Listeria monocytogenes in the Chinese food system: strain characterization through partial *actA* sequencing and tissue-culture pathogenicity assays

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Human listeriosis is generally caused by consumption of ready-to-eat (RTE) foods that are stored for extended periods of time at refrigeration temperatures and that permit the growth of the causative agent, *Listeria monocytogenes*. Food-consumption patterns in China are undergoing rapid changes and more regular consumption of refrigerated-storage RTE foods may increase the risk of human listeriosis. In total, 40 *L. monocytogenes* isolates were obtained from food ($n = 32$) and sewage ($n = 6$) samples and from two human listeriosis cases that occurred in China. All isolates were characterized into molecular subtypes by DNA sequencing of the 597 bp 3'-terminal region of the virulence gene *actA*. Sequence data were used to classify the 40 Chinese *L. monocytogenes* isolates into sequence types and phylogenetic lineages, and to compare the sequence types of the Chinese isolates with those of isolates from the USA. Phylogenetic analyses showed that the Chinese isolates could be separated into two genetic lineages, with 14 and 26 isolates belonging to lineages I and II, respectively. Lineage II could be subdivided further into two clusters, IIA and IIB. Lineages I and II were identical to the two lineages described previously among US *L. monocytogenes* isolates. In total, 14 *actA* sequence types could be differentiated among the 40 Chinese *L. monocytogenes* isolates; two specific *actA* sequence types were found among both Chinese and US isolates. Isolates belonging to lineage II showed a significantly lower ability to invade and multiply within human intestinal epithelial Caco-2 cells than lineage I isolates. It was concluded that DNA sequencing of the 3'-terminal region of *actA* appears to be an effective method for rapid subtype and lineage classification of *L. monocytogenes*. As strains belonging to lineages I and II have previously been found among isolates from Europe and North America, these results show that *L. monocytogenes* clonal groups found in China are very similar to those found in the USA. Many *L. monocytogenes* strains may thus represent globally distributed clonal types. Together with the first description of two human listeriosis cases in China, these data indicate that changes in food-distribution and -consumption patterns in China and other countries will probably lead to the emergence of human listeriosis as a food-safety issue, as virulent strains of this pathogen appear to be present in the Chinese food supply.

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INTRODUCTION

Listeria monocytogenes is a facultative, intracellular, Gram-positive bacterium that can cause severe food-borne illness in immunocompromised patients, pregnant women and newborns. Symptoms of listeriosis include encephalitis, meningitis and stillbirths (Altimira *et al.*, 1999; Vázquez-Boland *et al.*, 2001). A variety of DNA-based subtyping methods, such

as randomly amplified polymorphic DNA (RAPD), ribotyping, PFGE and amplified-fragment length polymorphisms, have been used for genotyping of *L. monocytogenes*. Based on these methods, *L. monocytogenes* strains can be divided into two or three distinct lineages (Borucki *et al.*, 2004; Brosch *et al.*, 1994; Call *et al.*, 2003; Keto-Timonen *et al.*, 2003; Wiedmann *et al.*, 1997; Wiedmann, 2002). Whilst a fully automated system for ribotyping (RiboPrinter microbial characterization system; DuPont Qualicon) allows highly reproducible subtyping of *L. monocytogenes*, ribotyping is relatively costly and can be less discriminatory than PFGE (Aarnisalo *et al.*, 2003; Sauders *et al.*, 2003). PFGE and RAPD show high discriminatory abilities, but the inter-laboratory

Abbreviations: CSF, cerebrospinal fluid; MLST, multilocus sequence typing; RAPD, randomly amplified polymorphic DNA; RTE, ready-to-eat.

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reproducibility of these methods, particularly of RAPD, can be limited (Destro *et al.*, 1996; Zhou & Jiao, 2004). Although PFGE shows a high level of sensitivity for discrimination of *L. monocytogenes* strains (Aarnisalo *et al.*, 2003; Yde & Genicot, 2004) and is often considered to be the gold standard for discriminatory ability (Sauders *et al.*, 2003), it may also sometimes detect small genetic differences that may not be epidemiologically significant (Tenover *et al.*, 1995). Furthermore, DNA fragment size-based subtyping methods (such as PFGE and RAPD) are difficult to standardize and, as a consequence, the ease of exchanging and comparing subtype data among laboratories can be severely limited.

DNA sequencing-based methods are increasingly being developed and used for subtyping and characterizing bacterial isolates. The advantages of DNA sequence-based subtyping methods over DNA fragment size-based typing methods include their ability to generate unambiguous data that are portable through web-based databases and that can be used for phylogenetic analyses (Chan *et al.*, 2001; Enright *et al.*, 2001). Cai *et al.* (2002) showed that *actA*, a virulence gene that encodes a protein facilitating cell-to-cell spread of *L. monocytogenes* between host cells, is highly polymorphic among *L. monocytogenes* isolates; sequencing of *actA* can thus be used to differentiate *L. monocytogenes* strains effectively. Specifically, sequencing of the 600 bp 3'-terminal region of *actA* allowed discrimination of the 15 *L. monocytogenes* isolates used into 13 sequence types (Cai *et al.*, 2002).

The goal of the study reported here was to define the phylogenetic diversity of *L. monocytogenes* strains found in China by using DNA sequencing-based subtyping methods and to characterize the virulence potential of selected strains by using a tissue-culture assay with human intestinal epithelial cells. In total, 40 *L. monocytogenes* isolates obtained from food and sewage samples and from two human listeriosis cases in China were characterized by DNA sequencing of a 597 bp 3' fragment of *actA*. Whilst multilocus sequence typing (MLST) methods have been reported for *L. monocytogenes* (Cai *et al.*, 2002), sequencing of a single, highly variable gene was chosen as a highly economical alternative to MLST methods. Phylogenetic analyses and comparisons of partial *actA* sequences for Chinese *L. monocytogenes* isolates with previously reported *actA* sequences for *L. monocytogenes* isolates collected in the USA showed that *L. monocytogenes* isolates found in China represent the same two major *L. monocytogenes* lineages found previously among isolates from the USA and Europe (Ericsson *et al.*, 2000; Rasmussen *et al.*, 1995; Wiedmann *et al.*, 1997). Lineage I isolates showed higher invasiveness and cell-to-cell spread characteristics than lineage II isolates, consistent with the predominance of lineage I strains among human cases (Wiedmann *et al.*, 1997; Wiedmann, 2002).

METHODS

***L. monocytogenes* strains.** In total, 38 *L. monocytogenes* isolates were obtained from food products ($n = 32$) and sewage samples ($n = 6$) collected in China (Table 1). In addition, two *L. monocytogenes* isolates

from human listeriosis cases that occurred in China were included in this study (Table 1).

The *actA* sequences for *L. monocytogenes* isolates collected predominantly in the USA were obtained from Cai *et al.* (2002). Eleven of the 15 isolates used by Cai *et al.* (2002) represented human and food isolates from the USA and one isolate was obtained from Europe (FSL X1-002). The geographical origins of the remaining three isolates (FSL J1-022, FSL W1-110 and FSL W1-111) were not available.

PCR. PCR primers were designed based on the published nucleotide sequence for *actA* (GenBank accession no. NC_003210). Primers 01 (5'-GCTGATTAAAGAGATAGAGGAACA-3') and 02 (5'-TTTATGTGGT TATTGCTGTC-3') amplified an 827 bp DNA fragment that corresponded to the 3' end of *actA*. Primers were synthesized by TaKaRa Biotechnology Co. PCR was performed in 100 µl reaction volumes containing 96 µl PCR mastermix (4 µl each primer at 12.5 µM, 10 µl 10× PCR buffer, 6.0 µl 25 mM MgCl₂, 5.0 µl 1 mM dNTP mix, 67 µl ddH₂O) and 4 µl *L. monocytogenes* cell lysate. Thermocycling conditions included an initial hold of 2 min at 94 °C, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min. A final extension step of 72 °C for 10 min was followed by a hold at 4 °C.

Purification and sequencing of PCR products. A 3 µl aliquot of each PCR product was run on a 1.5% agarose gel to confirm successful amplification of *actA*. The remaining 97 µl of each PCR product was purified by using a QIAquick PCR Purification kit (Qiagen). DNA sequencing was performed at TaKaRa Biotechnology Co., using both PCR primers to obtain forward and reverse sequences. Where necessary, PCR sequencing was repeated to confirm DNA sequence polymorphisms.

Sequence analysis and construction of a phylogenetic tree.

Partial *actA* sequences obtained for the 40 Chinese *L. monocytogenes* isolates were aligned with the corresponding *actA* sequences for the 15 isolates reported by Cai *et al.* (2002) by using MEGALIGN in the Lasergene software package (DNASTar). Neighbour-joining trees were constructed by using MEGA v. 2.1 (Kumar *et al.*, 2001).

Tissue-culture invasion assay and intracellular growth assays.

Enterocyte-like Caco-2 cells (obtained from P. Cossart, Institut Pasteur, Paris, France) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat-inactivated (30 min at 56 °C) fetal bovine serum (FBS) and 10 µg gentamicin ml⁻¹. For invasion and intracellular growth assays, Caco-2 cells were trypsinized and seeded into six-well plates at a concentration of 1×10^6 cells per well. Cells were grown without gentamicin to form a monolayer (72–76 h at 37 °C) in DMEM supplemented with 15% heat-inactivated FBS. The medium was changed every 24 h. *L. monocytogenes* isolates were grown on brain–heart infusion (BHI) broth (Difco) at 37 °C for 24 h prior to infection. Bacteria were harvested, adjusted to a concentration of approximately 3×10^6 bacteria ml⁻¹ in DMEM and added to each well, resulting in an m.o.i. of approximately 30 bacteria per host cell. Following 2 h incubation at 37 °C, cells were washed three times with PBS to remove non-adherent bacteria. To kill extracellular bacteria, 2 ml DMEM containing gentamicin (10 µg ml⁻¹) was added to the wells and the cells were then incubated for 1.5 h at 37 °C with 5% CO₂. Cells were then lysed with 1 ml ice-cold 0.1% Triton X-100 and the number of viable intracellular bacteria was determined by plating serial dilutions onto BHI agar plates. Invasion efficiency for each isolate was calculated as the ratio of the number of bacteria internalized at 3.5 h post-infection to the number of bacteria used for infection. To measure intracellular growth, DMEM supplemented with 5 µg gentamicin ml⁻¹ was used as the growth medium after infection and infected cells were incubated for 19.5 h after removal of extracellular bacteria (Van Langendonck *et al.*, 1998). The number of intracellular bacteria was then enumerated at 21.5 h post-infection as described above and intracellular doubling

Table 1. Chinese *L. monocytogenes* strains used in this study

Strain	Source	Lineage	<i>actA</i> sequence type
Lm1579	Human CSF	I	3
Lm1191	Human CSF	IIA	14
Lm03062501	Fresh fish	I	1
Lm03041601	Fresh fish	I	5
Lm03061101	Fresh fish	IIA	13
Lm03040701	Fresh fish	IIA	14
Lm03033101	Fresh fish	IIA	14
Lm03041602	Fresh fish	IIA	14
Lm03042101	Fresh fish	IIB	10
Lm03060601	Fresh fish	IIB	9
Lm03061802	Fresh meat	I	1
Lm03042102	Fresh meat	I	1
Lm03042103	Fresh meat	I	1
Lm03061803	Fresh meat	I	2
Lm03062502	Fresh meat	I	3
Lm03040702	Fresh meat	I	4
Lm03032002	Fresh meat	IIB	8
Lm03041105	Fresh meat	IIA	11
Lm03032001	Fresh meat	IIA	14
Lm03061801	Fresh meat	IIA	14
Lm03032102	Dairy product	IIA	14
Lm03060602	Dairy product	IIB	8
Lm03041501	Vegetable	I	3
Lm03061103	Vegetable	I	6
Lm03040704	RTE food	I	1
Lm03061104	RTE food	I	7
Lm03061808	RTE food	IIA	14
Lm03062505	RTE food	IIA	12
Lm03041603	RTE food	IIA	14
Lm03041102	RTE food	IIA	14
Lm03042104	RTE food	IIB	10
Lm03032101	RTE food	IIB	10
Lm03060604	RTE food	IIB	10
Lm03061807	RTE food	IIB	10
Lm03060104	Sewage	I	6
Lm03060603	Sewage	IIA	14
Lm03062503	Sewage	IIB	8
Lm03040703	Sewage	IIB	10
Lm03040705	Sewage	IIB	10
Lm03061102	Sewage	IIB	10

times between 3.5 and 21.5 h post-infection were calculated. In total, 24 selected isolates, representing 12 lineage I and 12 lineage II isolates (see Table 3), were tested in duplicate on two separate occasions.

RESULTS

Human listeriosis cases

The two human *L. monocytogenes* were isolated in April 2001 from the cerebrospinal fluid (CSF) of two patients showing symptoms of meningitis. Both patients showed high temperatures (39.4 and 38.8 °C, respectively). The first patient was a 49-year-old male; symptoms in this patient included

vomiting, drowsiness, seizures, headache and dislike of light. The second patient was a 35-year-old female, who showed symptoms of high fever, headache, stiff neck, nausea, vomiting, discomfort when looking into bright lights, confusion and sleepiness.

Phylogenetic analysis of partial *actA* sequences

actA sequences, encompassing a total of 597 bp at the 3' end of *actA*, were obtained for all 40 *L. monocytogenes* isolates from China. A neighbour-joining tree constructed by using these 40 *actA* sequences, as well as corresponding partial *actA* sequences obtained by Cai *et al.* (2002), showed that these

sequences could be separated into two major lineages (Fig. 1), consistent with the major lineages described previously (Wiedmann *et al.*, 1997). Lineage II could be separated further into two clusters, IIA and IIB (Fig. 1).

Distribution of *actA* sequence types and *L. monocytogenes* lineages among US and Chinese isolates

Among the 40 *L. monocytogenes* isolates obtained from China, 14 grouped into lineage I and 26 grouped into lineage II. Grouping of *L. monocytogenes* into clusters IIA and IIB was also found among both Chinese and US *L. monocytogenes* isolates. No Chinese *L. monocytogenes* isolates grouped with the lineage III isolates included in the 15 isolates used by Cai *et al.* (2002). The two human clinical isolates collected in China (Lm1579 and Lm1191) grouped into lineages I and II, respectively. All Chinese isolates from ready-to-eat (RTE) food products, except Lm03040704 and Lm03061104, grouped into lineage II (Table 1). Isolates from fresh food products were found in both lineages, with 10 isolates belonging to lineage I and 12 isolates belonging to lineage II.

In total, 14 *actA* sequence types could be differentiated among the 40 *L. monocytogenes* isolates from China. Two lineage I *actA* sequence types (10 and 14; Table 1) were represented among both Chinese and US isolates.

Nucleotide and deduced amino acid sequence similarities among *actA* 3'-end sequences

Nucleotide and deduced ActA amino acid sequences for the 597 bp 3'-end sequences of *actA* were compared for the 40 Chinese *L. monocytogenes* isolates, as well as for the 15 isolates reported by Cai *et al.* (2002). Nucleotide sequence similarities within clusters IIA and IIB varied from 99.7 to 100 % and from 99.3 to 100 %, respectively. Nucleotide sequence similarities within lineage I and lineage II isolates ranged from 98.8 to 100 % and from 90.8 to 100 %, respectively. Nucleotide sequence similarity between different lineages varied from 89.4 to 96.3 % (89.4 to 90.3 % between lineages I and II; 90.5 to 96.3 % between lineages I and III; 87.1 to 90.3 % between lineages II and III).

Amino acid sequence similarities within lineages I, II and III ranged from 97.5 to 100, 98.0 to 100 and 91.5 to 98.5 %, respectively. Amino acid sequence similarities between lineages I and II, I and III, and II and III ranged from 84.3 to 85.9 %, from 83.8 to 85.9 % and from 84.8 to 85.4 %, respectively. Nineteen amino acid sites showed consistent differences among lineages I, II and III (Table 2).

Caco-2 cell invasion and intracellular growth phenotypes for selected lineage I and II isolates

The ability of 12 lineage I and 12 lineage II isolates to invade and multiply in intestinal epithelial cells was evaluated by using an *in vitro* model with human enterocyte-like Caco-2 cells (Table 3). The mean invasion efficiency for lineage I isolates (10.2 %) was significantly higher (*t*-test; *P* < 0.05)

Fig. 1. Unrooted neighbour-joining tree for partial *actA* sequences from Chinese *L. monocytogenes* isolates and from Cai *et al.* (2002). For isolate designations, refer to Table 1 and, for five-digit designations, to Cai *et al.* (2002), e.g. J1047 represents FSL J1-047 in the study by Cai *et al.* (2002).

Table 2. Amino acid residues that differ consistently among *L. monocytogenes* lineages

Lineage	Amino acid residue																		
	435	447	466	470	489	493	504	505	506	508	519	522	523	535	537	541	545	574	594
I	T	P	I	N	G	A	N	R	P	D	U	R	N	U	G	P	U	U	U
II	S	S	P	K	R	T	K	Q	S	N	A	S	D	M	E	S	A	G	F
III	S	P	I	N	G	A	N	Q	P	D	U	R	N	M	G	P	U	U	U

Table 3. Invasion efficiency and multiplication rate of selected lineage I and II isolates in Caco-2 cells

All data represent the mean of two independent experiments.

Strain	Lineage	No. bacteria deposited	No. intracellular bacteria at 3·5 h	Invasion efficiency (%)	No. intracellular bacteria at 21 h	Intracellular doubling time (h)
Lm03061104	I	$2·05 \times 10^7$	$1·86 \times 10^6$	9·1	$9·8 \times 10^7$	3·06
Lm03060104	I	$1·98 \times 10^7$	$1·83 \times 10^6$	9·2	$1·15 \times 10^8$	2·93
Lm03061103	I	$2·01 \times 10^7$	$1·58 \times 10^6$	7·9	$1·32 \times 10^8$	2·74
Lm1579	I	$2·25 \times 10^7$	$2·17 \times 10^6$	9·6	$2·08 \times 10^8$	2·66
Lm03062502	I	$2·18 \times 10^7$	$2·30 \times 10^6$	10·5	$1·96 \times 10^8$	2·73
Lm03041501	I	$1·85 \times 10^7$	$2·32 \times 10^6$	12·5	$1·82 \times 10^8$	2·78
Lm03061601	I	$1·97 \times 10^7$	$2·42 \times 10^6$	12·2	$1·92 \times 10^8$	2·77
Lm03040702	I	$2·12 \times 10^7$	$2·21 \times 10^6$	10·4	$1·80 \times 10^8$	2·76
Lm03061802	I	$2·40 \times 10^7$	$2·30 \times 10^6$	9·50	$9·25 \times 10^7$	3·28
Lm03062501	I	$2·28 \times 10^7$	$1·44 \times 10^6$	6·60	$1·40 \times 10^8$	2·65
Lm03040704	I	$2·19 \times 10^7$	$2·72 \times 10^6$	12·32	$1·66 \times 10^8$	2·95
Lm03042102	I	$2·32 \times 10^7$	$2·93 \times 10^6$	12·60	$2·36 \times 10^8$	2·76
Lm1191	II	$2·08 \times 10^7$	$1·20 \times 10^6$	5·8	$5·61 \times 10^7$	3·15
Lm03061101	II	$2·10 \times 10^7$	$8·31 \times 10^4$	0·39	$7·98 \times 10^5$	5·36
Lm03061808	II	$1·99 \times 10^7$	$6·81 \times 10^4$	0·34	$8·58 \times 10^5$	4·79
Lm03041602	II	$2·00 \times 10^7$	$5·32 \times 10^4$	0·27	$5·98 \times 10^5$	5·01
Lm03040701	II	$1·89 \times 10^7$	$7·30 \times 10^4$	0·38	$8·60 \times 10^5$	4·92
Lm03032102	II	$2·22 \times 10^7$	$7·80 \times 10^4$	0·35	$7·30 \times 10^5$	5·42
Lm03032001	II	$2·16 \times 10^7$	$7·70 \times 10^4$	0·36	$2·00 \times 10^5$	12·71
Lm03041603	II	$1·99 \times 10^7$	$6·20 \times 10^4$	0·31	$4·85 \times 10^5$	5·90
Lm03041102	II	$2·18 \times 10^7$	$9·00 \times 10^4$	0·41	$1·19 \times 10^6$	4·70
Lm03060603	II	$1·98 \times 10^7$	$4·80 \times 10^4$	0·24	$2·30 \times 10^5$	7·74
Lm03033101	II	$2·13 \times 10^7$	$1·10 \times 10^5$	0·51	$1·20 \times 10^6$	5·08
Lm03061801	II	$2·28 \times 10^7$	$8·60 \times 10^4$	0·37	$1·10 \times 10^6$	4·76

than that for lineage II isolates (0·8 %). The mean intracellular doubling time for lineage I isolates (3·2 h) was significantly shorter (*t*-test, $P < 0·05$) than that for lineage II isolates (5·8 h).

DISCUSSION

Human listeriosis is generally caused by consumption of RTE foods that are stored for extended periods of time at refrigeration temperatures and that permit the growth of the causative agent, *L. monocytogenes*. Food-consumption patterns in China are undergoing rapid changes and more regular consumption of refrigerated-storage RTE foods may increase the risk of human listeriosis in China. In order to understand the epidemiology and ecology of *L. monocytogenes*

in China in more detail, we characterized 38 *L. monocytogenes* isolates obtained from food and sewage samples collected in China, as well as two isolates from human listeriosis cases that occurred in China. All isolates were characterized into molecular subtypes by using DNA sequencing of a 597 bp fragment of the *L. monocytogenes* virulence gene *actA*. We showed that sequencing of this single, highly variable gene represents an economical alternative to more expensive subtyping methods (e.g. MLST or PFGE). Phylogenetic and subtype analyses showed that the predominant *L. monocytogenes* lineages reported previously among isolates obtained from foods and human clinical cases in Europe and the USA are also present in China. Changing food-consumption and -distribution patterns in China, with increased consumption of RTE foods with extended refri-

gerated storage times, will thus probably result in an increased number of human listeriosis cases in this country. We also showed that isolates belonging to lineage I expressed, on average, a significantly higher ability to invade and multiply within human intestinal epithelial cells than lineage II isolates, further supporting the suggestion that lineage I isolates may be characterized by enhanced virulence.

Partial *actA* sequencing represents an economical and discriminatory subtyping and lineage-classification method for *L. monocytogenes*

Whilst a variety of molecular subtyping methods for *L. monocytogenes* have been described and are used for scientific studies and surveillance purposes, many methods are expensive and time-consuming and, thus, do not lend themselves to routine application for subtyping of large numbers of isolates in many public-health systems. In addition, many of the commonly used DNA banding pattern-based subtyping methods (e.g. ribotyping, PFGE and RAPD) are difficult to standardize and do not provide information suitable for evolutionary analyses. Initial data reported by Cai *et al.* (2002) showed that DNA sequencing of a 600 bp fragment representing the 3' end of *actA* could differentiate 15 *L. monocytogenes* isolates, representing eight *EcoRI* ribotypes, into 13 *actA* sequence types. Thus, *actA* sequencing, as used here, provides higher subtype discrimination than *EcoRI* ribotyping, a commonly used molecular subtyping method for *L. monocytogenes* (Aarnisalo *et al.*, 2003; Mereghetti *et al.*, 2002; Nadon *et al.*, 2001; Wiedmann *et al.*, 1997). Discrimination of 40 *L. monocytogenes* isolates collected in China into 14 *actA* sequence types further supports the use of *actA* sequencing as a sensitive molecular subtyping method.

Our data also showed that DNA sequencing of the 3' portion of *actA* not only provides sensitive subtype discrimination, but also allows for evolutionarily meaningful lineage classification of *L. monocytogenes* isolates. Phylogenetic analyses of partial *actA* sequences for the 40 Chinese *L. monocytogenes* isolates and the corresponding *actA* sequences for the 15 isolates reported by Cai *et al.* (2002) showed a clear separation of *L. monocytogenes* into the previously reported two main *L. monocytogenes* lineages, I and II (Fig. 1) (Call *et al.*, 2003; Ericsson *et al.*, 2000; Wiedmann *et al.*, 1997). These lineages correlated with *L. monocytogenes* serotypes: lineage I comprised serotypes 1/2b, 3b, 3c and 4b and lineage II comprised serotypes 1/2a, 1/2c and 3a (Nadon *et al.*, 2001). In addition, we showed that isolates representing lineage III clearly formed two separate groups that were closer to lineage I than to lineage II isolates in an *actA* neighbour-joining tree (Fig. 1). Thus, *actA* sequencing allowed reliable isolate classification into the three *L. monocytogenes* lineages that have previously been defined by a variety of other subtyping methods, including PFGE (Brosch *et al.*, 1994; Chasseignaux *et al.*, 2001), ribotyping (Wiedmann *et al.*, 1997) and DNA sequence data for *sigB* (Moorhead *et al.*, 2003), *flaA*, *iap* and *hly* (Rasmussen *et al.*, 1995).

Three other *L. monocytogenes* virulence genes (*hly*, *plcA* and

inlA) have previously been characterized in two serovar 4b strains and two serovar 1/2b strains (Vines & Swaminathan, 1998), which belong to lineage I (Nadon *et al.*, 2001). The nucleotide and amino acid similarities among these lineage I strains and strains of serovars 1/2a and 1/2c, which belong to lineage II (Nadon *et al.*, 2001), ranged from 97.3 to 98.8 % and from 97.8 to 99.4 %, respectively. In the present study, the corresponding similarity values among lineage I and II isolates were 89.4 to 90.3 % and 84.3 to 85.9 %, respectively. This indicates that partial *actA* sequencing may be better suited to differentiation and classification of *L. monocytogenes* into lineages than sequencing of other virulence genes (*hly*, *plcA* and *inlA*). Whilst *actA* sequencing allowed the differentiation of two clusters within lineage II (IIA and IIB), similar subdivisions could not be defined based on *inlB* sequencing (Ericsson *et al.*, 2000), further supporting the suggestion that partial sequencing of the *actA* gene may provide a more sensitive subtyping and lineage classification than other virulence genes. A study by Moorhead *et al.* (2003) also showed that *L. monocytogenes* could be divided into three lineages based on the nucleotide sequence for *sigB*, which encodes a stress-responsive alternative sigma factor in *L. monocytogenes*. The deduced sigma B amino acid sequence, however, was highly conserved and showed only one polymorphic site (Phe216Tyr) (Moorhead *et al.*, 2003). By comparison, we have shown that the deduced protein sequence of the partial *actA* gene is highly polymorphic, with 19 consistent amino acid differences between lineages I and II. These data not only support the suitability of *actA* sequencing for sensitive *L. monocytogenes* subtype discrimination, but also show that ActA is a highly polymorphic virulence protein; it is thus tempting to speculate that these polymorphisms may contribute to the proposed virulence differences between strains of lineages I and II (Wiedmann *et al.*, 1997).

***L. monocytogenes* subtypes found in China and in Europe and North America represent the same lineages and similar clonal groups**

A PubMed search, conducted on 4 April 2004, revealed very limited information available in English-language journals on the epidemiology of listeriosis in China. In particular, no published reports on human listeriosis cases in China were found. This current study not only represents one of the first reports on the *L. monocytogenes* molecular subtypes present in the food system in China, but also reports the occurrence of two human listeriosis cases in China. Our data show that the two main *L. monocytogenes* lineages (I and II) that have previously been described among human, food and animal isolates collected in the USA and Europe (Ericsson *et al.*, 2000; Piffaretti *et al.*, 1989; Rasmussen *et al.*, 1995; Wiedmann *et al.*, 1997) also represent the predominant lineages present in China. Similarly to previous studies in other countries (Norton *et al.*, 2001), lineage II strains, which include serotypes 1/2a and 1/2c, were found more frequently among RTE foods than lineage I strains. Lineage I strains were predominant among isolates from meats and fresh

vegetables. None of the *L. monocytogenes* isolates from China grouped into lineage III, which is not surprising, as Wiedmann *et al.* (1997) showed that strains of lineage III are rare among isolates from humans and food samples and are predominantly associated with animals; no animal isolates from China were included in this study. Interestingly, two *actA* sequence types (10 and 14; Table 1) were found among both Chinese isolates and human isolates obtained in the USA (Cai *et al.*, 2002). In summary, our data suggest strongly that the *L. monocytogenes* strains present in China are very similar to those found in the USA and other countries. *L. monocytogenes* lineages and clonal groups thus appear to be distributed globally, even in countries that have previously not reported human and animal listeriosis cases.

Virulence potential of isolates representing the genotypically distinct lineages I and II

The discrepancy between the widespread distribution of *L. monocytogenes* in RTE food products (Inoue *et al.*, 2000; Wiedmann, 2002) and the low incidence of listeriosis (e.g. 2500 cases per year in the USA) (Mead *et al.*, 1999) led to the hypothesis that some *L. monocytogenes* strains in food products may have attenuated virulence and may thus have limited potential to cause human disease (Hof & Rocourt, 1992; Rasmussen *et al.*, 1995). Comparative genomics showed that virulent *L. monocytogenes* strains may possess genes that are not present in avirulent isolates, and these markers can be used for PCR assessment of *L. monocytogenes* virulence (Liu *et al.*, 2003). As the virulence of *L. monocytogenes* depends critically on the organism's ability to invade human intestinal epithelial cells, we evaluated the ability of selected Chinese lineage I and II *L. monocytogenes* isolates to invade and multiply within the human enterocyte cell line Caco-2. Our results showed that lineage I isolates had a significantly higher invasion efficiency and significantly faster doubling times than lineage II strains in this cell-culture assay. Interestingly, the one human clinical lineage II isolate showed a higher invasion efficiency (5.8%) and shorter intracellular doubling time (3.15 h) than the other lineage II isolates (Table 3), which were obtained from food. These findings are consistent with previous studies showing that certain genetic types of *L. monocytogenes* found commonly in food are less invasive than others to Caco-2 cells (Larsen *et al.*, 2002) and mouse L cells (Norton *et al.*, 2001). As isolates from RTE food products belonged predominantly to lineage II (Table 1), we hypothesize that RTE food products in China may predominantly harbour less invasive *L. monocytogenes* strains. Possible reasons why RTE food products may harbour more low-virulence strains than fresh food products need to be further investigated.

Conclusions

Based on and confirming a previous small study on 15 *L. monocytogenes* isolates (Cai *et al.*, 2002), we have shown that 3'-end partial nucleotide sequencing of *actA* provides an economical and sensitive approach for subtype and lineage classification of *L. monocytogenes*. By using this subtyping

approach, we have also shown that *L. monocytogenes* isolates from foods and environmental samples collected in China represent the same major *L. monocytogenes* genetic lineages that have previously been identified in North America and Europe, including human listeriosis cases. The frequency of these lineages among food isolates was also comparable with that reported in many other countries, with a predominance of lineage II strains among food isolates (Norton *et al.*, 2001). In combination with the first description of two human listeriosis cases in China, our data suggest that changes in food-distribution and -consumption patterns in China and other countries will lead to the emergence of human listeriosis as a food-safety issue, as virulent strains of this pathogen appear to be present in food systems worldwide. Furthermore, our results showed that *L. monocytogenes* occurring in RTE food products are predominantly lineage II strains and that lineage II strains, on average, may be less virulent than lineage I strains. This phenomenon may partly contribute to the low incidence of human listeriosis, despite the frequent human exposure to *L. monocytogenes* through contaminated food products.

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REFERENCES

- Aarnisalo, K., Autio, T., Sjöberg, A. M., Lunden, J., Korkeala, H. & Suihko, M. L. (2003). Typing of *Listeria monocytogenes* isolates originating from the food processing industry with automated ribotyping and pulsed-field gel electrophoresis. *J Food Prot* **66**, 249–255.
- Altamira, J., Prats, N., López, S., Domingo, M., Briones, V., Domínguez, L. & Marco, A. (1999). Repeated oral dosing with *Listeria monocytogenes* in mice as a model of central nervous system listeriosis in man. *J Comp Pathol* **121**, 117–125.
- Borucki, M. K., Kim, S. H., Call, D. R., Smole, S. C. & Pagotto, F. (2004). Selective discrimination of *Listeria monocytogenes* epidemic strains by a mixed-genome DNA microarray compared to discrimination by pulsed-field gel electrophoresis, ribotyping, and multilocus sequence typing. *J Clin Microbiol* **42**, 5270–5276.
- Brosch, R., Chen, J. & Luchansky, J. B. (1994). Pulsed-field fingerprinting of listeriae: identification of genomic divisions for *Listeria monocytogenes* and their correlation with serovar. *Appl Environ Microbiol* **60**, 2584–2592.
- Cai, S., Kabuki, D. Y., Kuaye, A. Y., Cargioli, T. G., Chung, M. S., Nielsen, R. & Wiedmann, M. (2002). Rational design of DNA sequence-based strategies for subtyping *Listeria monocytogenes*. *J Clin Microbiol* **40**, 3319–3325.
- Call, D. R., Borucki, M. K. & Besser, T. E. (2003). Mixed-genome microarrays reveal multiple serotype and lineage-specific differences among strains of *Listeria monocytogenes*. *J Clin Microbiol* **41**, 632–639.
- Chan, M.-S., Maiden, M. C. J. & Spratt, B. G. (2001). Database-driven multi locus sequence typing (MLST) of bacterial pathogens. *Bioinformatics* **17**, 1077–1083.
- Chasseignaux, E., Toquin, M.-T., Ragimbeau, C., Salvat, G., Colin, P. &

- Ermel, G. (2001). Molecular epidemiology of *Listeria monocytogenes* isolates collected from the environment, raw meat and raw products in two poultry- and pork-processing plants. *J Appl Microbiol* **91**, 888–899.
- Destro, M. T., Leitão, M. F. F. & Farber, J. M. (1996). Use of molecular typing methods to trace the dissemination of *Listeria monocytogenes* in a shrimp processing plant. *Appl Environ Microbiol* **62**, 705–711.
- Enright, M. C., Spratt, B. G., Kalia, A., Cross, J. H. & Bessen, D. E. (2001). Multilocus sequence typing of *Streptococcus pyogenes* and the relationships between *emm* type and clone. *Infect Immun* **69**, 2416–2427.
- Ericsson, H., Unnerstad, H., Mattsson, J. G., Danielsson-Tham, M.-L. & Tham, W. (2000). Molecular grouping of *Listeria monocytogenes* based on the sequence of the *inlB* gene. *J Med Microbiol* **49**, 73–80.
- Hof, H. & Rocourt, J. (1992). Is any strain of *Listeria monocytogenes* detected in food a health risk? *Int J Food Microbiol* **16**, 173–182.
- Inoue, S., Nakama, A., Arai, Y. & 7 other authors (2000). Prevalence and contamination levels of *Listeria monocytogenes* in retail foods in Japan. *Int J Food Microbiol* **59**, 73–77.
- Keto-Timonen, R. O., Autio, T. J. & Korkeala, H. J. (2003). An improved amplified fragment length polymorphism (AFLP) protocol for discrimination of *Listeria* isolates. *Syst Appl Microbiol* **26**, 236–244.
- Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: molecular evolutionary genetic analysis software. *Bioinformatics* **17**, 1244–1245.
- Larsen, C. N., Nørnung, B., Sommer, H. M. & Jakobsen, M. (2002). In vitro and in vivo invasiveness of different pulsed-field gel electrophoresis types of *Listeria monocytogenes*. *Appl Environ Microbiol* **68**, 5698–5703.
- Liu, D., Ainsworth, A. J., Austin, F. W. & Lawrence, M. L. (2003). Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes. *J Med Microbiol* **52**, 1065–1070.
- Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. & Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerg Infect Dis* **5**, 607–625.
- Mereghetti, L., Lanotte, P., Savoye-Marczuk, V., Marquet-Van Der Mee, N., Audurier, A. & Quentin, R. (2002). Combined ribotyping and random multiprimer DNA analysis to probe the population structure of *Listeria monocytogenes*. *Appl Environ Microbiol* **68**, 2849–2857.
- Moorhead, S. M., Dykes, G. A. & Cursons, R. T. (2003). An SNP-based PCR assay to differentiate between *Listeria monocytogenes* lineages derived from phylogenetic analysis of the *sigB* gene. *J Microbiol Methods* **55**, 425–432.
- Nadon, C. A., Woodward, D. L., Young, C., Rodgers, F. G. & Wiedmann, M. (2001). Correlations between molecular subtyping and serotyping of *Listeria monocytogenes*. *J Clin Microbiol* **39**, 2704–2707.
- Norton, D. M., Scarlett, J. M., Horton, K., Sue, D., Thimothe, J., Boor, K. J. & Wiedmann, M. (2001). Characterization and pathogenic potential of *Listeria monocytogenes* isolates from the smoked fish industry. *Appl Environ Microbiol* **67**, 646–653.
- Piffaretti, J.-C., Kressebuch, H., Aeschbacher, M., Bille, J., Bannerman, E., Musser, J. M., Selander, R. K. & Rocourt, J. (1989). Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. *Proc Natl Acad Sci U S A* **86**, 3818–3822.
- Rasmussen, O. F., Skouboe, P., Dons, L., Rossen, L. & Olsen, J. E. (1995). *Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes. *Microbiology* **141**, 2053–2061.
- Sauders, B. D., Fortes, E. D., Morse, D. L., Dumas, N., Kiehlbauch, J. A., Schukken, Y., Hibbs, J. R. & Wiedmann, M. (2003). Molecular subtyping to detect human listeriosis clusters. *Emerg Infect Dis* **9**, 672–680.
- Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233–2239.
- Van Langendonck, N., Bottreau, E., Bailly, S., Tabouret, M., Marly, J., Pardon, P. & Velge, P. (1998). Tissue culture assays using Caco-2 cell line differentiate virulent from non-virulent *Listeria monocytogenes* strains. *J Appl Microbiol* **85**, 337–346.
- Vázquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J. & Kreft, J. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clin Microbiol Rev* **14**, 584–640.
- Vines, A. & Swaminathan, B. (1998). Identification and characterization of nucleotide sequence differences in three virulence-associated genes of *Listeria monocytogenes* strains representing clinically important serotypes. *Curr Microbiol* **36**, 309–318.
- Wiedmann, M. (2002). Molecular subtyping methods for *Listeria monocytogenes*. *J AOAC Int* **85**, 524–531.
- Wiedmann, M., Bruce, J. L., Keating, C., Johnson, A. E., McDonough, P. L. & Batt, C. A. (1997). Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infect Immun* **65**, 2707–2716.
- Yde, M. & Genicot, A. (2004). Use of PFGE to characterize clonal relationships among Belgian clinical isolates of *Listeria monocytogenes*. *J Med Microbiol* **53**, 399–402.
- Zhou, X. & Jiao, X. (2004). Investigation of *Listeria monocytogenes* contamination pattern in local Chinese food market and the tracing of two clinical isolates by RAPD analysis. *Food Microbiol* **21**, 695–702.