Typing of *Pseudomonas aeruginosa* isolated from patients with VAP in an intensive Care Unit

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SUMMARY

Aim of this study was to characterize isolates of *Pseudomonas aeruginosa* responsible for ventilator-associated pneumonia (VAP) in patients admitted to an ICU in order to evaluate a possible strain clonality.
The study was performed from October 2004 to June 2005 in one Southern Italy ICU and 29 patients suspected of having VAP were enrolled. The etiology of VAP was established by quantitative cultures of endotracheal aspirations. Molecular characterization was carried out by PFGE. *P. aeruginosa* was responsible for 51% of all cases of VAP (15/29) and 12/15 strains were multi-drug resistant. High mortality (44.8%) was connected to this pathogen and evidence of strain clonality was found. The early identification of strain clonality and the application of infection control procedures are necessary to avoid the spread of pathogens such as *P. aeruginosa* involved in nosocomial infections.

KEY WORDS: Intensive Care, Ventilator-associated pneumonia, Pseudomonas aeruginosa, Quantitative endotracheal aspirate, PFGE

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INTRODUCTION

Among nosocomial infections, pneumonia associated with mechanical ventilation (ventilator-associated pneumonia, VAP) is the most common complication for patients admitted to intensive care units (ICU). This infection prolongs the hospital stay, with a consequent increase in hospitalisation costs and contributes to the mortality of ICU patients. The classification of VAPs in early-onset VAP and late-onset VAP is necessary because the causative pathogens differ and the disease is usually less severe and with a better prognosis in early-onset than in late-onset VAP. Several studies have indicated that Gram-negative bacteria are the most common pathogens causing late-onset VAP, and among them, *P. aeruginosa* is the most frequently isolated microorganism (Alp *et al*., 2004; Weber *et al*., 2007; Valles *et al*., 2009).
The primary aim of this work was to use pulsed field gel electrophoresis (PFGE) to characterize *P. aeruginosa* strains isolated from patients with VAP in one ICU in Southern Italy. In addition, the ICU-acquired *P. aeruginosa* infection rate compared to *P. aeruginosa* colonization rate at admission was investigated.

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MATERIALS AND METHODS

Study population
For this prospective study, a total of 29 patients with suspected VAP out of 400 patients admitted between October 2004 and June 2005 to the ICU of "Federico II" University Hospital in Naples (Italy) were enrolled. About 450-500 patients per year are admitted to this ICU, which, at the time of the investigation, was divided into one large

room with 6 beds and 5 two-bed rooms; patients are admitted from any department of the hospital and from the regional emergency system network. All the patients included in this study underwent central venous catheterization and positioning of 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 to the ICU, several biological samples, i.e. urine and lower respiratory tract samples are obtained from all patients for qualitative bacterial and mycete cultures. Patients who are found to be colonized or infected by methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant *Enterococcus spp* or Gram-negative non-fermentative bacteria are isolated in the 5 two-bed rooms available in the ICU.
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 criteria were used for defining nosocomial infections. Therefore, a case of infection was defined as "hospital acquired" if the patient's first positive culture occurred 72 hours or more after admission. Standard CDC definitions were used to differentiate active infection from colonization (Garner *et al*., 1988).

Diagnosis of VAP
VAP was defined as any lower respiratory tract infection that developed after 2 days of mechanical ventilation (MV).
The criteria for clinical suspicion of pneumonia were: the presence of a new, persistent or progressive lung infiltrate on chest radiographs, plus two of the following:
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 the infection developed within the first four days of MV it was considered early-onset VAP, while if the infection developed five or more days after the start of MV it was considered late-onset VAP (Weber *et al*., 2007).

Processing of endotracheal aspirate, quantitative culture of microrganisms and phenotypic analysis
In this study, from the 29 patients suspected of having VAP, endotracheal aspirates were obtained as samples of choice and these underwent quantitative cultures (quantitative endotracheal aspirate, QEA). All endotracheal aspirates were mechanically homogenized with glass beads (1 min), followed by centrifugation (3000 rpm/10 min). Specimens were serially diluted in sterile saline solution (0.9%), at final concentrations of 10^4, 10^5, 10^6 and 10^7. Dilutions were then plated on sheep blood agar, CNA agar, chocolate agar, MacConkey agar, and Sabouroad agar. All plates were incubated overnight at 37°C in the presence of oxygen or in the presence of 5% CO_2 atmosphere. The diagnostic cut-off for QEA was fixed at 10^6 cfu/mL (Wu *et al*., 2002; Jourdain *et al*., 1995).
Strains underwent identification by means of the automated Vitek II system (BioMerieux). Preparation of suspensions, inoculations, incubation times, temperatures and interpretation of reactions were in agreement with the manufacturer's instructions.

Antimicrobial susceptibility testing methods
For the determination of sensitivity of the bacterial strains to antimicrobial agents, a microbroth dilution assay using the Vitek II system was used. Interpretation was in agreement with CLSI criteria (CLSI, 2002). Bacterial strains were considered multi-drug resistant based on the definition given by CDC of 2006 (Siegel *et al*., 2006).

Genotyping by PFGE
DNA fingerprinting was carried out using the method of Grothues (Grouthes *et al*., 1998) for *P. aeruginosa* strains.
Briefly, isolates were grown overnight on nutrient agar and then suspended in SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.5).
The cell suspension (2 McFarland) was mixed with 1.6% low-melting point agarose, molded into plugs at 4°C, and lysed with lysis buffer (1% N-lauryl sarcosine, EDTA 0.5 M, pH 8.00) with the addition of Proteinase K (500 g/mL); the DNA inserts were digested with *SpeI*, according to the supplier's instructions (New England Biolabs). Macro-restriction fragments were separated using CHEF III (Biorad, Inc.) 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. Fragment patterns were compared according to the criteria of Tenover (Tenover *et al.*, 1995).

Statistical analysis

Data were collected by analysis of database case records of all patients enrolled in the study. Statistical analysis was carried out with t-test and χ^2 test. A $p<0.05$ was considered statistically significant. Statistical analysis was performed using the SPSS software package.

RESULTS

Etiology of VAP

Suspected VAPs were confirmed in all 29 patients by microbiological analysis. In particular, in 15 patients (51.7%) the etiological agent was *P. aeruginosa*, while in 14 (48%) other bacteria were isolated i.e. *Enterobacter spp* (5), *Acinetobacter baumannii* (5), *Staphylococcus aureus* (1), *Klebsiella pneumoniae* (1), *Escherichia coli* (1), and *Haemophilus influenzae* (1).

More than half of the VAPs caused by *P. aeruginosa* were identified as late-onset VAP (10/15, 66.6%); the remaining cases were defined as early-onset VAP. The features of patients with early-onset VAP were similar to those of patients with late-onset VAP. Table 1 shows the distribution of

TABLE 1 - *Distribution of microrganisms isolated in patients with early-onset and late-onset VAP.*

Microrganism	*Patients with early-onset VAP*	*Patients with late-onset VAP*
P. aeruginosa	5	10
A. baumannii		5
Enterobacter spp	2	3
S. aureus	1	
K. pneumoniae	1	
E. coli	1	
H. influenzae	1	

TABLE 2 - *Features of patients with VAP caused by P. aeruginosa (group 1) compared to patients with VAP caused by other bacteria (Enterobacter spp, Acinetobacter baumannii, Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, and Haemophilus influenzae; group 2).*

	Group 1	*Group 2*	*P values*
Number of patients	15	14	
Sex (male/female)	7/8	11/3	0.166
Mean age (years)	64.87±10.42	65.50±12.44	0.883
GCS mean value	8.53±5.19	4.93±4.01	<0.05
SOFA mean value	9±2.33	9.71±4.20	0.912
SAPSII mean value	52.93±11.81	60.21±14.86	0.176
Mean LOS in ICU (days)	51.67±46.90	24.14±17.56	<0.05
Mean time of VM (days)	49.27±48.18	22.14±16.88	0.056
Underlying diseases			
Pulmonary disease	10	7	
Neurologic disease	3	2	
Cardiac disease	2	5	
Exitus	13	12	

TABLE 3 - *Results of pulsed-field and chemosusceptibility (MIC values in µg/mL and interpretation) studies for P.aeruginosa strains. Colistin was tested only with the Kirby-Bauer method.*

Strain No	*Pulsed-type*	*VAP-type*	*AMK*	*ATM*	*FEP*	*CAZ*	*CIP/LVX*	*TOB/GEN*	*IMP*	*MEM*	*PIP/TZP*	*COL*
1	A	Late-onset	16 (S)	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>16 (R)	>16 (R)	>16 (R)	>128 (R)	S
2	B1	Late-onset	<2 (S)	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>16 (R)	>16 (R)	>16 (R)	>128 (R)	S
3	B1	Late-onset	<2 (S)	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>16 (R)	>16 (R)	>16 (R)	>128 (R)	S
4	A	Late-onset	16 (S)	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>16 (R)	>16 (R)	>16 (R)	>128 (R)	S
5	B1	Late-onset	<2 (S)	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>16 (R)	>16 (R)	>16 (R)	>128 (R)	S
6	B2	Late-onset	<2 (S)	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>16 (R)	>16 (R)	>16 (R)	>128 (R)	S
7	C1	Early-onset	<2 (S)	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>16 (R)	>16 (R)	>16 (R)	>128 (R)	S
8	A	Late-onset	16 (S)	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>16 (R)	>16 (R)	>16 (R)	>128 (R)	S
9	C2	Early-onset	<2 (S)	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>16 (R)	>16 (R)	>16 (R)	>128 (R)	S
10	D	Late-onset	16 (S)	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>16 (R)	>16 (R)	>16 (R)	>128 (R)	S
11	D	Late-onset	16 (S)	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>16 (R)	>16 (R)	>16 (R)	>128 (R)	S
12	D	Late-onset	16 (S)	>64 (R)	>64 (R)	>64 (R)	>4 (R)	>16 (R)	>16 (R)	>16 (R)	>128 (R)	S
13	E	Early-onset	8 (S)	2 (S)	<1 (S)	<1 (S)	0.5 (S)	<1 (S)	<0.5 (S)	<0.25 (S)	<4 (S)	S
14	F	Early-onset	<2 (S)	4 (S)	2 (S)	<1 (S)	<0.25 (S)	<1 (S)	<0.5 (S)	8 (I)	<4 (S)	S
15	G	Early-onset	<2 (S)	2 (S)	>64 (R)	2 (S)	>4 (R)	>16 (R)	<0.5 (S)	<0.25 (S)	>128 (R)	S

AMK=Amikacin; ATM=Aztreonam; FEP=Cefepime; CAZ=Ceftazidime; CIP=Ciprofloxacin; LVX=Levofloxacin; TOB=Tobramycin; GEN=Gentamicin; IPM=Imipenem; MEM=Meropenem; PIP=Piperacillin; TZP=Piperacillin/tazobactam; COL=Colistin.

microrganisms isolated in patients with early-onset and late-onset VAP. On the basis of the bacterial-screening policy adopted by the ICU, no case of patients colonized by *P. aeruginosa* was found. Patients enrolled were divided into two groups:
Group 1: patients with a diagnosis of VAP by *P. aeruginosa*.
Group 2: patients with a diagnosis of VAP by other microorganisms (*Enterobacter spp*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Haemophilus influenzae*).
The distribution of clinical features in the two groups under study is shown in table 2.

Drug resistance
The system used for the study of chemo-susceptibility showed that all *P. aeruginosa* strains, except strains 13, 14 and 15, were considered multi-drug resistant. Aminoglycosides showed poor activity, except for amikacin. Cefepim and ceftazidime were relatively active against these strains as well as quinolones, carbapenems, piperacillin and piperacillin/tazobactam. Because of this multi-drug resistance, these strains were also tested with colistin: this antibiotic was active *in vitro* and therefore it was considered the drug of choice. Results of the sensitivity study are summarized in table 3.

PFGE typing
According to the interpreting criteria of Tenover for pulsed-type analysis, the 15 strains of *P. aeruginosa* were subdivided into seven different pat-

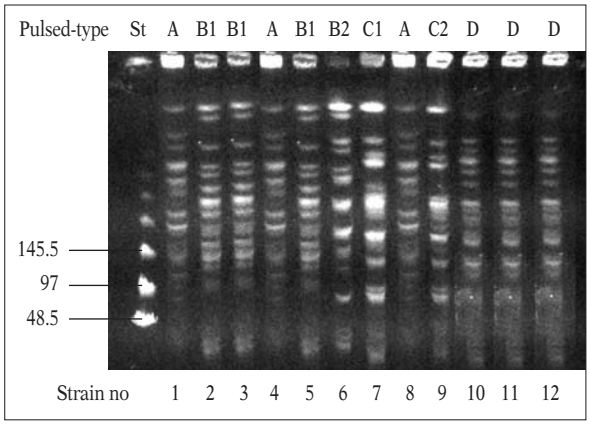

FIGURE 1 - *PFGE fingerprinting of some strains of P. aeruginosa. The numbers indicate the strains under study (data shown in results and table 3). Molecular size markers (a concatemer ladder of lambda phage DNA) were run in lane St. Sizes are indicated in kilobases.*

terns (A, B, C, D, E, F, and G; Fig. 1). Pulsed-type A was found in three strains, considered indistinguishable. Pulsed-type B was found in four closely related strains identified as B1 (three strains) and B2 (one strain). Pulsed-type C was found in two closely related strains, identified as C1 (one strain) and C2 (one strain). Pulsed-type D was found in three strains, considered indistinguishable. The other three pulsed-types (E-F-G) were represented by unique strains. Five strains were responsible for early-onset VAP (strains 7, 9, 13, 14, 15) and of these, only two (7 and 9) were closely related (pulsed-type C1 and C2). Strains responsible for late-onset VAP (1-6, 8, 10-12) were all indistinguishable or closely related (Table 3).
Strains with the same pulsed-type also shared the same antimicrobial-susceptibility profile.

DISCUSSION

Infections by multi-drug resistant bacteria complicate the therapeutic management of patients admitted to ICUs. Data reported in this study indicate that out of 29 episodes of VAP, only one was due to Gram-positive bacteria (MRSA): this high percentage of VAP due to Gram-negative bacilli is in agreement with other reports (Markowicz *et al.*, 2000; Chastre *et al.*, 2002; Weber *et al.*, 2007; De Rosa *et al.*, 2008).
Among Gram-negative bacilli, *P.aeruginosa* (followed by *A. baumannii* and *Enterobacter spp*) was the predominant pathogen (51.7%), as reported in several studies (Foca *et al.*, 2000; Chastre *et al.*, 2002; Valles *et al.*, 2004).
In agreement with other studies (Foca *et al.*, 2000; Valles *et al.*, 2004; Agodi *et al.*, 2007) indicating high rates of *P. aeruginosa*, *Acinetobacter* spp., and multi-resistant Gram-negative bacilli especially in late-onset VAP, and *H. influenzae*, *S. pneumoniae*, *S. aureus*, in early-onset VAP, we found that 10/15 VAP episodes by *P.aeruginosa* were considered late-onset VAP.
No case of *P. aeruginosa* colonized patient was found: so, our results suggest that the ICU personnel/environment served as reservoirs for cross-transmission. This is in agreement with a recent study by Agodi *et al.* (Agodi *et al.*, 2007) indicating higher rates of infection than those of colonization. So, we emphasize the importance of exogenous acquisition of resistant *P. aeruginosa*. It is very interesting to note that, although no colonized patient was found, we observed 5 cases of early-onset VAP by *P. aeruginosa*.
Comparing the parameters of groups 1 and 2, we found statistically significant differences in the GCS parameter ($p<0.05$) and in the mean LOS in the ICU parameter ($p<0.05$). The lack of significant differences between the two groups suggests that VAP infections due to any multi-resistant bacteria may be important regardless of whether *P.aeruginosa* is the etiological agent or not.
Once VAP is suspected, appropriate therapy should be initiated: therefore the role of cultures in assisting antibiotic selection is very important. The etiological diagnosis of VAP is critical for the choice of antibiotic therapy. It is known that the simple qualitative culture of endotracheal aspirates is a technique with a high percentage of false-positive results due to bacterial colonization of the proximal airways. It has been reported that the most reliable technique for this diagnosis is the quantitative culture of bronchoscopic protected specimen brush (PSB) or of broncho-alveolar lavage (BAL). BAL is considered the gold standard for the microbiological diagnosis of VAP but bronchoscopy, being invasive, is associated with complications, in particular when high respiratory support (elevated FiO_2 and PEEP) is needed. Wu *et al.* investigated the diagnostic reliability of quantitative cultures of PSB and BAL in comparison with quantitative endotracheal as-

pirate (QEA), and showed that QEA is characterized by acceptable sensitivity and specificity; it is also a non-invasive and easily repeatable technique (Wu *et al*., 2002).

In our study, QEA was the respiratory specimen of choice. Based on this technique, we recognized 29 cases of VAP, and the diagnosis was also confirmed by the clinical criteria. Using QEA at a cut-off point of 10^6 cfu/mL, we found only monomicrobial VAP infection: this is very important for the management of the chemotherapy of these patients.

In conclusion, to select the microbiological technique, we suggest carrying out this assay as a first step to reduce the number of PSB and BAL to those patients who failed to respond after the first antibiotic treatment.

Twelve out of the fifteen *P.aeruginosa* strains were multi-drug resistant and sensitive only to colistin, that was considered the drug of choice, and amikacin. This high resistance of *P.aeruginosa* is problematic for the therapeutic management of patients, therefore combination therapy was the choice for empiric therapy followed by descalation to monotherapy for definitive treatment. Colistin was administered as monotherapy (3-6000000 UI/die in three administrations) for 10 days, without any severe collateral effect: in particular, no renal failure was recorded.

As concerns the investigation of clonally-related *P.aeruginosa* strains, on the basis of ORION's indications (Stone *et al*., 2007), this part of the study is an outbreak report.

The results of PFGE indicated seven pulsed-types and the evidence of spread in twelve patients. The source of these pathogens is most probably exogenous (ICU personnel and environment) since the results of the bacterial screening-policy showed the absence of colonization for patients that developed VAP by this pathogen.

Our results, documenting a clonal outbreak with extensively-resistant strains and high fatality rates, suggest the adoption of drastic control measures (Siegel *et al*., 2006). Several studies indicate the measurable impacts on the incidence of ICU-acquired infections using infection control practices. Most recently, an Italian study indicated a statistically significant decrease in the incidence of VAP infection after application of an intervention designed to improve respiratory infection control practices in an ICU, showing an incidence rate of 36.9/1000 MV-days for a total of 27 cases of VAP in a period of four months (January-April, 2006) with respect to an incidence rate of 22.5/1000 MV-days for a total of 17 cases of VAP in a period of the same length (May-August 2006) (Prospero *et al*., 2008).

The observations indicated in this work have a strong clinical relevance and should be taken into consideration to adopt control measures in hospital units at high risk of infections, to select a non-invasive technique for diagnosis of VAP, and to address guidelines for empirical antibiotic regimes.

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