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ORIGINAL ARTICLE

Evolution of carbapenem resistance in *Acinetobacter baumannii*: An 18-year longitudinal study from a medical center in northern Taiwan

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IS*Aba1*-bla$_{OXA-51-like}$;
Tn*2006*;
Tn*2008*

Background: Carbapenem-resistant *Acinetobacter baumannii* has emerged as an important cause of nosocomial infections with high morbidity and mortality. The carbapenemases, especially class D carbapenem-hydrolyzing oxacillinases (CHDLs), play an important role, but the relationship between their prevalence trend and carbapenem resistance remains unclear.
Materials and methods: Between 1995 and 2012, we collected 667 isolates of *A. baumannii* from a single medical center in northern Taiwan. Pulsed-field gel electrophoresis (PFGE) was used to determine clonality. Antimicrobial susceptibility was determined. Carbapenemase genes and associated genetic structures were detected by polymerase chain reaction.
Results: Isolates were heterogeneous on PFGE. Susceptibility to carbapenem decreased steadily over the study period from 88.1% (2001–2003) to <25% (2010–2012), whereas the isolates remained susceptible to colistin (nearly 100%) and partially susceptible to tigecycline

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(80%). Starting in 2001, isolates carrying the IS*Aba1*-$bla_{OXA-51-like}$ allele were consistently identified. Isolates containing the transposons Tn*2006* or Tn*2008* first appeared in 2007 with increasing carriage rates from 17.5% (2007–2009) to 50.0% (2010–2012). The IS*1008*-ΔIS*Aba3*-$bla_{OXA-58-like}$, bla_{OXA-72} and metallo-β-lactamase genes were detected only sporadically. Isolates carrying CHDL genes were resistant to multiple drugs, including carbapenem, but remained susceptible to colistin (100.0%).

Conclusion: Increased carbapenem resistance in *A. baumannii* may be caused by the increased prevalence of isolates containing the IS*Aba1*-$bla_{OXA-51-like}$ allele and the transposons Tn*2006* and Tn*2008*.

Introduction

During the past decade, *Acinetobacter baumannii* has emerged as a central cause of nosocomial infections with high morbidity and mortality.[1,2] Carbapenem resistance in this pathogen is increasing steadily worldwide.[3] Studies by the Taiwan Surveillance of Antimicrobial Resistance program have documented an increase in carbapenem resistance rates among *A. baumannii-Acinetobacter calcoaceticus* complex (Abc) isolates from 3.4% in 2002 to 58.7% in 2010.[4] Previous studies also report decreased susceptibility to second-line antimicrobial agents such as ampicillin/sulbactam, tigecycline, and colistin in carbapenem-resistant *A. baumannii* (CRAB).[5,6]

The most common mechanisms of carbapenem resistance in *A. baumannii* are the overexpression of intrinsic β-lactamases and the acquisition of exogenous carbapenemases such as the class D carbapenem-hydrolyzing oxacillinases (CHDLs).[3,7] These oxacillinases were first discovered in 1993 and are important enzymes that contribute to the mechanism of carbapenem resistance in this species; four different clusters of CHDLs have been identified in *A. baumannii* to date.[8,9] Several cross-sectional studies show that different CHDL genes contribute differentially to antimicrobial resistance in *A. baumannii* in Taiwan.[10–13] The prevalence of different CHDLs in *A. baumannii* dictates the susceptibility to various antimicrobial agents, but the time-course of acquisition of resistance genes remains unknown.

In this study, we investigated trends in antimicrobial resistance and correlated the trends to the acquisition of CHDL genes in *A. baumannii*. Isolates obtained during an 18-year period were collected from a single large medical center in northern Taiwan. We also determined the resistance profile of isolates carrying different CHDL genes.

Materials and methods

Bacterial isolates and species identification

The study was conducted at the Taipei Veterans General Hospital, a 2900-bed tertiary care medical center in Taiwan. Between 1995 and 2012, nonrepetitive blood isolates from bacteremic patients were collected and phenotypically assessed by using the API ID 32 GN system (bioMérieux, Marcy l'Etoile, France) or the Vitek 2 bacterial identification system (bioMérieux). *A. baumannii* was identified at the genomic species level by a multiplex polymerase chain reaction (PCR) assay for the detection of a specific 16S-23S rRNA intergenic spacer, as described previously.[14,15]

Pulse-field gel electrophoresis

The clonality of randomly selected isolates from different years was determined with pulsed-field gel electrophoresis (PFGE), as previously described.[16] In brief, after digestion with *Apa*I, the DNA fragments were subjected to PFGE in 1% SeaKem Gold agarose gels (Cambrex Bio Science, Rockland, ME, USA) in 0.5× Tris/borate/ EDTA buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA, pH 8.0). The stained gel was photographed and analyzed by BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) to generate a dendrogram of relatedness among the isolates. Isolates with >85% similarity were considered clonal.[17]

Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC) for tigecycline was determined by Etest (AB BIODISK, Solna, Sweden) and for colistin, by agar dilution using colistin sulfate (Sigma-Aldrich, St. Louis, MO, USA). All other MICs, including MICs for imipenem and meropenem, were determined by broth dilution by using the automated Sensititre Susceptibility Plate (TREK Diagnostic Systems Ltd., East Grinstead, United Kingdom). Results were interpreted in accordance with the Clinical and Laboratory Standards Institute[18]; however, for tigecycline the susceptibility/resistance breakpoints were interpreted in accordance with the U.S. Food and Drug Administration criteria (i.e., susceptible, ≤2 mg/L; resistant, ≥8 mg/L).

Detection of CHDLs and transposons

A multiplex PCR assay was used to detect CHDL genes (i.e., $bla_{OXA-23-like}$,, $bla_{OXA-24-like}$, $bla_{OXA-51-like}$, $bla_{OXA-58-like}$, and $bla_{OXA-143}$), as previously described.[13,19,20] The upstream locations of the insertion sequences (ISs) were mapped by PCR with forward primers within the ISs and reverse primers within the carbapenemase genes.[3,10,13,21] Metallo-β-lactamases (MBLs) were detected with the imipenem and imipenem-EDTA

combined-disk test, the imipenem and EDTA-sodium mercaptoacetic acid double-disk synergy test, and PCR assays using primers specific for the bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{SPM}, and bla_{GIM-1} genes, as previously described.[22–25] To detect the transposons Tn*2006* and Tn*2008*, PCR mapping was used. The common regions of Tn*2006* and Tn*2008* (923 bp from bla_{OXA-23} to Δ*ATPase*) were amplified by using the primer P3 (Tn2006OXA23: 5′-GTCTATCAGG AACTTGCGCG-3′) and the primer P5 (TN2008ATPase; 5′-GGCTCATTAC AGTCAGGTAC AAGT-3′). For Tn*2006*, PCR was performed with primers P3 and P4 (Tn2006ISAba1; 5′-GCAAGGCTTT AGATGCAGAA GA-3′) to amplify the 2237-bp region between $bla_{OXA-23-like}$ and IS*Aba1* in Tn*2006*.[12,26]

Antimicrobial use

Information regarding the defined daily dose (DDD) and days of therapy per patient was collected. The DDD was developed by the World Health Organization to standardize the usage of drugs among healthcare environments. The DDD is defined as the assumed average maintenance dose per day for a drug used for its main indication. It is adjusted for bed occupancy and is presented as the DDD per 1000 patient-days.[27] The data on imipenem and meropenem use have only been available since 2005.

Results

Isolates and clonality determined by PFGE

During the study period, 668 unique isolates of *A. baumannii* were collected. We excluded one isolate from analysis because of a lack of antimicrobial susceptibility data. We performed PFGE on two randomly selected isolates for each year in the study period to determine whether clonal relationships existed. A heterogeneous pattern was revealed, but without the detection of any major pulsotype (Fig. 1).

Trends of susceptibility to different antimicrobial agents

Fig. 2 shows the susceptibility trends of different antimicrobial agents between 1995 and 2012. The susceptibility to carbapenem (e.g., imipenem and meropenem) started to decline during the 2001–2003 period. Susceptibility decreased steadily over the rest of the study period from 88.1% (2001–2003) to <25% (2010–2012). There were no difference between the susceptibility rates of imipenem and meropenem, except for the 2007–2009 period, during which time *A. baumannii* was slightly less susceptible to meropenem than to imipenem.

Tigecycline was introduced in this hospital in September 2007. Susceptibility to tigecycline remained stable at approximately 80% until the end of the study period. *A. baumannii* remained susceptible to colistin (approximately 100%) throughout the study period. Susceptibility rates to other antimicrobial agents were low (<60%) with the exception of amikacin, which increased gradually from 18.7% (1998–2000) to 65.2% (2010–2012).

Detection of oxacillinase and metallo-β-lactamase genes

Because of increased resistance to imipenem and meropenem over time, we investigated possible mechanisms. Carbapenemase genes, including CHDL genes (i.e., $bla_{OXA-143}$, $bla_{OXA-51-like}$, $bla_{OXA-58-like}$, $bla_{OXA-24-like}$, and $bla_{OXA-23-like}$) and MBL genes (i.e., bla_{IMP}, bla_{VIM}, bla_{SIM}, bla_{SPM}, and bla_{GIM-1}) were detected by PCR mapping. Fig. 3 shows the carriage trends of different clusters of CHDL genes. Isolates carrying the IS*Aba1*-$bla_{OXA-51-like}$ allele have been identified consistently since the 2001–2003 period. The carriage rate peaked (23.3% of all the isolates collected) during 2004–2006. Isolates carrying transposons Tn*2006* and/or Tn*2008* were absent until 2007, after which a sharp increase in carriage rate was observed (17.5% for Tn*2006* and/or Tn*2008* during 2007–2009 vs. 50.0% for Tn*2006* and/or Tn*2008* during 2010–2012). The other CHDL genes (i.e., IS*1008*-ΔIS*Aba3*-$bla_{OXA-58-like}$ and bla_{OXA-72},) were detected only sporadically. The MBL genes were detected in only seven isolates. Six isolates contained bla_{VIM} alone, and one isolate harbored both bla_{IMP} and bla_{VIM}. Only four isolates carried the MBL and CHDL genes. Three isolates contained the IS*Aba1*-$bla_{OXA-51-like}$ gene and one isolate carried the IS*1008*-ΔIS*Aba3*-$bla_{OXA-58-like}$ gene.

Antibiotic susceptibility of *A. baumannii* carrying different carbapenemase genes

We further analyzed the antibiotic susceptibility of isolates carrying CHDL genes (Table 1). *A. baumannii* isolates with the IS*Aba1*-$bla_{OXA-51-like}$ allele were resistant to carbapenem and multiple other antimicrobial agents, including aminoglycosides, quinolones, ampicillin/sulbactam, and other β-lactams. However, they remained fully susceptible to colistin (100.0%) and partially susceptible to tigecycline (83.3%). In the isolates carrying the IS*1008*-ΔIS*Aba3*-$bla_{OXA-58-like}$ and the bla_{OXA-72} alleles also had multidrug-resistance patterns. Isolates with the bla_{OXA-72} allele were highly resistant to almost every tested antimicrobial agent, including tigecycline; however, both isolates remained fully susceptible to colistin (100.0%).

Isolates carrying the transposons Tn*2006* and/or Tn*2008* also exhibited resistance to amikacin, ciprofloxacin, levofloxacin, ampicillin/sulbactam, various β-lactams, and carbapenem. However, the susceptibility to amikacin was slightly higher (42.9%) in these isolates than in isolates carrying other CHDL alleles. The isolates remained fully susceptible to colistin (100.0%) and were partially susceptible to tigecycline (70.6%).

The seven *A. baumannii* isolates carrying the MBL genes (four of which also harbored CHDL genes) had low susceptibility to carbapenem (40.0% for imipenem and 0.0% for meropenem), amikacin (0.0%), ciprofloxacin (14.3%), ampicillin/sulbactam (57.1%), piperacillin/tazobactam (40.0%), ceftazidime (0.0%), and cefepime (28.6%). Only three isolates were tested for resistance to colistin, and all three isolates were susceptible.

Trends of carbapenem susceptibility and antimicrobial consumption

We compared the susceptibility rates of *A. baumanii* to imipenem and meropenem by using data on carbapenem

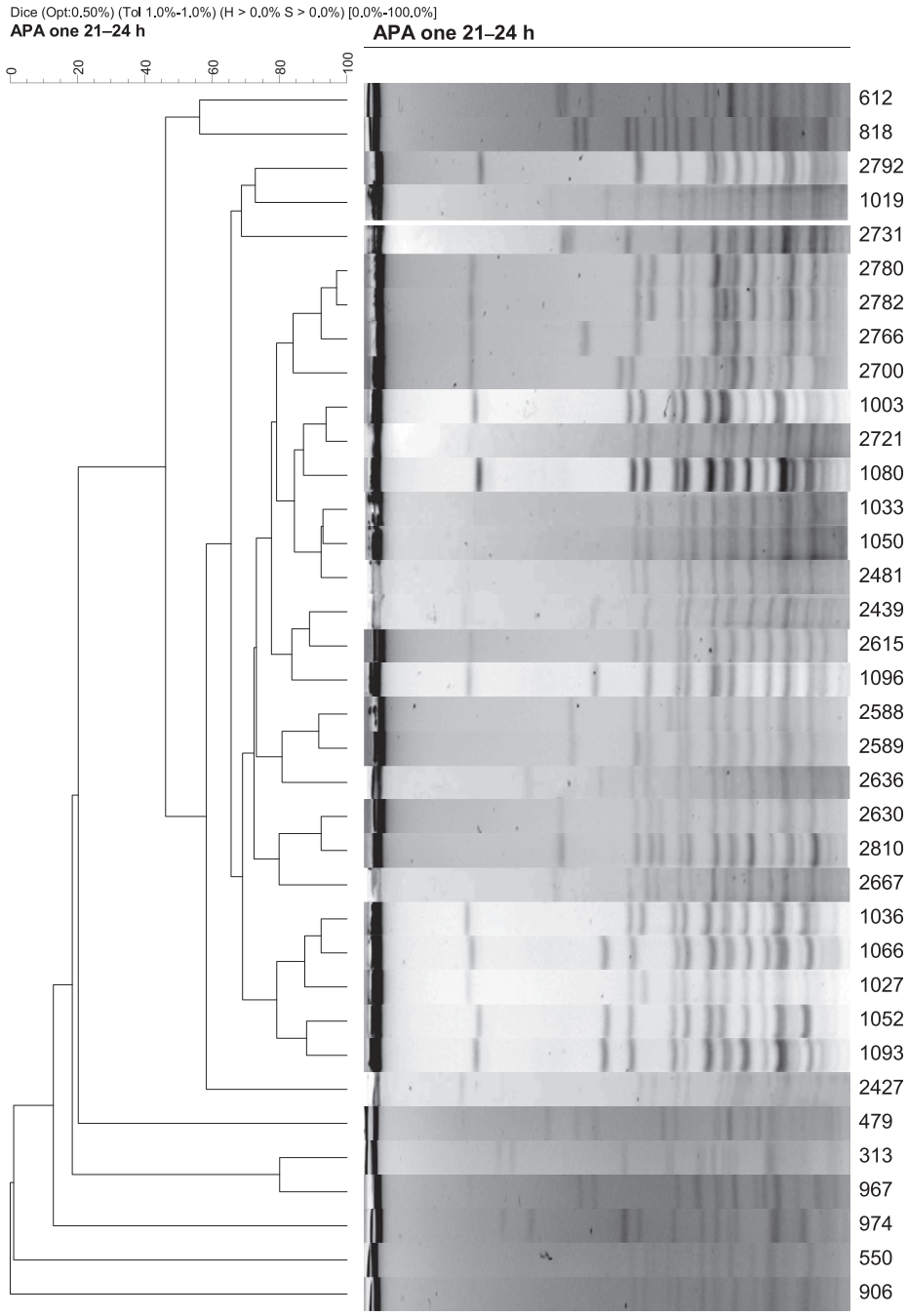

Figure 1. Pulsed-field gel electrophoresis profiles of *Apa1*-digested genomic DNA from randomly selected isolates of *Acinetobacter baumannii*. The numbers on the right represent the isolate number.

use (depicted as DDD per 1000 patient-days; Fig. 4). The steady increase of imipenem and meropenem use from 2006 (5.1 DDD/1000 patient-days for imipenem and 8.2 DDD/1000 patient-days for meropenem) to 2010 (10.1 DDD/1000 patient-days and 10.8 DDD/1000 patient-days, respectively) coincided with a steep decrease in the susceptibility of *A. baumanii* to these antimicrobial agents. Reduced consumption of imipenem from 2010 to 2012 (8.2 DDD/1000 patient-days and 6.4 DDD/1000 patient-days in 2011 and 2012, respectively) was correlated with a slight rebound of susceptibility to imipenem and meropenem from 19.0% to nearly 30.0% during that period.

Discussion

This 18-year longitudinal study of 667 isolates of genomic species identified as *A. baumannii* revealed a continuous increase in resistance to carbapenem. Increased resistance may be caused by the appearance and acquisition of antibiotic resistance genes during this time. The IS*Aba1*-$bla_{\text{OXA-51-like}}$ gene appeared in 2001, whereas the IS*1008*-ΔIS*Aba3*-$bla_{\text{OXA-58-like}}$ and $bla_{\text{OXA-72}}$ genes appeared sporadically throughout the study period. In the past 5 years, isolates have emerged that carry the IS*Aba1*-$bla_{\text{OXA-23-like}}$ gene and the transposons Tn*2006* and Tn*2008*.

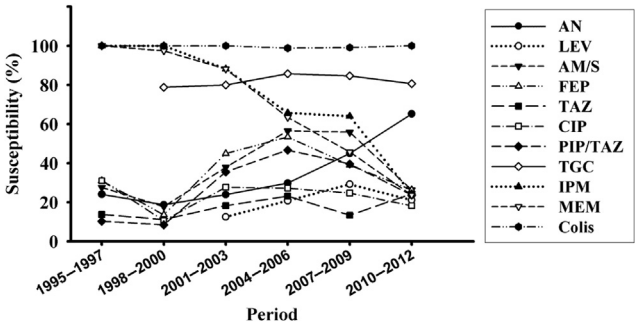

Figure 2. Susceptibility of *Acinetobacter baumannii* to antimicrobial agents from 1995 to 2012. The number of isolates in each interval was 33 isolates (1995–1997); 116 isolates (1998–2000); 84 isolates (2001–2003); 172 isolates (in 2004–2006); 157 isolates (2007–2009); and 105 isolates (2010–2012). AM/S = ampicillin/sulbactam; AN = amikacin; CIP = ciprofloxacin; Colis = colistin; FEP = cefepime; IPM = imipenem; LEV = levofloxacin; MEM = meropenem; PIP/TAZ = piperacillin/tazobactam; TAZ = ceftazidime; TGC = tigecycline.

The upstream insertion of IS*Aba1* to the intrinsic allele $bla_{\text{OXA-51-like}}$ has been associated with carbapenem resistance in *A. baumannii*.[3] Our study confirmed this finding. We determined that the emergence of these resistance-associated genetic structures, which appeared in our institution in 2001, correlated with the decreased susceptibility of *A. baumannii* to carbapenem. Because of the chromosomal location of $bla_{\text{OXA-51-like}}$ in *A. baumannii*, IS*Aba1*-$bla_{\text{OXA-51-like}}$ may persist in the genome and may be transferred to daughter cells during reproduction, thereby contributing to its consistent existence, compared to other plasmid-borne CHDL genes that were detected only sporadically. A recent nationwide study by Chuang et al[28] determined that the most commonly detected carbapenemase gene in *A. baumannii* was IS*Aba1*-$bla_{\text{OXA-51-like}}$, and the results suggested that the increase in this resistance gene occurred because of clonal spreading.

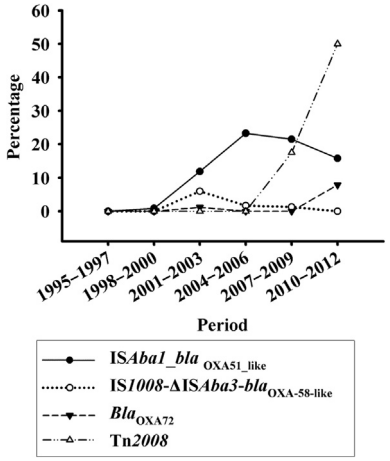

Figure 3. CHDL gene carriage in *Acinetobacter baumannii* from 1995 to 2012.

Table 1 Antimicrobial susceptibility in *Acinetobacter baumannii* isolates carrying different CHDL genes

Resistance gene	Total number	Susceptible isolates									
		AN	LEV	AM/S	FEP	TAZ	CIP	PIP/TAZ	TGC	IPM	MEM	Colis
IS*Aba1*-$bla_{\text{OXA-51-like}}$	105	27/101 (26.7)	5/40 (12.5)	46/98 (46.9)	31/97 (32.0)	2/94 (2.1)	13/91 (14.3)	28/90 (31.1)	25/30 (83.3)	43/105 (41.0)	28/105 (26.7)	69/69 (100.0)
IS*1008*-ΔIS*Aba3*-$bla_{\text{OXA-58-like}}$	11	2/11 (18.2)	N/A	4/11 (36.4)	6/11 (54.5)	0/11 (0.0)	1/11 (9.1)	1/8 (12.5)	N/A	2/11 (18.2)	5/11 (45.5)	7/7 (100.0)
$bla_{\text{OXA-72}}$	3	0/1 (0.0)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	0/3 (0.0)	0/2 (0.0)	0/3 (0.0)	1/3 (33.3)	0/2 (0.0)	0/3 (0.0)	2/2 (100.0)
Tn*2006* or Tn*2008*	58	21/49 (42.9)	2/46 (4.3)	7/57 (12.3)	6/55 (10.9)	2/51 (3.9)	2/41 (4.9)	4/55 (7.3)	24/34 (70.6)	11/57 (19.3)	2/58 (3.4)	34/34 (100.0)

Data are presented as *n* (%) unless otherwise indicated.
AM/S = ampicillin/sulbactam; AN = amikacin; CIP = ciprofloxacin; Colis = colistin; FEP = cefepime; IPM = imipenem; LEV = levofloxacin; MEM = meropenem; N/A = not available; PIP/TAZ = piperacillin/tazobactam; TAZ = ceftazidime; TGC = tigecycline.

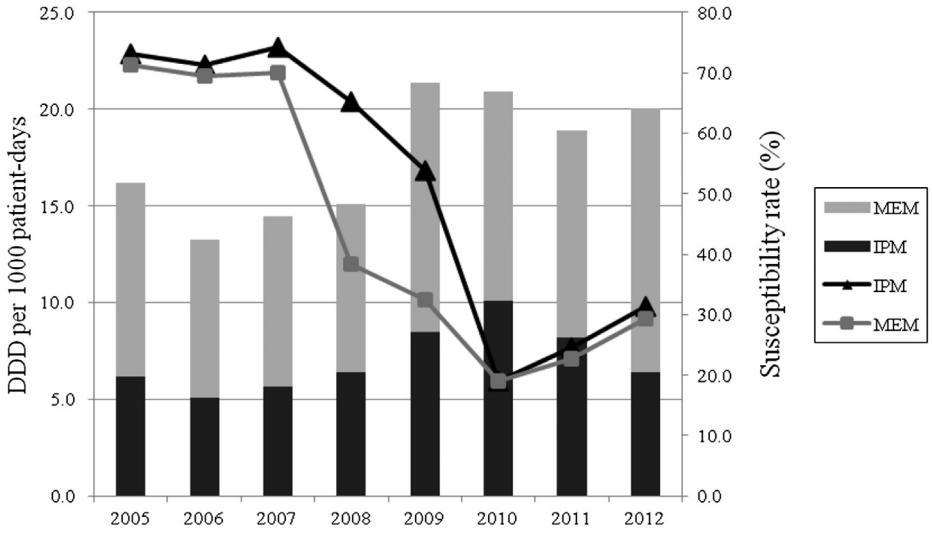

Figure 4. Susceptibility of *Acinetobacter baumannii* to imipenem and meropenem (curves) and carbapenem use, depicted as the defined daily dose per 1000 patient-days (bars) from 2005 to 2012. DDD = defined daily dose; IPM = imipenem; MEM = meropenem.

A. baumannii strains carrying the $bla_{\text{OXA-23-like}}$ gene have been discovered worldwide and are especially prevalent in Asian-Pacific regions.[29–32] A previous study by Lee et al[12] revealed that CRAB, which harbors the $bla_{\text{OXA-23-like}}$ gene, was clustered more frequently in central Taiwan in 2007 than in other areas of the island. Starting in 2007, we observed an interesting increase of Tn2006-bearing and Tn2008-bearing isolates in the study hospital, which was located in northern Taiwan. Lee et al[33] also suggest that the $bla_{\text{OXA-23-like}}$ gene replaced the IS*Aba1*-$bla_{\text{OXA-51-like}}$ gene as the primary resistance mechanism in other medical centers in northern Taiwan in 2009. To our knowledge, the $bla_{\text{OXA-23-like}}$ gene is mobilized by Tn*2006*, Tn*2007*, and Tn*2008* and the dissemination of the $bla_{\text{OXA-23-like}}$ gene is caused by transposons, rather than by clonal spreading.[12,34] We believe that the $bla_{\text{OXA-23-like}}$ gene was also spread to northern Taiwan by a transposon-mediated mechanism and that its dissemination between different geographic regions was facilitated by patient transfer among hospitals and nursing homes and between China and Taiwan. Increased use of carbapenem in this institution may have also facilitated the selection of strains of *A. baumannii* carrying the carbapenem resistance genes. Previous studies from China also demonstrated that the increased use of carbapenem contributed to the development of *A. baumannii* resistance to imipenem and meropenem and to possible cross-resistance with other β-lactams, aminoglycosides, and fluoroquinolones.[35]

Treatment of multidrug resistant *A. baumanii* has been a great challenge to clinicians, and inappropriate antimicrobial therapy has led to increased mortality.[36] In other studies, antimicrobial agents such as sulbactam, tigecycline, and colistin, had variable efficacy against *A. baumanii*.[5,6] In our study, most isolates were resistant to ampicillin/sulbactam and only 80% were susceptible to tigecycline. Lower susceptibility rates were observed in isolates harboring CHDL genes such as $bla_{\text{OXA-72}}$, Tn*2006*, and Tn*2008*. By contrast, colistin showed a uniformly high susceptibility in CRAB and in carbapenem-susceptible isolates. Newer antimicrobial agents targeting oxacillinase may be a viable choice against CRAB infection.

Our study was conducted in a single medical center in northern Taiwan and spanned almost two decades. We collected a large number of isolates, each having a complete panel of CHDL and MBL genes. We conducted assays to detect resistance-associated genes other than CHDLs and metallo-β-lactamases. Other nonenzymatic mechanisms such as changes in outer membrane protein and multidrug efflux pump expressions may contribute to the declining susceptibility to carbapenem.[3,6] Specific experiments should be designed to look at these factors.

In conclusion, our 18-year study, which contains a large number of isolates revealed increasing resistance to carbapenem in *A. baumannii*. This resistance was likely caused by the increased prevalence of the IS*Aba1*-$bla_{\text{OXA-51-like}}$ gene beginning in 2001, the sporadic appearance of the IS*1008*-ΔIS*Aba3*-$bla_{\text{OXA-58-like}}$ and $bla_{\text{OXA-72}}$ genes, and the rapid emergence of isolates carrying the transposons Tn*2006* and Tn*2008* in the past 5 years.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgments

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