DISSEMINATION OF CLASS I INTEGRON IN *ACINETOBACTER BAUMANNII* ISOLATED FROM VENTILATOR-ASSOCIATED PNEUMONIA PATIENTS AND THEIR ENVIRONMENT

Suntariya Sirichot[1,2], Pornphan Diraphat[3], Fuangfa Utrarachkij[3], Chanwit Tribuddharat[4] and Kanokrat Siripanichgon[3]

[1]Faculty of Graduate Studies, Mahidol University, [2]Ramathibodi Hospital, Mahidol University, [3]Department of Microbiology, Faculty of Public Health, Mahidol University, [4]Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Abstract. Multidrug resistant *Acinetobacter baumannii* has become the most common cause of health care-associated infections at Maharaj Nakhon Si Thammarat Hospital, Thailand. The objective of the study was to detect integrons using PCR-based method from 96 *A. baumannii* isolates from ventilator-associated pneumonia (VAP) patients and their environment. Antibiotic susceptibility was determined using a disk diffusion technique. Forty-six isolates exhibited integrase genes, with only class I and class II integron detected in 43 and 3 *A. baumannii* isolates, respectively. Twenty-seven of 52 clinical and 19 of 44 environmental isolates were integron-positive. Detection rate of integron-positive *A. baumannii* isolated from VAP patients increased from 25% to 83% over the 4 month study period. The majority (91%) of integron-positive *A. baumannii* showed resistance to 6 or more of 11 antibiotics tested and 72% of class I integron-positive isolates were imipenem-resistant. Thus, class I integron-positive *A. baumannii* had spread among the VAP patients and into hospital environment, the latter acting as reservoirs of potential pathogens possessing drug resistance genes.

INTRODUCTION

Acinetobacter spp cause ever-increasing problems of nosocomial infections around the world, with many infections from multiple resistant strains (Afzal-Shah and Livermore, 1998; Kuo *et al*, 2003). Studies of antibiotic resistance mechanism in *Acinetobacter baumannii* have demonstrated the presence of specific antimicrobial resistance genes located mainly in class I and class II integrons which are often associated with epidemic clones (Koeleman *et al*, 2001; Gaur *et al*, 2007).

Recently, isolation of multi-drug resistant (MDR) *A. baumannii* at Maharaj Nakhon Si Thammarat Hospital, Thailand among ventilator-associated pneumonia (VAP) patients in several intensive care units raises a concern about the spread of MDR strains in the hospital (Chaladchalam *et al*, 2008). The transfer of integrons into new bacteria and insertion of gene cassettes encoding resistance genes result in the emergence of multiple antibiotic-resistant strains (Fournier *et al*, 2006). The purpose of this study was to

Correspondence: Dr Kanokrat Siripanichgon, Department of Microbiology, Faculty of Public Health, 420/1 Ratchawithi Road, Bangkok 10400, Thailand.
Tel: 66 (0) 2354 8543 ext 6505; Fax: 66 (0) 2354 8538
E-mail: phksr@mahidol.ac.th

detect and analyze integrons of *A. baumannii* previously isolated from the clinical specimens of VAP patients and their environment at Maharaj Nakhon Si Thammarat Hospital using PCR-based method. Epidemiological relatedness of the integron-positive isolates was analyzed.

MATERIALS AND METHODS

Bacterial strains

Ninety-six isolates of *A. baumannii*, previously isolated from tracheal aspirates of VAP patients and their environment at Maharaj Nakhon Si Thammarat Hospital during March to the beginning of July in 2006 (Chaladchalam *et al*, 2008), were kept in 20% glycerol-Luria-Bertani broth at -80ºC. These were 52 isolates from patients, 16 from bed rails, 16 from endotracheal tube connectors, 6 from condensates in the ventilator tubes, and 6 from nurses' hands.

Culture and identification

Stock cultures were revived on MacConkey agar and after incubation at 37ºC overnight, single colonies were inoculated in tryptic soy broth and incubated at 37ºC for 4 hours. *A. baumannii* was identified by Gram staining and biochemical tests including oxidase, motility, glucose O/F, and citrate tests, and growth at 44ºC (Bergogne-Berezin and Towner, 1996).

Antimicrobial susceptibility

Antimicrobial susceptibility was determined using a disk diffusion technique according to CLSI guidelines (CLSI, 2007), with *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218 (obtained from National Institute of Health, Ministry of Public Health, Thailand) and *Pseudomonas aeruginosa* ATCC 27853 as controls. Antimicrobial susceptibility testing was performed on Mueller-Hinton agar with the following antimicrobial discs (Oxoid, UK): ampicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), amikacin (30 μg), ciprofloxacin (5 μg), cefotaxime (30 μg), ceftazidime (30 μg), gentamicin (10 μg), imipenem (10 μg), netilmicin (30 μg), cefoperazone/sulbactam (75/30 μg), and colistin sulphate (10 μg). MDR *A. baumannii* was defined as an isolate with intermediate or complete resistance to at least 3 of the following classes of antibiotics: beta-lactam, aminoglycoside, carbapenem and fluoroquinolone (Zapantis *et al*, 2007).

Detection of integron and gene cassette

Genomic DNA was extracted using NucleoSpin® Tissue kit following manufacturer's instruction. PCR amplification was carried out in 50 μl volume containing 200 ng of purified DNA, 0.2 mM (each) dNTP, 1X ThermoPol buffer, 1 U of *Taq* polymerase (NEB, MA, USA), and 200 nM of each primer. Primers used in PCR were IntF/IntR for integron class I, Int2F/Int2R for integron class II, and Int3F/Int3R for integron class III (Table 1). PCR amplification was performed in a PTC-100 Peltier thermal cycler (MJ Research, MA). Amplification cycle was as follows: initial denaturation at 95ºC for 2 minutes; followed by 35 cycles at 94ºC for 1 minute, 59ºC for 1 minute, 72ºC for 1 minute; and a final extension at 72ºC for 5 minutes (Dillon *et al*, 2005). Amplification products were resolved by electrophoresis at 100 V for 50 minutes in 1% agarose gel in 1X Tris-Borate-EDTA buffer containing ethidium bromide (0.2 μg/ml) and detected by an UV transilluminator (BIS 303 PC, Jerusalem, Israel). All PCR amplifications were performed in duplicate.

Integron-positive strains were screened for presence of gene cassette by PCR as described previously (White *et al*, 2000). In brief, each reaction (50 μl) contained 200 ng of purified DNA, 0.2 mM (each) dNTP, 1X ThermoPol buffer, 1.5 U of *Taq* polymerase (NEB, MA, USA), and 0.5 μM of each primer.

Table 1
Primers used for multiplex PCR amplification and sequencing of integrons.

Primer	Target	Sequence (5´ to 3´)	Position	Product size
Integron				
IntF[a]	*intI I*	CAG TGG ACA TAA GCC TGT TC	2734-2751	160
IntR[a]	*intI I*	CCC GAG GCA TAG ACT GTA	2874-2891	
Int2F[b]	*intI II*	CAC GGA TAT GCG ACA AAA AGG T	12291-12312	
Int2R[b]	*intI II*	GTA GCA AAC GAG TGA CGA AAT G	11524-11545	788
Int3F[b]	*intI III*	GCC TCC GGC AGC GAC TTT CAG	738-758	979
Int3R[b]	*intI III*	ACG GAT CTG CCA AAC ATG ACT	1697-1717	
Gene cassette				
Hep58[c]	Array	TCA TGG CTT GTT ATG ACT GT	2847-2866	Variable
Hep59[c]	Class I	GTA GGG CTT ATT ATG CAC GC	3941-3960	
Hep74[c]	Array	CGG GAT CCC GGA CGG CAT GCA CGA TTT GTA	1-30	Variable
Hep51[c]	Class II	GAT GCC ATC GCA AGT ACG AG	2205-2224	

[a]Koeleman *et al*, 2001; primer intI I position with respect to GenBank Acc. No. U12441
[b]Mazel *et al*, 2000; primer intI II position with respect to GenBank Acc. No. AP002527
[c]White *et al*, 2000; primer intI III position with respect to GenBank Acc. No. AF416297

Primers used for gene cassette amplification of integron class I were Hep58/Hep59, and Hep74/Hep51 for integron class II (Table 1). PCR cycle was as follows: initial denaturation at 95ºC for 2 minutes; followed by 30 cycles of 94ºC for 20 seconds, 55 ºC, for 30 seconds, 72ºC for 4 minutes; and a final extension at 72ºC for 5 minutes. PCR amplicons of class I and class II integron were purified, sequenced (1st Base, Malaysia) and compared with the National Center for Biotechnology Information (NCBI) database.

RESULTS

Detection of integron and gene cassettes

Forty-six out of 96 *A. baumannii* isolates (48%) exhibited the presence of integrase genes (Fig 1). Integron-positive *A. baumannii* were found among 52% (27/52) of clinical and 43% (19/44) of environmental isolates. Class I and class II integron was detected in 45% (43/96) and 3% (3/96) of isolates, respectively. Class III integron was not detected

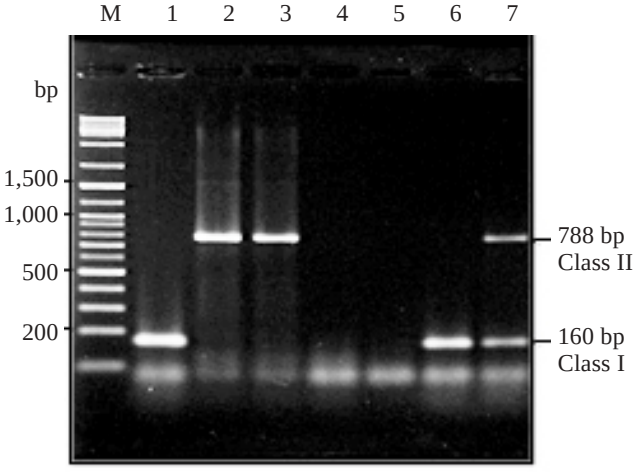

Fig 1–Detection of integrase genes by multiplex PCR. PCR amplifications were performed as described in Materials and Methods. Lane M, DNA size marker; lane 1, 6, *intI I*-associated (class I; 160-bp); lane 2, 3, *intI II*-associated (class II; 788 bp); lane 4, 5, negative for integron; lane 7, positive control.

Table 2
Distribution of integrons among *Acinetobacter baumannii* isolates.

Source of *A. baumannii*	Total isolates no. (%)	Integron-positive, No. (%)		Total integron-positive isolate no. (%)
		Class I	Class II	
Patient	52 (54)	24 (46)	3 (6)	27 (52)
Environment	44 (46)	19 (43)	0 (0)	19 (43)
Endotracheal tube connector	16 (17)	10 (23)		10 (62)
Bed rail	16 (17)	7 (16)		7 (44)
Nurse's hand	6 (6)	2 (4)		2 (12)
Condensate	6 (6)	0 (0)		0 (0)
Total	96 (100)	43 (45)	3 (3)	46 (48)

and class II integron was not found in the environmental isolates (Table 2). Amplicons of class I and II integrons were sequenced indicating the presence of *IntI I* and *IntI II* (data not shown). The inserted gene cassettes of class I integron ranged in size from 1.0 kb to 2.8 kb and those of class II integron was 2.0 kb. Gene cassettes were detected in 15 of 43 isolates (35%) of class I integron-positive *A. baumannii*, and in all 3 isolates of class II integron-positive. Detection rate of integron-positive *A. baumannii* isolated from the patients increased from 25% in March to 83% in June-July, while detection of integron-positive *A. baumannii* isolated from the patients' environment varied from 0 to 67%.

Antimicrobial susceptibility pattern of integron-positive *A. baumannii*

Among 63 MDR *A. baumannii*, 67% were integron-positive, and 91% (42/46) of integron-positive isolates were resistant to 6 or more of the 11 antibiotics tested (Table 3). All 3 isolates of class II integron-positive *A. baumannii* showed resistance to 8 of 11 test antibiotics. Among 49 imipenem-resistant *A. baumannii* isolates, 16 (33%) did not have integron, and 31 (67%) and 2 (4%) isolates carried class I and class II integron, respectively. Moreover, 72% (31/43) of all class I integron-positive isolates were imipenem-resistant *A. baumannii* (44% from patient's and 28% from environmental isolates). Antibiograms that showed resistance to ≤3 antibiotics were found only among integron-negative isolates, while resistant patterns of 4 or more antibiotics were found in integron-positive isolates (Table 3).

Resistance to 10 different antibiotics of *Acinetobacter* isolates was related to the presence of integron. Table 4 shows the antibiotic susceptibilities of integron-positive and integron-negative isolates to each of the test antibiotics. All integron-positive *A. baumannii* isolates were resistant to ampicillin, cefotaxime, and ceftazidime, with 96% resistant to amoxicillin/clavulanic acid and gentamicin. In general, clinical isolates showed higher antibiotic resistance than environmental isolates. The majority of integron-positive (74%) and integron-negative (98%) isolates were sensitive to cefoperazone/sulbactam. The presence of integron was significantly associated to tested drug resistance ($p < 0.05$), except for colistin, to which all isolates were sensitive.

Integron and epidemiological characteristics

Molecular typing of these 96 isolates

Table 3
Antibiotic resistance of integron-positive *A. baumannii* isolates from the VAP patients and their environment.

Pattern	Antibiotic											Patient isolate		Environmental isolate		Total
	AMP	AMC	CTX	CTZ	GN	AN	NET	CIP	IPN	CPZ	CT	Isolate	Integron+ (Class[a])	Isolate	Integron+ (Class)	
1	R	R	R	R	R	R	R	R	R	R	S	2	2(I)			2
2	R	R	R	R	R	R	R	R	R	S	S	8	7(I)	8	8(I)	16
3	R	R	R	R	R	R	R	R	R	I	S	7	7(I)	3	3(I)	10
4	R	R	R	R	R	R	S	R	R	S	S	7	2(II)	2		9
5	R	R	R	R	R	R	R	R	S	S	S	1	1(II)			1
6	R	R	R	R	R	R	S	R	R	I	S	1				1
7	R	R	R	R	R	R	I	R	R	S	S	1				1
8	R	R	R	R	R	S	S	R	R	S	S	1	1(I)	1	1(I)	2
9	R	R	R	R	S	R	S	R	R	S	S	1				1
10	R	R	R	R	R	R	S	R	S	S	S	1				1
11	R	R	I	S	R	R	R	R	R	S	S	2				2
12	R	R	R	R	R	I	S	R	R	S	S	1		2		3
13	R	R	R	R	R	S	S	R	S	S	S	2	2(I)	4	4(I)	6
14	R	R	R	R	R	S	S	S	R	S	S	2	2(I)			2
15	R	S	R	R	R	R	R	S	S	S	S	1	1(I)			1
16	R	S	R	R	R	R	S	R	S	S	S			1		1
17	R	I	R	R	R	R	R	S	S	S	S	2	1(I)			2
18	R	I	R	R	R	R	S	R	S	S	S	2		1		3
19	R	I	R	R	R	S	S	R	S	S	S			1	1(I)	1
20	R	R	R	R	R	S	S	S	I	S	S	1	1(I)			1
21	R	R	R	R	S	S	S	S	S	S	S			2	2(I)	2
22	R	I	I	S	R	S	S	R	S	S	S			2		2
23	R	S	I	S	R	S	S	R	S	S	S	1				1
24	R	R	I	S	S	S	S	R	S	S	S			2		2
25	R	I	I	S	R	S	S	S	S	S	S			1		1
26	I	S	I	S	R	S	S	R	S	S	S	1				1
27	R	S	I	S	R	S	S	S	S	S	S			1		1
28	R	R	I	S	S	S	S	S	S	S	S	1				1
29	R	I	S	S	S	S	S	S	S	S	S			1		1
30	R	S	I	S	S	S	S	S	S	S	S	1		4		5
31	R	I	I	S	S	S	S	S	S	S	S			6		6
32	I	R	I	S	S	S	S	S	S	S	S	1				1
33	I	S	I	S	S	S	S	S	S	S	S	3				3
34	I	S	S	S	S	S	S	S	S	S	S	1				1
35	S	S	I	S	S	S	S	S	S	S	S			2		2
Total isolates												52	27	44	19	96

AN, amikacin; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CIP, ciprofloxacin; CT, colistin; CPZ, cefoperazone/sulbactam; CTX, Cefotaxime; CTZ, Ceftazidime; GN, Gentamicin: IPN, Imipenem; NET, Netilmicin; S, susceptible; I, intermediate; R, resistant

[a] Roman number in parenthesis (I or II) indicates integron class

Table 4
Antimicrobial susceptibility of integron-positive and integron-negative *A. baumannii*.

Antibiotic	Integron-positive isolate (n = 46)			Integron-negative isolate (n = 50)			Total isolate (n = 96)			p[a]
	R %	I %	S %	R %	I %	S %	R %	I %	S %	
Ampicillin	100	0	0	84	12	4	92	6	2	0.029
Amikacin	69	0	30	38	6	56	53	3	44	0.007
AM/CA[b]	96	4	0	40	28	32	67	17	17	<0.001
Ciprofloxacin	85	0	15	56	0	44	70	0	30	0.002
Cefotaxime	100	0	0	40	56	4	69	29	2	<0.001
Ceftazidime	100	0	0	40	0	60	69	0	31	<0.001
Gentamicin	96	0	4	54	0	46	74	0	26	<0.001
Netilmicin	65	0	35	6	2	92	34	1	65	<0.001
Imipenem	72	2	26	32	0	68	51	1	48	<0.001
CEF/SUL[c]	4	22	74	0	2	98	2	11	86	0.003
Colistin	0	0	100	0	0	100	0	0	100	NA

[a]Chi-square test. R, resistant; I, intermediate; S, susceptible
[b]AM/CA, Amoxicillin/clavulanic acid; [c]CEF/SUL, Cefoperazone/sulbactam

was previously reported and found that genotype 2 was the most common cause of *A. baumannii* VAP, 70% (67/96) of all isolates and among them 48% (32/67 isolates) carried class I integron (Chaladchalam *et al*, 2008). Nine cases (41%) of *A. baumannii* VAP were admitted to medical sub-ICU, where the first case of integron-positive *A. baumannii* VAP (genotype 2) was found during the study. Genotype 2 integron-positive *A. baumannii* was endemic in this medical sub-ICU as it was the cause of VAP in 7 out of 9 cases admitted to this ward over 4 months of the study period. In addition, 3 out of these 7 cases had their environmental samples contaminated with genotype 2 integron-positive *A. baumannii.* Of 31 genotype 2 isolates from the environment (bed rails, endotracheal tube connectors, and condensates), 68% (21 isolates) were isolated from the medical sub-ICU, and 38% were positive for integron class I. Thus, epidemiological related isolates had contaminated the patients' environment in this ward.

DISCUSSION

Class I integron is significantly present among clinical isolates of gram-negative bacteria, such as *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Acinetobacter baumannii* (Martinez-Freijo *et al*, 1998; Koeleman *et al*, 2001; White *et al*, 2001; Mathai *et al*, 2004; Gaur *et al*, 2007; Gu *et al*, 2007). This study demonstrated detection of class I integron in *A. baumannii* from 52% of the clinical isolates, similar to other reports (Koeleman *et al*, 2001; Gu *et al*, 2007). A detection rate as high as 75% of the patients admitted in ICU of a teaching hospital with more than 1,000 beds also has been reported with high levels of multidrug resistance (Lin *et al*, 2009). However, very few studies has reported the presence of class I integron in *A. baumannii* isolated from a hospital environment (Kraniotaki *et al*, 2006; Ferreira *et al*, 2007). Detection of class I integrons in *A. baumannii* was as high as 43% (19/44) from the patient's environment in this study and

more than half were from contaminated endotracheal connectors of the VAP patients. In addition, an increase of integron-positive *A. baumannii* VAP cases from 25% in March to 83% in June-July indicated an emergence of MDR - *A. baumannii* with an increased carriage rate of integron.

Antimicrobial susceptibility results showed that class I integron-positive *A. baumannii* were more resistant to all tested antibiotics (except for colistin). Imipenem resistance was found in 51% of all isolates and in 72% of class I integron-positive isolates. Therefore, the use of carbapenems, previously recognized as drug of choice for *A. baumannii* infection (Montefour *et al*, 2008), should not be prescribed without a susceptibility test.

In this study, the first integron-positive isolate (class I integron) was detected in March 2006 from a VAP patient and it was resistant to 6 antibiotics, namely ampicillin, amoxicillin/clavulanic acid, ciprofloxacin, cefotaxime, ceftazidime, and gentamicin. Subsequent isolates in April of the same year with the same genotype and class I integron from VAP patients admitted to the same ward of the first isolate (Chalardchalam *et al*, 2008) were resistant to 9-10 drugs (now including amikacin, netilmicin, imipenem, and cefoperazone/sulbactam). Antibiograms showing resistance to 3 or less antibiotics were found only among integron-negative isolates, while resistant patterns to 4 or more antibiotics were found in integron-positive isolates. The results supported that the presence of integron was significantly associated with multiple antibiotic resistance (Bergogne-Berezin and Towner, 1996; Oh *et al*, 2002; Huang *et al*, 2008). Other studies have found antibiotic resistance genes located in integrons in *Acinetobacter* spp (Gombac *et al*, 2002; Navia *et al*, 2002; Nemec *et al*, 2004; Fournier *et al*, 2006). More epidemic strains of *A. baumannii* were found to contain integrons than non-epidemic strains. It has been suggested that epidemic potential among *A. baumannii* isolates may be linked to the presence of integrons (Koeleman *et al*, 2001). However, some integron-negative isolates were resistant to 6 or 9 drugs with the same antibiogram patterns of integron-positive strains. The antibiotic resistance genes of these isolates could be acquired by plasmid or other mobile elements (Perez *et al*, 2007). In addition, possibility of the presence of other integrase gene homologues could not be excluded as those genes may not be amplified by the primers used in this study.

PCR amplification of the resistance gene cassettes failed to produce amplicons in 65% of class I integron-positive isolates. These results could be due to the fact that inserted gene cassettes were too large to be amplified by conventional PCR method, or that such integrons may lack the 3' conserved sequence generally associated with class I integron (Hall and Collis, 1995), or no cassette was present (Sallen *et al*, 1995). However, the role of integrons and gene cassette systems in the evolution of bacterial and plasmid genomes is now known to be much broader than their roles in the dissemination of antibiotic resistance genes (Fournier *et al*, 2006).

In summary, the results of this study indicated that class I integron-positive *A. baumannii* had increasingly spread among VAP patients and hospital environment. The integron-carrying *A. baumannii* in the patient's environment could be an important pool of horizontally transferred drug resistant genes. Integrons and other mobile DNA elements carrying antibiotic resistant genes should also be determined among other common nosocomial pathogens, such as *Pseudomonas aeruginosa*, *E. coli*, or *Klebsiella*

pneumoniae, which often co-exist with *Acinetobacter* sp. Appropriate cleaning of patients' environment also could eliminate reservoir of integron-carrying *A. baumannii* and may help to control the spread of multi-resistant genes.

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