GENOTYPING OF DIFFERENT *PSEUDOMONAS AERUGINOSA* MORPHOTYPES ARISING FROM THE LOWER RESPIRATORY TRACT OF A PATIENT TAKEN TO AN INTENSIVE CARE UNIT

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***Pseudomonas aeruginosa* is an opportunistic pathogen and an ubiquitous environmental bacterium. Fifty-seven days after hospitalization, we isolated three distinct *P. aeruginosa* morphotypes (smooth, rough and mucoid) from the lower respiratory tract of a patient admitted to a Cardiology Intensive Care Unit (ICU). Moreover, a group of nine colony variants, arising from the three *P. aeruginosa* isolates growing in laboratory growth media, were also isolated. The resulting 12 isolates were characterised for antibiotic resistance profile and subjected to genotypic analysis by fluorescent-Amplified Fragment Length Polymorphism (f-AFLP) and automated repetitive extragenic palindromic-PCR (rep-PCR) fingerprinting. The three smooth, rough and mucoid morphotypes presented different antibiotic resistance profiles and genotyping analysis showed that they belonged to distinct clones, indicating that at day 57 after the admission the patient was simultaneously colonized by three distinct *P. aeruginosa* isolates. On the other hand, the nine colony variants presented heterogeneous antibiotic resistance profiles and clustered together with the three parental isolates. The understanding of the link between genotype plasticity and antibiotic resistance may contribute to improving our knowledge of this life-threatening pathogen.**

Members of the species *Pseudomonas aeruginosa* are found in large numbers in many ecological niches of natural environments as well as in infections of humans, animals and plants. This opportunistic pathogen is an important causative agent of nosocomial airway infections, particularly for immune-compromised individuals who are hospitalized and have severe underlying diseases, where it is a major cause of infectious-related mortality principally because of its ability to grow as biofilm and of its high antibiotic-resistance (1-4). Recent reports indicate that distinct strains of *P. aeruginosa* may be isolated at the same time within the same patient and that colony morphology variants (phenotypic variability) of *P. aeruginosa* may also arise from acute and chronic lower respiratory tract infections of the human host (5-9).

Since genome sequences of different *P. aeruginosa*

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strains of human and environmental origin have become available, *in silico* analysis showed that *P. aeruginosa* is endowed with high genetic plasticity and that strains able to cause human infections have acquired genomic islands in order to increase their fitness within susceptible hosts (10-17).

In cystic fibrosis (CF) patients, a distinguishing microbiological feature of *P. aeruginosa*, which chronically colonizes the lungs of these patients, is the ability ("hypermutable phenotype") of the persisting clone to segregate different colony variants endowed with different antibiotic resistance and virulence profiles (5-6, 18). Moreover, the isolation of these variants also in non-CF patients hospitalized in ICUs, is becoming an emerging threat (4, 11).

In this paper we describe a case of a pulmonary bacterial colonization of a non-CF patient admitted in a cardiology ICU and colonized by three different variants of *P. aeruginosa*. Namely, at day 57 after the admission, three different *P. aeruginosa* isolates presenting different colony morphologies (smooth, S; rough, R; and mucoid, M) were isolated from a bronchoalveolar lavage (BAL) fluid. Furthermore, on solid growth medium, each of the three different morphotypes segregated colony variants different from those of parental strains and a total of nine colonies were isolated. To assess genetic relatedness of the strains (the three parental and the nine different morphotypes), we evaluated the antibiotic resistance profiles of the twelve isolates together with a genotypic fingerprinting analysis using conventional fluorescent-Amplified Fragment Length Polymorphism (f-AFLP) and repetitive-sequence-based PCR (rep-PCR) methodologies (19-21).

MATERIALS AND METHODS

In July 2006, a 73-year-old male was admitted to the Emergency Department of the Sant'Andrea Hospital ("Sapienza" University of Rome, Rome, Italy) with dyspnoea and symptoms related to *angina pectoris* and was consequently taken to a Cardiology ICU. The patient had a history of type II *diabetes mellitus*, dilated cardiomyopathy and left ventricular dysfunction. Upon admission, the patient underwent coronariography and doppler echocardiography examinations which revealed the presence of a stenosis of 50% of right coronary. Consequently, the patient underwent angioplastic stent surgery. Bronchoalveolar lavage (BAL) fluids, sputum, urine, wound swabs were cultured to detect growth of aerobic and/or anaerobic microrganisms. Blood samples were evaluated for the presence of bacteria by using the Bactec System (Becton Dickinson). *P. aeruginosa* identification and antimicrobial susceptibility tests were performed by using Vitek 2.0 system with GN and AST-N022 cards, respectively (BioMerieux). Susceptibility profiles of *P. aeruginosa* isolates were evaluated by the Advance Expert System of Vitek 2 instrument according to Clinical and Laboratory Standards Institute (CLSI) notes released on 2007.

P. aeruginosa isolates were streaked on tryptic soy agar plates supplemented with 5% sheep blood. Well-isolated colonies were used for direct DNA extraction using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories), as recommended by the manufacturer. Genotyping of *P. aeruginosa* isolates was performed by using both f-AFLP Microbial fingerprinting (Applied Biosystems) and DiversiLab *Pseudomonas* rep-PCR (BioMerieux) kits, following manufacturer instructions. f-AFLP fingerprinting was performed using T4 DNA ligase, *Eco*RI and *Mse*I restriction enzymes (New England BioLabs), and *P. aeruginosa* specific primers *Eco*RI-0 (carboxyfluorescein labelled) and *Mse*I-C, as described (21). f-AFLP profiles were resolved by a ABI3130/XL automated fluorescence genetic analyzer (Applied Biosystems). Normalization and fragment sizing were carried out using the GeneScan-500 ROX size standard (Applied Biosystems). f-AFLP profiles were analyzed by using BioNumerics ver 5.00 (Applied Maths) software. Only DNA fragments in the range of 80-450 bp were considered. Similarity was calculated using product-moment Pearson correlation coefficient for banding pattern interpretation. Relationship amongst groups was displayed by a dendrogram based on Unweighted Pair Group Method with Arithmetic mean (UPGMA) elaboration. A threshold of 95% similarity was used to assess clustering.

For what it concerns rep-PCR fingerprinting, separation of amplified fragments and profile analysis were carried out using the DiversiLab technological platform (BioMerieux) which consists of an Agilent 2100 bioanalyzer (Agilent Technologies) and the DiversiLab web-based software (v. 2.1.66). The distance matrix and the clustering analysis were based on Kullback-Leibler correlation coefficient and UPGMA method, respectively. The threshold was fixed at 97% similarity, as described (19-20).

RESULTS

At day 57 after the admission, three distinct *P. aeruginosa* isolates presenting smooth, rough and mucoid morphotypes (S, R and M, respectively),

Table I. *Antibiotic resistance profiles and f-AFLP and rep-PCR genotype features of the twelve P. aeruginosa strains isolated at day 57 after admission.*

P. aeruginosa isolate	Colony morphology (texture, elevation, edge)	Antibiotic resistance profiles [a]												f-AFLP [b]	Rep-PCR [c]
		AMK	CIP	ATM	FEP	CAZ	GM	IPM	MEM	MEZ	NET	PIP	TZP		
S	smooth, convex, entire	R	S	R	R	R	I	R	R	R	I	R	R	2	1
S1	smooth, flat, entire	R	S	R	R	R	I	R	R	R	I	R	R	2	1
S2	smooth, convex, undulate	S	S	R	R	R	I	R	R	R	I	R	R	2	1
S3	smooth, raised, undulate	R	S	R	R	R	I	R	R	R	I	R	R	2	1
R	rough, raised, undulate	I	S	S	S	I	S	R	R	R	I	R	R	1	2
R1	rough, convex, irregular	S	S	R	R	R	I	R	R	R	I	R	R	1	3
M	mucoid, convex, irregular	S	S	S	S	S	S	S	S	S	S	S	S	3	3
M1	mucoid, flat, entire	S	S	R	R	R	I	R	R	R	I	R	R	3	3
M2	mucoid, raised, undulate	I	S	S	S	I	S	I	I	S	I	S	S	3	3
M3	mucoid, raised, irregular	I	S	S	S	I	S	R	R	R	I	R	R	3	3
M4	mucoid, flat, irregular	S	S	R	R	R	I	R	R	R	I	R	R	3	3
M5	mucoid, convex, entire	S	S	S	S	S	S	S	S	S	S	S	S	3	3

[a] *AMK, amikacin; CIP, ciprofloxacin; ATM, aztreonam; FEP, cefepime; CAZ, ceftazidime; GM, gentamicin; IPM, imipenem; MEM, meropenem; MEZ, mezlociclin; NET, netilmicin; PIP, piperacillin; TZP, piperacillin/tazobactam; S, susceptible; I, intermediate; R resistant.*

[b] *f-AFLP, Fluorescent-Amplified Fragment Length Polymorphism fingerprinting clusters*

[c] *rep-PCR, repetitive extragenic palindromic-PCR fingerprinting clusters*

were isolated from the lower respiratory tract of the 73-year old patient taken to the Cardiology ICU of the "Sant'Andrea" Hospital (Table I). The S, R and M isolates showed three different susceptibility profiles, particularly the S and R isolates resulted susceptible to 1 and 4 antibiotics, respectively, while the M isolate was susceptible to the entire 12 antibiotic panel (Table I). A retrospective analysis revealed that during the course of hospitalization the lower respiratory tract of the patient was colonized by *P. aeruginosa* during the first days of hospitalization (BAL fluids were positive for *P. aeruginosa* at day 12 and 42 after internalization; Fig. 1). Interestingly, the S isolate, but not the R and M isolates, showed the same antibiotic resistance profile of the *P. aeruginosa* isolates collected at days 12 and 42 (data not shown), indicating that probably the *P. aeruginosa* isolate with an S morphotype colonized the patient before day 57.

Therefore, a detailed analysis of S, R and M *P. aeruginosa* isolates was undertaken. On blood agar plates, the S, R and M isolates generated colony variants presenting different phenotypic traits (S-, R- and M-like variants; Table I). Namely, the S isolate generated three different S-like colony variants (S1 to S3); the R isolate one R-like variant (R1); and the M isolate five M-like variants (M1 to M5) (Table I). Regarding the antibiotic resistance profile, the S-, R- and M-like variants presented a certain degree of heterogeneity, if compared to parental isolates. Namely, the S-like variants S1 and S3 presented the same antibiotic resistance profile of parental S primary isolate, while S2 was susceptible to amikacin; the R1 variant presented an antibiotic resistance profile identical to the S2 variant and significantly different from that of parental R isolate; the M-like variants M2 and M5 were highly susceptible, as the parental M isolate, while M1 and M4 isolates showed an antibiotic resistance profile similar to S2 and M3 identical to R (Table I). All the isolates were susceptible to ciprofloxacin. These results likely indicate genetic variability among isolates upon cultivation on blood-agar plates. To assess the genetic relatedness of the twelve *P. aeruginosa* isolates (S and S1 to S3; R and R1; and M and M1 to M5), a fingerprinting analysis was performed using f-AFLP and a new commercial rep-PCR genotyping system (see Materials and Methods for details). The f-AFLP and rep-PCR fingerprinting analysis showed that the S, M and R isolates clustered into three distinct genetic clusters (Table I and Figs. 2 and 3), indicating that the patient was simultaneously colonized by three distinct strains of *P. aeruginosa*.

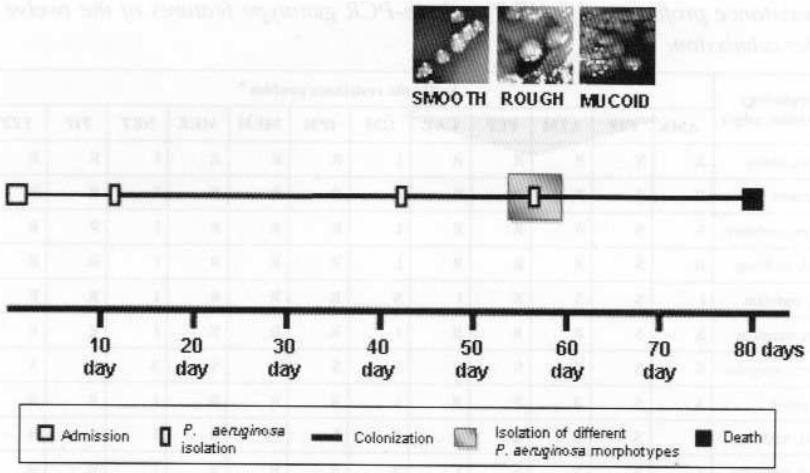

Fig. 1. *Time course of P. aeruginosa colonization as evidenced by the clinical history of the patient. BAL fluid samples were collected at days 12, 42 and 57 after the admission (white rectangles). The grey square indicates the simultaneous appearance of the three P. aeruginosa morphotypes (smooth, rough and mucoid) at day 57. The patient died at day 80.*

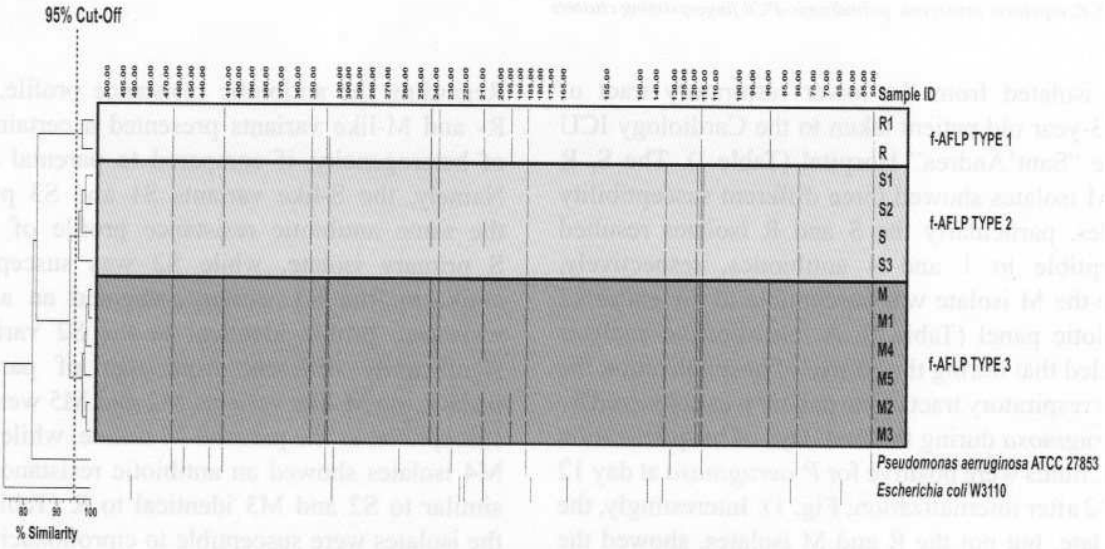

Fig. 2. *Normalized f-AFLP profiles and relative dendrogram of the twelve P. aeruginosa isolates. Scale bar (shown on the bottom left) indicates the percentage of genetic similarity, while the vertical dotted line represent the Pearson cut-off value (95%) used to group similar profiles (according to the differentiation criteria proposed by Speijer et al., 1999). Molecular sizes (Kb) are shown on the top. The grey rectangles indicate the three different fingerprinting clusters. P. aeruginosa strain ATCC 27853 and Escherichia coli strain W3110 were used as controls.*

f-AFLP analysis of the S1 to S3, R1 and M1 to M5 colony variants showed that, independently of their antibiotic resistance profiles, they cluster together with their primary S, R and M isolates (f-AFLP TYPE 1 to 3) (Fig. 2 and Table I). The results obtained with rep-PCR were consistent with those obtained with the f-AFLP fingerprinting analysis with the exception of the R1 isolate which clusters within the group of M-like variants M1 to M5 (rep-PCR TYPE 3), instead of the parental R isolate (rep-

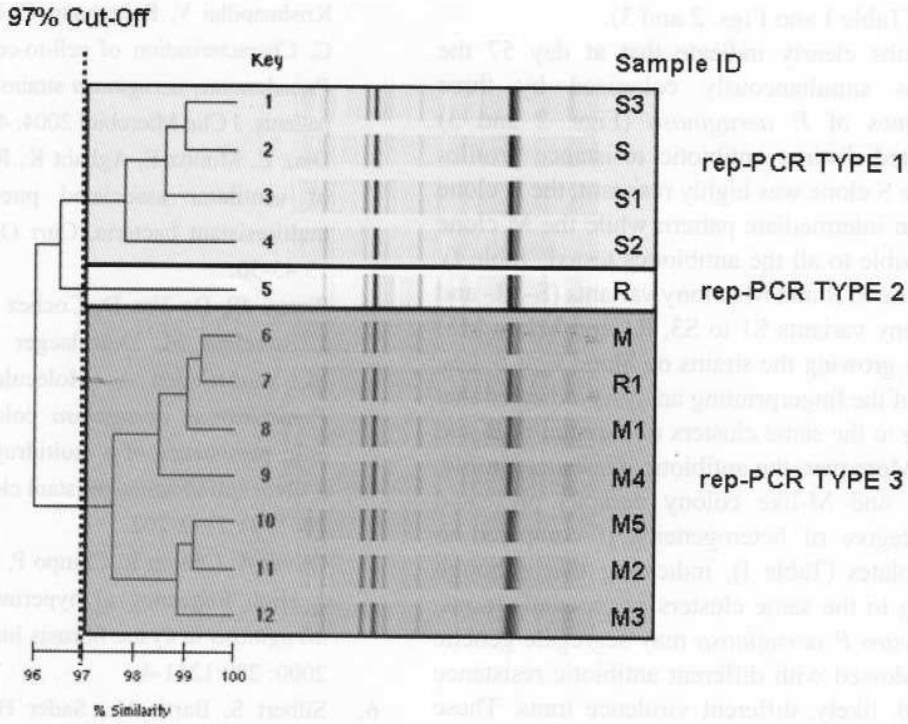

Fig. 3. *Normalized Rep-PCR fingerprinting patterns and dendrogram of the twelve P. aeruginosa isolates. Scale bar (shown on the bottom left) indicates the percentage of genetic similarity, while the vertical dotted line represents the Kullback-Leibler cut-off value (97%) used to group similar rep-PCR profiles (Harrington et al., 2007; Ross et al., 2005). The grey rectangles indicates the three different fingerprinting clusters.*

PCR TYPE 2) (Fig. 3 and Table I).

These results indicate that, although some minor discrepancies between the antibiotic resistance profiles and the f-AFLP and the rep-PCR fingerprinting analysis, the S-, R- and M-like colony variants have most probably originated from parental S, R and M parental isolates.

DISCUSSION

Although most patients, once colonized with *P. aeruginosa,* tend to harbour a conserved clone during the course of the disease, the lower respiratory tract of chronically infected patients (cystic fibrosis patients, ventilated patients taken to ICUs, etc.) may also harbour distinct strains and/or variants of the original clones, which may present different virulence and/or antibiotic resistance profiles, as well as minor differences when subjected by genome fingerprinting analysis (4, 6-7, 22-24). The insurgence of variants mainly depends on the genetic variability of *P. aeruginosa* and on the different type of environmental pressure (for instance, antibiotic therapy) (4, 22).

Our report depicts a case of a patient admitted to the Cardiology ICU of the Sant'Andrea Hospital, "Sapienza" University of Rome, Rome, Italy, who, at day 57 after admission, presented the lower respiratory tract simultaneously infected by three *P. aeruginosa* isolates showing smooth, rough or mucoid (S, R and M. respectively) morphotypes. To evaluate whether the three morphotypes originated from the same strain or were distinct clones, the antibiotic resistance profiles and the genetic relatedness (f-AFLP and rep-PCR fingerprinting analysis) were determined. Moreover, upon culturing on blood-agar plates, the three morphotypes segregated colony variants different from parental S, R and M isolates. A total of nine colony variants were also isolated. The antibiotic resistance profiles and the genetic relatedness of the nine isolates was also determined and compared to those obtained for parental S, R and

M isolates (Table I and Figs. 2 and 3).

The results clearly indicate that at day 57 the patient was simultaneously colonized by three distinct clones of *P. aeruginosa* (Figs. 2 and 3) that presented distinct antibiotic resistance profiles (namely, the S clone was highly resistant, the R clone presented an intermediate pattern while the M clone was susceptible to all the antibiotics tested; Table I). Regarding the S, R and M colony variants (S-, R- and M-like colony variants S1 to S3, R1 and M1 to M5) obtained by growing the strains on blood agar plates, the results of the fingerprinting analysis indicated that they belong to the same clusters of parental S, R and M clones. Moreover, the antibiotic resistance profile, the S-, R- and M-like colony variants presented a certain degree of heterogeneity, if compared to parental isolates (Table I), indicating that although they belong to the same clusters as parental strains, at least *in vitro P. aeruginosa* may segregate genetic variants endowed with different antibiotic resistance profiles and, likely, different virulence traits. These data confirm and extend previous reports indicating that *P. aeruginosa* may segregate variants presenting different antimicrobial susceptibility and virulence profiles (6-7). Whether or not these variants may have originated *in vivo* within the patient lower respiratory tract remains an open question and further studies are certainly needed to clarify this point.

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