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Identification of *Listeria* Species by Microarray-Based Assay

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We have developed a rapid microarray-based assay for the reliable detection and discrimination of six species of the *Listeria* genus: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi*. The approach used in this study involves one-tube multiplex PCR amplification of six target bacterial virulence factor genes (*iap*, *hly*, *inlB*, *plcA*, *plcB*, and *clpE*), synthesis of fluorescently labeled single-stranded DNA, and hybridization to the multiple individual oligonucleotide probes specific for each *Listeria* species and immobilized on a glass surface. Results of the microarray analysis of 53 reference and clinical isolates of *Listeria* spp. demonstrated that this method allowed unambiguous identification of all six *Listeria* species based on sequence differences in the *iap* gene. Another virulence factor gene, *hly*, was used for detection and genotyping all *L. monocytogenes*, all *L. ivanovii*, and 8 of 11 *L. seeligeri* isolates. Other members of the genus *Listeria* and three *L. seeligeri* isolates did not contain the *hly* gene. There was complete agreement between the results of genotyping based on the *hly* and *iap* gene sequences. All *L. monocytogenes* isolates were found to be positive for the *inlB*, *plcA*, *plcB*, and *clpE* virulence genes specific only to this species. Our data on *Listeria* species analysis demonstrated that this microarray technique is a simple, rapid, and robust genotyping method that is also a potentially valuable tool for identification and characterization of bacterial pathogens in general.

The genus *Listeria* consists of six species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi*. All *Listeria* species are ubiquitously distributed in nature and can often be found in soil, decaying plants, sewage, silage, dust, and water (3, 19). Two species, *L. monocytogenes* and *L. ivanovii*, are considered pathogenic for animals, with *L. monocytogenes* being predominantly associated with human illnesses such as meningitis, encephalitis, and sepsis (39, 40, 45). Among the risk groups (infants, pregnant women, elderly persons, and immunocompromised individuals), the worldwide case fatality rate for listeriosis is estimated to be as high as 36% (41). Infection of humans predominantly occurs as a result of occasional contamination of ready-to-eat and raw food products. Outbreaks and sporadic cases of listeriosis have been associated with contamination of different food items, including coleslaw, milk, pâté, soft cheese, meat, and seafood products (14, 30, 40).

Conventional methods for detecting *L. monocytogenes* involve multiple selective enrichment steps followed by biochemical identification and serotyping (15, 27–29). These methods are time consuming, generally requiring at least 2 to 3 days (13, 16). A number of alternative biochemical, immunological, and molecular methods that shorten the time for *Listeria* sp. detection in food and environmental samples have been reported (1, 4). Molecular methods used for the genetic identification and characterization of *Listeria* isolates include PCR with primers specific for characteristic genes (6, 23, 31), restriction fragment length polymorphism analysis (42, 44), amplified fragment length polymorphism analysis (34), random amplification of polymorphic DNA (47), and pulsed-field gel electrophoresis (25).

Several genetic markers, including 16S and 23S rRNA genes, 16 S-23S intergenic spacer regions, and certain virulence factor genes, have been reported to be useful targets for the PCR amplification and for differentiation among *Listeria* species (2, 6, 12, 21, 24, 31, 35, 48). Although PCR amplification followed by separation and characterization of DNA products by gel electrophoresis is a simple and sensitive method, this approach has a number of inherent shortcomings. Highly sensitive PCR amplification tends to generate more nonspecific DNA products, which complicate interpretation of results. Furthermore, in cases where multiplex PCR is used for the simultaneous detection of several genetic markers, these nonspecific products may be a significant problem (17). Therefore, there is a need for improved high-throughput methods for genotyping that are sensitive, highly discriminating, and robust.

A combination of PCR with DNA-DNA hybridization instead of gel electrophoresis significantly improves the specificity of target sequence detection in the presence of nonspecific PCR products (10). Microchip technology might provide an ideal solution to this problem. DNA and oligonucleotide microchips (microarrays) are widely used for genomic studies, including gene expression, genotyping, and single-nucleotide polymorphisms (22, 32, 33, 37, 38). Unlike other hybridization tools, such as microplates or dot blots for DNA-DNA hybridization with membrane-bound probes, miniature glass microchips are capable of containing DNA probes specific to thousands of individual target DNAs. Potentially, microarray technology allows the rapid determination of a complete genetic profile of a microorganism in one hybridization experiment. Thus, this approach may be a valuable tool for genetic screening and identification of microorganisms.

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Previous studies employed oligonucleotide arrays for detection of genetic markers associated with bacterial and viral pathogenesis and drug resistance (9, 10). In the present study we used the combined PCR-microarray approach for reliable and accurate discrimination among six *Listeria* species.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the origins and descriptions of the *Listeria* strains used in this study. The strains were obtained from the Special Listeria Culture Collection, Institute of Hygiene and Microbiology, University of Würzburg, Würzburg, Germany, the American Type Culture Collection, Manassas, Va., the Centers for Disease Control and Prevention, Atlanta, Ga., and the collections of Anthony Hitchins and Farukh Khambaty of the Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Md. Bacteria were grown overnight on brain heart infusion plates (Difco, Detroit, Mich.) at 37°C.

Total DNA preparation. Freshly grown bacteria were boiled in 1× Tris-EDTA buffer (approximately 10^8 cells/ml) for 10 min followed by centrifugation at 14,000 × *g* for 10 min to remove denatured proteins and bacterial membranes. The presence of genomic DNA in all prepared samples was confirmed by 1% agarose gel electrophoresis followed by staining with ethidium bromide.

Design of microarray oligoprobes. A BLAST search was used to identify and retrieve the sequences of homologues of each of the six genes analyzed (Table 2). The sequences of the *iap* gene of *L. monocytogenes* isolates used in this study were determined by using an ABI Prism 310 genetic analyzer. The sequences were aligned by using ClustalX software (43). Sequences of regions highly conserved among all alleles of each gene were selected to design the gene-specific oligonucleotide probes (oligoprobes) listed in Table 3. The 5′ end of each oligonucleotide was modified during synthesis with TFA Aminolink CE reagent (PE Applied Biosystems, Foster City, Calif.) to enable their immobilization on silylated glass slides (CEL Associates, Inc., Houston, Tex.).

Microchip design and fabrication. Each bacterial gene was identified by hybridization with 10 independent oligonucleotides (Table 3) covering entire DNA amplicons. Oligoprobes specific for each gene were spotted on individual rows of the array. Marker spots for array positioning on the slide were made by using 1× spotting solution (ArrayIt, Sunnyvale, Calif.) in 0.25 M acetic acid.

Microchips were printed with the PIXSYS 5500 contact microspotting robotic system (Cartesian Technologies, Inc., Calif.) equipped with a microspotting pin (CMP7; ArrayIt). The average size of spots was 250 μm. The spotting solution contained a mixture of specific oligonucleotide probe (80 μM) and quality control (QC) oligonucleotide (8 μM) in 50% dimethyl sulfoxide. Printed slides were dried for at least 20 min at 80°C and treated for 15 min with a freshly prepared 0.25% $NaBH_4$ solution in water. Slides were washed once for 5 min with 0.2% sodium dodecyl sulfate in water and five times for 1 min each with distilled water to remove unbound oligonucleotides.

PCR amplification. Table 4 lists the primers used to amplify genes for different *Listeria* virulence factors. The standard PCR mixture (30 μl) contained 1.5 U of HotStarTaq DNA polymerase, 1× reaction buffer supplemented with 2.5 mM $MgCl_2$ (Qiagen, Chatsworth, Calif.), 600 nM (each) forward and reverse primers, a 200 μM concentration of each deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dTTP), and 1 to 2 μl of DNA template (approximately 0.2 μg of bacterial DNA). PCR was performed with a Gene Amp PCR system 9700 thermocycler (Applied Biosystems, Foster City, Calif.) with the following conditions: initial activation at 95°C for 15 min, 40 cycles at 94°C for 20 s, 60°C for 20 s, and 72°C for 50 s, and a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis in 2% agarose gels containing 1× Tris-acetate-EDTA buffer and visualized by staining with ethidium bromide.

Synthesis of Cy5-labeled ssDNA. Single-stranded-DNA (ssDNA) samples for microarray assay were synthesized by using 40 cycles of the primer extension reaction driven by *Taq* polymerase in the presence of only reverse primers for amplification of six virulence factor genes (Table 4). The reaction was performed in a volume of 50 μl containing 10 U of *Taq* DNA polymerase (Sigma, St. Louis, Mo.), 1× reaction buffer with 3.0 mM $MgCl_2$, 600 nM (each) reverse primer, 200 nM dGTP, dATP, and dTTP, 40 nM dCTP, 40 nM Cy5-dCTP, and 2 to 4 μl of double-stranded DNA template from the first round of multiplex PCR purified using QIAquick PCR purification kit (Qiagen). The protocol included an initial activation step at 95°C for 1 min followed by 40 cycles at 94°C for 20 s, 58°C for 20 s, and 72°C for 2 min, with a final extension step at 72°C for 10 min. The fluorescently labeled ssDNA products were purified from nonincorporated reagents with a QIAquick PCR purification kit according to the manufacturer's protocol, dried in a vacuum, and solubilized in 10 μl of water.

TABLE 1. Origin and description of reference strains used in this study

Species	Strain (serotype)[a]	Source[b]	Microarray identification
L. monocytogenes	LM1250 (NI)	FK	*L. monocytogenes*
L. monocytogenes	CEB2776 (4b)	AH	*L. monocytogenes*
L. monocytogenes	LM1253 (NI)	FK	*L. monocytogenes*
L. monocytogenes	H6900 (1/2a)	CDC	*L. monocytogenes*
L. monocytogenes	SLCC 5957 (NI)	SLCC	*L. monocytogenes*
L. monocytogenes	SE106 (1a)	AH	*L. monocytogenes*
L. monocytogenes	LM37 (NI)	FK	*L. monocytogenes*
L. monocytogenes	F5034 (1/2b)	CDC	*L. monocytogenes*
L. monocytogenes	LM1251 (NI)	FK	*L. monocytogenes*
L. monocytogenes	LM1248 (NI)	FK	*L. monocytogenes*
L. monocytogenes	LM82 (1/2b)	AH	*L. monocytogenes*
L. monocytogenes	LM1249 (NI)	FK	*L. monocytogenes*
L. monocytogenes	LM1252 (NI)	FK	*L. monocytogenes*
L. monocytogenes	LM38 (NI)	FK	*L. monocytogenes*
L. monocytogenes	ATCC 15313 (NI)	ATCC	*L. monocytogenes*
L. monocytogenes	Murray B (4b)	AH	*L. monocytogenes*
L. monocytogenes	Scott A (4b)	AH	*L. monocytogenes*
L. monocytogenes	V37 (4b)	AH	*L. monocytogenes*
L. monocytogenes	ATCC 35152 (3d)	ATCC	*L. monocytogenes*
L. monocytogenes	SE31 (3b)	AH	*L. monocytogenes*
L. monocytogenes	SF-014 (3a)	AH	*L. monocytogenes*
L. ivanovii	ATCC19119	ATCC	*L. ivanovii*
L. ivanovii	LA29	AH	*L. ivanovii*
L. ivanovii	SLCC 6965	AH	*L. ivanovii*
L. ivanovii	SLCC 6966	AH	*L. ivanovii*
L. ivanovii	SLCC 7926	AH	*L. ivanovii*
L. ivanovii	SLCC 2379	AH	*L. ivanovii*
L. ivanovii	SLCC 6032	AH	*L. ivanovii*
L. ivanovii	F6983	AH	*L. ivanovii*
L. ivanovii	ATCC 8243	ATCC	*L. ivanovii*
L. seeligeri	ATCC 35967	ATCC	*L. seeligeri*
L. seeligeri	SE139	AH	*L. seeligeri*
L. seeligeri	BS27	AH	*L. seeligeri*
L. seeligeri	F7295 (1/2b)	AH	*L. seeligeri*
L. seeligeri	F7334 (1/2b)	AH	*L. seeligeri*
L. seeligeri	G906 (1/2b)	AH	*L. seeligeri*
L. seeligeri	SLCC 5921	AH	*L. seeligeri*
L. seeligeri	F7896	AH	*L. seeligeri*
L. innocua	ATCC 33090	ATCC	*L. innocua*
L. innocua	LS027	AH	*L. innocua*
L. innocua	LA-01	AH	*L. innocua*
L. innocua	DA-15	AH	*L. innocua*
L. innocua	23	AH	*L. innocua*
L. welshimeri	BA84	AH	*L. welshimeri*
L. welshimeri	LABSTR	AH	*L. welshimeri*
L. welshimeri	4889A	AH	*L. welshimeri*
L. welshimeri	LS-156	AH	*L. welshimeri*
L. welshimeri[c]	SE116	AH	*L. seeligeri*
L. welshimeri[c]	2436KA	AH	*L. seeligeri*
L. welshimeri[c]	LS-166	AH	*L. seeligeri*
L. grayi	ATCC 25401	ATCC	*L. grayi*
L. grayi	ATCC 19120	AH	*L. grayi*
L. grayi	37A	AH	*L. grayi*

[a] NI, not identified.
[b] AH, A. Hitchins, Food and Drug Administration; ATCC, American Type Culture Collection; CDC, Centers for Disease Control; FK, F. Khambaty, Food and Drug Administration; SLCC, Special Listeria Culture Collection, Institute of Hygiene and Microbiology, University of Würzburg.
[c] Misidentified strain.

Hybridization conditions. Hybridization of the fluorescently labeled ssDNA samples to the microarray was performed in 1× hybridization buffer composed of 5× Denhardt's solution, 6× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 0.1% Tween 20 at 45°C for 30 min. Before hybridization,

TABLE 2. Accession numbers of the sequences used for the microarray target sequence design

Gene	Organism	Accession no.
iap	*L. monocytogenes*	M80351, AL591975, X52268
	L. ivanovii	M80350
	L. innocua	NC_003212, AL596165, M80349
	L. seeligeri	M80353
	L. welshimeri	M80354, M80348
	L. grayi	M80352, M95579
hly	*L. monocytogenes*	AF253320, X60035, X15127, M24199
	L. ivanovii	X60461
	L. seeligeri	X60462
inlB	*L. monocytogenes*	AF121024–AF121047, AL591975, AJ012346, M67471
plcA	*L. monocytogenes*	U25444, U25447, U25450, U25453, AL591974, X54618, M24199
plcB	*L. monocytogenes*	AL591974, M82881, X59723
clpE	*L. monocytogenes*	AL591977, AF076664
	L. innocua	AL596167

1 μl of the Cy5-labeled ssDNA sample was mixed with equal volume of 2× hybridization buffer containing 0.1 μM Cy3-labeled QC probe (Table 3), followed by denaturing at 95°C for 1 min and chilling on ice. Each sample was placed on the microchip and covered with a 5- by 5-mm plastic coverslip to prevent evaporation of the probe during incubation. After hybridization, the slides were washed once for 1 min with 6× SSC containing 0.2% Tween 20, three times for 1 min with 6× SSC buffer, twice with 2× SSC buffer, and once with 1× SSC buffer and dried in a stream of air.

Microarray scanning. Fluorescent images of the microarrays were obtained by scanning the slides with ScanArray 5000 (Perkin-Elmer, Boston, Mass.). The fluorescent signals from each spot were measured and compared by using QuantArray software (Perkin-Elmer, Boston, Mass.). Fluorescent signals that differed from the average background at a statistically significant level ($P < 0.01$) were considered positive.

Sequencing. In some cases, sequences of the *iap* gene for some *L. monocytogenes* strains were determined experimentally. The PCR-amplified DNA fragments were purified by electrophoresis in an agarose gel, extracted with a QIAquick gel extraction kit (Qiagen) according to the manufacturer's protocol, and sequenced by using the ABI Prism 310 genetic analyzer system (PE Applied Biosystems, Foster City, Calif.).

Nucleotide sequence accession numbers. GenBank accession numbers of the deposited sequences are AF500174 (strain F5034), AF500175 (strain LM1250), AF500176 (strain SLCC5957), AF500177 (strain CEB2776), AF500178 (strain LM1248), AF500179 (strain LM82), AF500180 (strain LM1249), AF500181 (strain LM1252), AF500182 (strain SE106), AF500183 (strain LM1253), AF500184 (strain H9600), AF500185 (strain LM37), and AF50018 (strain LM38).

RESULTS

Species identification based on the *iap* gene sequence. The two universal PCR primers, LisF and LisR (Table 4), recognizing conserved sequences of the *iap* gene, amplified this gene from all six *Listeria* species (Fig. 1A). The size of the PCR product generated with these primers varied from 644 to 716 bp depending on the species (Table 4).

The presence of multiple species-specific sequences within the selected region of the *iap* gene enabled us to design 10 individual oligoprobes (Table 3) per species.

As shown in Fig. 1B, all designed oligoprobes hybridized with homologous DNA, although in some cases, sporadic and low cross-hybridization with the single oligoprobes of heterologous species was observed (e.g., *L. innocua* spot 7 with *L. seeligeri* samples *or L. seeligeri* spot 3 with *L. innocua* samples [Fig. 1B, panels 5 and 3, respectively]).

Identification of hemolytic *Listeria* species. As with the *iap* gene, conserved sequences within *hly* were used to design a pair of universal primers, IsoF and IsoR, for amplification of the 708-bp DNA segment from any hemolytic *Listeria* strain. The primers enabled us to amplify the segment from each of the hemolytic species (Fig. 2A, lanes 1 to 3) but, as expected, did not amplify any DNA from nonhemolytic *Listeria* species (Fig. 2A, lanes 4 to 6). The genetically divergent region within the amplicons was used to design 10 individual oligoprobes for discriminating between the three species (Table 3). All the probes specifically recognized Cy5-labeled fluorescent samples only from hemolytic species (Fig. 2B). No cross hybridization between amplicons from *L. monocytogenes*, *L. ivanovii*, *or L. seeligeri* was detected.

Microarray analysis of L. monocytogenes-specific virulence factors. The sequences of all PCR primers and probes for the virulence factors *inlB*, *plcA*, *plcB*, and *clpE*, specific only for *L. monocytogenes* (20), are summarized in Tables 4 and 3, respectively. The primers ClpEF and ClpER, selected for amplification of the *clpE* gene of *L. monocytogenes* (Table 4), also amplified the fragment from another species, *L. innocua* (Fig. 3A, lane 2). However, the PCR product from *L. innocua* did not hybridize to *L. monocytogenes*-specific probes of the microarray (Fig. 3B).

Simultaneous microarray analysis of *Listeria* virulence factors using multiplex PCR. To simplify microarray identification, detection, and analysis of *Listeria* species using the virulence factors described above (*iap*, *hly*, *inlB*, *plcA*, *plcB*, and *clpE*), we amplified all six genes in a single multiplex PCR.

The *Listeria* strains listed in Table 1 were tested by using the multiplex PCR followed by hybridization with the "universal" *Listeria* microchip containing the probes for all six virulence factor genes. Results of the microarray analysis showed that DNA from each *Listeria* species hybridized only with homologous probes (representative data are shown in Fig. 3B). We found that the use of more than one gene for characterizing pathogenic *Listeria* species might reduce bacterial misidentification associated with genomic instability. Three strains, SE116, 2436KA, and LS-166, previously characterized as *L. welshimeri* (Table 1) were identified by microarray as *L. seeligeri* on the basis of hybridization with the *iap*-specific oligonucleotides. However, these strains did not hybridize with *L. seeligeri hly*-specific oligonucleotides. The direct sequencing of the *iap*, *prfA*, and 16S rRNA genes of these strains showed that these three strains belonged to *L. seeligeri* but lost the *hly* gene due to a deletion (about 6,000 bp) in the central virulence gene cluster (LIPI-1) (data not shown).

DISCUSSION

In this paper, we describe the use of oligonucleotide microarray hybridization for the rapid detection and identification of six species of the genus *Listeria*: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi*. Sequence analysis of different virulence factor genes and bacterial genetic markers previously used for characterization and discrimination among *Listeria* species allowed us to select two

TABLE 3. Oligonucleotide probes for detection and discrimination among *Listeria* species

Type	Name	Sequence	Length (nt)	G+C (%)	T_m[a] (°C)
iap specific	Iap-Mc1	AGTTGCACCAACACAAGAA	19	42	47
	Iap-Mc2	TACTACTCAACAAGCTGCA	19	42	47
	Iap-Mc3	GCCTAAAGTAGCAGAAACG	19	47	49
	Iap-Mc4	ACTACACCTGCGCCTAAA	18	50	48
	Iap-Mc5	TACTACACACGCTGTCAAAA	20	40	48
	Iap-Mc6	CTACACCTGCGCCTAAAGT	19	53	51
	Iap-Mc7	AGTATTATTACGTCCATCAAAG	22	32	47
	Iap-Mc8	CAACCGAATCTAACGGC	17	53	47
	Iap-Mc9	CCAGTAATAGATCAAAATGCTA	22	32	47
	Iap-Mc10	AAAACAAACTACACAAGCAACTA	23	30	48
	Iap-Ivan1	ACTGTTCTTCCTAAAGCGGAA	21	43	50
	Iap-Ivan2	AAGTGCCCCTGCACCAGAA	19	58	53
	Iap-Ivan3	CTGTTACTGCTACTTGG	17	47	45
	Iap-Ivan4	TCCAGTAAATATGGCACTTC	20	40	48
	Iap-Ivan5	TTAGGTACTACTGTAACA	18	33	41
	Iap-Ivan6	AACTGCTTCAACATACACTGTT	22	36	49
	Iap-Ivan7	GTTCAAAACATAATGTCAT	19	26	40
	Iap-Ivan8	TGGTACAAAATTTCATATG	19	26	40
	Iap-Ivan9	AAACAGAAACTCCTGCTGTA	20	40	48
	Iap-Ivan10	CTGCTGAAACGAAACCA	17	47	45
	Iap-Wsh1	GTTAAATGTTCGTTCTGGT	19	37	45
	Iap-Wsh2	CAATAGTATTGTTACTTCCCTAA	23	30	48
	Iap-Wsh3	TCGAAGCAGCTGAATCCAA	19	47	49
	Iap-Wsh4	AAGCAGCTGAATCCAATGG	19	47	49
	Iap-Wsh5	ATAAAATTTCTTATGGCGAAGGAA	24	29	49
	Iap-Wsh6	CTCCTGTTGCTAAACAAGA	19	42	47
	Iap-Wsh7	ATACTAATGCTACTACTCATACT	23	30	48
	Iap-Wsh8	CCCTACTGCTCCAAAAGC	18	56	50
	Iap-Wsh9	GAAACGACAAAACAAACTGCA	21	38	49
	Iap-Wsh10	ACTAAAACAGCACCAGTAGTA	21	38	49
	Iap-Innc1	GACACAACAAGTTAAACCT	19	37	45
	Iap-Innc2	TTGCTACTGAAGAAAAAGCA	20	35	46
	Iap-Innc3	ACCACAGCATTCTTACTTC	19	42	47
	Iap-Innc4	AAAACAACCAACTACACAACAAA	23	30	48
	Iap-Innc5	AACGCTACTACACATAACGTT	21	38	49
	Iap-Innc6	ACTGCTCCTGCACCAAAAG	19	53	51
	Iap-Innc7	TTGACCACAGCATTCTTACTT	21	38	49
	Iap-Innc8	AAAGCAACTAGCACTCCA	18	44	46
	Iap-Innc9	AGTTGTTAAACAAGAAGTGAAA	22	27	46
	Iap-Innc10	GCTACAGAAGCAAAAACAGA	20	40	48
	Iap-Seel1	AGATAATGGCACAACTG	17	41	42
	Iap-Seel2	CATTAAAAAAAGCTAACAAAC	21	24	43
	Iap-See13	AAATCACTTATGGTGAAGG	19	37	45
	Iap-See14	AAAAACAGGCTACGTTAATG	20	35	46
	Iap-See15	AAGTTAAACAAGAGGAAACTA	21	29	45
	Iap-See16	TACACAAGCGGCTCCTG	17	59	49
	Iap-See17	TCCTGCTCAACAAACTAAAAC	21	38	49
	Iap-See18	CAGCAACTACTGAAAAAGATG	21	38	49
	Iap-See19	ATGCAACAACTCATACCGTTAA	22	36	49
	Iap-See110	CTGAAAAAGATGCAGTAGA	19	37	45
	Iap-Gry1	CCATCGGTTGTCTCAGCAA	19	53	51
	Iap-Gry2	ATACAGTGGTTGTCGCATC	19	47	49
	Iap-Gry3	CTCCTGACGCAAATGAAAAAAT	22	36	49
	Iap-Gry4	TTTCCGCTGCTGGAATAG	18	50	48
	Iap-Gry5	CTTCCAAAACTGGTACTAC	19	42	47
	Iap-Gry6	GACCAACTAAAACAACTCAAT	21	33	47
	Iap-Gry7	CTCAATAAACTTGACTCTGAT	21	33	47
	Iap-Gry8	ATCTGACGCAAAAGTCGCT	19	47	49
	Iap-Gry9	TGTCGTTACGAAAGCAGTG	19	47	49
	Iap-Gry10	TTCAAAAATTGATCGAATGGAA	22	27	46
hly specific	Hly-Mc1	TTTTTCGGCAAAGCTGTTAC	20	40	48
	Hly-Mc2	AAACAATAAAAGCAAGCTAGC	21	33	47

Continued on following page

TABLE 3—*Continued*

Type	Name	Sequence	Length (nt)	G+C (%)	T_m[a] (°C)
	Hly-Mc3	TTTTGATGCTGCCGTAAGT	19	42	47
	Hly-Mc4	TCCTTCAAAGCCGTAATTTAC	21	38	49
	Hly-Mc5	AATCTGTCTCAGGTGATGT	19	42	47
	Hly-Mc6	ACTGGAGCGAAAACAATAAAAGCAAGCTAGC	31	42	60
	Hly-Mc7	AATTATGATCCTGAAGGTAACGAAATTGTTCAAC	34	32	58
	Hly-Mc8	ACTAATTCCCATAGTACTAAAGTAAAAGCTGCT	33	33	58
	Hly-Mc9	ATTTTTCGGCAAAGCTGTTACTAAAGAGCAGTT	33	36	59
	Hly-Mc10	TAAAAGACAATGAATTAGCTGTTATTAAAAAC	32	22	53
	Hly-Ivan1	ATTCTTTGGTAAAAGTGTTAC	21	29	45
	Hly-Ivan2	AAAGAAAACTTGCAAGCGCT	20	40	48
	Hly-Ivan3	TCGTGACATTTTCGTGAAATT	21	33	47
	Hly-Ivan4	TCACACAGCACCAGAGTGA	19	53	51
	Hly-Ivan5	TACTGCATTTAAGGGTAAATC	21	33	47
	Hly-Ivan6	TGAAGGCTGCATTCGATAC	19	47	49
	Hly-Ivan7	GGAGATTTAAGCAAATTACGA	21	33	47
	Hly-Ivan8	CATTTTACGACATCAATCTATTT	23	26	46
	Hly-Ivan9	TATTAATATTCATGCGAAAGAAT	23	22	45
	Hly-Ivan10	TTGCGAGATTCAATGTTACTT	21	33	47
	Hly-Seel1	AACAACTAGATGCTTTAGGTG	21	38	49
	Hly-Seel2	ATGACGAGAACGGAAATGAA	20	40	48
	Hly-Seel3	ATTAACATCTATGCAAGAGAAT	22	27	46
	Hly-Seel4	GAAATGAAATAAAAGTTCATAAGAA	25	20	46
	Hly-Seel5	CAATTTAGGCGAACTTCGAG	20	45	50
	Hly-Seel6	ACTATCCTCTAGCTCGCAT	19	47	49
	Hly-Seel7	GTGGTTATGTAGCCCAATT	19	42	47
	Hly-Seel8	GGTCCACTTATGATAGAGAA	20	40	48
	Hly-Seel9	AGTAACAAAGTTAAAACTGCTTT	23	26	46
	Hly-Seel10	TCGAGGCGGCGATGAGTGG	16	68	58
inlB specific	InlB1	ATGGGAGAGTAACCCAACCACTGAA	25	48	58
	InlB2	GAAAAGCAAAAGCAAGATTTCATGGGAGAG	30	40	59
	InlB3	ACGTTTAACTAAGCTGGATACTTTGTCTC	29	38	57
	InlB4	GACTCCAGAAATAATAAGTGATGATGGC	28	39	57
	InlB5	AATAAGTGATGATGGCGATTATGAAAAACC	30	33	56
	InlB6	CCAAATTAGTGATATTGTGCCACTTG	26	38	55
	InlB7	CAGAAATAATAAGTGATGATGGCGATTATG	30	33	56
	InlB8	TGGAAAGTTTGTATTTGGGAAATAAT	26	27	50
	InlB9	AAATGGCATTTACCAGAATTTATAAAT	27	22	49
	InlB10	TTTAAGTAAGAATCACATAAGCGATTTAAG	30	27	53
plcA specific	PlcA1	CTAATATCGATGTACCGTATTCCTGCT	27	41	57
	PlcA2	CCATGGTAAATGTTGAGATTGTCTTTTGC	29	38	57
	PlcA3	TTTCTTTAAAAATTGAGTAATCGTTTCTAATACA	34	21	54
	PlcA4	CGCATAATAATGGTTTCTTTTGGATTTTTC	30	30	55
	PlcA5	TATCGTTGCTGTTTTGCTCGTCTTTTAAA	29	34	56
	PlcA6	TTGATTAGTGGTTGGATCCGATAATCAAA	29	34	56
	PlcA7	AGTTCTGGGAGTAGTGTAAAAATAATCTTT	30	30	55
	PlcA8	GTAGGGATTTTATTGCTCGTGTCAG	25	44	56
	PlcA9	GTAATAATATTTTTCCGCGGACATCTTTTA	30	30	55
	PlcA10	AATGGCTTTTTTGTGTGGTTCTCTGAAA	28	36	56
plcB specific	PlcB1	AATATCATACCCTCCAGGCTACCACT	26	46	58
	PlcB2	AGTCAACCTATGCACGCCAATAATTTT	27	37	55
	PlcB3	GCCTAGCAATCCATTATTATACGGATATT	29	34	56
	PlcB4	GAAATTTGACACAGCGTTTTATAAATTAG	29	28	53
	PlcB5	ATTTCAATCAATCGGTGACTGATTACCGA	29	38	57
	PlcB6	GCTAATGCGAAAATAACAGGAGCAAAG	27	41	57
	PlcB7	TGATAGAGATAATACTTATTTGCCGGGTTT	30	33	56
	PlcB8	AGTACATTTTTATCTCATTTTTATAATCCTGATAG	35	23	55
	PlcB9	ATGCGGATCATAAAAATCCATATTATGATACT	32	28	55
	PlcB10	GATAAACAAATAGCTCAAGGAATATATGATG	31	29	55
clpE specific	ClpEm1	CTTACTTTTTTTATAAAAAAAACGCCCCTATA	32	25	54
	ClpEm2	GTAAGGCTGCGAGTAACAAAAACGCA	26	46	58

Continued on following page

TABLE 3—*Continued*

Type	Name	Sequence	Length (nt)	G+C (%)	T_m[a] (°C)
	ClpEm3	GATAAATATAGCATAAGTAGGCTTGGAC	28	36	56
	ClpEm4	ACGAGCGAAGCGACAAAGATACCAAT	26	46	58
	ClpEm5	GATACCAATAATCGCTGCAAAATTGC	26	38	55
	ClpEm6	CTCCCGGGATGATGGCAACGATAA	24	54	59
	ClpEm7	GCAACGATAAGTAGTAGCCAAATTATC	27	37	55
	ClpEm8	CATAATAACGCTGCAAAAACATTCGAGAA	29	34	56
	ClpEm9	TAGCCAAATTATCAAAGAATGCGTCGAA	28	36	56
	ClpEm10	ATGATATTCCATACGAGCGAAGCGA	25	44	56
QC	QCprb	TTGGCAGAAGCTATGAAACGATATGGG	27	44	58
	Cy3-QC	CCCATATCGTTTCATAGCTTCTGCCA	26	46	58

[a] Basic melting temperature (T_m) was calculated with the Oligonucleotide Properties Calculator (http://www.basic.nwu.edu/biotools/oligocalc.html).

of them, *iap* and *hly*, as the most suitable for development of the microarray-based assay. The first gene, *iap*, encodes the 60-kDa secreted invasion-associated protein p60 (26). Homologues of this gene have been found in all *Listeria* species (8). Previously, the *iap* gene was successfully used for *Listeria* species discrimination by methods based on PCR (6, 7). Minor differences in the sequences of *L. monocytogenes* serovars can also be used for their identification using restriction enzyme-PCR (11). Another gene selected, *hly*, encodes a virulence factor that is involved in release of *Listeria* from primary phagosomes of host cells (5). This gene is specific only to *L. monocytogenes*, *L. ivanovii*, *and L. seeligeri* and can be used for distinguishing among clinically important serotypes of *L. monocytogenes* (46). However, in the present study we did not pursue the determination of *L. monocytogenes* types, and serotype-specific oligonucleotides for the *iap* and *hly* genes were not included in the microchip.

The last four genes, *inlB*, *plcA*, *plcB*, and *clpE*, were included in the microarray to verify the identity of *L. monocytogenes*.

Some genetic markers, including rRNA and 16S-23S rRNA intergenic spacers, previously used for distinguishing among *Listeria* species (18, 21, 36), were found to be highly conserved among *Listeria* species and unsuitable for development of multiple oligoprobes for microarray-based discrimination. Other genes, like *prfA* and *actA* (45), were not included in the assay because they are missing from some *Listeria* species or are highly divergent, making it difficult to select universal primers for PCR amplification.

Thus, our approach is based on identification of six virulence factor genes in the bacterial chromosome: *iap*, *hly*, *inlB*, *plcA*, *plcB*, and *clpE*. These factors were simultaneously amplified by one-tube multiplex PCR in the presence of primers for all six genes. *Listeria* species were discriminated on the basis of the hybridization profile of the fluorescent DNA sample with the universal *Listeria* microarray, which contained immobilized species-specific oligoprobes.

Microarray hybridization has the potential to become the most efficient approach for rapid PCR-based detection and

TABLE 4. Primers used for amplification of various *Listeria* genes

Primer	Nucleotide sequence (5′–3′)	Target gene	GenBank accession no.	PCR product size (bp)	T_m[f] (°C)
LisF	ATGAATATGAAAAAAGCAACKATC	*iap*	M80351, M80350, M80349, M80353, M80354, M80352	644,[a] 707,[b] 713,[c] 716[d]	47–49
LisR	ACATARATIGAAGAAGAAGAWARATTATTCCA				52–55
IsoF	GTTAATGAACCTACAAGHCCTTCC	*hly*	AF253320, X60461, X60462	708	54–56
IsoR	AACCGTTCTCCACCATTCCCA				54
PlcBF	GATAACCCGACAAATACTGACGTAAATAC	*plcB*	AL591977, AL591974	503	57
PlcBR	TCATCTGAGCAAAATCTTTTTGCTACCATGTC				59
ClpEF	CTCCTTTTAAAAATGAGAAATGAAAGGTCTTG	*clpE*	AL596167	517,[e] 518[f]	57
ClpER	TTAAAGTAATGCCAGGCGGTCTAGAACATTC				60
InlBF	AATCACTTTCTTTGGAGCATAATGGTATAAGTG	*inlB*	AF121047	562	58
InlBR	GTTCCATCAACATCATAACTTACTGTGTAAACC				59
PlcAF	AAGTTGAGTACGAAYTGCTCTACTTTGTTG	*plcA*	U25453	600	58–59
PlcAR	AACACAAACGATGTCATTGTACCAACAACTAG				59

[a] *L. grayi*.
[b] *L. ivanovii* and *L. welshimeri*.
[c] *L. seeligeri*.
[d] *L. innocua*.
[e] *L. monocytogenes*.
[f] T_m, melting temperature.

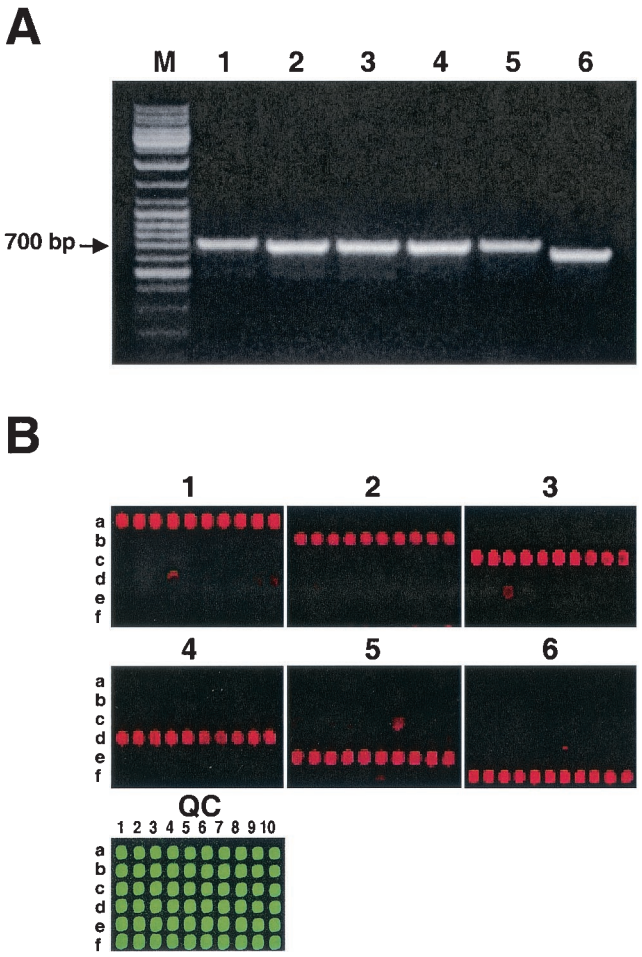

FIG. 1. Species identification based on differences in *iap* gene sequences. Genomic DNAs of six reference strains were amplified by using universal *iap*-specific primers followed by separation of PCR products in a 1.5% agarose gel (A). Lanes: M, 100-bp DNA ladder mix; 1, *L. monocytogenes* (H9600 strain); 2, *L. ivanovii* (ATCC 19119); 3, *L. welshimeri* (LABSTR strain); 4, *L. innocua* (ATCC 33090); 5, *L. seeligeri* (ATCC 35967); 6, *L. grayi* (ATCC 25401). Species-specific DNAs were hybridized with the *iap* microchip (B) containing individual oligonucleotides specific to *L. monocytogenes* (row a), *L. ivanovii* (row b), *L. welshimeri* (row c), *L. innocua* (row d), *L. seeligeri* (row e), and *L. grayi* (row f). Microarray image panels are numbered in accordance with the species numeration in Fig. 1A. QC, Cy3 microarray QC image (see Materials and Methods). For panel QC, 10 individual species-specific oligoprobes were spotted on each row and labeled 1 through 10.

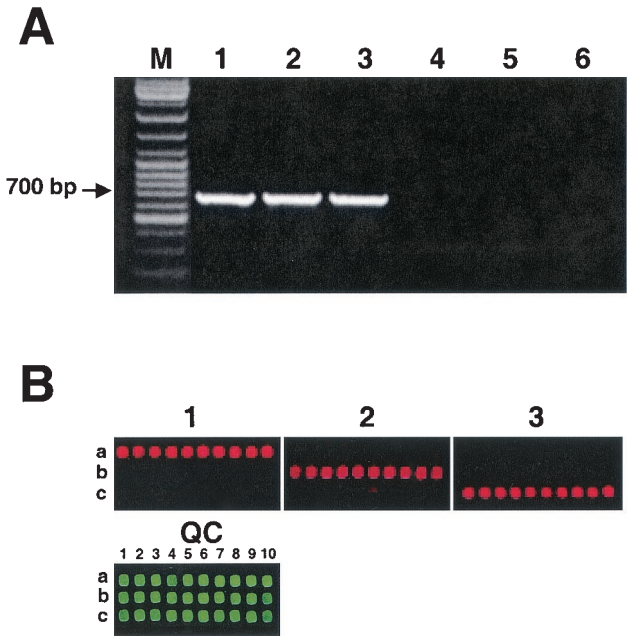

FIG. 2. Identification of hemolytic *Listeria* species. The DNAs of six reference strains were amplified by using universal *hly*-specific primers and separated in a 1.5% agarose gel (A). Lanes: M, 100-bp DNA ladder mix; 1, *L. monocytogenes* (H9600 strain); 2, *L. ivanovii* (ATCC 19119); 3, *L. seeligeri* (ATCC 35967); 4, *L. welshimeri* (LABSTR strain); 5, *L. innocua* (ATCC 33090); 6, *L. grayi* (ATCC 25401). (B) Hybridization images of amplified DNAs with the *hly* microchip. Oligonucleotides were specific to *L. monocytogenes* (a), *L. ivanovii* (b), and *L. seeligeri* (c). Microarray image panels are numbered in accordance with the species numbering in panel A. For panel QC, 10 individual species-specific oligoprobes were spotted on each row and labeled 1 through 10.

characterization of viral and bacterial contamination in a variety of samples, including food products. The PCR by itself is a very sensitive method and allows the amplification of single DNA molecules. However, the sensitivity and specificity of PCR amplification tend to be inversely related, such that DNA generated in a highly sensitive PCR assay often contains nonspecific products which complicate the interpretation of the electrophoresis data (17). Although gel electrophoresis, usually used for the analysis of PCR products, is simple and quick, it is often inadequate for accurate characterization of multiple PCR products. In addition, species-specific PCR primers occasionally fail to amplify DNA from a particular isolate if there is a spontaneous mutation(s) in the primer-binding site. However, it may be possible to avoid these shortcomings and improve PCR-based genotyping significantly by replacing gel electrophoresis analysis of PCR products by DNA-DNA hybridization in microarray format. Firstly, the specificity of DNA-DNA hybridization allows the unambiguous detection of target sequences regardless of the potential presence of nonspecific DNA products. Secondly, microarray technology permits the use of universal degenerate primers capable of amplifying all the genetic variants of the target gene(s), thereby increasing the sensitivity and efficiency of the assay. Thirdly, the miniature size of the microarray enables simultaneous analysis of a large number of the genetic markers in one experiment. Fourthly, the use of multiple oligonucleotides for each target gene increases the certainty of detection and discrimination between closely related species. Therefore, species can be identified by recognizing the hybridization pattern of bacterial DNA with species-specific microarray probes. Moreover, hybridization with short oligonucleotides (20 to 25 nucleotides) is sensitive enough to detect a single nucleotide mismatch between the template DNA and the oligoprobes, thus allowing one to monitor minor genetic variability of target genes in a bacterial population.

In previous studies (9, 10) in which DNA samples were labeled with fluorescent dyes during PCR amplification, it was found that in order to maximize assay sensitivity, the labeling

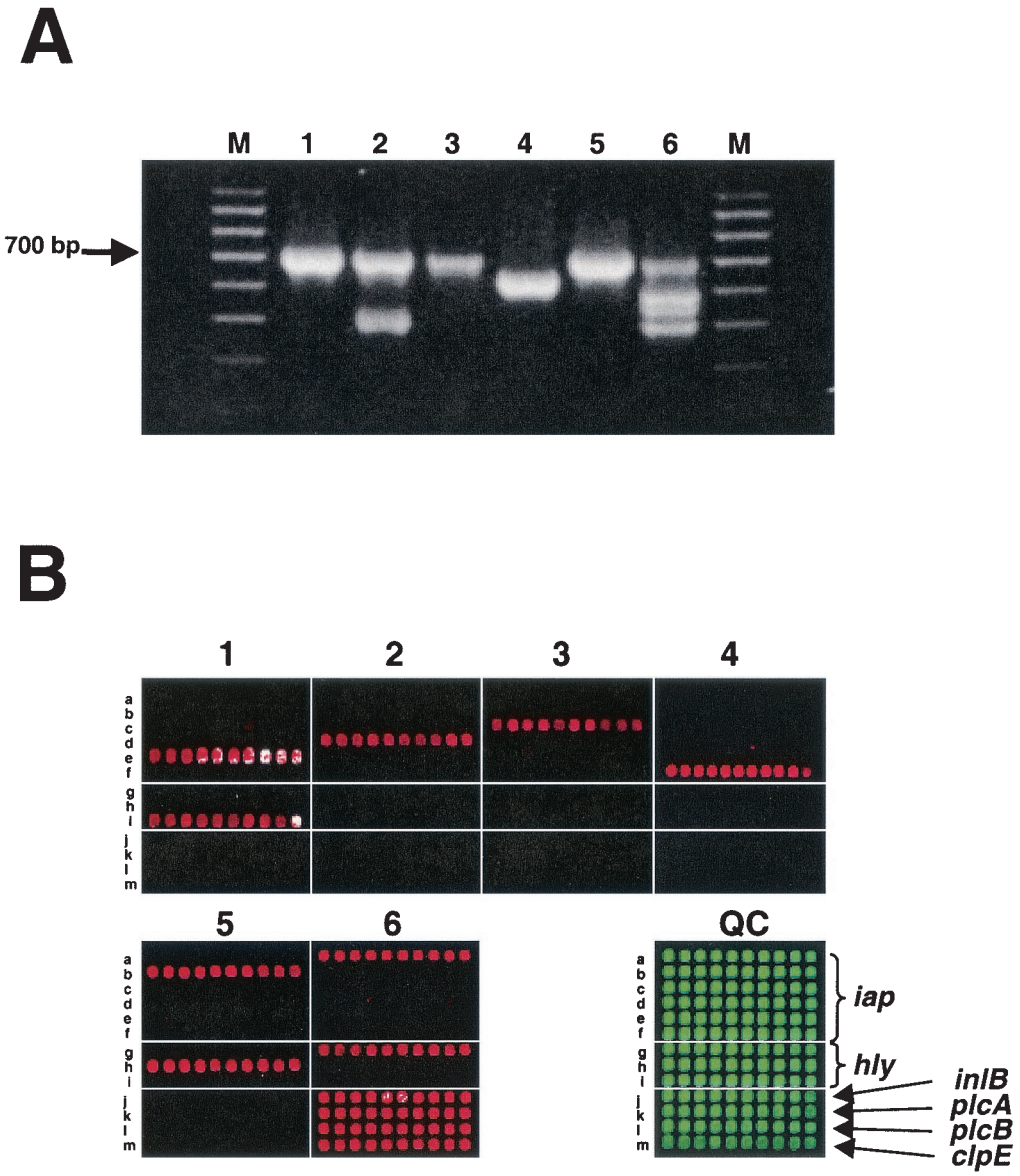

FIG. 3. Identification of *Listeria* species using the universal *Listeria* microchip. Bacterial DNAs were amplified by one-tube multiplex PCR (A) and analyzed by microarray assay (B). The multiplex PCR was performed in the presence of the *iap*-, *hly*-, *inlB*-, *plcA*-, *plcB*-, and *cplE*-specific primers. Lanes: M, 100-bp DNA ladder; 1, *L. seeligeri* (ATCC 35967); 2, *L. innocua* (ATCC 33090); 3, *L. welshimeri* (BA84 strain); 4, *L. grayi* (ATCC 25401); 5, *L. ivanovii* (ATCC 19119); 6, *L. monocytogenes* (LM82 strain). Rows a to f contain *iap*-specific oligonucleotides (identical to Fig. 1B), rows g to i contain *hly*-specific oligonucleotides (identical to Fig. 2B), and rows j to m contain oligonucleotides specific to the other four *L. monocytogenes* virulence factors. Microarray image panels are numbered in accordance with the species numbering in panel A. QC, composition microarray for *Listeria* species identification.

step and PCR step should be separated. The presence of Cy5(3)-dCTP was observed to diminish the yield of the PCR products. Therefore, in the present study, we replaced the method of ssDNA synthesis with a primer extension of DNA synthesized in the first round of PCR. This approach ensured adequate quantity and quality of fluorescent ssDNA and enabled unambiguous identification of DNA by microarray hybridization even in cases where we failed to detect the presence of the respective PCR product by gel electrophoresis (data not shown).

The approach described in this study is one of the first attempts to use oligonucleotide microarray technology combined with PCR amplification for monitoring the safety of the food supply. It is noteworthy that previously developed PCR-based protocols for bacterial identification can be easily adapted to take advantage of the specificity and speed of microarray hybridization to produce powerful tools for rapid, high-throughput screening and accurate genotyping of a variety of viral and bacterial pathogens.

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