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ORIGINAL PAPER

Specific detection of the most prevalent five Listeria strains and unspecific detection of 15 Listeria using multiplex real-time PCR

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Abstract
Listeria in food, are a serious risk to consumer's health that may even have a fatal outcome. To analyze Listeria in food, the methods used must provide reliable results and detect all strains of Listeria. Several qPCR systems have been published for the identification of *Listeria monocytogenes*, *innocua*, *ivanovii*, *welshimeri* and *seeligeri*. PCR systems for Listeria spp. have also been published. However, they do not detect all known Listeria. To achieve this, we have developed a novel multiplex real-time PCR method. This multiplex qPCR system was able to determine DNA specifically from the five most common Listeria as well as 15 other known Listeria strains simultaneously after cultivation on selective plates.

Keywords Multiplex qPCR · *Listeria spp* · *Listeria monocytogenes*

Introduction

Listeria are a common bacterial food contaminant found mainly in cheese and meat products [1–8]. These bacteria pose a serious health hazard. *Listeria monocytogenes* is considered the most threatening food born infection. Classical microbiology uses enrichment techniques like colony counting to determine the bacterial load in food samples. Such methods are recommended and certified by the International Organization for Standardization (ISO). Specific culture plates for Listeria are available, but often they do not detect Listeria other than *L. monocytogenes*. Less selective plates can lead to unclear results as, e.g., it is reported for some *Bacillus cereus* strains on the chromogenic isolation medium ALOA [9].

Many PCR and qPCR systems are available specifically *for Listeria monocytogenes* and *Listeria* species (spp.) [10–14]. Specific qPCR protocols have also been published for other Listeria strains [15]. Multiplex qPCR systems that detect also other bacteria species beside Listeria have also been published, making qPCR more efficient and allowing easy screening for unexpected bacteria in food [16–19]. Recently, a multiplex qPCR system was published that enables the detection of six of the most common strains of Listeria at once [20]. Other techniques, like MALDI-TOF was also applied to identify *Listeria* spp. [21]. Unfortunately, MALDI-TOF is not capable to identify specific bacteria in bacterial mixes. However, subtyping of Listeria strains from clones is possible [22].

The detection of any Listeria in food is interpreted as prerequisite also for the growth of pathogenic listeria and has serious implications. Such food is considered a possible health hazard for consumers. Production must be improved immediately to eliminate this risk. It is, therefore, important to exclude false negative results when using PCR systems with limited specificity.

We, therefore, decided to develop an efficient multiplex PCR system detecting the most prevalent listeria strains like *Listeria monocytogenes*, *innocua*, *ivanovii*, *welshimeri* and *seeligeri using specific primers and probes.* In addition, the system should detect *Listeria spp* covering 15 other available Listeria strains as to be: *Listeria costaricensis*, *Listeria thailandensis*, *Listeria grayi*, *Listeria rocourtiae*, *Listeria marthii*, *Listeria weihenstephanensis*, *Listeria fleischmannii*, *Listeria riparia*, *Listeria aquatica*, *Listeria floridensis*, *Listeria grandensis*, *Listeria cornellensis*, *Listeria booriae*, *Listeria newyorkensis*, *Listeria goaensis*. This was achieved using additional primers and probes, detecting all these strains in one single detection channel.

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Materials and methods

Bacterial strains

For the experiments the following strains from German Collection of Microorgansims and Cell Cultures (DSM) were used: *Listeria seeligeri* DSM 20,751, *Listeria innocua* DSM 20,649, *Listeria costaricensis* DSM 105,474, *Liseria thailandensis* DSM 107,638, *Listeria grayi* DSM 20,601, *Listeria rocourtiae* DSM 22,097, *Listeria marthii* DSM 23,813, *Listeria weihenstephanensis* DSM 24,698, *Listeria fleischmannii* DSM 24,998, *Listeria riparia* DSM 26,685, *Listeria aquatica* DSM 26,686, *Listeria floridensis* DSM 26,687, *Listeria grandensis* DSM 26,688, *Listeria cornellensis* DSM 26,689, *Listeria booriae* DSM 28,860, *Listeria newyorkensis* DSM 28,861, *Listeria goaensis* DSM 29,886 and *Listeria sp.* DSM 29,997. Additionally, the strain from the American Type Culture Collection (ATCC) *Listeria monocytogenes* ATCC 7644 and strains of *Listeria ivanovii* and *Listeria welshimeri* identified by the National Reference Centre for Enteropathogenic Bacteria and Listeria (NENT) were utilized.

The strains *Candida albicans* ATCC 10,231, *Escherichia coli* ATCC 11,775, *Enterococcus faecalis* ATCC 19,433, *Salmonella enteritidis* ATCC 13,076, *Staphylococcus aureus* ATCC 25,923 and *Bacillus cereus* DSM 2302 were used as negative controls. These were complemented with additional strains listed in Table 4

Listeria monocytogenes, *innocua*, *ivanovii*, *welshimeri* and *seeligeri* and the non-target Bacteria strains were enriched in tryptic soy broth (TSB) by incubation at aerophilic conditions for about 24 h, but at least until an obvious turbidity was visible. Afterwards, these listeria strains were transferred on separate blood agar plates and the other bacteria on plate count agar plates. The remaining listeria species were plated on blood agar as described in the instruction for first-time application from the DSM. All plates were incubated at aerophilic conditions for about 24 h, but at least until single colonies reached a sufficient size. *Listeria rocourtiae*, *Listeria weihenstephanensis*, *Listeria riparia*, *Listeria aquatica*, *Listeria floridensis*, *Listeria grandensis*, *Listeria cornellensis*, *Listeria booriae*, *Listeria newyorkensis*, *Listeria goaensis* and *Listeria sp.* were incubated at 30 °C, the others at 37 °C.

Bacterial lysis and DNA extraction

For DNA extraction 100 µl PCR-water was transferred to a 1.5 ml Tube. With a one-way inoculation loop, a single colony was picked from an agar plate and added to the water in the tube. To lyse the bacteria a shaking and heating step in a heating block at 300 rpm and 95 °C was applied for 5 min. From each sample strain, DNA from several colonies were extracted individually in this way.

Primers and probes

Primers and probes of the multiplex PCR system named AllList, were taken from previous publications or developed in this work (see Table 1). All primers and probes were synthesized by Microsynth AG, Balgach, Switzerland. Labelling of probes with fluorescent markers was done according to the recommendations of the Rotorgene 6000 manual and are listed in Table 1.

Primer development for the *Listeria* spp. system

A series of primers and probes targeting the gene for the bifunctional UDP-*N*-acetylglucosamine diphosphorylase/glucosamine-1-phosphate *N*-acetyltransferase (GlmU, Reference CP044432.1:206,781–206,942 *Listeria monocytogenes* strain FDA00009448 chromosome, complete genome) in all the tested Listeria strains were developed. Beside targeted selection of possible sequences, it was mainly a "try and error" approach leading to the successful amplification without cross reactions to other bacteria strains. The selected sequences, primer and probe positions are listed in Table 1. The label FAM was taken as summary channel for all *Listeria* spp amplicons.

Real-time PCR procedure

5 µl DNA extracts were added to 20 µl of reaction mix containing Sensifast Probe no ROX Kit (Bioline, Meridian Bioscience, United Kingdom), and all primers and probes of AllList (primers and their concentrations are listed in Table 1). PCR was performed on a Rotorgene 6000 real-time PCR system (Corbett, Australia) according to the following cycling protocol: Initial step of 5 min at 95 °C; followed by 40 cycles of 5 s at 94 °C and 15 s. at 60 °C and 7 s. at 72 °C.

Validation data to determine the performance characteristics of AllList

DNA-dilution rows were used to assess the performance characteristics of AllList (Tables 2 and 3). We estimate the amount of DNA from one clone to be easily sufficient to produce a positive signal by PCR.

Table 1 Overview of the primers and probes used for the multiplex qPCR system AllList for the simultaneous determination of 21 Listeria strains

Primer/ Probe	Final conc µM	Sequence 5' 3'	Amplicon	GenBank acc.no / source/Labelling
Specific system for Listeria monocytogenes				
Limo F	0.2	CGGCGCACCTAACCAAGTAA	80 bp	MH375831.1 [20]
Limo R	0.2	CAGTCTGGACAATCTCTTTGAATTTT		
LimoAlx350	0.08	TCAAGATGACTACAATGGTCCGAGTGT GAAAA		
Specific system for Listeria welshimeri				
Liwe F	0.1	CTCCCACATTGGTGCTACTC	95 bp	LT906444.1 [20]
Liwe R	0.1	GATTCCGTTCACTAATCCATCAG		
LiweRox	0.08	ACAAGTCCGGCGAATGGCATGATTAAG		
Specific system for Listeria seelingeri				
Lise F	0.1	CTGATTTTGTCGTTAAATCTTCAG	155 bp	NC_013891 [20]
Lise R	0.1	GTTAAATTAATTTGAACGAAATGAGGG		
LiseCy5	0.08	CAGTTGTTTCTTCCGCGACGGCTAAAG		
Specific system for Listeria innocua				
Liin f	0.1	CTACAAGTAAACGAGGTTGCTAC	108 bp	NZ_CP045743.1 [20]
Liin R	0.1	GGAAGTAAGAATGCTGTGGTC		
LiinDY681	0.08	CTCCAGCGCCAGAACGTACATTAAGCC		
Specific system for Listeria ivanovii				
Livan F	0.1	CAGGGATTATTATACTCATTGTGG	102 bp	NZ_CP009577.1 [20]
Livan R	0.1	GCTGCGAACTTAACTCAACTTG		
LivanHex	0.08	CCTGATTATCACCCGTTTCTGCTCCAAC		
Specific system for Listeria grayi				
Ligra F	0.2	CTGCACGATCAAGGTCAATC	140 bp	NZ_LR134483.1 [20]
Ligra R	0.2	GGAACTGCCATCAAGTCCG		
		no probe, signal is covered by the probes for Listeria spp, see below		

Primer/Probe	Final conc µM	Sequence 5' 3'	Amplicon	source
Primers for Listeria spp				
Lis spp F	0.4	CAGGRTTACTCGTTGATTGAATAAC	~137 bp	[12]
Lis spp R	0.4	GCTGAAGAGATTGCGAAAGAAG		
Jasm3 R	0.5	GCGGCGGAGGTCGCGGAAGTGG		This work
Jasm11 F	0.5	CCGGAAAACCAGTGGACTGCACAAC		This work
Jasm7 R	0.5	TAGCGGAAGAGATTGCGAAGGAAGT		This work
Jasm10 F	0.5	ACTGGGTAGCTTGTTGATTGWACGACA		This work
Jasm12 F	0.5	ACAGGATAACTCGTCGATTGAACCACATAAA		This work
Jasm8R	0.5	GCAGAAGAAATTGCACGCGAAGTAGG		This work
Jasm9 F	0.5	GGATTGCTSGTCGACTGCACGAC		This work
Jasm9 R	0.5	CWGGAAAACCAGTGGACTGCACGAC		This work
Jasm14 F	0.5	GCGGCGGAAATCGCGGAAGT		This work
Jasm10R	0.5	CWGGGTAGCTTGTCGACTGGACAAC		This work
Jasm13 F	0.5	AGCGGAAGACATTGCGAAGGAAGTAGG		This work
Laqu R	0.5	GCGGAAGAAATTGCGAAAGAAGTAGGG		This work
Laqu F	0.5	ACAGGATAACTCGTCGATTGAACCACAT		This work
Lrip R	0.5	GCAGCTGAGGTTGCTGAAGTAGC		This work

Table 1 (continued)

Primer/Probe	Final conc µM	Sequence 5' 3'	Amplicon	source
Lrip F	0.5	CAGGAAAACCAGTGGACTGCACAAC		This work
Primer/ Probe	Final conc µM	Sequence 5' 5'		Sequence
Probes for Listeria spp				
Lis spp Fam L	0.07	CATGACAACCACGGATACTTTCTTCAATGTTAA5TTG		Fam BHQ1 5 = LNA-T [11]
CosFam L	0.07	CAA6TCAACATTGAAGAAAGTATCCGCGGCTGCCACG		Fam BHQ1 6 = LNA-A this work
ThaiFam	0.07	CAAATCAATATCGAGGAAAGTATCCGTGGCTGTCACG		Fam BHQ1 this work
FlorFam	0.07	CATCGCTGAAATGAGTGACACTTGATTTCCCCAGCTC		Fam BHQ1 this work
RipFam	0.07	CAGATTAATATTGAAGAGAGTATCCGTGGTTGTCACG		Fam BHQ1 this work
NewFam	0.07	CATCACTAAAATGGGTCACGCTGGACTTCCCTAAATT		Fam BHQ1 this work
RocFam	0.07	CAGATTAATATTGAAGAAAGTATCCGTGGTTGCCACG		Fam BHQ1 this work

The modification LNA is a Looked Nucleotide Acid (LNA), e.g., as Adenin (A) or T for Thymin resulting in an augmented annealing temperature leading to a stronger signal. The targeted strains are mentioned in the name of the probe (e.g., CosFam L targets *L. costaricensis*)

Table 2 Multiplex serial dilution of DNA standard rows for AllList in the range from 0.2 to 100 ng DNA per assay

AllList ng/assay	*Listeria ivano*	*Listeria welshi*	*Listeria seeli*	*Listeria innoc*	*Listeria mono*	*Listeria spp*
Standard 1	20	20	20	20	20	100
Standard 2	6.25	6.25	6.25	6.25	6.25	32
Standard 3	2	2	2	2	2	10
Standard 4	0.63	0.63	0.63	0.63	0.63	3.2
Standard 5	0.2	0.2	0.2	0.2	0.2	1

At all dilutions, the listeria species were clearly detectable (crossing point cp < 35)

Table 3 Performance of AllList

AllList	*Listeria. spp*	*Listeria ivano*	*Listeria welshi*	*Listeria seeli*	*Listeria innoc*	*Listeria mono*
amplification efficiencies	1.08	0.99	1.01	0.98	0.99	1.13
correlation R2	0.86	0.96	0.93	0.98	0.94	0.78
performance	0.93	0.95	0.94	0.96	0.93	0.88
relative standard deviation	13.5	13.8	13.5	12.6	12.2	17.3
measurement uncertainty	14	15	14	14	13	18

To evaluate these properties the multiplex serial dilution was used as calibrator. The correlation and amplification efficiencies were calculated by the Rotorgene algorithm directly. The numbers are mean values from 12 single experiments, where the lowest dilution exhibited a positive signal in all runs. As AllList is used only as qualitative assay, these numbers serve only to characterize the performance

Comparing of results from classical microbiological ISO-method with AllList using proficiency trial

Table 4 Specificity testing results for target and non-target bacteria strains

Bacteria	Signal for Listeria spp	Listeria ivano	Listeria welshi	Listeria seeli	Listeria innoc	Listeria mono
L. ivanovii NENT	positive	positive	neg	neg	neg	neg
L. welshimeri NENT	positive	neg	positive	neg	neg	neg
L. seeligeri DSM 20,751	positive	neg	neg	positive	neg	neg
L. inncocua DSM 20,649	positive	neg	neg	neg	positive	neg
L. monocytogenes ATCC 7644	positive	neg	neg	neg	neg	positive
L. costaricensis DSM 105,474	positive	neg	neg	neg	neg	neg
L. thailandensis DSM 107,638	positive	neg	neg	neg	neg	neg
L. grayi DSM 20,601	positive	neg	neg	neg	neg	neg
L. rocourtiae DSM 22,097	positive	neg	neg	neg	neg	neg
L. marthii DSM 23,813	positive	neg	neg	neg	neg	neg
L. weihenstephanensis DSM 24,698	positive	neg	neg	neg	neg	neg
L. fleischmanii DSM 24,998	positive	neg	neg	neg	neg	neg
L. riparia DSM 26,685	positive	neg	neg	neg	neg	neg
L. aquatica DSM 26,686	positive	neg	neg	neg	neg	neg
L. floridensis DSM 26,687	positive	neg	neg	neg	neg	neg
L. grandensis DSM 26,688	positive	neg	neg	neg	neg	neg
L. cornellensis DSM 26,689	positive	neg	neg	neg	neg	neg
L. booriae DSM 28,860	positive	neg	neg	neg	neg	neg
L. newyorkensis DSM 28,861	positive	neg	neg	neg	neg	neg
L. goaensis DSM 29,886	positive	neg	neg	neg	neg	neg
L. sp DSM 29,997	positive	neg	neg	neg	neg	neg
Arcobacter butzleri DSM 7301	neg	neg	neg	neg	neg	neg
Arcobacter skirrowii DSM 7302	neg	neg	neg	neg	neg	neg
Aeromonas hydrophila DSM 30,187	neg	neg	neg	neg	neg	neg
Aspergillus niger in house strain	neg	neg	neg	neg	neg	neg
Bacillus thuringensis DSM 350	neg	neg	neg	neg	neg	neg
Bacillus cereus DSM 2302	neg	neg	neg	neg	neg	neg
Bacillus pumilus HPA 234,242,244	neg	neg	neg	neg	neg	neg
Candida albicans ATCC 10,231	neg	neg	neg	neg	neg	neg
Campylobacter jejuni ATCC 33,291	neg	neg	neg	neg	neg	neg
Clostridium perfringens ATCC 13,124	neg	neg	neg	neg	neg	neg
Clostridium botulinum LGL Bayern	neg	neg	neg	neg	neg	neg
Cronobacter muytjensii ATCC 51,329	neg	neg	neg	neg	neg	neg
Cryptosporidium in house strain	neg	neg	neg	neg	neg	neg
E. coli ATCC 11,775	neg	neg	neg	neg	neg	neg
Enterobacter cloacae inhouse strain	neg	neg	neg	neg	neg	neg
Enterococcus faecalis ATCC 19,433	neg	neg	neg	neg	neg	neg
Enterobacter sakazaki inhouse strain	neg	neg	neg	neg	neg	neg
Geobacillus spp. DSM 3299	neg	neg	neg	neg	neg	neg
Klebsiella pneumoniae inhouse strain	neg	neg	neg	neg	neg	neg
Kocuria rhizophilia DSM 348	neg	neg	neg	neg	neg	neg
Lactobacillus plantarum DSM 20,205	neg	neg	neg	neg	neg	neg
Lactobacillus casei DSM 7469	neg	neg	neg	neg	neg	neg
Legionella dumoffi inhouse strain	neg	neg	neg	neg	neg	neg
Micrococcus ruber inhouse strain	neg	neg	neg	neg	neg	neg
Micrococcus luteus inhouse strain	neg	neg	neg	neg	neg	neg
Pseudomonas aeruginosa ATCC 27,853	neg	neg	neg	neg	neg	neg
Proteus hauseri ATCC 13,315	neg	neg	neg	neg	neg	neg
Salmonella enteritidis ATCC 13,076	neg	neg	neg	neg	neg	neg

Table 4 (continued)

Bacteria	Signal for Listeria spp	Listeria ivano	Listeria welshi	Listeria seeli	Listeria innoc	Listeria mono
Salmonella typhimurium ILS 26.2.10	neg	neg	neg	neg	neg	neg
Shigella dysenteriae inhouse strain	neg	neg	neg	neg	neg	neg
Shigella sonnei inhouse strain	neg	neg	neg	neg	neg	neg
Staphylococcus aureus ATCC 25,923	neg	neg	neg	neg	neg	neg
Staphylococcus leutens ATCC 700,403	neg	neg	neg	neg	neg	neg
Streptococcus agalactiae DSM 2134	neg	neg	neg	neg	neg	neg
Streptococcus faecalis DSM 20,060	neg	neg	neg	neg	neg	neg
Streptococcus lactis DSM 20,481	neg	neg	neg	neg	neg	neg
Vibrio parahaemolyticus HPA 224	neg	neg	neg	neg	neg	neg
Vibrio cholerae inhouse strain	neg	neg	neg	neg	neg	neg
Yersinia enterocolitica DSM 4780	neg	neg	neg	neg	neg	neg

The here listed results for target Listeria strains represent signals of 8 assays (8 single pick and boil DNA isolations). No unspecific signal was observed

samples

Template DNA from stored proficiency tests were analyzed by AllList. The Listeria strains were chosen by the proficiency test provider and preferred *Listeria monocytogenes*. However, this is a stress-test for the method, as it does not use DNA from enriched clones as done for the validation of this method, but often from enrichment broth directly spiked with low concentrations of Listeria.

Comparing three methods

Food samples were analyzed by routine cultural ISO-method and AllList. AllList was applied after enrichment and cultivating on culture plates (clone picking). In addition, as a confirmation method the clones were analyzed by MALDI-TOF.

Results and discussion

AllList is based on the published PCR system for the six most common Listeria strains [1]. For the detection of the other Listeria strains several primer pairs were developed at the locus for Listeria spp. (GlmU or ribose-phosphate diphosphokinase gene). The sequences of the primers and probes were adapted to run under the same conditions. No special primer design software was used for this purpose, as the behavior is not precisely predictable and has to be evaluated with empirical data. The primers and probes that performed best (amplification efficiency and specificity) were used in multiplex format and tested with DNA from reference bacterial strains.

AllList is designed for screening of single colonies. Therefore, only one bacteria species is expected and competition by another template DNA does not take place. For this application competition between the PCR-systems does not occur. The applied DNA dilution row takes this in account (Table 2). The results are compiled in Table 3. As all dilutions revealed a positive signal at 0.2 ng/µl we assume that the LOD can be estimated to be at least at this level. From experience, the amount of DNA from one clone can be expected to be much higher. The performance characteristics presented here confirm these expectations. The estimated maximal measurement uncertainty of ± 18% is acceptable but does not include the isolation of the DNA. However, these data serve to characterize the PCR system AllList and has no impact on the final qualitative results.

The specificity of AllList was tested using the DNA from a wide range of bacteria (see Table 4). No false positive signals were observed and only the target species and strains gave a positive signal.

Proficiency test samples from the Health Protection Agency (HPA, UK) over a period of 1 year, were analyzed in parallel with classical microbiological methods and AllList. In total 36 samples were analyzed and most of the samples contained several species at once, predominantly *Listeria monocytogenes* with another strain (Table 5). The resulting match between AllList and the spiked bacteria according to the proficiency test was 48 detects of 52 spikes, which is 92%. We believe this to be a high degree of confirmation considering the prolonged storage of the sample material (isolated DNA) and that the sample material was not from a picked clone but directly from an enrichment broth.

Table 5 Compilation of the results from proficiency test program of Public Health England (PHE)

	Species of spike		Measured	Species		Difference
PHE 304 S0645	Mono	Innocua	spp pos	Mono	Innocua	None
PHE 304 S0646	Mono		spp pos	Mono		None
PHE 308 S0653	Mono	Seelingeri	spp pos	Mono	Seelingeri	None
PHE 308 S0654		Welshimerii	spp pos		Welshi	None
PHE 310 S0657	Mono	Ivanovii	spp pos	Mono	Ivanovii	None
PHE 310 S0658			spp neg			None
PHE 316 S0669	Mono	Innocua	spp pos	Mono	Innocua	None
PHE 316 S0670	Mono		spp pos	Mono		None
PHE 320 S0677	Mono		spp pos	Mono		None
PHE 320 S0678	Mono	Welshi	spp pos	Mono	Welshi	None
PHE 322 S0681	Mono	Innocua	spp pos	Mono	Innocua	None
PHE 322 S0682	Mono	Welshi	spp pos	Mono	Welshi	None
PHE 298 S6034	Mono	Seelingeri	spp pos	Mono	(seelingeri)	None
PHE 298 S6033			spp neg			None
PHE 292 S0621	Mono	Welshi	spp pos	Mono		Welshimeri not detected
PHE 292 S0621	Mono	Welshi	spp pos	Mono	Welshi	None
PHE 286 S0610	Mono	Welshi	spp pos	Mono	Welshi	None
PHE 286 S0609	Mono		spp pos	Mono		None
PHE 284 S0606	Mono	Ivanovii	spp pos	Mono	Ivanovii	None
PHE 284 S0605	Mono	Ivanovii	spp pos	Mono	Ivanovii	None
PHE 280 S0598	Mono		spp pos	Mono		None
PHE 280 S0597		Ivanovii	spp neg			Ivanovii not detected
PHE 274 S0586	Mono	Welshi	pos	Mono	Welshi	None
PHE 274 S0585	Mono		pos	Mono		None
PHE 272 S0582		Innocua	spp neg			Innocua not detected
PHE 272 S0581	Mono	Ivanovii	pos	Mono	Ivanovii	None
PHE 268 S0574		Welshi	spp neg			Welshimeri not detected
PHE 268 S0573	Mono		spp pos	Mono		None
PHE 266 S0570	Mono		spp pos	Mono		None
PHE 266 S0569	Mono	Ivanovii	spp pos	Mono	Ivanovii	None
PHE 260 S0558		Seelingeri	spp pos		Seelingeri	None
PHE 260 S0557	Mono	Innocua	spp pos	Mono	Innocua	None
PHE 256 S0550		Innocua	spp pos		Innocua	None
PHE 256 S0549	Mono		spp pos	Mono		None
PHE 254 S0546	Mono	Innocua	spp pos	Mono	Innocua	None
PHE 254 S0545	neg		spp neg			None

The spiked species of the bacteria genus Listeria and the results measured by AllList are compared and the differences determined. Total of 36 samples were analyzed mostly spiked with two species, some with none or only *L. monocytogenes*. In total 72 Results were compared. 4 of them were analyzed false negative by all List. No false positive result was noticed

Table 6 shows the results of routine samples which were analyzed by classical microbiology (ISO method), MALDI-TOF and AllList direct from enrichment broth and after cultivating on culture plate followed by clone picking and AllList. The ISO-method often gave unclear results, while AllList and MALDI-TOF did not. After enrichment AllList was sometimes positive for Listeria but after growing on the culture plate the Listeria was lost. This may indication false negative results by cultivation methods. The results of AllList match the results of MALDI-TOF better than the ISO-method. The influence of the matrices is comparable to the ISO method, as the sample is first cultivated on ISO recommended plates.

Conclusion

AllList was developed to enable the fast and accurate determination of 5 listeria strains and the Listeria species in general including a total of 15 Listeria strains. This method was

Table 6 The 8 samples of prepared green salads were analyzed

Probe	Result AllList after enrichment	Result AllList from clones of plates	Result from ISO method	Result from Maldi-TOF
A	Contains Listeria	No Listeria	No Listeria	*Staphylococcus lentus*
B	Contains Listeria	No Listeria	Unclear	*Microbacterium spp*
C	Contains Listeria seelingeri	No Listeria	Unclear	*Listeria seelingeri*
D	Contains Listeria	No Listeria	Unclear	*Microbacterium lacticum*
E	Contains Listeria	No Listeria	No Listeria	*Staphylococcus sciuri*
F	Contains Listeria	Contains Listeria seelingeri	Unclear	*Listeria seelingeri*
G	No Listeria	No Listeria	Unclear	*Bacillus pumilis*
H	No Listeria	No Listeria	Unclear	*Leucobacter chromiiresistens*

Results were gained by AllList, cultivating by ISO-method and Maldi-TOF (by service laboratory Mabritec AG, Switzerland). AllList was used after enrichment and for confirming of clones grown on culture plates. In parallel the clones from culture plates were analyzed by Maldi-TOF

challenged using DNA from reference strains, proficiency test samples and routine samples. In all settings the performance was satisfying. The tests were performed over a period of 1 year by several technical assistants, demonstrating the robustness of the method. First attempts were made to use AllList directly after enrichment without cultivation on cultivating plates. However, in this case the LOD needs to be determined in future work. In short, AllList can characterize the most common listeria clones and extends the range of detectable Listeria to all strains of the species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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