



Polymorphism of the rDNA and tDNA loci in clinical isolates of *Pseudomonas aeruginosa*: A perspective for molecular epidemiology surveillance

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Abstract

The Gram-negative bacterium *Pseudomonas aeruginosa* has a wide environmental and ecological distribution. It is an opportunistic pathogen that acquires resistance to multiple antimicrobial agents and can infect plants, animals and humans. We used rDNA and tDNA PCR markers to characterize the bacterial diversity of *P. aeruginosa* strains isolated at a Brazilian teaching hospital (Oswaldo Cruz University Hospital, Recife, Brazil) between March 2003 and February 2004. Clonal groups of *P. aeruginosa* clinical isolates were identified from different patients in different hospital units using either rDNA or tDNA markers, or a combination of both in a duplex PCR. These PCR-typing methods together with drug-resistance profiles were used to trace the distribution of antibiotic resistant *P. aeruginosa* clones and to identify cross-infection of the same patient with a different bacterial clone after being moved to a different hospital unit. The data presented here demonstrates a rapid, reliable and useful method for epidemiological surveillance that can contribute to the control of *P. aeruginosa* infections in hospital environments.

Key words: antibiotic resistance, molecular typing, nosocomial infection, *P. aeruginosa*, rDNA-PCR, tDNA-PCR.

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Introduction

The routine identification of hospital isolates of *Pseudomonas aeruginosa* is based on phenotypic analysis using characteristic biochemical reactions. However, horizontal dissemination cannot be traced properly using phenotypic methods based only on genus and specie identification. On the other hand, methods based on the polymerase chain reaction (PCR) have been widely used for the analysis of the genetic diversity of many microorganisms (Agodi *et al.*, 2000; Lopes *et al.*, 2005). Depending on the primers and amplification conditions employed, the results allow the discrimination of organisms at not only the genus and species level but also at the level of individual strains. By using molecular typing methods it is possible to identify genetic similarities between both phenotypically unrelated and phenotypically analogous strains. These molecular approaches provide the precise information necessary for the monitoring and control of hospital infections. Moreover,

epidemiological relationships can be detected and/or confirmed and clonal groups can be delimited (Severino *et al.*, 1999).

The fact that the complete *P. aeruginosa* genome sequence is known allows the identification of polymorphic regions present in different bacterial strains and the design of methods to discriminate isolates based on such polymorphisms (see The *Pseudomonas* Genome Project at www.pseudomonas.com).

The *P. aeruginosa* rRNA cluster (5S-23S-(ISR)-tRNA^{ALA}-(ISR)-tRNA^{ILE}-(ISR)-16S) is distributed four times throughout the genome of the sequenced *P. aeruginosa* strain Pa01. The intergenic spacer regions (ISRs) are subject to lower evolutionary pressure, and therefore show wider genetic variation (Gürtler and Stanisich, 1996) that is dependent on the number and type of both tRNA genes and *boxA* sequence between 23S and 16S sequences and by the presence of enzyme-recognition sites at the ISRs, besides the length of the ISRs themselves (García-Martínez *et al.*, 1999). Two PCR-based methods have been proposed for identifying this cluster. Intergenic Transcribed Spacer PCR ribotyping (PCR-ribotyping) uses specific primers that amplifies sequences between the 16S and 23S gene and has

been applied for molecular identification of bacteria at the species level (Jensen *et al.*, 1993; Daffonchio *et al.*, 1998; Agodi *et al.*, 2000) and the discrimination of bacterial strains (Kostman *et al.*, 1992; Clementino *et al.*, 2001; Pereira *et al.*, 2002). This method detects both the number of tRNA genes and the spacer length within the cluster. Another highly promising method for bacterial identification is based on the PCR length polymorphisms of the intergenic spacers between tRNA genes (tDNA-PCR) spread along the bacterial genome. The tRNA genes are highly conserved among eubacteria and occur in multiple copies throughout the bacterial genome, within and outside rDNA gene clusters. These genes are generally clustered and are separated by spacers whose length and sequences are subjected to a higher degree of variations (Welsh and McClelland, 1992). The tDNA-PCR method uses consensus primers complementary to the highly conserved edges of the flanking tRNA genes that are directed outwards (Welsh and McClelland, 1991). This method is regarded as producing species-specific banding patterns and has been applied to the differentiation of various bacterial species (Vanechoutte *et al.*, 1998; De Gheldre *et al.*, 1999; Clementino *et al.*, 2001).

A significant increase in the number of multi-drug resistant (MDR) *P. aeruginosa* infections has been detected by our group at the Oswaldo Cruz University Hospital, Recife, Brazil (unpublished results). However, biochemical routine identification was not able to distinguish clonal groups of multi-drug resistant strains. We tested rDNA-PCR and tDNA-PCR for their capacity to type *P. aeruginosa* hospital isolates, our aim being to contribute to the epidemiological control of *P. aeruginosa* infections. Our results indicate that rDNA/tDNA duplex PCR might be useful for *P. aeruginosa* typing of isolates from different hospital units. Moreover, the sequence of acquisition and/or expression of antimicrobial resistance by cells of distinct clonal groups could be identified.

Material and Methods

Bacterial strains and microbiological methods

The *Pseudomonas* Genome Project (Boston, USA) kindly provided *Pseudomonas aeruginosa* strain PA01 and The Oswaldo Cruz Foundation Microbial Collection (INCQS, Rio de Janeiro, Brazil) provided the *Pseudomonas* reference strains *P. aeruginosa* ATCC 9027, *P. putida* ATCC 15175 and *P. fluorescens* ATCC 13525.

We collected 34 *P. aeruginosa* isolates from nosocomial infections of patients hospitalized in different units of the Oswaldo Cruz University Hospital (Recife, Brazil) between March 2003 and February 2004, the isolates being obtained from a variety of clinical samples (Table 1). The isolates were identified by colony pigmentation, grape-like odor, motility and biochemical tests (carbohydrate fermentation (-), citrate assimilation (+), lysine decar-

boxylase (-), indol (-), oxidase (+), beta-hemolysis on blood-agar (+), DNase (-). Identifications were confirmed using the ID32 Mini-Api automatic system (BioMerieux, France). Antibiogram data were registered by disk diffusion method according to the criteria of *National Committee for Clinical Laboratory Standards* (NCCLS M100). Strains were maintained on nutrient agar (pH 7.4) containing (g L⁻¹) beef extract, 10; tryptone, 5; NaCl, 5; bacteriological agar 12 g or by freezing in 15% (v/v) glycerol at -80 °C. All reagents were of at least analytical purity, and beef extract, tryptone and bacteriological agar were supplied by Oxoid (Oxoid Brasil Ltda, São Paulo, Brazil).

Of the 34 *P. aeruginosa* hospital isolates collected, 33 were confirmed by automated identification except for isolate 22 (Table 1) which gave an unacceptable profile for *P. aeruginosa*. All the isolates, including isolate 22, were submitted to antibiogram assays with nine antibacterial agents suitable for *Pseudomonas* (Pellegrino *et al.*, 2002). Isolates 6, 22 and 26 were susceptible to all drugs tested while the other isolates displayed different individual susceptibility profiles, with some showing multiple drug resistance (MDR) to up to eight different antibacterial agents. No correlation was observed between bacterial antibiogram susceptibility profiles and site of infection or hospital unit (Table 1). Genomic DNA from each isolate was extracted for further molecular analysis.

Genotyping

Genomic DNA was extracted according to Ausubel *et al.* (1989), separated on 0.8% (w/v) agarose gel, stained using ethidium bromide and visualized under UV light.

We carried out rDNA-PCR by amplification of ISRs regions between the rRNA16S-23S genes with the 03 primer (5'-TTGTACACACCGCCCGTCA-3') complementary to the rRNA 23S conserved region and the 04 primer (5'-GGTACCTTAGATTGTTTCAGTTC-3'), complementary to the rRNA 16S conserved region according to Kostman *et al.* (1992). The PCR reactions were prepared containing 50 ng of genomic DNA, 20 pmol of each primer, 4 pmols of dNTP, 37.5 pmols of MgCl₂, 1U of *Taq* DNA polymerase and 1x reaction buffer (Invitrogen) in a final volume of 25 µL and the amplifications carried out in a Hybaid Touchdown PCR thermocycler (Thermo Electron Corp., Waltham, MA) using an initial denaturing step of 2 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C with a final 7 min extension at 72 °C. Amplification products were separated on 1.6% (w/v) agarose gel in 0.5x TBE buffer at 10 V.cm⁻¹ and stained with ethidium bromide.

The tDNA-PCR consisted of amplification of ISRs regions between the tDNA genes using the conserved primers T5A (5'-AGT CCG GTG CTC TAA CCA ACT GAC-3') and T3B (5'-AGG TCG CGG GTT CGA ATC C-3') described by Welsh and McClelland (1991). The reaction mix was prepared as described for the rDNA-PCR. Ampli-

Table 1 - Phenotypic and molecular characterization of *Pseudomonas aeruginosa* clinical isolates.

Clinical isolate ¹	Isolate code ²	Antibacterial resistance profile ²									PCR pattern	
		cp	gn	am	pt	cf	cm	im	az	pl	rRNA-PCR	tRNA-PCR
GICU-6Mar03-Sk	1	r	r	r	s	r	r	r	s	s	R1	R1T1
CU-16Apr03-Rt	2	r	r	r	r	r	s	r	r	s	R1	R1T2
CU-23Apr03-Sk	3	s	s	s	s	r	r	s	s	s	R1	R1T1
CU-30Apr03-BI	4	-	r	r	s	r	r	r	s	s	R1	R1T1
ASU-27Jun03-Be	5	r	r	r	s	r	r	r	s	s	R1	R1T1
IDU-23Jul03-Ur	6	s	s	s	s	s	s	s	s	s	R1	R1T1
IDU-11Sep03-BI	7	r	r	r	r	r	r	r	r	s	R1	R1T1
IDU-18Sep03-Ur	8	s	r	s	s	s	s	s	s	s	R1	R1T1
GICU-6Oct03-Rt	9	r	r	r	s	r	r	r	r	s	R2	R2T5
GICU-7Oct03-Rt	10	r	r	r	s	r	r	r	s	s	R1	R1T1
CU-10Oct03-BI	11	s	r	s	s	r	s	s	s	s	R1	R1T1
AU-23Oct03-Ur	12	r	r	s	r	r	r	s	r	s	R1	R1T1
CU-31Oct03-Sk	13	s	r	s	s	r	r	s	s	s	R1	R1T1
IDU-31Oct03-Rt	14	r	r	r	s	r	r	r	s	s	R1	R1T1
IDU-4Nov03-BI	15	s	r	s	r	r	r	r	r	s	R4	R4T7
CEU-10Nov03-Sk	16	r	r	r	r	r	r	r	s	s	R1	R1T1
IDU-11Nov03-Ur	17	r	r	r	r	r	r	r	s	s	R1	R1T1
GPU-18Nov03-Ur	18	r	r	r	r	r	r	r	s	s	R1	R1T1
GPU-26Nov03-Ur	19	r	r	r	s	r	r	r	s	s	R1	R1T1
CU-28Nov03-Sk	20	r	r	r	r	r	r	r	s	s	R1	R1T1
PU-28Nov03-Rt	21	r	r	r	s	r	r	r	s	s	R1	R1T4
CU-2Dec03-BI*	22	s	s	s	s	s	s	s	s	s	R1	R1T1
CEU-3Dec03-Ur	23	r	r	r	s	r	r	r	s	s	R1	R1T1
GICU-3Dec03-Sk	24	s	s	s	s	r	r	s	s	s	R5	R5T8
PU-4Dec03-Rt	25	r	r	r	s	r	r	s	s	s	R1	R1T1
IDU-5Dec03-BI	26	s	s	s	s	s	s	s	s	s	R1	R1T1
PU-13Dec03-Rt	27	r	r	r	s	r	r	s	s	s	R1	R1T1
OU-18Dec03-Sk	28	s	r	s	s	r	s	s	s	s	R1	R1T1
IDU-31Dec03-Ur	29	s	r	s	s	r	s	s	s	s	R1	R1T1
GICU-9Jan04-BI	30	-	r	r	r	r	-	r	r	s	R3	R3T6
ASU-16Jan04-Rt	31	s	s	s	s	r	s	s	s	s	R1	R1T1
OU-19Jan04-BI	32	s	r	s	s	r	s	r	s	s	R1	R1T3
GPU-22Jan04-Ur	33	s	r	r	s	r	r	r	s	s	R1	R1T1
IDU-27Jan04-Sk	34	r	r	r	r	r	r	r	s	s	R1	R1T1

¹The initial letters give the hospital unit from which the clinical isolate was obtained (GICU- = General Intensive Care Unit; IDU = Infectious Diseases Unit; CU = Cardiology Unit; ASU = Abdominal Surgery Unit; AU = Ambulatory Unit; CEU = Cardiology Emergency Unit; CU = Coronary Unit; GPU = General Practice Unit; IDU = Infectious Diseases Unit; OU = Oncology Unit; and PU = Pneumology Unit) followed by the isolation date and the clinical sample (Be = Bile; BI = Blood; Sk = Skin, Rt = Respiratory tract; and Ur = Urine).

²This simplified code was used in the phylogenetic analysis and the text.

³Antibacterial agent code: Amicacin = am; Aztreonam = az; Cefepime = cm; Cefotaxime = cf; Ciprofloxacin = cp; Gentamicin = gn, Imipinem = im; Piperacilin/Tazobactam = pt; and Polimixin = pl. Resistance codes: Resistant = r; Susceptible = s; and 'Not Determined' is represented by a hyphen (-).

*Not positively identified as *P. aeruginosa* by automated biochemical identification.

fication conditions consisted of denaturation for 2 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 2 min at 72 °C with a final 10 min extension at 72 °C. Amplification products were separated and visualized as described above.

Duplex PCR used both sets of primer pairs, rDNA and tDNA markers, and the reaction was conducted according to the rDNA-PCR protocol described above, with minor modifications (2 min for the cyclic extensions at 72 °C and 10 min final extension). Amplification products were sepa-

rated on 1% (w/v) agarose gel and stained as described above.

Computational analysis

The bacterial isolates were analyzed using the Net-Work program version 4.1.0.8 (Fluxus Technology Ltd, www.fluxus-engineering.com) designed for phylogeny analysis and showing the geographic distribution of haplotypes. Binary data, grouped according median analysis, consisted of susceptibility (0) and resistance (1) to the antibiotics tested.

Results

Genetic typing

For the rDNA marker the reference strains *P. aeruginosa* PA01 and ATCC 9027 showed the same amplification profile (banding pattern) and a characteristic 800 base pair (bp) band, which was not shown by *P. putida* ATCC 15175 and *P. fluorescens* ATCC 13525 (Figure 1A). We included *P. putida* because it had been reported in cases of bacteremia in intensive care units for newborn babies (Bouallegue, *et al.*, 2004) while *P. fluorescens* was selected due to reports of its presence in oncology units (Hsueh, *et al.*, 1998). We found that isolate 22 showed an rDNA marker banding pattern similar to the two *P. aeruginosa* reference strains (Figure 1B), suggesting that was indeed a *P. aeruginosa* isolate. The rDNA marker banding patterns for the remaining 33 isolates were classified in five distinct rDNA-PCR banding patterns (profiles R1 to R5, Figure 1B) of which the R1 pattern was observed in 88% of the isolates (34/30) and the two *P. aeruginosa* reference strains. The other four patterns were observed for only one isolate each.

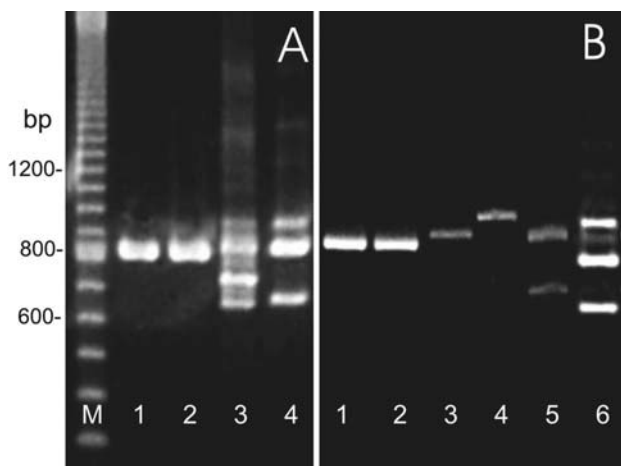


Figure 1 - Amplification products generated by rDNA-PCR. A. lane 1 = *P. aeruginosa* PA01, lane 2 = *P. aeruginosa* ATCC 9027, lane 3 = *P. putida* ATCC 15175 and lane 4 = *P. fluorescens* ATCC 13525. B. Reference strains: lane 1 = *P. aeruginosa* PA01, lane 2 = isolate 22 rDNA pattern 1, lanes 3 to 6 one representative of the other four rDNA-PCR patterns produced by automated identified *P. aeruginosa* isolates. Lane M = 100 bp ladder.

The rDNA marker showed 15% of genetic variability among the isolates (5/34).

For the tDNA marker the two *P. aeruginosa* reference strains again presented the same banding pattern, including four common bands (365, 250, 170 and 125 bp). As was seen for the rDNA marker banding patterns, the tDNA marker banding patterns for the *P. putida* and *P. fluorescens* reference strains also differed from those observed for the *P. aeruginosa* reference strains (Figure 2A). The tDNA marker banding pattern of isolate 22 was similar to the reference strain *P. aeruginosa* ATCC 9027 (Figure 2B), as also observed for the rDNA-PCR marker. Analysis

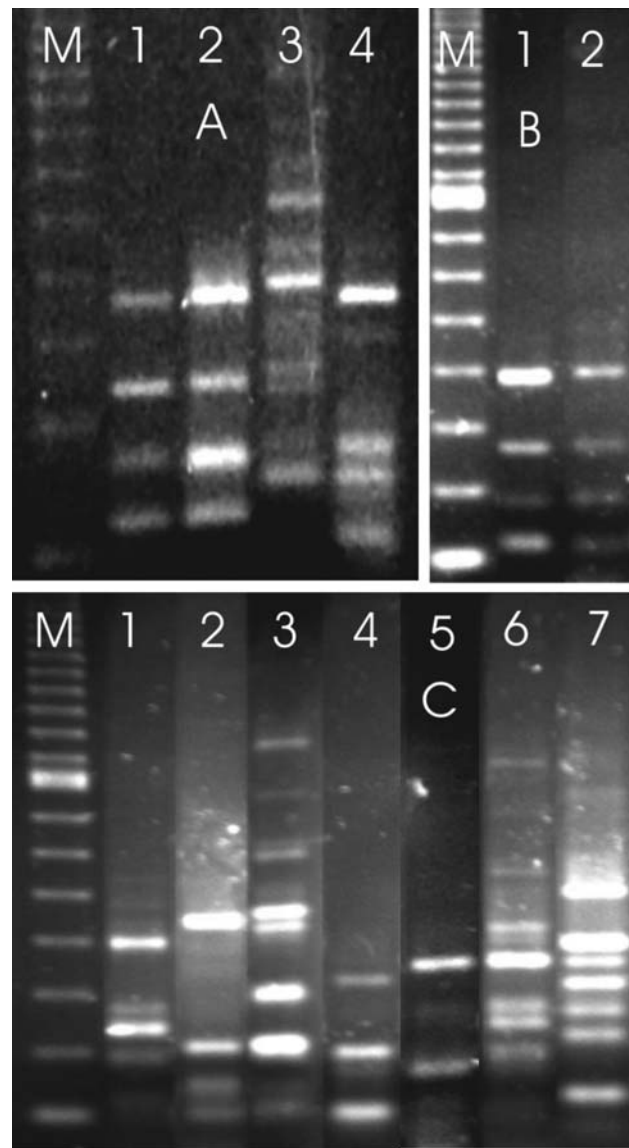


Figure 2 - Amplification products generated by tDNA-PCR. A. lane 1 = *P. aeruginosa* PA01, lane 2 = *P. aeruginosa* ATCC 9027; lane 3 = *P. putida* ATCC 15175 and lane 4 = *P. fluorescens* ATCC 13525. B. Reference strains: lane 1 = *P. aeruginosa* PA01 and lane 2 = isolate 22 (both representing the T1 amplification pattern). C. lane 1 = T2 amplification pattern (AP), lane 2 = T3 AP, lane 3 = T4 AP, lane 4 = T5 AP, lane 5 = T6 AP, lane 6 = T7 AP and lane 7 = T8 AP. Lane M = 100 bp ladder.

of the other 33 isolates revealed eight different tDNA-PCR banding patterns (profiles T1 to T8, Figure 2C) of which the T1 pattern was seen in 79% of our bacterial isolates (27/34) as well as the two *P. aeruginosa* reference strains. The tDNA marker showed 23% of genetic variability among the isolates (8/34).

Using the R1 rDNA profile and four of the tDNA profiles together resulted in four additional tDNA profiles (profiles R1T1, R1T2, R1T3 and R1T4), with R1T1 representing the most common pattern found among the isolates. Although the polymorphism occurring for both primers cannot be considered species-specific for *P. aeruginosa* it can, however, be concluded that any bacterial isolate presenting the R1T1 profile can be identified as belonging to this species, even, as the case of isolate 22, with ambiguous identification by the biochemical tests.

The results showed the discrimination power of both markers, which can be used as an epidemiological tool for detection of strain dispersion. In this sense, the results of PCR typing and the resistance profiles showed that bacterial isolates 16, 17, 118, 20 and 34 may correspond to a unique *P. aeruginosa* strain which was isolated from four different hospital units and infected at least five different patients during the period of study (Table 1). Moreover, in one case two genotypically different isolates were collected from the same patient. Isolate 21 (genotype R1T4) was isolated first but five days after the patient had been transferred to another hospital unit isolate 23 (genotype R1T1) was recovered from the same patient (Figure 3), suggesting that isolate 23 may represent a second nosocomial infection episode.

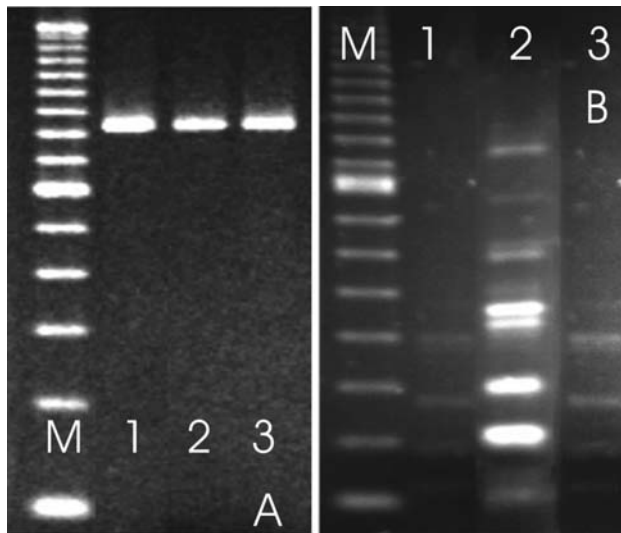


Figure 3 - Amplification products of two bacterial isolates from the same patient. A. rDNA-PCR of *P. aeruginosa* ATCC 9027 (lane 1), bacterial isolate 21 (lane 2) and bacterial isolate 23 (lane 3). B. tDNA-PCR of *P. aeruginosa* ATCC 9027 (lane 1), bacterial isolate 21 (lane 2) and bacterial isolate 23 (lane 3). The molecular marker 100 bp ladder is shown in lane M.

Duplex-PCR typing

The results above suggested the possibility of combining both markers in a duplex PCR for use in typing and screening of *P. aeruginosa* clinical isolates. Duplex PCR of the two *P. aeruginosa* reference strains and the *P. putida* and *P. fluorescens* reference strains generated the expected profiles resulted from the combination of the both markers (data not shown). When duplex PCR was used for typing the 34 isolates, the profiles observed corresponded to the eight profiles detected, showing the same percentage of genetic variability observed for the tDNA marker (data not shown). Duplex rDNA/tDNA-PCR may thus be used as a reliable approach to follow the spread of different bacterial clonal lineages across different hospital units.

Evolution of the resistance to antibiotics

The molecular typing results suggested that some strains were disseminated to different units in the hospital. In addition, such strains may suffer distinct selective pressures at the different units, resulting in the acquisition and/or expression of differential resistance to antibiotics. In such a context, the isolates of the clonal lineage R1T1 were submitted to computational analysis that revealed a haplotypic distribution among its isolates and the emergence of antibiotic resistance (Figure 4). In this analysis, isolates 6, 22 and 26 (clonal group 6) were susceptible to all the antibiotics tested (Table 1) and could be the basal isolates from which isolate 8 (gentamicin resistant) and 31 (cefotaxime resistant) evolved by distinct events of acquiring or expressing resistance. The expression of resistance to cefotaxime by isolate 8 or to gentamicin by isolate 31 could

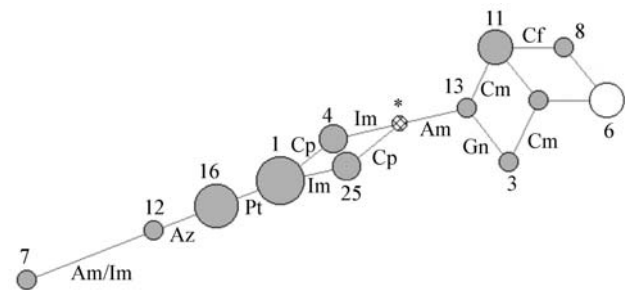


Figure 4 - Computational simulation of the evolution of antibiotic resistance by the *P. aeruginosa* clinical isolates investigated in this study. Each antibiogram pattern is considered to be one phenotype (haplotype) group that includes one or several bacterial isolates. The numbers represent group designations and the area of the circle corresponds to the number of isolates included in the group: 6 (isolates 6, 22 and 26), 11 (11, 28 and 29), 4 (4 e 33), 25 (25 and 27), 1 (1, 5, 10, 14 19 and 23) and 16 (16, 17, 18, 20 and 34). The remaining circles represent groups with only one isolate. The white circle was considered the base of the group network gray circles, while the dashed circle corresponds to a representative isolate of the R1T1 genotype that was not isolated among our bacterial samples (*). The distances between each group are proportional to the number of steps in which one group evolved to the next, represented by expression of resistance to the following antibiotics: Amicacin (am), Aztreonam (az), Cefepime (cm), Cefotaxime (cf), Ciprofloxacin (cp), Gentamicin (gn), Imipinem (im) and Piperacilin/Tazobactam (pt).

have generated clonal group 11 (isolates 11, 28 and 29) and, likewise, expression of resistance to cefepime could have generated isolate 3. The expression of resistance to cefepime by group 11 isolates or to gentamicin by isolate 3 could have generated the isolate 13 and so on. Our analysis suggests that isolate 7 emerged from group 6 after sequential expression of resistance to eight different antibiotics. Moreover, this *in silico* analysis suggests that at least one representative of the R1T1 genotype has not been isolated among our bacterial samples (Figure 4). This clone, showing resistance to four antibiotics, could have been the basal strain for clonal groups 4 and 25 and may be present, but occult, in one of the hospital units. Obviously, this analysis is an attempt to establish the relatedness among the observed clonal lineages, which does not excluded the possibility that new unrelated R1T1 clones could enter the hospital.

Discussion

At 6.3 Mb the *P. aeruginosa* genome is the largest bacterial genome so far sequenced, and this size, coupled with the high number of regulatory genes and subsequent complex metabolism, is consistent with its versatility in adapting to different environments (Stover *et al.*, 2000). Based on this genomic versatility we used two PCR markers that were sufficiently polymorphic to discriminate bacterial isolates from different clonal origins, our results being reproducible and allowed the establishment of common amplification patterns detected for the type strains and for the majority of hospital isolates. However, because of the polymorphism presented by both markers, we cannot suggest their use for routine bacterial identification, except, as in the case of the isolate 22, when bacterial isolates present the common pattern generated by type-strains.

Many techniques have been described for typing *P. aeruginosa*, such as arbitrarily primed PCR (Kersulyte *et al.*, 1995; Bennekov *et al.*, 1996), pulsed field gel electrophoresis (Kersulyte *et al.*, 1995; Agodi *et al.*, 2000; Nakamura *et al.*, 2001), traditional ribotyping (Bennekov *et al.*, 1996; Agodi *et al.*, 2000) and more recently multi-locus sequence typing (MLST; Curran *et al.*, 2004) and enterobacterial repetitive intergenic consensus-based PCR (ERIC-PCR; Yang *et al.*, 2005). One of these methods, rDNA-PCR typing, has been reported as a rapid and accurate method for typing *P. aeruginosa* (Liu *et al.*, 1996; Agodi *et al.*, 2000) and *Burkholderia (Pseudomonas) cepacia* (Dasen *et al.*, 1994; Kostman *et al.*, 1992). In our present work the rDNA marker revealed four distinct clonal groups among our *P. aeruginosa* clinical isolates. Our technique was based on the amplification of ITS loci at rDNA gene clusters and any polymorphism detected may have been due to significant genetic variation such as nucleotide deletions or insertions of tRNA genes or ISRs. Therefore, two bacterial isolates presenting the same amplification pattern could be considered as originating from the same

clonal origin. The use of ITS locus restriction analysis and RFLP-ribotyping has been proposed for strain discrimination and clustering by evaluating genetic differences caused by point mutations that create or remove restriction sites (Gruner *et al.*, 1993).

Several authors have used tDNA-PCR typing to identify bacterial genera and species, *e.g.* *Lactobacillus* (Baele *et al.*, 2002) and other bacteria (Welsh and McClelland, 1991) but our results suggest that this approach is more appropriated for intraspecific discrimination and we have found similar results for clinical isolates of *Klebsiella pneumoniae* (manuscript in preparation). Our work is the first report of the application of tDNA to *P. aeruginosa* typing, which increased the discriminatory power of the rDNA marker and the combination of both markers in a duplex PCR proved to be a reliable tool with high discriminatory power for typing *P. aeruginosa* isolates. The combination of both markers generated amplification patterns that varied from three to ten bands, which may be enough for strain discrimination.

Strain discrimination and clustering can also be obtained using RAPD analysis (Matar *et al.*, 2005), which generates more bands than tDNA-PCR typing. However, RAPD analysis has been criticized because of its low reproducibility and reliability and the fact that amplification can be influenced by factors other than the genetic variation between bacterial isolates. Therefore, it can be difficult to reproduce RAPD results between different laboratories, which is a great disadvantage for epidemiological surveillance studies. On the other hand, the specific primer annealing which occurs with both rDNA-PCR and tDNA-PCR ensures reproducibility wherever these techniques are used.

Gencer *et al.* (2002) have pointed out that *P. aeruginosa* strains are the third most prevalent pathogen isolated from cases of hospital infections, while Pellegrino *et al.* (2002) reported that in some hospitals it is the main bacterial species isolated. The Intensive Care Unit (ICU) at the hospital where our isolates were collected has shown an increase in the number of cases of *P. aeruginosa* infections over the last five years as well as in the antibiotic resistance (data not shown) and diversity of the clonal groups of the *P. aeruginosa* isolates recovered (Table 1). Similar genetic variability in ICU *P. aeruginosa* clinical isolates has been reported by Sarwari *et al.* (2004) and Stover *et al.* (2000) have noted that the genome complexity of *P. aeruginosa* seems to be responsible for the versatility, environmental adaptation and intrinsic drug resistance exhibited by this organism.

Nosocomial infections related to *P. aeruginosa* are frequently caused by multi-drug resistant (MDR) strains (Sarwari *et al.*, 2004), which we also detected in our study (Table 1). However, no correlations between clonal groups and susceptibility profiles were detected. The same finding was observed when the different units of the hospital or the sites of infection were compared to the genotype profiles

(Table 1). Nevertheless, data from susceptibility tests and amplification profiles could be used to follow the spread of clonal groups through the hospital units. In addition, such data could be useful for analysis of the evolution of the phenotype of resistance to antibiotics, which may represent the expression of intrinsic mechanisms or the acquisition of resistance mechanisms by horizontal genetic transmission (Poole and Srikumar, 2001). Analysis of the distribution of antibiotic resistance could help in developing an effective control of nosocomial infections by identifying the origin of contamination. By assuming that all the isolates in the R1T1 group were genetically similar we were able to follow the spread of members of this group (and of the other seven groups) to the various hospital units and infection sites. This was the case for clonal group 16 (isolates 16, 17, 18, 20 and 34), members of which were detected in five different patients in distinct units of the hospital, indicating that members of this group were dispersed in different points within the hospital. On the other hand, some isolates seemed to be confined to a particular unit (Table 1). Our results support those of Gales *et al.* (2001) and suggest that informative molecular markers can be a powerful method for following the dispersion of clonal groups and to detect the occurrence of cross-infections (Blanc *et al.*, 1997; Ruiz *et al.*, 2004). Another example of informative data produced by our analysis is the identification of the two clones which were collected from the same patient at different times and in different hospital units, these clones showing the same antibiotic resistance phenotype but distinct tDNA profiles. These results indicate that phenotypic convergence for antibiotics resistance can occur in different bacterial strains submitted to similar selective pressure.

In conclusion, our results indicate that the use of rDNA and tDNA markers for *P. aeruginosa* typing was sufficiently discriminatory to generate very informative data regarding the clonal dispersion of nosocomial hospital strains of *P. aeruginosa*. Moreover, results from the duplex-PCR associated to antibiotic susceptibility analysis may be used as an epidemiological approach to prevent *P. aeruginosa* outbreaks.

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