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Molecular Epidemiology of *Pseudomonas aeruginosa* Clinical Isolates from Portuguese Central Hospital

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ABSTRACT. The relatedness between clinical isolates of *P. aeruginosa* obtained from patients during their stay in a *Portuguese Central Hospital* was evaluated. Genotypic fingerprinting (M13-PCR), phenotypic methods (biotyping and antibiotyping) and epidemiological information (spatial and temporal links) were used to evaluate the relatedness between 88 clinical isolates (68 patients), selected randomly out of 189. Sixty-two M13 types were found, 12 of them containing isolates from more than one patient. Thirty-four antibiotypes were found, as well as a significant association ($p < 0.05$) between epidemic isolates and multiresistance patterns. The nosocomial transmission of *P. aeruginosa* strains may be limited since M13 typing demonstrated a high degree of diversity among all the isolates, suggesting the occurrence of mainly independent infectious episodes. The results show the possible occurrence of cross-acquisition, cross-colonization and cross-infection and suggest an epidemic population structure for *P. aeruginosa* in this hospital.

Abbreviations

ABT(s)	antibiotype(s)	MDR	multidrug-resistant
ATCC	*American Type Culture Collection*	PFGE	pulse field gel electrophoresis
BS	bronchial secretion	R	resistant
CA	catheter	REF	reference strain
EX	exudate	S	susceptible
HMS	*Harvard Medical School*	UR	urine
ICU	intensive care unit		

Pseudomonas aeruginosa is an opportunistic pathogen recognized as a leading cause of nosocomial infections (Morales *et al*. 2002; Hoštacká *et al.* 2006), which are associated with increased mortality and longer hospital stay mainly because of their high antibiotic resistance profile. In order to understand the source of infections and to recognize the routes of transmission, there is a need to establish clonal relationships between individual isolates, which is accomplished by strain typing techniques (Speijer 2001). Phenotypic methods have been traditionally used but have been substituted by methods with higher discriminatory power, *i.e*., genotypic methods (Pirnay *et al*. 2002). Nevertheless, phenotypic methods, such as antibiotic susceptibility typing, can produce important epidemiological additional data, although they can not confirm clusters of nosocomial cross infections (Bergmans *et al*. 1997).

Production of pigments can be considered biotyping because of the variability of response, thus enabling different profiles (Hunter *et al*. 1989). Different molecular techniques, such as PFGE and DNA probe for exotoxin A, can be used to study the molecular epidemiology of bacteria (Nogueira 1995; Speijer *et al*. 1999). Although PFGE is the "gold standard" typing method for *P. aeruginosa* (Bertrand *et al*. 2001), it is expensive, complex and requires prolonged turnaround times for obtaining results. Alternative methods that use stringent conditions and therefore are easily standardized (Olive 1999) can be suitable options. PCR fingerprinting technique (M13-PCR) has been shown to combine maximum discrimination power, stability, reproducibility and type ability with cost-effective use of operator time and reagents, thus being an important tool in routine epidemiological surveillance (Brízio *et al*. 2006*a*; Buchholz *et al*. 1995; Elaichouni *et al*. 1994); it has also been used to study genetic relatedness in other species (*cf*. Brízio *et al*. 2006*b*; Grundmann *et al*. 1997).

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The aim of the present work was to gain insight in the epidemiology of *P. aeruginosa* at a large teaching affiliated *Portuguese Central Hospital* (*Hospital S. João*), namely sources of infections and routes of transmission, by using genotypic fingerprinting (M13-PCR), phenotypic methods (biotyping and antibiotyping) and epidemiological information (spatial and temporal links).

MATERIALS AND METHODS

Strains and growth conditions. *Hospital S. João* is a 1200-bed teaching (*Faculty of Medicine*) affiliated hospital in Porto. Ninety-six isolates (88 clinical, 8 reference) were used. The clinical isolates were selected randomly out of 189 isolates from patients admitted to this hospital (Dec 2001–Dec 2002): 51 from BS, 14 from UR, 13 from CA and 10 from EX, each isolate being obtained from a different infection case. The reference set was constituted by five clinical isolates from different sources (REF 94 and REF 53 from urine, REF 48 and REF 51 from blood and REF 43 from cystic fibrosis) obtained from S. Lory (HMS), as well as three reference strains, PAO1 (REF 74) (S. Lory, HMS), AK1 (REF 96) (H.C. van der Mei, *Groningen University*) and ATCC 27853^T (REF 81). Bacteria were stored at –70 °C in brain-heart infusion medium (*Merck*, Germany) with 20 % glycerol. All isolates were biochemically identified by Vitek auto microbic system (*bioMérieux*) and growth characterization on cetrimide agar (*BioRad*, Portugal) (Kiska *et al*. 1999). For analysis of genotypic and phenotypic parameters, isolates were first transferred from stock cultures onto Luria–Bertani agar (*Difco*) plates and incubated at 37 °C. These fresh cultures were subsequently used for phenotypic assays and DNA extraction.

Phenotypic characterization. Pyocyanin and pyoverdine assay: isolates were analyzed for production of pyoverdine by incubation for 18–24 h in King's B agar, for production of pyocyanin by incubation for 18–24 h in King's A agar (both *BioRad*, Portugal) (King *et al*. 1954; Huston *et al*. 2004). The results were assessed as production of pigment (+) and no production (–) and strains were grouped in four biotypes:

production of pyocyanin and pyoverdine (biotype *1*),
no production of pigments (biotype *2*),
no production of pyocyanin, production of pyoverdine (biotype *3*),
production of pyocyanin, but not pyoverdine (biotype *4*).

For antimicrobial susceptibility the strains were tested at the hospital microbiology laboratory using its routine automated system according to procedures and reporting protocols recommended by the manufacturers' (*bioMérieux*). The *Pseudomonas* susceptibility cards (GNS-PA) were loaded and filled in accordance with manufacturer's instructions. The antimicrobial agents tested included amikacin, gentamicin, piperacillin/tazobactam, ceftazidime, imipenem, ciprofloxacin, aztreonam and ticarcillin/clavulanic acid. The ATCC 27853^T strain was incorporated as control strain.

Genotypic characterization by M13-PCR. Genomic DNA was isolated using a Puregene DNA Isolation Kit (*Gentra Systems*, USA) following the protocol for G$^-$ bacteria. M13-PCR fingerprinting using the "core sequence" from M13 bacteriophage (Huey *et al*. 1989; Elaichouni *et al*. 1994; Buchholz *et al*. 1995; Brízio *et al*. 2006*a*) was applied to characterize the *P. aeruginosa* isolates. M13 primer (5′-GAG GGT GGC GGT TCT-3′) was used in PCR amplifications after bacterial DNA extraction, by applying the following PCR amplification program in a thermocycler (Robocycler 96; *Stratagene*): 5 min 94 °C, 40 cycles (1 min 94 °C, 1 min 50 °C, 2 min 72 °C), and the last cycle (6 min 72 °C). All PCR reactions were performed in 25 μL using 40 ng of template DNA, 1 mmol/L of primer, 0.2 mmol/L of dNTPs, 1 U of Taq DNA polymerase, 1× PCR buffer and 3 mmol/L of $MgCl_2$ (all *Gibco*).

The resulting amplified DNA fragments were electrophoresed through agarose gels (1 %, *W*/*V*) in 0.5× TBE buffer (mmol/L: Tris 45, boric acid 45, EDTA 0.5), stained with ethidium bromide (2.5 mg/L) and visualized using UV light. Digital images were obtained using *Kodak* 1D 2.0 software.

Data analysis. (*i*) Data from the antimicrobial susceptibility testing were analyzed by the χ^2 method. Significance was defined as $p \leq 0.05$. (*ii*) The BioNumerics software (*Applied Maths*) was used to perform the densitometric analysis of M13-PCR fingerprints and to generate a phenogram to evaluate relationships among strains. Pearson's correlation coefficient was used as a similarity measure and agglomeration was based on unweighted pair group method with arithmetic average. The criteria for related clones for all assays was taken as profiles with ≥90 % similar bands.

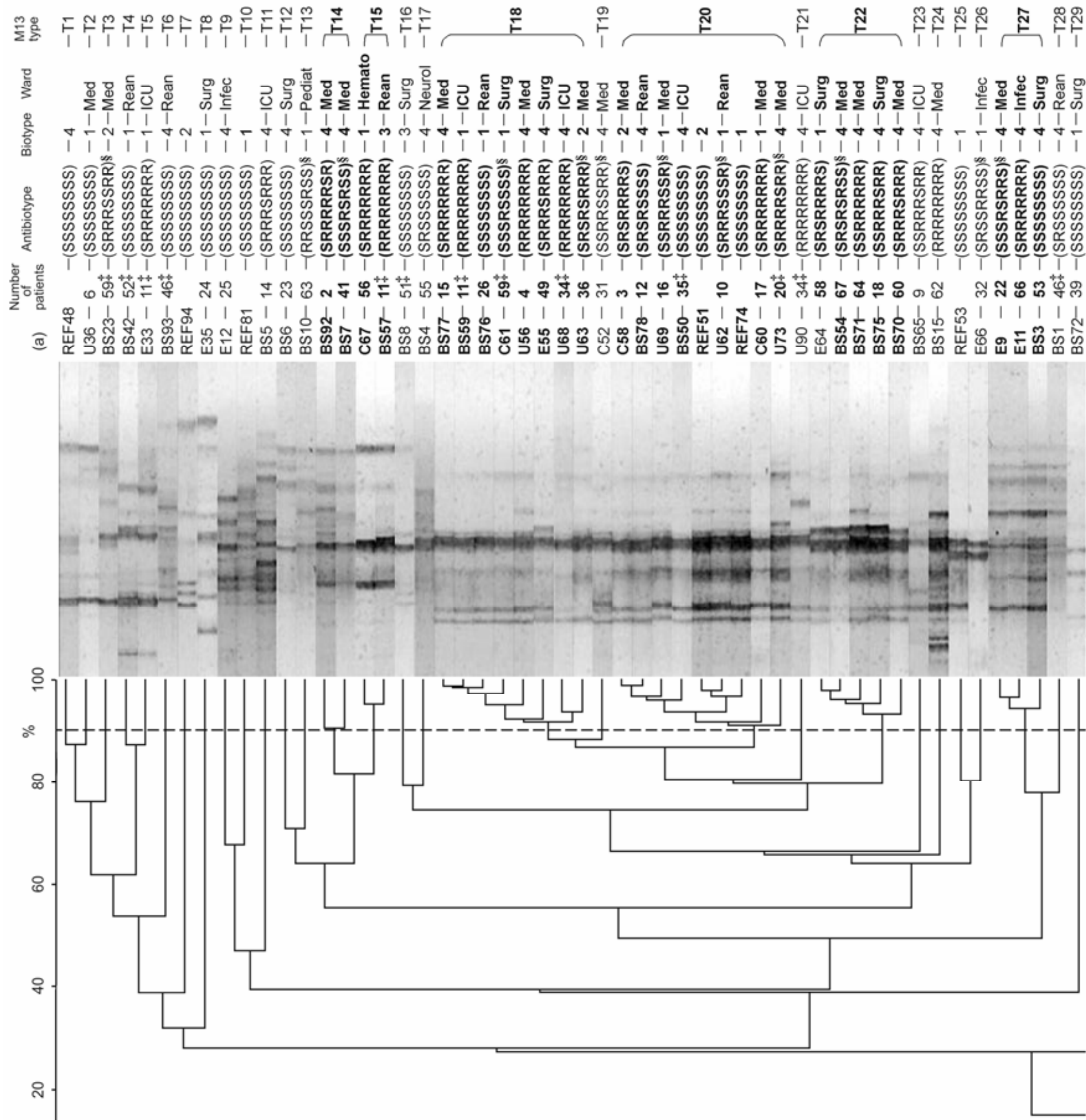
RESULTS

Fifteen % of the isolates were obtained from CA, 11 from EX, 58 from BS and 16 % from UR. M13-PCR fingerprint, ABTs and biotypes were also determined for this set of isolates (*see Materials and Methods*).

Given that PFGE is considered to be the "gold standard" typing method for *P. aeruginosa*, a group of 16 isolates (epidemic and with diverse similarity values by M13 fingerprinting) were submitted to macro-restriction with *Xba*I and resolved by PFGE (*data not shown*). The concordance between PFGE and M13-PCR was evaluated visually by comparing the groupings obtained by both techniques. Eleven out of 16 strains have concordance between the two typing methods. Moreover, from the 10 epidemic strains obtained with M13-PCR seven were also considered epidemic with PFGE.

Fig. 1 shows the genomic relationships assessed by M13-PCR fingerprinting and the phenotypic characteristics among clinical isolates and 8 reference strains. A total of 62 different M13 types (similarity >90 %) were identified; 12 of them (T14, T15, T18, T20, T22, T27, T32, T38, T43, T48, T58, T61) containing isolates from more than one patient (epidemic strains; for their antimicrobial resistance *see* Table I).

Patients of types T14, T18, T20, T22, T43 and T48, belonging to the same ward or unit, had a maximum time lapse between dates of admission of 27 d, and patients of types T15, T18, T20, T22, T27, T32, T38 and T58 (belonging to different wards or units) had a maximum time lapse of three months. The two patients of type T61 had a time lapse of 1 d.



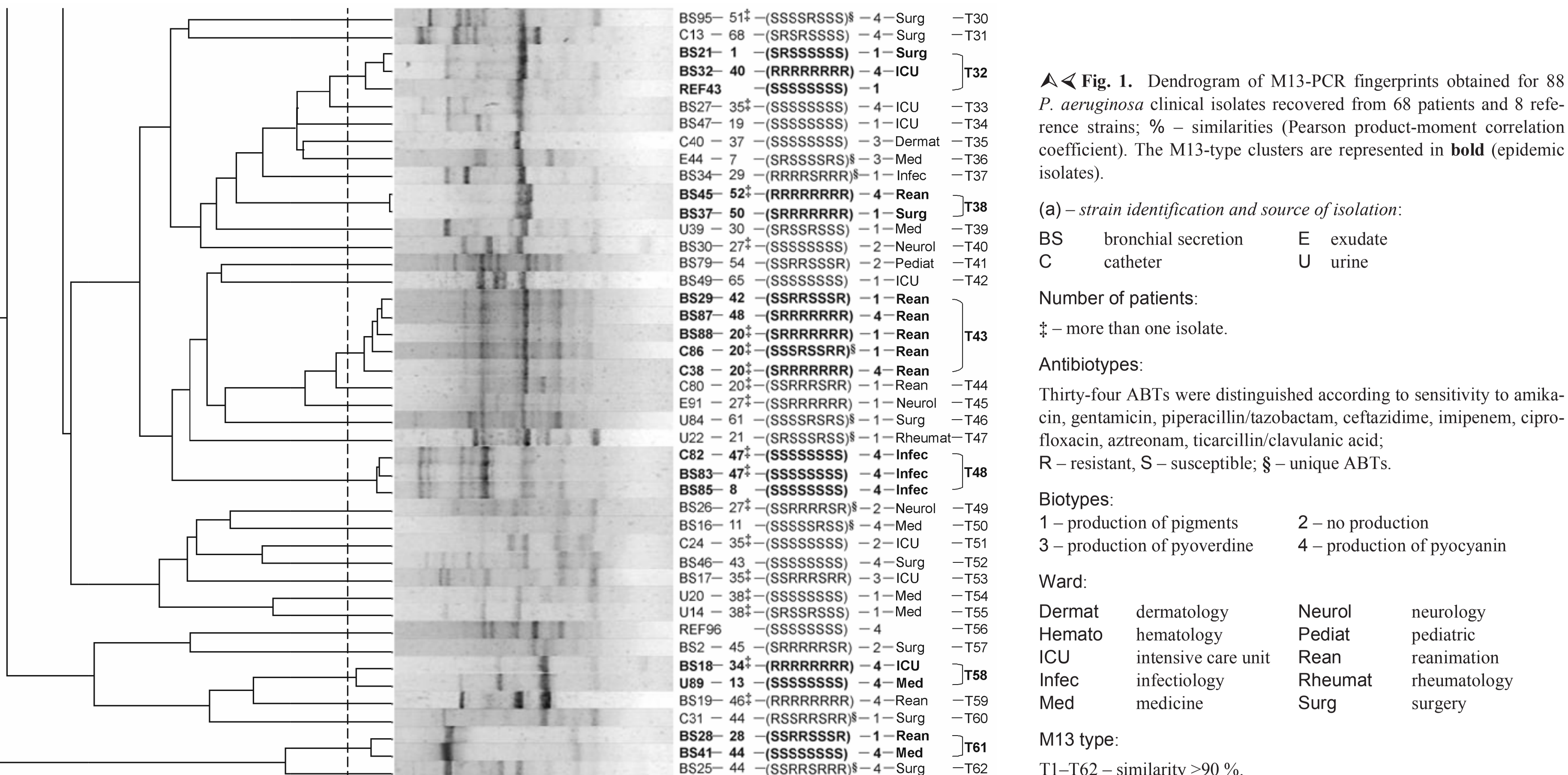


Fig. 1. Dendrogram of M13-PCR fingerprints obtained for 88 *P. aeruginosa* clinical isolates recovered from 68 patients and 8 reference strains; % – similarities (Pearson product-moment correlation coefficient). The M13-type clusters are represented in **bold** (epidemic isolates).

(a) – *strain identification and source of isolation*:

BS	bronchial secretion	E	exudate
C	catheter	U	urine

Number of patients:

‡ – more than one isolate.

Antibiotypes:

Thirty-four ABTs were distinguished according to sensitivity to amikacin, gentamicin, piperacillin/tazobactam, ceftazidime, imipenem, ciprofloxacin, aztreonam, ticarcillin/clavulanic acid;
R – resistant, S – susceptible; § – unique ABTs.

Biotypes:

1 – production of pigments	2 – no production
3 – production of pyoverdine	4 – production of pyocyanin

Ward:

Dermat	dermatology	Neurol	neurology
Hemato	hematology	Pediat	pediatric
ICU	intensive care unit	Rean	reanimation
Infec	infectiology	Rheumat	rheumatology
Med	medicine	Surg	surgery

M13 type:

T1–T62 – similarity >90 %.

Thirty-four resistance patterns were obtained allowing the establishment of 12 major ABTs (with 2–24 strains) and 22 unique profiles (Table II). Of the seven MDR epidemic isolates, four were collected in the ICU (57 %).

Table I. Antimicrobial resistance of the 88 clinical isolates

Antibiotic	Number of isolates (%)
Ceftazidime	52 (59)
Ticarcillin/clavulanic acid Gentamicin	43 (49)
Aztreonam Piperacillin/tazobactam	41 (47)
Ciprofloxacin Imipenem	39 (44)
Amikacin	13 (15)

Generally, isolates with identical ABTs belong to different M13 types and many isolates that are unrelated on the basis of ABTs had identical M13 types. Five of the 11 patients with >1 isolate had isolates with identical ABTs (Fig. 1). Isolates belonging to six types (T15, T18, T20, T22, T27, T58) were collected from different sources of isolation and different wards.

Production of pyoverdine (biotypes *1* and *3*) was observed in 37 isolates and pyocyanin (biotypes *1* and *4*) in 75 of 88 isolates, although isolates that produce pyocyanin, but not pyoverdine (biotype *4*) were the most frequent (49 %). From the epidemic isolates 95 % produced either one or both of the pigments, comparing to 87 % of the non-epidemic isolates (all the single clusters of Fig. 1).

The antibiotic resistance of the epidemic and non-epidemic isolates was also compared. Forty-nine % (43 out of 88) were epidemic isolates (T14, T15, T18, T20, T22, T27, T32, T38, T43, T48, T58, T61) in contrast to 51 % (45 out of 88) of non-epidemic isolates (T1–T13, T16, T17, T19, T21, T23–26, T28–T31, T33–T37, T39–T42, T44–T47, T49–T57, T59, T60, T62). Thirty-five % of the epidemic isolates were resistant to all the antibiotics or with the exception to amikacin whereas 47 % of the non-epidemic isolates were susceptible to all the antibiotics tested. The results of the χ^2 test showed a significant difference ($p \leq 0.05$) in resistance between epidemic and non-epidemic isolates for all the antibiotics, except amikacin and imipenem. Moreover, there was no significant difference ($p > 0.05$) in the source of isolation between epidemic and non-epidemic isolates.

Most M13 types contain strains from different sources. The variability within each ward of the hospital was nearly like within the whole population.

Table II. Resistance patterns (ABT[a]) for the 88 clinical isolates

Antibiotic[b]								Number of isolates (%)
Ami	Gen	Pip/Taz	Cef	Imi	Cip	Azt	Tic/Cla	
S	S	S	S	S	S	S	S	24 (27)
R	R	R	R	R	R	R	R	10 (11)
S	R	R	R	R	R	R	R	9 (10)
S	R	R	R	S	R	R	R	3 (3)
S	R	R	R	R	R	S	R	2 (2)
S	R	S	S	S	S	S	S	3 (3)
S	R	S	S	R	S	S	S	2 (2)
S	S	R	R	R	S	R	R	4 (5)
S	S	R	R	S	S	S	R	3 (3)
S	R	S	R	S	S	S	S	2 (2)
S	S	R	R	R	R	R	R	2 (2)
S	R	S	R	R	R	R	S	2 (2)

[a]S – susceptible, R – resistant.
[b]Ami amikacin; Azt aztreonam; Cef ceftazidime; Cip ciprofloxacin; Cla clavulanic acid; Gen gentamicin; Imi imipenem; Pip piperacillin; Taz tazobactam; Tic ticarcillin

DISCUSSION

P. aeruginosa is an important cause of nosocomial infections and this may result from its ability to colonize abiotic surfaces for prolonged periods of time (Grundmann *et* al. 1993; Černohorská and Votava 2008). There is only one report (Nogueira 1995) of a study employing molecular typing of *P. aeruginosa* in this large and teaching affiliated *Portuguese Central Hospital*.

The control of hospital infections through any microorganism is strongly impaired in the absence of knowledge about local epidemiological patterns (Morales *et al*. 2004; Nogueira 1995).

We found that 51 % of the patients were colonized or infected with isolates with unique genotypes. These patients may have been colonized *via* an endogenous source, and thus the isolates could not be detected on admission (Speijer *et al*. 1999). Spatial location (the same ward) and temporal relation (date of admission) could explain transmission of the same M13 type isolates between patients. We can state that there was transmission from patient to patient in the same ward (T14, T18, T20, T22, T43, T48) for a period of 27 d, which can be explained by survival of the *P. aeruginosa* in the same ward. Transmission of types T15, T18, T20, T22, T27, T32, T38 and T58 between patients from different wards could be explained by their survival *via* hospital staff, several hospital devices or from the environment and, consequently, during 1–3 months in a ward in order to colonize or infect another patient.

However, considering studies over a long time in a large set of bacteria, there are limits for the use of genotyping methods that make epidemiological relatedness unlikely (Tenover *et al*. 1995). Transmission of the T61-type isolates between two patients could be explained by survival of the *P. aeruginosa* on the hands or surfaces for one day (Jalaluddin *et al*. 1998). The isolation of the same M13 type from a catheter and bronchial secretions may suggest that there is a need for more control and care in manipulating invasive devices by hospital staff.

The DNA typing using M13-PCR is a discriminatory tool suitable for routine epidemiological studies, namely for the initial screening of large numbers of *Pseudomonas* strains. DNA-Macro restriction analysis could be used as confirmatory or for a more accurate answer to epidemiological questions on selected strains. With M13-PCR the detection of a different PCR pattern can lead to the conclusion that the isolates belong to a different clonal type (Fonseca *et al*. 2007). Moreover, with this technique, it was also possible to distinguish different *P. aeruginosa* genotypes with a similarity >90 %, pointing to its potential use in clinical settings to recognize epidemic *P. aeruginosa* clones over the short term. There is a sufficient justification to suggest that *P. aeruginosa* displays an epidemic population structure in this hospital (*see* also Pirnay *et al*. 2002; Fonseca *et al*. 2004).

In this work there are identical strains (T27, T32, T58) with absolutely opposite antibiotic susceptibility profiles; this may be due to the horizontal transfer of the resistance genes or possible induction of chromosomal β-lactamases. This opposite antibiotic susceptibility profile can be also due to the known errors (false positives and/or false negatives) associated with the use of Vitek to obtain *P. aeruginosa* antibiotic susceptibility profile (Juretschko *et al*. 2007). Nevertheless, by using routine molecular epidemiology tools, such as M13-PCR fingerprinting, it is also possible to detect eventual antibiotic susceptibility profile errors.

The nosocomial transmission of *P. aeruginosa* strains and cross-infection may be limited since M13 typing demonstrated a high degree of diversity among all the isolates tested, suggesting the occurrence of mainly independent infectious episodes. However, since there were six genomic types representing 27 isolates from both different wards and sources, the occurrence of cross-acquisition, cross-colonization and cross-infection is probable. Nevertheless, isolates belonging to the same clonal type can also be an independent acquisition of strains from diverse sources due to the fact that the majority of clinical relevant clones are in fact, highly successful clonal complexes which are widespread in the environment (Pirnay *et al*. 2005; Wiehlmann *et al*. 2007). This is the possible explanation for the inclusion of PAO1 in M13 type T20 and for the inclusion of other reference strains (REF 43 and REF 51) that are grouped within the detected epidemic clones. As a consequence of this, anti-nosocomial strategies should take into account the difficulty of a clear distinction between environmental and nosocomial routes of *P. aeruginosa*. The implementation of prophylactic measures, based on routine epidemiological surveillance data and on the reinforcement of education of hospital staff, is therefore a priority.

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