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BRIEF COMMUNICATION

First Report of Molecular Characterization of Carbapenem-Resistant *Acinetobacter baumannii* in Different Intensive Care Units in University Hospital Split, Croatia

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Acinetobacter baumannii is an important cause of nosocomial infections such as pneumonia, septicemia, urinary tract infections, and wound infections [1]. It may become resistant to a wide range of antibiotics, thus complicating the treatment of nosocomial infections [1]. The emergence of carbapenem (imipenem and/or meropenem) resistance in *A. baumannii* has become a global concern since these -lactams are often the only effective treatment left against many multiresistant strains [2]. Acquisition of IMP-like and VIM-like metallo- -lactamases (Ambler molecular class B) has been reported worldwide in *A. baumannii* [3]. A recent development has been the discovery of a novel group of narrow-spectrum OXA -lactamases (Ambler molecular class D) in carbapenem resistant strains, some of which have acquired the ability to hydrolyze the carbapenems [2].

The first of these (designated OXA-23) was found in a strain isolated in Edinburgh, England, before imipenem was in use in the hospital [4]. Between the years 2000 and 2004, at least six novels OXA-type enzymes were characterized from carbapenem-resistant strains collected worldwide [2].

According to a recent report, the overall resistance rate to imipenem in *A. baumannii* isolated from 17 representative laboratories in Croatia was 1% (range 0%-8%) [5]. However, the molecular basis of carbapenem resistance has not yet been investigated.

We report here a molecular investigation of carbapenem-resistant strains obtained from clinical samples from 11 infected patients in various Intensive Care Units (ICUs) in the University Hospital of Split, Croatia. During 2003 and 2004, 11 non-repetitive isolates of *A. baumannii* with an unusual resistance profile were isolated from three different ICUs (two surgical adult and one pediatric) in different locations inside the hospital, which is a 1651-bed university teaching hospital and the only hospital in the region. It serves a pediatric and adult population of about 500,000 and acts as a referral hospital for a wide area of southern Croatia, thus serving a population of about one million. Isolates were recovered from blood cultures, urine samples and bronchial secretions. Isolation of *A. baumannii* was performed in routine work on blood agar plates (Bio Rad). Identification was performed by conventional biochemical tests as well as with the API 20NE system (bioMerieux, Marcy-l'Etoile, France) and was confirmed as *A. baumannii* by tRNA spacer fingerprinting (personal communication, Dr. K. Towner, Nottingham). Routine susceptibility testing was done by the standard disc diffusion method and broth microdilution according to CLSI (earlier NCCLS) guidelines and *Pseudomonas aeruginosa* ATCC 27853 was used as control strain [6]. All isolates of *A. baumannii* displayed an intermediate or resistant profile to imipenem (minimum inhibitory concentrations, MICs 8-16 mg/L) and meropenem (MICs 8-64 mg/L). MICs were also determined for ceftazidime, cefepime, ceftriaxone, amikacin, gentamicin, ciprofloxacin and piperacillin-tazobactam by broth microdilution method according to CLSI recommendation [6]. All isolates were multidrug-resistant exhibiting high resistance to ceftazidime (>128 mg/L), cefepime (64-128 mg/L), ceftriaxone (>128 mg/L) piperacillin/tazobactam (64/4->128/4 mg/L), amikacin (128 mg/L), gentamicin (>128 mg/L), and ciprofloxacin (16-64 mg/L). No resistance to ampicillin/sulbactam was observed, probably because it has not been in use in our hospital for a long time.

The isolates of *A. baumannii* were genetically characterized using pulsed-field gel electrophoresis (PFGE) by macrorestriction with *Apa I* enzyme. A single colony of each isolate was transferred to brain-heart broth and incubated overnight at 37°C. Cells were then resuspended in 6 mM TrisCl and 1 M NaCl at pH 8.0 and a solution of lysozyme (100 mg/mL, Sigma Chemical Company, St. Louis, MO, USA) was added to the suspension. The cell suspen-

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sion was mixed with an equal amount of melted 1.7% low gelling point agarose (Bio-Rad Laboratories, Hercules, CA, USA) and the mixture was distributed into plug molds. The agarose plugs were then transferred to a solution containing proteinase K (1mg/mL, Sigma). Bacterial DNA was digested with 20 U of Apa I restriction enzyme (Sigma) for 3 hours at 30°C. DNA separation was performed in 0.5x TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) in a pulsed-field electrophoresis system (CHEF-DRIII; Bio-Rad Laboratories) with 5.5 V/cm^2 for 24 h and pulse times ranged from 1-30 s. DNA macrorestriction patterns were analyzed by visual inspection, based on the criteria of relatedness proposed by Tenover et al [7,8]. Image of ethidium bromide-stained gels are shown in *Figure 1*.

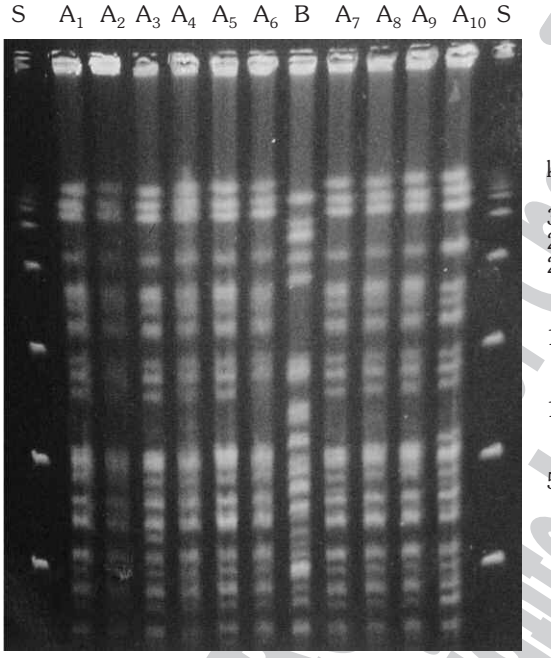

FIGURE 1 - Pulsed-field gel electrophoresis analysis of ApaI digested chromosomal DNA from eleven various *Acinetobacter baumannii* isolates on three different ICUs. PFGE profile A1-A6 were isolated from one adult surgical ICU, and the PFGE profile A7-A10 from a second adult neurosurgical ICU. The PFGE profile B was a strain isolated from a pediatric ICU. Lateral lane S contains multimers of phage lambda DNA molecular mass markers. Sizes of lambda DNA molecular mass markers are indicated on the right of the panel.

Bacterial DNA was extracted from isolates for PCR for each single gene analysis by boiling one to three colonies in 100 µL of sterile ultra-pure water for 10 min and centrifuging briefly. PCRs (50-µL final volumes) contained 2 µL of DNA extract, 20 mM Tris-HCL (ph 8.8), 10 mM KCL, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.8 mM dNTPs and 1.25 U DNA polymerase (Promega, Southampton, UK). The cycling conditions were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 2 min, with a final extension period of 72°C for 10 min. To determine the presence of bla_{OXA23}, bla_{OXA-24}, bla_{OXA-58}, bla_{OXA-51} and bla_{OXA-69} genes, specific primers were used as follows: for OXA-23- (5'-gat-gtg-tca-tag-tat-tcg-tcg-3' and 5'-tca-caa-caa-cta-aaa-gca-ctg-3'), for OXA-24-(5'-gta-cta-atc-aaa-gtt-gtg-aa-3' and 5'tcc-ccc-taa-cat-gaa-ttt-gt-3'), for OXA-58-(5'-cga-tca-gaa-tgt-tca-agc-gc-3' and 5'acg-att-ctc-ccc-tct-gcg-c-3'), for OXA-51-(5'-taa-tgc-ttt-gat-cgg-cct-tg-3' and 5'-tgg-att-gca-ctt-cat-ctt-gg-3'), and for OXA-69- (5'-cta-ata-att-gat-cta-ctc-aag-3' and 5'-cca-gtg-gat-gga-tgg-ata-gat-tat-c-3'). The sequences of consensus primers were kindly provided by Dr. S. Brown, University of Edinburgh, UK.

PCR products were detected by electrophoresis in 1% agarose gel electrophoresis after staining with ethidium bromide.

Production of metallo- -lactamases was screened by E test-MBL and PCR with primers specific for VIM (5'-cag-att-gcc-gat-ggt-gtt-tgg and 5'-agg-tgg-gcc-att-cag-cca-ga) and IMP -lactamases (5'ggc-agt-cgc-cct-aaa-aca-aa and 5'-aca-acc-agt-ttt-gcc-tta-cc). -lactamases were prepared from overnight broth cultures, released by sonication and cell debris was removed by centrifugation. Hydrolysis of imipenem was measured by UV spectrophotometric method at 294 nm. PCRs were positive for the OXA-69 and OXA-51 like enzyme in 10 *Acinetobacter* strains belonging to a unique PFGE profile characterized by pulsotype A diffused among surgical and neurosurgical adult intensive care units, while the strain characterized by pusotype B was the only strain isolated from the pediatric ICU and the carbapenemase gene was not found with tested primers. In spite of high carbapenem MICs no significant hydrolysis of imipenem was detected in all 11 strains.

It is assumed that resistance to carbapenems was due to production of OXA-51 and OXA-69 since no other oxacillinases were found. According to the bibliographical data OXA-69 was first described in 2004 and belongs to the novel class D carbapenemase (OXA-51/OXA-69), subgroup 3, sharing 56% and <63% amino-acid identity with subgroups 1 and 2, respectively [2]. However, the latter enzymes exhibit relatively weak hydrolytic activities against carbapenems compared with the metallo- -lactamases and it is likely that other combined mechanisms, such as changes in the outer membrane proteins or active efflux mechanism, contribute the most to the observed resistance [8,9]. This is in agreement with the results obtained by other authors who detected isolates with the highest carbapenem MICs (>128/ mg/L) expressing only the *bla* OXA-51-like gene [10]. It is not clear, therefore, whether these resistance genes are acquired or occur naturally in *Acinetobacter* spp [2].

Diffusion of the pulsotype A with OXA-69-type oxacillinases was due to clonal dissemination of multiresistant *A. baumanni*i between two different adult ICUs, probably by hospital staff during medical procedures.

To our knowledge, this is the first molecular characterization of clinical strains producing oxacillinases in Croatia. Similar findings in Argentina, Singapore, Greece and Turkey underscore the progressive emergence of these resistance determinants in different geographic areas [2,10,11]. Our *A. baumanii* isolates were multidrug resistant and posed serious therapeutic problems in our hospital. Since resistance can differ according to geographical location, continuous local monitoring of resistance patterns is necessary to adequately select empirical antimicrobial therapy. Consequently, the incidence and spread of multidrug-resistant *A. baumannii* nosocomial infections suggests the need for a surveillance program and enforcing adequate control measures in different hospital settings.

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