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Detection and Differentiation of *Listeria* spp. by a Single Reaction Based on Multiplex PCR

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The *iap* gene encodes the protein p60, which is common to all *Listeria* species. A previous comparison of the DNA sequences indicated conserved and species-specific gene portions. Based on these comparisons, a combination consisting of only five different primers that allows the specific detection and differentiation of *Listeria* species with a single multiplex PCR and subsequent gel analysis was selected. One primer was derived from the conserved 3′ end and is specific for all *Listeria* species; the other four primers are specific for *Listeria monocytogenes*, *L. innocua*, *L. grayi*, or the three grouped species *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*, respectively. The PCR method, which also enables the simultaneous detection of *L. monocytogenes* and *L. innocua*, was evaluated against conventional biotyping with 200 food hygiene-relevant *Listeria* strains. The results indicated the superiority of this technique. Thus, this novel type of multiplex PCR may be useful for rapid *Listeria* species confirmation and for identification of *Listeria* species for strains isolated from different sources.

The genus *Listeria* comprises six characterized species: *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* (25). Among these gram-positive, nonsporulating and motile species, only *L. monocytogenes* is a human and animal pathogen, capable of causing severe infections like septicemia, encephalitis, and meningitis, especially in immunocompromised individuals, newborns, and pregnant women (26). *L. monocytogenes* belongs to the facultative intracellular bacteria that invades, replicates, and multiplies in a variety of mammalian cells (22). A number of genes and gene products necessary for the intracellular survival of this pathogen have been previously reviewed (22).

Several large outbreaks of listeriosis have been associated with contaminated commercial foodstuffs, such as vegetables, milk, and meat products, on which these bacteria can multiply even at low temperatures (26). Contamination not only is caused during food processing but also begins with the production of raw food materials in the environment. Some *Listeria* species like *L. monocytogenes* and *L. innocua* have been isolated from various environmental samples, e.g., soil, vegetation, and human and animal feces, indicating the widespread presence of the pathogen in nature (26). Due to its frequent occurrence in food, *L. innocua* can be considered an indicator bacterium for the presence of *L. monocytogenes*. However, little is known about the occurrence and distribution of other *Listeria* species. Species-specific identification with biochemical standard methods which include sugar fermentations or the CAMP phenomenon (27) are laborious and time-consuming and can require up to 7 days according to International Dairy Federation (IDF) standard 143:1995 (16). Moreover, isolates which demonstrated significant differences in main biochemical features were described previously (1, 4). Other, faster procedures like PCR and immunological or bacteriophage lysis techniques which might allow a more rapid monitoring of all *Listeria* species are limited for this purpose because they detect only the genus *Listeria* or only *L. monocytogenes* (7, 9, 12, 19, 23), thus lacking the ability to simultaneously characterize species other than *L. monocytogenes*. Especially would the coidentification of *L. innocua* be beneficial, as this species can be found associated with the occurrence of *L. monocytogenes* (11, 18), which association may lead to typing of only one of these two species.

To overcome some of these problems, we developed a novel multiplex PCR containing a minimum number of different primers. For this purpose, we used the previously characterized *iap* gene common to all members of the genus *Listeria* as the target because the comparison of all *iap* genes indicated there were conserved gene portions at the 5′ and 3′ ends, while the internal portions are species-specific (8). The *iap* gene of *L. monocytogenes* encodes the major extracellular protein p60 (20), which has been shown to be basically an essential murein hydrolase required for septum separation in a late step in cell division (3, 28). In addition, the *L. monocytogenes* p60 plays a role in the adherence of this organism to certain eukaryotic cells and confers immune protection to mice following infection with this pathogen (5, 13, 14, 21).

In several studies, *iap*-derived primers have been applied for the specific identification of *L. monocytogenes* (1, 4, 8, 10, 12). Recently, we developed a simple method for the simultaneous specific identification and differentiation of *L. monocytogenes* isolates by PCR amplification and size comparison of a hypervariable internal *iap* gene fragment. The encoded so-called Thr-Asn repeat domain, which is located on this fragment, also shows an extended-length polymorphism in strains of the same serotype (6). However, the new method described here enables the simultaneous detection of *Listeria* species and differentiation between *L. monocytogenes*, *L. innocua*, and two groups

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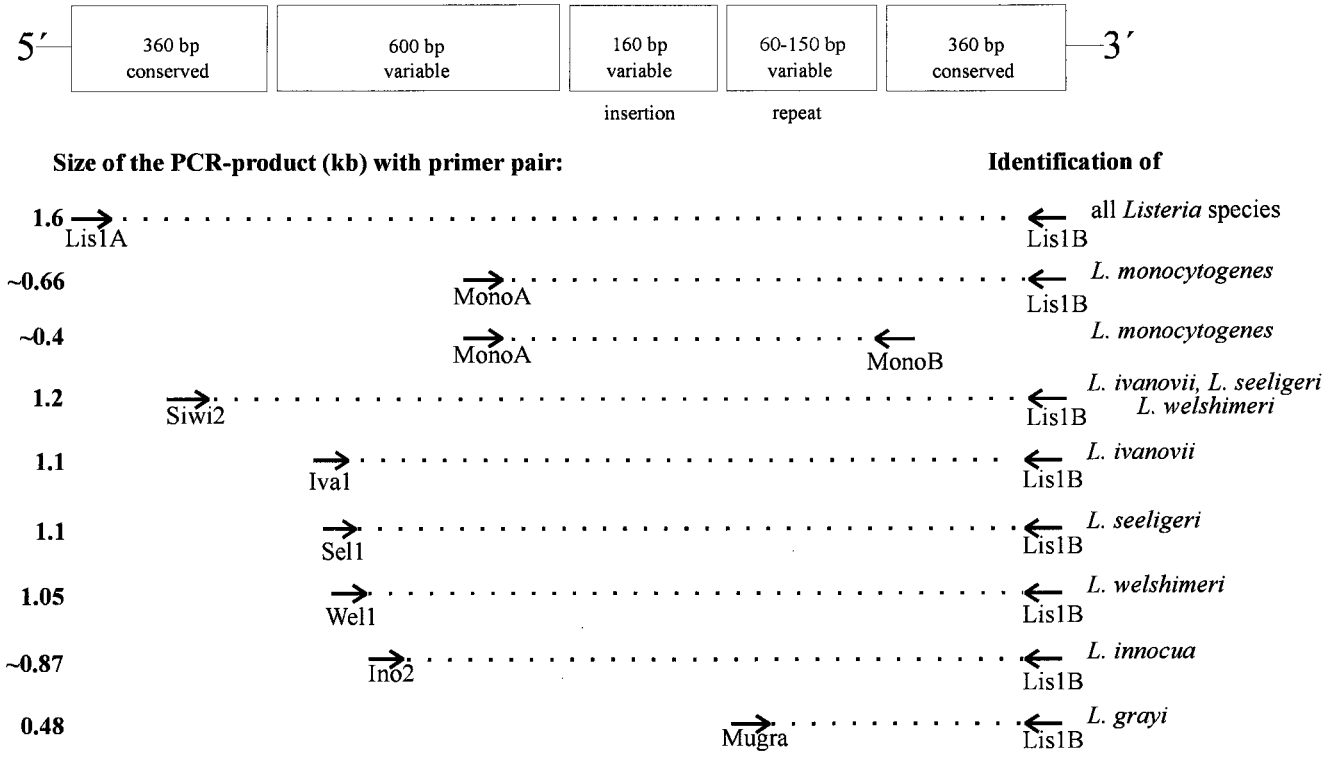

FIG. 1. Binding regions of the primers within the *iap* genes selected for PCR identification of *Listeria* spp. The conserved and variable gene portions are schematically summarized according to Bubert et al. (5). Note that the third gene portion from the left site codes for a putative additional substrate binding domain which is not present in the *iap* genes of *L. monocytogenes* and *L. innocua* (28).

containing very rarely occurring species, the first containing *L. seeligeri*, *L. welshimeri*, and *L. ivanovii* and the second containing *L. grayi* and *L. grayi* subsp. *murrayi*, by a single amplification reaction.

Based on the *iap* DNA sequence comparison, we previously selected primer combinations for the specific identification by PCR of all serotypes of *L. monocytogenes* (primers MonoA and MonoB) (Fig. 1) and all serotypes of *L. innocua* (primers Ino2 [5′-ACTAGCACTCCAGTTGTTAAAC-3′] and Lis1B [5′-TTATACGCGACCGAAGCCAAC-3′]) (8). PCR with the latter species leads to a specific product of 870 bp. In addition, the grouped species *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* could be specifically identified by using primer pair Siwi2 (5′-TAACTGAGGTAGCGAGCGAA-3′) and Lis1B, which yields a PCR product comprising approximately 1.2 kb (8). Since primer Lis1B binds to the 3′ end of all listerial *iap* genes (8), this primer was selected to represent the fixed downstream primer along with four specific upstream primers in a multiplex PCR mix.

Initially, we ensured that primer combination MonoA (5′-CAAACTGCTAACACAGCTACT-3′) and Lis1B also identified all *L. monocytogenes* serotypes. Chromosomal DNA of bacteria used for amplification was prepared as described earlier (4). Reaction mixtures each contained 100 ng of each primer, 200 μM (each) deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1× PCR buffer, 50 to 100 ng of chromosomal DNA, and 1.5 U of *Taq* polymerase (Promega, Mannheim, Germany) according to standard protocols (15). PCR conditions are indicated in the figure legends. As shown in Fig. 2, *L. monocytogenes* strains belonging to all known serotypes could be specifically identified by a PCR product of approximately 660 bp. The slight size differences of the PCR products are due to the length polymorphism found in this amplified gene portion (see above). No cross-reactions with other listerial DNA were observed. We next selected a primer pair (MugraI and Lis1B) specific for the species *L. grayi* and *L. grayi* subsp. *murrayi*. As shown in Fig. 3, this primer pair yielded a specific PCR product of 480 bp in size only with these two species, while all other *Listeria* species gave no PCR product.

The fact that all specific PCR products can be easily distinguished by size comparisons in agarose gels enabled the generation of a multiplex PCR consisting of the four species-specific upstream primers MugraI, MonoA, Ino2, and Siwi2 and the conserved downstream primer Lis1B. The amplification procedures were the same as indicated above. As shown in Fig. 4, the presence of *L. grayi* or *L. grayi* subsp. *murrayi* led to the expected 480-bp product. When chromosomal DNA of *L. monocytogenes* was added to the reaction mix, the expected band of approximately 660 bp was observed. The species *L. innocua* was identified by the 870-bp DNA fragment, and the *L. seeligeri-L. welshimeri-L. ivanovii* group was identified by the occurrence of the 1.2-kb PCR product. No cross-reactions or additional bands were observed with other gram-positive or gram-negative bacterial species (data not shown). The control group included *Bacillus subtilis*, *Brochothrix thermosphacta*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium*. The three rare species *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* are not distinguishable by this method but can be easily differentiated on blood agar plates. However, to complete the list of specific identifications of all *Listeria* species by PCR, upstream primers in combination with Lis1B specific for each of these three species were selected. Primer Iva1 (5′-CTACTCAAGCGCAAGCGGCAC-3′) for *L. ivanovii*, primer Sel1 (5′-TACACAAGCGGCTCCTGCTCAA

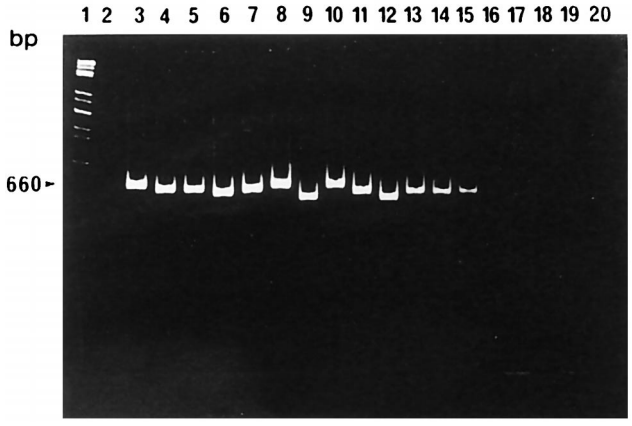

FIG. 2. *L. monocytogenes*-specific PCR products with the primer pair MonoA and Lis1B. PCR conditions were as follows: 30 cycles, each at 95°C for 15 s, 58°C for 30 s, and 72°C for 45 s. Lanes: 1, molecular weight standard; 2, control reaction (all reagent ingredients except chromosomal DNA); 3, *L. monocytogenes* EGD serovar 1/2a (sv1/2a); 4, *L. monocytogenes* SLCC 2755 sv1/2b; 5, *L. monocytogenes* NCTC 5348 sv1/2c; 6, *L. monocytogenes* NCTC 5105 sv3a; 7, *L. monocytogenes* SLCC 5543 sv3b; 8, *L. monocytogenes* SLCC 2479 sv3c; 9, *L. monocytogenes* L 99 sv4a; 10, *L. monocytogenes* SLCC 4561 sv4ab; 11, *L. monocytogenes* SLCC 4013 sv4b; 12, *L. monocytogenes* ATCC 19116 sv4c; 13, *L. monocytogenes* ATCC 19117 sv4d; 14, *L. monocytogenes* ATCC 19118 sv4e; 15, *L. monocytogenes* SLCC 2482 sv7; 16, *L. innocua* sv6a; 17, *L. welshimeri* SLCC 5334; 18, *L. seeligeri* SLCC 3945; 19, *L. ivanovii* ATCC 19119; 20, *L. grayi*. PCR products were separated in a 4% polyacrylamide gel and stained with ethidium bromide.

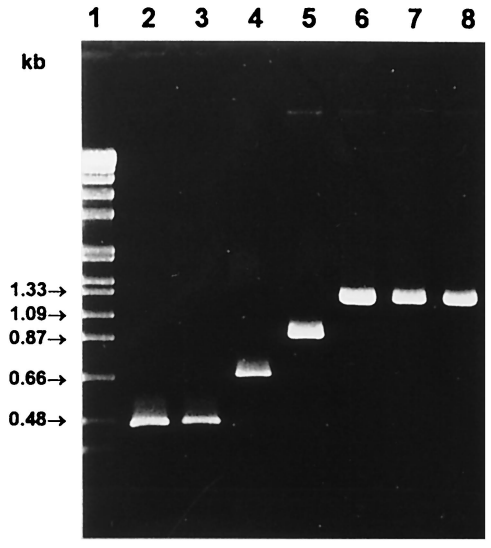

FIG. 4. Identification and differentiation of *Listeria* species by multiplex PCR containing the five primers MugraI, MonoA, Ino2, and Siwi2, and Lis1B (LisMix). Reaction conditions were as follows: 30 cycles, each at 95°C for 15 s, 58°C for 30 s, and 72°C for 50 s. Lanes: 1, molecular weight standard; 2, *L. grayi*; 3, *L. grayi* subsp. *murrayi*; 4, *L. monocytogenes* EGD sv1/2a; 5, *L. innocua* sv6a; 6, *L. ivanovii*; 7, *L. seeligeri*; 8, *L. welshimeri*. PCR products were separated in a 1.2% agarose gel and stained with ethidium bromide.

C-3′) for *L. seeligeri*, and primer Wel1 (5′-CCCTACTGCTCC AAAAGCAGCG-3′) for *L. welshimeri* were derived from species-specific internal *iap* gene portions. As shown in Fig. 5, all primer combinations led to the specific identification of the three species. In addition, specificity was confirmed with six different isolates of each species (data not shown).

To test the suitability of the multiplex PCR for coidentification of different *Listeria* species with a single reaction, we applied PCRs containing the chromosomal DNAs of *L. innocua* and *L. monocytogenes*. As shown in Fig. 6, the 660- and 870-bp bands which lead to the identification of both species could be obtained. The multiplex PCR also led to two clear products of the expected sizes when other bacterial combinations, such as *L. innocua* and *L. grayi*, *L. monocytogenes* and *L. grayi*, and *L. monocytogenes* and *L. ivanovii*, were used. However, when PCR was performed with the chromosomal DNA of a third *Listeria* species, unclear bands occasionally appeared; thus, the method might not be advisable for the simultaneous identification of more than two *Listeria* species from the four different groups. These additional amplification products might occur by in vitro recombination of PCR products, which can be generated by the *Taq* DNA polymerase jumping between templates during amplification (24).

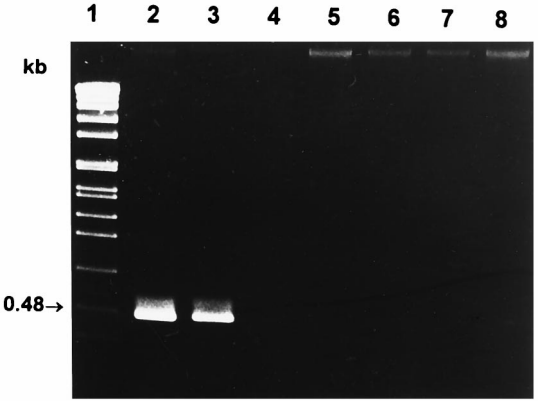

FIG. 3. *L. grayi*-specific PCR products with the primer combination MugraI and Lis1B. PCR conditions were as follows: 30 cycles, each at 95°C for 15 s, 58°C for 30 s, and 72°C for 45 s. Lanes: 1, molecular weight standard; 2, *L. grayi* (Institute for Milk Hygiene culture collection); 3, *L. grayi* subsp. *murrayi* (Institute for Milk Hygiene culture collection); 4, *L. monocytogenes* EGD; 5, *L. innocua* NCTC 11288 sv6a; 6, *L. ivanovii* ATCC 19119; 7, *L. seeligeri* SLCC 3945; 8, *L. welshimeri* SLCC 5334. PCR products were separated in a 1% agarose gel and stained with ethidium bromide.

Next, the multiplex PCR was evaluated with pure cultures derived from the type culture collection of the Institute for Milk Hygiene, Vienna, Austria. All isolates were characterized by using either IDF method 143:1995 (16) or International Organization for Standardization (ISO) method 11290-1 (17) as the standard method to demonstrate the species-specific biochemical features of relevance. A total of 199 pure cultures, which had been isolated mostly from foodstuffs but also from clinical samples, were screened by the *iap*-specific multiplex technique. The numbers of isolates were 100 and 49 for *L. monocytogenes* and *L. innocua*, respectively, and a total of 50 for the *L. seeligeri-L. welshimeri-L. ivanovii* and *L. grayi-L. grayi* subsp. *murrayi* groups. All strains from the type culture collection were grown in tryptone soy broth at 37°C overnight and subjected to standard detection procedures according to IDF or to multiplex PCR analyses.

Overall conformity of microbiological versus multiplex PCR results was found in 100% of the analyzed *L. monocytogenes* and *L. innocua* strains. In a single case the multiplex PCR detected *L. innocua* in addition to *L. monocytogenes*. Microbiological reinvestigation revealed that the *L. monocytogenes* culture was indeed contaminated with *L. innocua*. With regard to conformity between results from both methods, the *L. seeligeri-L. welshimeri-L. ivanovii* group results proved to be slightly divergent. Out of 50 isolates tested, 35 strains showed concor-

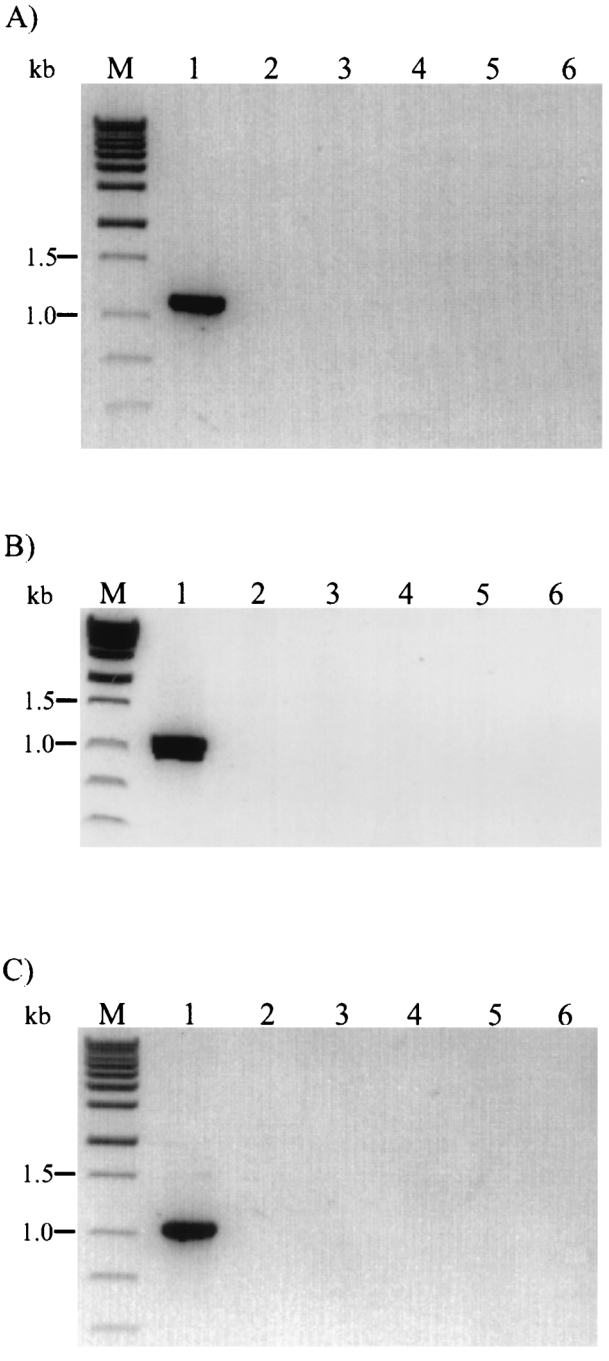

FIG. 5. Specific identification of *L. ivanovii* (A), *L. seeligeri* (B), and *L. welshimeri* (C) by PCR with primer pairs Iva1 and Lis1B, Sel1 and Lis1B, and Wel1 and Lis1B, respectively. PCR conditions were as follows: 35 cycles, each at 95°C for 15 s and 62°C for 30 s. Lanes in panel A: 1, *L. ivanovii*; 2, *L. seeligeri*; 3, *L. welshimeri*; 4, *L. grayi*; 5, *L. monocytogenes* EGD; 6, *L. innocua*. Lanes in panel B: 1, *L. seeligeri*; 2, *L. ivanovii*; 3, *L. welshimeri*; 4, *L. grayi*; 5, *L. monocytogenes* EGD; 6, *L. innocua*. Lanes in panel C: 1, *L. welshimeri*; 2, *L. ivanovii*; 3, *L. seeligeri*; 4, *L. grayi*; 5, *L. monocytogenes* EGD; 6, *L. innocua*. Lanes M, molecular weight markers. PCR products were separated in a 1.2% agarose gel and stained with ethidium bromide.

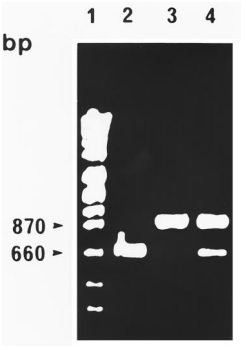

FIG. 6. Parallel identification and differentiation of *L. monocytogenes* and *L. innocua* by multiplex PCR containing the Lis-Mix primers. Reaction conditions were the same as indicated in the legend of Fig. 4. Lanes: 1, molecular weight marker; 2, *L. monocytogenes* EGD; 3, *L. innocua* sv6b; 4, *L. monocytogenes* EGD and *L. innocua* sv6b. PCR products were separated in a 1.2% agarose gel and stained with ethidium bromide.

dant results after a first PCR trial. One isolate was shown to be *L. monocytogenes* whereas four isolates proved to be *L. innocua* by PCR. However, the results obtained by PCR were confirmed by repeated microbiological investigation by plating the cultures onto Rapid Lis-Agar and confirmation of suspect colonies was obtained by CAMP reaction. Triplicate PCR trials did not lead to a positive amplification of 10 isolates although microbiological investigation by IDF standard 143:1995 identified the presence of five *L. seeligeri* strains, four *L. welshimeri* strains, and one *L. ivanovii* strain. Repeated microbiological investigation proved these cultures contained nonlisterial cells, thus confirming results obtained by PCR. PCR identified conclusively all 50 strains for this group, whereas only 35 strains were correctly identified by standard biotyping.

In conclusion, we have developed a novel multiplex PCR for the specific identification and differentiation of *L. monocytogenes*, *L. innocua*, *L. grayi*, and the three grouped species *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*. The latter three can be differentiated by subsequent PCRs; thus, a complete PCR identification protocol specific for each *Listeria* species is now available. In contrast to what is required by other typing methods, e.g., API *Listeria* (2), no pure cultures are required for accurate typing of *Listeria* spp. However, this method reaches its limit when more than two *Listeria* species from the four different groups are present. Moreover, the comparison with conventional biotyping indicates that identification of *Listeria* species by PCR with *iap* as a stable chromosomal target gene is more reliable, less time-consuming, and less laborious, and thus very cost-effective. In addition, this multiplex PCR may also allow a more systematic study of the occurrence of *Listeria* spp. in food or in the environment. The application for the direct detection of *Listeria* spp. in food samples by this method is in progress.

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