Dissemination of class D carbapenemase resistance genes and plasmid profiles of *Acinetobacter baumannii* isolated from Chiangrai Prachanukroh hospital, Thailand

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Abstract

   Acinetobacter baumannii is a gram negative bacteria causing nosocomial infection, especially in intensive care unit (ICU). In Thailand, the prevalence of carbapenem resistance *A. baumannii* (CR-AB) has been increasing in the past decades. The aims of this study were to determine the antibiotic susceptibility patterns, the plasmid profiles of extensively drug resistant *A. baumannii* (XDR-AB) and to detect the class D carbapenemase resistance genes in *A. bauamannii* isolated from Chiangrai Prachanukroh hospital, Thailand. The specimens were collected from July to October 2014. All *A. baumannii* isolates were determined antibiotic susceptibility pattern by the disk diffusion method. Our results showed the prevalence of carbapenem resistance *A. baumannii* (CR-AB) to be 85.32%. The percentage of antibiotic resistence were 49.54% (amikacin), 56.88% (cefepime), 79.82% (cefotaxime), 12.84% (cefpopera/sulbactam), 77.98% (ceftazidime), 80.73% (ceftriazone), 81.65% (ciprofloxacin), 64.22% (gentamicin), 85.32% (imipenem), 85.32% (meropenem), 78.90% (piperacillin/tazo-bactam), 69.72% (tetracycline) and 42.20% (trimethoprim/sulfamethoxazole). All isolates were sensitive to colistin and tigecycline. Class D carbapenemase genes were investigated by multiplex polymerase chain reaction (multiplex-PCR) amplification. Most isolates carried OXA carbapenemase genes, including *bla*$_{OXA51}$ 97.25%, *bla*$_{OXA23}$ 75.32%, *bla*$_{OXA58}$ 7.34%. XDR-AB strains were selected to study the plasmid profiles. Each strain carried two plasmids, ranging in size from 20 to 23 Kb. The *bla*$_{OXA23}$ gene presented in all XDR-AB plasmids. Data obtained from our study showed the evaluation of antibiotic resistance genes in *A. baumannii* which is necessary to prevent the further spread of antibiotic resistance bacteria.

INTRODUCTION

       Acinetobacter baumannii is gram negative bacteria that play an important role in nosocomial infection, mainly affecting patients in the intensive care unit (ICU). *A. baumannii* is generally found naturally in soil, water and in the hospital environment*,* where it can survive on inanimate surfaces, instruments etc. It can colonize on multiple site of human skin, thus being a danger to inpatients (Towner 2009, Peleg, Seifert, and Paterson 2008). The types of infections caused by this pathogen are pneumonia, bacteremia, wound infection and meningitis (Howard et al. 2012). In addition, *A. baumannii* strains are usually resistant to antimicrobial agents, including aminoglycosides, fluoroquinolones, tetracyclines and β-lactams such as carbapenems (McConnell, Actis, and Pachon 2013). Carbapenems are drugs used for treatment of infection caused by multidrug-resistant *A. baumannii* (MDR-AB). In the past decades, the prevalence of carbapenem-resistant *A. baumannii* (CR-AB) has been increasing in hospitals worldwide (Higgins et al. 2010). In Thailand, previous studies reported that extensively drug resistant *A. baumannii* (XDR-AB) has increased from 46.0% in 2001 to 67.5% in 2009 (Chaiwarith et al. 2004, Aimsaad et al. 2009). The mechanisms of drug resistance such as efflux pump overexpression*,* decrease in membrane permeability and especially production of antibiotics-modifying enzymes were found in *A. baumannii* strains (Rice 2006). Major mechanism to carbapenem resistance in *A. baumannii* is the production of class D carbapenemases, which are referred to oxacillinase. These enzymes are encoded by bla_{OXA23}, bla_{OXA24}, bla_{OXA58} and intrinsic bla_{OXA51} genes (Zarrilli et al. 2009). Previous studies have reported that the most common acquired class D carbapenemase gene in Asia is bla_{OXA23} and it is categorized as a high dissemination in Thailand, including Bangkok, Phitsanulok, Prachuap Khiri Khan, Songkla, Pathum Thani and Ubon Ratchathani (Karunasagar et al. 2011, Lee et al. 2013, Niumsup et al. 2009, Santimaleeworagun et al. 2014, Jantama et al. 2013, Thapa et al. 2010, Teo et al. 2015). Some of these genes such as bla_{OXA23}, bla_{OXA24} and bla_{OXA58} have been reported to be encoded on plasmids (Evans

and Amyes 2014). Generally, plasmids are circular double stranded extra-chromosomal DNA, that replicates independently from the host chromosome. Plasmids have contributed to the spread of antibiotics resistant genes to other bacterial strains (Potron, Poirel, and Nordmann 2011, Yang, Nam, and Lee 2014). Plasmid carry antibiotic resistant genes which can be spread by the mechanism called conjugation and transformation while the genome based resistant genes are spread by replication (Huddleston 2014). Therefore, plasmid have an important role in bacterial evolution and adaptability (Heuer and Smalla 2012). However, there have been only a few studies into the occurrence of CR-AB isolates in northern part of Thailand. No previous studies have been reported on plasmid profiles in XDR-AB strains. In this present study, we determined the antimicrobial susceptibility patterns and detected the class D carbapenemase resistance genes in the *A. baumannii* strains isolated from Chiangrai Prachanukroh hospital, Thailand. We further characterized the plasmid profiles of XDR-AB using plasmid extraction method.

MATERIAL AND METHODS

Bacterial isolation and identification of *A. baumannii*

  A. baumannii isolates were collected from patients hospitalized between July to October 2014 in Chiangrai Prachanukroh hospital, Chiangrai. All *A. baumannii* isolates were identified by biochemical methods, including oxidase, triple sugar iron (TSI), motile indole lysine (MIL), citrate and urease test. Polymerase chain reactions (PCR) were performed to confirm *Acinetobacter* spp. using 16s rRNAs gene as described by Misbah et al. (2005)

Determination of antibiotic susceptibility

  The antibiotic susceptibility of *A. baumannii* isolates were determined using disc diffusion method. The results were interpreted according to CLSI (2014). Disc diffusion test were performed with the following 15 antibiotics : amikacin (30 µg), cefepime (30 µg), cefotaxime (30 µg), cefpopera/sulbactam (105 µg), ceftazidime (30 µg), ceftriazone (30 µg), ciprofloxacin (5 µg), colistin (10 µg) gentamicin (10 µg), imipenem (10 µg), meropenem (10 µg), piperacillin/tazobactam (100/10 µg), tetracycline (30 µg), tigecycline (15 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg). Multidrug resistant *A. baumannii* (MDR-AB), Carbapenem resistant

A. baumannii (CR-AB) and extensively drug resistant *A. baumannii* (XDR-AB) were classified as previously described by Magiorakos et al. (2012)

Detection of class D carbapenemase genes

Multiplex polymerase chain reaction (Multiplex PCR) assay was used to detect *bla*$_{OXA23}$, *bla*$_{OXA24}$, *bla*$_{OXA58}$ and intrinsic *bla*$_{OXA51}$ genes. The primers used are shown in Table 1. Amplification reactions were performed in a final volume of 20 µL. Each reaction mixture consisted of 2 µL of 10X buffer, 2.5 mM of dNTPs, 25 mM of MgCl$_2$, 20 mM of each primer, 5U of *Taq* DNA polymerase and 2 µL of lysate cells as the template. Amplification was carried out under the following Multiplex PCR conditions, 95ºC for 5 minutes, followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 52ºC for 40 seconds, extension at 72ºC for 50 seconds and then final extension at 72ºC for 5 minutes. PCR products were electrophoresed though 1% agarose in 0.5X Tris-Boric acid-EDTA buffer at 100 V for 35 minutes.

Table 1. Primer for detection of class D carbapenemase genes (Woodford et al. 2006)

Target genes	Primer name	Sequence 5'-3'	Size (bp)
bla$_{OXA-23}$	bla$_{OXA-23}$-F	GATCGGATTGGAGAACCAGA	501
	bla$_{OXA-23}$-R	ATTTCTGACCGCATTTCCAT	
bla$_{OXA-24}$	bla$_{OXA-24}$-F	GGTTAGTTGGCCCCCTTAAA	246
	bla$_{OXA-24}$-R	AGTTGAGCGAAAAGGGGATT	
bla$_{OXA-51}$	bla$_{OXA-51}$-F	TAATGCTTTGATCGGCCTTG	353
	bla$_{OXA-51}$-R	TGGATTGCACTTCATCTTGG	
bla$_{OXA-58}$	bla$_{OXA-58}$-F	AAGTATTGGGGCTTGTGCTG	599
	bla$_{OXA-58}$-R	CCCCTCTGCGCTCTACATAC	

Plasmid profiles analysis

Plasmids of extensively drug resistant *A. baumannii* (XDR-AB) strains were extracted using a Plasmid Mini Kit (RBC Bioscience, Taiwan) according to the manufacturer instructions. XDR-AB plasmids were separated by electrophoresis in 1% agarose gel containing 0.5 µg ethidium bromide at 100 V for 50 minutes. Plasmid

profiles were visualized under UV transilluminator using gel documentation instrument (Bio-Rad Laboratories, USA).

Detection of class D carbapenemase genes in XDR-AB plasmids

All XDR-AB plasmid isolates were checked for the presence of class D carbapenemase genes such as bla_{OXA23}, bla_{OXA24} and bla_{OXA58} genes (Table 1) by multiplex PCR. PCR products were separated by 1% agarose gel electrophoresis with a molecular weight marker of 100 bp DNA Ladder.

RESULTS

Bacterial strains

Chiangrai Prachanukhoh hospital is a large hospital with a total of 756 beds, located in Northern part of Thailand. We collected 109 *A. baumannii* clinical isolates from different inpatients wards. All *A. baumannii* isolates were taken from medicine ward (43.12%), ICU (25.68%), surgical ward (16.60%) and some other wards (15.60%) (monk ward, coronary care unit (CCU), trauma ward, pediatric ward and outpatient department (OPD)). All sample were recovered from sputum. All of isolates were positive for 16s rRNA gene of *Acinetobacter* spp. by PCR method.

Antimicrobial susceptibility patterns

All antimicrobial susceptibility results of *A. baumannii* isolates are shown in Fig. 1. Drug resistance rates for amikacin, cefepime, cefotaxime, cefpopera/ sulbactam, ceftazidime, ceftriazone, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillin/tazobactam, tetracycline and trimethoprim/sulfamethoxazole were 49.54, 56.88, 79.82, 12.84, 77.98, 80.73, 81.65, 64.22, 85.32, 85.32, 78.90, 69.72 and 42.20%, respectively. All isolate were sensitive to colistin (CT) and tigecycline (TGC). The prevalence of MDR-AB, CR-AB and XDR-AB were 87.16% (95/109), 85.32% (93/109) and 5.50% (6/109), respectively. Among all isolates, 14(12.84%) isolates were non antibiotic resistance strains.
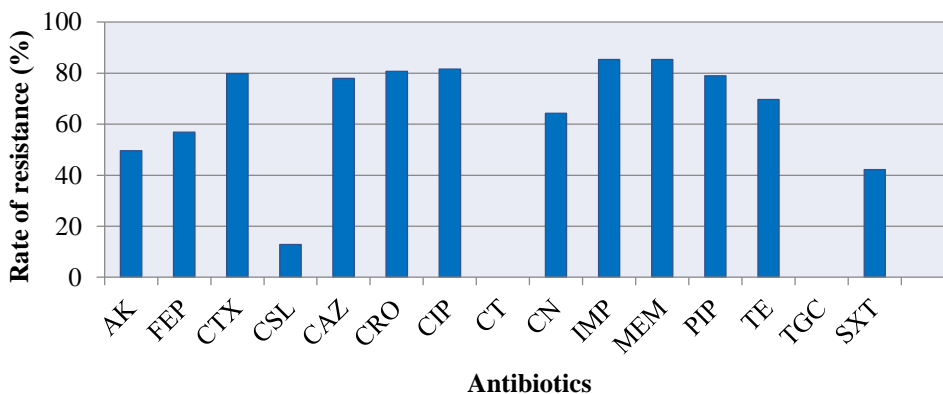
Fig.1 Antibiotic susceptibility patterns of *A. baumannii* isolates in Chiangrai Prachanukroh hospital. Amikacin (AK), cefepime (FEP), cefotaxime (CTX), cefpopera/sulbactam (CSL), ceftazidime (CAZ), ceftriazone (CRO), ciprofloxacin (CIP), colistin (CT), gentamicin (CN), imipenem (IMP), meropenem (MEM), piperacillin/tazobactam (PIP), tetracycline (TE), tigecycline (TGC) and trimethoprim/sulfamethoxazole (SXT).

Detection of class D carbapenemase genes

Multiplex PCR were used to detect the presence of bla_{OXA23}, bla_{OXA24}, bla_{OXA58} and intrinsic bla_{OXA51} genes. The amplicon sizes for bla_{OXA23}, bla_{OXA24}, bla_{OXA58} and intrinsic bla_{OXA51} genes were 501 bp, 246 bp, 599 bp and 353 bp, respectively (Fig. 2). Eighty two isolates were found to carry acquired bla_{OXA23} gene (75.23%). The bla_{OXA58} gene was detected in eight isolates (7.34%) and the intrinsic bla_{OXA51} gene was detected in one hundred and six isolates (97.25%). While, the bla_{OXA-24} gene was not detected in any of the isolates. Among 109 *A. baumannii* isolates, 81(74.31%) contained both bla_{OXA23} and intrinsic bla_{OXA51} genes, 8(7.34%) contained both bla_{OXA58} and intrinsic bla_{OXA51} genes and 2(1.83%) isolates are co-existence of acquired bla_{OXA23} and bla_{OXA58} genes.
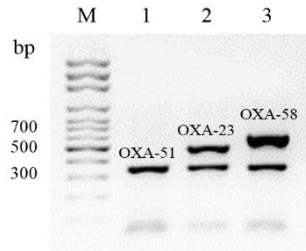
Fig. 2 Amplification of OXA carbapenemase genes. M: 100 bp DNA Ladder; Lane 1: bla$_{OXA-51}$ gene; Lane 2: bla$_{OXA-51}$ gene and bla$_{OXA-23}$ gene; Lane 3: bla$_{OXA-51}$ gene and bla$_{OXA-58}$ gene.

Plasmid profiles analysis

All XDR-AB strains were selected to study the plasmid profiles (Fig. 3). Each strain carried two plasmids, ranging in size from 20-23 Kb. There were no different between the plasmid profiles of each XDR-AB isolates.
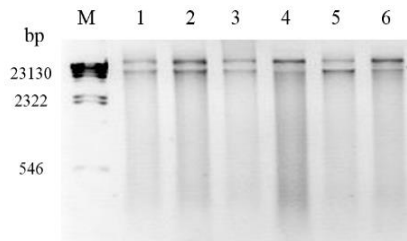

Fig. 3 Plasmid profiles of XDR-AB strains. M: VC Lambda/*Hind* III Marker; Lane 1-6: Plasmid of *A. baumannii* 95,180, 183, 241, 246, 269, respectively.

Detection of class D carbapenemase genes in XDR-AB plasmids

Overall XDR-AB plasmids were screened for the presence of bla_{OXA23}, bla_{OXA24} and bla_{OXA58} by multiplex PCR. There were positive for bla$_{OXA-23}$ gene, while bla$_{OXA-24}$ and bla$_{OXA-58}$ gene were not detected in all strains (Fig. 4). The PCR products were observed as common in 6 of all plasmids. Our finding also indicated that class D carbapenemase genes were found in plasmids and maybe related with XDR-AB plasmid profiling among tested strains.
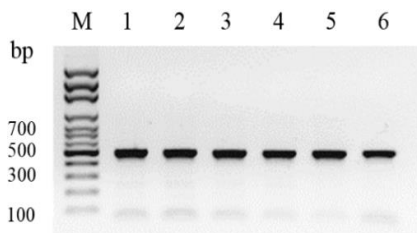
Fig. 4 Amplification of class D carbapenemase genes were found in XDR-AB plasmids. M: 100 bp DNA Ladder; Lane 1-6: bla$_{OXA-23}$ gene was found in plasmids of *A. baumannii* 95, 180, 183, 241, 246, 269, respectively.

DISCUSSION

All of the specimens in our study were isolated from sputum which recovered from the respiratory infection. The results of this present study similar to previous study that sputum was the major specimen of *A. baumannii* in our country (Santimaleeworagun et al. 2014). Prevalence of carbapenem-resistant *A. baumannii* (CR-AB) has been increasing in the hospitalized patients worldwide. Carbapenems are the first choice for treatment of *A. baumannii* infection (Poirel and Nordmann 2006). In Thailand, prevalence of CR-AB were found to have an incidence of 84.2 % in Phramongkutklao hospital (Aimsaad et al. 2009). The antimicrobial resistance patterns of *A. baumannii* strains are shown in Fig. 1. Eighty-five percent were resistant to the group of carbapenem antibiotics, including imipenem and meropenem. Our finding indicated that the CR-AB demonstrated high resistant rate. Therefore, therapeutic antibiotics should be specific and used as little as possible.

Mechanism of carbapenem resistance in *A. baumannii* is production of class D carbapenemase are mostly associated with OXA-type β-lactamase, including bla$_{OXA23}$, bla$_{OXA24}$, bla$_{OXA58}$ and intrinsic bla$_{OXA51}$ genes (Zarrilli et al. 2009). Our study showed that the most common of acquired bla$_{OXA}$ presented in 109 isolates was OXA23 encoding gene. Previous studies from Thailand revealed that CR-AB carried bla$_{OXA23}$, bla$_{OXA24}$, bla$_{OXA58}$ and intrinsic bla$_{OXA51}$ genes were reported. The bla$_{OXA-23}$ gene was found in 80-100% among *A. baumannii* isolates. (Niumsup et al. 2009, Santimaleeworagun et al. 2014, Jantama et al. 2013, Thapa et al. 2010, Teo et al. 2015). Jumroon *et al.* studied the prevalence of bla$_{OXA}$ gene and they reported that bla$_{OXA-58}$ was found in 7.69% (Jumroon and Santanirand 2013). This study

demonstrated the second occurrence of bla_{OXA58} in *A. baumannii* clinical isolates in Thailand. The bla_{OXA24} gene was not detected in any of the isolates. However, the report in 2014 have demonstrated that the occurrence of bla_{OXA-24} was found in 2.32% of CR-AB isolated from Hua Hin hospital (Santimaleeworagun et al. 2014).

In this study, XDR-AB isolates were screened for the presence of the plasmids using plasmid extraction kit. It was found that all XDR-AB strains harbored two plasmids sized approximately between 20 and 23 kb. Plasmid profiles of XDR-AB showed no different between each strains (Fig. 3). All the isolates that had plasmids resistant to amikacin, cefepime, cefotaxime, cefpopera/sulbactam, ceftazidime, ceftriazone, ciprofloxacingentamicin, imipenem, meropenem, piperacillin/tazobactam, tetracycline and trimethoprim/ sulfamethoxazole. This result was different from the finding in western India, where *Acinetobacter* spp. isolates were carried three plasmid with molecular weight ranging from 1.5 to 40 kb. *Acinetobacter* spp. carried 40 kb plasmid showed intermediate to low level resistance to some antibiotics (Pardesi, Yavankar, and Chopade 2007). While, the results of the detection class D carbapenemase genes displayed the six strains contained bla$_{OXA-23}$ gene. Saranathan *et al*, reported similar finding. In their study, plasmids of *A. baumannii* strains isolated from clinical specimens of patients admitted in India hospital showed a size ranged from 500 bp to ≥ 25 kb. The high prevalence of bla_{OXA-23} (42%) in plasmids from *A. baumannii* was previously reported (Saranathan et al. 2014). The results of our present study suggested that the multiple antibiotic resistances in bacteria may be commonly association with the presence of plasmids. Therefore, plasmids are the major carrier for the spreading of antibiotic resistant genes via horizontal gene transfer between bacterial populations by transformation and conjugation (Sadeghifard et al. 2010, Valenzuela et al. 2007, Bertini et al. 2010). Our studies indicated that the prevalence of antibiotic resistant bacteria are increasing in currently.

CONCLUSION

The present study showed the high prevalence CR-AB and high dissemination of class D carbapenemase resistance genes. XDR-AB plasmids carried bla_{OXA-23} gene, which can be transferred antibiotic resistance gene between bacteria via transformation

and conjugation mechanism. Therefore, strategies to control CR-AB infections are need in Thai hospitals.

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