MOLECULAR TAXONOMY AND EPIDEMIOLOGY

The use of RAPD-PCR as a typing method for *Serratia marcescens*

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Serratia marcescens has emerged in the last few years as an important nosocomial pathogen. Many methods for typing this organism have been described. In this study the random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) was shown to be a convenient typing method for *S. marcescens*. Different combinations of primers previously used for typing other gram-negative bacilli were assessed. The combination of primer HLWL-74 and 1254 gave distinguishable patterns for different serotypes and proved to be the most satisfactory. By applying this combination to 175 isolates of *S. marcescens*, which could be classified into 38 groups on the basis of serotyping and phage typing, 73 different RAPD patterns with good reproducibility were obtained. This is, to our knowledge, the first application of the method to a large collection of *S. marcescens* representing a wide range of serotypes.

Introduction

Over the last 30 years *Serratia marcescens* has emerged as an opportunist pathogen and a cause of significant nosocomial infections of varying severity. These include urinary tract infection [1], meningitis [2], endocarditis [3] and septicaemia [4, 5]. Some of these infections have proved difficult to treat because the strains involved are resistant to a wide range of antibiotics [6]. Many outbreaks of infection have been caused by *S. marcescens*, of which the first was reported by Wheat *et al.* [7] in 1951, with most caused by the serotype O14, K14 [8, 9]. No common environmental source has yet been determined. It is essential to be able to distinguish between isolates to demonstrate the source of infection, and to determine the relationship between isolates from different patients and from the environment. It may also be important to characterise multiple isolates from the same patient to distinguish relapsing infection from re-infection. For this a satisfactory typing method is required.

Different methods have been used to type this organism including biotyping [10], antibiogram [8], ribotyping [11, 12], serotyping [13, 14], phage typing [15], bacteriocin production and susceptibility [8, 16], and protein fingerprinting [17, 18]. However, none of these methods has been found to be satisfactory because of insufficient discrimination, poor reproducibility,

lack of availability of specific reagents, or through technical difficulties. Others require a considerable amount of time, expertise or equipment; for example, pulsed-field gel electrophoresis (PFGE) which has been found recently to be of value in detecting an outbreak of infection due to *S. marcescens* in a neonatal intensive care unit [19].

The polymerase chain reaction (PCR) has been demonstrated as a useful method for typing many micro-organisms [20]. The PCR with random amplified polymorphic DNA (RAPD) analysis has been used successfully for typing different organisms, including some gram-negative bacteria [21–25]. However, the method has been applied to only a small number of isolates of *S. marcescens* [26].

S. marcescens originally emerged in a hospital served by our laboratory in the early 1980s as a cause of infection and in 1984 two simultaneous outbreaks were reported [27]. Following an interval of 3 years, *S. marcescens* re-appeared and has been isolated regularly from a range of patients in different hospitals served by the laboratory. Rapid discrimination between isolates is clearly important to the understanding of this new endemic organism. The present study assessed RAPD-PCR with different combinations of five 10-mer primers for this purpose. The most discriminatory combination was applied to the characterisation of 175 isolates of *S. marcescens*, some of different serological, phage and antibiotic susceptibility patterns, to build up a library of banding patterns for this organism.

Materials and methods

Strains and identification

The 175 *S. marcescens* isolates tested were from patients admitted to one of four Dublin hospitals over a period of 3 years (1993–1995). At least one isolate from each colonised or infected patient was included. All isolates were routinely identified in the diagnostic laboratory by the API 20E system. A short series of carbohydrate fermentation tests, sorbitol, raffinose and arabinose, was necessary to confirm the identification [10]. Urease and DNAase tests, as recommended by Grimont and Grimont [28], were also used.

Serotyping and phage typing

All isolates were sent to the Laboratory of Hospital Infection, Central Public Health Laboratory, 61 Colindale Avenue, London, for serological and phage typing.

The isolates used in the study were divided into different groups according to their serological types. Some of those of the same O and K serological types could be subdivided into subgroups according to their phage types (Table 1). Five strains were chosen on the basis of their serological and phage types to assess the combinations of five different primers (Table 2).

Extraction of genomic DNA

S. marcescens was harvested from a MacConkey agar plate, which had been incubated aerobically for 24 h at 37°C. The bacteria were washed twice in Tris-HCl buffer (pH 8.0) containing 10 mM EDTA (TE). DNA was extracted with phenol:chloroform:isoamylalcohol (PCI) (25:24:1) by a method similar to that described by Xia *et al.* [21]. Briefly, the pellet was resuspended

Table 1. *S. marcescens* isolates used in the RAPD-PCR study for the assessment of banding patterns

O serotype	K serotype	Phage type	Number of isolates	Number of RAPD patterns
14	14	1, 4, 10	16	5
14	14	NT	10	4
14	14	10	15	4
14	14	11 ...	11	2
14	14	4, 10	3	1
14	14	5, 10	2	1
14	14	9 ...	18	5
14	14	V	5	1
14	ND	NA	3	2
14	NT	NA	4	3
14	13	NA	2	2
16	3255	4	21	1†
16	3255	1, 4	4	1†
16	3255	NT	6	3*
16	3255	11	1	1
16	3255	4, 11	1	1
NT	14	NA	13	5
NT	3255	NA	3	2
NT	12	NA	1	1
NT	7	NA	1	1
NT	22	NA	1	1
NT	NT	NA	1	1
22	ND	NA	1	1
22	22	NA	1	1
5	15	NA	1	1
5	3255	NA	1	1
1	1	NA	1	1
2	13	NA	1	1
2	2	NA	3	3
2	ND	NA	1	1
6	14	NA	2	1
8	14	NA	2	1
9	14	NA	2	2
21	ND	NA	1	1
21	3	NA	4	4
21	14	NA	7	3
3255	3255	NA	4	4
1254	14	NA	1	1
Totals	13	12	175	73

NT, not typable; ND, not done; NA, not applicable; 11 ..., different phages shared with phage 11; 9 ..., different phages shared with phage 9; V, these isolates had variable phages which were different from the above.
*Two isolates of O16, K3255 that were not phage typable shared RAPD patterns with those of the same serotype susceptible to phage 4 and two others shared the RAPD pattern with those susceptible to phages 1 and 4. The remaining two were indistinguishable from each other but otherwise distinct.
†These two patterns were considered as subgroups because of only one band difference (Fig. 5).

Table 2. *S. marcescens* isolates used in choosing the most suitable combination of primers

Isolate no.	Susceptibility to									Phage type	O serotype	K serotype	Represented in lanes
	Amp	Imp	Ctx	Atm	Caz	Cip	Gen	Tic	Aug				
57	R	S	M	S	S	S	S	M	R	10	14	14	2 and 8 (Fig. 1), 2 (Fig. 2)
37	R	S	S	S	S	S	S	R	R	4	16	3255	3 and 9 (Fig. 1), 3 (Fig. 2)
73	R	S	S	S	S	S	S	S	R	11	3255	3255	4 and 10 (Fig. 1), 4 (Fig. 2)
207	R	S	S	S	S	S	S	S	R	1, 2	18	4	5 and 11 (Fig. 1), 5 (Fig. 2)
251	R	S	S	S	S	S	S	S	R	1, 2	18	4	6 (Fig. 2)
NCTC	R	S	S	S	S	S	S	S	R	NA	NT	14	6 and 12 (Fig. 1)

Isolates 207 and 251 are of the same strain and were isolated from a patient with septicaemia and from the blood bag from which he was transfused.

Amp, ampicillin; Imp, imipenem; Ctx, cefotaxime; Atm, aztreonam; Caz, ceftazidime; Cip, ciprofloxacin; Gen, gentamicin; Tic, ticarcillin; Aug, augmentin; NA, not applicable; R, resistant; M, moderately resistant; S, susceptible; NT, not typable.

in 450 μ l of TE buffer and lysed with 50 μ l of lysozyme (10 μ g/ μ l). After storage at room temperature for 30 min, 50 μ l of SDS 10% was added and the mixtures were incubated at 37°C in a water bath for 30 min. Proteinase K (10 μ l) was added to a final concentration of 2.5 μ g/ μ l and the tubes were incubated in a water bath at 56°C for 1 h with occasional mixing. DNA was extracted with PCI and precipitated by cold absolute ethanol (−20°C) and 0.3 M sodium acetate. The pellet was washed with cold ethanol (−20°C) 70% and subsequently resuspended in 30–200 μ l of sterile water depending on the quantity of DNA obtained. Two different methods of target preparation by boiling the cells, as described previously [24, 29], were also assessed. The DNA content and purity of DNA extracts were determined, for DNA extracted by either PCI or boiling the cells, by measuring the absorbance at 260 and 280 nm. Values for OD₂₆₀/OD₂₈₀ $\geq$ 1.7 were considered suitable for the RAPD-PCR.

PCR conditions

RAPD fingerprinting was by the method of Akopyanz *et al.* [30], in a final volume of 25 μ l containing 2.5 μ l of reagent buffer; 3 μ l of 25 mM MgCl₂ (final concentration 3 mM), 1 μ l of a mixture containing 10 mM each of dATP, dCTP, dGTP, dTTP (Boehringer Mannheim) to give the final concentration of 400 μ M, 20 pmol of each primer and 2.5 U of *Taq* DNA polymerase (Promega, WI, USA). Reagent buffer and MgCl₂ were supplied with the *Taq* polymerase. A bulk reaction mix was made, dispensed in 20- μ l volumes, overlaid with 40 μ l of sterile mineral oil and the tubes were exposed to shortwave UV for 10 min with the caps open. Extracted DNA (5 μ l containing 500 ng of DNA), was added under the mineral oil. A second experiment was performed with the same amounts of reagents except that 5 U of *Taq* polymerase were used instead of 2.5 U.

The amplification parameters were similar to those described by Riain *et al.* [31] and included four cycles of 94°C, 5 min; 36°C, 5 min; 72°C, 5 min, 30 cycles of 94°C, 1 min; 36°C, 1 min; 72°C, 2 min, and finally 72°C for 10 min. Electrophoresis of 15 μ l of the

mixture was done as described by Xia *et al.* [21] with agarose 2% gel in 0.5 $\times$ TBE buffer. A 100-bp DNA ladder (5 μ l; Gibco, BRL) was used as a size marker. DNA was detected by staining with ethidium bromide and gels were photographed under UV light.

Primers

Five primers used in RAPD typing of other gram-negative organisms were used for this study. These were primers 1281 (3'-AACGCGCAAC-5') [21, 22, 24], 1283 (3'-GCGATCCCCA-5') [21, 22, 24], PH1 (3'-TCACGATGCA-5') [23], 1254 (3'-CCGCACC-CAA-5') [24] and HLWL 74 (3'-ACGTATCTGC-5') [25]. To determine the best combination, all primers were applied to five strains, including *S. marcescens* NCTC 1193 (Table 2). The primers were applied individually and in all possible pair-wise combinations. Finally, a library of banding patterns was built up with the combination of HLWL-74 with 1254.

Reproducibility

To determine whether RAPD patterns for a strain were stable over time, DNA was extracted from four isolates – from nutrient agar slopes (NAS) kept at room temperature and from isolates stored in Protect beads (Technical Services Consultants Ltd, Bury, Lancs) at −70°C – on 3 days and subjected to the RAPD technique. In addition, different colonies of the same culture were tested by subculturing six colonies from the same pure culture and extracting the DNA on the following day and subsequently applying the technique. Lastly, a number of isolates of serotype O16 which were epidemiologically related were typed simultaneously.

Results

Identification

In the present study, only 85% of isolates gave an API 20E profile corresponding to a 'good identification' at the species level. Therefore, the identification was confirmed by arabinose fermentation in a separate tube [10].

Primer selection

The most suitable combination of primers was chosen on the basis of clarity and number of bands yielded for a selection of isolates. None of the five primers applied individually to the strains shown in Table 2 yielded any bands. Initially the combination of primers 1281 and 1283 was applied on two different occasions to nine (seven *S. marcescens* and two *S. liquefaciens*) and 13 (12 *S. marcescens* and one *S. liquefaciens*) isolates. This combination gave good results for the discrimination between different species and different serological types, but not between different strains of the same serotype. However, some of the primer combinations appeared discriminatory for some of the strains listed in Table 2 but not for all. This can be seen in Fig. 1 which shows the use of the combinations of HLWL-74 and 1281, and HLWL-74 and 1283. The bands obtained with these and other combinations were not sufficient to allow comparison between the strains. With some combinations some strains did not produce any bands.

Fig. 2 shows the result of using the combination of HLWL-74 and 1254 which was applied to the five strains shown in Table 2. The banding patterns were clearly distinguishable for different serotypes. Two related isolates yielded identical patterns. These two isolates were from a patient with a *S. marcescens* septicaemia and from the blood bag from which he was transfused. Because of its apparent usefulness, this combination of HLWL-74 and 1254 was used subsequently to build up the library of different banding patterns, discernible by eye, of 175 *S. marcescens* isolates.

Establishing the library of banding patterns

On the basis of one or more band differences, 23 different banding patterns were obtained for the 80 isolates of serotype O14, K14 which had been subdivided into eight groups on the basis of their

Fig. 1. Different primer combinations for the RAPD analysis of strains shown in Table 2. Lanes 1, 14, markers; 7, 13, negative controls; 2–6, combination of primer HLWL-74 with 1281; 8–12, combination of primer HLWL-74 with 1283; 6, 12, NCTC *S. marcescens*.

Fig. 2. The combination of primers HLWL-74 with 1254 for RAPD analysis of *S. marcescens*. Lanes 1, 8, markers; 7, negative control; 2–4, different O serotypes (57, 37, 73: Table 2); 5, 6, isolates from a recent septicaemia case (five from the patient and six from the blood bag: 207 and 251 in Table 2).

phage susceptibility. If patterns were considered different on the basis of at least two band differences, 12 distinct banding patterns were recognised for these 80 isolates. Some strains of the same phage type gave different banding patterns while others were indistinguishable or differed by not more than one band.

Among 33 isolates of serotype O16, K3255, five banding patterns which differed by one or more bands were obtained. However, 29 isolates differed only in one reproducible band and so could be classified into two subgroups (containing 23 and six isolates, respectively). Two of the remaining four isolates were indistinguishable from each other and two more gave different banding patterns to these and to each other. Therefore, on the basis of at least two band differences, four RAPD types could be assigned to this serotype. Other isolates of different O and K serotypes gave banding patterns which differed by at least two bands from each other and from those given by the O14 and O16 isolates. In all, 73 different banding patterns were obtained, or 61 patterns differing by two or more bands, for the 175 isolates of *S. marcescens* tested.

Reproducibility

Extracting the DNA from a single organism grown and stored on NAS on three occasions did not yield reproducible results for the four strains tested, as shown in Fig. 3a. In contrast, extraction of DNA from the same strains stored at -70°C yielded reproducible results (Fig. 3b) with the exception of one strain (Fig. 3b lanes 3–5). Extraction of DNA from six colonies of the same culture of two strains resulted in good reproducibility (Fig. 4). Lastly, isolates of the same

Fig. 3. Effect of storage on RAPD patterns. Lanes 1, 15, markers; 14, negative control; 2–13, four strains stored (a) on NAS or (b) at -70°C from which DNA was extracted three times.

strain defined by another typing method (O16 serological type) gave the same banding patterns when they were typed simultaneously (Fig. 5).

Discussion

A number of methods for typing *S. marcescens* have been described previously including biotyping, serotyping, phage typing, antibiograms and bacteriocin typing [8]. Ribotyping has also been used and has been reported to be more discriminatory than biotyping, serotyping and bacteriocin typing and has equivalent discriminatory power to total DNA analysis [11, 12]. However, because of technical difficulties and the prolonged time needed for southern blot analysis, its clinical use has been limited. A PCR-based method

which amplifies DNA between enterobacterial repetitive intergenic consensus (ERIC) sequences has been applied successfully to *S. marcescens* [29]. Biotyping cannot always distinguish between epidemiologically unrelated strains [8]. Many strains give very similar reactions while others can give irregular results for certain tests like the arabinose fermentation test in the API 20E system. However, this test gives reproducible results in peptone water separately from the API 20E system. Bacteriocin typing has been useful for typing other species, but when applied to *S. marcescens* it has not always been able to distinguish between epidemiologically unrelated strains [8]. Phage typing may be of value only in subdivision of strains of the same O group from the same incident of infection [10]. Serotyping is the standard method available for typing *S. marcescens*, but has some limitations which lie in

Fig. 4. RAPD analysis of different colonies of the same strains. Lanes 1, 15, markers; 14, negative control; 2–7, different colonies of the first strain; 8–13, different colonies of the second strain.

Fig. 5. RAPD analysis of isolates of a single strain, defined by serotyping (O16, K3255). (a) Lanes 1 and 20, markers; 2, negative control. (b) Lanes 1 and 20, markers; 19, negative control. Different strains were clearly distinguishable: (a) lanes 7, 8, 16; (b) lanes 5, 7, 8, 12, 13, 18.

the tedious determination of both O and K antigens and the presence of untypable mutants within a strain [8].

The RAPD-PCR method (which is sometimes referred to as arbitrarily-primed PCR), has not been used, to our knowledge, to type this organism except for the recent study by Bosi *et al.* [26] which was applied to only a small number of isolates. The RAPD method is straightforward and does not require previous genetic

knowledge of the target organism, and there are few technical difficulties. Furthermore, it is very quick and convenient to perform, as a strain can be typed within 48 h of harvesting the cells from the agar medium. Although some authors have boiled the cells to extract the DNA [24, 29] in order to simplify the method, the present study was not able to detect any PCR products with two such methods, although extraction of the DNA by PCI followed by alcohol precipitation was always successful.

Clearly the successful use of RAPD-PCR depends on the choice of the primers [32]. The application of the combination of HLWL-74 and 1254 to all isolates (Table 1) gave good results, except for the O21 isolates. Doubling the concentration of *Taq* polymerase appeared to give satisfactory results for this serotype and differentiated between different serotypes and between unrelated strains of the same serotype. This might be explained by the fact that, for some target sequences, increasing the *Taq* concentration results in a more efficient reaction and hence more product [33]. The ability of the technique to differentiate between and within a variety of serotypes is important because of the predominance of some serotypes, not only in these hospitals, but also in others [15].

The study showed that the technique is clearly reproducible on the basis of three parameters: testing isolates at different times; testing different colonies of the same culture; and, more importantly, testing the same serotypes from different patients. Interpretation of typing patterns obtained from any molecular method is subjective. Genetic events such as point mutations may change the DNA patterns by two or three bands, disguising a relatedness between two isolates [34]. Different centres are liable to give

entirely different interpretations of relatedness of strains [34]. The nearest that can be achieved in standard conditions is by running the DNA from the isolates under investigation on the same gel [35]. The number of band differences that can be allowed depends on the total number of bands obtained by the method used and the reliability and reproducibility of the method. However, normally more than two band differences are considered sufficient to distinguish between strains. To ensure reproducibility, the present study showed that isolates should be tested immediately after isolation from the clinical sample or stored at -70°C prior to DNA extraction. The discrimination afforded by RAPD and its reproducibility gives confidence in this approach to typing *S. marcescens* and indicate that this method should facilitate future epidemiological investigations.

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