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Species-Specific Detection of *Listeria monocytogenes* by DNA Amplification

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The polymerase chain reaction was used to detect and specifically identify *Listeria monocytogenes*. A 174-bp region of the listeriolysin O gene was shown to be specifically amplified in *L. monocytogenes* but not in other species of *Listeria* or in a number of other gram-positive and gram-negative organisms. Less than 50 organisms could routinely be detected by a procedure involving two rounds of 35 amplification cycles each and without the need for subsequent hybridization with labeled probes.

Food and dairy products contaminated with *Listeria monocytogenes* have been implicated as the source of several recent severe outbreaks of listeriosis (6, 14, 19). However, questions regarding the epidemiology of the disease, the extent of food contamination, and the importance of the foodborne route of transmission remain unanswered partly due to the lack of simple, rapid, and sensitive isolation and identification procedures for *L. monocytogenes*. Standard methods which rely on cultivation of presumptive *Listeria* colonies can take 3 to 4 weeks before a species identification is possible (12). Identification of *L. monocytogenes* by colony hybridization with a specific DNA probe has been reported (4, 5), but here also prior cultivation of the organisms was necessary. A sensitive, quantitative hybridization assay using riboprobes against listerial 16S rRNA has been described recently (16), although the specificity for *L. monocytogenes* was not reported. The polymerase chain reaction (PCR) (17) offers an alternative approach for the specific and sensitive detection of environmental organisms. For example, Bej et al. (1) were able to detect less than five coliform bacteria in 100 ml of water by using DNA amplification followed by hybridization with radiolabeled probes. Similarly, Starnbach et al. (20) have applied PCR to the detection of *Legionella pneumophila* in water samples. In this study, we show that the PCR reaction can be used specifically to detect very low numbers of *L. monocytogenes* without the need for prior enrichment or cultivation of the organisms. Recently, Bessesen et al. (2) have also applied PCR technology to the detection of *L. monocytogenes*, although they did not specify the primers used or use a two-stage amplification procedure as we describe below.

Bacterial strains used in this study are listed in Table 1. All strains were streaked onto Trypticase soy agar or blood agar plates and incubated overnight at 35°C, and DNA was purified from isolated colonies by two procedures. In the first procedure, three to four colonies were suspended in 50 μl of 10 mM Tris–1 mM EDTA (pH 8.0)–20% sucrose with 10 mg of freshly added lysozyme (Sigma) per ml. After incubation at 37°C for 45 min, 150 μl of 10 mM Tris–1 mM EDTA (pH 8.0)–1% sodium dodecyl sulfate (SDS)–proteinase K (1 mg/ml; GIBCO, Burlington, Ontario) was added and incubation was continued for 1 h. Proteins were removed by phenol-chloroform extraction (13), and the DNA was precipitated by adding one-third volume of 7.5 M ammonium acetate and 2 volumes of ethanol. DNA was collected by centrifugation and resuspended in 20 μl of filter-sterilized water. For the second method, two to three colonies were suspended in 25 μl of the Tris-EDTA-sucrose-lysozyme solution described above, incubated at 37°C for 45 min, and then mixed with 75 μl of Tris-EDTA-SDS with 5 mg of proteinase K per ml. This suspension was incubated for 1 h at 37°C followed by 15 min at 60°C. After cooling, 300 μl of 4 M NaCl was added, followed by 10 μl of Glass-milk suspension (GeneClean kit; Bio 101, La Jolla, Calif.). This mixture was incubated on ice for 10 min with occasional mixing to keep the Glass-milk in suspension and allow the DNA to bind to the glass particles. Subsequent washing of the Glass-milk suspension and elution of the bound DNA was exactly as described in the GeneClean procedure (Bio 101). Bound DNA was eluted in a total volume of 20 μl of sterile water. Both procedures gave DNA which was readily amplifiable by PCR and gave equivalent results, but the second method, while being slightly more labor intensive, was considerably faster and avoided the use of hazardous organic solvents. Other methods of DNA extraction, such as the direct lysis method of Li et al. (11), were found to give poor yields of PCR-amplified product in our hands. For some experiments, DNA from a known number of cells was extracted as follows. Bacteria were grown overnight in Trypticase soy broth, and total (viable plus nonviable) cell numbers were determined by counting cells in a Petroff-Hausser chamber (8). Cells were diluted appropriately in filter-sterilized water, and 1 ml of each dilution was centrifuged for 15 min at 16,000 × *g* to pellet the cells. These cell pellets were resuspended in Tris-EDTA-sucrose-lysozyme and processed as described above.

PCR amplification was performed by using a DNA thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, Conn.). The reaction mixture contained 1× PCR buffer [50 mM Tris (pH 8.0), 6.7 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 5 μM EDTA, 10 mM β-mercaptoethanol, 150 μg of bovine serum albumin per ml], 200 μM concentration of each of the deoxynucleoside triphosphates, 10 pM concentration of each of the primers, up to 20 μl of the DNA preparation (typically containing about 500 ng of DNA), and 3 U of *Taq* DNA polymerase (GIBCO) in a total volume of 50 μl. Template DNA was initially denatured at 95°C for 4 min, and then 35 cycles of PCR amplification were run under the following conditions: denaturation at 95°C for 45 s, primer annealing at 60°C for 45

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TABLE 1. Bacterial strains used

Strain	Source[a]	174-bp PCR product
Listeria monocytogenes		
35152 (H^+)[b]	ATCC	+
35152 (H^-)[b]	ATCC	+
15313	ATCC	+
19113	ATCC	+
43256	ATCC	+
90-106 (serotype 1/2b)	Clinical isolate, RUH	+
D-20 (serotype 4b)	Clinical isolate, RUH	+
058 (serotype 1/2b)	Clinical isolate, RUH	+
Listeria ivanovii	LCDC	−
Listeria innocua	LCDC	−
Listeria seeligeri	LCDC	−
Listeria grayi	LCDC	−
Listeria welshimeri	LCDC	−
Aeromonas hydrophila	Clinical isolate, RUH	−
Bacillus cereus	Clinical isolate, RUH	−
Bacillus subtilis 168	G. Spiegelman	−
Campylobacter jejuni	Clinical isolate, RUH	−
Clostridium difficile	Clinical isolate, RUH	−
Clostridium perfringens	Clinical isolate, RUH	−
Escherichia coli (enterotoxigenic)	Clinical isolate, RUH	−
Escherichia coli C600	G. Spiegelman	−
Lactobacillus acidophilus 137	L. Mead	−
Lactobacillus brevis BS031	L. Mead	−
Lactobacillus casei BS035	L. Mead	−
Lactobacillus frigidis NCIB8518	L. Mead	−
Leuconostoc mesenteroides BS0117	L. Mead	−
Micrococcus luteus	Clinical isolate, RUH	−
Propionibacterium acnes	Clinical isolate, RUH	−
Salmonella thompson	Clinical isolate, RUH	−
Salmonella typhimurium	Clinical isolate, RUH	−
Serratia marcescens	Clinical isolate, RUH	−
Shigella flexneri	Clinical isolate, RUH	−
Shigella sonnei	Clinical isolate, RUH	−
Staphylococcus aureus	Clinical isolate, RUH	−
Streptococcus pneumoniae	Clinical isolate, RUH	−
Streptococcus pyogenes	Clinical isolate, RUH	−
Vibrio parahaemolyticus	Clinical isolate, RUH	−
Yersinia enterocolitica	Clinical isolate, RUH	−

[a] ATCC, American Type Culture Collection, Rockville, Md.; RUH, Royal University Hospital, Saskatoon, Saskatchewan; LCDC, Laboratory Center for Disease Control, Ottawa, Ontario.
[b] H^+, Hemolytic strain 35152; H^-, nonhemolytic variant of 35152 (17).

s, and DNA extension at 72°C for 1 min, with a final extension at 72°C for 5 min. In some cases a second round of PCR amplification was performed by removing 10 μl of the amplified reaction products, adding buffer, deoxynucleoside triphosphates, primers, and *Taq* polymerase as described above, and subjecting this to an additional 35 cycles of amplification. Amplified products were detected by electrophoresing 15 μl of the reaction mixture through a 1.4% agarose gel in Tris-acetate-EDTA buffer (13) and staining with ethidium bromide.

Synthetic oligonucleotide primers (Regional DNA Synthesis Lab, Calgary, Alberta, Canada) were designed to amplify a 174-bp region of the *L. monocytogenes* listeriolysin O (*hlyA*) gene, based on the sequence reported by Mengaud et al. (15). We chose to target the *hlyA* gene because of its critical role in the virulence of *L. monocytogenes* (3, 7) and because it is apparently only present in two other species of

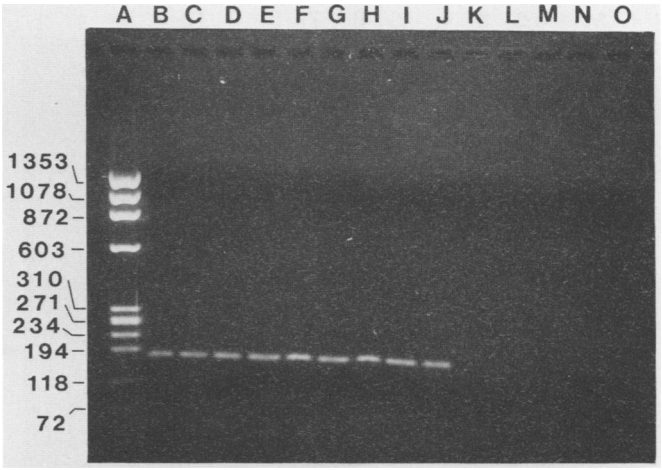

FIG. 1. Agarose gel (1.4%) electrophoretic analysis of PCR-amplified product from Glass-milk-purified DNA from various *Listeria* species, using primers Lis-1 and Lis-2. Lanes: A, molecular size markers with sizes, in base pairs, given on left; B, *L. monocytogenes* 35152 (H^+); C, *L. monocytogenes* 35152 (H^-); D, *L. monocytogenes* 35152 (mixture of H^+ and H^-); E, *L. monocytogenes* 43256; F, *L. monocytogenes* 15313; G, *L. monocytogenes* 19113; H, *L. monocytogenes* 90-106; I, *L. monocytogenes* D-20; J, *L. monocytogenes* 058; K, *L. grayi*; L, *L. welshimeri*; M, *L. ivanovii*; N, *L. seeligeri*; O, *L. innocua*. The amplified 174-bp DNA fragment from the *L. monocytogenes hlyA* gene is seen in lanes B to J.

Listeria, *L. ivanovii* and *L. seeligeri* (10). Primer Lis-1 (5'-GCATCTGCATTCAATAAAGA) was located between bp 130 and 149 and primer Lis-2 (5'-TGTCACTGCATCTCCG TGGT) was located between bp 284 and 303 within the coding region at the 5' end of the *hlyA* gene. This region was chosen because of its lack of homology with the corresponding streptolysin O and pneumolysin gene sequences (15).

The specificity of these primers was tested by amplifying DNA from various species of *Listeria* as described above. DNA from all strains of *L. monocytogenes* gave a single amplified product of the expected size (174 bp) (Fig. 1). Even the nonhemolytic variant of *L. monocytogenes* 35152 (18) showed a 174-bp fragment, indicating that the *hlyA* gene is present but nonfunctional in this strain. PCR applied to DNA from other species of *Listeria* failed to generate products of any size, including the DNA from *L. seeligeri* and *L. ivanovii*, two hemolytic species which have been shown to carry a gene homologous to the *L. monocytogenes hlyA* gene (10). This suggests that there is some sequence heterogeneity among the *hly* determinants of these species of *Listeria* and that the Lis-1 and Lis-2 primer pair is specific only for the *L. monocytogenes hlyA* gene. Only when the PCR reaction conditions were changed significantly (primer annealing temperature lowered below 50°C; annealing time of >1.5 min) could several nonspecific DNA fragments be weakly amplified from other species of *Listeria*, although none were in the 174-bp size range (data not shown). This would indicate some limited sequence homology between the Lis-1 and Lis-2 primers and chromosomal sequences from other *Listeria* spp. However, the use of the more stringent reaction conditions described above ensures that the Lis-1, Lis-2 primer pair is specific for *L. monocytogenes*.

The specificity of these primers was further demonstrated by attempting to amplify target sequences from a number of gram-negative and gram-positive bacteria (Table 1), includ-

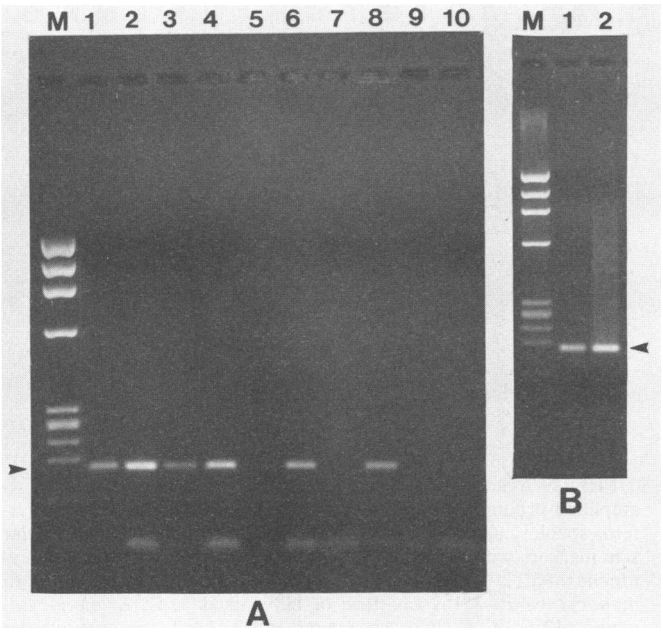

FIG. 2. (A) Agarose gel (1.4%) electrophoretic analysis of PCR-amplified products from Glass-milk-purified DNA derived from serially diluted *L. monocytogenes* 35152. Lane M, Molecular size markers with sizes as given in the legend to Fig. 1; lanes 1 to 10, amplified products from serially diluted cells. The first lane of each pair shows products after one round of 35 amplification cycles, while the second lane of the pair shows products after reamplification of 10 μl of the first sample for an additional 35 cycles (see text for details): 1 and 2, 5.42×10^4 cells; 3 and 4, 5.42×10^3 cells; 5 and 6, 542 cells; 7 and 8, 54 cells; 9 and 10, 5.4 cells. The location of the 174-bp amplified product is shown by the arrow. The lowermost band seen in some of the lanes is a "primer-dimer" artifact of the PCR reaction. (B) Amplification of *L. monocytogenes* 35152 DNA in the presence of nonlisterial DNA. Lane M, Molecular size markers; lane 1, amplified product (35 cycles) obtained from DNA from 5.42×10^3 *L. monocytogenes* cells mixed with 10^5 *E. coli* C600 cells and 10^5 *B. subtilis* 168 cells; lane 2, as lane 1 but 10 μl of the reaction material was reamplified for an additional 35 cycles (see text for details).

ing hemolytic bacteria, clinically relevant pathogens, and bacteria likely to be found in the same environmental niche as *L. monocytogenes* (food and dairy products). With the stringent reaction conditions described above, no amplified products were detected from any of the strains examined (Table 1).

As well as having the required specificity, a PCR-based detection system must be at least as sensitive as traditional culture methods in detecting low numbers of *L. monocytogenes* in environmental samples, often in the presence of nonlisterial organisms. In this case, sensitivity will be a function of the number of bacteria present as well as the efficiency of cell lysis and isolation of semipurified DNA suitable for amplification. Therefore, to determine the practical levels of sensitivity, *L. monocytogenes* 35152 was grown in Trypticase soy broth and the total (viable and nonviable) cell number was determined by direct counting. DNA prepared by the Glass-milk procedure from serially diluted cells was then used in the PCR assay and the products were visualized by agarose gel electrophoresis and ethidium bromide staining (Fig. 2A). The specific 174-bp fragment was faintly seen after a single round of 35 amplification cycles when as few as 542 bacteria were used as the starting material (lane 5, Fig. 2A). To see whether the sensitivity could be improved without resorting to dot-blotting and hybridization with labeled probes, an aliquot of the amplified material was removed and reamplified for a further 35 cycles after addition of fresh reagents (see above). This second round of amplification increased the sensitivity such that 54 cells could routinely be detected (lane 8, Fig. 2A). As few as 5.4 cells could sometimes be detected (lane 10, Fig. 2A), but this was not reliable or predictable. Therefore, the practical level of sensitivity with this two-phase amplification scheme must be considered to be between 5 and 50 cells. Finally, we observed that, even in the presence of large numbers of nonlisterial bacteria, the specificity and sensitivity of the PCR assay for *L. monocytogenes* were retained (Fig. 2B), indicating that the presence of excess competing DNA had no inhibitory effect. The effects of even greater numbers of nonlisterial bacteria on specificity and sensitivity will be examined further when the PCR assay is applied to actual food samples.

In summary, we have described the application of PCR technology to the detection of *L. monocytogenes*. Isolation of amplifiable DNA from low numbers of bacterial cells was easily and quickly achieved by adsorption to Glass-milk reagent, a procedure which would be applicable to other bacterial species as well. Oligonucleotide primers targeted to the *L. monocytogenes hlyA* gene were highly species specific and provide a means of easily differentiating *L. monocytogenes* from other hemolytic species of *Listeria*. The procedure outlined here is rapid, requiring <12 h to go from whole bacteria to a final result, and very sensitive when two rounds of amplification are performed. Sensitivity could potentially be improved by applying dot-blotting and hybridization with appropriately labeled oligonucleotide probes, but this is time-consuming and adds an additional level of complexity to the assay whereas we sought to keep this detection system as simple as possible. From a food safety point of view, however, care must be taken in interpreting results generated by PCR analysis since this technique is only semiquantitative (17) and cannot distinguish between viable and nonviable organisms. In addition, the use of a two-phase amplification system as described here requires that stringent measures be taken to prevent possible contamination with amplified products and the generation of false-positive results (9). We hope to refine the PCR assay so that more quantitative results are obtained and to adapt it for the direct examination of food and dairy products. Clearly, the overall utility of a PCR-based detection system can best be assessed by the direct testing of a variety of food products. Nevertheless, the rapid species-specific identification of *L. monocytogenes* as described here would be of benefit to the food industry as well as to health care workers in aiding both diagnostic and epidemiological studies.

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