行政院國家科學委員會專題研究計畫　成果報告

多重藥物或現有全部藥物抗藥性 Acinetobacter baumannii 引起之院內感染：臨床表現、預後、菌株之藥物感受性

計畫類別：個別型計畫
計畫編號：NSC91-2314-B-002-172-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：國立臺灣大學醫學院檢驗醫學科

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報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 92 年 10 月 6 日

Dissemination of A Clone of Unusual Phenotype of Pandrug-Resistant *Acinetobacter baumannii* at a University Hospital in Taiwan

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Running head: PANDRUG-RESISTANT *A. BAUMANNII WITH UNUSUAL PHENOTYPE*

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ABSTRACT

From December 2002 to February 2003, 15 isolates of pandrug-resistant unidentified *Acinetobacter* species were recovered from seven patients with nosocomial infections or colonizations treated at different wards or intensive care units at the National Taiwan University Hospital. These isolates, which were glucose- and lactose-non-acidifiers, failed to recognize to the species level using three commercial identification systems: the Vitek GNI, API 20NE system (bioMerieux, Marcy L'Etoile, France) and the Phoenix System (Becton-Dickinson, Sparks, Md.), and 16S rRNA gene sequence analysis. However, 16S-23S rRNA intergenic spacer PCR-restriction fragment length polymorphism profiles and the sequence analysis of these isolates both identified as *A. baumannii*. All these isolates were uniformly resistant to ampicillin-sulbactam (MICs, 128->128 «g/ml), ceftazidime (MICs, 64->128 «g/ml), piperacillin-tazobactam (MICs, 128->128 «g/ml), cefepime (MICs, 16-32 «g/ml), aztreonam (MICs, 64-128 «g/ml), ciprofloxacin (MICs, 64-128 «g/ml), trovafloxacin (MICs, 8-16 «g/ml), moxifloxacin (MICs, 4 «g/ml), garenoxacin (MICs, 16-32 «g/ml), amikacin (MICs, >128 «g/ml), imipenem (MICs, 8-16 «g/ml), and meropenem (MICs, 128->128 «g/ml). The identity of the pulsed-field

gel electrophoresis patterns and antibiotypes among these isolates from the same patients with an interval of 4-8 weeks and different patients indicated that this pandrug-resistant *A.baumannii* with unusual phenotype could have long-term persistence in humans and has widely disseminated at the hospital.

INTRODUCTION

Bacteria that constitute the genus *Acinetobacter* were originally identified in the early twentieth century, but it was appreciated as a ubiquitous pathogen only in the last decade (2). *Acinetobacter* species are aerobic, encapsulated, nonmotile, and gram-negative organisms. Among them, *A. baumannii* is the species most commonly involved in infections. Nosocomial infections caused by multidrug-resistant *A. baumannii* have been reported in recent years (4, 5, 12, 13, 24). The emergence of carbapenem-resistant *A. baumannii* (CRAB) was reported in the United States in 1991 (8). Since then, CRAB infections and hospital-wide outbreaks have been reported worldwide (1, 5). Isolates of pandrug-resistant *A. baumannii* (PDRAB), which was resistant to all commercially available antibiotics, were first recovered in May 1998 at the National Taiwan University Hospital (NTUH) (14). Since then, clusters of PDRAB infections and nosocomial outbreaks persisted, although the incidence of nosocomial infections caused by PDRAB declined in the past two years (12, 13, 15).

In December 2002, an isolate of PDR *Acinetobacter* species was recovered from respiratory secretions of a hospitalized patient. This isolate was identified presumptively to be an unusual phenotype of PDR *A. baumannii* (PDRABup) because of its negative reaction to 10% lactose. Further biochemical profile studies using three commercial identification kits failed to identify the organism to species level. Since

then, a total of 15 isolates of this unusual phenotype of PDR *Acinetobacter* strains were recovered various clinical specimens of seven hospitalized patients in the following three months at the hospital. The aim of this study is to determine the species level of this unusual phenotype of *Acinetobacter* species, their in vitro susceptibility, and the clonality of these organisms.

PATIENTS AND METHODS

Clinical data. From 1 December 2002 through 28 February 2003, 15 isolates of PDRABup recovered from various clinical specimens of seven patients who were treated at the National Taiwan University Hospital were identified. These isolates were resistant to all commercially available antibiotics tested (i.e. ampicillin-sulbactam, ceftazidime, piperacillin-tazobactam, cefepime, aztreonam, ciprofloxacin, trovafloxacin, moxifloxacin, garenoxacin, amikacin, imipenem, and meropenem). Relevant information on the clinical presentation of these patients was collected. These data included the underlying diseases, associated medical condition (the use of an indwelling catheter and administration of chemotherapy), clinical syndromes, days of positive culture for this organism after hospitalization, preceding antibiotic regimens before acquisition of this organism, and outcome.

Identification of bacterial isolates. The 15 isolates were initially identified as PDRABup on the basis of colonial morphotypes, gram staining characteristics, oxidase reaction, and growth on triple-sugar agar. These isolates were intended to identify to species level by their biochemical profiles obtained with the Vitek GNI and API 20NE system (bioMerieux, Marcy L'Etoile, France) and Phoenix System (Becton-Dickinson, Sparks, Md.).

PCR amplification of the complete 16S rRNA gene and direct sequencing of the

amplification product was performed according to previous description. PCR amplification of 16S-23S intergenic spacer sequences (ITS) was also performed and the amplicons were digested by *Alu*I and then subjected to restriction fragment length polymorphism (RFLP) analysis and direct sequencing study based on the protocol described previously.

Antimicrobial susceptibility testing. The minimum inhibitory concentrations (MICs) of antimicrobial agents for the 15 isolates of PDRABup were determined using the agar dilution method according to guidelines established by the National Committee for Clinical Laboratory Standards (NCCLS) (21, 22). The following 14 antimicrobial agents were obtained as standard reference powders of known potency for laboratory use: ampicillin-sulbactam, and trovafloxacin (Pfizer Inc., New York, NY); cefotaxime (Aventis Pharma, Romainville, France); ceftazidime (Glaxo, Greenford, U.K.); flomoxef (Shiohogi & Co., Ltd. Osaka, Japan); aztreonam, cefepime, amikacin, and garenoxacin (Bristol-Myers Squibb, Princeton, N.J.); piperacillin-tazobactam (Wyeth-Ayerst Laboratories, Pearl River, N.Y.); imipenem (Merck Sharp & Dome, Rahway, N.J.); meropenem (Sumitomo Pharmaceuticals, Osaka, Japan); and ciprofloxacin and moxifloxacin (Bayer Co., West Haven, Conn.). The isolates were grown overnight on trypticase soy agar plates supplemented with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) at 37^{o}C. Bacterial

inocula were prepared by suspending the freshly grown bacteria in sterile normal saline and adjusting to a 0.5 McFarland standard. Using a Steers replicator, an organism density of 10^4 CFU/spot was inoculated onto the unsupplemented Mueller-Hinton agar (BBL Microbiology Systems) with various concentrations of antimicrobial agents and then incubated at 35^{o}C in ambient air.

For synergy analysis, five pairs of antimicrobial disks (ceftazidime and amikacin, cefepime and amikacin, imipenem and amikacin, imipenem and ampicillin-clavulanate, and imipenem-ciprofloxacin) were applied onto unsupplemented Mueller-Hinton agar with a distance of 15 mm (center-to-center) of two disks. Synergy between two antimicrobial agents was identified if the presence of enhanced inhibition zone between two antimicrobial disks.

Molecular typing. Genotypes of the 15 isolates of PDRABup and five PDRAB isolates, one each from clones 1 to 5 reported previously (14), were determined by the PFGE. For PFGE, DNA extraction and purification were also carried out as described previously (14). The DNA was digested by the restriction enzyme *Sfi*I and the restriction fragments were separated in a CHEF-DRIII unit (Bio-Rad, Hercules, Calif.). Interpretation of the PFGE profiles followed the description by Tenover, *et al.* (26) PFGE profiles of the isolates were considered derived from a common ancestor (closely related isolates), if the numbers of fragment differences were three or less

Definitions. Antibiotypes were considered identical if the MICs of all antimicrobial agents tested were identical or within a 1-dilution discrepancy. Isolates were defined as the same strain or originating from a single clone if they had identical antibiotypes and PFGE profiles.

RESULTS

Characteristics of the patients. The clinical characteristics of the 7 patients with infections caused by PDRABup are provided in Table 1. Their mean age was 64 years old (range, 14-90 years). Three patients (43%) were male. One patient had underlying malignancy (cholangiocarcinoma) and none had hematological malignancies or immunodeficiency. All except one patient (patient 5) had fever as the presentation of the infection. Six patients were in bed-ridden state due to stroke or head injury. All except patients 2 and 5 received endotracheal mechanical ventilation. All infections or colonizations due to PDRABup were all hospital-acquired, occurring 9 to 38 days after admission.

The most common source of infection was respiratory tract. PDRABup was isolated from multiple sites in three patients (patients 3, 4, and 7). Co-isolates were identified from the same specimen in 6 patients, including MRSA (4 patients), *Stenotrophomonas maltophilia* (3 patients), and *Pseudomonas aeruginoma* (2 patients). All but one (patient 1) received various antimicrobial agents (extended-spectrum cephalosporins, glycopeptides, ciprofloxacin, clindamycin, or metronidazole) for seven to 14 days before acquisition of PDRABup. After notification of positive culture for PDRABup, two patients (patients 2 and 5) did not receive any parenteral antimicrobial agents and five were treated with meropenem,

ciprofloxacin, or extended-spectrum cephalosporins, according to the susceptibility results of the co-isolates. Five patients survived.

(i) **Patient 1**. A 68-year-old woman diagnosed with ischemic stroke and underlying congestive heart failure and diabetes mellitus. Antibiotic with amoxicillin-clavulanate was administered empirically for aspiration pneumonia. On the 9th day of antibiotic treatment, PDRABup was recovered from a sputum specimen. There was no co-isolate yielded from the first two samples (A1, A2). Urinary tract infection with *Klebsiella pneumoniae* developed later and was successfully treated by ceftazidime and then cefepime. PDRABup was repeatedly recovered from sputum at an interval of one month.

(ii) **Patient 3.** A 76-year-old woman diagnosed with cerebellar hemorrhage and respiratory failure with endotracheal intubation. Nosocomial pneumonia developed and PDRABup was identified on the ninth day of hospitalization. *Stenotrophomonas maltophilia*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were co-isolate bacteria. After successful treatment with flomoxef, ciprofloxacin and then meropenem, the pneumonia improved and the patient survived. But persistent recovery of the PDRABup from sputum and urine was noted for an interval of 3 months.

(iii) **Patient 6.** A 90-year-old woman with head injury and intracranial

hemorrhage. Ventilator-associated pneumonia, cardiogenic shock and acute renal failure developed during hospitalization. PDRABup, *P. aeruginosa* were identified from sputum and *Candida* species and *P. aeruginosa* from urine. The patient died of severe sepsis and deteriorated multiple organ failure.

Bacterial isolates. All these isolates were gram-negative bacilli, oxidase-negative, non-hemolytic, lactose (10%)-negative and glucose-non-acidifiers. Colonies on trypticase soy agar supplemented with 5% sheep's blood were mucoid and slightly pink. They were identified as *A. lwoffii* by the Vitek GNI system (97% presumptive identification) and as *A. baumannii/calcoaceticus* complex by the API 20NE (76.8 to 83% identification) and as *Acinetobacter* species by the Phoenix system (90% confidence value). A control stain, *A. baumannii* ATCC 19606, was 10% lactose-positive and glucose-acidifier, and was identified as *A. baumannii/calcoaceticus* complex by the Vitek GNI system (99% presumptive identification) and the API 20NE (99% identification) and as *Acinetobacter* species by the Phoenix system (92% confidence value) (table 2).

The 16S rRNA sequencing data of these isolates (650 nucleotides) were identical and were comparable with identification of *A. baumannii/calcoaceticus* complex or *A. junii.* The 16S-23S ITS PCR-RFLP and the following sequencing analysis (883 nucleotides) were identical and confirmed the identification of *A.*

baumannii. There was only one nucleotide difference of the 16S-23S ITS sequence between our isolates and *A. baumannii* ATCC 19606. The percentage of match to GenBank sequence U60279 (*A. baumannii*), U60280 (*Acinetobacter* ATCC19004, genomospecies 3), U60281 (*Acinetobacter* ATCC17903, genomospecies 13), and U60278 (*A. calcoaceticus*, genomospecies 1) was 99.0%, 95.7%, 95.7%, and 95.7%, respectively.

Susceptibility testing. All these isolates were uniformly resistant to ampicillin-sulbactam (MICs, 128->128 μg/ml), ceftazidime (MICs, 64->128 μg/ml), piperacillin-tazobactam (MICs, 128->128 μg/ml), cefepime (MICs, 16-32 μg/ml), aztreonam (MICs, 64-128 μg/ml), ciprofloxacin (MICs, 64-128 μg/ml), trovafloxacin (MICs, 8-16 μg/ml), moxifloxacin (MICs, 4 μg/ml), garenoxacin (MICs, 8-32 μg/ml), amikacin (MICs, >128 μg/ml), imipenem (MICs, 8-16 μg/ml), and meropenem (MICs, 16->128 μg/ml).

Among the five pairs of antimicrobial agents tested for synergy, only imipenem and ampicillin-sulbactam showed an enhanced zone of inhibition between the two disks for all 15 isolates (Fig. 1).

PFGE profiles. All the 15 isolates of the PDRABup had an identical PFGE profile, which was different from those of the five PDRAB isolates (only one isolate was shown) (Fig. 2). Four isolates from sputum specimens recovered from patient 1 at

an interval of one month had identical PFGE profiles. The similar scenario was also found among five isolates (four from sputum specimens and one from a urine sample) recovered from patient 3 at an interval of two months.

DISCUSSION

Over the past two decades, *Acinetobacter* species has been increasingly associated with nosocomial infection and colonization, particularly those occurred in patients with respiratory tract infections who hospitalized in intensive care units. Many nosocomial outbreaks due to *A. baumannii,* particularly carbapenem-resistant isolates or PDRAB isolates have been reported. Infections caused by PDRAB pose a clinical dilemma because of the lack of appropriate antimicrobial therapy at present. According to our previous reports, the mortality rate in patients with bacteremia due to PDRAB was as high as 60% (17). The most common sources of infection are derived from respiratory tract, indwelling catheters, and wounds. Furthermore, a few clones of PDRAB were implicated in nosocomial *Acinetobacter* infections at the NTUH since 1998 and a major clone (clone 5) has widely circulated in different settings of the hospital with widespread carbapenem use (14).

This report describes a PDRAB clone with a phenotype different from PDRAB clones found at the NTUH before and characterizes a nosocomial outbreak due to these organisms during a 3-month period. Our results show three important facets. First, although glucose-nonoxidizing *A. baumannii* accounts for 5% of all clinical *A. baumannii* isolates **(**3, 25**)**, isolates of *A. baumannii* (including PDRAB isolates) with negative reaction to both glucose and lactose was never seen at the hospital. Because

of problems in the routine clinical microbiology laboratories in speciation of *Acinetobacter* species, a phenotypic scheme for identification of genomospecies 1 to 12 has previously been described (3), however, by using this system, discrepancies with identifications by DNA-DNA hybridization, 16S rRNA sequencing, and 16S-23S ITS PCR-RFLP and sequencing have been found **(**6, 10, 16**)**. In the present study, the biochemical profiles of these asaccharolytic isolates (glucose-, lactose-, xylose-, and mannitol-non-oxidizing strains) of *Acinetobacter* species generated by the three commercial biochemical identification kits could not categorize to any genomospecies of *Acinetobacter* **(**3, 7, 25**).** Although the 16S rRNA sequencing data only suggested the identification of *A. baumannii/calcoaceticus* complex or *A. junii,* the 16S-23S ITS PCR-RFLP and the following sequencing analysis confirmed the identification of *A. baumannii.*

Second, this PDRABup had a PFGE profile different from those of 10 clones of PDRAB recognized in our previous study indicating that these PDRABup isolates belonged to a newly emerging clone. As seen in other PDRAB and other non-fermentative gram-negative bacteria illustrated before, this clone isolates could also have long-term persistence (infection or colonization) in humans for weeks and months.

Finally, all isolates were highly resistant to extended-spectrum cephalosporins,

carbapenems, fluoroquinolones, aminoglycosides, and ampicillin-sulbactam. The high MICs of these agents exceeded the levels achievable in plasma and tissue suggesting their limited role as treatment regimen. Recent studies have demonstrated that sulbactam alone at higher doses or in combinations with other agents provide efficacy against nosocomial infections caused by multiresistant *A. baumannii*. Although synergy was detected by disk method for combination of only imipenem plus ampicillin-sulbactam, more studies including time-kill and in vivo animal studies should be performed to establish the treatment options.

The environmental source and mode of spread of these PDRABup is obscure. Previous studies have clearly demonstrated that air humidifiers, intravascular institution fluids, feeding syringe, ventilator monitor board, resuscitation bags, hands of medical staff, and intestinal flora have been found to be reservoirs and associated with nosocomial outbreaks (14). During the period of this outbreak (from December 2002 to February 2003), several small clustering of PDRAB still occurred in intensive care units and environmental surveillance (samples from air humidifiers, hands of medical staff, mattresses, stock solution, sinks, taps, and portable X-ray machine) failed to find this organisms. Fortunately, this clone circulated in the hospital for three months and disappeared spontaneously, however, the classic PDRAB isolates was still occurring.

In this study, the majority of patients acquired PDRABup infections or colonizations one week to more than one months after hospitalization, particularly those received mechanical ventilation therapy. Clinically, it is difficult to determine the pathogenic role of this organism because of the poor underlying medical condition of the patients, the polymicrobial entity in specimens of infected sites and absence of concurrent bacteremia due to this organism. In the two patients (patients 6 and 7) who died, two well-known pathogens (*P. aeruginosa* and MRSA, respectively) were also identified.

In summary, we report a nosocomial outbreak due to a novel PDRAB clone occurred in seven patients at the hospital during a 3-month period. Because of the lack of sufficient phenotypic discriminating criteria for identification of *Acinetobacter* species, molecular methods should be conducted, particularly in isolates with unusual phenotype.

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TABLE 2. Biochemical profiles of *Acinetobacter baumannii* ATCC19606 and the clone of pandrug-resistant *A. baumannii* with unusual phenotype (PDRABup)

	Biochemical profiles	
	A. baumannii	The clone
Reaction	ATCC19606	(PDRABup)
Growth at		
37°C	+	+
41°C	+	+
Acid from		
Glucose	+	−
Lactose	+	−
Sucrose	−	−
Galactose	−	−
Fructose	−	−
Mannitol	−	−
α-ketoglutaric acid	+	+
Tiglic acid	+	+
Utilization of		
Acetate	+	+

Citrate	+	+
Malonate	+	+
Glycine	+	+
Leucine	+	+
Arginine dihydrolase	+	−
Orinithine decarboxylase	−	−
Urea	−	−
Esculin hydrolysis	−	−

FIG. 1. An enhanced zone of inhibition between imipenem and ampicillin-sulbactam disks for the pandrug-resistant *A. baumannii* isolates with unusual phenotype.

FIG. 2. Profiles obtained by PFGE for *A. baumannii* after digestion with *Sfi*I. Lane M, molecular size marker; lanes 1 to 11, pandrug-resistant *A.baumannii* with unusual phenotype (PDRABup) isolates of A1. A3, A4, B, C1, C4, C5, D, E, F, and G (see table 1 for designation of isolates); and lane 12, a PDRAB isolate belonging to clone 5, the major clone of PDRAB shown in the previous study (14).

Table 1. Clinical characteristics of seven patients with positive cultures for pan-drug resistant *Acinetobacter baumannii* with unusual phenotype who were treated at the National Taiwan University Hospital

Patient no.	Sex/Age (yr)	Underlying diseases[a]	Clinical syndrome	Isolation of *A. baumannii*			Co-isolates
				Site	designation	Date (d/mo/yr)	
1	F/68	Ischemic stroke, RHD, CHF, DM, UTI, pneumonia	Fever	Sputum	A1	2/12/2002	*A. baumannii*
				Sputum	A2	18/12/2002	*S. malsophili*
				Sputum	A3	23/12/2002	MRSA
				Sputum	A4	2/1/2003	
2	M/81	Old stroke, BPH, recurrent UTI	Fever	Urine	B	5/12/2002	None
3	F/76	Cerebellar hemorrhage, post tracheostomy, hypertension, pneumonia, UTI	Fever	Sputum	C1	9/12/2002	*S. maltophilia*
				Sputum	C2	30/12/2002	*P. mirabilis,*
				Sputum	C3	10/1/2002	*P. aeruginos*
				Sputum	C4	20/1/2003	*K. pneumonia*
				urine	C5	27/1/2003	*C. albicans*
4	F/14	Encephalitis, status epilepticus,	Fever	Sputum, pressure	D	12/12/2002	MRSA

		respiratory failure, UTI		sore			
5	M/60	Old stroke, DM, CHF, renal insufficiency, hypoxic encephalopathy, UTI	Shock (no fever)	Wound (amputation site)	E	21/1/2003	MRSA
6	F/90	Head injury, intracranial hemorrhage, CHF, cardiogenic shock, cellulites, acute renal failure, pneumonia	Fever	Sputum	F	2/2/2003	*P. aeruginosa*
7	M/65	Chronic renal failure, UTI, cholangiocarcinom	Fever, shock	Sputum, urine, PTCD	G	20/2/2003	MRSA, *C. menigosep* *S. maltophili*

	a with obstructive jaundice and BTI, operated

[a]BPH, benign prostatic hypertrophy; BTI, biliary tract infection; CHF, congestive heart failure; DM, diabetes mellitus; MRSA, methicillin-resistant *Staphylococcus aureus*; PTCD, percutaneous transhepatic cholangial drainage; RHD, rheumatic heart disease; UTI, urinary tract infection.