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Identification of *Listeria innocua* by PCR targeting a putative transcriptional regulator gene

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Abstract

Listeria innocua is a common, non-pathogenic bacterial species that shares morphological, biochemical and molecular characteristics with the pathogenic species *L. monocytogenes*. The presence of *L. innocua* may cause difficulty or confusion in the laboratory identification of *L. monocytogenes* or other *Listeria* spp. In this report, through examining the recently published genome sequence of *L. innocua* strain CLIP 11262 (serovar 6a), we identified a *L. innocua*-specific gene (*lin0464*) encoding a putative transcriptional regulator and evaluated its efficacy for species-specific detection by polymerase chain reaction (PCR). The specificity of the oligonucleotide primers (lin0464F and lin0464R) derived from this gene was confirmed with the formation of a 749-bp fragment in PCR from genomic DNA of *L. innocua* strains only. We expect that this assay will be useful in confirming identification of *L. innocua* or in studies where rapid detection of *L. innocua* is necessary.
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Keywords: Polymerase chain reaction; Transcriptional regulator; *Listeria innocua*

1. Introduction

The genus *Listeria* comprises a group of Gram-positive, non-spore forming coccobacilli, within which six closely related species are recognized taxonomically: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri* and *L. grayi*. Of these six species, only *L. monocytogenes* is pathogenic in humans, causing gastroenteritis, abortions, meningitis, encephalitis, and sepsis, and it is a significant cause of foodborne infections in man [1]. *Listeria* species are found in a variety of environments and are resistant to high concentrations of salt (up to 10% NaCl) and relatively wide pH (4.0–9.5) and temperature ranges (< 1–45°C) [2,3]. Although it is non-pathogenic for mice [4], *L. innocua* is isolated from foodstuffs and in food processing plants, often in conjunction with *L. monocytogenes* [5–7].

Differentiation of *Listeria* species on the basis of in vitro culture with enrichment and selective media is time-consuming, requiring 6–10 days to generate a definitive result. Indeed, a pre-enrichment in phosphate-buffered broth medium containing inhibitors as well as an indicator system is required for isolation of *Listeria* that has been injured during food processing [8]. Another problem is that *Listeria* species demonstrate close resemblances in terms of morphological, biochemical, and serological characteristics [1,4]. Sugar fermentation patterns are not reliable for differentiating *L. innocua* from *L. monocytogenes* due to strain variation. Therefore, *L. innocua* is distinguished phenotypically from *L. monocytogenes* based on negative CAMP test and failure to cause β-hemolysis [4] or by failure to detect phosphatidylinositol phospholipase C activity through the use of chromogenic media [9,10]. Serologically, *L. innocua* overlaps with *L. monocytogenes*, *L. welshimeri*, and *L. seeligeri*. In one study, several misclassifications of *Listeria* species by biochemical methods were documented, including three *L. monocytogenes* strains that were biochemically classified as *L. innocua* [11].

Polymerase chain reaction (PCR) assays have been developed for differentiation of *Listeria* species, including one that is based on *Listeria* 16S and 23S rRNA genes and intergenic spacer regions [12]. In this assay, PCR products are only obtained from *Listeria* spp., and the

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Table 1
List of bacterial strains examined by PCR using *L. innocua*-specific primers (*lin0464*F and lin0464R)

Strain	Serovar	Source	lin0464 (749 bp)
L. innocua ATCC 33090	6a	Cow brain	+
L. innocua ATCC 33091	6b	Human	+
L. innocua ATCC 43547	6b	Bovine brain	+
L. innocua ATCC 51742		Cabbage	+
L. innocua CLIP 11262	6a		+
L. innocua RM2213		Food	+
L. innocua RM2442		Oysters	+
L. innocua RM2443		Beef	+
L. innocua RM2444		Food	+
L. innocua RM2445		Crab salad	+
L. innocua RM2446		Turkey slice	+
L. innocua RM3007			+
L. innocua RM3304	4b	Plant	+
L. innocua RM3315	4b	Plant	+
L. innocua RM3318		Cheese	+
L. innocua 415		Turkey burger	+
L. innocua 416		Veal/beef patty	+
L. innocua 417		Beef steak	+
L. innocua 662		Raw milk	+
L. innocua 1419		Ground cheese	+
L. innocua 1425		Pecorino Romano	+
L. innocua 1720		Chicken	+
L. innocua 1944		Ground turkey	+
L. innocua F12-1		Catfish	+
L. innocua F12-2		Catfish	+
L. innocua F13-2		Catfish	+
L. innocua F14-2		Catfish	+
L. innocua F15-1		Catfish	+
L. innocua F15-2		Catfish	+
L. innocua F16-1		Catfish	+
L. innocua F16-2		Catfish	+
L. grayi ATCC 19120		Chinchilla feces	−
L. grayi ATCC 25400		Corn leaves/stalks	−
L. murrayi ATCC 25401		Corn leaves/stalks	−
L. ivanovii ATCC 19119		Sheep	−
L. ivanovii RM3325		Cheese	−
L. seeligeri ATCC 35967		Soil	−
L. seeligeri RM3008		Soil	−
L. seeligeri RM3321		Cheese	−
L. welshimeri ATCC 35897		Plant	−
L. welshimeri ATCC 43550	1/2b	Soil	−
L. welshimeri ATCC 43551	6a	Soil	−
L. welshimeri CCF4		Catfish brain	−
L. welshimeri 1471		Environment	−
L. monocytogenes ATCC 19111	1	Poultry	−
L. monocytogenes ATCC 19112	2	Human	−
L. monocytogenes ATCC 19113	3	Human	−
L. monocytogenes ATCC 19114	4a	Human	−
L. monocytogenes ATCC 19115	4b	Human	−
L. monocytogenes ATCC 19116	4c	Chicken	−
L. monocytogenes ATCC 19117	4d	Sheep	−
L. monocytogenes ATCC 19118	4e	Chicken	−
L. monocytogenes ATCC 15313	1	Rabbit	−
L. monocytogenes EGD (NCTC 7973)	1/2a	Guinea pig	−
L. monocytogenes HCC7	1	Catfish brain	−
L. monocytogenes HCC8	1	Catfish brain	−
L. monocytogenes HCC12	4	Catfish brain	−
L. monocytogenes HCC13	4	Catfish kidney	−
L. monocytogenes HCC16	4	Catfish brain	−
L. monocytogenes HCC17	4	Catfish brain	−
L. monocytogenes HCC18	4	Catfish spleen	−
L. monocytogenes HCC19	4	Catfish spleen	−
L. monocytogenes HCC23	4	Catfish brain	−

Table 1 (*Continued*).

Strain	Serovar	Source	lin0464 (749 bp)
L. monocytogenes HCC24	4	Catfish spleen	–
L. monocytogenes HCC25	4	Catfish kidney	–
L. monocytogenes 168		Aborted calf fetus	–
L. monocytogenes 180		Human outbreak	–
L. monocytogenes 418		Freezer study	–
L. monocytogenes 742		Ground beef	–
L. monocytogenes 874		Cow brain	–
L. monocytogenes 1002		Pork sausage	–
L. monocytogenes 1084		Chicken	–
L. monocytogenes 1400		CDC/Jalisco outbreak	–
Aeromonas hydrophila ATCC 35654			–
Clostridium perfringens		Clinical	–
Enterococcus faecalis ATCC 29212			–
Escherichia coli ATCC 25922			–
Flavobacterium indolegenes		Clinical	–
Klebsiella pneumoniae ATCC 13883			–
Proteus vulgaris ATCC 13315			–
Pseudomonas aeruginosa ATCC 27853			–
Salmonella typhimurium ATCC 14028			–
Serratia marcescens ATCC 8100			–
Staphylococcus aureus ATCC 25923			–
Streptococcus pneumoniae		Clinical	–
Streptococcus pyogenes ATCC 19615			–
Vibrio cholerae		Clinical	–
Yersinia pseudotuberculosis		Clinical	–

species are differentiated by subsequent *Rsa*I digestion of the amplicons. Another PCR assay has been reported that is based on the *iap* (invasion-associated protein) gene, which is shared by all *Listeria* species. This assay uses a multiplex strategy to amplify different sized amplicons from *L. monocytogenes*, *L. innocua*, and *L. grayi* [13]. The *iap* gene has also been used to establish a quantitative PCR method for detection of *L. monocytogenes* and *L. innocua* [14].

The availability of a PCR assay based on a gene present uniquely in *L. innocua* would be useful as a confirmatory method where standard techniques do not allow for clear differentiation of *Listeria* species. In addition, this type of assay could provide an independent confirmation of *L. innocua* in cases where identification is based on PCR assays that are based on the selective amplification of a common gene (such as 16S/23S rRNA genes or *iap*). In particular, results from PCR assays based on a common gene may be confusing in environmental or epidemiological studies where multiple *Listeria* species are present because competition would occur between genus- and species-specific primers and multiple products would be generated.

The *L. innocua* and *L. monocytogenes* genomes contain a relatively high proportion of transcriptional regulators (7.3% of the predicted coding sequences) [15]. This high proportion of regulatory genes reflects *Listeria*'s ability to survive and grow in a broad range of environmental conditions, and we considered it likely that the *L. innocua* genome would contain transcriptional regulator gene(s) that are unique to this species and allow it to adapt to its specific environment.

Here, we report the identification of a gene encoding a transcriptional regulator that is uniquely present in *L. innocua*. PCR primers based on this gene amplify a product only from *L. innocua* and not from other *Listeria* or other bacterial species, making this assay useful as a confirmatory method for identification of *L. innocua* or as a species-specific detection tool for environmental or epidemiological studies.

2. Materials and methods

2.1. Bacteria

Some of the *Listeria* and other bacterial strains were obtained from the American Type Culture Collection (ATCC) or the National Collection of Type Culture (NCTC). Environmental isolates were also used [16,17], along with other food or clinical isolates. A total of 73 *Listeria* strains were examined. These included 31 *L. innocua*, two *L. grayi*, one *L. murrayi*, two *L. ivanovii*, three *L. seeligeri*, five *L. welshimeri*, and 29 *L. monocytogenes* strains (Table 1). In addition, 15 common Gram-positive and negative bacterial strains were included in the study to verify the specificity of the PCR primers developed (Table 1).

2.2. DNA isolation

Several colonies of *Listeria* and other bacterial strains grown on 5% sheep blood agar plates (TSA II, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) were transferred to flasks containing 25 ml of brain heart infusion broth (Difco Laboratories, Detroit, MI, USA). The bacterial cultures were incubated at 37°C overnight with shaking. Genomic DNA was prepared from the overnight cultures using a standard procedure [18]. Namely, bacteria were pelleted and resuspended in 2.5 ml of lysis solution (0.1 M Tris–HCl, pH 8.0, 2% Triton X-100, and 0.25% sodium azide) containing 2 mg ml^{-1} lysozyme (Sigma, St. Louis, MO, USA). Following incubation at 37°C for 30 min, 25 μl of 10 mg ml^{-1} proteinase K (Sigma) was added. After incubation at 56°C for 2 h, one volume of phenol/chloroform/isoamyl-alcohol (25:24:1) (Sigma) was added. Following extraction and centrifugation, the supernatant was transferred to a new tube, and one volume of isopropyl alcohol and 150 μl of 5 M NaCl were added. The DNA was pelleted by centrifugation and washed with 3 ml of 70% ethanol. The purified DNA was dissolved in 1×TE (10 mM Tris–HCl and 1 mM EDTA, pH 8.0), and the DNA concentrations were determined at UV 260/280 nm in a GeneSpecI (Hitachi Genetic Systems, Japan). A small amount of DNA from each bacterial strain was diluted in distilled water at 10 ng μl^{-1} for PCR analysis.

2.3. Identification of L. innocua-specific gene(s)

From the list of *L. innocua* genes that have no *L. monocytogenes* ortholog [15], all 10 of the chromosomal genes encoding putative transcriptional regulators were screened using BLAST searches to identify unique sequences. This search yielded two unique gene candidates (*lin0464* and *lin0864*). To provide additional candidate genes, a selected number of *L. innocua* genes encoding proteins with unknown function from the same list were also screened using BLAST searches, and two additional genes were selected (*lin1451* and *lin0455*). Primers specific for the selected genes were designed with Primer3 software (Whitehead Institute for Medical Research, Cambridge, MA, USA), and synthesized by Sigma Genosys (The Woodlands, TX, USA).

2.4. PCR

PCR amplification was performed in a volume of 25 μl using a GeneAmp PCR System 2400 (Perkin Elmer, Foster City, CA, USA). The reaction mixture consisted of 0.5 U Taq DNA polymerase (Fisher Scientific, Houston, TX, USA), 1× PCR buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl and 1.5 mM $MgCl_2$), 50 μM dNTPs, 25 pmol primers each and 15 ng DNA. The reaction mixture with no template DNA was used as a negative control. The cycling programs consisted of one cycle of 94°C for 2 min; 25 cycles of 94°C for 20 s, 60°C for 20 s and 72°C for 45 s; and a final incubation at 72°C for 2 min. After completion of all cycles, 3 μl of 10× DNA loading buffer was added to each tube, and the amplified products were examined in 1.0% agarose gel electrophoresis in the presence of ethidium bromide (0.5 μg ml^{-1}). The stained gels were visualized under UV light and results recorded using a ChemiImager 5500 (BSI, Stafford, TX, USA).

2.5. Determination of PCR detection limit

Serial dilutions of DNA from a *L. innocua* strain (ATCC 33090) ranging from 10 ng μl^{-1} to 0.1 pg μl^{-1} were examined by PCR (described above) employing specific primers developed. The PCR detection limit was determined to be the lowest amount of DNA that was sufficient to amplify the *L. innocua*-specific PCR fragment as detectable in ethidium bromide-stained agarose gel.

3. Results and discussion

Out of the four selected *L. innocua* genes (*lin0464*, *lin0864*, *lin1451* and *lin0455*), preliminary PCR results indicated that only one of these genes, *lin0464*, showed potential for species-specific identification of *L. innocua*. Primers derived from the other three genes failed to recognize all *L. innocua* strains under investigation (data not shown).

lin0464 is located between nucleotide sequences 28719 to 29715 in the *L. innocua* genome and encodes a protein similar to a transcriptional regulator [15]. Although a nucleotide BLAST search yielded no significant homology with bacterial genes, BLAST search with the 310-amino acid deduced protein sequence revealed limited, partial homologies with hypothetical or unknown proteins from *Bacillus subtilis* and *Enterococcus faecium*, as well as putative transcriptional regulators from *L. innocua* (*lin0395*) and *L. monocytogenes* (*lmo0376*). Oligonucleotide primers (lin0464F: 5′-CGCATTTATCGCCAAAACTC-3′ and lin0464R: 5′-TCGTGACATAGACGCGATTG-3′), which are complementary to *lin0464* at nucleotide positions 28847–28866 and 29595–29576, respectively, were designed to amplify a 749-bp fragment.

When primers lin0464F and lin0464R were screened against the panel of bacteria, only DNA from *L. innocua* strains produced the predicted 749-bp amplicon by PCR (Table 1). DNA from other *Listeria* species and 15 common Gram-positive and Gram-negative species did not form detectable bands on ethidium bromide-stained agarose gels (Table 1). The detection limit of PCR employing lin0464F and lin0464R was relatively low: as little as 10 pg of *L. innocua* DNA was readily detected (data not shown).

L. monocytogenes and *L. innocua* occupy similar ecological niches; in fact, it has been suggested that *L. innocua*

could be used as an indicator for improper hygiene techniques that lead to *L. monocytogenes* contamination of food [13]. This is reflected in the conservation of transcriptional regulators between the genomes: greater than 95% of the 203 putative transcriptional regulators in the *L. innocua* genome have *L. monocytogenes* orthologs [15]. However, apart from positive regulating factor (PrfA), which is a pleiotropic virulence gene regulator in *L. monocytogenes* [1,19], very little has been reported on *Listeria* transcriptional regulators and their functions. PrfA has no ortholog in *L. innocua* [20] and regulates expression of genes that allow *L. monocytogenes* to adapt and survive in mammalian hosts [21,22]. Our data indicate that *lin0464* is well conserved in *L. innocua* and is highly species-specific, which suggests that it regulates a conserved function in this species.

This is the first report of a PCR assay for specific detection of *L. innocua* that is based on a gene unique to this species. We expect that it could be useful as a tool for confirming identification of this species based on results from other assays or in studies where rapid, consistent detection of *L. innocua* is needed.

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