isolates were $bla_{\text{OXA-58}}$- and $bla_{\text{OXA-51}}$-positive and $bla_{\text{OXA-23}}$- and $bla_{\text{OXA-24}}$-negative (Table 2). The analysis of insertion sequences revealed the presence of IS*Aba1* and IS*Aba2*, but they were not located upstream of $bla_{\text{OXA-51}}$ nor $bla_{\text{OXA-58}}$ genes in all isolates. IS*Aba4* and IS18 were not detected. IS*Aba3* was detected in all isolates (Table 2). However, only isolate DN050 possessed a $bla_{\text{OXA-58}}$ gene bracketed by two IS*Aba3* elements (Figures 1 and 2). The promoter region of $bla_{\text{OXA-58}}$ gene in this isolate (Figure 2) was similar to sequences described by Poirel and Nordmann [38]. The genetic structure of $bla_{\text{OXA-58}}$ upstream sequences which led to overexpression of this gene displayed a remarkable variability [38–40]. Hybrid promoters constituting an IS*Aba3* sequence truncated by other insertion sequences were generally considered strong promoters [22, 41]. However, in this study, isolate DN050 bearing possible-intact IS*Aba3* sequence upstream of $bla_{\text{OXA-58}}$ gene was not interrupted by inserted sequences, provided -35 and -10 promoter sequences as already described [38]. This structure probably drove high level carbapenemase production. The acquisition of insertion sequences by an imipenem-susceptible $bla_{\text{OXA-58}}$ harboring isolate can lead to carbapenem resistance in *A. baumannii* [38]. Our results highlighted the threat of undetected reservoirs of carbapenem-resistant determinants and mechanisms in Vietnamese *A. baumannii* isolates.

3.3. Relative Quantitation of $bla_{\text{OXA-58}}$ and $bla_{\text{OXA-51}}$ mRNA Level. We chose three isolates (DN050, TN341, and TN345) to study the relative expression of $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-58}}$ under condition with oxacillin as an inducer and without oxacillin induction. They all had high β-lactamase activity in periplasmic fractions as shown in the following experiment (Table 3). The mRNA level of $bla_{\text{OXA-58}}$ and $bla_{\text{OXA-51}}$ genes in all isolates was determined by quantitative real-time RT-PCR. Under oxacillin induction, DN050 showed a significantly higher level of $bla_{\text{OXA-58}}$ mRNA expression than isolates TN341 and TN345 (Figure 3). $bla_{\text{OXA-51}}$ expression was also upregulated, but not comparable to that of $bla_{\text{OXA-58}}$. Interestingly, the high expression level of $bla_{\text{OXA-58}}$ from DN050 could be associated with the presence of an upstream IS*Aba3* sequence as previously suggested [38]. Furthermore, in this study, the possible intact IS*Aba3* sequence might be customized to $bla_{\text{OXA-58}}$ gene to drive a very strong gene expression, as seen in IS*Aba1* for $bla_{\text{OXA-23}}$ and AmpC genes [42]. The other isolates lacked upstream IS*Aba3* sequence.

Table 2: β-Lactamase susceptibility profiles and genotypes of five *A. baumannii* isolates.

Isolate	ID	DN050	TN078	DN014	TN341	TN345
Phenotype	Imipenem	+	−	−	−	−
	Meropenem	+	N/A	+	+	+
	Ceftazidime	+	+	−	+	+
	Cefotaxime	+	+	+	N/A	+
	Ceftriaxone	+	+	+	+	+
	Cefpodoxime	N/A	+	N/A	+	+
	Cefepime	+	−	+	+	+
	Piperacillin	+	N/A	−	N/A	N/A
	Ampicillin/sulbactam	+	−	−	+	+
	Piperacillin/tazobactam	+	−	−	+	+
	Ticarcillin/clavulanic acid	N/A	+	N/A	+	+
	Amikacin	+	+	N/A	N/A	N/A
	Gentamicin	+	+	N/A	+	+
	Ankamycin	N/A	N/A	+	+	−
	Netilmicin	N/A	N/A	−	+	+
	Ciprofloxacin	+	−	+	+	+
	Levofloxacin	+	−	−	N/A	N/A
Genotype	$bla_{\text{OXA-51}}$	+	+	+	+	+
	$bla_{\text{OXA-23}}$	−	−	−	−	−
	$bla_{\text{OXA-24}}$	−	−	−	−	−
	$bla_{\text{OXA-58}}$	+	+	+	+	+
	IS*Aba1*	+	+	−	+	−
	IS*Aba2*	+	+	+	−	−
	IS*Aba3*	+	+	+	+	+
	IS*Aba4*	−	−	−	−	−
	IS18	−	−	−	−	−
	IS*Aba3*_*bla*OXA-58	+	−	−	−	−
	NDM	+	+	−	−	−
	KPC	−	−	−	−	−
	MLVA profile*	6-0-7-1-17-5-0-3	6-0-7-14-17-6-15-3	9-0-7-1-7-5-14-3	9-0-5-15-15-6-0-2	9-0-5-15-15-6-0-2

N/A: not determined; −: assay negative (susceptible/absence); +: assay positive (resistant/presence). *MLVA profile according to the surveyed loci: 3468-1988-3002-845-2396-5350-826-2240.

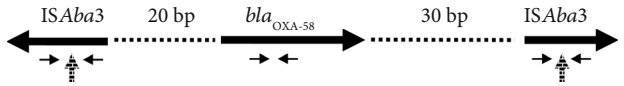

Figure 1: Genetic structures identified in the IS*Aba3*-$bla_{\text{OXA-58}}$-positive *A. baumannii* isolate, DN050. IS*Aba3* and $bla_{\text{OXA-58}}$ genes were indicated by horizontal bold arrows. Horizontal dash lines indicated sequences separating IS*Aba3* and $bla_{\text{OXA-58}}$. Vertical arrows were for the truncated sites previously reported that did not exist in this isolate. Positions of primers were indicated as referred to Table 1 with short thin arrows. The figure is not to scale.

3.4. Analysis of Periplasmic β-Lactamase Activity in Association with $bla_{\text{OXA-51/-58}}$ Relative Expression. Under the condition of oxacillin induction, the $bla_{\text{OXA-58}}$ expression of isolate DN050 ($\text{MIC}_{\text{imipenem}} \geq 32\ \mu\text{g}\cdot\text{ml}^{-1}$) was also significantly higher than the expression of other four isolates, TN078, DN014, TNA341, and TN345 with $\text{MIC}_{\text{imipenem}}$ which were 0.5, 0.19, 0.75, and 0.5, respectively (Table 3). All isolates expressed a low level of $bla_{\text{OXA-51}}$, confirming that the presence of $bla_{\text{OXA-51}}$, without an upstream IS*Aba1*, did not confer a resistance phenotype [16]. Furthermore, in variants harboring $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-58}}$ genes, carbapenem resistance only correlated with $bla_{\text{OXA-58}}$ [43], which is in agreement with the results of this study.

The enzyme activity of extracellular fractions was not significantly different ($p = 0.2187$) while one of the periplasmic fractions exhibited a significant difference among isolates ($p < 0.0001$). Extracellular fractions possessed lower enzyme activity than periplasmic fractions ($p = 0.0355$) in most cases. The periplasmic fraction recovered from all isolates exhibited variable β-lactamase activity, with very high activity corresponding to isolate DN050. Isolates TN341 displayed the highest β-lactamase activity though weakly expressed $bla_{\text{OXA-58}}$ gene. This high enzyme activity probably corresponded to other β-lactamases responsible for the multidrug resistance phenotypes of the isolate, such as

Figure 2: Promoter structure of bla_{OXA-58} gene from isolate DN050. The -35 and -10 putative promoter sequences and the +1 transcription initiation site within IS*Aba3* are boxed. The bla_{OXA-58} start and stop codons, ATG (M) and TAA (∗), respectively, are underlined. Upstream IS*Aba3*/bla_{OXA-58} sequences and downstream IS*Aba3*/bla_{OXA-58} gene junctions are indicated by arrows. Full sequences obtained are deposited in GenBank (accession number KY660721).

Table 3: Relative quantitation of bla_{OXA-51} and bla_{OXA-58} mRNA level and β-lactamase activity in five *A. baumannii* isolates.

Isolate		DN050	TN078	DN014	TN341	TN345
MIC imipenem ($\mu g \cdot ml^{-1}$)		≥32	0.5	0.19	0.75	0.5
Relative expression of bla_{OXA-51}	ΔCt	8.87 ± 0.39	12.87 ± 0.30	7.54 ± 0.66	9.64 ± 0.71	8.80 ± 0.60
	Expression (time)	1.00 (0.76-1.31)	0.06 (0.05-0.08)	2.51 (1.58-3.98)	0.59 (0.36-0.96)	1.04 (0.69-1.59)
Relative expression of bla_{OXA-58}	ΔCt	4.18 ± 1.18	8.64 ± 0.48	7.99 ± 2.23	8.45 ± 0.53	7.28 ± 0.24
	Expression (time)	25.69 (11.3-58.37)	1.17 (0.84-1.64)	1.84 (0.39-8.61)	1.33 (0.93-1.93)	3.01 (2.55-3.56)
Total β-lactamase activity ($U \cdot mg^{-1}$)	Extracellular	10.8 ± 3.3	17.7 ± 5.2	13.9 ± 4.1	10.8 ± 3.3	12.9 ± 3.8
	Periplasmic	44.7 ± 12.8	15.3 ± 4.3	28.5 ± 8.2	49.6 ± 16.0	27.4 ± 10.0

extended-spectrum AmpCs [44]. The presence of other β-lactamases could explain the high enzyme activity in periplasmic fractions of the other isolates. Particularly, bla_{NDM} gene detected in both isolates DN050 and TN078, but the corresponding β-lactamase activities as well as the antimicrobial susceptibilities were different between the two isolates. The mechanism that a strain carrying a bla_{NDM} gene is not resistant to carbapenems needs to be discovered further in *A. baumannii*. It might need a unique genetic structure for bla_{NDM} gene to be expressed as seen in *K. pneumoniae* [45].

In a transformed *A. baumannii* strain with a bla_{OXA-58} plasmid-borne vector, this carbapenemase is selectively released via outer membrane vesicles (OMV) after periplasmic

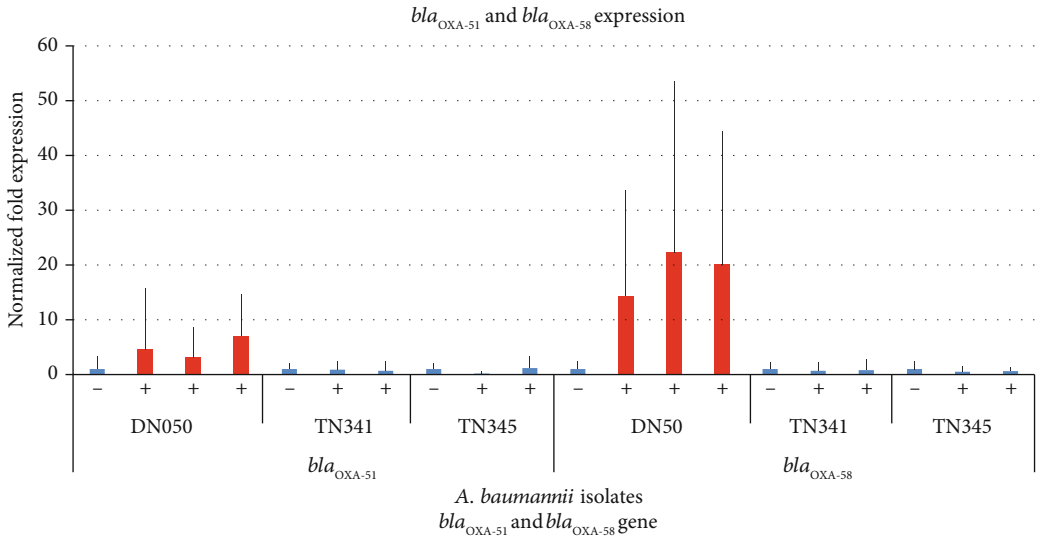

Figure 3: Duplex real-time RT-PCR analysis of the $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-58}}$ mRNA relative expression in three *A. baumannii* isolates. The error bars represent the deviation for the normalized fold expression of $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-58}}$ in three isolates which were positive or negative for the IS*Aba3* upstream of the $bla_{\text{OXA-58}}$ gene. −: not induced; +: induced.

translocation through Sec-dependent system [32]. Furthermore, overexpression of $bla_{\text{OXA-58}}$ gene increases its periplasmic enzyme concentration and extracellular release leading to efficient carbapenem hydrolysis [32]. The $bla_{\text{OXA-58}}$ high mRNA level and high periplasmic β-lactamase activity of the DN050 isolate in this study suggested a similar overexpression, periplasmic translocation, and release mechanism of $bla_{\text{OXA-58}}$ carbapenemase, even though our experimental work did not directly show the selection of OMV after being translocated to a periplasmic space. The high periplasmic β-lactamase activity of the isolates, especially TN341 in this study, also suggested a possible translocation and release of other β-lactamases with a mechanism similar to that identified with $bla_{\text{OXA-58}}$. Further studies should be carried out to prove the suggested mechanism in clinical isolate similar to the transformed *A. baumannii* strain. To the best of our knowledge, our study is the first report on the overexpression of $bla_{\text{OXA-58}}$ gene of *A. baumannii* clinical isolates from Vietnam.

This study had some limitations. The first limitation involved the small sample size due to the low prevalence of clinical isolates harboring $bla_{\text{OXA-58}}$ gene in the population surveyed. The screening has been done in previous studies [17]. Secondly, we did not characterize other resistance mechanisms in *A. baumannii* such as the overexpression of efflux pump genes or existence of multicopy $bla_{\text{OXA-58}}$ gene [7, 11, 46]. In addition, the presence of other β-lactamase genes such as bla_{IMP}, bla_{VIM}, bla_{GES}, $bla_{\text{OXA-143}}$, and $bla_{\text{OXA-235}}$ was not excluded. Furthermore, we did not carry out an alternative experimental approach, such as western blotting against $bla_{\text{OXA-58}}$ to unequivocally determine if the increase in β-lactamase activity is mainly due to this protein.

4. Conclusions

This study identified a mechanism of imipenem resistance related to the overexpression of $bla_{\text{OXA-58}}$ gene preceded by IS*Aba3* and its corresponding periplasmic enzyme present at high concentration in a multidrug-resistant clinical isolate recovered from a hospital in Vietnam.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Supplementary Materials

Figure S1: result of imipenem *E*-test for five clinical isolates of A. baumannii (blaOXA-58). Figure S2: electrophoresis results of PCR screening for the presence/absence of ISAba1, ISAba2, ISAba3, ISAba4, and IS18 in five clinical isolates of A. baumannii (blaOXA-58). Figure S3: electrophoresis results of PCR for the presence/absence of insertion sequence (IS) upstream of blaOXA-58 gene. Figure S4: duplex real-time RT-PCR analysis of the blaOXA-51 and blaOXA-58 mRNA relative expression compared with 16S rRNA in five A. baumannii isolates. Figure S5: Bradford assay standard curve of concentration versus absorbance for protein

quantification. Figure S6: nitrocefin standard curve. Table S1: duplex real-time RT-PCR analysis of the blaOXA-51 and blaOXA-58 mRNA relative expression in three A. baumannii isolates under conditions with oxacillin as an inducer or without oxacillin induction. Table S2: results for protein quantification of supernatant and periplasmic fractions. Table S3: results for β-lactamase activity of supernatant and periplasmic fractions. *(Supplementary Materials)*

References

[1] P. E. Fournier, D. Vallenet, V. Barbe et al., "Comparative genomics of multidrug resistance in *Acinetobacter baumannii*," *PLoS Genetics*, vol. 2, no. 1, 2006.

[2] C. H. Rodriguez, M. Nastro, and A. Famiglietti, "Carbapenemasas en *Acinetobacter baumannii*. Revisión de su diseminación en América Latina," *Revista Argentina de Microbiología*, vol. 50, no. 3, pp. 327–333, 2018.

[3] B. A. Evans and S. G. B. Amyes, "OXA β-Lactamases," *Clinical Microbiology Reviews*, vol. 27, no. 2, pp. 241–263, 2014.

[4] I. Rossi, S. Royer, M. L. Ferreira et al., "Incidence of infections caused by carbapenem-resistant *Acinetobacter baumannii*," *American Journal of Infection Control*, vol. 47, no. 12, pp. 1431–1435, 2019.

[5] WHO, "Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis," pp. 1–87, 2017.

[6] V. Manchanda, S. Sanchaita, and N. Singh, "Multidrug resistant *Acinetobacter*," *Journal of Global Infectious Diseases*, vol. 2, no. 3, pp. 291–304, 2010.

[7] L. Poirel and P. Nordmann, "Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology," *Clinical Microbiology and Infection*, vol. 12, no. 9, pp. 826–836, 2006.

[8] P. G. Higgins, C. Dammhayn, M. Hackel, and H. Seifert, "Global spread of carbapenem-resistant *Acinetobacter baumannii*," *Journal of Antimicrobial Chemotherapy*, vol. 65, no. 2, pp. 233–238, 2010.

[9] J. Walther-Rasmussen and N. Hoiby, "OXA-type carbapenemases," *Journal of Antimicrobial Chemotherapy*, vol. 57, no. 3, pp. 373–383, 2006.

[10] T. Martinez, I. Martinez, G. J. Vazquez, E. E. Aquino, and I. E. Robledo, "Genetic environment of the KPC gene in *Acinetobacter baumannii* ST2 clone from Puerto Rico and genomic insights into its drug resistance," *Journal of Medical Microbiology*, vol. 65, no. 8, pp. 784–792, 2016.

[11] S. Brown, H. K. Young, and S. G. Amyes, "Characterisation of OXA-51, a novel class D carbapenemase found in genetically unrelated clinical strains of _*Acinetobacter baumannii*_ from Argentina," *Clinical Microbiology and Infection*, vol. 11, no. 1, pp. 15–23, 2005.

[12] N. Woodford, M. J. Ellington, J. M. Coelho et al., "Multiplex PCR for genes encoding prevalent OXA carbapenemases in _*Acinetobacter*_ spp.," *International Journal of Antimicrobial Agents*, vol. 27, no. 4, pp. 351–353, 2006.

[13] C. Heritier, L. Poirel, and P. Nordmann, "Cephalosporinase over-expression resulting from insertion of IS*Aba1* in *Acinetobacter baumannii*," *Clinical Microbiology and Infection*, vol. 12, no. 2, pp. 123–130, 2006.

[14] S. Figueiredo, L. Poirel, J. Croize, C. Recule, and P. Nordmann, "In Vivo Selection of Reduced Susceptibility to Carbapenems in *Acinetobacter baumannii* Related to IS*Aba1*-Mediated Overexpression of the Natural bla$_{\text{OXA-66}}$ Oxacillinase Gene," *Antimicrobial Agents and Chemotherapy*, vol. 53, no. 6, pp. 2657–2659, 2009.

[15] S. Figueiredo, L. Poirel, A. Papa, V. Koulourida, and P. Nordmann, "Overexpression of the Naturally Occurring bla$_{\text{OXA-51}}$ Gene in *Acinetobacter baumannii* Mediated by Novel Insertion Sequence IS*Aba9*," *Antimicrobial Agents and Chemotherapy*, vol. 53, no. 9, pp. 4045–4047, 2009.

[16] J. F. Turton, M. E. Ward, N. Woodford et al., "The role of ISAba_1 in expression of OXA carbapenemase genes in *Acinetobacter baumannii*," *FEMS Microbiology Letters*, vol. 258, no. 1, pp. 72–77, 2006.

[17] N. Tuan Anh, T. V. T. Nga, H. M. Tuan et al., "Molecular epidemiology and antimicrobial resistance phenotypes of *Acinetobacter baumannii* isolated from patients in three hospitals in southern Vietnam," *Journal of Medical Microbiology*, vol. 66, no. 1, pp. 46–53, 2017.

[18] E. C. Clímaco, M. L. de Oliveira, A. Pitondo-Silva et al., "Clonal complexes 104, 109 and 113 playing a major role in the dissemination of OXA-carbapenemase-producing *Acinetobacter baumannii* in Southeast Brazil," *Infection, Genetics and Evolution*, vol. 19, pp. 127–133, 2013.

[19] P. G. Higgins, F. J. Pérez-Llarena, E. Zander, A. Fernández, G. Bou, and H. Seifert, "OXA-235, a novel class D β-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*," *Antimicrobial Agents and Chemotherapy*, vol. 57, no. 5, pp. 2121–2126, 2013.

[20] M. B. Schultz, D. Pham Thanh, N. Tran Do Hoan et al., "Repeated local emergence of carbapenem-resistant *Acinetobacter baumannii* in a single hospital ward," *Microbial Genomics*, vol. 2, no. 3, article e000050, 2016.

[21] S. D'Arezzo, L. Principe, A. Capone, N. Petrosillo, A. Petrucca, and P. Visca, "Changing carbapenemase gene pattern in an epidemic multidrug-resistant *Acinetobacter baumannii* lineage causing multiple outbreaks in Central Italy," *Journal of Antimicrobial Chemotherapy*, vol. 66, no. 1, pp. 54–61, 2011.

[22] W. Wu, Y. He, J. Lu, Y. Lu, J. Wu, and Y. Liu, "Transition of *bla*$_{\text{OXA-58-like}}$ to *bla*$_{\text{OXA-23-like}}$ in *Acinetobacter baumannii* Clinical Isolates in Southern China: An 8-Year Study," *PLoS One*, vol. 10, no. 9, article e0137174, 2015.

[23] S. Pournaras, A. Markogiannakis, A. Ikonomidis et al., "Outbreak of multiple clones of imipenem-resistant *Acinetobacter baumannii* isolates expressing OXA-$_{58}$ carbapenemase in an intensive care unit," *Journal of Antimicrobial Chemotherapy*, vol. 57, no. 3, pp. 557–561, 2006.

[24] A. Giordano, P. Varesi, A. Bertini et al., "Outbreak of *Acinetobacter baumannii* Producing the Carbapenem-Hydrolyzing Oxacillinase OXA-58 in Rome, Italy," *Microbial Drug Resistance*, vol. 13, no. 1, pp. 37–43, 2007.

[25] N. Ozen, A. Ergani, T. Naas et al., "Outbreak of carbapenem-resistant Acinetobacter baumannii producing the carbapenemase OXA-58 in Turkey," *The Open Antimicrob Agents*, vol. 1, no. 1, pp. 1–8, 2009.

[26] C. Q. Hoang, H. D. Nguyen, H. Q. Vu et al., "Emergence of New Delhi Metallo-Beta-Lactamase (NDM) and *Klebsiella pneumoniae* Carbapenemase (KPC) Production by *Escherichia coli* and *Klebsiella pneumoniae* in Southern Vietnam and Appropriate Methods of Detection: A Cross-Sectional Study," *BioMed Research International*, vol. 2019, Article ID 9757625, 2019.

[27] L. Poirel, E. Lebessi, C. Héritier, A. Patsoura, M. Foustoukou, and P. Nordmann, "Nosocomial spread of OXA-58-positive carbapenem-resistant *Acinetobacter baumannii* isolates in a paediatric hospital in Greece," *Clinical Microbiology and Infection*, vol. 12, no. 11, pp. 1138–1141, 2006.

[28] M. S. Wilson, C. Bakermans, and E. L. Madsen, "In situ, real-time catabolic gene expression: extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater," *Applied and Environmental Microbiology*, vol. 65, no. 1, pp. 80–87, 1999.

[29] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[30] C. Pourcel, F. Minandri, Y. Hauck et al., "Identification of variable-number tandem-repeat (VNTR) sequences in *Acinetobacter baumannii* and interlaboratory validation of an optimized multiple-locus VNTR analysis typing scheme," *Journal of Clinical Microbiology*, vol. 49, no. 2, pp. 539–548, 2011.

[31] N. T. K. Nhu, N. P. H. Lan, J. I. Campbell et al., "Emergence of carbapenem-resistant *Acinetobacter baumannii* as the major cause of ventilator-associated pneumonia in intensive care unit patients at an infectious disease hospital in southern Vietnam," *Journal of Medical Microbiology*, vol. 63, no. 10, pp. 1386–1394, 2014.

[32] Y. T. Liao, S. C. Kuo, M. H. Chiang et al., "*Acinetobacter baumannii* extracellular OXA-58 is primarily and selectively released via outer membrane vesicles after sec-dependent periplasmic translocation," *Antimicrobial Agents and Chemotherapy*, vol. 59, no. 12, pp. 7346–7354, 2015.

[33] G. F. Ames, C. Prody, and S. Kustu, "Simple, rapid, and quantitative release of periplasmic proteins by chloroform," *Journal of Bacteriology*, vol. 160, no. 3, pp. 1181–1183, 1984.

[34] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[35] C. H. O'Callaghan, A. Morris, S. M. Kirby, and A. H. Shingler, "Novel method for detection of beta-lactamases by using a chromogenic cephalosporin substrate," *Antimicrobial Agents and Chemotherapy*, vol. 1, no. 4, pp. 283–288, 1972.

[36] K. Montgomery, L. Raymundo Jr., and W. L. Drew, "Chromogenic cephalosporin spot test to detect beta-lactamase in clinically significant bacteria," *Journal of Clinical Microbiology*, vol. 9, no. 2, pp. 205–207, 1979.

[37] A. P. Magiorakos, A. Srinivasan, R. B. Carey et al., "Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance," *Clinical Microbiology and Infection*, vol. 18, no. 3, pp. 268–281, 2012.

[38] L. Poirel and P. Nordmann, "Genetic structures at the origin of acquisition and expression of the carbapenem-hydrolyzing oxacillinase gene $bla_{\text{OXA-58}}$ in *Acinetobacter baumannii*," *Antimicrobial Agents and Chemotherapy*, vol. 50, no. 4, pp. 1442–1448, 2006.

[39] Y. Fu, J. Jiang, H. Zhou et al., "Characterization of a novel plasmid type and various genetic contexts of $bla_{\text{OXA-58}}$ in *Acinetobacter* spp. from multiple cities in China," *PLoS One*, vol. 9, no. 1, article e84680, 2014.

[40] L. Al-Hassan, A. Opazo, B. S. Lopes, H. El Mahallawy, and S. G. B. Amyes, "Variations in IS6 promoters alter the expression of carbapenem resistance in related strains of *Acinetobacter baumannii*," *Journal of Global Antimicrobial Resistance*, vol. 3, no. 1, pp. 5–8, 2015.

[41] T. L. Chen, R. C. Wu, M. F. Shaio, C. P. Fung, and W. L. Cho, "Acquisition of a plasmid-borne bla_{OXA-58} gene with an upstream IS*1008* insertion conferring a high level of carbapenem resistance to *Acinetobacter baumannii*," *Antimicrobial Agents and Chemotherapy*, vol. 52, no. 7, pp. 2573–2580, 2008.

[42] M. A. Rezaee, O. Pajand, M. R. Nahaei et al., "Prevalence of Ambler class A β-lactamases and *amp*C expression in cephalosporin-resistant isolates of *Acinetobacter baumannii*," *Diagnostic Microbiology and Infectious Disease*, vol. 76, no. 3, pp. 330–334, 2013.

[43] A. Tsakris, A. Ikonomidis, S. Pournaras, N. Spanakis, and A. Markogiannakis, "Carriage of OXA-$_{58}$ but not of OXA-$_{51}$ beta-lactamase gene correlates with carbapenem resistance in *Acinetobacter baumannii*," *Journal of Antimicrobial Chemotherapy*, vol. 58, no. 5, pp. 1097–1099, 2006.

[44] J.-M. Rodríguez-Martínez, P. Nordmann, E. Ronco, and L. Poirel, "Extended-spectrum cephalosporinase in *Acinetobacter baumannii*," *Antimicrobial Agents and Chemotherapy*, vol. 54, no. 8, pp. 3484–3488, 2010.

[45] D. Yong, M. A. Toleman, C. G. Giske et al., "Characterization of a New Metallo-β-Lactamase Gene, $bla_{\text{NDM-1}}$, and a Novel Erythromycin Esterase Gene Carried on a Unique Genetic Structure in *Klebsiella pneumoniae* Sequence Type 14 from India," *Antimicrobial Agents and Chemotherapy*, vol. 53, no. 12, pp. 5046–5054, 2009.

[46] A. Bertini, L. Poirel, S. Bernabeu et al., "Multicopy $bla_{\text{OXA-58}}$ Gene as a Source of High-Level Resistance to Carbapenems in *Acinetobacter baumannii*," *Antimicrobial Agents and Chemotherapy*, vol. 51, no. 7, pp. 2324–2328, 2007.