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Research Note

Temperature Gradient Gel Electrophoresis of the Amplified Product of a Small 16S rRNA Gene Fragment for the Identification of *Listeria* Species Isolated from Food

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ABSTRACT

The development of a rapid method for the identification of *Listeria* spp. is described. It is based on the polymerase chain reaction amplification of a small fragment from the 16S rRNA gene followed by temperature gradient gel electrophoresis. Forty-five strains of *Listeria* spp. (*Listeria monocytogenes, Listeria innocua, Listeria ivanovii, Listeria seeligeri,* and *Listeria welshimeri*) were used for the optimization of the protocol. No differences were observed between the results of the identification of the strains tested using traditional methods and those obtained by polymerase chain reaction–temperature gradient gel electrophoresis analysis.

Listeria spp. have long been recognized as animal pathogens, and *Listeria monocytogenes* is now well established as a foodborne human pathogen. Previous results of DNA/DNA hybridization experiments unambiguously identified five genomic groups: *L. monocytogenes, Listeria ivanovii* (responsible for abortion in animals), *Listeria innocua, Listeria welshimeri,* and *Listeria seeligeri (18)*. This subdivision has proven its usefulness for practical and epidemiological purposes, especially when problems have emerged from food contamination at the origin of outbreaks of human listeriosis. In addition to this situation, the present status of *Listeria grayi* and *Listeria murrayi* remains unclear. On the basis of DNA homology, Stuart and Welshimer *(21)* proposed to consider them as a single species, a proposal that was later supported by numerical taxonomy results *(22)* but not considered when the Approved List of Bacterial Names was established *(20)*. Standard methods based on the cultivation of *Listeria* cells take 1 to 2 weeks before species identification is possible. The biochemical tests used rely on the expression of phenotype, which may not always allow discrimination between species or strains within a species. Because of the high phenotypic similarity between these species, only a small number of biochemical markers are routinely used to identify *Listeria* isolates *(17)*. Of these tests, hemolysis is the only marker that can be used to differentiate *L. monocytogenes* from *L. innocua,* but in some rare cases it is unsatisfactory *(1)*. Several molecular typing methods have also been applied to the genus *Listeria*: restriction endonuclease analysis *(9)*, multilocus enzyme electrophoresis *(2)*, pulsed-field gel electrophoresis *(8)*, randomly amplified polymorphic DNA *(4)*, repetitive element sequence-based polymerase chain reaction (PCR) *(10)*, and PCR on specific genes *(7, 14)*. This study describes the development of a new method, based on the amplification of a short fragment of the 16S rRNA gene and temperature gradient gel electrophoresis (TGGE) analysis, to identify *Listeria* spp. strains isolated from food.

MATERIALS AND METHODS

Bacterial strains. Two *L. monocytogenes* strains and one *L. innocua* strain came from the American Type Culture Collection (Manassas, Va.); two *L. monocytogenes* strains came from the National Collection of Typed Cultures (London, England); two *L. monocytogenes* strains came from the Collection Institut Pasteur (Paris, France); one *L. monocytogenes,* one *L. innocua,* one *L. ivanovii,* and one *L. seeligeri* strain came from the Istituto Lattiero Caseario (Lodi, Italy); one *L. monocytogenes,* one *L. innocua,* three *L. ivanovii,* two *L. seeligeri,* and one *L. welshimeri* strain came from the Istituto Lattiero Caseario (Thiene, Italy); two *L. monocytogenes* strains came from the Azienda Ospedaliera Policlinico di Modena (Modena, Italy); and six *L. monocytogenes,* seven *L. innocua,* three *L. ivanovii,* three *L. seeligeri,* and four *L. welshimeri* strains collected in our laboratory from meat, sausages, and cheese were used.

Listeria strains obtained from food were isolated by the traditional method according to the U.S. Department of Agriculture, Food Safety and Inspection Service *(3)*. This method consists of a first enrichment step in LEB1 (Oxoid, Milan, Italy) for 24 h at 30°C and a second subculture in Fraser broth (Oxoid) for 24 h at 37°C. Single colonies spread onto Oxford agar (Oxoid) were isolated and identified using morphological, cultural, and biochemical criteria as described in the U.S. Department of Agriculture, Food Safety and Inspection Service protocol. Api-Listeria (BioMerieux, Florence, Italy) was also used for biochemical tests. All

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strains were grown on brain heart infusion agar (Oxoid) for 24 h at 37°C and used to evaluate the PCR-TGGE method.

DNA extraction. The DNA was extracted from *Listeria* cells using proteinase K as described by Manzano et al. *(13)*. A total of 10 μl/ml of proteinase K (Roche Diagnostics, Milan, Italy) was added to 200 μl of a suspension containing 10^7 cells/ml and incubated at 65°C for 1 h. After this period, the suspension was boiled for 10 min and then kept at 4°C. A total of 5 μl was used for the PCR assay.

DNA amplification. Primers U968-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and L1401 (5′-GCG TGT GTA CAA GAC CC-3′) *(16)* were used to amplify the V6 to V8 regions of the *Listeria* spp. 16S rRNA. The GC clamp in primer U968-GC creates PCR products suitable for separation by TGGE *(15)*. The addition of a 30- to 40-bp GC clamp to one of the PCR primers ensured that the region screened was in the lower melting domain and that the DNA remained partially double-stranded. PCR was performed in a final volume of 50 μl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 1.25 U *Taq*-polymerase (Roche), and 10 pmol of the primers and the DNA template. The samples were amplified in a MiniCycler (MJ-Genenco, Watertown, Mass.) using the following program: 95°C for 5 min, 30 cycles of 94°C for 1 min, 42°C for 1 min, 68°C for 1 min, and finally 68°C for 7 min. Aliquots of 10 μl were analyzed by standard electrophoresis on a 2% (wt/vol) agarose gel containing ethidium bromide to verify the sizes and amount of the amplicons.

TGGE analysis. The DCode Universal Mutation Detection System (Bio-Rad, Richmond, Calif.) was used for the sequence-specific separation of the PCR products. Electrophoresis was performed in a 0.8-mm polyacrylamide gel (8% [wt/vol] acrylamide: bisacrylamide 37.5:1, 7 M urea) in 1.25× Tris-acetate buffer (pH 8) at 120 V for 4 h. A temperature ramp of 0.5°C/h from 67 to 69°C was applied during the electrophoresis. The gel was stained in 1.25× Tris-acetate buffer containing 1× SYBR Green (Sigma, Milan, Italy) and photographed using a DS34 camera system (Polaroid, St. Louis, Mo.).

RESULTS AND DISCUSSION

This study describes the optimization of a PCR-TGGE method to identify *Listeria* spp. isolated from food. The method is based on the amplification of the V6 to V8 regions on 16S rRNA gene followed by TGGE analysis to describe the differences in the PCR product sequence of the analyzed bacteria. The primers used in this method are able to amplify a highly conserved region of the 16S rRNA gene in the genus *Eubacterium,* resulting in a 434-bp PCR product. The *Listeria* spp. used in the study gave PCR products of the same weight (about 434 bp), which were analyzed by TGGE.

Methods such as denaturing gradient gel electrophoresis *(6)* or TGGE *(19)* have been developed to rapidly analyze microbial communities by sequence-specific separation of 16S rRNA gene amplicons *(5, 23)*. Separation in TGGE is based on the decreased electrophoretic mobility of a partially melted DNA molecule in polyacrylamide gels compared with that of the completely helical form of the molecule. The melting of fragments proceeds in discrete melting domains. Once the melting domain with the lowest melting temperature reaches its melting temperature, a transition from helical to partially melted molecules occurs, and migration of the molecule will practically halt at a particular position in the TGGE gel. Sequence variations present within such domains cause their melting temperatures to differ so that sequence variants of particular fragments stop migrating at different positions in the gel during the denaturing temperature gradient, allowing the separation of the PCR products by TGGE *(11)*.

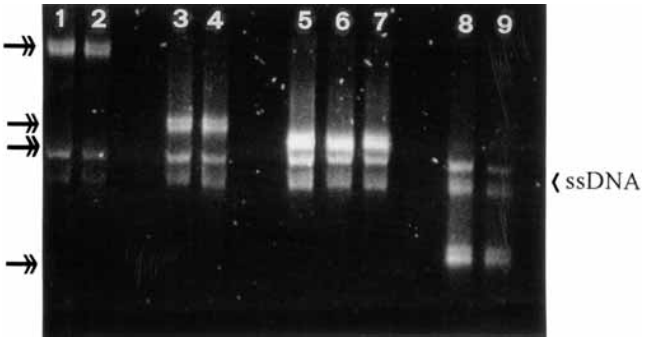

FIGURE 1. *TGGE analysis of the PCR products obtained from the 16S rRNA gene of different* Listeria *spp. Lanes 1 and 2,* L. monocytogenes*; lanes 3 and 4,* L. seeligeri*; lanes 5 and 6,* L. innocua*; lane 7,* L. welshimeri*; lanes 8 and 9,* L. ivanovii. *ssDNA indicates single-stranded DNA. The arrows indicate the position of the specific* Listeria *spp. PCR product.*

In this study, the differences within the 16S rRNA gene were exploited to identify *Listeria* spp. As shown in Figure 1, three species of *Listeria* (*monocytogenes*, *seeligeri*, and *ivanovii*) were distinguished on the basis of the different migration of the PCR products in a polyacrylamide gel subjected to a temperature increase. On the other hand, *L. innocua* and *L. welshimeri* fragments migrate to the same position in the denaturing gel, and for this reason they could be confused. However, this result loses importance when we consider that the *Listeria* spp. that must be absent from food products is *L. monocytogenes* and that by using the method involved it is identified without doubt. In the gel, single-stranded DNA bands are also present. They are probably produced as a consequence of the denaturation of the PCR products that lack the GC region, which is obtained during the first cycles of the amplification because of the use of the reverse primer without the GC clamp. Given the different migratory properties of the single-stranded DNA with respect to the double-stranded DNA (of the PCR product), its presence did not hinder species identification. No differences were observed when we compared the results obtained with the traditional identification method with those obtained by PCR-TGGE analysis.

The protocol described is simple, reproducible, and rapid and could be used for the routine identification of *Listeria* spp. isolated from food to confirm the identity of the colonies and presence or absence of *L. monocytogenes.* In 8 h it is possible to obtain the identification results compared with the 1 to 2 weeks required by the traditional methods. The use of the PCR technique in food studies, after further improvement of the method, could permit the identification of *Listeria* spp. directly on food enrichment

cultures. It would need about 24 h to complete the process, and replacing the primers described in the earlier protocols used to detect foodborne pathogens, such as *L. monocytogenes* in food *(12, 13)*, with those (added with the GC clamp) used in this study, it would be possible to determine PCR products with TGGE. Thus, a protocol would be available to directly detect *Listeria* spp. in food samples, without previous isolation and biochemical characterization, using only short, selective enrichment cultures.

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