IDENTIFICATION AND TYPING OF BACTERIA

Epidemiological investigation of *Pseudomonas aeruginosa* nosocomial bacteraemia isolates by PCR-based DNA fingerprinting analysis

Y. LIU, A. DAVIN-REGLI, C. BOSI, R. N. CHARREL and C. BOLLET

Laboratoire d'Hygiène et de Microbiologie et Comité de Lutte contre l'Infection Nosocomiale, Hôpital Salvator, 249 Bd Ste Marguerite, 13009 Marseille, France

Between July 1994 and March 1995, 64 isolates of *Pseudomonas aeruginosa* were implicated in bacteraemia in 25 cancer patients in five wards of two hospitals. These, together with 24 environmental isolates and one isolate from a bacteraemia in a non-cancer patient were examined by three PCR-based DNA fingerprinting methods: random amplified polymorphic DNA (RAPD), enterobacterial-repetitive intergenic consensus (ERIC)-PCR, and 16S–23S spacer region-based RAPD. These methods were reproducible, discriminatory and showed close agreement; all indicated that 47 isolates that had caused bacteraemia in 19 cancer patients were indistinguishable. Seventeen other isolates that had caused bacteraemia in 10 cancer patients were discriminated into eight further groups, and the 24 environmental and non-cancer patient isolates into further distinct groups. No environmental source of the epidemic strain was found, but it was suspected that the outbreak was related to infusion implants.

Introduction

Pseudomonas aeruginosa is an opportunist pathogen frequently isolated in the hospital from moist environmental sources and equipment [1]. Septicaemic infections are an important cause of mortality in immunocompromised patients [2], particularly in neutropenic cancer patients [1]. Investigations of the nosocomial epidemiology of *P. aeruginosa* have been hampered by the inadequate discriminatory capacity of classical phenotypic methods such as serotyping, phage typing, pyocin typing and biotyping. Modern DNA-based techniques, including restriction fragment length polymorphism (RFLP) demonstrated by hybridisation with species-specific coding regions such as the exotoxin A gene, the pilin gene and a broad-spectrum probe of *Escherichia coli* rDNA [3–7], macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) [5, 8–10] or field inversion gel electrophoresis (FIGE) [11, 12] and restriction endonuclease analysis (REA) [13] have been applied in epidemiological studies of *P.*

aeruginosa. However, these techniques are expensive, slow, and some require specific gene probes.

Of the PCR-based techniques, random amplified polymorphic DNA (RAPD) [14] is based on the amplification of DNA segments with a single primer of arbitrary nucleotide sequence, and has been widely used for the epidemiological investigation of many micro-organisms [15] including *P. aeruginosa* [10, 16, 17]. The 126-bp enterobacterial repetitive intergenic consensus (ERIC) elements contain a highly conserved central inverted repeat located in extragenic regions; this has been used as a primer in PCR (ERIC-PCR) to obtain DNA fingerprints for several bacterial species [18, 19]. The spacer regions separating the genes coding for 16S, 23S and 5S rRNA are characterised by a high degree of sequence variation at the genus and species level [20]. The diversity is mainly due to variations in the number and type of tRNA sequences found within the spacer. PCR amplification of spacer regions between the 16S and 23S RNA genes has been used for characterisation of a broad range of bacteria [20]. Spacer region PCR products of *Mycobacterium tuberculosis* strains have been used as target DNA for RAPD amplification that gave discriminatory results [21].

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Corresponding author: Dr C. Bollet.

In this study, RAPD, ERIC-PCR and 16S–23S spacer region polymorphism-based RAPD analysis were compared in the investigation of a possible outbreak of *P. aeruginosa* bacteraemia in cancer patients in two hospitals in Marseille.

Materials and methods

Isolates and patients

A total of 89 *P. aeruginosa* strains was selected for investigation. Sixty-four isolates from 25 cancer patients were implicated in bacteraemic episodes between July 1994 and March 1995. These patients had various malignancies, including breast cancer, cerebral tumour, lymphoma, hepatocellular carcinoma and uterine cancer, and all developed bacteraemia during chemotherapy administered *via* an infusion implant. They were in five wards (oncology, radiotherapy, gynaecology, dermatology and neurosurgery) at two hospitals. A further 24 strains from the hospital environment (water, siphon and surfaces) in these five wards, and one isolate implicated in bacteraemia in a non-cancer patient with no infusion implant, who was hospitalised for surgery in another ward, were collected during the same period.

Identification, susceptibility testing and O serotyping

All isolates were identified as *P. aeruginosa* according to colony morphology, Gram's stain appearance, oxidase reaction, growth at 41°C, and reaction patterns in the API 20 NE system (bioMérieux, Marcy l'Etoile, France) and API 100 carbon auxanogram strips (bioMérieux). Antibiotic susceptibility testing was performed with the Walkaway 40 System (Sanofi-Diagnostics Pasteur, Marnes-la-Coquette, France). O serotypes were determined by the International Antigenic Typing System (Diagnostic Pasteur) [22]. Reactions with commercial O antisera were controlled with reference strains.

DNA extraction

Isolates were grown in trypticase-soya broth at 37°C for 24 h and DNA was extracted by the Chelex technique as described by De Lamballerie *et al.* [23].

RAPD and ERIC-PCR conditions

Amplification reactions were performed in a total volume of 47 µl containing 100 µM each of dATP, dCTP, dGTP and dTTP, 0.2 µM of primer AP12H (5'-CGGCCCCCTGT-3') or ERIC-2 (5'-AAGTAAGT-GACTGGGGTGAGCG-3'), 25 ng of template DNA and 1.25 units of *Taq* polymerase (Perkin-Elmer/Cetus, Norwalk, CT, USA) in PCR buffer (20 mM Tris-HCl, pH 8.3; 50 mM KCl, 2.5 mM MgCl₂). A negative control without template DNA was included in each

experiment. The reaction mixtures were overlaid with mineral oil and subjected to amplification in a DNA thermal cycler (TR 2, Cera-Labo, Aubervilliers, France) programmed for 45 cycles of 1 min at 94°C, 1 min at 45°C and 1 min at 74°C. After the last cycle, samples were maintained at 74°C for 10 min. Amplification products were compared by electrophoresis of 10-µl samples on agarose 1.2% gels (Eurogentec, Angers, France) in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.2), stained with ethidium bromide and photographed on a UV light transilluminator. The presence or absence of bands in a gel lane was determined by visual inspection of the patterns independently by two observers. Fingerprint patterns were interpreted without knowledge of epidemiological data. Fingerprint patterns were considered similar (indistinguishable) if all bands had the same migration distance or if there were no more than two band differences for patterns comprising 6–11 products or three band differences for patterns comprising 12–18 products. These criteria for classifying isolates as indistinguishable or different were established in previous studies [24, 25]. Variation in intensity and shape of bands were not considered to be differences. In addition, photographs of the gels were scanned and analysed with a Bio Image wholeband system, Scanner 3+ (Millipore). Mol. wt standards were included (Marker V, Boehringer, Boehringer Mannheim, Germany). The mol. wts of sample bands were calculated automatically from reference bands allowing evaluation of mol. wts to *c.* 1%.

PCR amplification of 16S–23S spacer region and RAPD based upon this region

PCR was performed as follows: a 50-µl volume of reaction mixture containing 25 ng of isolate DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, Triton X-100 0.1%, 100 µM of each deoxynucleotide triphosphate (Boehringer Mannheim), 62.5 ng of each of two oligonucleotide primers G1 (5'-GAAGTCG-TAACAAGG-3') and L1 (5'-CAAGGCATCCACCGT-3') previously selected [20] and 1.3 units of *Taq* DNA polymerase (Perkin-Elmer/Cetus) was subjected to 25 amplification cycles of denaturation at 94°C for 1 min, annealing at 55°C for 7 min and extension at 72°C for 2 min. After the last cycle, samples were maintained at 72°C for 7 min. The PCR products (10 µl) were separated by electrophoresis on agarose 1.2% gel (Eurogentec, Angers) in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.2). The gels were stained with ethidium bromide and photographed on an UV light transilluminator. A mol. wt standard was included (Marker V, Boehringer). The remaining PCR product was precipitated with ethanol and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) for use as target DNA for RAPD; 15 ng of this, and 0.25 µM of primer AP12H or AP4 (5'-TCA CGA TGC A-3'), were amplified under the conditions described above for RAPD.

Reproducibility of DNA fingerprints

Fingerprint profiles generated from independent DNA preparations extracted from single-colony cultures at different times and amplified separately were run side by side on an agarose gel to determine the reproducibility of the three PCR-based DNA fingerprinting methods.

Results

Identification

All isolates grew at 41°C. Colonies from the 47 clinical isolates were rough and dwarf after growth for 24 h on blood agar. They were identified as *P. aeruginosa* with the API 20 NE system and all clinical isolates yielded the same pattern (0154575). All isolates showed the pattern characteristic of *P. aeruginosa* in API 100 carbon auxanogram strips.

Antibiograms

All clinical isolates were susceptible to azlocillin, piperacillin, ticarcillin, cefsulodin, ceftazidime, aztreonam, imipenem, gentamicin, tobramycin, amikacin and ciprofloxacin. They were of intermediate susceptibility to moxalactam, cefoperazone and aztreonam, and

resistant to chloramphenicol and trimethoprim-sulphamethoxazole.

Serotyping

All clinical strains were non-agglutinable. Of the 24 environmental isolates, eight were of serotype O6, two of serotype O1, one of serotype O10 and one of serotype O12; three were non-agglutinable and nine were polyagglutinable.

PCR analyses

In RAPD with the AP12H primer, nine distinct patterns were found. RAPD pattern 1 comprised 48 cancer patient isolates; patterns 2–5 comprised the remaining 16 isolates from cancer patients. The non-cancer patient isolate belonged to RAPD pattern 6, and the 24 environmental isolates yielded patterns 7, 8 and 9 (Fig. 1; Table 1).

ERIC-PCR yielded 12 typing patterns. Pattern 1 comprised 51 cancer patient isolates; patterns 2–8 comprised the remaining 13 cancer patient isolates. The non-cancer patient isolate gave pattern 9 and the 24 environmental isolates yielded three further patterns (Fig. 2; Table 1).

Fig. 1. Representative paired replicate lanes of the nine RAPD types obtained with primer AP12H. Each strain was selected to represent a specific type. Types 1–5 were clinical isolates; type 6 was a clinical control strain; types 7–9 were environmental isolates. Lane M, mol. wt marker (bp).

Table 1. Typing results of three PCR-based DNA fingerprinting methods for 64 *P. aeruginosa* clinical isolates from five wards

Number of patients	Number of isolates	RAPD pattern	ERIC-PCR pattern	16S–23S spacer RAPD pattern	Combined group
19	47	1	1	1	I
3	6	2	1, 5, 7	3	II
2	2	2	2, 6	2	III
1	3	5	3	3	IV
1	1	4	4	5	V
1	2	4	5	6	VI
1	1	1	8	3	VII
1	1	3	1	3	VIII
1	1	5	1	4	IX

Fig. 2. Representative paired replicate lanes of the 12 ERIC types obtained with primer ERIC 2. Each strain was selected to represent a specific type. Types 1–8 were clinical isolates; type 9 was a clinical control strain; types 10–12 were environmental isolates. Lane M, mol. wt marker (bp).

All isolates shared three common fragments of 356, 570 and 1033 bp in the 16S–23S spacer region amplification products and were indistinguishable (data not shown).

However, when RAPD with AP4 primer was performed with the amplified spacer products as the target, nine patterns were resolved. Pattern 1 comprised 47 cancer patient isolates; patterns 2–6 comprised the remaining 17 cancer patient isolates. The non-cancer patient isolate gave pattern 7 and the 24 environmental isolates were distributed among typing patterns 8 and 9 (Fig. 3; Table 1).

The profiles obtained with AP4 primer RAPD of the spacer-region products were less complex and easier to interpret than those obtained with RAPD of whole-cell DNA, or ERIC-PCR. However, RAPD of the spacer product with primer AP12H yielded a single uniform pattern, so the choice of the primer seems to be critical.

Reproducibility of DNA fingerprints

The reproducibility of fingerprint profiles from different DNA preparation of the same strain was investi-

gated by analysis of DNA from separate cultures extracted at different times. In each case, identical profiles were generated, although differences in band intensity were present.

Combined interpretation of the results of the three fingerprint methods

The results showed good agreement. In combination, the three methods allowed classification of the isolates into 13 groups. Group I comprised 47 isolates from 19 cancer patients in five wards of the two hospitals. Groups II–IX comprised the remaining 17 cancer patient isolates. Fig. 4 shows the distribution of the 64 clinical isolates in the five wards. The non-cancer patient isolate belonged to group X (Table 1). The 24 environmental isolates were distributed among three other groups (data not shown), and were considered to be clearly different from the clinical strains.

Epidemiological investigation

During 8 months, an epidemic type of *P. aeruginosa* was responsible for bacteraemia in 19 cancer patients during their chemotherapy. The results of this study

Fig. 3. Representative paired replicate lanes of the nine RAPD profiles with primer AP4 of the 16S–23S spacer region products of *P. aeruginosa* isolates. Types 1–6 were clinical isolates; type 7 was a clinical control strain; types 8 and 9 were environmental isolates. Lane M, mol. wt marker (bp).

Fig. 4. Diagrammatic representation of bacteraemic *P. aeruginosa* isolates from 25 cancer patients between July 1994 and March 1995 in five wards. Each symbol corresponds to a specific combined group: ■, I; ▨, VI(N) or VII(R); □, II; ▤, III; ▥, IV; ▦, V; ▧, VIII; ▨, IX. N, neurosurgery ward; R, radiotherapy; O, oncology; G, gynaecology; D, dermatology.

indicated that the *P. aeruginosa* bacteraemia cases might be related to the presence of infusion implants. These were implanted by two surgeons who shared the same materials and antiseptic solutions in the same unit. Contamination from the hands of one or both surgeons, the shared equipment or an environmental source could have explained the outbreak. However, no positive fingertip impression cultures from the surgeons, environment samples or equipment samples were obtained. As bacteraemia cases were observed 2 weeks–6 months after surgical implantation of the devices, contamination could have occurred during manipulation of infusion implants for the administration of anticancer therapy. However, bacteriological culture of some infusion implants removed from infected patients did not yield *P. aeruginosa*. Similarly, *P. aeruginosa* was not isolated from anticancer therapy infusates. No other case of *P. aeruginosa* bacteraemia was observed in these patients and the outbreak stopped after hospital personnel were informed about the risk of bacterial contamination during implantation and manipulation of infusion implants.

Discussion

Investigations of outbreaks of nosocomial infection due to *P. aeruginosa* are hampered by the inadequate discriminatory capacity of phenotypic markers, but with the advent of DNA-based techniques, stable and discriminatory epidemiological markers have become available. Typing systems used for epidemiological investigation should have a high degree of typability and reproducibility and be sensitive enough to distinguish between isolates that are similar but not identical. First DNA-based approaches used DNA

fragment patterns after restriction endonuclease cleavage [13]. Difficulties in interpretation of the complex banding patterns led to the use of specific gene probes or rare-cutting restriction endonucleases to generate patterns with few fragments. Chromosomal fingerprinting with DNA probes or macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) have been employed for epidemiological typing of *P. aeruginosa* [4–12]. However, Grundmann *et al.* were the first to compare the discriminatory power and the reproducibility of hybridisation of restricted chromosomal DNA with a specific (tox A) probe, ribotyping and PFGE [5]. They concluded that PFGE was the most powerful tool for studying the epidemiology of *P. aeruginosa* because of its high discriminatory capacity, good reproducibility and ease of interpretation. Moreover, Martin *et al.* [7] used ribotyping for molecular typing of strains of *P. aeruginosa* from patients with cystic fibrosis and observed a low degree of heterogeneity of rRNA operons. They considered that ribotyping would not be a valuable method for epidemiological studies with *P. aeruginosa*.

At the same time, the labour-intensive nature of macrorestriction typing prompted Kersulyte *et al.* to compare PFGE with a PCR-based typing method, arbitrarily primed-PCR (AP-PCR), which was easier to perform [10]. They found an excellent correlation between PFGE and AP-PCR. For a similar result with equivalent discrimination, AP-PCR presented advantages of rapidity and simplicity and was recommended by the authors for initial screening with many strains of *P. aeruginosa* are to be typed.

In the present study, three PCR-based DNA fingerprinting methods were compared for investigating the

epidemiological relationship between *P. aeruginosa* isolates that had caused bacteraemia in cancer patients. This paper reports the first application of ERIC-PCR and 16S–23S spacer region-based RAPD to the epidemiological typing of *P. aeruginosa* and the first comparison between three PCR typing methods. RAPD and ERIC-PCR methods generated reproducible fingerprint profiles from extracted DNA of isolates cultured at different time. However, experimental parameters such as DNA isolation methods, buffer composition, primer or DNA template concentration affect the production of reproducible fingerprints. Investigations of the reproducibility of random PCR typing methods have enabled us to standardise those parameters routinely in our laboratory [26, 27].

Combination of the results of the three methods suggested that most of the *P. aeruginosa* isolates causing bacteraemia in cancer patients were indistinguishable and represented an epidemic clone. It also showed that the 24 isolates from the environment of the cancer patients were dissimilar from the isolates causing bacteraemia. Consequently, no environmental reservoir of the outbreak strain was found, although it was suspected that the outbreak was related to infusion implants.

RAPD and ERIC-PCR are generally used together in our laboratory and they always gave results in close agreement. The results of this study have shown that the analysis of polymorphism of the 16S–23S RNA spacer region by RAPD is a useful alternative, despite the requirement of a double procedure. Despite problems of reproducibility, which can be circumvented with experience, RAPD and ERIC-PCR are very useful in the epidemiological investigation of nosocomial outbreaks of *P. aeruginosa* infection for most clinical laboratories.

In conclusion, the three PCR-based typing methods showed similar discrimination for *P. aeruginosa* isolates. PCR-based typing methods were simple to do and to interpret and had the advantages of typability and discriminatory capacity equivalent to macrorestriction analysis.

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