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Studies of common antibiotic resistance-associated genes of *Acinetobacter baumannii*

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Abstract:

This study, the presence of 4 common beta-lactamase encoding genes including OXA-23, OXA-51, VIM and IMP was determined simultaneously by multiplex PCR, either directly with sputum samples or with purified DNA, giving results within 3-4 hours instead of 2 days compared to the culture method. Within 58 samples positive with *Acinetobacter baumannii* collected from the hospital, OXA-51 fragment was dominant (81%), whereas, VIM fragment was only identified in one sample (DNA and sputum samples) (1.7%). The antibiograms indicated for 19 samples were used to find correlation between genotype and resistance phenotype.

Keywords: ***Acinetobacter baumannii*, antibiotic resistance-associated genes, beta-lactamase, multiplex PCR, susceptibility.**

Classification number: **3.5**

Introduction

Pneumonia is a form of acute respiratory infection that affects the lungs. When an individual has pneumonia, the alveoli are filled with pus and fluid, which make breathing painful and limits oxygen intake. Pneumonia is the single largest infectious cause of death in children worldwide. Pneumonia killed 920,136 children under the age of five around the world in 2015, accounting for 16% of all deaths of children under five years old [1]. Pneumonia is caused by a number of infectious agents, including viruses, bacteria, and fungi. Antibiotics can be effective for many of the bacteria that cause pneumonia. Unfortunately, antibiotic resistance is growing amongst the bacteria that cause pneumonia, often arising from the overuse and misuse of antibiotics [2]. The most dangerous antibiotic-resistant bacteria that causes pneumonia is *Acinetobacter baumannii.*

A. baumannii is a pleomorphic aerobic gram-negative bacillus belonging to the *Moraxellaceae* family of the *Gammaproteobacteria* class. It can survive on surfaces for a long time. *A. baumannii* has several resistance mechanisms to many classes of antimicrobials, including beta-lactamase, multidrug efflux pumps, aminoglycoside-modifying enzymes, permeability defects, and alteration of target sites. The beta-lactam-resistant mechanism plays the most important role in multidrug resistance in *A. baumannii*. It involves the hydrolysis of beta-lactam-based drugs by beta-lactamases. These enzymes are divided into four classes: A, B, C, D, and their antibiotic-resistant abilities are not equivalent. Classes B and D are more concerning because beta-lactamases in class B are mostly strong penicillinase, and, for genes in class D, some OXAs can hydrolyse wide-range cephalosporins and carbapenem [3]. Studying genes of this class is essential because this can help doctors reduce the time of diagnosis and treatment for patients suffering from *A. baumannii* pneumonia.

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Recently, there have been several antimicrobial-susceptibility testing methods available for *A. baumannii*, such as cultured-based methods (disk diffusion, E-test [4]), genotypic methods (polymerases chain reaction, DNA hybridisation), and single-cell imaging method [5]. These all have the same goal: to provide a reliable prediction of the sensitivity of *A. baumannii* to respond therapeutically to a particular antibiotic treatment. Cultured-based methods are the gold standard and are routinely used in hospitals; however, they require considerable time for analysis. Single-cell imaging is a novel form of testing prototypes, with very short time for analysis; however, it has been applied only in research, not in practice. Genotyping methods can be an effective alternative to these two methods, with shorter test result turn-around time.

In Vietnam, most studies have focused only on the level of resistance of *A. baumannii* to antimicrobials often used in hospital. In the study of V.Q. Nguyen (2013) on 32 strains of *A. baumannii* isolated from 98 patients suffering from pneumonia at Hue Central Hospital, the results showed that this bacteria resistant to all of the 17 antibiotics tested [6]. T.H.P. Ngo, et al. (2013) generated a similar result: 100% of the *A. baumannii* specimens were resistant to most of the common antibiotics, except Colistin [7]. H.H. Tran, et al. (2017) investigated the presence of beta-lactamases in classes B and D, including *NDM-1, OXA-23, OXA-51, IMP,* and *VIM,* in *A. baumannii* from patients at three major hospitals in Hanoi [8]. However, this study does not mention the correlation between gene sequences and antibiogram to support diagnosis. These studies of the antibiotic resistance of *A. baumannii* are only in the initial phase and are not systematic. Therefore, our study focuses on four beta-lactamase-encoding genes, *OXA-23* and *OXA-51* in class D and *VIM* and *IMP* in class B, that may affect the susceptibility of *A. baumannii* to antibiotics.

Materials and methods

Materials

Specimens: a total 58 samples, including purified DNA (52/58) and sputum (6/58), identified positive for *A. baumannii* were collected by the National TB Reference Lab at the National Lung Hospital. DNA samples were extracted from cultured *A. baumannii*. Sputum was treated with NaLC-NaOH and then heated at 95°C for 10 minutes before being stored at -20°C.

Primers: the list of primers used for the detection of the beta-lactamase-encoding genes of *A. baumannii* are listed in Table 1.

Table 1. Primer list for *OXA-23, OXA-51, IMP,* and *VIM* fragments.

Beta-lactamase gene	Product size (bp)	Sequence (5'-3')
OXA-23	501	F- GATCGGATTGGAGAACCAGA R- ATTTCTGACCGCATTTCCAT [9]
OXA-51	353	F- TAATGCTTTGATCGGCCTTG R- TGGATTGCACTTCATCTTGG [9]
VIM	390	F- GATGGTGTTTGGTCGCATA R- CGAATGCGCAGCACCAG [10]
IMP	188	F- GGAATAGAGTGGCTTAAYTCTC R- CCAAACYACTASGTTATCT [10]

Methods

Multiplex PCR for identifying the four target genes simultaneously: identification of the four beta-lactamase-encoding genes' co-presence was achieved by means of multiplex PCR. The multiplex PCR was designed to include 1U Dream Taq polymerase (Thermo Scientific), 1X Green Taq Buffer (Thermo Scientific), dNTPs, four primer pairs at concentrations of 0.25-0.5 µM, DNA and sputum samples, and sterile deionised water. The reaction was conducted in a volume of 12.5 µl and occurred according to the following thermal cycles: 94°C, 5 minutes; 30 cycles of 94°C, 30 seconds, 50°C, 45 seconds, 72°C, 50 seconds, and finally, 72°C, 5 minutes. Four pairs of primers used in the amplified reaction mixture: the 501 bp fragment corresponding to the *blaOXA-23* gene, the 353 bp gene coded for the *blaOXA-51* gene, the 390 bp gene encoding the *blaVIM* gene, and the 188 bp fragment within the *blaIMP* gene.

Analysis of PCR products by means of agarose electrophoresis: in this study, 5 µl of multiplex PCR product was analysed using 3% agarose gel electrophoresis (3 g/100 ml) (Clever Scientific, England) with 1X RedSafe DNA staining solution (Intron Biotechnology, Korea), which replaced the traditional Ethidium bromide stain. Following gel electrophoresis, the gel was observed under UV light (GelDoc, BIO-RAD).

Results and discussion

Optimisation of multiplex PCR for detecting the four beta-lactamase coding genes, *OXA-51, OXA-23, VIM,* and *IMP:* primer specificity was initially confirmed through the presence of single bright bands at calculated molecular weights for each target gene in a single PCR. To establish the multiplex PCR, which simultaneously amplified the four fragments in one amplification reaction, the annealing temperature (50°C, 52°C) (Fig. 1A), primer concentration (Fig. 1B), and DMSO concentrations (0-10%, 2% interval) were examined. As a result, the optimal condition was conducted at 50°C annealing temperature, 0.2 µM of *OXA-23* and *OXA-51* primer, and 0.3 µM for VIM, 0.4 µM for IMP,

and DMSO concentration of 8%. The results showed four specific bands corresponding to the four target genes of *A. baumannii* and no extra band. Moreover, when performing PCR with DNA and sputum samples, the quality of PCR products was comparable; therefore, sputum could be used directly for the multiplex PCR, reducing testing time.

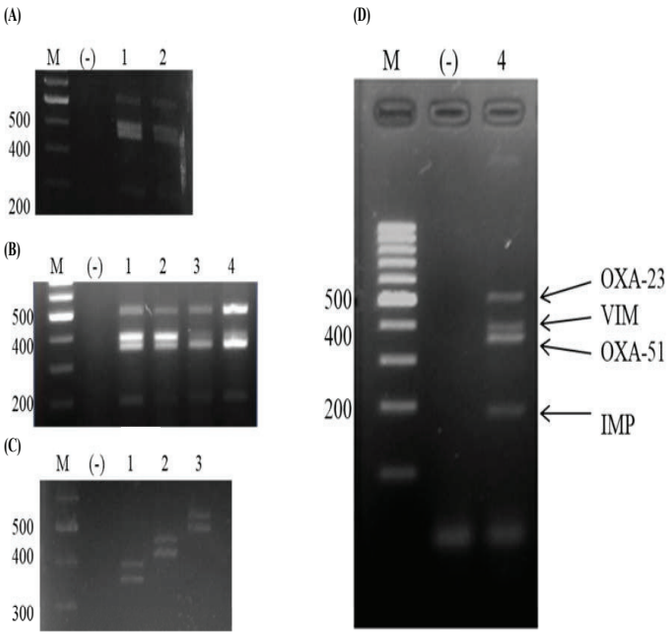

Fig. 1. Optimisation of multiplex PCR for the four targets. (**A**) Annealing temperature optimisation at 500C (1) and 520C (2) was examined. (**B**) Primer optimisation with different concentrations of *OXA-23*, *OXA-51*, *VIM*, and *IMP*, 0.2; 0.2; 0.3; 0.4 µM (1); 0.2; 0.1; 0.1; 0.3 µM (2); 0.3; 0.1; 0.1; 0.3 µM (3); 0.3; 0.3; 0.2; 0.4 µM (4). (**C**) Electrophoresis running for 50 minutes (1), 45 minutes (2), 40 minutes (3). (**D**) Final multiplex PCR. (M): marker; (-): negative.

In order to resolve the 353-bp and 390-bp bands of *OXA-51* and *VIM*, respectively, three different running times (40-50 minutes, 5-minute interval) were tested and the final electrophoresis condition was at 100 volts for 45 minutes.

Prevalence of the four targeted resistance-associated genes

Screening of the 58 samples (52 DNA and 6 sputum samples) using the established multiplex PCR showed that the presence of *OXA-51* was the highest (47/58, 81%), following by *OXA-23*: (63.8%, 37/58), and *IMP* (8.6%, 5/58); *VIM* (1.7%), the lowest, was found in only one sample (Fig. 2). Such prevalence is in line with the findings of other studies [9, 10]. However, compared to others, the sample size in our study was relatively small; therefore, the study should be expanded to obtain more statistically significant data.

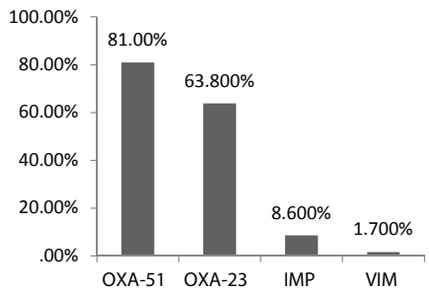

Fig. 2. Percentages of the four genes, *OXA-51, OXA-23, IMP,* and *VIM* in the 58 samples.

The consistently lower percentages of *VIM* and *IMP* are explained by the ineffective amplification of these fragments. Our established multiplex PCR also showed the unequal intensity of the four bands in one sample carrying all four genes, in which the *VIM* band was the faintest (Fig. 1D). In future studies, RT-PCR may be a method for increasing the sensitivity of detection and also allowing investigation of the copy number of the gene.

Correlation between genomic profiling and antibiotic susceptibility

The results of the antibiotic sensitivity test of eight β-lactam antibiotics, as indicated in 19/58 samples, revealed that three antibiotics, CRO, FEP (a cephalosporin), and

Table 2. Genomic profiling and antibiotic susceptibility.

No	PCR		Culture-based method							
	OXA-23	*OXA-51*	*IMP*	*DOR*	*MEM*	*CAZ*	*CRO*	*FEP*	*TCC*	*TZP*
30	-	+								
31	-	-								
36	+	+								
37	+	+								
38	-	+								
39	+	+								
41	-	+								
45	+	+								
48	+	+								
49	+	+								
50	+	+								
51	+	+								
52	+	+								
53	+	+								
54	+	+								
55	+	+								
56	+	+								
57	+	+								
58	+	+								
∑	15	18	15		16	16	17	17	17	15

: No information : Sensitive : Intermediate : Resistant

IMP: imipenem; DOR: doripenem; MEM: meropenem; CAZ: ceftazidime; CRO: ceftriaxone; FEP: cefepime; TCC: tic + A. clavulanic; ZP: pipe + tazobactam.

TCC were the most resisted (17/19), while IMP and TZP were less resisted (15/19); however, the difference between the two groups was insignificant (Table 2). An examination of the molecular characteristics of these 19 samples showed the presence of *OXA-51* in 18 samples and *OXA-23* in 15 samples, whereas no samples carried *VIM* and *IMP*.

The sample designated 31, lacking both *OXA-51* and *OXA-23*, was resistant to only TCC and intermediately to CRO, while being sensitive to all the other six antibiotics. The remaining 14 samples containing both genes were resistant to all seven or eight antibiotics tested. The results confirms the association of *OXA-51* and *OXA-23* with β-lactam resistance, which had been addressed in previous studies. The results also promote the importance of examining these resistant genes for supporting drug prescription and treatment in addition to the conventional antibiotic sensitivity test.

Conclusions

The established multiplex PCR was successful in simultaneously detecting four beta-lactamase-encoding genes: *OXA-23, OXA-51, VIM,* and *IMP*. In a total of 58 samples, the *OXA-51* gene predominated, with more than 80% carrying the target. *VIM* was identified in only a single sample, approximately 1.7%. The presence of *OXA-51* and *OXA-23* is likely linked to the β-lactam resistance of *A. baumannii,* a finding that could be used for prescribing effective antibacterial drugs.

The authors declare that there is no conflict of interest regarding the publication of this article.

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