Typing of *Listeria* spp. by random amplified polymorphic DNA (RAPD) analysis

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Summary. Random amplified polymorphic DNA (RAPD) analysis, a variation of the polymerase chain reaction (PCR) in which a single primer is used, was evaluated for use as a simple and reliable method with which to type *Listeria* spp. Representatives of six species of *Listeria* were studied. Five isolates of *L. innocua* and four isolates of *L. seeligeri* were all distinguishable from one another, but the four isolates of *L. ivanovii* tested, although distinguishable from other *Listeria* spp., were not differentiated. Among *L. monocytogenes* serovars 1/2a (eight isolates), 1/2b (eight isolates) and 4b (10 isolates), at least six, three and six RAPD patterns were observed, respectively. Fourteen neonatal cross-infection sets of *L. monocytogenes* isolates, shown to be indistinguishable by serotyping and phage typing, were examined with three different primers. With one primer, three of the sets were shown to consist of closely related, but distinguishable, strains. In the other 11 cases, each set of strains was indistinguishable with all three primers. These preliminary data indicate that RAPD analysis has promise as a method for typing *Listeria* spp.

Introduction

Typing of *Listeria* spp., especially *L. monocytogenes*, is of considerable importance in the surveillance of possible community food-borne outbreaks or nosocomial cross-infection episodes of listeriosis, and as a research tool.^{1,2} Several typing systems have been developed, but none has given more than a partial understanding of the ecology and epidemiology of *Listeria* spp. Biotyping, resistogram and monocolin typing seem to be of little value, or have not been investigated extensively.^{3–5} Serotyping has been used since the early 1940s,⁶ and seems to be a reliable, but not especially discriminatory method. Although there are 13 serovars of *L. monocytogenes*, almost 90% of isolates in the UK from human infection belong to serovar 1/2a, 1/2b or 4b, with c. 60% belonging to serovar 4b.⁷ Accordingly, the level of discrimination achieved with serotyping is low.^{7,8} Bacteriophage typing has been employed to confirm clusters of community infection and hospital cross-infection, and to monitor human isolates.⁹ However, only c. 64% of human isolates obtained in the UK between 1967 and 1983 were typable—37% of serogroup 1/2 and 82% of serogroup 4.⁹ Phage typing has also been used to type *L. innocua*.¹⁰ Multilocus enzyme separation by electrophoresis,¹¹ and analysis of plasmid and chromosomal DNA, have also been evaluated. Deter-

mination of plasmid profiles provides useful epidemiological information, but only 1.7% of *L. monocytogenes* isolates from human infection in the UK were found to contain plasmids (unpublished observations). Although *L. monocytogenes* isolates from other countries, and *L. innocua* isolates have a higher incidence of plasmid carriage,^{12–14} the incidence of plasmid carriage is insufficient to serve as a base for a general typing method. Investigation of chromosomal DNA, either by restriction endonuclease analysis (REA) or by the use of digoxigenin-labelled DNA probes, has been used to investigate strains obtained from food and hospital outbreaks of listeriosis, and to distinguish strains of *L. monocytogenes* which cannot be phage typed. This approach has been shown to discriminate among isolates of *L. monocytogenes* serovar 4b.^{15–18}

Previous studies have shown that the polymerase chain reaction (PCR), with gene-specific primers, can detect *L. monocytogenes* present in or on foods.^{19,20} In a variation of the PCR, termed random amplified polymorphic DNA (RAPD) analysis,²¹ primers of arbitrarily chosen sequence, rather than two specifically designed primers, are used. Any short sequence will suffice and one or more can be used in a reaction mixture.²² The method has potential for typing since it exploits the fact that, for any given short oligonucleotide sequence, the genomes of bacteria and higher organisms are likely to contain many sequences with partial, rather than complete, homology to the

primer. Under non-stringent conditions the primer will anneal to these sequences with varying degrees of stability, as determined by the number of H-bonds that can be formed between the primer and a particular partially homologous sequence. If two such complementary sequences are located close together on the genome on opposite strands, and both have the same polarity, then PCR amplification of the intervening sequence can occur under conditions that permit the primer to anneal to both sequences. The distribution of these partially complementary sequences is random, and hence the result of PCR is a set of random amplified polymorphic DNA sequences.^{21, 23} Single base changes may destroy the ability of a sequence to anneal to the primer, or may create a new primer binding site. Hence, the pattern of amplified sequences obtained is primer and strain specific, and constitutes an identity profile of the organism. Clonal identity is reflected in isolates having the same band patterns with any particular primer. Genetic drift is seen as discrete changes to the band patterns.²⁴ Different species yield different band patterns.²⁵ In this paper we report on the application of RAPD analysis to the typing of *Listeria* spp.

Materials and methods

Bacterial strains

The following strains were used: *L. grayi* subsp. *murrayi* strain NCTC 10812; *L. grayi* subsp. *grayi* strain NCTC 10815; *L. monocytogenes* serovar 1/2a strains DMRQC 757, 875, 874, 581, 765, 737, 757, 911, 1048, 890 and 882; *L. monocytogenes* serovar 1/2b strains DMRQC 1018, 992, 983, 1018, 1066, 987, 1033, 1004, 1114 and W5462; *L. monocytogenes* serovar 4b strains DCES1, BRI C360, BRI C401, Frenchay 188, DMRQC 591, 524, 477, 270, 662 and 20; *L. monocytogenes* serovar 4b(x) strain DMRQC 3077; *L. ivanovii* strains W5379, W4379, ATCC 18118 and CIP 7843; *L. innocua* strains SLCC 4213, 4202, 5326, CIP 8011 and NCTC 11289; *L. seeligeri* strains CIP 4642, CLP 100100 and BDS1; *L. welshimeri* strain Abdn. In addition, the following sets of strains from neonates were examined. *L. monocytogenes* DMRQC strains 449, 504; 696, 697a; 834, 835; 856, 857; 976a, 977a; 1252, 1253; 1261, 1263; 1490, 1491; 1810, 1811; 1877, 1878; 1881, 1882; 2789, 2796, 2801; 2963, 2964, 2965 and 3200, 3199. These strains were related epidemiologically and by bacteriophage type.

Bacterial strain storage, culture and DNA preparation

Strains of *Listeria* spp. were stored on Dorset Egg Medium (Southern Group Laboratories, Corby) at 4°C or at -70°C on plastic beads. Cultures were streaked on to Tryptose Phosphate Agar (Oxoid) and incubated for 18 h at 37°C. Single colonies were picked, streaked on to the same medium and incubated for 24 h at 37°C. Several colonies were picked and

suspended in 15–20 µl of sterile water. The suspension was then boiled for 3–5 min to lyse the cells and release DNA. Heated samples were centrifuged for 20 s in a bench microfuge to pellet cell debris. The supernate was used directly as the source of DNA template. No attempt was made to quantitate DNA concentrations.

DNA primers

The primers used in this work (synthesised in the Department of Biochemistry, University of Bristol) were designed for DNA sequencing studies in other projects unrelated to this work and were chosen at random from several available. The primers used were: no. 1, 5'-TTATGTAAAACGACGGCCAGT-3' (universal primer); no. 2, 5'-GGGCGTTGTCGG-TGTTTCATG-3' (primer 43423); no. 3, 5'-AC-AGGTCCAACAAAAGCTGG-3' (primer 59644). Primer 1 is the universal forward sequencing primer; primer 2 is a sequencing primer for *Tn21*; and primer 3 is a sequencing primer for the *ampC* gene of *Citrobacter diversus* (primers 2 and 3 were obtained by courtesy of Miss Ramila Patel and Mr M. Jones, respectively, Department of Pathology and Microbiology, University of Bristol).

Amplification conditions

Amplification reactions were performed in a reaction mixture (final volume of 50 µl) containing: 10 mM Tris-HCl, pH 9.0; 1.5 mM MgCl₂; 50 mM KCl; gelatin 0.1% w/v; Triton X100 0.1% w/v; 200 µM each of dATP, dGTP, dTTP and dCTP (Pharmacia); 0.5 µM DNA primer; 0.1 unit super-Taq DNA polymerase (Strattech Scientific, Luton); and 5–10 µl of cell supernate (with H₂O added to 50 µl, as appropriate). A single primer was added to each reaction mixture, which was then overlaid with liquid paraffin (c. 20 µl). Amplification was performed in a Thermal Reactor (Hybaid, Teddington), with temperature ramping as follows: 94°C for 3 min to denature template DNA; four low stringency cycles of 94°C for 45 s, 26°C for 2 min, and 72°C for 2 min, with a ramp setting of 2; 30 cycles of 94°C for 45 s, 36°C for 1 min, and 72°C for 2 min (ramp default setting); and finally, 72°C for 5 min.

Analysis of PCR products

A loading mixture (comprising bromophenol blue 0.25% w/v and sucrose 40% w/v in distilled water) was added (2 µl) to 10 µl of the PCR mixture. The amplification products were then analysed by electrophoresis in agarose 1% w/v gels, with TBE buffer (0.089 M Tris base; 0.089 M orthoboric acid; 0.002 M EDTA; pH 8.0). DNA bands were visualised by soaking gels in a solution of ethidium bromide 0.5 mg/L and viewing over a UV-Transilluminator (LKB-Pharmacia). Results were recorded on Polaroid type 665 instant positive/negative film.

Results

Discrimination between *Listeria* species

Fig. 1 shows examples of RAPD profiles obtained with oligonucleotide primer no. 1 and representatives of *L. grayi* subsp. *murrayi*, *L. grayi* subsp. *grayi*, *L. seeligeri*, *L. welshimeri*, *L. innocua*, *L. ivanovii* and *L. monocytogenes* serovars 1/2a, 1/2b, 4b and 4b(x). Clear discrimination between each of the isolates examined was obtained.

Discrimination within species and serovars

Fig. 2 shows RAPD profiles obtained with five strains of *L. innocua* and four strains of *L. seeligeri*. The five *L. innocua* strains all gave similar but distinguishable profiles, as did the four strains of *L. seeligeri*. In contrast, the five strains of *L. ivanovii* tested gave profiles that were indistinguishable from one another (data not shown).

Figs. 3, 4 and 5 show the RAPD profiles of *L. monocytogenes* serovars 1/2a, 1/2b and 4b, respectively, all obtained with primer no. 1. At least six different profiles were apparent among the eight profiles from the serovar 1/2a isolates, at least three profiles were obtained from the eight serovar 1/2b isolates, and six profiles from the 10 serovar 4b isolates.

Fig. 1. Examples of RAPD profiles obtained with oligonucleotide primer no. 1 and different species of *Listeria*. Tracks: 1, molecular size markers (λ -BglII digest); 2, *L. grayi* subsp. *murrayi*; 3, *L. grayi* subsp. *grayi*; 4, *L. seeligeri*; 5, *L. welshimeri*; 6, *L. innocua*; 7, *L. ivanovii*; 8, *L. monocytogenes* serovar 1/2a; 9, *L. monocytogenes* serovar 1/2b; 10, *L. monocytogenes* serovar 4b; and 11, *L. monocytogenes* serovar 4b(x).

Fig. 2. Examples of discrimination by RAPD within species. Tracks: 1, molecular size markers (λ -BglII digest); 2-6, *L. innocua*; 7-10, *L. seeligeri*. Oligonucleotide primer no. 1 was used with each strain examined.

Fig. 3. RAPD analysis with primer no. 1 of *L. monocytogenes* serovar 1/2a isolates (tracks 2-9). Track 1 contains molecular size markers (λ -BglII digest).

Although these profile sets have bands of the same size, each is distinguishable from the others, while the core patterns appear to be serovar related.

Fig. 4. RAPD analysis with primer no. 1 of *L. monocytogenes* serovar 1/2b isolates (tracks 2-9). Track 1 contains molecular size markers (λ -BglII digest).

Fig. 5. RAPD analysis with primer no. 1 of *L. monocytogenes* serovar 4b isolates (tracks 2-11). Track 1 contains molecular size markers (λ -BglII digest).

Discrimination between and among epidemiologically-related *L. monocytogenes* isolates

Fourteen sets of *L. monocytogenes* isolates from neonates were examined with all three primers. The strains in each set were indistinguishable with primers 1 and 2. Primer no. 3 identified three cases in which the

organisms that constituted the set were not identical (DMRQC strains 976a, 977a; 1881, 1882; and 2789, 2796, 2801) (fig. 6). The isolates that comprised the other sets were not distinguishable by this primer.

Discussion

No single typing system for *Listeria* spp. is ideal. The two main methods used until the mid-1980s were either not sufficiently discriminatory (e.g., serotyping) or left a significant number of strains untypable (e.g., 63% of serovar 1/2 strains and 18% of serovar 4b strains were not phage-typable).²⁶ While phage typing gives rise to a large number of patterns, serovar 1/2 strains form three main divisions and serovar 4b strains form five.⁹ Although the original typing phages were not useful for typing species other than *L. monocytogenes*, further panels have been developed that allow > 80% of *L. innocua* strains to be phage typed.¹⁰

Other methods have also been used. Multilocus enzyme electrophoresis proved of value in typing 300 strains of *L. monocytogenes*, producing 56 electrophoretic types, and this method has been used to investigate common source outbreaks.¹¹ However, it is both expensive and time-consuming. Plasmid typing is of little value in the UK (unpublished observations), but methods based on chromosomal DNA are more promising. Restriction endonuclease analysis (REA) offers discrimination, but complex DNA patterns are produced with multiple bands, which makes minor differences difficult to evaluate when REA digests are run on different gels.¹⁸ However, REA has been used successfully to examine pairs of strains and confirm the occurrence of episodes of neonatal cross-infection. REA has also indicated that serovar 4b(x) strains from a recent food-associated outbreak are indistinguishable, thereby adding to data obtained from monoclonal antibody and phage-typing studies.² In addition, 10 stable and reproducible REA profiles were produced from among 40 strains of *L. monocytogenes*,¹⁵ and it has been shown that it is possible to type non-phage-typable strains by this technique.²⁷ Gene probes have also been evaluated as typing aids,¹⁷ however, most serovar 4b isolates of *L. monocytogenes* from the UK have been shown to have the same profile.¹⁶ This method does have the advantage of producing gels which are simpler to interpret, but the technique is slightly more complex.

In the present study, RAPD analysis with randomly chosen primers was shown to discriminate between different species of *Listeria*, although this can also be done biochemically. However, RAPD analysis was capable of typing within the four main species of *Listeria*, and only *L. ivanovii* showed non-discriminatory patterns among the strains tested with a single primer. DNA products produced in RAPD analysis depend on the primer used, with different primers producing different banding patterns. Therefore, the

Fig. 6. RAPD analysis with primer no. 3 of epidemiologically related *L. monocytogenes* isolates. Tracks 1, 14 and 25 contain molecular size markers (λ -BglII digests). Tracks 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 26, 27, 28, 29, 30, 31, and 32, 33 represent, respectively, DMRQC isolates 499, 504, 696, 697a, 834, 835, 856, 857, 976a, 977a, 1252, 1253, 1261, 1263, 1490, 1491, 1810, 1811, 1877, 1878, 1881, 1882, 2789, 2796, 2810, 2963, 2964, 2965, and 3200, 3199.

more primers used, the greater the likelihood of demonstrating strain dissimilarities. Thus, among the 14 neonatal cross-infection clusters studied, three showed different banding patterns, but none was shown to be different with two of the primers used. It is possible that other primers would separate the pairs which were indistinguishable.

In conclusion, RAPD analysis promises a discriminatory, easy to interpret, and low cost method with

which to type *Listeria* spp. A more formal comparison with phage typing is required to validate the method, but the provisional data presented here indicate that RAPD typing is a valuable approach, either when used on its own or in conjunction with other established methods such as serotyping and phage typing.

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