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Nucleic acid-based, cultivation-independent detection of *Listeria* spp. and genotypes of *L. monocytogenes*

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Abstract

Based on comparative analysis of 16S rRNA gene sequences, two oligonucleotide probes for in situ detection of all members of the genus *Listeria* were designed. These probes allowed fast and reliable in situ detection of *Listeria* spp. even in complex samples like raw milk. Almost full-length *iap* (invasion-associated protein) gene sequences were determined for 69 *Listeria monocytogenes* strains of all 13 known serotypes. A comparison of these sequences revealed that the *L. monocytogenes* strains can be grouped into three distinct genotypes. These clusters correlate well with distinct serotypes. Thus, strains of serotypes b and d belong to genotype I, a and c to genotype II, and 4a and 4c, which are rarely isolated from humans, group together within genotype III. These results could be corroborated by further comparative sequence analysis of genes encoding two phospholipases – *plcA* and *plcB*. Based on the *iap* gene sequences, a highly specific and reproducible competitive PCR detection method was developed. Primer pairs targeting genotype-specific regions of the *iap* gene were designed. The amplification of non-specific PCR products from DNA of non-target strains was prevented by adding competitive primers. By applying this method, the rapid and reliable distinction of the three *L. monocytogenes* genotypes was possible.

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Keywords: *Listeria monocytogenes*; Fluorescence in situ hybridization; Competitive polymerase chain reaction; Strain differentiation

1. Introduction

Listeria monocytogenes belongs to the Gram-positive bacteria with a low DNA G+C content [1] and is associated with potentially severe food-borne diseases characterized by meningitis, encephalitis or septicemia in immunocompromised patients, infants or the elderly [2,3]. Due to the ability of *L. monocytogenes* to replicate even at refrigeration temperatures [2], it has become increasingly important as one of the major pathogens accounting for an estimated 28% of all food-related deaths [4]. Only three (1/2a, 1/2b and 4b) of the 13 known *L. monocytogenes* serotypes are responsible for over 90% of all listeriosis cases [5,6]. Moreover, serotype 4b strains were responsible for all epidemic outbreaks reported in Europe and North America during the last two decades [7–9].

Species- or strain-specific detection of *L. monocytogenes* from environmental or food samples requires labor-intensive isolation and biochemical identification methods and is therefore very time-consuming. However, for epidemiological studies, a reliable and fast method for the detection of a pathogen is essential, especially with regard to contaminated food as a main cause of listeriosis. PCR-based methods are useful for fast and reliable identification of *L. monocytogenes* [10–15].

Differentiation of *L. monocytogenes* strains can be performed by both genetic methods (e.g. pulsed-field gel electrophoresis [16], ribotyping, random amplification of polymorphic DNA [17–19], restriction fragment length

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polymorphism [20], genomic subtraction [21]) and phenotypic subtyping methods (e.g. phage typing [22–25], serotyping [26–28] or multilocus enzyme electrophoresis [29]). All these methods require pure cultures of *L. monocytogenes* and are therefore very time-consuming. In addition, many techniques are characterized by a low reproducibility or typeability.

Nowadays, culture-independent approaches such as the fluorescence in situ hybridization (FISH) technique [30] have become important tools for the specific and fast detection of human pathogens [31–35]. In the present study we applied two 16S rRNA-targeted probes for the in situ detection of all members of the genus *Listeria*.

Several attempts have been undertaken to get a better insight into the phylogeny of the genus *Listeria* and especially of *L. monocytogenes* because it is known that there are significant strain differences between a number of *L. monocytogenes* isolates (e.g. [36]). Strains of *L. monocytogenes* can be subdivided into three distinct clusters based on partial sequences of some *L. monocytogenes* functional and virulence genes (*iap*, *hly*, *flaA*, *actA* [36,37]). However, since only partial sequences were compared, it was not possible to retrieve meaningful phylogenetic conclusions from these studies.

The gene of choice for phylogenetic investigations is the 16S rDNA gene, but a classification of *L. monocytogenes* strains cannot be based on ribosomal RNA sequences alone since the gene is too conserved to distinguish even *Listeria* species [38]. Therefore, we used the *iap* (invasion-associated protein) gene for phylogenetic studies of all members of the genus *Listeria*. This gene is less conserved than the 16S rRNA gene and its highly variable central domain may allow a subdifferentiation of *L. monocytogenes* isolates.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains used in this study were isolated from environmental sources (soil and waste processing plants) or kindly provided by W. Goebel, University of Würzburg and Martin Wagner, University of Vienna and are listed in Table 1. All strains were grown aerobically at 37°C in brain heart infusion broth (BHI; Difco Laboratories) and were maintained on BHI agar. Stock cultures were stored at −80°C.

2.2. Isolation of genomic DNA

Two milliliters of an overnight culture were incubated with penicillin G (500 U ml^{-1}, 1 h, 37°C), then pelleted by centrifugation (5 min, 14 000 rpm). After washing with phosphate-buffered saline, DNA was extracted according to the following protocol. Bacterial pellets were resuspended in 500 µl distilled H_2O and, after an initial incubation with 300 U ml^{-1} mutanolysin (45 min, 37°C), were digested with 70 U ml^{-1} proteinase K. Subsequently, cells were incubated in 1% sodium dodecyl sulfate at 60°C for 30 min, and cell lysis was completed by the addition of NaCl to a final concentration of 1 M. Lysis was detectable by the clearing of the solution. DNA was further extracted by adding 1 vol chloroform–isoamyl alcohol (24:1). After vortexing, the mixture was centrifuged at 25 000 × *g* at 4°C. The aqueous phase was carefully transferred to a fresh tube, mixed with 0.1 vol 3 M sodium acetate, and the DNA was precipitated with 0.56 vol isopropanol for 30 min at room temperature. After centrifugation (25 000 × *g*, 15 min, 4°C), pellets were washed with 200 µl 70% ethanol, dried and resuspended in 50–100 µl H_2O. DNA isolation was furthermore performed from exponentially growing *Listeria* cells (OD_{600} of 0.6–1.0) using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Isolation was performed according to the manufacturer's instructions. The amount and quality of the DNA was determined spectrophotometrically and by gel electrophoresis.

2.3. PCR amplification of iap genes

Complete *iap* gene fragments were amplified from DNA of *Listeria* spp. using the Lis1A/Lis1B primer pair [39], resulting in a PCR product of about 1500 bp. Positive controls with pure DNA from *L. monocytogenes* EGD type strain as well as a negative control omitting DNA were included in the PCR reaction. Sizes of the PCR products were determined by agarose gel electrophoresis. Amplification of *plcB* fragments was performed as described by Vasquez-Boland et al. [40].

2.4. Competitive PCR amplification of iap gene fragments for the specific detection of L. monocytogenes genotypes

For the specific amplification of *iap* gene fragments from the three *L. monocytogenes* genotypes, a competitive PCR method was developed. Competitive primers (containing a 2′,3′-dideoxynucleotide (ddNTP)), having no mismatches for priming regions on the DNA of non-target organisms, were added to the reaction mixture. The addition of competitive primers in excess was a prerequisite to obtain specific PCR signals for each genotype. PCR reactions were performed in a capillary cycler (Idaho Technology); appropriate annealing temperatures were determined using a gradient master PCR cycler (Eppendorf, Hamburg, Germany). Primers and competitors for the detection of the three *L. monocytogenes* genotypes and the respective PCR conditions are listed in Tables 2 and 3.

2.5. Construction of competitive primers

Competitive primers (competitors) were purchased from

the supplier (Interaktiva, Ulm, Germany) shortened by one 3′-terminal base. The respective ddNTP was added by a terminal transferase reaction according to the manufacturer's instructions (Roche, Mannheim, Germany).

2.6. Cloning, sequencing and phylogenetic investigation

Full-length *iap* PCR products were ligated into the vector pCR®2.1-TOPO according to the manufacturer's instructions (Invitrogen, San Diego, CA, USA) and transformed into chemocompetent INVαF′ *Escherichia coli* cells.

Nucleotide sequences were determined using the chain termination method [41], sequencing purified plasmid preparations or purified PCR products. Sequencing reactions employed the Thermo Sequenase Cycle sequencing kit (Amersham, Little Chalfont, UK) with infrared dye (IRD-800)-labelled, plasmid (M13)- or *iap*-targeted sequencing primers (Table 2). Nucleic acid sequences obtained were aligned according to predicted amino acids to a database comprising previously described *iap* gene sequences [39] using the ARB program package (http://www.arb-home.de). Phylogenetic analysis was performed using distance matrix, maximum likelihood and maximum parsimony methods in the ARB and PHYLIP (Phylogeny Inference Package) software package. To apply *iap* gene sequences for phylogenetic studies, filters were constructed that allowed the exclusion of *iap* TN (threonine-asparagine) repeats because of the low value phylogenetic information within repetitive domains of a protein.

2.7. Nucleotide sequence accession numbers

Nucleic acid sequences were submitted to GenBank. Nucleotide sequence accession numbers are listed in Table 1.

2.8. Enrichment of Listeria cells

Raw milk (25 ml) was aseptically sampled and transferred into 225 ml buffered *Listeria* enrichment broth supplemented with selective reagents provided by the manufacturer (Oxoid, Wesel, Germany). After 1, 2 and 7 days of incubation, 1 ml was transferred into 9 ml Fraser Bouillon with selective supplement (Oxoid). After 24 and 48 h incubation, an inoculation loop was streaked onto Palcam and Oxford agar, both with selective supplement (Oxoid). All incubation steps followed the manufacturer's recommendations. Samples for FISH were harvested 1, 2, 3 and 7 days after incubation from the first enrichment step in buffered *Listeria* enrichment broth and fixed as described below.

2.9. Probe design, evaluation, synthesis and labeling

Probe design was performed with the PROBE DESIGN tool included in the software package ARB (http://arb-home.de). Oligonucleotide probes were labeled at the 5′-end with the fluorescent dyes Cy3, Cy5 or FLUOS and were purchased from Interactiva (Ulm, Germany). Conditions for in situ hybridization of the newly designed probe were optimized with fixed reference strains by gradually increasing the formamide concentration of the hybridization buffer [42]. The optimal formamide concentration is the highest concentration which still yields good signals with the target cells but allows the discrimination of non-target cells. All probes used in this study are summarized in Table 4.

2.10. Fluorescence in situ hybridization

Cells from exponentially growing cultures or from samples after pre-enrichment were fixed with ethanol as described by Amann et al. [30]. Hybridizations with fluorescently labelled oligonucleotide probes were performed according to Stoffels et al. [43].

3. Results

3.1. In situ detection of Listeria with 16S rRNA-targeted oligonucleotide probes

The standard technique of identifying *Listeria* and especially the human pathogen *L. monocytogenes* relies on a number of selective enrichment steps. To minimize the time-consuming enrichment and isolation procedure, oligonucleotide probes for in situ detection of *Listeria* spp. were designed. Probe design and specificity analysis were performed using the ARB software package (http://arb-home.de) and the implemented tools PROBE DESIGN and PROBE MATCH. The probe Lis-1255 was constructed in a previous study [35]. This probe has no mismatch within the 16S rRNA binding region of all *Listeria* species, and it also completely matches the binding region of the 16S rRNA molecules of *Brochothrix thermosphacta* and *Brochothrix campestris*. The newly designed probe Lis-637 perfectly matches the target region of all members of the genus *Listeria* except *L. grayi*. Moreover, it did not react with members of the genus *Brochothrix*. The applicability and specificity of both fluorochrome-labelled probes for in situ hybridization of whole cells was proven using ethanol-fixed reference strains of all *Listeria* species, all serotypes of *L. monocytogenes* and representative non-target reference organisms. Hybridization conditions were adjusted and optimized by increasing the formamide concentration. The formamide concentrations recommended for in situ hybridization were the highest that still yielded strong signals with the target organisms and prevented binding of the probe to non-target reference strains. Using 35% deionized formamide in the hybridization buffer, only target organisms were detected after hybridization with

Table 1
List of all *Listeria* strains used in this study and the affiliation of *L. monocytogenes* strains to the respective genotypes

Species	Serotype [sv]	Culture collection	GenBank accession numbers of *iap* gene sequences	*iap* TN repeats	GenBank accession numbers of *plcA* gene sequences	GenBank accession numbers of *plcB* gene sequences	Genotype
L. grayi	n.d.	TUMLis1	M80352	–	–	–	–
L. innocua	6a	NCTC11288	M80349	20	–	–	–
L. ivanovii	5	ATCC19119	M80350	–	X72685	U19035	–
L. seeligeri	n.d.	SLCC3954	M80353	–	X97014	AY150839	–
L. welshimeri	n.d.	SLCC5828	M80354	–	–	–	–
L. monocytogenes EGD-e	1/2a	TUMLis18	X52268	19	AL591974	X59723	I
L. monocytogenes Mackaness	1/2a	SLCC 5764	AF532236	19	–	–	I
L. monocytogenes Aachen	1/2a	TUMLis17	AF532253	18	–	–	I
L. monocytogenes LL141	1/2a	TUMLis26	AF532242	18	–	–	I
L. monocytogenes LL365	1/2a	TUMLis27	AF532244	18	–	–	I
L. monocytogenes LL93	1/2a	TUMLis28	AF532237	19	–	–	I
L. monocytogenes	1/2a	IMVW1433	AF532243	19	–	–	I
L. monocytogenes	1/2a	IMVW1449	AF532230	16	–	–	I
L. monocytogenes	1/2a	IMVW1454	AF532252	21	–	–	I
L. monocytogenes	1/2a	IMVW1632	AF532232	17	–	–	I
L. monocytogenes	1/2a	IMVW34564	AF532247	17	–	–	I
L. monocytogenes	1/2a	IMVW349	AF532246	17	–	–	I
L. monocytogenes	1/2a	IMVW761	AF532248	24	–	–	I
L. monocytogenes	1/2c	IMVW310	AF532260	19	–	–	I
L. monocytogenes	1/2c	IMVW351	AF532255	20	–	–	I
L. monocytogenes	1/2c	NCTC 5348	AF532254	20	AF532206	AY150830	I
L. monocytogenes F strain	3a	TUMLis19	AF532251	19	–	AY150838	I
L. monocytogenes	3a	NCTC5105	AF532259	19	AF532207	AY150831	I
L. monocytogenes	3a	IMVW1630	AF532231	16	–	AY150840	I
L. monocytogenes	3c	SLCC 2479	AF532258	19	AF532208	–	I
L. monocytogenes	4ab	SLCC 4561	AF532257	19	AF532210	–	I
L. monocytogenes Iso3	n.d.	TUMLis22	AF532223	17	–	–	I
L. monocytogenes R479a	n.d.	TUMLis30	AF532234	18	–	–	I
L. monocytogenes R62a	n.d.	TUMLis31	AF532235	18	–	–	I
L. monocytogenes V527a	n.d.	TUMLis33	AF532240	17	–	–	I
L. monocytogenes V5a	n.d.	TUMLis34	AF532238	16	–	–	I
L. monocytogenes	n.d.	IMVW1302	AF532302	18	–	–	I
L. monocytogenes	n.d.	IMVW1436	AF532239	17	–	–	I
L. monocytogenes	n.d.	IMVW1516	AF532241	17	–	–	I
L. monocytogenes	n.d.	IMVW1602	AF532297	18	–	–	I
L. monocytogenes	n.d.	IMVW567	AF532249	19	–	–	I
L. monocytogenes	n.d.	IMVW568	AF532250	19	–	–	I
L. monocytogenes	1/2b	SLCC 2755	AF532277	17	AF532205	AY150836	II
L. monocytogenes	1/2b	IMVW1464	AF532283	17	–	–	II
L. monocytogenes	1/2b	IMVW1726	AF532284	17	–	–	II
L. monocytogenes	1/2b	IMVW1727	AF532285	17	–	–	II
L. monocytogenes	3b	SLCC 5543	AF532261	17	AF532216	AY150837	II
L. monocytogenes	3b	IMVW1603	AF532298	17	–	–	II
L. monocytogenes	4b	SLCC4013	AF532287	16	AF532211	AY150833	II
L. monocytogenes ScottA	4b	TUMLis32	AF532288	16	–	–	II
L. monocytogenes	4b	IMVW1724	AF532294	16	–	–	II
L. monocytogenes	4b	IMVW1725	AF532292	16	–	–	II
L. monocytogenes	4d	ATCC 19117	AF532291	16	AF532213	AY150834	II
L. monocytogenes	4e	ATCC 19118	AF532293	16	AF532214	–	II
L. monocytogenes	4e	IMVW1446	AF532272	16	–	–	II
L. monocytogenes	7	SLCC 2482	AF532276	17	AF532215	AY150835	II
L. monocytogenes	n.d.	IMVW1427	AF532280	17	–	–	II
L. monocytogenes	n.d.	IMVW1428	AF532281	17	–	–	II
L. monocytogenes	n.d.	IMVW1447	AF532289	16	–	–	II
L. monocytogenes	n.d.	IMVW1517	AF532273	16	–	–	II
L. monocytogenes	n.d.	IMVW1577	AF532282	13	–	–	II
L. monocytogenes	n.d.	IMVW2291	AF532262	13	–	–	II
L. monocytogenes	n.d.	IMVW2296	AF532290	16	–	–	II
L. monocytogenes	n.d.	IMVW2309	AF532271	16	–	–	II
L. monocytogenes	n.d.	IMVW2311	AF532269	16	–	–	II
L. monocytogenes	n.d.	IMVW2312	AF532268	16	–	–	II

Table 1 (*Continued*).

Species	Serotype [sv]	Culture collection	GenBank accession numbers of *iap* gene sequences	*iap* TN repeats	GenBank accession numbers of *plcA* gene sequences	GenBank accession numbers of *plcB* gene sequences	Genotype
L. monocytogenes	n.d.	IMVW2313	AF532270	16	–	–	II
L. monocytogenes	n.d.	IMVW2314	AF532264	16	–	–	II
L. monocytogenes	n.d.	IMVW478	AF532279	16	–	–	II
L. monocytogenes	n.d.	IMVW479	AF532300	16	–	–	II
L. monocytogenes Iso1	n.d.	TUMLis20	AF532263	16	–	–	II
L. monocytogenes Iso11	n.d.	TUMLis21	AF532295	13	–	–	II
L. monocytogenes Iso45	n.d.	TUMLis23	AF532267	16	–	–	II
L. monocytogenes Iso46	n.d.	TUMLis24	AF532266	16	–	–	II
L. monocytogenes Iso50	n.d.	TUMLis25	AF532278	17	–	–	II
L. monocytogenes NV4700	n.d.	TUMLis29	AF532274	16	–	–	II
L. monocytogenes	4a	SLCC2374	AF532229	11	AF532211	AY150841	III
L. monocytogenes	4a	TUMLis6	AF532228	11	–	–	III
L. monocytogenes	4c	ATCC 19116	AF532227	11	AF532212	AY150832	III

ATCC: American Type Culture Collection, Rockville, MD, USA; IMVW: Institute for Milk Hygiene, Veterinary University of Vienna, Austria; NCTC: National Collection of Type Cultures; SLCC: Special Listeria Culture Collection, Institute of Hygiene and Microbiology, University of Würzburg, Germany; TUM: Technical University of Munich, Institute for Microbiology, Germany.
n.d.: not determined. TN: threonine-asparagine repeats within the *iap* gene.
Strain *L. monocytogenes* sv 4a (L99) was obtained from T. Chakraborty, Institute for Medical Microbiology, Gießen, Germany.

Table 2
Primers used in this study

Primer	5′-3′ Sequence	Target	Reference
iap gene-specific primers			
Lis1A	ATG-AAT-ATG-AAA-AAA-GCA-AC	*iap* gene	[39]
Lis1B	TTA-TAC-GCG-ACC-GAA-GCC-AA	*iap* gene	[39]
IRD_{800}-labelled sequencing primers			
iap-F-IR	AAA-AAA-GCA-ACT-ATC-GCG-GC	*iap* gene	this study
iap-F-IR	GCC-AAC-TAG-ATA-TTT-ACC-CC	*iap* gene	this study
iap-int-F-IR	CTG-TTA-TCA-ACA-CCA-GCG-CC	*iap* gene	this study
M13-universal	TGT-AAA-ACG-ACG-GCC-AGT	vector pCR®2.1	Invitrogen
PCR primers for each evolutionary genotype			
iap-412-I-F	TAC-TTA-ACT-GAC-AAA-GCA-GT	genotype I	this study
iap-1047-I-R	ATT-CGT-ATT-AGT-ATT-TGA-GTT-TG	genotype I	this study
iap-820-II-F	ACT-AAC-ACT-AAC-ACA-AAT-GC	genotype II	this study
iap-1047-II-R	GGA-GTT-TGT-ATT-AGT-ATT-GGT-A	genotype II	this study
iap-217-III-F	AAT-GAG-GTC-GCT-AAA-AAC-AC	genotype III	this study
iap-1047-III-R	TGT-GTT-CGT-GTT-TGT-ATT-TGT-G	genotype III	this study
Competitive primers			
iap-1-dd-II/III-F	TAC-TTA-ACT-GAC-AAA-GTA-G*	genotype I	this study
iap-1-dd-II-R	ATT-CGT-ATT-GGA-GTT-TGT-ATT-A*	genotype I	this study
iap-1-dd-III-R	AGC-ATT-TGT-GTT-CGT-GTT-T*	genotype I	this study
iap-2-dd-I/III-F	ACK-AAY-ACA-AAT-ACW-GCT-M*	genotype II	this study
iap-2/3-dd-I-R	AGT-ATT-TGA-GTT-TGT-ATT-AGT-AT*	genotype II	this study
iap-2-dd-III-R	TGT-GTT-CGT-GTT-TGT-ATT-TGT*	genotype II	this study
iap-3-dd-I/II-F	GAG-GTK-GCT-GCT-GCT-G*	genotype III	this study
iap-3-dd-II-R	GGA-GTT-TGT-ATT-AGT-ATT-GGT*	genotype III	this study
plcA gene-specific primers			
plcA-F	ACC-AGG-TAC-ACA-TGA-ATA-CGA	*plcA* gene	this study
plcA-R	CCG-AGG-TTG-CTC-GGA-GAT-AT	*plcA* gene	this study
plcB gene-specific primers			
plcB-F	ATG-AAA-TTC-AAA-AAG-GT	*plcB* gene	[40]
plcB-R	ATT-TGT-TTT-TTT-AGA-CC	*plcB* gene	[40]

Nomenclature of competitors: Arabic numbering indicates *L. monocytogenes* genotypes intended to be amplified; Roman numbering indicates target genotypes which are discriminated within a PCR reaction by binding of the competitors; asterisks indicate the presence of a 2′,3′-ddNTP.
IRD_{800}: infrared dye for detection of nucleic acid strands after sequencing reaction.
Primer M13-universal was used to sequence *iap* genes inserted into a pCR-2.0 TOPO-TA vector (Invitrogen).

Table 3
PCR conditions for the detection of the three *L. monocytogenes* genotypes

Primer pair	Specificity	Primer concentration (pmol µl^{-1})	$MgCl_2$ concentration (mM)	DNA concentration (ng µl^{-1})	Competitor concentration (pmol µl^{-1})	Initial denaturation	Denaturation	Annealing temperature (°C)	Elongation time (s)	Final elongation
iap-820-I-V/*iap*-1047-I-R	genotype I	15	40	100	30	94°C/4 min	94°C, 20 s	57	15	72°C, 1 min
iap-412-II-V/*iap*-1047-II-R	genotype II	15	40	100	30	94°C/4 min	94°C, 20 s	60	20	72°C, 1 min
iap-217-III-V/*iap*-1047-III-R	genotype III	15	25	100	0	94°C/4 min	94°C, 20 s	52	40	72°C, 1 min

probes Lis-1255 and Lis-637 (results not shown). After specificity evaluation, the probes were tested regarding their in situ applicability within complex samples. Several raw milk samples were investigated using classical enrichment techniques. Aliquots were harvested every day, fixed with ethanol and analyzed by the FISH technique, applying both oligonucleotide probes. It was possible to detect *Listeria* cells in situ with FISH after 2 days of enrichment (Fig. 1).

3.2. *Subtyping and phylogeny of* L. monocytogenes *strains according to* iap *gene sequences*

3.2.1. *Sequence analysis and phylogenetic inferences*

Comparative analysis of complete *iap* gene sequences of 69 *L. monocytogenes* strains belonging to all 13 serotypes was performed. *Iap* gene sequences were aligned according to Bubert et al. [39] and compared for phylogenetic analysis using the ARB software program package (http://www.arb-home.de).

A characteristic feature of the *iap* gene is a highly variable central domain which contains a stretch of TN repeats. These repeats were not taken into consideration for phylogenetic studies since they are of low evolutionary relevance. Phylogenetic trees reconstructed from both nucleic and amino acid sequences enabled subdivision of *L. monocytogenes* strains into three lineages which we termed *iap* genotypes (Fig. 2). The three genotypes agree well with previous proposals based on single nucleotide differences of partially sequenced *L. monocytogenes* genes as well as on band patterns obtained by restriction enzyme analysis. Trees derived from amino acid sequences also reflected the three *iap* genotypes, but could not, in contrast to the nucleic acid-based analysis, distinguish between *L. monocytogenes* strains within one lineage.

3.2.2. *Repetitive elements within the* iap *gene*

Based on the alignment, significant length differences were detected between the *L. monocytogenes* strains. This is due mainly to the varying number of TN repeats. The phenomenon of repetitive threonine-asparagine elements within the *iap* gene was first described by Köhler et al. [44] who determined 19 TN repeats for *L. monocytogenes* EGD type strain. Many isolates are also characterized by varying numbers of TN repeats which were already assumed from length differences according to their different electrophoretic mobility in polyacrylamide gels [10] and from partial sequencing of the respective region within the *iap* gene [37]. The full sequence analysis of *iap* genes of 71 *L. monocytogenes* isolates showed that the length of the TN repeats differs from 11 to 24. Interestingly, the affiliation of an individual *L. monocytogenes* strain to one of the three *iap* genotypes is reflected by the number of TN repeats present within the *iap* gene. We were able to assign strains with 11 TN repeats to genotype III, whereas strains of genotype II had the highest number of TN re-

Table 4
Oligonucleotide probes used for FISH

Name	Sequence	Target organisms	Reference
EUB-338-I	5′-GCT-GCC-TCC-CGT-AGG-AGT-3′	Bacteria[a]	[43]
EUB-338-II	5′-GCA-GCC-ACC-CGT-AGG-TGT-3′	Planctomycetales	[44]
EUB-338-III	5′-GCT-GCC-ACC-CGT-AGG-TGT-3′	Verrucomicrobiales	[44]
Lis-1255	5′-ACC-TCG-CGG-CTT-CGC-GAC-3′	Genera *Listeria*, *Brochothrix*	[35]
Lis-637	5′-CAC-TCC-AGT-CTT-CCA-GTT-TCC-3′	Genus *Listeria*, except *L. grayi*	This study

[a]Probe EUB-338-I reacts with most bacteria except Planctomycetales and Verrucomicrobiales.

peats (16–24). Members of genotype I were characterized by a TN number of 13–17.

3.2.3. Correlation to serotype

Our results furthermore substantiate the correlation between *iap* genotypes and *L. monocytogenes* serotypes [16,36,37,45]. Genotype I consists of strains belonging to flagellar antigen types b and d, whereas isolates typed as antigen type a or c belong to genotype II. Rarely detected serotypes 4a and 4c cluster together within the third and most distinct genotype III.

3.2.4. Confirmation of L. monocytogenes iap genotypes by partial sequencing of plcA and plcB genes

The genes *plcA* and *plcB* encode two phospholipases and may also be useful phylogenetic markers. Primers targeting the *plcA* gene were designed. For the amplification of *plcB*, the primers described by Vasquez-Boland et al. [40] were used. Even partial sequences of these virulence genes were sufficient to confirm the three genotypes. The tree derived from the *plcA* gene is shown in comparison to the *iap*-derived tree including all *L. monocytogenes* serotypes (Fig. 3).

3.2.5. Detection of L. monocytogenes genotypes by PCR

Highly variable regions within *iap* make this an ideal gene not only for a phylogenetic differentiation of *L. monocytogenes* strains, but also for the development of a routine method to detect the different genotypes of *L. monocytogenes* in environmental samples. An essential prerequisite for the design of specific primers for each genotype was the generation of an extensive *iap* gene sequence database. Primer pairs specific for the respective genotypes were designed, targeting different regions within the *iap* gene. These regions are conserved among strains of one genotype, but have significant mismatches with strains of the other two genotypes. The PCR products obtained by the respective primer combinations had a length of 615–645 bp (genotype I), 195–201 bp (genotype II) and 804 bp (genotype III). Minor length differences occur due to the varying number of TN repeats in the different *L. monocytogenes* strains. PCR amplificates for all three *L. monocytogenes* genotypes are shown in Fig. 4. Specificity of the primers was confirmed using DNA isolated from all 71 *L. monocytogenes* isolates which were obtained from various food (Romadur-style cheese, smoked salmon), environmental (soil, wastewater) and clinical samples. Five

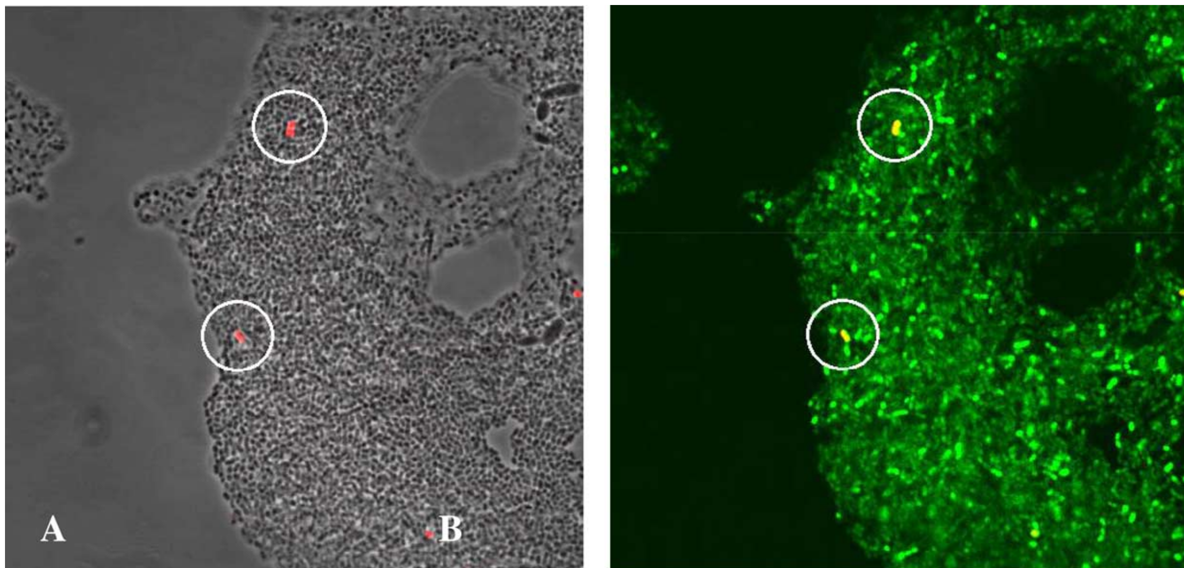

Fig. 1. Detection of *Listeria* spp. with probes Lis-637-Cy3 (red signal) and EUB-338-Fluos (green signal) after 2 days enrichment of raw milk samples. A: Overlay of phase contrast and red signals derived from probe Lis-637. B: Overlay of red and green signals derived from probes Lis-637 and EUB-338; the yellow color results from an overlay of red and green fluorescence.

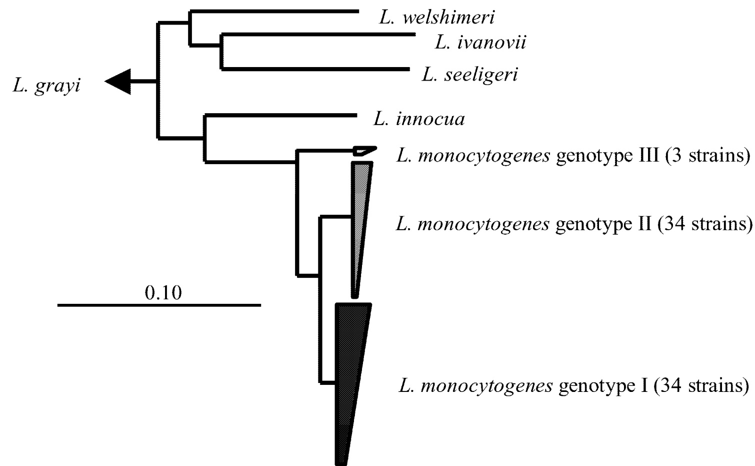

Fig. 2. *L. monocytogenes* subclassification based on *iap* gene nucleic acid sequences; the bar indicates 10% estimated sequence divergence.

L. monocytogenes strains isolated from patients showing gastroenteritic symptoms (strains IMVW 1724, 1725, 1726, 1727 and 34563, see Table 1) could be affiliated to genotypes I (1724–1727) and II (34563) (Fig. 2). PCR reactions with the genotype-specific primers allowed the assignment of the isolates to the respective genotypes (Fig. 5).

The intended amplification of a gene of a particular group of organisms directly from environmental samples often leads to the formation of non-specific PCR products due to a mispairing of the specific primers used. Similarly, binding to priming regions of non-target DNA of other genotypes complicated the specific detection of *L. monocytogenes* genotypes. Therefore, competitive primers were added to the PCR reaction mixture. They were constructed complementary to priming regions of non-target organisms. The characteristic feature of the competitors is that their 3′-end was terminated by a 2′,3′-ddNTP. The lack of the 3′-OH group prevents the elongation of non-target DNA strands trapped by competitors. The distinct effect of the competitors is exemplified in Fig. 6. Competitive primers were added in excess to the reaction mix and did not influence detection efficiency of target strains (Fig. 6). Furthermore, it was possible to enrich *iap* DNA of a particular *L. monocytogenes* strain by PCR out of artificial mixtures of DNAs from *L. monocytogenes* isolates belonging to different *iap* genotypes.

4. Discussion

4.1. *In situ detection of* Listeria *sp. using FISH*

The FISH technique has become a widely used and

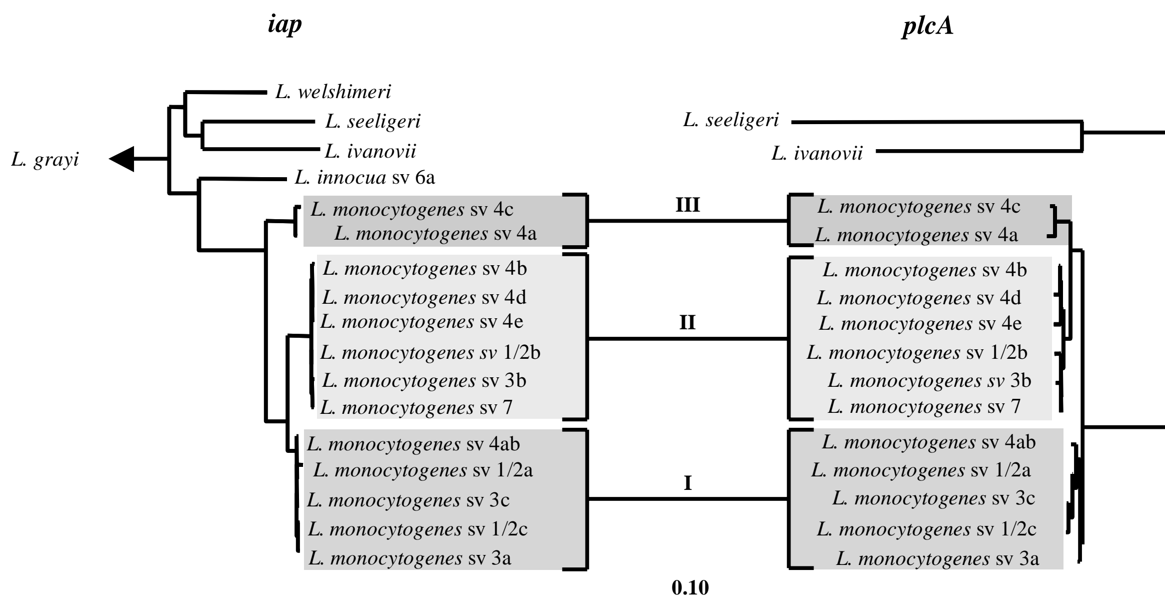

Fig. 3. Comparison of dendrograms derived from *iap* and *plcA* gene nucleic acid sequences for classification of *L. monocytogenes* genotypes; the bar indicates 10% estimated sequence divergence.

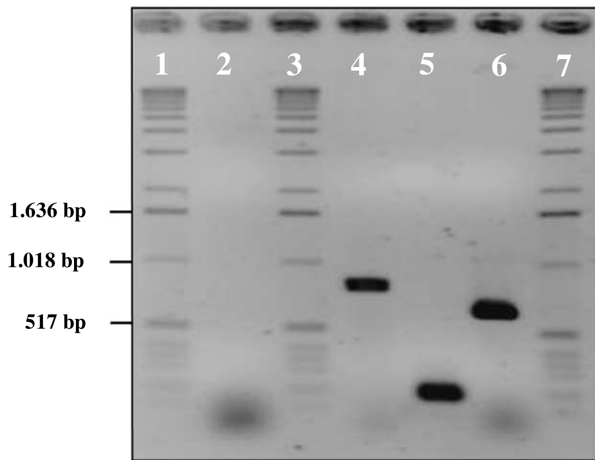

Fig. 4. Positive PCR reaction for *L. monocytogenes* genotypes I, II and III. Lanes 1, 3, 7: length standard; 2: negative control without the addition of DNA; 4: detection of *L. monocytogenes* genotype III; 5: detection of *L. monocytogenes* genotype II; 6: detection of *L. monocytogenes* genotype I.

powerful tool for culture-independent detection of bacteria, even of human pathogens and of difficult or as yet uncultured bacteria [31,34]. The classical detection of *Listeria* spp. in food samples relies on several time-consuming enrichment and isolation steps. In this study we were able to detect *Listeria* spp. with the FISH technique within 2 days in raw milk samples after pre-enrichment in buffered *Listeria* enrichment broth (Fig. 1). The probe Lis-1255 has no mismatch within the binding region to *B. thermosphacta* and *B. campestris*. The two *Brochothrix* species, which are closely related to the genus *Listeria*, have also been found in silage, feces and in milk products [46], but they were never isolated from clinical samples. They are not known to be pathogenic for humans or animals. Probe Lis-637 did not react with *Brochothrix* spp. and is suitable for detecting all *Listeria* species except *L. grayi*. This species is non-pathogenic for animals and humans and is, in addition, a very unusual isolate of *Listeria*-contaminated samples.

4.2. Phylogenetic classification of L. monocytogenes

Previous studies [16,36,37,47–53] allowed the subdivision of *L. monocytogenes* strains into three distinct lineages according to ribotypes, multilocus enzyme electrophoresis types, pulsed field gel electrophoresis band patterns and monocin typing. These typings were compared with the respective serotypes. Thereby, strains of serotypes 4a and 4c appeared to represent a distinct cluster of uncommon isolates which seemed to have a significantly lower pathogenic potential against mammalian hosts. Furthermore, these analyses consistently grouped the remaining *L. monocytogenes* serotypes into two clusters represented by their flagellar antigens: cluster I consisted of strains characterized by flagellar antigens b and d, whereas cluster II strains were defined by flagellar antigens a or c with the exception of sv 4a and 4c strains.

These results are highly consistent, but little attention has been directed to the phylogenetic relationships among the three *L. monocytogenes* lineages. Rasmussen et al. [37] suggested an evolutionary difference between a number of *L. monocytogenes* strains based on partial sequences derived from *iap*, *hly* and *flaA* gene sequences. For *iap* gene sequences, they took into account the highly variable TN repeat region. However, repetitive elements like the TN repeat region normally descend from insertion and duplication events, which makes an interpretation concerning the evolutionary relevance of this region very difficult.

Evidence for the phylogenetic distribution of three genotypes within the species *L. monocytogenes* can only be obtained by taking into account exclusively non-repetitive sequences of the *iap* gene. The highly variable TN elements are normally created by duplications, do not reflect evolutionary history and were therefore excluded from

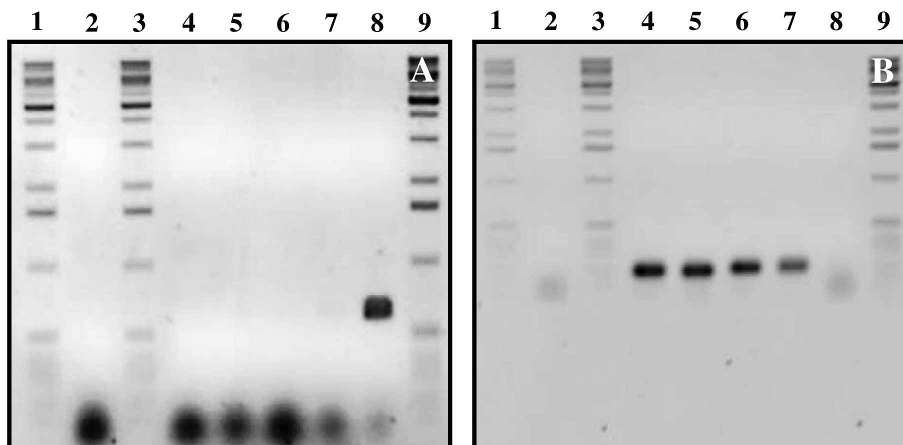

Fig. 5. Specific detection of *L. monocytogenes* strains of clinical origin causing gastroenteritis and their affiliation to genotypes I and II. No clinical isolates were affiliated to genotype III. A: PCR reaction with primers targeting genotype I. B: PCR reaction with primers targeting genotype II. Lanes 1, 3, and 9: length standard; 2: negative control without the addition of DNA; 4–8: PCR reactions with DNA of *L. monocytogenes* IMVW 1724, IMVW 1725, IMVW 1726, IMVW 1727 and IMVW 34564, respectively.

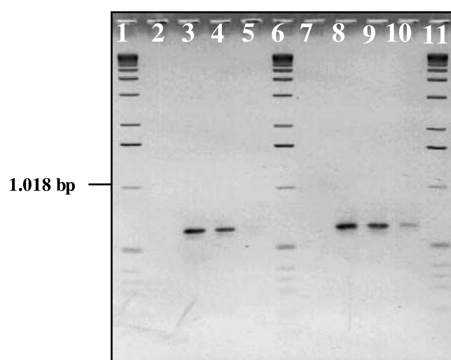

Fig. 6. Specific detection of *L. monocytogenes* genotype I. Lanes 1, 6, 11: length standard; 2, 7: negative control without the addition of DNA; 3, 8: positive control with DNA from *L. monocytogenes* sv 4ab; 4, 9: detection of *L. monocytogenes* sv 4ab in a mixture of five strains from genotypes II and III; 5, 10: control for specificity of the PCR using mixed DNAs of two strains from genotypes II and III. In PCR reactions on the left (lanes 2–5), competitors were added to the PCR reaction mixes; in lanes 7–10, no competitors were added.

phylogenetic analysis. This was facilitated by the construction of appropriate filters within the ARB software that exclude the TN repeat domain.

4.3. *Competitive PCR detection method for* L. monocytogenes *genotypes*

Based on the extensive *iap* gene sequence data set we were able to design a highly specific and rapid PCR-based method to easily distinguish the three *L. monocytogenes* genotypes. PCR reactions yielded amplificates of different lengths for each of the genotypes (see Figs. 4 and 5), but weak mismatches to non-target genotypes may cause non-specific PCR products. This could be avoided by the addition of competitive primers (Fig. 6). The competitors do not allow amplification of the respective non-target DNA strand due to the lack of the –OH group at their 3′-end. The competitive PCR method allows the specific amplification of highly similar DNAs. It is applicable for the differentiation of closely related organisms as well as for their detection in highly complex material such as food or environmental samples. Therefore, it could become a prerequisite on the one hand for epidemiological studies prior to high-resolution methods such as ribotyping or phage typing; on the other hand, it may be a suitable tool for studying differences in the environmental persistence of the various *L. monocytogenes* genotypes.

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