Molecular characteristics and virulence potential of *Listeria monocytogenes* isolates from Chinese food systems

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ABSTRACT

In this study, we examined *Listeria monocytogenes* isolates from Chinese food sources in an attempt to gain further insights on the molecular characteristics and virulence potential of this important foodborne pathogen. Of the 88 *L. monocytogenes* food isolates recovered, 42 (47.7%) were of serovars 1/2a or 3a; 23 (26.1%) of serovars 1/2b or 3b; 15 (17.0%) of 1/2c or 3c; 6 (6.8%) of serovars 4b, 4d or 4e; and 2 (2.2%) of serovars 4a or 4c. In contrast to *inlAB* locus conserved in all serovars, internalin cluster between *ascB* and *dapE* varies with different serovars, with *inlC2DE*, *inlGC2DE* and *inlGHE* predominantly in serovars 1/2b or 4b, serovar 1/2a and serovar 1/2c. While *inlF* existed in all the *inlGHE*- and *inlGC2DE*-containing isolates but 17.4% of those having *inlC2DE*, *lmo2026* existed in all the *inlGHE*-containing isolates but 20.0% of those bearing *inlGC2DE*, suggesting that *inlF* might have co-evolved with *inlGC2DE* and *inlGHE* while *lmo2026* with *inlGHE* only. With the exception of serovar 4a isolate, most serovar isolates demonstrated remarkable ability to form plaques on L929 cells and produced significant mouse mortality irrespective of the internalin gene organization and whether an intact *actA* gene is present or not. These results indicate that majority of these food isolates may have the potential to cause human diseases if ingested via contaminated foods. Given that serovar 4b accounts for nearly half of human clinical listeriosis cases documented, the relative low proportion of serovar 4b food isolates suggests that this serovar is probably more tolerant of the adverse conditions in the host's stomach and/or more efficient in entering host cells than serovars 1/2a, 1/2b and 1/2c.

1. Introduction

Listeria monocytogenes is an intracellular bacterium that has the capability to infect a range of cell types, including professional phagocytes and non-phagocytes (e.g. epithelial cells, endothelial cells, hepatic cells and fibroblasts), and to cross the intestinal, blood-brain and placental barriers. Due to its widespread nature and its ability to tolerate wide pH, temperature and salt ranges, *L. monocytogenes* readily enters food processing facilities and survives and grows in a variety of food stuffs such as milk, seafood, vegetables and meat products. Accordingly, this bacterium has been responsible for an increasing proportion of human foodborne diseases, especially in infants, pregnant women, the elderly and immunosuppressed individuals, with a mortality rate approaching to 30% (Vazquez-Boland et al., 2001; Liu et al., 2006a,b).

L. monocytogenes infection process comprises several distinct stages: adhesion and invasion of host cells, escape from vacuole, intracellular multiplication and intercellular spread (Vazquez-Boland et al., 2001). *L. monocytogenes* adheres to and invades host cells both passively through phagocytosis and actively through actions of internalins, a complex family of leucine-rich repeat-containing (LRR) proteins (Bierne et al., 2007). Of the 25 members in the multigene internalin family, InlA binds to host adhesion protein E-cadherin for entering into epithelial cells and InlB interacts with a wide range of cell receptors for gaining entry into other cell types (Bergmann et al., 2002; Geese et al., 2002). InlC has been shown to be involved in the post-intestinal stage of infection (Engelbrecht et al., 1996), and InlJ plays a role in assisting *L. monocytogenes* crossing the intestinal barrier and also functions as an adhesin (Sabet et al., 2005, 2008). The genes encoding InlC and InlJ have recently been proved to be excellent markers for rapid determination of *L. monocytogenes* virulence (Liu et al., 2003, 2007a,b). In addition, *inlH*, which is contiguous with *inlG* and *inlE* between *ascB* and *dapE*, is also involved in listerial virulence (Dramsi et al., 1997; Bierne et al., 2007). Remarkably, some strains carry clusters *inlGC2DE* or *inlC2DE* in this region, with *inlC2* and

inlD instead of *inlH* (Nelson et al., 2004; Tsai et al., 2006). In contrast, *inlF* (which mediates invasion and virulence under specific conditions) and *lmo2026* (which possibly affects listerial multiplication in the brain) are located discretely in chromosomal regions (Bierne et al., 2007; Kirchner and Higgins, 2008). Once inside host cells, *L. monocytogenes* readily escapes from vacuole, replicates in the cytoplasms, and spreads to the neighboring cells to initiate a new infection cycle. Here, a *L. monocytogenes* surface protein ActA encoded by *actA* contributes significantly to the actin-based intra- and intercellular mobility in addition to being an adhesion factor (Suarez et al., 2001).

Because the usual route of *L. monocytogenes* infection in humans is via contaminated food, we conducted a survey of *L. monocytogenes* isolates recovered from Chinese food systems, and examined 12 internalin genes as well as *actA* gene in these isolates by PCR and DNA sequencing. This was followed by evaluation of selected *L. monocytogenes* food isolates for *in vitro* adhesion and cell-plaque formation as well as *in vivo* mouse virulence.

2. Materials and methods

2.1. Bacterial strains and DNA manipulations

A total of 88 *L. monocytogenes* isolates from various food sources in southeastern China were analyzed. These included 9 from milk products, 20 from seafood, 3 from vegetables, 22 from pork, 21 from chicken, 4 from beef, 3 from mutton, 3 from duck, 2 from cony meat and 1 from venison (Table 1). The food samples were homogenized and *Listeria* bacteria were enriched in 225 ml half Fraser broth for 4 h at 30 °C followed by full Fraser broth for 48 h at 37 °C before verification with *Listeria* selective agar plate (CHROMAgar Listeria, Paris, France) and multiplex PCR (Zeng et al., 2006). Additionally, 10 *L. monocytogenes* strains representing serovars 1/2a, 1/2b, 1/2c, 4a and 4b from reference collections were examined as controls in the study (Table 1). While the 88 *L. monocytogenes* food isolates were derived from different samples of given food types, there was a possibility that some isolates might belong to similar clones as they were all originated from a relatively small geographic region (surrounding Hangzhou city) in southeast China.

Listeria strains were retrieved from frozen glycerol stocks and cultured on brain heart infusion broth (BHI; Oxoid, Hampshire, England) at 37 °C. Genomic DNA was extracted from these strains using a protocol reported previously (Jiang et al., 2008). Briefly, *Listeria* cell pellet from 5 ml BHI culture was resuspended in 1 ml lysis buffer consisting of 2.0% Triton X-100 plus 2.5 mg sodium azide/ml in 0.1 M Tris–HCl buffer at pH 8.0 and proteinase K (10 μl of 20 mg/ml stock). After incubation at 55 °C overnight, *Listeria* DNA was extracted with phenol–chloroform and precipitated in isopropanol. Oligonucleotide primers were synthesized by Invitrogen Biotechnology (Shanghai, China) (Table 2), and Taq DNA polymerase (TaKaRa Biotech Co. Ltd., Dalian, China) was used for PCR amplification. PCR was conducted using a thermal cycler (MJ Research Inc. MA, Boston, U.S.A.), with annealing temperatures depending on specific primer pairs (Table 2), and the duration of extension depending on the expected length of amplicon (1 min per kb, at 72 °C). For DNA sequencing analysis, PCR fragments were purified with the AxyPrep DNA Gel Extraction Kit (Axygen Inc., USA) and their sequences determined by dideoxy method on ABI-PRISM 377 DNA sequencer.

2.2. Phylogenetic analysis of actA gene

A 537 bp (or 432 bp in some cases) fragment (corresponding to the *actA* nucleotide positions between 775 and 1313, which covers four proline-rich repeats or PRRs) was amplified from the 88 *L. monocytogenes* food isolates as well as reference strains by PCR using *actA* gene primers and its nucleotide sequence analyzed (Table 2). The deduced amino acid sequences were aligned for lineage classification (Wiedmann et al., 1997) by using the Molecular Evolutionary Genetics Analysis software (MEGA version 3.0) (http://www.megasoftware.net).

2.3. Serotype identi cation

A multiplex PCR with primers from ORF2819, ORF2110, *lmo0737* and *lmo1118* was performed for typing the food isolates (Doumith et al., 2004a). Specifically, ORF2819 primers recognize serovars 1/2b, 3b, 4b, 4d and 4e; ORF2110 primers further separate serovars 4b, 4d and 4e from serovars 1/2b and 3b; *lmo0737* primers identify serovars 1/2a, 3a, 1/2c and 3c strains; and *lmo1118* further distinguish serovars 1/2c and 3c from 1/2a and 3a (Doumith et al., 2004a). Also, *lmo1134* primers with specificity for all *L. monocytogenes* strains except serovars 4a and 4c were utilized (Liu et al., 2003). Serotyping based on agglutination between somatic/flagellar antigens and specific antisera (Schonberg et al., 1996) was applied to selected *L. monocytogenes* isolates, including all from milk and seafood, and several from vegetable, pork and chicken along with the reference strains, to confirm the accuracy of serogrouping results based on multiplex PCR.

2.4. Examination of internalin genes

As the internalin genes are known to be scattered in different sections of *L. monocytogenes* genome and contribute to bacterial adhesion and/or virulence (Bierne et al., 2007), we investigated the presence or absence of 12 internalin genes in the 88 *L. monocytogenes* food isolates by PCR with specific primers outlined in Table 2. Due to the conserved repeats present in internalin multigene family (Bierne et al., 2007), primers were designed based on the distinguishable regions through sequence comparison. As *inlH* and *inlC2* shared highly identical nucleotide sequences, a common primer set was employed. To further examine the internalin organization between *ascB* and *dapE*, upstream primer (u, targeting *inlG*) was combined with downstream primer (d1, targeting *inlE*; d2, targeting *inlD*) respectively (Table 2). Bridging PCR using primer pair u/d1 was expected to produce 4000 bp fragment from strains harboring *inlGC2DE*, 2241 bp fragment from strains harboring *inlGHE* cluster and no fragment from those harboring *inlC2DE* or being empty between *ascB* and *dapE*, while PCR using primer pair u/d2 only yield 2241 bp fragment from strains harboring *inlGC2DE*.

2.5. Adhesion assay

The ability of selected *L. monocytogenes* isolates to adhere to HeLa epithelial cells was examined (Olier et al., 2002; Jiang et al., 2006). As for plaque-forming and mouse virulence assays, food isolates were selected for adhesion assay on the basis of their distinct or unusual internalin and *actA* gene profiles in addition to the control strains. Briefly, HeLa monolayers at 80% confluence in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) in 12-well plates (Corning, USA) were inoculated with 300 μl of fresh bacterial suspension (10^7 colony forming units or CFU per ml) to obtain a multiplicity of infection (MOI) of 10:1 for 1 h at 37 °C in the presence of 5% CO_2. The cell monolayers were then washed three times with 10 mM phosphate-buffered saline (PBS) pH 7.2 to remove nonadherent bacteria. Adherent bacteria were harvested after lysis of the cell monolayers with 250 μl Triton X-100 (0.25% in cold PBS) and 250 μl trypsin for 10 min at 4 °C. The CFU values for viable bacteria were determined by plating suitable dilutions of the lysates onto BHI plates. The plates were subsequently incubated for 36 h at 37 °C. We set the adhesion rate of the serovar 4b strain M5

Table 1
Characteristics of *L. monocytogenes* reference and food isolates under investigation.

Stain/isolate	Source	Lineage[a]	Multiplex PCR[b]	Agglutination[c]	*actA*[a]	*inlA*	*inlB*	*inlC*	*inlD*	*inlE*	*inlF*	*inlG*	*inlH/C2*	*inlI*	*inlJ*	*lmo2026*	*inlG-E*[d] (bp)	*inlG-D*[e] (bp)	*ascB dapE* structure
ScottA	Reference	I	4b,4d or 4e	4b	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
NICPBP 54007	Reference	I	4b,4d or 4e	4b	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
SLCC2755	Reference	I	1/2b or 3b	1/2b	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
EGD-e	Reference	II	1/2a or 3a	1/2a	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
10403S	Reference	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	+	+	+	+	+	4000	2241	*inlGC2DE*
NICPBP 54003	Reference	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	+	+	+	+	+	4000	2241	*inlGC2DE*
AB2483	Reference	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	+	+	+	+	+	4000	2241	*inlGC2DE*
NICPBP 54002	Reference	II	1/2c or 3c	1/2c	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
NCTC 5348	Reference	II	1/2c or 3c	1/2c	537	+	+	+	+	+	+	+	+	+	+	+	4000	2241	*inlGC2DE*
NICPBP 54006	Reference	III	4a or 4c	4a	432	+	+	−	−	−	−	−	−	−	−	−	–	–	–
M1	Milk	I	1/2b or 3b	1/2b	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
M2	Milk	I	1/2b or 3b	1/2b	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
M3	Milk	I	1/2b or 3b	1/2b	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
M4	Milk	II	1/2a, or 3a	1/2a	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
M5	Milk	I	4b,4d or 4e	4b	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
M6	Milk	II	1/2a, or 3a	1/2a	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
M7	Milk	III	4a or 4c	4a	432	+	+	−	−	−	−	−	−	−	−	−	–	–	–
M8	Milk	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	−	+	+	+	−	–	–	*inlC2DE*
M9	Milk	II	1/2c or 3c	1/2c	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
S1	Seafood	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
S2	Seafood	I	4b,4d or 4e	4b	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
S3	Seafood	II	1/2c or 3c	1/2c	537	+	+	+	+	+	+	+	+	+	+	+	4000	2241	*inlGC2DE*
S4	Seafood	I	4b,4d or 4e	4b	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
S5	Seafood	I	1/2b or 3b	1/2b	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
S6	Seafood	I	4b,4d or 4e	4ab	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
S7	Seafood	I	1/2b or 3b	1/2b	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
S8	Seafood	I	1/2b or 3b	1/2b	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
S9	Seafood	II	1/2a or 3a	1/2a	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
S10	Seafood	I	1/2b or 3b	1/2b	432	−	−	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
S11	Seafood	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	−	+	+	+	−	–	–	*inlC2DE*
S12	Seafood	I	1/2b or 3b	1/2b	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
S13	Seafood	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	+	+	+	+	+	4000	2241	*inlGC2DE*
S14	Seafood	I	1/2b or 3b	1/2b	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
S15	Seafood	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	+	+	+	−	−	4000	2241	*inlGC2DE*
S16	Seafood	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	+	+	+	−	−	4000	2241	*inlGC2DE*
S17	Seafood	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	+	+	+	−	−	4000	2241	*inlGC2DE*
S18	Seafood	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	+	+	+	−	−	4000	2241	*inlGC2DE*
S19	Seafood	III	4a or 4c	4b	537	+	+	+	+	+	−	+	+	+	+	−	4000	2241	*inlGC2DE*
S20	Seafood	II	1/2c or 3c	1/2c	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
V1	Vegetable	II	1/2c or 3c	1/2c	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
V2	Vegetable	I	4b,4d or 4e	4b	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
V3	Vegetable	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
P1	Pork	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
P2	Pork	I	1/2b or 3b	1/2b	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
P3	Pork	II	1/2a or 3a	1/2a	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
P4	Pork	I	4b,4d or 4e	4b	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
P5	Pork	I	1/2b or 3b	ND	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
P6	Pork	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	−	+	+	+	−	–	–	*inlC2DE*
P7	Pork	II	1/2a or 3a	ND	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
P8	Pork	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
P9	Pork	II	1/2c or 3c	ND	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
P10	Pork	I	1/2b or 3b	ND	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
P11	Pork	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
P12	Pork	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
P13	Pork	I	1/2b or 3b	ND	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
P14	Pork	II	1/2c or 3c	ND	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
P15	Pork	I	1/2b or 3b	ND	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
P16	Pork	I	1/2b or 3b	ND	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
P17	Pork	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
P18	Pork	II	1/2a or 3a	ND	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
P19	Pork	II	1/2c or 3c	1/2c	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
P20	Pork	II	1/2c or 3c	ND	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
P21	Pork	II	1/2c or 3c	ND	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
P22	Pork	II	1/2c or 3c	ND	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
C1	Chicken	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
C2	Chicken	II	1/2a or 3a	1/2a	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
C3	Chicken	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	+	4000	2241	*inlGC2DE*
C4	Chicken	II	1/2c or 3c	ND	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
C5	Chicken	II	1/2a or 3a	ND	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
C6	Chicken	II	1/2a or 3a	ND	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
C7	Chicken	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
C8	Chicken	I	1/2b or 3b	ND	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
C9	Chicken	II	1/2a or 3a	ND	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
C10	Chicken	II	1/2c or 3c	ND	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*

(*continued on next page*)

Table 1 (*continued*)

Stain/isolate	Source	Lineage[a]	Multiplex PCR[b]	Agglutination[c]	*actA*[a]	*inlA*	*inlB*	*inlC*	*inlD*	*inlE*	*inlF*	*inlG*	*inlH/C2*	*inlI*	*inlJ*	*lmo2026*	*inlG-E*[d] (bp)	*inlG-D*[e] (bp)	*ascB dapE* structure
C11	Chicken	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
C12	Chicken	I	1/2b or 3b	ND	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
C13	Chicken	I	1/2b or 3b	ND	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
C14	Chicken	I	1/2b or 3b	ND	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
C15	Chicken	II	1/2c or 3c	ND	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
C16	Chicken	II	1/2c or 3c	ND	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
C17	Chicken	I	1/2b or 3b	ND	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
C18	Chicken	II	1/2a or 3a	1/2a	537	+	+	+	+	+	+	+	+	+	+	+	4000	2241	*inlGC2DE*
C19	Chicken	II	1/2a or 3a	ND	537	+	+	+	+	+	+	−	+	+	+	−	–	–	*inlC2DE*
C20	Chicken	II	1/2a or 3a	ND	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
C21	Chicken	II	1/2a or 3a	ND	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
B1	Beef	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
B2	Beef	I	1/2b or 3b	ND	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
B3	Beef	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	+	4000	2241	*inlGC2DE*
B4	Beef	II	1/2a or 3a	ND	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
G1	Mutton	II	1/2a or 3a	ND	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
G2	Mutton	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
G3	Mutton	II	1/2a or 3a	ND	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
D1	Duck	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
D2	Duck	II	1/2c or 3c	ND	537	+	+	+	−	+	+	+	+	+	+	+	2241	–	*inlGHE*
D3	Duck	I	1/2b or 3b	ND	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
R1	Cony meat	I	1/2b or 3b	ND	537	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*
R2	Cony meat	II	1/2a or 3a	ND	537	+	+	+	+	+	+	+	+	+	+	−	4000	2241	*inlGC2DE*
E1	Vension	II	1/2a or 3a	ND	432	+	+	+	+	+	−	−	+	+	+	−	–	–	*inlC2DE*

[a] The lineage of *L. monocytogenes* strains/isolates was ascertained on the basis of the *actA* gene sequences.
[b] The multiplex PCR employs primers from ORF2819, ORF2110, *lmo0737* and *lmo1118*, and divides into five serotyping groups: 1/2a or 3a; 1/2b or 3b; 1/2c or 3c; 4b, 4d or 4e; 4a or 4c.
[c] The agglutination assay was performed as previously described by Schonberg et al. (1996).
[d] PCR employing primers u and d1 (Table 2) yield 4000 bp fragment from strains harboring *inlGC2DE*, 2241 bp fragment from strains harboring *inlGHE* or none from strains harboring *inlC2DE* or being empty between *ascB* and *dapE*.
[e] PCR employing primers u and d2 (Table 2) yield 2241 bp fragment from strains harboring *inlGC2DE* or none from other strains.

Table 2
Identity and sequence of PCR primers used in this study.

Gene	Specificity	Forward and reverse primers (5′–3′)	Product size (bp)	Annealing temperature (°C)	Reference
lmo0737	*L. monocytogenes* serovars 1/2a, 3a, 1/2c and 3c	AGGGCTTCAAGGACTTACCC ACGATTTCTGCTTGCCATTC	691	53	Doumith et al., 2004a
lmo1118	*L. monocytogenes* serovars 1/2c and 3c	AGGGGTCTTAAATCCTGGAA CGGCTTGTTCGGCATACTTA	906	53	Doumith et al., 2004a
ORF2819	*L. monocytogenes* serovars 1/2b, 3b, 4b, 4d, 4e and 7	AGCAAAATGCCAAAACTCGT CATCACTAAAGCCTCCCATTG	471	53	Doumith et al., 2004a
ORF2110	*L. monocytogenes* serovars 4b, 4d and 4e	AGTGGACAATTGATTGGTGAA CATCCATCCCTTACTTTGGAC	597	53	Doumith et al., 2004a
lmo1134	All *L. monocytogenes* serovars but 4a, 4c and 7	ACCCGATAGCAAGGAGGAAC AACTTCTCTCGATACCCATCCA	367	53	Liu et al., 2003
actA	All *L. monocytogenes* serovars	GGTACGTGATAAAATCGACGA TAGTTATGTCACTTATCAGAGC	537 or 432[a]	55	Wiedmann et al., 1997
inlA	All *L. monocytogenes* serovars	TAATATAAGTGATATAAGCCCAG TTTATCCGTACTGAAATTCC	606	60	This study
inlB	All *L. monocytogenes* serovars	ATCACTTTCTTTGGAGCATAATGGT GCCATCATCACTTATTATTTCTGGA	394	60	This study
inlC	All *L. monocytogenes* but serovar 4a and some 4c	CCATCTGGGTCTTTGACAGTA CAAATAAGTGACCTTAGTCCTT	398	55	This study
inlD	Some lineages I and II strains	CTGTAGTAATGGCAATTAGCTT TGTTATTAGGGACCACAAGCT	870	52	This study
inlE	All *L. monocytogenes* but serovar 4a and some 4c	AGCTCAAAAGAAGTACAAGCA GTGCAATAAGCTCACCAGAAA	787	55	This study
inlF	Some lineage II strains	TGACTTATTTGCAGTTGGGGT TTGGTTCAGGAATAAGCGCG	1119	55	This study
inlG	Some lineage II strains	GTGAAGACGGAACTTGGAAA GCTTCTACTATCGGTTGAACA	668	52	This study
lnlH/C2	All *L. monocytogenes* but serovar 4a and some 4c	ATAGCTACTTTATCAGCATTT ATATCACTTATTTTATTATCATC	437	52	This study
inlI	All *L. monocytogenes* but serovar 4a and some 4c	GTTTCCAGACGACAATCTTGCTA AATCGGTACAGTTACTCGCATCA	635	58	This study
inlJ	All *L. monocytogenes* but serovar 4a and some 7	TAGATGTGACACCACAAACTCAA TGTATTATGCGTGACATCAAGCT	401	58	This study
lmo2026	Some lineage II strains	CGGTTGTTCCTGATGTGTTGCTT TCGACGAACTCTAATCCTTTTGC	837	58	This study
ascB dapE region	All *L. monocytogenes* but serovar 4a and some 4c	u-TGATGATTCAAGTATGATTCCTA d1-ATCAGTAAGCACTGGATCAGTA d2-CGTTTGCTAAATTCTCATCTGTA	Variable[b]	55	This study

[a] Some *L. monocytogenes* strains exhibit a deletion of 105 nucleotides in *actA* gene, leading to removal of 35 amino acids in the ActA protein.
[b] Primer pairs u/d1 and u/d2 both yield variable product sizes from different strains (for more information see Table 1).

at 100%. For statistical analysis, the two-tailed Student's *t*-test was applied and *P*-values of ≤ 0.05 were considered as statistically significant.

2.6. Plaque-forming assay

The ability of selected *L. monocytogenes* isolates to form plaques on mouse fibroblasts L929 cells was assessed (Roberts et al., 2005; Jiang et al., 2006). Cell monolayers were grown to 80% confluence in 2 ml DMEM containing 10% fetal bovine serum in six-well plates (Corning, USA). The overnight *Listeria* cultures were centrifuged and resuspended in PBS. For each strain tested, one well was infected with 5×10^5 CFU and the other was infected with 1.5×10^5 CFU. Upon 1-h incubation at 37 °C, the cell monolayers were washed three times with PBS and overlaid with 3 ml of DMEM containing 20 μg/ml gentamicin and 1.4% agarose (Oxoid Ltd., Hampshire, England). Following 3-day incubation at 37 °C, a second 2-ml overlay of DMEM containing 0.02% neutral red solution and 1.4% agarose was added. After a final day of incubation, plaques were photographed by a digital camera. The diameters of 25 plaques were measured using adobe photoshop software for each strain. The plaque size of serovar 4b strain M5 was set at 100%.

2.7. Virulence in mice

The ability of selected *L. monocytogenes* isolates to cause pathogenic effects on mice was assessed (Jiang et al., 2006, 2007). Five groups (six per group) of female ICR mice at 20–22 g (Zhejiang College of Traditional Chinese Medicine, Hangzhou, China) were inoculated intraperitoneally with 0.2 ml aliquots of appropriately diluted *Listeria* strain resuspended in PBS. Mice in the control group were injected with 0.2 ml PBS. The LD_{50} values for mice were calculated by using the trimmed Spearman–Karber method on the basis of mouse mortality data recorded during a ten-day post-injection period. Relative virulence (%) of *L. monocytogenes* isolates

Table 4
Genotypic and phenotypic characteristics of atypical *L. monocytogenes* food strains in comparison with reference strains.

Strain	PCR[a]		Carbohydrate fermentation[b]				Hemolytic activity[b]	Lecithinase activity[b]
	lmo1134	*ORF2819*	L-Rhamnose	D-Xylose	D-Mannitol	Glucose		
NICPBP 54007	+	+	+	−	−	+	+	−
EGD-e	+	−	+	−	−	+	+	−
10403S	+	−	+	−	−	+	+	−
NICPBP 54006	−	−	+	−	−	+	+	+
S10	+	+	+	-	−	+	+	−
S15	+	−	+	−	−	+	+	−
S16	+	−	+	−	−	+	+	−
S17	+	−	+	−	−	+	+	−
S18	+	−	+	−	−	+	+	−
M7	−	−	+	−	−	+	+	+
S19	−	−	+	−	−	+	+	−

[a] The presence (+) or absence (−) of a band of the indicated size by PCR amplification with the specific primers.
[b] The positive (+) or negative (−) results obtained by carbohydrate fermentation tests and hemolytic and lecithinase reactions.

1/2a (or 3a), exhibited great diversity of internalin profiles in this locus. While the majority of serovar 1/2c (or 3c) isolates carried *inlGHE* (14/15) cluster except one containing *inlGC2DE*, serovar 1/2a (or 3a) isolates harbored either *inlGHE* (1/42), *inlGC2DE* (24/42) or *inlC2DE* (17/42) cluster. In addition, serovar 4a isolate M7 carried nothing between *ascB* and *dapE*, and putative serovar 4c isolate S19 contained *inlGC2DE* in this locus (Tables 1 and 3). Interestingly, the status of *inlF* and *lmo2026* was also related to *ascB dapE* structures. The gene *inlF* existed in all the *inlGHE*-containing and *inlGC2DE*-containing isolates but 17.4% of *inlC2DE*-containing ones of lineage II, while *lmo2026* existed in all the *inlGHE*-containing isolates, 20.0% of *inlGC2DE*-containing ones, but none of *inlC2DE*-containing isolates (Table 5).

Examination of 20 selected *L. monocytogenes* food isolates along with reference serovar 4a strain NICPBP 54006 in the adhesion assay using HeLa epithelial cells revealed that relative adhesion rates of these strains ranged from 6.7% to 101.3% (Table 6). While strain S10 lacking the *inlAB* locus showed a significant impaired adhesion ability (6.7%) compared to other strains ($P < 0.01$), strains S17 and S18 without *inlJ* exhibited a slightly lower mean adhesion rate at 14.8% ($P < 0.05$ to other strains except 54006). Although strains lacked all internalins other than *inlA* and *inlB*, M7 and reference strain 54006 showed a comparable mean adhesion rate at 20.6% to other strains ($P > 0.05$). Within serovars 1/2a (3a) and 1/2c (3c) isolates, no significant difference was observed due to different *ascB dapE* structures (Table 6). Similarly, in the plaque-forming assay based on L929 cells, apart from serovar 4a strain M7 that formed no plaques in the cell monolayer, the differences among other 20 selective *L. monocytogenes* food strains in their plaque-forming ability were insignificant (Table 6), regardless of whether they possess an intact *actA* gene or harbor a 105 bp deletion in this gene.

The ability of *L. monocytogenes* to adhere to epithelial cells and actin-based motility has been shown to correlate with virulence (Jaradat and Bhunia, 2003; Liu et al., 2007a). Thus we assessed the virulence of 22 *L. monocytogenes* food-related strains and five reference strains in mouse models. Serovar 4a strain M7 with impaired cell-to-cell motility exhibited low virulence in mice (log LD_{50} 8.21, relative virulence 7%) (Table 6). The reference serovar 4a strain NICPBP 54006 had log LD_{50} 8.35 and relative virulence 3%. The other 20 food strains displayed log LD_{50} ranging from 3.86 to 6.83 and relative virulence from 33% to 93% in mice (Table 6).

Table 5
Relationship between *ascB dapE* structure and existence of

4. Discussion

As listeriosis is essentially a foodborne disease, it is important to investigate the molecular characteristics and virulence potential of *L. monocytogenes* strains recovered from various food sources for designing and implementing more effective prevention strategies. In this study, we examined 88 *L. monocytogenes* isolates from Chinese food systems, including milk, meat (e.g. pork, beef, mutton, cony meat and venison), seafood, poultry (e.g. chicken and duck) and vegetables by using various molecular and phenotypic procedures, and obtained several interesting findings.

It is notable that of the 88 *L. monocytogenes* food isolates from southeastern China, 42 (47.7%) were serovars 1/2a or 3a isolates, followed by serovars 1/2b or 3b (23, 26.1%), serovars 1/2c or 3c (15, 17.0%), serovars 4b, 4d, 4e or 4ab (6, 6.8%), and serovars 4a or 4c (2, 2.4%). The serovar compositions of these Chinese food isolates appear to be similar to those of French food isolates collected between 2000 and 2001, where 58% (132/226) were identified as serovars 1/2a or 3a, 18% (41/226) as serovars 1/2b or 3b, 13% (27/226) as serovars 1/2c or 3c and 12% (26/226) as serovars 4b, 4d or 4e (Hong et al., 2007). *L. monocytogenes* serovars 4b, 1/2a, 1/2b and 1/2c have been responsible for 49% (294/603), 27% (163/603), 20% (120/603) and 4% (22/603) of human clinical listeriosis cases in France during 2001–2003 (Goulet et al., 2006), suggesting that serovar 4b strains are somehow more capable of successfully establishing infections in humans than serovars 1/2a, 1/2b and 1/2c although in murine intragastric model, serovar 1/2a strains are as infective as serovar 4b strains (Barbour et al., 2001).

Next, *L. monocytogenes actA* located in the PrfA-regulated virulence gene cluster has been found to be important for its spread to neighboring cells and maintenance of infection (Vazquez-Boland et al., 2001). Previously, a serovar 4a strain L99 harboring an altered *actA* gene (with a 105 bp deletion) has been shown to produce a possibly non-functional ActA protein that is 5 kDa smaller than that of serovar 1/2a strain EGD, and this strain had limited ability to undergo cell-to-cell spread in the plaque-forming assay (Chakraborty et al., 1994), as deletion of 35 amino acids in effect removes 2 copies of PRR required for binding with the focal contact proteins VASP and Mena to stimulate actin-based motility (Geese et al., 2002). In this study, serovar 4a strain M7 (possessing a 105 bp deletion in its *actA* gene) failed to spread to neighboring cells as assessed in the plaque-forming assay and caused negligible mouse mortality (with relative virulence of 7%). The impaired intercellular spread ability of serovar 4a strain M7 might be due to the substitute of an alanine for a proline at position 267 of *actA* as well as the absence of other genes (e.g. *inlC*

Table 6
Relationship between *L. monocytogenes actA*, internalin gene status and *in vitro* and *in vivo* virulence.

Strain	Serovar	*actA* PCR (bp)	*inlC* PCR	*inlJ* PCR	*ascB dapE* structure	Relative adhesion rate ± SD[a] (%)	Relative size of plaque ± SD[a] (%)	Mice	
								log LD_{50}	Relative virulence (%)
ScottA	4b	537	+	+	*inlC2DE*	ND	ND	5.50	60
NICPBP 54007	4b	537	+	+	*inlC2DE*	ND	ND	6.79	37
EGD-e	1/2a	537	+	+	*inlGHE*	ND	ND	6.64	37
10403S	1/2a	537	+	+	*inlGC2DE*	ND	92.3 ± 5.8	5.49	60
NICPBP 54006	4a	432	−	−	−	16.2 ± 2.2	ND	8.35	3
S5	1/2b	432	+	+	*inlC2DE*	ND	94.5 ± 3.5	5.94	53
S7	1/2b	432	+	+	*inlC2DE*	ND	84.9 ± 3.1	5.79	57
S8	1/2b	432	+	+	*inlC2DE*	47.6 ± 1.5	92.7 ± 2.2	5.08	70
M1	1/2b	537	+	+	*inlC2DE*	54.1 ± 4.8	94.9 ± 8.2	6.46	40
P2	1/2b	537	+	+	*inlC2DE*	ND	82.0 ± 1.3	6.45	43
C17	1/2b or 3b	537	+	+	*inlC2DE*	ND	ND	5.83	53
M2	1/2b	432	+	+	*inlC2DE*	ND	77.3 ± 0.4	6.43	40
M3	1/2b	432	+	+	*inlC2DE*	ND	89.6 ± 0.7	6.32	43
S10	1/2b	432	+	+	*inlC2DE*	6.7 ± 1.1	ND	ND	ND
S2	4b	537	+	+	*inlC2DE*	101.3 ± 5.2	78.0 ± 3.9	6.74	37
S4	4b	537	+	+	*inlC2DE*	ND	79.0 ± 4.5	6.72	37
M5	4b	537	+	+	*inlC2DE*	100 ± 0	100 ± 0	3.86	93
S6	4ab	537	+	+	*inlC2DE*	99.7 ± 10.5	83.2 ± 0.7	4.40	83
S9	1/2a	537	+	+	*inlGHE*	50.8 ± 3.2	91.2 ± 1.4	6.31	43
M4	1/2a	537	+	+	*inlGC2DE*	31.3 ± 3.2	82.5 ± 2.3	5.45	60
M6	1/2a	537	+	+	*inlGC2DE*	ND	88.4 ± 13.1	5.55	60
S1	1/2a	537	+	+	*inlGC2DE*	26.2 ± 3.7	95.8 ± 7.8	5.53	60
S17	1/2a	537	+	−	*inlGC2DE*	13.7 ± 6.0	ND	ND	ND
S18	1/2a	537	+	−	*inlGC2DE*	15.9 ± 3.8	ND	ND	ND
P1	1/2a	537	+	+	*inlGC2DE*	ND	88.9 ± 1.2	6.26	47
P3	1/2a	537	+	+	*inlC2DE*	23.7 ± 2.2	90.8 ± 3.4	6.07	50
C18	1/2a	537	+	+	*inlGC2DE*	34.4 ± 4.5	ND	ND	ND
S11	1/2a	537	+	+	*inlC2DE*	40.4 ± 3.0	ND	ND	ND
P6	1/2a	537	+	+	*inlC2DE*	46.6 ± 7.2	ND	ND	ND
C2	1/2a	432	+	+	*inlC2DE*	38.7 ± 4.8	ND	ND	ND
P19	1/2c	537	+	+	*inlGHE*	28.5 ± 3.1	ND	ND	ND
V1	1/2c	537	+	+	*inlGHE*	45.6 ± 3.6	78.5 ± 1.3	6.11	50
S3	1/2c	537	+	+	*inlGC2DE*	26.2 ± 3.2	84.9 ± 0.6	6.19	47
M7	4a	432	−	−	−	25.0 ± 3.1	0	8.21	7
S19	(4c)	537	+	+	*inlGC2DE*	65.6 ± 7.3	ND	6.83	33

ND, not done.
[a] The adhesion rate and plaque size of serovar 4b strain M5 was set at 100%.

and *inlJ*). On the other hand, the fact that five serovar 1/2b strains (S5, S7, S8, M2 and M3) with a 105-bp deletion in the *actA* gene had no difficulty spreading to other cells, and causing significant mouse mortality (with relative virulence of 40–70%) undermines the role of *actA* in listerial virulence and highlights the possible existence of other mechanisms for *L. monocytogenes* spreading than the requirement of a fully functional ActA protein. Indeed, a serovar 4b strain F2365 from Jalisco cheese outbreak of 1985 in California (Nelson et al., 2004) has been shown to contain a 105-bp deletion in its *actA* gene, which did not seem to stop the bacterium causing listeriosis in humans. As a number of internalins (e.g. *inlC*, *inlJ* and *lmo2470*), transcriptional regulators (e.g. *lmo1134*, *lmo1116* and *lmo2470*) are absent in serovar 4a strain M7, but present in other serovars (Liu et al., 2003, 2007a,b), the distinct genomic background including the presence of additional internalin and transcriptional regulator genes in *L. monocytogenes* serovar 1/2b strains S5, S7, S8, M2 and M3 may have contributed partially to their cell-to-cell spread and virulence.

Furthermore, the *inlAB* locus encodes critical proteins for *L. monocytogenes* entry into epithelial and other cells types (Bierne et al., 2007), and there is evidence that nucleotide alterations in this locus may reduce its virulence in mammalian hosts (Olier et al., 2002). Indeed, several *L. monocytogenes* strains producing truncated InlA protein have been isolated from human carriers, and these strains displayed reduced ability to cause disease in murine models (Olier et al., 2002). Isolation of a serovar 1/2b strain (S10) from seafood in this study represents the first instance of *L. monocytogenes* bacterium which loses the whole *inlAB* locus in the food environment.

Although the organization of internalin genes between *ascB* and *dapE* failed to further delineate listerial adhesion and intercellular abilities and virulence (Table 6), *ascB dapE* structure offered a potential marker for lineages and serovars from phylogenic perspective. Clusters *inlC2DE*, *inlGC2DE* and *inlGHE* were supposed to be the ancestral versions of lineage I (including serovars 1/2b, 3b, 4b, 4d, and 4e), serovar 1/2a (or 3a) and serovar 1/2c (or 3c) respectively. Accordingly, serovar 1/2a (or 3a) strains carrying *inlGHE* (e.g. EGD-e and S9) and serovar 1/2c (or 3c) strains carrying *inlGC2DE* (e.g. NICPBP 54002, NCTC 5348 and S3) appeared as the atypical replacements. In fact, EGD-e was hinted to be an atypical serovar 1/2a strain in previous reports (Hain et al., 2007). Moreover, *inlF* existed in all the *inlGHE*-containing and *inlGC2DE*-containing isolates but 17.4% of *inlC2DE*-containing ones of lineage II, while *lmo2026* existed in all the *inlGHE*-containing isolates, 20.0% of *inlGC2DE*-containing ones but none of *inlC2DE*-containing isolates, suggesting that *inlF* might have co-evolved with *inlGC2DE* and *inlGHE* while *lmo2026* with *inlGHE* only.

If we consider gene deletion as an important force in *Listeria* evolution, *L. monocytogenes* serovar 1/2c (or 3c) strains, containing a complete set of internalins together with intact ActA (Table 3), is possibly more ancestral than those of other serovars, and evolved to serovars 1/2a (or 3a), 1/2b (or 3b), and 4b (or 4d, 4e and 4ab), and then to serovar 4a via gene deletion and/or horizontal gene transfer. Previously, upon examination of 264 genes from 113 *L. monocytogenes* strains by microarray, Doumith et al. (2004b) have also shown the possibility of *L. monocytogenes* serovars evolving from serovar 4b, to serovar 4c, and then to serovar 4a. Given the absence

of *inlGC2DE* and other genes in *Listeria innocua* in comparison with *L. monocytogenes* serovar 4a, the authors further hypothesized the transition from *L. monocytogenes* serovar 4a to *L. innocua*. At this stage, the whole genome sequences of *L. monocytogenes* serovars 1/2a and 4b, *L. innocua* and *Listeria welshimeri* (Glaser et al., 2001; Nelson et al., 2004; Hain et al., 2007) have been published, and the sequencing analyses of other *L. monocytogenes* serovars and *Listeria* species are in progress. There is no doubt that the evolutionary and phylogenetic links among *L. monocytogenes* serovars and *Listeria* species will be much clearer when the full genomic sequence data of representative strains become available in the near future.

In addition, while conventional agglutination assay is useful for serotyping *L. monocytogenes* strains, previous reports have indicated that this typing procedure is unable to correctly identify serovars 4a, 4b and 4c (Liu et al., 2006a,b; Schonberg et al., 1996). The fact that strain S19 was recognized as serovar 4b, but as 4c by other molecular typing techniques (e.g. *actA* gene sequence analysis and PCR targeting ORF2819, *lmo1134* and *inlJ* genes), reinforces again the continuing need to develop improved serotyping procedures for diagnostic and epidemiological applications. Toward this end, several lineage- and group-specific genes have been utilized for more precise serotyping of *L. monocytogenes* (Doumith et al., 2004a). Additionally, a multiplex PCR assay targeting *inlA, inlC* and *inlJ* genes has been devised for species- and virulence-specific determination (Liu et al., 2007b). The results from this study offer support for the use of *inlC* and *inlJ* as virulence markers since food strains harboring these genes were capable of causing severe mouse mortality via intraperitoneal route, whereas food strain M7 without these genes produced negligible mouse mortality.

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