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Use of Dcode™ System To Detect the Food-Borne Bacterial Pathogen *Listeria monocytogenes*

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Abstract

Amplification of a region of the 16S rRNA gene of *L. monocytogenes*, *Listeria innocua*, and *Listeria seeligeri* by the polymerase chain reaction (PCR)* using primers that anneal to conserved sequences, yielded multiple copies of gene fragments that, when evaluated by density gradient gel electrophoresis (DGGE), produced banding patterns that distinguished *L. monocytogenes* from the other two species. One set of primers yielded fragments from the 16S rRNA gene(s) of *L. monocytogenes* that gave rise to two distinct DGGE bands: one of which co-migrated with the single bands produced from the other two species, and another not produced by the other two species, which migrated to a location higher on the gel than the other band.

Introduction

Detection and quantification of food-borne pathogenic bacteria such as *Listeria monocytogenes* have historically depended on culture techniques that require an incubation period of several days. Use of procedures that involve culture and incubation for positive identification of causative agents of disease delays implementation of preventive action to control the spread of disease. New molecular techniques that provide positive identification of the causative agent of disease without the need to culture the suspect microorganism(s) facilitate a more rapid response to the problem. Using the polymerase chain reaction (PCR), positive identification of *L. monocytogenes* can be established within 12–48 h of sample collection.[1-3]

Numerous PCR protocols have been developed for detection of *L. monocytogenes*, but all utilize a primer set that is specific for this species. In such cases the primers typically target a hypervariable region of the 16S rRNA gene (16S rDNA), or a sequence of a gene coding for a virulence factor unique to this bacterium.[4] There are instances, however, where one may wish to screen for more than one type of bacteria in a sample, or for uncultivable bacteria whose unique sequences have not yet been identified. In such cases one can use a primer set that anneals to a sequence of the 16S rDNA that is common to many different types of bacteria. The PCR products from the different bacteria are the same size, but contain intervening regions where the base sequence is unique. The 16S rRNA of *L. monocytogenes* has 99.2 % and 98.5% nucleotide homology with the corresponding molecule of the closely-related species *L. innocua* and *L. seeligeri*, respectively. This corresponds to an 11 bp difference in the 16S rDNA of *L. monocytogenes* and *L. innocua*, and a 22 bp difference in the 16S rRNA molecule of *L. monocytogenes* and *L. seeligeri*.[5]

Density gradient gel electrophoresis (DGGE) can be used to separate DNA fragments of the same size when the fragments display differences in the sequence of the base pairs that yield different G+C content. These fragments may be resolved as discrete bands on a gel. This approach has been used to detect bacteria, which have not yet been cultured in the laboratory.[6] The different fragments responsible for each band in the gel can be sequenced and the sequence used to construct oligonucleotide probes specific to each type of organism present in the sample. In this note, we describe how the DCode system was used to detect a unique base pair sequence in a fragment of the16S rDNA of *L. monocytogenes* amplified by PCR using a general primer set and the Gene Cycler™. The approach permitted detection of *L. monocytogenes* in the presence of two closely-related species, *L. innocua* and *L. seeligeri*.

Methods and Materials

THEORY OF DGGE

DGGE is a technique that separates DNA fragments of the same length but with different guanine + cytosine (G+C) base content. Separation is based on the concentration of a denaturant such as urea or formamide needed to effect separation (melting) of double-stranded helical fragments into partially-separated, single-stranded fragments. The G+C content of the fragment determines the denaturant concentration at which domains of the fragment will melt. A partially-melted fragment migrates more slowly through a polyacrylamide gel than does an undegraded, helical, double-stranded DNA molecule. The denaturation reaction can be controlled by establishing a gradient of DNA denaturants such as urea and formamide within the gel. During

electrophoresis, the migrating double-stranded fragment begins to melt upon exposure of various domains of the fragment to an increasing denaturant concentration. Domains with a low %G+C will melt at a low denaturant concentration, while domains with high %G+C melt at higher denaturant concentrations further down the gel.

A 40 base pair (bp), GC-rich oligonucleotide referred to as a "GC-clamp" can be attached to the fragments using cloning techniques to retard migration of melted fragments through the gel to achieve better resolution of fragments with small differences in sequence variation. The denatured molecules with the same G+C content accumulate at the same location in the gel to form a discrete band.

Bands tend to sharpen as their rate of migration is reduced because molecules at the trailing edge of a band move faster than molecules at the leading edge of the band. As a result, DGGE appears to be less sensitive to loading conditions (volume of sample loaded, ionic strength of sample, etc.) than other polyacrylamide gel electrophoresis procedures.[7] To further sharpen the bands, an acrylamide gradient can be incorporated into the gel. After electrophoresis, the bands are visualized by reacting the gel with fluorogenic, chromogenic, or radioactive molecules that bind to DNA. Fragments containing sequences that differ by as little as one base-pair have been resolved in this manner.[6] This sensitivity offers the opportunity to detect and distinguish closely-related bacteria present in a sample.

There are two different ways to perform DGGE: perpendicular and parallel gels. Perpendicular gels have an increasing gradient of denaturants from left to right, perpendicular to the direction of electrophoresis. Parallel gels have an increasing gradient of denaturants from top to bottom, parallel to the direction of electrophoresis. Perpendicular gels are normally used to determine the optimal denaturant gradient to use in the gel, while parallel gels are normally used for final separation of different fragments. Determination of the denaturant gradient can also be achieved using parallel gels.

BACTERIAL DNA EXTRACTION AND QUANTIFICATION

Pure cultures of *L. monocytogenes* (ATCC 15313), *Listeria innocua* (ATCC 33090), and *Listeria seeligeri* (ATCC 35967) served as the source of 16S rDNA for this study. Broth cultures of the three species of bacteria were serially diluted and lysed by heating at 94 °C for 10 min and stored at -40 °C until used. DNA concentration in the various dilutions was determined by reacting 25 ml of sample with 5 ml of a 50 ul/ml volume of 4', 6-diamidino-2-phenylindole (DAPI), a fluorogenic molecule that selectively binds DNA. The fluorescence intensity of the reaction product was determined in a fluorescence spectrometer at 435 nm using an excitation wavelength of 360 nm. Calf thymus DNA was used as a standard.

POLYMERASE CHAIN REACTION

PCR was performed with a Bio-Rad Gene Cycler using Pfu™ or PfuTurbo polymerase. Two different primer pairs, obtained from Macromolecular Resources, Colorado State University, Fort Colins, CO, were used: 341F (with GC-clamp) and 534R, which target the V3 region and 1055 F and 1406 R (GC-clamp), which target the V9 region of the 16S rRNA gene. The DNA fragments produced using these primers contained sequences unique to *L. monocytogenes*. Amplification was carried out in a mixture containing 5 ml of 10x cloned Pfu polymerase buffer (Strategene, San Diego, CA), 50 mM of each dNTP (Sigma Chemical Co., St. Louis, MO) 0.6 mM of each primer pair, 1 ml containing 2.5 units of cloned Pfu or PfuTurbo (DNA polymerase Strategene, San Diego, CA), 1 ml of sample DNA, and deionized water (Sigma Chemical Co., St. Louis, MO) added to achieve a final reaction mixture volume of 50 ml.

To minimize non-specific annealing of the primers, Pfu or PfuTurbo polymerase was added after the first denaturing step, at a temperature of 80 °C, a technique referred to as "Hot Start".[6] Another technique, referred to as "Touchdown", was also used to reduce the formation of spurious by-products.[8] This involved setting the annealing temperature (T_a) 10 °C higher than the calculated primer melting temperature for the first cycle, then decreasing it by 0.5 °C every cycle for 20 cycles, and finally annealing at the T_m during 9 subsequent cycles. When the reaction was carried out with primer pair 341F (with GC clamp) and 534R, the annealing temperature (Touchdown) was set at 60 °C for the first cycle and decreased by 0.5 °C each cycle thereafter for 20 cycles, with the final 9 cycles performed at T_m of 50 °C as shown below.

"Hot Start" & "Touchdown" PCR

94 °C	3 min	
80 °C	1 min	
60 °C	1 min	Repeated 20 times, with the annealing temperature decreased by 0.5 °C every cycle
75 °C	45 sec	
94 °C	1 min	
50 °C	1 min	Repeated 9 times at T_m.
75 °C	45 sec	
94 °C	1 min	
55 °C	45 sec	
75 °C	10 min	

When the reaction was carried out with primer set 1055F and 1406R (with GC-clamp), the annealing temperature was initially set at 65 °C for the first cycle and decreased by 0.5 °C each cycle thereafter for 20 cycles, with the final 9 cycles performed at T_m of 55 °C as shown below.

"Hot Start" & "Touchdown" PCR

94 °C	3 min	
80 °C	1 min	
65 °C	45 sec	Repeated 20 times. The annealing temperature decreased by 0.5 °C every cycle
75 °C	2 min	
94 °C	1 min	
55 °C	45 sec	Repeated 9 times at T_m.
75 °C	2 min	
94 °C	1 min	
55 °C	45 sec	
75 °C	10 min	

In the final step of the reaction, the mixture was held at 75 °C for 10 min to allow extension of incomplete products.[9] Amplified products were analyzed by agarose gel electrophoresis as follows: 5 ml product was mixed with 1 ml gel loading buffer (40% sucrose, Mallinckrodt Inc., St. Louis, MO; 0.5% Brom Phenol Blue, Allied Signal Specialty Chemical, Michigan Center, MI) and the mixture applied to a 1% agarose low melt preparative grade gel (Bio-Rad Laboratories, Hercules, CA) in Tris-boric acid-EDTA buffer (89 mM Sigma 7-9 Tris[hydroxymethyl]aminomethane, Sigma Chemical Co, St. Louis, MO; 89 mM boric acid, Mallinckrodt Inc., St. Louis, MO; 2 mM ethylenediamine tetraacetic acid, Fisher Scientific, Pittsburgh, PA). Ethidium bromide (Bio-Rad Laboratories, Hercules, CA) was added to the agarose solution at a concentration of 0.5 mg/ml before the gel was poured in the mold. The gel was run for 1 hr at 50 V, and the PCR product was visualized under UV-light. The amplified products were stored at -20 °C until they were used for DGGE.

DGGE

DGGE was performed with a DCode denaturing gel electrophoresis system (Bio-Rad Laboratories, Hercules, CA). A parallel gel was prepared with a denaturing gradient ranging from 15 to 40% as described in Appendix 1 (where 100% denaturant contained 7 M urea (Bio-Rad Laboratories, Hercules, CA) and 40% formamide (Fisher Scientific, Pittsburgh, PA). The gel also contained a gradient of polyacrylamide ranging from 10 to 20%, made from a 40% stock solution of acrylamide/bisacrylamide (37.5:1) (Bio-Rad Laboratories, Hercules, CA). The gel was cast using a Bio-Rad Model 385 Gradient Delivery System (Bio-Rad Laboratories, Hercules, CA) and a non-denaturing, 6% acrylamide loading gel (Appendix 1) was cast on top of the gradient gel. The gel was placed in 0.5x strength Tris/Acetic Acid/EDTA buffer (TAE) (Bio-Rad Laboratories, Hercules, CA) equilibrated to 60 °C. Five µl PCR product was mixed with 5 µl loading buffer and applied to the gel wells. Electrophoresis was carried out at 60 °C for 15 h at 200 V. The gel was subsequently soaked for 30 min in 1/10,0000x strength SYBR Green I nucleic acid gel stain (Molecular Probes, Inc., Eugene, OR) and visualized under UV-light.

Results and Discussion

Primer pair 1055F and1406R with GC-clamp yielded a single band on agarose gel corresponding to a 400 bp DNA fragment when the PCR reaction was carried out with Pfu polymerase on DNA extracted from *L. monocytogenes*. Primer pair 341F with GC-clamp and 534 R yielded a single band corresponding to a 250 bp DNA fragment when the PCR reaction was carried out with Pfu polymerase in the presence of DNA from *L. monocytogenes*. That a single band was obtained with each primer pair indicates that only the target gene was amplified, with no non-specific primer attachment or hetroduplexes formed.

PCR of DNA extracted from different dilutions of the three *Listeria spp.* was undertaken to determine the minimal number of cells required for successful amplification of the conserved region of their 16S rRNA gene using PfuTurbo polymerase. A detectable PCR product was obtained using either set of primer pairs with as little as 1.3 ng DNA from 1.3×10^4 colony forming units (cfu) of *L. innocua*. A detectable PCR product was obtained with as little as 1.6 ng DNA from 5.2×10^3 cfu of *L. monocytogenes* using primer pair 1055F and 1406R with GC-clamp. Ten times this amount of *L. monocytogenes* DNA was required to obtain a detectable PCR product using the primer pair 341F with GC-clamp and 534R. A detectable PCR product was obtained with as little as 7.7 ng DNA from 6.9×10^3 cfu of *L. seeligeri* using primer pair 1055F and 1406R with GC-clamp, whereas, the other primer pair required ten times this amount of *L. seeligeri* DNA for a detectable product. The minimum quantity of DNA from these *Listeria spp.* that was required for successful amplification of fragments by PCR was generally less than that (25–500 ng) recommended by the suppliers of the polymerase.

L. monocytogenes was distinguished from *L. innocua* and *L. seeligeri* when fragments of their 16S rRNA gene(s), amplified by PCR using PfuTurbo polymerase and primer pair 1055F and 1406R (with GC-clamp), were resolved as discrete bands by DGGE. This primer set yielded two bands rather than one band for *L. monocytogenes* (Figure 1). One of the two bands migrated to the same location in the gel as the single band obtained from *L. innocua* and *L. seeligeri*. The second band produced from a 16S rDNA fragment from *L. monocytogenes*, which was not obtained from the other two species, migrated more slowly through the gel than the band common to all three species. The two bands suggest that *L. monocytogenes* has two 16S rRNA genes with slightly different sequences within the targeted region. This phenomenon is referred to as "sequence heterogeneity."[9] It has long been known that bacteria have more than one gene coding for 16S rRNA to increase the rate of ribosome

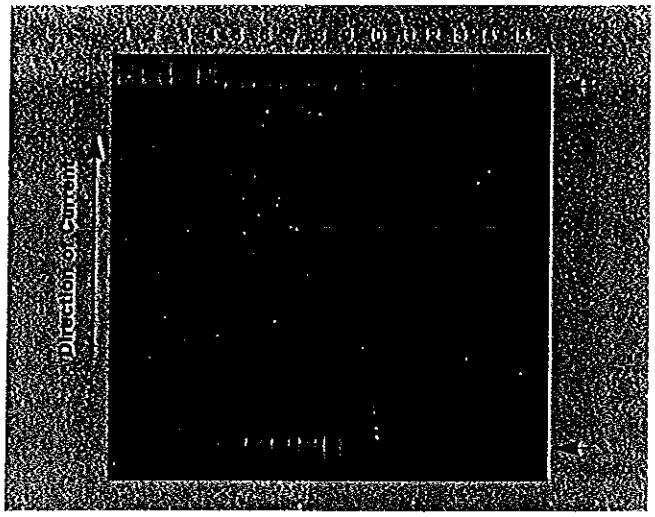

Fig. 1. DGGE of 16S rDNA fragments from L. innocua *(lanes 1-4),* L. monocytogenes *(lanes 5-9),* L. seeligeri *(lanes 10-14) and the 3 combined (lane 15). General bacterial primer set 1055F/1406R (with GC-clamp) was used to obtain fragments. Horizontal arrows show location of bands.*

production in the cell. Normally the genes have the same sequence but for reasons not yet understood, some genes within the same organism have different sequences that, when transcribed, yield a ribosome with a slightly altered structure.

Alignment of the base sequences of the 16S rRNA molecules from the three *Listeria spp.* for the region corresponding to the gene fragment targeted by the primer pair 1055F and 1406R with GC-clamp reveals the 3 bases that differ in the fragment obtained from *L. monocytogenes* (Figure 2). This difference yields a DNA fragment with lower %G+C, melting at a lower denaturant concentration to produce a DGGE band that migrates a shorter distance through the gel than the bands produced from fragments from *L. innocua* and *L. seeligeri*. DGGE therefore appears to be quite sensitive to slight differences in base sequence.

Co-migration of gene fragments from different organisms to the same location on the gel, as displayed in Figure 1, does not necessarily imply that the fragments share the same sequence. Using a different primer pair (341F with GC-clamp and 534 R), we produced fragments from another region of the 16S rDNA from *L. innocua* and *L. seeligeri*, which differ in sequence by 2 bases (Figure 3). In this case however, no difference in the migration rate and final gel positions of the respective DGGE bands was observed (Figure 4). Co-migration may be explained, in this instance, in that the base differences did not change the %G+C of the melting domain in the fragments.

The PCR fragments generated from the DNA of *L. innocua* and *L. seeligeri*, using primer pair 1055F and 1406R with GC-clamp, also migrated to the same location in the gel (Figure 1). Examination of the base sequences of the fragments from these two species reveals sequence homology where base assignments have been made (Figure 2). Since base assignments have yet to be made at 9 locations on the fragment, whether the co-migrating fragments possess sequence homology or the same G+C content remains to be determined.

As was the case with primer pair 1055F and 1406R with GC-clamp, the gene fragment obtained from *L. monocytogenes* with primer pair 341F with GC-clamp and 534 R yielded a DGGE band that was distinguishable from the bands obtained from the other two *Listeria spp.* (Figure 4). The base sequence of the fragment from *L. monocytogenes* differed from that of *L. innocua* by no more than 2 bases and no less than 1 base, and from that of *L. seeligeri* by no more than 1 base (Figure 3). The difference in migration rate of the band from *L. monocytogenes* compared to that of the bands from the other two species may be due to a difference in %G+C of the fragments arising from the possible base differences cited above. This remains to be confirmed, however.

The general bacterial primers used in this study amplify target sequences within the 16S rRNA gene of many different bacteria. Competition for primer, enzyme and dNTPs often occurs with DNA from the different bacteria in the sample

5' GAUAGUACAAAGGGUCGCGAAGCCGCGAGGUGGAGCUAAUCCAUAAAACUAUUC 3' *L. monocytogenes*
5' GAUGGUACAAAGGGUCGCGAAGCCGCGAGGUGGAGCCAAUCCAUAAAACCAUUC 3' *L. innocua*
5' GAUGGUACAAAGGGUCGCGAAGCCGCGAGGUGGAGCCAAUCCAUAAAACCAUUC 3' *L. seeligeri*

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Fig. 2. Segment of 16S rDNA fragment amplified by PCR using primer pair 1055F/1406R (with GC-clamp) where differences in base sequence occur for the three Listeria spp. *Sequence data obtained from Collins et al. (1991).*

5' CUGUUGUUAGAGAAGAACAAGGAUAAGAGUAACUGCUGCUKGUCC 3' *L. monocytogenes*
5' CUGUGGUUAGAGAAGAACAAGGAUAAGAGUAACUGCUGCUUGUCC 3' *L. innocua*
5' CUGUUGUUAGAGAAGAACAAGGAUAAGAGUAACUGCUGCUGGUCC 3' *L. seeligeri*

450

Fig. 3. Segment of 16S rDNA fragment amplified by PCR using primer pair 341F (with GC-clamp)/534R where differences in base sequence occur among the three Listeria spp. *Sequence data obtained from Collins et al. (1991).*

during PCR amplification. When the copy number of a gene from one type of bacterium is considerably higher than the copy number of the same gene from another type of bacterium (*i.e.*, an abundance of cells of one species over cells of another), the gene with the greater copy number will be amplified preferentially over the gene present in lower copy number. An experiment was conducted to determine the minimal ratio of *L. monocytogenes* DNA to *L. innocua* and *L. seeligeri* DNA that still enabled detection of the unique band from *L. monocytogenes* in DGGE gels after fragment amplification by PCR using PfuTurbo polymerase and primer pair 1055F and 1406R with GC-clamp. The results indicate that *L. monocytogenes*-specific band could still be detected in the presence of 2–3 times its weight of DNA contributed by the other two species. When the difference in relative amounts were above this, the *L. monocytogenes*-specific band was undetectable.

In summary, DGGE using the DCode system facilitates detection of variations in 16S rDNA sequence that result in different G+C content among closely-related bacterial species as well as among multiple alleles of the same gene in a single species. Separation based on subtle differences in G+C content by DGGE into discrete visible bands facilitates subsequent isolation of fragments from the gel for further PCR amplification and sequencing.

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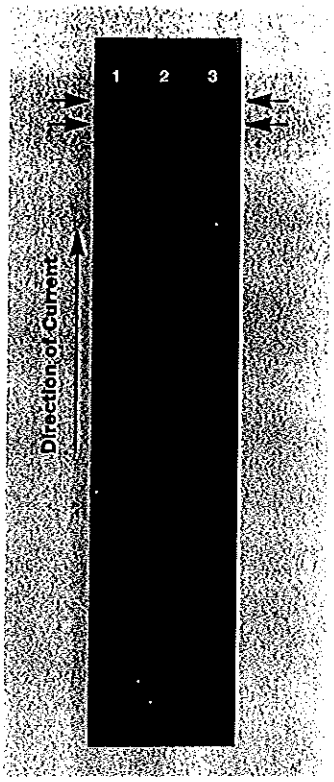

Fig. 4. DGGE of 16S rDNA fragments from L. innocua (lane 1), L. monocytogenes (lane 2) and L. seeligeri (lane 3). General bacterial primer set 341F (with GC-clamp)/534R was used to obtain fragments. Horizontal arrows indicate location of bands.

Appendix

15% denaturant / 10% Acrylamide
5% Formamide (Fischer Scientific)
1.05 M Urea (Bio-Rad)
10% Acrylamide (40% Acrylamide/Bis solution 37.5:1, Bio-Rad)
0.5x TAE (50x Tris/Acetic Acid/EDTA, Bio-Rad)
0.03125% APS Ammonium Persulfate (Bio-Rad)
0.0625% TEMED N,N,Nl,Nl-Tetramethylethylenediamine (Bio-Rad)
Dissolved in Millipore water: 6.306 g urea, add 6 ml Formamide, 25 ml 40% acrylamide, 1 ml of 50x TAE, and bring final volume to 100 ml with Millipore water. APS and TEMED are added just before use.

40% denaturant / 20% Acrylamide
Dissolve in Millipore water: 16.8168 g urea, add 16 ml Formamide, 50 ml 40% acrylamide, 1 ml of 50x TAE and bring final volume to 100 ml with Millipore water. The APS and TEMED are added just before use.

* *The Polymerase Chain Reaction (PCR) Process is covered by patents owned by Hoffman-LaRoche. Use of the PCR process requires a license.*