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Brief Report

Characterization of *Pseudomonas aeruginosa* in Burn Patients Using PCR- Restriction Fragment Length Polymorphism and Random Amplified Polymorphic DNA Analysis

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Abstract

One of the major opportunistic pathogens in patients with burn injuries is *Pseudomonas aeruginosa*, which causes severe infections in burned patients. The objective of the study was to examine the molecular epidemiology of *P. aeruginosa* colonization in the burn unit of Shahid Motahari Hospital in Tehran, Iran. Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analysis were employed to study 127 clinical and two environmental *P. aeruginosa* isolates collected from January to June 2008. In RFLP, the PCR products of *16S rRNA* gene were digested with restriction enzyme *Alu* I, *Hae* III, and *Rsa* I, and the fragments generated were analyzed by agarose electrophoresis. Molecular typing by RFLP did show no discriminatory power for *P. aeruginosa* isolates, but RAPD-PCR revealed eight different genotypes; RAPD1to RAPD8 in clinical and environmental isolates. RAPD1 was the major genotype in clinical (n=64, 50.4%) and environmental isolates (n=1, 50%). The findings suggest that RAPD might have a superior typeability and discriminatory power over RFLP to study *P. aeruginusa.* Moreover, they highlight the need for further attention to the control of infection sources in Burn Units to prevent the transmission of the bacterium.

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Introduction

Pseudomonas aeruginosa is a human opportunistic pathogen that infects burned patients with immunological system defects, and causes a wide range of infections.[1] Burn injury is one of the significant public health problems in many areas of the world, particularly in developing countries.[1,2] Since *P. aeruginosa* is naturally resistant to many drugs and is able to gain resistance to all effective antibiotics, the infection with this organism is a particularly problematic condition for patients.[3-6]

For determining the source of an infection, detecting the cross-transmission of nosocomial pathogens and recognizing the outbreaks of the infection, the process of the identification of subtypes of the bacteria is important epidemiologically. Any subtyping method must have a high differentiation power, and demonstrate the relationship of all organisms isolated from individuals infected from the same source.[7] Another concern for subtyping methodologies is reproducibility. Reproducibility is especially significant for the construction of reliable

databases containing all known strains within a species to which unknown organisms can be compared. Variable expression of phenotypic characteristics can contribute to problems with reproducibility.[7]

Molecular epidemiologic studies have an important role in determining the transmission routes of a pathogen. This kind of information can be used in clinical settings to differentiate continuing epidemic of an infectious agent from incidentally increased infection rates.[8]

DNA-based typing methods are known as the most appropriate approaches for epidemiological studies. Such methods may be divided into two major categories. The first one is direct sequencing-based techniques such as multilocus sequence typing and DNA microarray. The second category includes indirect methods of sequence analysis such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and pulsed field gel electrophoresis (PFGE).[8] Restriction fragment length polymorphism refers to the polymorphic nature of the locations of restriction enzyme sites within defined genetic regions. Specific genetic loci are routinely amplified and examined for differences indicative of strain variation and antimicrobial resistance. The specific locus to be examined is amplified with gene-specific primers, and is subjected to RFLP analysis. Vila and colleagues found that compared with other typing techniques, RAPD assay was more discriminating than RFLP analysis of either the *16S rRNA* genes or the 16S-23S rRNA spacer region but less discriminating than Rep-PCR for *Acinetobacter calcoaceticus* and *A. baumannii*.[9]

Despite the major significance of molecular epidemiological studies in the effective management of nosocomial infections in burn injuries, there are only few studies in regards to the molecular epidemiology of *P. aeruginosa* in Iran.[10] Therefore, this study was planned to investigate the routes of transmission of *P. aeruginosa*, and to determine by molecular epidemiology the relationship between *P. aeruginosa* isolates in the Burn Unit (BU) of Shahid Motahari Hospital, one of the referral BUs in Tehran, Iran.

Material and Methods

Sampling and Patients Demography

One hundred and twenty seven *P. aeruginosa* isolates from burned patients and 2 isolates from the Hospital environment were collected between February and June 2008. Patients had been hospitalized in BU for different types of burn injuries. The clinical samples included burn wound swabs or biopsy specimens. The environmental samples included water from faucets, antiseptics, hand-washing solutions, and swabs from sinks, hydrotherapy equipments, floors, and other damp surfaces with potential for cross-contamination throughout the BU. All samples were cultured on the Mueller-Hinton agar. *P. aeruginosa* were isolated from samples by standard microbiology procedures.[7] Each isolate, originated from a single colony of each patient's culture, was identified as *P. aeruginosa* by API 20NE (bioMérieux, Lyon, France). *P. aeruginosa* isolates were stored in Luria-Bertani broth medium (Merck KGaA, Darmstadt, Germany) containing 30% glycerol at -80°C.

Isolation of Genomic DNA

Genomic DNA of the isolates was extracted from 2 ml of cultures acquired from a single colony.The cells were harvested by centrifugation (3000 g, 8 min). Afterwards, the bacterial pellet was suspended in 567 µl of TE buffer (50 mM Tris, 50 mM EDTA, pH 8.0) plus 30 µl 10% SDS and 3 µ1 of proteinase K (20 mg/ml), and incubated foronehour at 37°C. Then 80 µ1 of 10% CTAB in 0.7% NaCl was added, and the mixture was incubated for 10 min at 65°C. The solution was extracted with 750 µl of chloroform/isoamylalcohol (24:1), spun, and the aqueous phase was re-extracted with phenol/chloroform/isoamylalcohol (25:24:1). DNA was precipitated with 500 µ1 isopropanol from the aqueous phase. The DNA pellet was then washed with 70% ethanol, dried briefly and resuspended in 100 µl of TE buffer. The DNA concentration was determined by measuring the absorbance of the samples at 260 nm using a UV spectrophotometer.[11]

Oligonucleotide Primers

The oligonucleotide primers were designed using primer 3 version 0.6 software.[12] The primers sequences were designed so to amplify the complete sequence of 16S *rDNA* gene. The product size synthesized by left and right primers was 1658 bp. The sequences of forward and reverse oligonucleotide primers were 3'TTGCTGAGCCAAGTTTAGGG5' and 3'TGAGCTACAGACCCAATCGTC5', respectively (MWG Biotech, Ebersberg, Germany).

DNA Amplification

Reaction mixtures for PCR contained 1X PCR buffer, 250 µM of each deoxynucleoside triphosphates (dNTPs), 1.5 mM $MgCl_2$, each oligonucleotide primers at a final concentration of 30 pmol, 1 unit of *Taq* DNA polymerase (MBI Fermentas, Vilnius, Lithuania). DNA amplification was performed with a ASTEC PCR

Thermal Cycler PC 707-02 (ASTEC, Fukuoka, Japan) with the cycling conditions as follows: a 5 min hot start at 94°C, followed by; 94°C for 40 s, 58°C for 45 s, and 72°C for 1 min, for 32 cycles, followed by a final extension step at 72°C for 10 min. PCR products (one-third of each reaction mixture) were analyzed by horizontal gel electrophoresis in 1.5% (wt/vol) agarose gels (Invitrogen, Carlsbad, CA, USA) with tris-borate-EDTA (TBE) running buffer at 80 V for 1 hour. DNA molecular size markers were included in all gels (GeneRuler 100-bp DNA ladder, MBI Fermentas). After the gels were stained with ethidium bromide, they were observed in Gel-Documentation system (UVItec, Cambridge, UK).

RFLP Analysis

Three used enzyme were selected based on the analysis of restriction patterns that was acquired from bioinformatics software (www.restrictionmapper.org) and on previous works.[13,14] Ten microlitres of the PCR product were removed and digested with 1 U of *Alu* I and 10 U of *Hae* III and *Rsa* I restriction enzymes as recommended by the manufacturer (Takara Bio Inc., Otsu, Japan). RFLP products were separated by 2% (wt/vol) agarose gels (Invitrogen, Carlsbad, CA, USA) with tris-borate-EDTA (TBE) running buffer at 80 V in TBE buffer for 1.5-2 h. DNA molecular size markers were included in all gels (GeneRuler 100-bp DNA ladder, MBI Fermentas). After being stained with ethidium bromide, the gels were observed in Gel-Documentation system (UVItec, Cambridge, UK). Patterns resulting from RFLP typing were analyzed by eyes. Isolates with the same RFLP patterns were considered identical, otherwise, they were considered different.

RAPD Analysis

RAPD-PCR was done as described previously.[15] Briefly, reactions mixtures (25 µl) contained 40 ng of genomic DNA, 40 pmol of oligonucleotide primer 272 (3'AGCGGGCCAA5') (MWG Biotech, Ebersberg, Germany), 1 unit of *Taq* DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 250 mM of each deoxynucleoside triphosphate (MBI Fermentas), 1x reaction buffer supplied by the manufacturer, and 3 mM $MgCl_2$ (MBI Fermentas). DNA was amplified in ASTEC PCR Thermal Cycler PC 707-02 (ASTEC, Fukuoka, Japan) with the cycling conditions as follows: (i) 4 cycles of 94°C for 5 min, 36°C for 5 min, and 72°C for 5 min, and (ii) 30 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, followed by a final extension step of 72°C for 10 min.

RAPD products were separated by 1.5% (wt/vol) agarose gels (20 wells; 11 by 14 cm) with tris-borate-EDTA (TBE) running buffer at 9 V/cm for 4 hours. DNA molecular size markers were included in all gels (GeneRuler 100-bp DNA ladder, MBI Fermentas). After being stained with ethidium bromide, the gels were observed in Gel-Documentation system (UVItec, Cambridge, UK). In the pattern resulting from RAPD assays, only major reproducible bands that were detected by UV fluorescence, and comprised of 500 to 1,500 bp were taken into account for analysis, as previously described.[16] Isolates with RAPD patterns that were different by one or more discrete bands were considered different; otherwise, the isolates were considered identical.[16]

Results

Patients Demographics

Patients included 14 (11.02%) under 15 year old and 113 (88.98%) over 15 years old individuals. 98 (77.2%) of the patients were males and the rest (n=29, 22.8%) were females. The main causes of burn injuries were; gasoline (n=41, 32.3%), petroleum (n=24, 18.9%), liquid gas (n=22 17.3%), boiling water (n=17, 13.4%), electricity (n=7, 5.5%), flame (n=5, 4%), tar (n=4, 3.1%), alcohol (n=4, 3.1%), and acid (n=3, 2.4%).

Bacterial Isolates

127 *P. aeruginosa* isolates were obtained from clinical specimens and two environmental isolates were obtained from inanimate samples. Environmental isolates were recovered from a sink (n=1) and a floor (n=1). All cultures from the hands of the medical staffs were negative for *P. aeruginosa*. All isolates were identified as *P. aeruginosa* by API 20NE.

RFLP Analysis of 16S rDNA Gene

In contrast to other studies based on PCR-RFLP,[13] analysis of RFLP with these restriction enzymes in 2% agarose gels showed no typeability and discriminatory power for this technique. Each restriction enzyme had only one digested pattern on the agarose gel for the isolates, consequently no typeable results were obtained using such a technique. In summary, this kind of genotyping may not be suitable for fingerprinting of *P. aeruginosa* strains. The results of RFLP analysis are shown in figure 1.

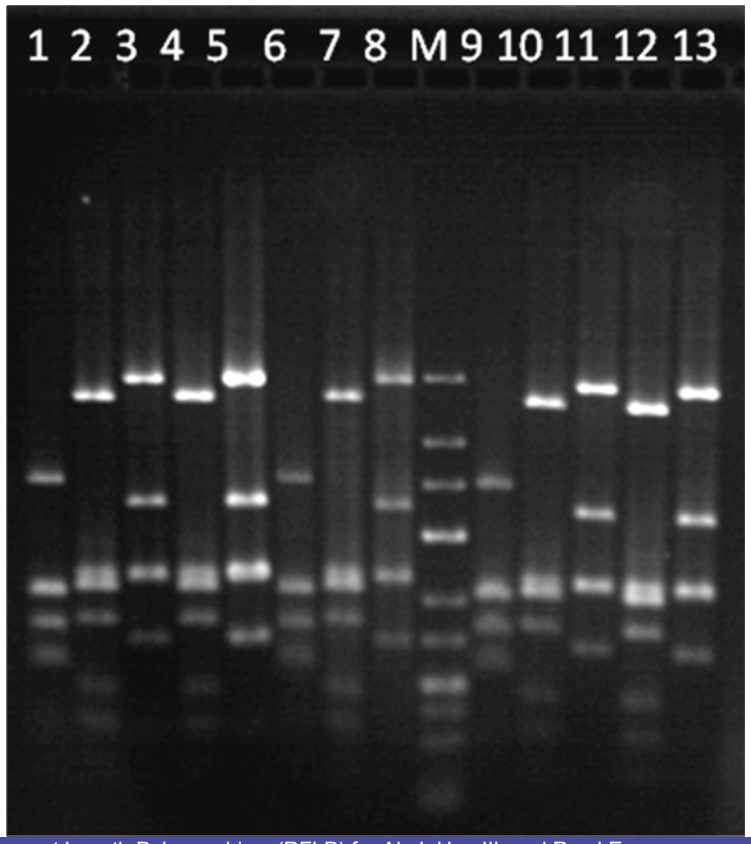

Figure 1: Restriction Fragment Length Polymorphism (RFLP) for Alu I, Hae III, and Rsa I Enzymes
PCR products of 16S rDNA gene digested by Alu I, Hae III, and Rsa I enzymes are shown in lanes 1, 6, and 9; lanes 2, 4, 7, 10, and 12; and lanes 3, 5, 8, 11, and 13, respectively. M is DNA molecular size marker (GeneRuler 100-bp DNA ladder).

RAPD Fingerprinting of P. aeruginosa

The RAPD fingerprinting technique identified 8 different genotypes, RAPD1 to RAPD8, in burn patients. Sixty-four patients were colonized with RAPD type 1 isolates, 29 patients with RAPD type 2 isolates, and 12, 7, 5, 5, 3 and 2 patients were colonized with RAPD type 3, 4, 5, 6, 7 and 8, respectively. In addition, the technique identified two genotypes of RAPD type 1 and RAPD type 4 in the two environmental samples. Having different numbers, sizes, and intensities of amplified DNA bands, the RAPD profiles were discriminatory among BU isolated strains.. RAPD profiles for some *P. aeruginosa* isolates which were obtained by primer 272 are shown in figure 2.

Discussion

P. aeruginosa isolated from burn wounds contributes significantly to the mortality and morbidity in hospitalized burned patients in developing countries.[1] Genotyping is an important technique to determine infection routes and to choose the appropriate approach for managing the infection. This report has investigated the genotypes of *P. aeruginosa* isolates using RFLP and RAPD methods to, explore any possible relationship among isolates, identify routes of the infection, and help find appropriate methods of eliminating the infections.

RAPD-PCR is a genotypic identification and characterization system with a great specificity and sensitivity to define a wide range of bacterial isolates. RAPD typing could be used effectively for typing *P. aeruginosa*,[15] Eftekhar and colleagues used RAPD-PCR for fingerprinting *P. aeruginosa* obtained from patients with cystic fibrosis, and found 13 different genotypes.[17] The present study used the same primer as that used in theirs.[17] Therefore, it was useful for fingerprinting of *P. aeruginosa.* All 129 *P. aeruginosa* isolates described in this study were typeable by RAPD method.

The study also used RFLP of *16S rRNA* gene as another technique for genotyping. PCR-RLFP is sensitive,typeable , and has a high degree of discriminatory capability. It is also a simple and inexpensive technique, and has been used successfully for the typing of *Haemophilus parasuis*.[13] However, the present study failed to show discriminatory power of RFLP of *16S rRNA* gene by three restriction enzymes, *Alu* I, *Hae* III, and *Ras* I, for *P. aeruginosa* isolates. These enzymes were chosen according to the PCR product size

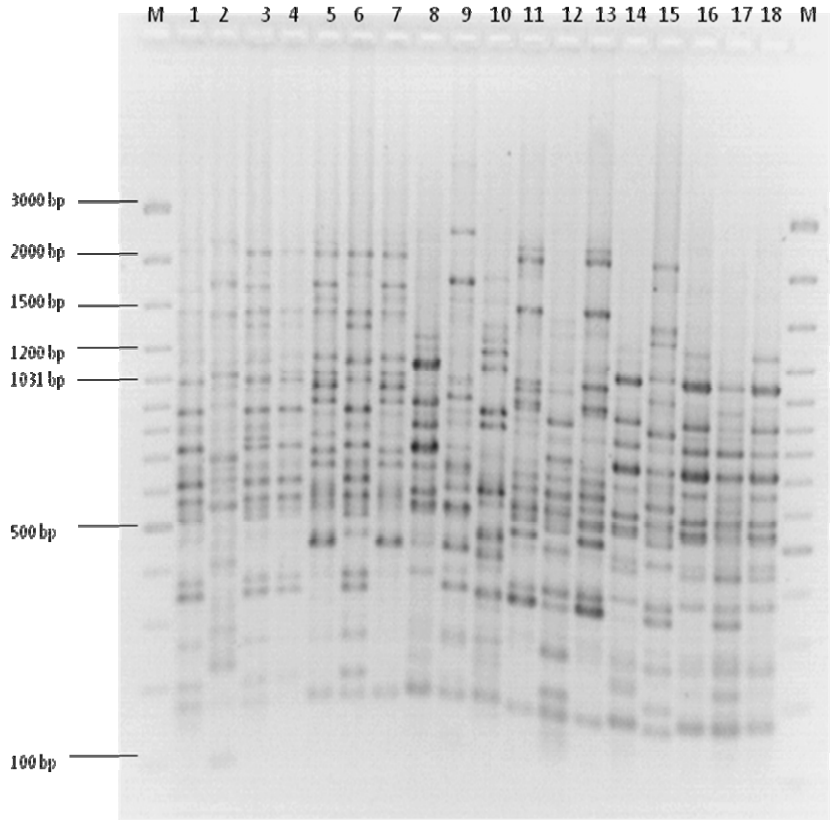

Figure 2: The Random Amplified Polymorphic DNA (RAPD) fingerprinting of *P. aeruginosa* isolates
Lanes 13 and 18 are from environmental isolates and the rest are from clinical ones. Lanes 12, 14, 16, 17, and 18 are RAPD1; lanes 1, 3, 4, 6 and 15 are RAPD2; lanes 5, and 7 are RAPD3; lanes 11 and 13 are RAPD4, lane 10 is RAPD5, lane 8 is RAPD6; lane 9 is RAPD7; and lane 2 is RAPD8 profile; lane M is DNA molecular size marker (GeneRuler 100-bp DNA ladder).

(~1600 bp) and bioinformatics analyses.

P. aeruginosa colonization may originate from endogenous sources such as intestinal tract, or from exogenous sources such as contaminated equipment or other patients colonized with *P. aeruginosa*. Understanding the routes of colonization is crucial to the development of effective preventive measures against infection. The large number of unique genotypes observed in the patients, however, suggests that most patients had been colonized from an exogenous source. Furthermore, 64 patients were colonized with the RAPD1 strain, 29 patients were colonized with the RAPD2 strain, and 12, 7, 5, 5, 3 and 2 patients were colonized with RPAD3 to RPAD8 isolates, respectively. Colonized patients represent a continuous reservoir of strains from which other patients can be colonized via cross-acquisition. Several studies have demonstrated that cross-acquisition can play an important role in the epidemiology of nosocomial colonization and infection with *P. aeruginosa*.[18] In the present study the origin of infection transmitted to 74 patients (RAPD1 and RAPD4 profiles) were from the environmental sources. Remaining isolates might originate from the staffs, equipments or other sources in the BU. Even if the overall rate of *P. aeruginosa* colonization is not significantly reduced, it is important to recognize cross-infecting strains, especially if they exhibit resistance to a variety of antibiotics and give rise to severe infections.

In contrast with a previous study,[18] the present study did isolate two *P. aeruginosa* strains from the inanimate hospital environment. Such isolates were important sources of patient's infections. In addition, a thorough survey of the inanimate hospital environment succeeded in identifying two ongoing reservoirs of RAPD1 and RAPD4 strains.

Conclusion

The findings of the present study highlight the need for further attention to the control of infection sources in BUs to prevent the transmission of *P. aeruginosa*. Moreover, they demonstrated that RFLP of *16S rDNA* gene did have no typeability and discriminatory power for *P. aeruginusa* by *Alu* I, *Hae* III, and *Ras* I enzymes.

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Conflict of Interest: None declared

References

1 Church D, Elsayed S, Reid O, et al. Burn wound infections. *Clin Microbiol Rev* 2006; 19: 403-34.
2 Polavarapu N, Ogilvie MP, Panthaki ZJ. Microbiology of burn wound infections. *J Craniofac Surg* 2008; 19: 899-902.
3 Rastegar Lari A, Bahrami Honar H, Alaghehbandan R. Pseudomonas infections in Tohid Burn Center, Iran. *Burns* 1998; 24: 637-41.
4 Ahangarzadeh-Rezaee M, Behzadiyan-Nejad Q, Owlia P, et al. In vitro activity of imipenem and ceftazidime against mucoid and non-mucoid strains of *Pseudomonas aeruginosa* isolated from patients in Iran. *Arch Iran Med* 2002; 5: 251-4.
5 Behzadiyan-Nejad Q, Souri E, Owlia P. *In vitro* effects of sub-inhibitory concentrations of gentamicin on *P. aeruginosa* alginate production. *Pharm Pharmacol Commun* 1998; 4: 489-91.
6 Rezaee MA, Behzadiyan-Nejad Q, Pirayeh SN, et al. Higher aminoglycoside resistance in mucoid Pseudomonas aeruginosa than in non-mucoid strains. *Arch Iran Med* 2002; 5: 108-10.
7 Patrick R, Murray RP, Baron EJ, Jorgensen J, Landry ML: Manual of Clinical Microbiology. 9th ed. Washington, DC, ASM, 2007.
8 Gürtler V, Mayall BC. Genomic approaches to typing, taxonomy and evolution of bacterial isolates. *Int J Syst Evol Microbiol* 2001; 51: 3-16.
9 comparative study of different PCR-based DNA fingerprinting techniques for typing of the *Acinetobacter calcoaceticus-A. baumannii* complex. *J Med Microbiol* 1996; 44: 482-9.
10 Ranjbar R, Owlia P, Saderi H, et al. Isolation of clinical strains of *Pseudomonas aeruginosa* Harboring Different Plasmids. *Pak J Biol Sci* 2007; 10: 3020-2.
11 Sambrook J. Molecular cloning: A laboratory manual. 3rd ed. New York, NY, Cold Spring Harbor Laboratory, 2001.
12 Rosen S, Skaletsky HJ. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, editors. Bioinformatics Methods and Protocols: Methods in Molecular Biology.Totowa, NJ, Humana Press; 2000. p. 365-86.
13 de la Puente Redondo VA, Navas Méndez J, García del Blanco N, et al. Typing of *Haemophilus parasuis* strains by PCR–RFLP analysis of the *tbpA* gene. *Vet Microbiol* 2003; 92: 253-62.
14 Flemming L, Rawlings D, Chenia H. Phenotypic and molecular characterisation of fish-borne *Flavobacterium johnsoniae*-like isolates from aquaculture systems in South Africa. *Res Microbiol* 2007; 158: 18-30.
15 Mahenthiralingam E, Campbell ME, Foster J, et al. Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *J Clin Microbiol* 1996; 34: 1129-35.
16 Renders N, Römling Y, Verbrugh H, et al. Comparative typing of *Pseudomonas aeruginosa* by random amplification of polymorphic DNA or pulsed-field gel electrophoresis of DNA macrorestriction fragments. *J Clin Microbiol* 1996; 34: 3190-5.
17 Eftekhar F.,Khodadad A.,Henry D, Speert D.P. Isolation and genetic fingerprinting of *Pseudomonas aeruginosa* from Iranian patients with cystic fibrosis using RAPD-PCR. *Iranian Journal of Biotechnology* 2003; 1: 95-100.
18 Bertrand X, Thouverez M, Talon D, et al. Endemicity, molecular diversity and colonisation routes of *Pseudomonas aeruginosa* in intensive care units. *Intensive Care Med* 2001; 27: 1263-8.