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A multiplex PCR for species- and virulence-specific determination of *Listeria monocytogenes*

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Abstract

Listeria monocytogenes internalin gene *inlJ* has been described previously for differentiation of virulent from avirulent strains. However, a recent report indicated that there exist some unusual lineage IIIB strains (*e.g.*, serotype 7 strain R2-142) that possess no *inlJ* gene but have the capacity to cause mouse mortality *via* intraperitoneal inoculation. Therefore, a multiplex PCR incorporating *inlA*, *inlC* and *inlJ* gene primers was developed in this study for rapid speciation and virulence determination of *L. monocytogenes*. Although *inlB* gene was also assessed for species-specific recognition, it was not included in the multiplex PCR due to the negative reaction observed between the *inlB* primers and serotypes 4a–e strains. The species identity of the 36 *L. monocytogenes* strains under investigation was verified through the amplification of an 800 bp fragment with the *inlA* primers and the virulence of these strains was ascertained by the formation of 517 bp and/or 238 bp fragments with the *inlC* and *inlJ* primers, respectively. Whereas *L. monocytogenes* pathogenic strains with capacity to cause mortality (showing relative virulence of 30–100%) in A/J mice *via* the intraperitoneal route were invariably detected by the *inlC* and/or *inlJ* primers, naturally non-pathogenic strains (showing relative virulence of 0%) were negative with these primers. While 8 of the 10 *L. ivanovii* strains reacted with the *inlC* primers, they could be effectively excluded as non-*L. monocytogenes* through their negative reactions with the *inlA* primers in the multiplex PCR. Thus, the use of the multiplex PCR targeting *inlA*, *inlC* and *inlJ* genes facilitates simultaneous confirmation of *L. monocytogenes* species identity and virulence.

Keywords: *Listeria monocytogenes*; Virulence; Internalin; PCR; Identification

1. Introduction

The genus *Listeria* is composed of six Gram-positive bacterial species (i.e., *Listeria monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri* and *L. grayi*) that are distributed widely in the environment. While *L. monocytogenes* is pathogenic to both humans and animals, infection with *L. ivanovii* (previously known as *L. monocytogenes* serotype 5) is generally limited to ungulated animals such as sheep and cattle (Low and Donachie, 1997; Vazquez-Boland et al., 2001). The other four *Listeria* species are essentially saprophytes that have adapted for survival in soil and decaying vegetation. Owing to their remarkable tolerance of external stresses such as extreme pH, temperature and salt concentrations (Sleator et al., 2003), *Listeria* species are able to survive many food manufacturing processes. Not surprisingly, with a recent trend toward increased consumption of ready-to-eat and heat-to-eat food products, *L. monocytogenes* has emerged as a significant foodborne pathogen, causing serious illness in infants, pregnant women, elderly and immnuno-suppressed individuals, with symptoms ranging from septicemia, meningitis, encephalitis, abortions, to occasional death (Mead et al., 1999).

Although *L. monocytogenes* is an important foodborne pathogen, the species encompasses a spectrum of strains with varied virulence and pathogenicity. Whereas many *L. monocytogenes* strains are naturally virulent and capable of producing significant morbidity and mortality, others are avirulent and unable to establish an infection within mammalian hosts (Roche et al., 2001; Liu et al., 2003). Of the 11 common *L. monocytogenes* serotypes (i.e., 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, and 4e), over 98% of clinical isolates from human

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listeriosis belong to only four serotypes (i.e., 4b, 1/2a, 1/2b and 1/2c) (Jacquet et al., 2002). Other serotypes (especially 4a) are found mostly in foods and animals and are rarely responsible for human *L. monocytogenes* infections, and show limited ability to cause mouse mortality even *via* intraperitoneal inoculation (Liu et al., 2003, 2006b). This notable diversity in the pathogenicity among *L. monocytogenes* strains necessitates the development of rapid and accurate laboratory procedures that readily distinguish virulent from avirulent strains. The availability of this information is critical to the effective control and prevention of listeriosis, in addition to providing valuable insight on the prevalence and distribution of naturally avirulent *L. monocytogenes* strains in food and environment.

Toward this end, many virulence testing protocols have been developed to enhance the laboratory assessment of *L. monocytogenes* virulence. These include *in vivo* bioassays, *in vitro* cell assays, and assays for key virulence-associated proteins and their corresponding genes. While the *in vivo* mouse virulence assay offers a comprehensive assessment of all known and uncharacterized virulence determinants of *L. monocytogenes*, its utility as a routine technique is questionable due to its expense and its use of animals. *In vitro* cell culture techniques measure the ability of *L. monocytogenes* to adhere, invade and grow within established mammalian cell lines, to spread to neighboring cells, to cause cytopathogenic effects and to form plaques in these cells, as well as to cause death of chicken embryos (Roche et al., 2001; Olier et al., 2002). Even though the *in vitro* adhesion, invasion, and intracellular growth assays have proven useful for separation of wild-type *L. monocytogenes* strains from mutant strains, these approaches are not effective in the differentiation of virulent from wild-type avirulent strains. The *in vitro* cell-to-cell spread (or plaque-forming) assay has shown promise for distinguishing virulent *L. monocytogenes* strains from naturally avirulent strains (Roche et al., 2001). Nonetheless, the widespread application of the cell-to-cell spread assay is hindered by its lack of desired reproducibility and by the time required to run the assay. Thus, attempts have been made to use key virulence-associated proteins and their corresponding genes as targets for improved discrimination of virulent from avirulent *L. monocytogenes* strains (Roche et al., 2001; Olier et al., 2002). Few such studies have resulted in satisfactory outcomes due principally to the fact that the key virulence proteins and genes are present in the whole spectrum of *L. monocytogenes* strains (Nishibori et al., 1995; Jaradat et al., 2002).

Among a panel of newly identified virulence-specific genes encoding putative internalins and transcriptional regulators, a putative internalin gene *lmo2821* was identified as a useful target for rapid differentiation of *L. monocytogenes* virulent strains capable of producing mouse mortality from avirulent strains that produce no mouse mortality (Liu et al., 2003, 2006b; Doumith et al., 2004b; Liu, 2004). The subsequent confirmation of *lmo2821* as a novel internalin gene (*inlJ*) involved directly in *L. monocytogenes* passage through the intestinal barrier as well as involvement in the subsequent stages of infection and virulence lent further support for the validity of using this gene for virulence assessment (Sabet et al., 2005).

While the presence or absence of internalin gene *inlJ* provides a valuable indicator for the potential virulence or avirulence of *L. monocytogenes* strains, a more recent study indicates that there exist some unusual lineage IIIB strains (*e.g.*, serotype 7 strain R2-142) that contain no *inlJ* gene as examined by PCR and Southern blot, and yet have the capacity to cause mouse mortality *via* intraperitoneal inoculation (Liu et al., 2006b; Roberts et al., 2006). Therefore, we sought to evaluate additional virulence-associated genes that can be applied for improved determination of *L. monocytogenes* pathogenicity. Given that internalin C (InlC) is a virulence marker that contributes to the post-intestinal stages of *L. monocytogenes* infection (Engelbrecht et al., 1996) and that internalin A (InlA) and internalin B (InlB) are species-specific surface proteins that play essential roles in listerial entry into host cells (Gaillard et al., 1991; Poyart et al., 1996; Jung et al., 2003), we investigated the feasibility of using their corresponding genes (*i.e.*, *inlA*, *inlB* and *inlC*) together with *inlJ* in a multiplex PCR format for improved species- and virulence-specific determination of *L. monocytogenes*. Multiplex PCR is a variant of PCR that introduces two or more sets of primer pairs with specificity for different gene or gene regions in one test tube, leading to the amplification of multiple products at the same time. Use of a multiplex PCR that selectively amplifies a shared *iap* gene has enabled discrimination of all six *Listeria* species in a single test (Bubert et al., 1999). In addition, multiplex PCR assays targeting group-specific genes have facilitated rapid differentiation of various *L. monocytogenes* serotypes (Borucki and Call, 2003; Doumith et al., 2004a). The results of this study indicate that a combined application of *inlA*, *inlC* and *inlJ* gene primers in a multiplex PCR makes it possible to rapidly and simultaneously confirm *L. monocytogenes* species identity and its potential virulence.

2. Materials and methods

2.1. Bacterial strains

A collection of 36 *L. monocytogenes* strains representing serotypes 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d and 4e as well as lineage subgroups IIIA, IIIB and IIIC were analyzed (Table 1). The virulence of representative *L. monocytogenes* strains was determined in an A/J mouse model through intraperitoneal inoculation per mouse (five per strain) with 0.1 ml (containing 4×10^7–1.2×10^8 colony-forming units) of a 1/200 dilution of the bacterial suspension ($OD_{540\ nm}=1.35$), and their relative virulence (%) calculated (Liu et al., 2003, 2006b; Liu, 2004). In addition, 22 non-monocytogenes *Listeria* strains representing *L. ivanovii* ($n=10$), *L. innocua* ($n=3$), *L. seeligeri* ($n=3$), *L. welshimeri* ($n=3$) and *L. grayi* ($n=3$) were included as controls for the PCR and Southern blot experiments (Table 1). Further, 15 other common Gram-positive and Gram-negative bacterial species were also used to verify the specificity of the *L. monocytogenes* internalin gene primers for the development of the multiplex PCR assay. These comprised one each of *Aeromonas hydrophila* (ATCC 35654), *Clostridium perfringens*, *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Flavobacterium indologenes*,

Table 1
Examination of *Listeria* strains by PCR and Southern blot with internalin gene primers and probes

Strain	Source	Serotype (subgroup)	Southern band(s) (kb)				Multiplex PCR			Relative virulence (%)[a]
			inlA	*inlB*	*inlC*	*inlJ*[b]	*inlA* (800 bp)	*inlC* (517 bp)	*inlJ* (238 bp)	
L. monocytogenes EGD	Guinea pig	1/2a	1.5	4.0	5.0	5.0	+	+	+	100
F6854	Turkey frank	1/2a	1.5	4.0	5.0	5.0	+	+	+	ND
RM3158 (J1-169)	Human blood, sporadic	1/2b	1.3	4.0	5.0	5.0	+	+	+	ND
RM3368	Environment	1/2b	1.3	4.0	5.0	5.0	+	+	+	ND
RM3017	Blood	1/2c	1.5	4.0	5.0	5.0	+	+	+	ND
RM3367	Environment	1/2c	1.5	4.0	5.0	5.0	+	+	+	ND
ATCC 19112	Human spinal fluid	1/2c	1.5	4.0	5.0	5.0	+	+	+	30
HCC7	Catfish brain	1	1.5	4.0	5.0	5.0	+	+	+	70
HCC8	Catfish brain	1	1.5	4.0	5.0	5.0	+	+	+	70
RM3026	Food	3a	1.3	4.0	5.0	4.0, 1.5	+	+	+	ND
RM3162 (M1-004)	Human hip, sporadic	3a	1.3	4.0	5.0	4.0, 1.5	+	+	+	ND
RM3836	Beef frank	3b	1.5	4.0	5.0	5.0	+	+	+	ND
RM3845	Hot dog	3b	1.5	4.0	5.0	5.0	+	+	+	ND
RM3027	Chicken	3c	1.5	4.0	5.0	5.0	+	+	+	ND
RM3159 (J1-049)	Human, sporadic	3c	1.5	4.0	5.0	5.0	+	+	+	ND
ATCC 19114	Ruminant brain	4a	1.3	1.5	−	−	+	−	−	0
HCC12	Catfish brain	4a	1.3	1.5	−	−	+	−	−	0
HCC18	Catfish spleen	4a	1.3	1.5	−	−	+	−	−	0
HCC23	Catfish brain	4a	1.3	1.5	−	−	+	−	−	0
HCC25	Catfish kidney	4a	1.3	1.5	−	−	+	−	−	0
ATCC 19115	Human	4b	1.3	1.5	5.0	5.0	+	+	+	70
RM3177	Human	4b	1.3	1.5	5.0	5.0	+	+	+	ND
ATCC 19116	Chicken	4c	1.5	1.5	−	1.5	+	−	+	100
874	Cow brain	4c	1.5	1.5	4.0	1.5	+	+	+	100
ATCC 19117	Sheep	4d	1.5	1.5	4.0	5.0	+	+	+	40
RM3108	Chicken	4d	1.5	1.5	4.0	5.0	+	+	+	ND
ATCC 19118	Chicken	4e	1.5	1.5	4.0	5.0	+	+	+	50
RM2218	Oyster	4e	1.5	1.5	4.0	5.0	+	+	+	ND
1002	Human	ND	1.5	1.5	4.0	5.0	+	+	+	60%
F2-458	Human blood, sporadic	(IIIA)	1.3	3.0, 1.5	5.0	1.5, 1.0	+	+	+	100
J2-074	Bovine	(IIIA)	1.3	3.0, 1.5	5.0	5.0	+	+	+	100
X1-002	Food	4a (IIIA)	1.3	3.0, 1.5	−	−	+	−	−	0
F2-086	Human blood, sporadic	(IIIB)	4.0	3.0	5.0	−	+	+	−	100
R2-142	Bagged salad	(IIIB)	4.0	3.0	5.0	−	+	+	−	100
F2-208	Human, sporadic	(IIIC)	1.3	3.0, 1.5	5.0	1.5, 1.0	+	+	+	100
F2-270	Human, sporadic	(IIIC)	1.3	3.0, 1.5	5.0	1.5, 1.0	+	+	+	100
*L. ivanovii*ATCC 19119	Sheep		−	−	3.0	−	−	+	−	ND
A8243	Unknown		ND	ND	ND	ND	−	+	−	ND
B4126	Unknown		ND	ND	ND	ND	−	+	−	ND
C1452	Unknown		ND	ND	ND	ND	−	+	−	ND
RM3325	Cheese		ND	ND	ND	ND	−	+	−	ND
SLCC2379	Unknown		ND	ND	ND	ND	−	+	−	ND
SLCC6965	Unknown		ND	ND	ND	ND	−	+	−	ND
SLCC6966	Unknown		ND	ND	ND	ND	−	+	−	ND
SLCC6032	Unknown		ND	ND	ND	ND	−	−	−	ND
SLCC7926	Unknown		ND	ND	ND	ND	−	−	−	ND
L. innocua ATCC 33090	Cow brain	6a	−	−	−	−	−	−	−	ND
ATCC 51742	Cabbage		ND	ND	ND	ND	−	−	−	ND
CLIP 11262	Corn		ND	ND	ND	ND	−	−	−	ND
L. seeligeri ATCC 35967	Soil		−	−	−	−	−	−	−	ND
AT-02	Unknown		ND	ND	ND	ND	−	−	−	ND
DA-41	Unknown		ND	ND	ND	ND	−	−	−	ND
L. welshimeri ATCC 43550	Soil	1/2b	−	−	−	−	−	−	−	ND
ATCC 43551	Soil		ND	ND	ND	ND	−	−	−	ND
ATCC 35897	Plant		ND	ND	ND	ND	−	−	−	ND
L. grayi ATCC 25400	Corn leaves/stalks		−	−	−	−	−	−	−	ND
ATCC 20402	Corn leaves/stalks		ND	ND	ND	ND	−	−	−	ND
ATCC 20403	Corn leaves/stalks		ND	ND	ND	ND	−	−	−	ND

[a] The relative virulence (%) was determined *via* intraperitoneal inoculation of A/J mice, and calculated by dividing the number of dead mice with the number of tested and then multiplying by 100 (Liu, 2004; Liu et al., 2006b). ND, not done.

[b] Southern blot analysis of *inlJ* gene was performed previously by using a PCR amplified *inlJ* fragment of 611 bp as probe (Sabet et al., 2005; Liu et al., 2006b). ND, not done.

Klebsiella pneumoniae (ATCC 13883), *Proteus vulgaris* (ATCC 13315), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enterica* serovar Typhimurium (ATCC 14028), *Serratia marcescens* (ATCC 8100), *Staphylococcus aureus* (ATCC 25923), *Streptococcus pneumoniae*, *Streptococcus pyogenes* (ATCC 19615), *Vibrio cholerae* and *Yersinia pseudotuberculosis*. *Listeria* and other bacterial strains were acquired from reference collections such as the American Type Culture Collection (ATCC, Manassas, VA) and International Life Sciences Institute (ILSI) North America *Listeria monocytogenes* Strain Collection (www.pathogentracker.com) (Fugett et al., 2006), or obtained from other sources.

2.2. Genomic DNA isolation

Listeria and other bacterial strains were retrieved from glycerol stocks kept at −80 °C and were cultured initially on 5% sheep blood agar plates (TSA II, Becton Dickinson Microbiology Systems, Cockeysville, MD), and several colonies of each strain were transferred into 10 ml of brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) in 50 ml Falcon tubes and incubated at 37 °C for 18 h with aeration to an $OD_{540\ nm}$ of 1.2. Genomic DNA was prepared from *Listeria* and other bacterial broth cultures after treatment with lysozyme and proteinase K and extraction with phenol/chloroform (Liu et al., 2003). The purified DNA was resuspended in 1× TE (10 mM Tris–HCl pH 8.0 and 1 mM EDTA pH 8.0), and the DNA concentrations were determined spectrophotometrically using a Nanodrop System (NanoDrop Technologies, Wilmington, DE). An aliquot of DNA from each *Listeria* and other bacterial strain was diluted in distilled water to 10 ng/μl for PCR amplification.

2.3. PCR

Oligonucleotide primers were designed from the *L. monocytogenes* internalin genes *inlA*, *inlB*, *inlC* and *inlJ* (GenBank Accession No. NC_003210) using Primer3 software (Whitehead Institute for Medical Research, Cambridge, MA) and synthesized commercially (Sigma Genosys, The Woodlands, TX) (Table 2). The *inlA* and *inlB* primers were intended for species-specific recognition, and the *inlC* and *inlJ* primers were designed for virulence determination of *L. monocytogenes*. These primers were first assessed in individual PCR and subsequently in a multiplex PCR format. PCR was conducted in a GeneAmp PCR System 9700 (Perkin Elmer, Boston, MA) in a volume of 25 μl containing 0.8 U *Taq* DNA polymerase (Fisher Scientific, Houston, TX), 1× PCR buffer, 200 μM dNTPs and 10 ng each *Listeria* or other bacterial DNA, together with 25 pmol each primer (for individual PCR assessment), or 40 pmol each *inlA*, 30 pmol each *inlC* and 20 pmol each *inlJ* primers (for multiplex PCR assessment). Reaction mixture with no DNA template was incorporated as a negative control in each run. The cycling program consisted of 1× 94 °C for 2 min; 30× 94 °C for 20 s, 55 °C for 20 s and 72 °C for 50 s; and 1× 72 °C for 2 min. PCR results were verified *via* agarose gel electrophoresis. Specific PCR fragments that were amplified from *L. monocytogenes* EGD strain with the *inlA*, *inlB* and *inlC* primers were excised, purified by a Geneclean kit (MP Biochemicals, Irving, CA) and used as probes in Southern blots.

2.4. Southern blot

Southern blot was performed as described previously (Liu et al., 2006a). Briefly, genomic DNA (20 μg) from each of the 36 *L. monocytogenes* and five non-monocytogenes *Listeria* strains was digested with 20 U of *Hin*dIII (Promega, Madison, WI) for 3 h at 37 °C. Approximately 5 μg of *Hin*dIII digested genomic DNA from each strain was loaded per well and separated by 1.0% agarose gel electrophoresis. After denaturation with NaOH and neutralization with Tris–HCl pH 8.0, DNA was transferred onto Hybond N+ membranes (Amersham Pharmacia, Piscataway, NJ) *via* capillary action, and cross-linked to the membrane in a GS Gene Linker UV Chamber (Bio-Rad, Hercules, CA). The bound DNA was then detected with chemically labeled *inlA*, *inlB* or *inlC* probes (800, 884 or 517 bp, respectively) using the ECL direct nucleic acid labeling and detection system (Amersham Pharmacia, Piscataway, NJ).

3. Results

As examined individually in PCR, the *inlA* gene primers (Table 2) formed an expected band of 800 bp with genomic DNA from all 36 *L. monocytogenes* strains, but not with DNA from 22 non-monocytogenes *Listeria* strains (Table 1). The *inlB* gene primers (Table 2) generated a 884 bp fragment from genomic DNA of *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c, 3a, 3b and 3c as well as lineage IIIA, IIIB and IIIC strains, but they did not react with *L. monocytogenes* serotypes 4a, 4b, 4c, 4d and 4e strains, nor with the 22 non-monocytogenes *Listeria* strains (data not shown). In addition to producing a band of 517 bp with

Table 2
Identities and nucleotide sequences of *L. monocytogenes* internalin gene primers

Gene	Coding sequences	Primer sequences (5′→3′)	Nucleotide positions	Expected PCR product (bp)
inlA	94534–96936	ACGAGTAACGGGACAAATGC	94612–94631	800
		CCCGACAGTGGTGCTAGATT	95411–95392	
inlB	97021–98913	TGGGAGAGTAACCCAACCAC	97957–97976	884
		GTTGACCTTCGATGGTTGCT	98839–98209	
inlC	107200–108090	AATTCCCACAGGACACAACC	107306–107325	517
		CGGGAATGCAATTTTTCACTA	107822–107802	
inlJ	188153–190708	TGTAACCCCGCTTACACAGTT	188989–189009	238
		AGCGGCTTGGCAGTCTAATA	189226–189207	

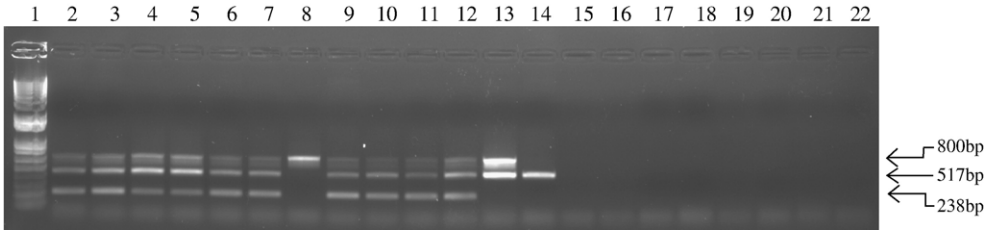

Fig. 1. Agarose gel electrophoresis of DNA products generated in multiplex PCR using internalin gene *inlA*, *inlC* and *inlJ* primers. Lane 1, DNA molecular weight marker (1 kb plus, Life Technologies, Invitrogen, Carlsbad, CA); lanes 2–13, products amplified from DNA of *Listeria monocytogenes* serotype 1/2a (EGD), serotype 1/2b (RM3368), serotype 1/2c (RM3017), serotype 3a (RM3026), serotype 3b (RM3845), serotype 3c (RM3159), serotype 4a (ATCC 19114), serotype 4b (ATCC 19115), serotype 4c (874), serotype 4d (ATCC 19117), serotype 4e (ATCC 19118), and lineage IIIB (R2-142) strains; lane 14, *L. ivanovii* (ATCC 19119); lane 15, *L. innocua* (ATCC 33090); lane 16, *L. seeligeri* (ATCC 35967); lane 17, *L. welshimeri* (ATCC 43550); lane 18, *L. grayi* (ATCC 25400); lane 19, *Enterococcus faecalis* (ATCC 29212); lane 20, *Salmonella enterica* serovar Typhimurium (ATCC 14028); lane 21, *Staphylococcus aureus* (ATCC 25923); and lane 22, negative control with no DNA template. The expected *inlA*, *inlC* and *inlJ* gene products are of 800, 517, and 238 bp in size, respectively (as indicated on the right).

the genomic DNA of 29 of the 36 *L. monocytogenes* strains (except for serotype 4a strains ATCC 19114, HCC12, HCC18, HCC23 and HCC25, one serotype 4c strain ATCC 19116, and one lineage IIIA strain X1-002) (Table 1), the *inlC* gene primers (Table 2) also recognized 8 of the 10 *L. ivanovii* strains analyzed (Table 1). However, this primer pair did not react with 2 *L. ivanovii* and 12 other non-monocytogenes *Listeria* strains (Table 1). While the *inlJ* gene primers (Table 2) identified a band of 238 bp with the genomic DNA of 28 of the 36 *L. monocytogenes* strains (excluding serotype 4a strains ATCC 19114, HCC12, HCC18, HCC23 and HCC25, one lineage IIIA strain X1-002, and lineage IIIB strains F2-086 and R2-142), they did not show any reaction with the 22 non-monocytogenes *Listeria* strains (Table 1 and data not shown). None of the *inlA*, *inlB*, *inlC* and *inlJ* primer pairs cross-reacted in PCR with the 15 Gram-positive and Gram-negative bacterial strains tested, nor with the no DNA template negative control (data not shown).

In view of the fact that the *inlB* gene primers failed to recognize serotypes 4a–e strains, a multiplex PCR based on the *inlA*, *inlC* and *inlJ* gene primers was conducted. As illustrated in Fig. 1 and summarized in Table 1, the multiplex PCR demonstrated excellent agreement with the individual PCR using the *inlA*, *inlC* and *inlJ* gene primer pairs separately. It was particularly noteworthy that the species identity of the 36 *L. monocytogenes* strains was validated through the formation of an 800 bp band with the *inlA* primers, and the virulence of these strains was confirmed by the production of 517 bp and/or 238 bp bands with the *inlC* and *inlJ* primers in the multiplex PCR (Table 1). That is, *L. monocytogenes* strains with capacity to cause mortality in A/J mice *via* intraperitoneal route (showing relative virulence of 30–100%) were identified by the *inlC* and/or *inlJ* primers, while the non-pathogenic strains with a relative virulence of 0% (*i.e.*, serotype 4a strains ATCC 19114, HCC12, HCC18, HCC23 and HCC25 as well as IIIA strain X1-002) were negative with these primers (Table 1) (Liu et al., 2006b). Furthermore, 8 of the 10 *L. ivanovii* strains were identified with the *inlC* primers, but not with the *inlA* and *inJ* primers in the multiplex PCR (Table 1 and Fig. 1, lane 14).

Southern blot analysis provided further confirmation to the PCR results. Using the 800 bp PCR fragment from *L. monocytogenes* EGD *inlA* gene as a probe, a *Hin*dIII band of 1.3, 1.5 or 4.0 kb in rare occasions (*i.e.*, lineage IIIB strains F2-086 and R2-142) was detected in the 36 *L. monocytogenes* strains tested (Table 1 and data not shown). On the other hand, using the 884 bp PCR fragment from *L. monocytogenes* EGD *inlB* gene as a probe, a *Hin*dIII band of 4.0 kb was observed in *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c, 3a, 3b and 3c strains, a band of 1.5 kb in serotypes 4a, 4b, 4c, 4d and 4e strains, and bands of 1.5 and/or 3.0 kb in lineage IIIA, IIIB and IIIC strains (Table 1). In addition, using the 517 bp PCR fragment from *L. monocytogenes* EGD *inlC* gene as a probe, a *Hin*dIII band of 4.0 or 5.0 kb was found in 29 of the 36 *L. monocytogenes* strains (except serotype 4a strains ATCC 19114, HCC12, HCC18, HCC23 and HCC25, serotype 4c strain ATCC 19116, and lineage IIIA strain X1-002) (Table 1). Using a 611 bp PCR fragment from *L. monocytogenes* EGD *inlJ* gene as a probe, *Hin*dIII bands of 1.0, 1.5, 4.0 and/or 5.0 kb were recognized in 28 of the 36 *L. monocytogenes* strains (except serotype 4a strains ATCC 19114, HCC12, HCC18, HCC23 and HCC25, one lineage IIIA strain X1-002, and lineage IIIB strains F2-086 and F2-142) (Table 1) (Liu et al., 2006a,b). Apart from the formation of a 3.0 kb band with DNA from *L. ivanovii* ATCC 19119, the *inlC* gene probe did not react with DNA from *L. innocua* ATCC 33090, *L. seelgeri* ATCC 35967, *L. welshimeri* ATCC 43550 and *L. grayi* ATCC 25400 as determined by Southern blotting (Table 1 and data not shown). Moreover, the *inlA*, *inlB* and *inJ* gene probes displayed no reaction with these five non-monocytogenes *Listeria* strains in Southern blot (Table 1 and data not shown) (Liu et al., 2006a,b).

4. Discussion

L. monocytogenes is made up of multiple serotypes/strains showing varied virulence potential. As a means to implement effective control and prevention measures against *L. monocytogenes* infections, it is necessary to identify virulent, disease-causing strains from avirulent, non-pathogenic strains that are relatively harmless. In spite of numerous past attempts including the use of *in vivo* bioassays, *in vitro* cell assays, and assays for key virulence-related proteins and genes, a fast, reliable and low-cost procedure for differentiation of virulent from avirulent *L. monocytogenes* strains has not been available. This may be due mainly to the fact that our understanding of the

molecular mechanisms of *L. monocytogenes* virulence and pathogenicity is not complete. The recent publication of the whole genome sequences of *L. monocytogenes* EGD-e (GenBank Accession No. NC_003210) has provided further targets for potential use as virulence markers. This is exemplified by the identification and application of a putative internalin gene *lmo2821*, designated *inlJ*, for rapid discrimination of virulent from naturally avirulent strains (of serotype 4a) (Liu et al., 2003; Doumith et al., 2004a). However, given that there appear to be some uncommon *L. monocytogenes* serotypes/strains (*e.g.*, lineage IIIB strains F2-086 and R2-142) with virulence potential and which also lack the *inlJ* gene, a search for additional virulence marker(s) to take account for these strains is warranted.

The present report describes the development of a multiplex PCR that incorporates the *inlA* gene primers for species-specific confirmation and the *inlC* and *inlJ* gene primers for virulence determination of *L. monocytogenes*. The multiplex PCR was evaluated with a collection of 36 *L. monocytogenes* strains covering 11 common serotypes and lineages IIIA, IIIB, and IIIC strains, along with 22 non-monocytogenes *Listeria* and 15 Gram-positive and Gram-negative bacterial strains. The species identity of *L. monocytogenes* was validated by the amplification of an 800 bp band using the *inlA* primers, whereas its potential virulence was determined by the production of 517 bp and/or 238 bp bands using the *inlC* and *inlJ* primers. The inclusion of both the *inlC* and *inlJ* gene primers in the multiplex PCR has added advantage in that it provides a double verification of *L. monocytogenes* virulence for most strains. The usefulness of this multiplex PCR for virulence determination is supported by the observations that *L. monocytogenes* virulent strains with the potential to cause mouse mortality *via* intraperitoneal inoculation of mice were positive with the *inlC* and/or *inlJ* primers, while naturally avirulent strains unable to produce mouse mortality were negative with these primers (Table 1). Even though 8 of the 10 *L. ivanovii* strains cross-reacted with *L. monoctyogenes* virulence-specific *inlC* primers, they could be readily excluded by their negative reaction with *L. monoctyogenes* species-specific *inlA* primers included in the multiplex PCR. The fact that 2 of the 10 *L. ivanovii* strains were undetected by the *L. monoctyogenes inlC* primers suggests that some *L. ivanovii* strains may possess an altered or no *inlC* gene. As InlC is a known virulence protein in *L. monocytogenes* with a role in the post-intestinal stages of listerial infection (Engelbrecht et al., 1996), *L. ivanovii* strains containing an altered or no *inlC* gene may possibly show lowered virulence compared to those with an intact copy of *inlC* gene. Further analysis is clearly required to clarify the precise role of InlC in *L. ivanovii* pathogenicity.

It should be noted that although the presence of the *inlC* and/or *inlJ* genes in a given *L. monocytogenes* strain implies its potential virulence and its ability to cause mouse mortality *via* the intraperitoneal route, it does not necessarily indicate the certainty of the strains harboring these genes to produce disease in humans *via* the conventional oral ingestion. Considering that only four serotypes (4b, 1/2a, 1/2b and 1/2c) are frequently isolated from human listeriosis cases, many serotypes (*e.g.*, 3a, 3b, 3c, 4c, 4d and 4e) that are recognized as being potentially virulent by the multiplex PCR may encounter difficulty crossing the host's intestinal or other barriers during infection. For a *L. monocytogenes* strain to cause infection in humans *via* oral ingestion, it requires involvement of many other known (*e.g.*, InlA, LLO, PlcA, PlcB, Mpl and ActA) and uncharacterized proteins. These undefined proteins require further characterization and examination. Nonetheless, an implication from the current study is that while *L. monocytogenes* serotypes other than 4b, 1/2a, 1/2b and 1/2c are not commonly associated with human listeriosis (due presumably to their inability to efficiently cross human intestinal and other barriers), they (with the exception of naturally avirulent serotype 4a strains) may have the potential to produce disease in humans whose immune functions are suppressed, or who are inadvertently exposed to these serotypes *via* intravenous transfusions or open wounds. The added value of this multiplex PCR lies in that it helps identify naturally avirulent serotype 4a strains, which are non-pathogenic in mice even through intraperitoneal inoculation, and which are therefore unlikely to cause problems to immunocompromised populations. Apart from the absence of *inlA* and *inlC* genes, serotype 4a strains do not have a number of virulence-specific transcriptional regulator genes (*e.g.*, *lmo0833*, *lmo1116*, *lmo1134* and *lmo2672*) and internalin *lmo2470* gene (Liu et al., 2003, 2006b). In addition, these strains harbor a mutated *actA* gene, in which a 105 bp deletion removes a stretch of 35 amino acids (Genbank accession No. DQ118415), effectively taking two out of the four copies of proline-rich repeats (DFPPPPTDEEL) in ActA. Ultimately, these changes, along with many other still uncharacterized genetic variations, decrease the capacity of *L. monocytogenes* serotype 4a strains to prosper in murine liver and spleen, to spread to neighboring cells and to cause mouse mortality even *via* intraperitoneal inoculation.

As human listeriosis usually results from ingestion of *L. monocytogenes*-contaminated foods, there is no question that an ideal way to induce listerial infection in experimental animals is through intragastric (or oral) route. This will enable a more close mimicking of human listeriosis biologically and pathologically. However, given that inbred mouse strains widely accessible contain a mutated E-cadherin, which is inappropriate for interaction with *L. monocytogenes* InlA, they often give inconsistent outcome upon intragastric inoculation. For these wild-type mouse strains, intraperitoneal inoculation produces consistent infection. Additionally, although serotypes 4b, 1/2a, 1/2b and 1/2c are mainly responsible for human listeriosis (*via* contaminated foods), our current understanding of *L. monocytogenes* genomics does not permit a clear differentiation of these pathogenic serotypes from those presumably non-pathogenic serotypes (*e.g.*, 3a, 3b, 3c, 4c, 4d and 4e except for 4a). From the limited data available, virtually all serotypes (but 4a) possess virulence-specific transcriptional regulator (*e.g.*, *lmo1116*, *lmo1134* and *lmo2672*) and internalin (*e.g.*, *inlJ*, *inlC*, *inlE*, *inlH*, *lmo0333* and *lmo2470*) genes (Liu et al., 2003, 2006b; Doumith et al., 2004b). The genetic differences between the highly pathogenic (*i.e.*, 4b, 1/2a, 1/2b and 1/2c) and low pathogenic serotypes (*i.e.*, 3a, 3b, 3c, 4c, 4d

and 4e) are not clearly demonstrable with an intragastric mouse model, but the potentially pathogenic serotypes (including 4b, 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4c, 4d and 4e) can be distinguished from naturally avirulent serotype (*i.e.*, 4a) with an intraperitoneal mouse model. As we learn more from genome-based studies of *L. monocytogenes* genomics, more appropriate virulence testing models will emerge in the future that will facilitate better genetic distinction among the highly pathogenic, low pathogenic, and naturally avirulent serotypes.

Previously, both *L. monocytogenes inlA* and *inlB* genes have been shown to have species-specificity (Poyart et al., 1996; Jung et al., 2003), suggesting their species-wide sequence conservation. Although some *L. monocytogenes* food isolates were found to harbor mutations in the *inlA* gene, leading to a significant virulence reduction in these strains (Nightingale et al., 2005), a recent study indicates that the proportion of *L. monocytogenes* isolated from ready-to-eat seafood having a nonsense-mutated *inlA* was negligible (1.7% or 1/59) (Handa-Miya et al., 2007). PCR and Southern blot analysis in the current study revealed that while the *inlA* gene remains largely unchanged in the 11 common serotypes (with the formation of a *Hin*dIII band of 1.3 or 1.5 kb), the *inlB* gene differs notably among these serotypes (with serotypes 1/2a, 1/2b, 1/2c, 3a, 3b and 3c showing a larger *Hin*dIII band of 4.0 kb in Southern blot and a positive reaction in PCR; and serotypes 4a, 4b, 4c, 4d and 4e showing a smaller *Hin*dIII band of 1.5 kb in Southern blot and a negative reaction in PCR). These observations suggest that *L. monocytogenes* serotypes 4a, 4b, 4c, 4d and 4e may possess an altered *inlB* gene in relation to serotypes 1/2a, 1/2b, 1/2c, 3a, 3b and 3c. A complete, fully functional InlB may be more important than InlA for *L. monocytogenes* entry into murine host cells, since wild-type mice are known to have an inappropriate E-cadherin receptor for InlA (Lecuit et al., 2001). There is evidence that InlB is critical for *L. monocytogenes*' colonization of murine liver and spleen, but not for its crossing of murine intestinal epithelium (Khelef et al., 2006). The fact that serotype 4b strain ATCC 19115 produced a somewhat lower mouse mortality than serotype 1/2a strain EGD in mice seems to be in contradiction with the human listeriosis where serotype 4b is associated with epidemic listeriosis while serotype 1/2a associated with sporadic listeriosis. It is possible that *L. monocytogenes* serotype 4b strains exhibit a higher pathogenicity in humans than in mice because they possess an intact *inlA* gene but a functionally imperfect *inlB* gene. The redundant role of InlB in human listeriosis is supported by the finding that a *L. monocytogenes* Δ*inlB* mutant strain with an intact *inlA* was not attenuated in guinea pigs and rabbits, which harbor a human-like E-cadherin receptor (Khelef et al., 2006). By contrast, *L. monocytogenes* serotype 1/2a strains display a higher virulence in mice than in humans as they possess a perfect copy of *inlB* gene that is vital for listerial growth in liver and spleen.

In conclusion, a multiplex PCR incorporating *inlA* gene primers for *L. monocytogenes* species-specific recognition, and *inlC* and *inlJ* gene primers for virulence determination was developed. Whereas *L. monocytogenes* pathogenic strains with capacity to cause mortality in A/J mice *via* intraperitoneal inoculation reacted with the *inlC* and/or *inlJ* primers, naturally non-pathogenic strains did not. Application of the multiplex PCR on the basis of the *inlA*, *inlC* and *inlJ* genes enables rapid, simultaneous confirmation of *L. monocytogenes* species identity and virulence.

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