Persistence of carbapenem-resistant *Acinetobacter baumannii* strains in an Italian intensive care unit during a forty-six month study period

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──────── SUMMARY ────────

The aims of this study were to analyze carbapenem-resistance *Acinetobacter baumannii* isolates (CRAB) and their molecular epidemiology in an ICU of Southern Italy. Clinical outcomes and therapeutic management of patients are also described. The study was performed from January 2007 to October 2010. The presence of carbapenemases was determined by PCR. Strains were typed by PFGE. All *A. baumannii* isolates were carbapenem-resistant with imipenem MIC$\geq$16 µg/mL. Molecular characterization showed the occurrence of a predominant clone. The most frequent infection by CRAB was ventilator-associated pneumonia; colistin was the drug of choice for this infection. The therapy was safe in all cases except in one where therapy was suspended due to the onset of acute renal failure. We documented the presence of CRAB in this ICU, besides the occurrence of a predominant clone, over all the study period. Despite the infection control procedures used, intra-facility *A. baumannii* transmission is evident as well as the significant capacity for long-term survival in the hospital environment.

KEY WORDS: Acinetobacter baumannii, Multidrug-resistant, Carbapenem-resistance, PFGE, Colistin

INTRODUCTION

Acinetobacter baumannii is a glucose non-fermentative Gram-negative cocco-bacillus considered a relevant nosocomial pathogen especially in immunocompromised and in Intensive Care Unit (ICU) patients (Weingarten *et al.*, 1999; Rello *et al.*, 2003; Bou *et al.*, 2007). The most frequent health-care associated infections are urinary tract infections, bacteremia, surgical-site infection and ventilator-associated pneumonia (VAP) (Lambiase *et al.*, 2009). *A. baumannii* isolates recovered in the nosocomial environment showed a broad spectrum of resistance to antimicrobial agents, which was most probably a consequence of the extensive use of antimicrobial molecules. Recent studies indicate that the mortality rate of patients infected by multidrug-resistant *A. baumannii* isolates is higher than that of patients infected by not multidrug-resistant isolates. Carbapenems have been reported as the most appropriate choice for the treatment of infections by multidrug-resistant *A. baumannii*. However, the resistance to these molecules has increased so much worldwide (Beck-Sague *et al.*, 1990; Smolyakov *et al.*, 2003) that only its presence is sufficient to define an *A. baumannii* isolate as highly resistant (CRAB: carbapenem-resistant *A. baumannii*) (Corbella *et al.*, 2000). The most clinically significant carbepenemases are metalloenzymes and, in *A. baumannii*, resistance to carbapenems is also conferred by some carbapenemases (Ambler class D) that weakly hydrolyze imipenem and meropenem (Nordmann *et al.*, 2002).

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Transmission of *A. baumannii* in association with contamination of hospital equipment or cross-transmission among patients have been reported (Corbella *et al.*, 2000; Takahashi *et al.*, 2000; Denton *et al.*, 2005). This spread is linked to the capacity of bacteria to survive in dry conditions and their need for simple growth conditions.

The present study has 3 goals:

1) to describe the prevalence of *A. baumannii* infections in an Italian ICU during a 46-month period;
2) to investigate the mechanisms of resistance to carbapenems;
3) to characterize the *A. baumannii* isolates by PFGE.

MATERIALS AND METHODS

Patients and setting

This study was conducted in the ICU of the University Hospital of Naples, Italy, in the period from January 2007 to October 2010. From 450 to 500 patients per year are admitted to this ICU; patients can be admitted from any department of the hospital as well as from the regional emergency system network. All patients included in this study underwent central venous catheterization and positioning of a bladder catheter.

The nurse-to-patient ratio in this ICU is 1:3. The bacterial-screening policy adopted by the ICU is the following: at admission, lower respiratory tract samples and urinary samples are obtained from all patients for bacterial and fungal cultures. Patients who are infected by methicillin-resistant *Staphylococcus aureus* (MRSA) or Vancomycin-resistant *Enterococcus spp* or Gram-negative non-fermentative bacteria are isolated in dedicated areas. Healthcare workers wear gloves, gowns and masks before entering the room of patients infected or colonized by the above-mentioned bacteria. They remove these protective garments and wash their hands immediately after leaving the room; nurses are also asked to dedicate the use of non-critical devices, such as stethoscopes, thermometers and sphygmomanometers only to these patients.

Standardized CDC criteria were used to define nosocomial infections (Garner *et al.*, 1988). For each patient, gender, age, Glasgow coma scale (GCS), sequential organ failure assessment (SO-FA) score at admission, simplified acute physiology (SAPSII) score, mean length of stay (LOS) in the ICU, mean time of mechanical ventilation (MV), reason for admission to the ICU, underlying diseases and mortality were recorded. Acute renal failure (ARF) was defined by glomerular filtration rate (GFR). Urinary Output (UO) criteria were: triplicated serum creatinine and decreased GFR <75% or serum creatinine >4 mg/dl and urine output <0.3 ml/kg/h in 24 h or anuria for the previous 12 h (Bellomo *et al.*, 2007).

Diagnosis of VAP

VAP was defined as any lower respiratory tract infection that developed after 2 days of MV. The criteria for clinical suspicion of pneumonia were: the presence of a new, or persistent, or progressive lung infiltrate on chest radiographs, plus two of the following items:

1) fever>38.3°C or hypothermia <36°C;
2) WBC count >10,000/mm^3 or <5,000/mm^3;
3) purulent endotracheal aspirate.

If infection developed within the first four days of MV, it was considered early-onset VAP, while if infection developed after five or more days from the start of MV, it was considered late-onset VAP (Weber *et al.*, 2007).

Identification of A. baumannii isolates

Isolates of *A. baumannii* were obtained from various samples (i.e. blood, urine, lower respiratory tract, venous catheters, urinary catheters). These were grown in McConkey agar plates at 37°C overnight. Bacteria were identified by a biochemical test (API 20 Strep, bioMérieux, France) and by an automatic system (Vitek II System, bioMérieux).

To confirm the phenotypic identification, the presence of $bla_{oxa-51-like}$ carbapenemase gene was investigated in all isolates using the followed primers: 5'-TAATGCTTTGATCGGCCTTG-3', 5'-TGGATTGCACTTCATCTTGG-3' (353 bp fragment) (Turton *et al.*, 2006). Negative control PCRs were employed for every experiment.

All *A. baumannii* isolates obtained during the study period were collected and stored in glycerol-broth at -80°C.

Antimicrobial susceptibility

In vitro susceptibility tests were performed using the microbroth dilution assay with a Vitek II au-

tomatic system using the AST-GN09 card. The criteria adopted were according to the National Committee for Clinical Laboratory Standards (CLSI) Interpretative Criteria, document M100-S20 (Clinical and Laboratory Standards Institute, 2010).

Susceptibility to colistin was tested by disk diffusion method and, to determine colistin MIC values, an automated microdilution method was used (card NMIC/ID-88, Phoenix System, Becton Dickinson). Susceptibility to imipenem was also confirmed using Etests (AB Biodisk). Isolates were considered multidrug-resistant according to the definition given by CDC of 2006 (Siegel *et al.*, 2006).

Molecular analysis of carbapenem-resistance

The presence of the $bla_{oxa-23-like}$, $bla_{oxa-24-like}$ and $bla_{oxa-58-like}$ genes encoding the Ambler class D carbapenemases were demonstrated by PCR. The primers used and PCR conditions were according to Afzal-Shah (Afzal-Shah *et al.*, 2001) and Woodford (Woodford *et al.*, 2006). Particularly, primers used were: OXA-23 (5'-GATGTGT-CATAGTATTCGTCG-3', 5'-TCACAACAAC-TAAAAGCACTG-3', 501 bp fragment); OXA-24 (5'-GTACTAATCAAAGTTGTGAA-3', 5'-TTCCCCTAA-CATGAATTTGT-3', 246 bp fragment); OXA-58 (5'-AAGTATTGGGGCTTGTGCTG-3', 5'-CCCCTCT-GCGCTCTACATAC-3', 599 bp fragment). Negative control PCRs were used for every experiment.

Preparation of chromosomal DNA for PFGE, restriction digestion and gel electrophoresis

DNA fingerprinting was carried out by the method described by Grothues (Grothues *et al.*, 1998). DNA inserts were digested with *SmaI* and *ApaI* (New England Biolabs). Macro-restriction fragments were separated using CHEF III (Biorad) at 10°C for 19 h, with a start time of 5s and an end-pulse time of 35s, at a field strength of 6V/cm. A concatemer ladder of lambda phage DNA was used as a size marker. Fragment patterns were compared according to Tenover's criteria (Tenover *et al.*, 1995). For the general molecular characterization of bacteria, one strain per patient was used. In addition, to confirm the presence of the same profile in more isolates of the same patient, we applied PFGE analysis on two up to five isolates per patient.

RESULTS

Throughout the study period, we obtained a total of 567 *A. baumannii* isolates from 46 patients (from 8 up to 16 isolates per patient). The presence of the $bla_{oxa-51-like}$ gene in all isolates confirmed the identification.

Co-infections with other bacteria and/or fungi were found in 30/46 patients. Bacterial species and fungi involved in co-infections were: *P. aeruginosa*, *K. pneumoniae*, *E. cloacae*, *E. coli*, *P. stuartii*, *E. faecalis*, *Haemophilus spp*, *C. albicans* and *C. glabrata*.

The mean interval between ICU admission and *A. baumannii* acquisition was 9 days (range 3 – 34 days).

VAP was diagnosed in 93% of patients. In three patients, central venous catheter-related sepsis by *A. baumannii* was diagnosed.

TABLE 1 - *Characteristics of patients infected by A. baumannii*

	Patients with infection by A. baumannii
Number of patients	46
Gender [male/female]	24/22
Mean age [years];	55.39±18.04
GCS mean value	7.22±3.87
SOFA mean value	7.59±4.37
SAPSII mean value	33.93±12.53
LOS in ICU [mean value, days]	23±14
Length of MV [mean value, days]	15±2
Reason for admission to ICU	
Pulmonary failure	28
Cardiac failure	10
Neurologic disease	8
Exitus	20

Legend: Glasgow coma scale (GCS), sequential organ failure assessment (SOFA), simplified acute physiology score II (SAPSII), mean length of stay (LOS), intensive care unit (ICU), mechanical ventilation (MV).
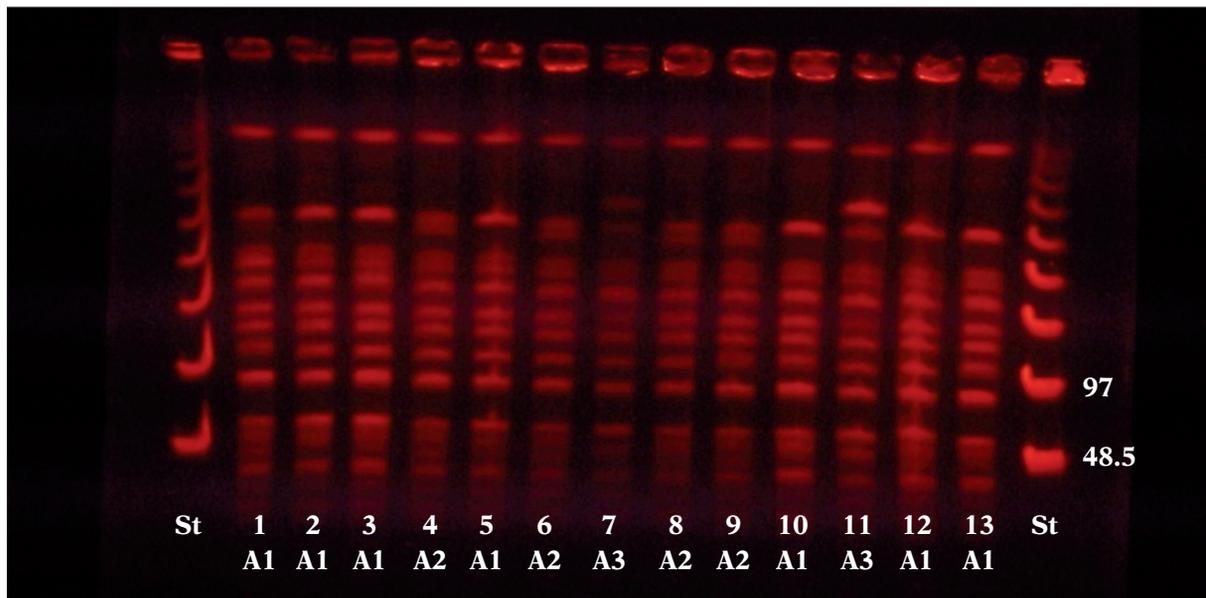
FIGURE 1 - *PFGE fingerprinting obtained by ApaI macrorestriction of some strains of A.baumannii. The numbers indicate the strains in the study. Molecular size markers (a concatemer ladder of lambda phage DNA) were run in lane St. Sizes are indicated in kilobases.*

The distribution of gender, mean age in years, GCS mean value, SOFA mean value at admission, SAPSII mean value, mean LOS in ICU, mean length of MV, reason for admission and death rate are shown in table 1.

The systems used for the study of antibiotics susceptibility showed that all *A. baumannii* isolates were multidrug-resistant. All isolates were resistant to cephalosporins; aminoglycosides showed poor activity, considering that some strains showed intermediate values of sensitivity for gentamicin, tobramycin and amikacin. Quinolones, piperacillin and piperacillin/tazobactam, trimethoprim-sulfametoxazole were inactive. All isolates were considered CRAB and they were positive for the $bla_{\text{oxa-58-like}}$ gene, and few isolates were positive for the $bla_{\text{oxa-23-like}}$ gene. Isolates involved in co-infections were sensitive to carbapenems. Isolates were sensitive to colistin, with MIC values between <0.5 and 1 µg/mL. Colistin was considered the drug of choice: mean dose of colistin was 50,000 UI/kg/die for 10 days, i.v. maximum. In 32 out of 46 patients (69%) a clinical cure was observed. The therapy was safe in all cases except one where the drug was suspended due to the onset of acute renal failure. Colistin was used as a "rescue" agent in 70% of patients

(in which the colistin therapy started after microbiology results) and as empirical therapy, in combination with linezolid, in 30% of cases. According to the presence/absence of Gram-positive microorganisms, linezolid was later suspended or continued, according to the "de-escalation therapy" protocol currently adopted in our Unit.

According to Tenover's criteria for pulsed-type analysis, we found, among the 46 strains in study (one per patient), a unique cluster (A), where possibly related and closely related strains were included (clones A1, A2, A3, A4) (Figure 1).

Strains with the same pulsed-type also shared the same antimicrobial-susceptibility profile. Moreover, strains sequentially isolated from the same patient (from two up to five) had identical antimicrobial susceptibility and PFGE profiles. Table 2 shows the results of the chemosusceptibility and pulsed-field studies of *A. baumannii* strains.

DISCUSSION

The importance of this study is the epidemiological description of *A. baumannii* acquisition dur-

ing ICU stay not based on short surveillance periods but on one long period. *A. baumannii* was not found at admission in our cohort study: therefore, our results suggest that the ICU personnel/environment served as reservoirs for cross-transmission. Consequently, we emphasize the importance of exogenous acquisition of multidrug-resistant *A. baumannii* in hospital units at

TABLE 2 - *Antimicrobial susceptibility of A.baumannii pulsed-types*

Antibiotics	Antimicrobial susceptibility of pulsed-types			
	A1	A2	A3	A4
Amikacin	I	R	R	R
Amoxicillin-clavulanic acid	R	R	R	R
Ampicillin	R	R	R	R
Ceflacor	R	R	R	R
Cefotaxime	R	R	R	R
Cefoxitin	R	R	R	R
Ceftazidime	R	R	R	R
Cefazolin	R	R	R	R
Ciprofloxacin	R	R	R	R
Gentamicin	I	R	R	R
Imipenem	R	R	R	R
Nalidixic acid	R	R	R	R
Netilmicin	R	R	R	R
Norfloxacin	R	R	R	R
Ofloxacin	R	R	R	R
Piperacillin-tazobactam	R	R	R	R
Ticarcillin	R	R	R	R
Ticarcillin-clavulanic acid	R	R	R	R
Tobramycin	I	R	R	R
Trimethoprim-sulfamethoxazole	R	R	R	R
Colistin	S	S	S	S

high risk of infections. To visualize the trend of isolation of *A. baumannii*, an epidemic curve was plotted over the time period (Figure 2).

In agreement with other studies, a high mortality (20/46 patients) was found in this cohort study. Several data indicate a directly attributable mortality in patients who acquired *A. baumannii* infection in an ICU. Lortholary *et al.* (Lortholary *et al.*, 1995) documented a directly attributable mortality of 25% in 40 patients who acquired *A. baumannii* in an ICU. Fagon *et al.* (Fagon *et al.*, 1996) reported a mortality of 42% in a group of patients with VAP caused by *Acinetobacter spp* and *Pseudomonas spp* and 29% when only colonized patients were considered. Kaul *et al.* (Kaul *et al.*, 1996) documented an attributable mortality of 23% in respiratory acquisitions of *A. baumannii*, with no difference between infected and not infected patients. Garcia-Garmendia *et al.* (Garcia-Garmendia *et al.*, 1999), in a case-control study, documented an attributable mortality for *A. baumannii* acquisition of 30% and concluded that this acquisition by ICU patients implies an increase in the LOS and the risk of death.

The extensive use of antimicrobial chemotherapy has contributed to the emergence of *A. baumannii* isolates resistant to a wide range of antibiotics, including broad-spectrum beta-lactams, aminoglycosides, fluoroquinolones and carbapenems. Although the problem of resistance to carbapemens is well known as very common for *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, this phenomenon is also increasing for enterobacteriaceae in different geographic areas. In particular, an Italian study of 2010 (Ambretti *et al.*, 2010) described a KPC-producing (*Klebsiella pneumoniae* carbapenemases-producing) *K. pneumoniae* isolated from the urinary tract in an elderly woman with recurrent urinary tract infections.

In our study, the mechanism of resistance to carbapenems was related to the presence of the $bla_{oxa-58-like}$ gene. All *A. baumannii* isolated in this study were multidrug-resistant and this is a problem for the therapeutic management of patients and presumably contributed to their mortality. Only the strains of the A1 clone showed an intermediate sensitivity versus aminoglycosides.

Colistin can have a direct toxic effect in kidneys with tubular necrosis and renal insufficiency or even failure: in this event, early discontinuation
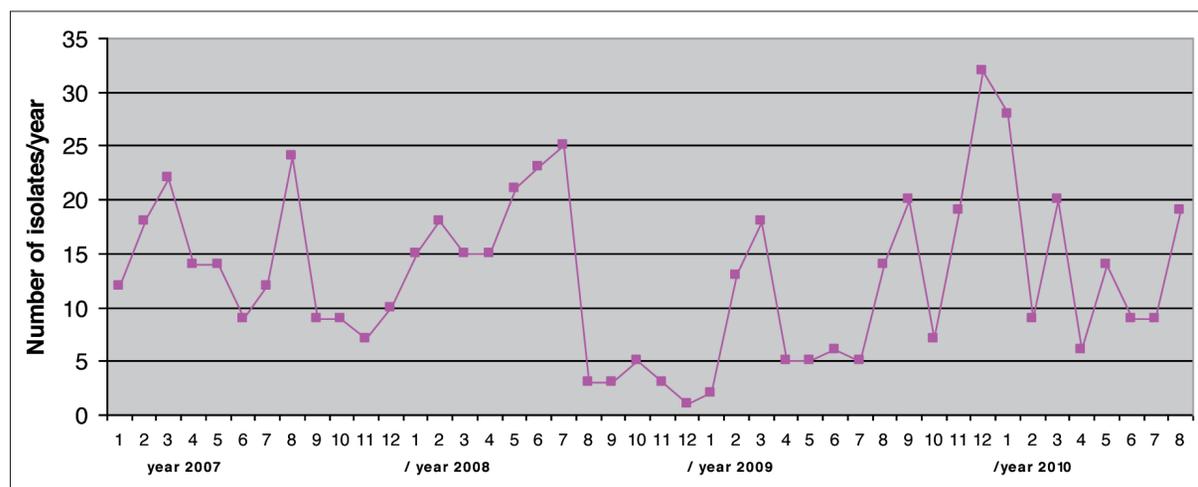
FIGURE 2 - *Epidemic curve shows the isolation trend of CRAB over the time period.*

of the regimen is necessary. Other major adverse events are neurotoxicity and neuromuscular blockade, which were not found in our cohort.

A. baumannii is a bacteria that frequently colonizes medical equipment, such as ventilators and veno-venous hemofiltration machines (Bernards *et al.*, 2004), gloves (Patterson *et al.*, 1991), resuscitation bags, pressure transducers, various surfaces, mattresses (Sheretz *et al.*, 1985), pillows (Weernink *et al.*, 1995), sinks, and bedside tables, and the skin of patients and health care personnel (Crombach *et al.*, 1989).

The usual transmission is via the hands of personnel. In addition, the use of contaminated nebulizer reservoirs or fiber optic bronchoscopes has been involved in several outbreaks (Webster *et al.*, 2000; Centers for Disease Control and Prevention, 2006). Moreover, *A. baumannii* has a significant capacity for long-term survival in the hospital environment, thus favoring the transmission between patients, either via human reservoirs or via inanimate materials.

Several methods have been used to investigate outbreaks of *Acinetobacter* spp and, among them, the macrorestriction of genomic DNA followed by PFGE is indicated as the gold standard. In our study, the macrorestriction was carried out by *SmaI* and *ApaI*. It is very important to note that the cut-capacity of *SmaI* was inferior with respect to *ApaI* (data not shown). In the study described by Borgmann *et al.* (Borgmann *et al.*, 2004), *SmaI* worked well only in non-multiresistant *A. bau-*

mannii strains, whereas *ApaI* worked well also in resistant strains. For these authors, this phenomenon was attributed to methylation of the *SmaI* restriction sites in the resistant clones, but they emphasized that the evolutionary and/or molecular link between methylation and multiple antibiotic resistance has not been well-investigated.

The results of PFGE indicated a unique epidemic clone, designated clone "A", where all strains were closely or possibly related. The presence of a unique clone persistently occurring in an ICU is also connected to the capability of the microrganism to survive for long periods in the hospital environment, even in dry conditions.

The observations from this work have a strong clinical relevance and medical staff should consider whether to adopt special control measures in hospital units at high risk of infections.

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