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Abstract

, the causative organism of listeriosis, is primarily transmitted to humans through contaminated food. In this study, we examined 1275 batches of aquatic products imported from 29 countries and found that 36 batches from 8 countries were contaminated by (2.8%), with . accounting for 2.6% (33/1275) and . for 0.2% (3/1275). Of the 23 selected . isolates (from the 33 identified), 15 (65.2%) were of serovar 4b complex (4b, 4d, or 4e), three (13.0%) of 1/2a or 3a, four (17.4%) of 1/2b or 3b, and one (4.4%) of 1/2c or 3c. Notably, four of the 23 isolates belonged to epidemic clone I (ECI) and another four were associated with epidemic clone II (ECII), two highly clonal 4b clusters responsible for most of the documented listeriosis outbreaks. In the multilocus sequence typing scheme based on the concatenated genes - - - - - - - - - , serovar 4b complex isolates from imported aquatic products exhibited significant genetic diversity. While the four ECI isolates were genetically related to those from Chinese diseased animals, both lacking one proline-rich repeat of ActA, the four ECII isolates were located between 1/2b or 3b strains. As the . isolates from imported aquatic products possessed a nearly complete set of major infection-related genes, they demonstrated virulence potential in mouse model.

Introduction

LISTERIA MONOCYTOGENES A F DB E bacterial pathogen that has the capability to adhere to and enter host cells, escape from vacuoles, multiply in cytoplasm, and spread to neighboring cells. Given its tolerance to arduous external conditions including wide pH, temperature, and osmolarity ranges, . is ubiquitously distributed in the environment, leading to its frequent occurrence on various food products and causing listeriosis. Although the initial clinical manifestations of listeriosis are mild and nonspecific (e.g., flu-like symptoms and gastroenteritis), the consequences can be extremely severe (e.g., meningitis, encephalitis, septicemia, and occasional death) in the absence of prompt therapeutical intervention (Vazquez-Boland ., 2001).

Based on phylogenetic analysis, . is separated into three lineages: lineage I covering serovars 1/2b, 3b, 4b, 4d, 4e, 4ab, and 7; lineage II including serovars 1/2a, 3a, 1/2c, and 3c; and lineage III containing serovars 4a and 4c (Wiedmann ., 1997; Doumith ., 2004a, b; Liu ., 2006). Remarkably, over 98% of the documented listeriosis outbreaks involved four serovars (4b, 1/2a, 1/2b, and 1/2c) (Swaminathan and Gerner-Smidt, 2007; Goulet ., 2008). In particular, serovar 4b strains are responsible for the majority of outbreaks and sporadic cases of listeriosis, and tend to cause a higher mortality (26%) than serovar 1/2 (16%), indicating that strains of serovar 4b may be more virulent than other serovars in humans (Gerner-Smidt ., 2005; Swaminathan and Gerner-Smidt, 2007). Further, serovar 4b strains are more often isolated from patients with meningoencephalitis than from patients with blood stream infection (Gerner-Smidt ., 2005).

Subsequent investigations uncovered the role of four major epidemic clones (EC) of . in listeriosis outbreaks (Chen and Knabel, 2007; Chen ., 2007). ECI, a serovar 4b cluster, is implicated in several major outbreaks in Canada (coleslaw, 1981), Switzerland (soft cheese, 1983–1987), United States (Mexican-style cheese, 1985), and France (pork tongue, 1992). ECII, a new genotype of serovar 4b, was

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TAB E 1. S B GA D V E CE T A F *LISTERIA* I A E

/						In vivo														-
				[a]		actA ( )[b]	ascB-dapE	(.) (n =)		(%)	gyrB	dapE	hisJ	sigB	ribC	purM	betL	gap	tuf	
NB9	Argentina	Squid	I	1/2b or 3b	–	537	3.7×10^7	5	100		1	1	1	1	1	1	1	1	1	1
NB20	Mexico	Squid	I	1/2b or 3b	–	537	5.3×10^7	5	100		2	2	1	1	2	1	2	1	1	2
NB26	India	Shrimp	I	1/2b or 3b	–	432	6.5×10^7	5	100		3	3	2	1	1	2	3	1	1	3
NB27	Uruguay	Squid	I	1/2b or 3b	–	537	5.5×10^7	4	80		2	2	1	1	2	1	4	2	1	4
M1	China	Milk	I	1/2b	–	537	3.0×10^7	5	100		2	4	3	1	2	3	5	3	1	5
S10	China	Squid	I	1/2b	–	432	ND	ND	ND		2	5	1	1	3	3	6	4	1	6
NB1	Argentina	Squid	I	4b complex	non	537	5.5×10^7	5	100		2	6	1	2	2	4	7	3	1	7
NB4	United States	Salmon	I	4b complex	I	432	2.1×10^7	5	100		2	7	4	2	2	1	7	1	1	8
NB6	Chile	Squid	I	4b complex	II	537	3.3×10^7	5	100		2	5	5	3	4	4	8	1	1	9
NB7	Chile	Squid	I	4b complex	II	537	4.6×10^7	5	100		2	5	5	3	4	4	9	1	1	10
NB8	Chile	Squid	I	4b complex	II	537	5.1×10^7	5	100		2	5	5	3	4	4	10	1	1	11
NB10	Chile	Squid	I	4b complex	II	537	2.0×10^7	5	100		2	5	6	3	4	4	11	1	1	12
NB11	United States	Sardine	I	4b complex	non	432	4.7×10^7	5	100		2	2	7	3	5	5	2	3	2	13
NB13	Chile	Squid	I	4b complex	I	432	2.5×10^7	5	100		2	8	4	2	2	1	7	1	1	14
NB14	Argentina	Squid	I	4b complex	non	537	4.7×10^7	5	100		2	6	1	2	2	4	7	3	1	7
NB15	Argentina	Squid	I	4b complex	non	537	4.2×10^7	5	100		2	6	1	2	2	4	7	3	1	7
NB16	Argentina	Squid	I	4b complex	non	537	4.6×10^7	5	100		2	6	1	2	2	4	7	3	1	7
NB22	Uruguay	Squid	I	4b complex	I	432	5.7×10^7	5	100		2	8	4	2	2	1	7	1	1	15
NB23	Peru	Squid	I	4b complex	I	432	1.5×10^7	5	100		2	8	4	2	2	1	7	1	1	15
NB25	India	Shrimp	I	4b complex	non	432	4.8×10^7	5	100		2	8	8	2	2	6	7	1	1	16
NB29	India	Shrimp	I	4b complex	non	432	5.7×10^7	5	100		2	8	9	2	2	6	7	1	1	17
S2	China	Red drum	I	4b	non	537	2.5×10^7	5	100		2	6	1	2	2	4	7	3	1	7
S4	China	Red drum	I	4b	non	537	4.0×10^7	5	100		2	6	1	2	2	4	7	3	1	7
S6	China	Shrimp	I	4b	non	537	2.5×10^7	5	100		2	6	1	2	2	4	7	3	1	7
M5	China	Milk	I	4b	non	537	1.3×10^7	5	100		2	6	1	2	2	4	7	3	1	7
V2	China	Vegetable	I	4b	non	537	4.5×10^7	5	100		2	6	1	2	2	4	7	3	1	7

XJ90	China	Sheep	I	4b	non	432		3.3×10^{7}	5	100	2	8	4	2	2	6	7	1	1	18
90SB1	China	Sheep	I	4b	non	432		5.5×10^{7}	5	100	2	8	4	2	2	6	7	1	1	18
125SL1	China	Sheep	I	4b	non	432		3.7×10^{7}	5	100	2	8	4	2	2	6	7	1	1	18
ScottA	–	Reference	I	4b	non	537		3.3×10^{7}	5	100	2	6	1	2	2	4	7	3	1	7
NB12	Peru	Squid	II	1/2a or 3a	non	537		5.1×10^{7}	5	100	4	9	10	4	6	7	12	5	3	19
NB21	Vietnam	Acaleph	II	1/2a or 3a	non	537		3.9×10^{7}	4	80	4	9	11	4	7	8	13	6	3	20
NB30	Uruguay	Squid	II	1/2a or 3a	non	537		6.0×10^{7}	5	100	4	9	12	4	8	9	13	7	3	21
P3	China	Pork	II	1/2a	non	432		4.0×10^{7}	5	100	4	4	13	5	9	10	14	6	3	22
S11	China	Squid	II	1/2a	non	537		ND	ND	ND	4	4	14	4	9	9	12	8	3	23
10403S	–	Reference	II	1/2a	non	537		5.0×10^{7}	5	100	4	9	10	4	6	7	12	9	3	24
EGD	–	Reference	II	1/2a	non	537		5.5×10^{7}	5	100	5	10	14	4	10	11	12	10	3	25
NB28	Peru	Squid	II	1/2c or 3c	–	537		4.1×10^{7}	5	100	5	4	14	4	10	11	12	11	4	26
V1	China	Vegetable	II	1/2c	–	537		3.0×10^{7}	5	100	5	4	14	4	10	11	12	10	4	27
P19	China	Pork	II	1/2c	–	537		5.0×10^{7}	5	100	5	11	14	4	10	11	12	10	4	28
J1-168	–	Reference	III	4a	–	537		1.2×10^{7}	4	80	6	12	15	6	11	12	15	12	1	29
J1-158	–	Reference	III	4b	–	432	–	2.2×10^{7}												

TAB E 2. PCR P E U ED T S D

				( )	( )	
A		Actin-assembly inducing protein precursor	GGTACGTGATAAAATCGACGA	TAGTTATGTCACTTATCAGAGC	537 or 432[a]	55	Wiedmann ., 1997
A	1	Actin-assembly inducing protein precursor	AATAACAACAGTGAACAAAGC	TATCACGTACCCATTTACC	373	56	Ward ., 2004
	2	Phosphatidylcholine-phospholipase C	TTGTGATGAATACTTACAAAC	TTTGCTACCATGTCTTCC	564	56	Ward ., 2004
A	3- 3	A virulence gene cluster region	CGGCGAACCATACAACAT	TGTGGTAATTTGCTGTCG	277	56	Ward ., 2004
	0	Putative transcriptional regulator	AGGGCTTCAAGGACTTACCC	ACGATTTCTGCTTGCCATTC	691	53	Doumith ., 2004
		Hypothetic protein	AGGGGTCTTAAATCCTGGAA	CGGCTTGTTCGGCATACTTA	906	53	Doumith ., 2004
	0	Hypothetic protein	AGTGGACAATTGATTGGTGAA	CATCCATCCCTTACTTTGGAC	597	53	Doumith ., 2004
		Putative transcriptional regulator	AGCAAAATGCCAAAACTCGT	CATCACTAAAGCCTCCCATTG	471	53	Doumith ., 2004
		Putative transcriptional regulator	ACCCGATAGCAAGGAGGAAC	AACTTCTCTCGATACCCATCCA	367	53	Liu ., 2003

lmo1134TD(regulator)Tj12.076G-275T665.8(ACGT)-27CTAACCA2TmCT , 2004TD(regulator)Tj12.076GAAin , 2004

		Bile salt hydrolase	CCCACGACTATAAGCATCCA	GCAGGACTCAATTTCTCAGGA	399	58	This study
		Class I heat-shock protein	ATTGGTCTGCATTGTTGCGA	TGGCGTAATCACTGGTGATGT	488	57	This study
		Chaperone protein	AATCGCCTGCTCCTTCTACGA	CTATCGCTCAAGTTGCTGCTA	585	57	This study
	2	Glutamate decarboxylase	CGGAGAAATGCCAACCATGCA	GTTCTTGAATAGAGGCTTGGA	412	58	This study
		Catabolic ornithine carbamoyltransferase	ATGAGTGATTACAACCATCCA	GCTTCATCAATAACAACTGAG	512	60	This study
		Arginine:ornithine antiporter	GTTGTTATCCAGTGAGCGATA	AATTTGCTACTAATGTATACA	459	60	This study
	00	Agmatine deiminase	TTGAAAAACACGCTGGTTGCT	CCAACCACTTCACAGTTTGGA	967	62	Chen ., 2009a
	A	Carbamate kinase	GCAATCTTTCTTGAGGATGCT	TGGGCAACATACTTCCTGCTG	379	60	This study
	A	Arginine deiminase	GGTCATTTATGCATTTGGACA	GATCGTTTGATACTTGATTTCGA	321	62	This study
		Internalin A	TAATATAAGTGATATAAGCCCAG	TTTATCCGTACTGAAATTCC	606	60	Chen ., 2009c
		Internalin B	CACTTTCTTTGGAGCATAATGGT	CATCATCACTTATTATTTCTGGA	394	60	Chen ., 2009c
		Internalin C	CCATCTGGGTCTTTGACAGTA	CAAATAAGTGACCTTAGTCCTT	398	55	Chen ., 2009c
		Internalin D	CTGTAGTAATGGCAATTAGCTT	TGTTATTAGGGACCACAAGCT	870	52	Chen ., 2009c
		Internalin E	AGCTCAAAAGAAGTACAAGCA	GTGCAATAAGCTCACCAGAAA	787	55	Chen ., 2009c
		Internalin F	TGACTTATTTGCAGTTGGGGT	TTGGTTCAGGAATAAGCGCG	1119	55	Chen ., 2009c
		Internalin G	GTGAAGACGGAACTTGGAAA	GCTTCTACTATCGGTTGAACA	668	52	Chen ., 2009c
	/ 2	Internalin H/ Internalin C2	ATAGCTACTTTATCAGCATTT	ATATCACTTATTTTATTATCATC	437	52	Chen ., 2009c
		Internalin I	GTTTCCAGACGACAATCTTGCTA	AATCGGTACAGTTACTCGCATCA	635	58	Chen ., 2009c
		Internalin J	AGATGTGACACCACAAACTCAA	TGTATTATGCGTGACATCAAGCT	401	58	Chen ., 2009c
	- -1	Internalin cluster between and	TGATGATTCAAGTATGATTCCTA	ATCAGTAAGCACTGGATCAGTA	Variable[b]	55	Chen ., 2009c
	- -2	Internalin cluster between and	TGATGATTCAAGTATGATTCCTA	CGTTTGCTAAATTCTCATCTGTA	Variable[b]	55	Chen ., 2009c
	A	Phosphatidylinositol-phospholipase C	ATTAACCAAACCACTGGCTCA	TTGATAAGCAGTCTGGACAAT	502	55	This study
		Listeriolysin O	GTTGCAAGCGCTTGGAGTGAA	ACGTATCCTCCAGAGTGATGG	420	58	This study
		Metalloenzyme	CAAGGACAGCTTAGGATTAC	TTCTTATTCGCCCATCTCGC	886	55	This study
		Phosphatidylcholine-phospholipase C	ATTAACCAAACCACTGGCTCA	TTGATAAGCAGTCTGGACAAT	502	55	This study
		Hexose phosphate transport	GATTTGTGCAATCACCAGGT	GAACCTAGCAATGCTCCAAT	529	58	This study
	A	Transcriptional regulator	CCATACACATAGGTCAGGATT	TTCGTTATAATGTCTGGCTTT	266	60	This study

[a]Some . strains/isolates harbor a 105-bp deletion in *A* gene, leading to removal of 35 amino acids in the ActA protein.
[b]Primer pairs targeting internalin cluster between and yield variable product sizes from different strains (Chen ., 2009c).

responsible for two multistate outbreaks in the United States, associated with contaminated hotdogs and turkey deli meat in 1998–1999 and 2002. ECIII, of serovar 1/2a, was associated with the outbreak caused by turkey deli meat in the country in 2000. ECIV, another serovar 4b cluster, caused outbreaks linked to the consumption of vegetable (United States, 1983) and pate (UK, 1987–1989) (Herd and Kocks, 2001; Kathariou, 2002; Swaminathan and Gerner-Smidt, 2007).

Whereas serovar 1/2a is most frequently isolated from foods, serovar 4b dominated listerial isolations from noncatfish seafood in a recent study (Chou and Wang, 2006). Considering the importance of various foods in . epidemiology, and the increasing internationalization of food production (Rocourt ., 2000; Liu, 2008), we investigated the incidence of contamination in imported aquatic products in China. We then determined the molecular and phylogenetic characteristics as well as virulence potential of the selected . strains from these products.

Materials and Methods

Bac e a a

A total of 1275 batches of raw aquatic products imported from 29 countries in Asia, Australia, Europe, North America, and South America during July 2007 to November 2008 were screened for as described previously (Chen ., 2009a). This led to the identification of 36 isolates, of which 23 . and three . were further analyzed in this study (Table 1). Ten . , including one acaleph isolate from Vietnam, six squid isolates from Argentina/Uruguay, and three squid isolates from Peru, were uncharacterized because they were from the same countries and continuous batches as others. Also analyzed were 21 . strains, 12 of which were from Chinese food systems (Chen ., 2009c), three from diseased animals in China, and six from reference collection. strains were retrieved from glycerol stocks maintained at −80°C and cultured in brain heart infusion broth (Oxoid, Hampshire, England) at 37°C.

DNA a a

Genomic DNA was extracted from these strains using a protocol reported previously (Jiang ., 2008). Oligonucleotide primers were synthesized by Invitrogen Biotechnology (Shanghai, China) (Table 2), and DNA polymerase (TaKaRa Biotech Co. Ltd., Dalian, China) was used for polymerase chain reaction (PCR) amplification. PCR was conducted using a PT200 thermal cycler (MJ Research Inc., Boston, MA), with annealing temperatures depending on specific primer pairs (Table 2) and the duration of extension depending on the expected length of amplicon (1 min/kb, at 72°C). For DNA sequencing analysis, PCR fragments were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Inc., Union City, CA) and their sequences determined by dideoxy method on ABI-PRISM 377 DNA sequencer.

L ea e ca ca

A 537 bp (432 bp in some cases) fragment (corresponding to the nucleotide sequence between 775 and 1313, which covers four proline-rich repeats or PRRs) was amplified from the . strains by PCR using gene primers, and its nucleotide sequence was analyzed (Table 2) (Wiedmann ., 1997). In addition, allele-specific oligonucleotide PCR based on virulence gene cluster sequence was employed to confirm the accuracy of lineage classification (Ward ., 2004).

Se e de ca

The serogrouping of . isolates was determined by a multiplex PCR targeting ORF2819, ORF2110, 0 , and (Doumith ., 2004a). Specifically, ORF2819 primers recognize serovars 1/2b, 3b, 4b, 4d, and 4e; ORF2110 primers further separate serovar 4b complex (4b, 4d, 4e) from serovars 1/2b and 3b; 0 primers identify serovars 1/2a, 3a, 1/2c, and 3c strains; and further distinguish serovars 1/2c and 3c from 1/2a and 3a (Doumith ., 2004a). The primers with specificity for all . - strains except serovars 4a and 4c were also used (Liu ., 2003). Due to the inability to distinguish by molecular techniques, serovars 4b, 4d, and 4e within lineage I are grouped together as "serovar 4b complex" (Doumith ., 2004a; Eifert ., 2005).

Rec a EC

Three separate PCR assays were conducted using primers targeting F2365_2798, H7858_0487. 8 to , and F6854_2463.4, specific for ECI, ECII, and ECIII, respectively (Chen and Knabel, 2007).

De ec ec - e a ed e e

PCR-based techniques were used to determine the presence or absence of 28 infection-related genes in selected . - strains classified under the following five categories: (i) stress response genes conferring tolerance to harsh conditions within the host (e.g., , , , , , and - - 00 - - bsdxus), (ii)332(distse4166301497223370BBmy-togen

numbers of strains analyzed (N), using the following equation (higher D.I. values indicate better discriminatory power) (Hunter and Gaston, 1988):

$$\text{D.I.} = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} nj(nj-1)$$

In addition, the chi-square test was used to test for significant associations between isolation frequency and origins.

V e ce e

The virulence potential of . isolates was assessed by using a previously reported protocol (Liu, 2004; Liu ., 2006). Briefly, five female ICR mice weighing 20–22 g (Zhejiang College of Traditional Chinese Medicine, Hangzhou, China) were inoculated intraperitoneally with 0.1 mL aliquot of a 1/200 dilution of . brain heart infusion broth cultures (adjusted to OD540 nm = 1.35) of each strain, washed, and resuspended in phosphate buffered saline. Mice in the control group were injected with 0.1 mL phosphate buffered saline. Daily observation was conducted and mortalities recorded until all of the mice inoculated with the virulent EGD strain died. Relative virulence (%) was calculated by dividing the number of dead mice with the total number of mice tested. On the 15th day after inoculation, all surviving mice were euthanized. EGD was used as a control because it is a well-characterized virulent strain whose whole genome has been published (Glaser ., 2001).

N c e de e e ce

Sequences generated in this study have been deposited in GenBank within the accession numbers FJ774122 to FJ774144 ( ), FJ774178 to FJ774200 ( ), FJ774274 to FJ774282, FJ774294, FJ774298, FJ774301 to FJ774312 ( ), FJ774345 to FJ774367 ( ), FJ774401 to FJ774423 ( ), FJ774458 to FJ774480 ( ), FJ774514 to FJ774536 ( ), FJ774569 to FJ774591 ( ), and FJ774626 to FJ774648 ( ).

Results and Discussion

Rec e ~Li ı â~ ed a a a c d c

Of the 1275 batches of raw aquatic products imported from 29 countries of 5 continents, 36 batches (2.8%) from 8 countries were found to harbor (including 33 [2.6%] . - and 3 [0.2%] .). Specifically, was recovered in aquatic products from Chile (31.3%), Argentina/Uruguay (13.1%), United States (11.1%), Vietnam (5.9%), India (2.5%), Peru (1.8%), and Mexico (1.0%). Overall, the contamination in aquatic products from South America was the highest (5.7%), followed by North America (2.4%) and Asia (1.0%). No was found in aquatic products from the other 21 countries under investigation (Table 3).

The recovery rate of in Chinese aquatic products between 2000 and 2007 was 2.7% (Chen ., 2009d), similar to that in imported aquatic products (2.8%) in this study. Among isolates from Chinese aquatic products, . accounted for the majority (66.7%) and . - only for 11.1% (Chen ., 2009d). By contrast, in the

TAB E 3. P E A E CE F *LISTERIA* I ED RA A A C P D C

/ [a]			Listeria *(%)*	L. monocytogenes *(%)*	L. innocua *(%)*
Asia	Burma	24	0	0	0
	India	163	4 (2.5)	3 (1.9)	1(0.6)
	Indonesia	163	0	0	0
	Japan	32	0	0	0
	Pakistan	29	0	0	0
	Republic of Korea	43	0	0	0
	Thailand	27	0	0	0
	Vietnam	34	2 (5.9)	2 (5.9)	0
	Others	63	0	0	0
	Subtotal	**578**	**6 (1.0)**	**5 (0.9)**	**1 (0.2)**
Australia	**Subtotal**	**13**	**0**	**0**	**0**
Europe	**Subtotal**	**25**	**0**	**0**	**0**
North America	Mexico	97	1 (1.0)	1 (1.0)	0
	United States	18	2 (11.1)	2 (11.1)	0
	Others	9	0	0	0
	Subtotal	**124**	**3 (2.4)**	**3 (2.4)**	**0**
South America	Argentina/Uruguay	122	16 (13.1)	14 (11.5)	2 (1.6)
	Chile	16	5 (31.3)	5 (31.3)	0
	Peru	338	6 (1.8)	6 (1.8)	0
	Subtotal	**476**	**27 (5.7)**	**25 (5.3)**	**2 (0.4)**
Others	Pacific Inlands	59	0	0	0
	Subtotal	**59**	**0**	**0**	**0**
Total		**1275**	**36 (2.8)**	**33 (2.6)**	**3 (0.2)**

Bold numbers indicate the subtotals of each category.
[a]Countries with less than 10 batches of aquatic products were included in the category "others."

TABLE 4. LINEAGE, SEROVAR, AND ECOTYPE OF *L. MONOCYTOGENES* ISOLATED FROM AQUATIC PRODUCTS

	. (%)		. (%)		. (%)
I	19/23 (82.6)	1/2b or 3b	4/23 (17.4)	–	–
		4b complex	15/23 (65.2)	I	4/23 (17.4)
				II	4/23 (17.4)
II	4/23 (17.4)	1/2a or 3a	3/23 (13.0)	III	0/23 (0)
		1/2c or 3c	1/23 (4.4)	–	–
III	0/23 (0)	4a or 4c	0/23 (0)	–	–

present study, the proportions of . and . - in imported aquatic products were 8.3% and 91.7%, respectively. This indicates that the contamination rate of . in imported aquatic products (2.6%) is significantly higher than in Chinese aquatic products (0.3%; < 0.05).

D a ce ~ e a 4b c e

Based upon *A* sequences and allele-specific oligonucleotide PCR patterns, 19 (82.6%) of the 23 selected . - isolates were classified as belonging to lineage I and four (17.4%) as lineage II. Using serotyping multiplex PCR, four (17.4%) . isolates were recognized as serovar 1/2b or 3b; 15 (65.2%) as serovar 4b complex; three (13.0%) as 1/2a or 3a; and one (4.4%) as 1/2c or 3c. Of the 15 serovar 4b complex strains, EC-specific PCR identified four isolates as ECI and four as ECII (4/23, 17.4%) (Table 4). The four ECI isolates included salmon isolate NB4 from the United States and squid isolates NB13 from Chile, NB22 from Uruguay, and NB23 from Peru. The four ECII isolates comprised squid isolates NB6, NB7, NB8, and NB10, all from Chile (Table 1).

The predominance of serovar 4b in American noncatfish seafood was also noted by Chou and Wang (2006). However, the serovar composition of . isolates from Chinese aquatic products was different, with serovar 1/2a or 3a being predominant (8/20, 40%), followed by 1/2b or 3b (6/20, 30%), 1/2c or 3c (2/20, 10%), 4b complex (3/20, 15%), and 4c (1/20, 5%), and no ECs present (Chen ., 2009c).

MLST a a

In the MLST scheme, the 9 genes sequenced in 47 isolates harbored a total of 928 polymorphic sites (15.88% on average; ranging from 2.06% to 27.90% per gene). The average nucleotide diversity (p) was 3.564%, ranging from 0.413% to 6.878% per gene. Combination of 34 alleles at 9 genes (ranging from 8 to 20 per gene) indicated that the overall D.I. of this MLST scheme was 0.95 (ranging from 0.57 to 0.90 per gene) (Table 5). The cladogram revealed two main . lineages (I and II) covering all the . isolates

TABLE 5. P F N E GE E U ED F M C SE E CE T G SC E E

	.		.	. (%)					
		( )					/	p	. .
	47	657	11	64 (9.74)	0.10349	0.00024	0.00232	0.02059	0.63
	47	669	17	124 (18.54)	0.10058	0.00917	0.09117	0.02690	0.90
	47	714	20	168 (23.53)	0.23946	0.02192	0.09154	0.06034	0.88
	47	642	11	81 (12.62)	0.14273	0.00268	0.01878	0.02952	0.78
	47	633	16	151 (23.85)	0.35267	0.01057	0.02997	0.06878	0.77
	47	693	17	144 (20.78)	0.23030	0.00998	0.04334	0.05139	0.88
	47	534	20	149 (27.90)	0.33195	0.01063	0.03202	0.06436	0.82
	47	621	14	19 (3.06)	0.01889	0.00103	0.05453	0.00526	0.82
	47	681	8	14 (2.06)	0.01405	0.00116	0.08256	0.00413	0.57
Concatenated	47	5844	34	928 (15.88)	0.14693	0.00753	0.05125	0.03564	0.95
Concatenated, .	44	5844	32	700 (11.98)	0.10823	0.00532	0.04916	0.02684	0.95
Concatenated, lineage I	30	5844	18	99 (1.69)	0.01298	0.00131	0.10092	0.00397	0.89
Concatenated, serovar 4b complex	24	5844	12	60 (1.03)	0.00940	0.00118	0.12553	0.00306	0.82
Concatenated, serovar 4b complex from imported aquatic products	15	5844	11	60 (1.03)	0.01298	0.00140	0.10786	0.00404	0.93
Concatenated, serovar 4b complex from Chinese food system	5	5844	1	0 (0)	0.00174	0.00110	0.63218	0	0
Concatenated, lineage II	10	5844	10	210 (3.59)	0.04287	0.00200	0.04665	0.01108	1.00
Concatenated, lineage III	4	5844	4	388 (6.64)	0.14213	0.00790	0.05558	0.03631	1.00
Concatenated, .	3	5844	2	46 (0.79)	0.01702	0.00179	0.10517	0.00528	0.67

D.I., discrimination index; p, average nucleotide diversity.

from imported aquatic products, and lineage III strains placed between . lineages I and II and . (Fig. 1). This was consistent with previous reports by Doumith . (2004b) and Chen . (2009b). Lineage III strains appeared to be the most genetically diverse population (p = 3.631%), while those of lineage I, especially 4b complex, showed significantly lower level of genetic diversity (p = 0.397% for lineage I and 0.306% for 4b complex) (Table 5).

The twenty-four 4b complex isolates, including 15 from imported aquatic products, five from Chinese food systems, three from Chinese diseased animal source, and one from reference collection, clustered in five subbranches. Although the isolates belonging to the same EC came from distinct regions at different times, they had similar genetic sequences. The four ECI isolates (NB4, NB13, NB22, and NB23) representing ST8, ST14, and ST15 differed from each other only in their sequences, and the four ECII isolates (NB6, NB7, NB8, and NB10) representing ST9 to ST12 differed in and sequences. ECI (ST8, ST14, and ST15) formed sister subbranch with three isolates from Chinese diseased animals (ST18) and two India shrimp isolates, NB25 (ST16) and NB29 (ST17). ECII (ST9 to ST12) branched off from the main cluster of 4b complex and was placed between 1/2b or 3b isolates NB9 (ST1) and NB26 (ST3). Four Argentina squid isolates (NB1, NB14, NB15, and NB16), five Chinese food isolates (S2, S4, S6, M5, and V2), and reference strain ScottA belonged to ST7, which occupied another subbranch not related to any EC. Additionally, the American sardine isolate NB11 (ST13) fell into the 1/2b or 3b cluster (Table 1 and Fig. 1). The 4b complex isolates prevalent in Chinese food systems might have come from certain non-EC (p = 0), while the 4b complex isolates from imported aquatic products showed a greater level of diversity (p = 0.404%) and included high-risk ECs (ECI and ECII) (Table 5).

P e e ce ~ a ~ec ı - e a ed e e

All . isolates from imported aquatic products and other lineages I and II strains contained 23 out of the 28 infection-related genes examined, that is, *A* , , , *A*, , , *00* , *A* , *A* , , , , , , , , , , , , , and . Lineage II isolates contained one additional internalin gene, .

Combined with the results from , , and / PCR and bridging PCR, these . isolates showed great diversity of internalin profiles in the - locus. Specifically, all lineage I isolates except NB9 (ST1) contained ; all 1/2a or 3a and 1/2c or 3c isolates harbored and , respectively; and lineage III strains carried or nothing (Table 1). Intriguingly, 1/2b or 3b isolate NB9 seemed atypical as having lineage II–specific internalin structure ( ) in this locus and being

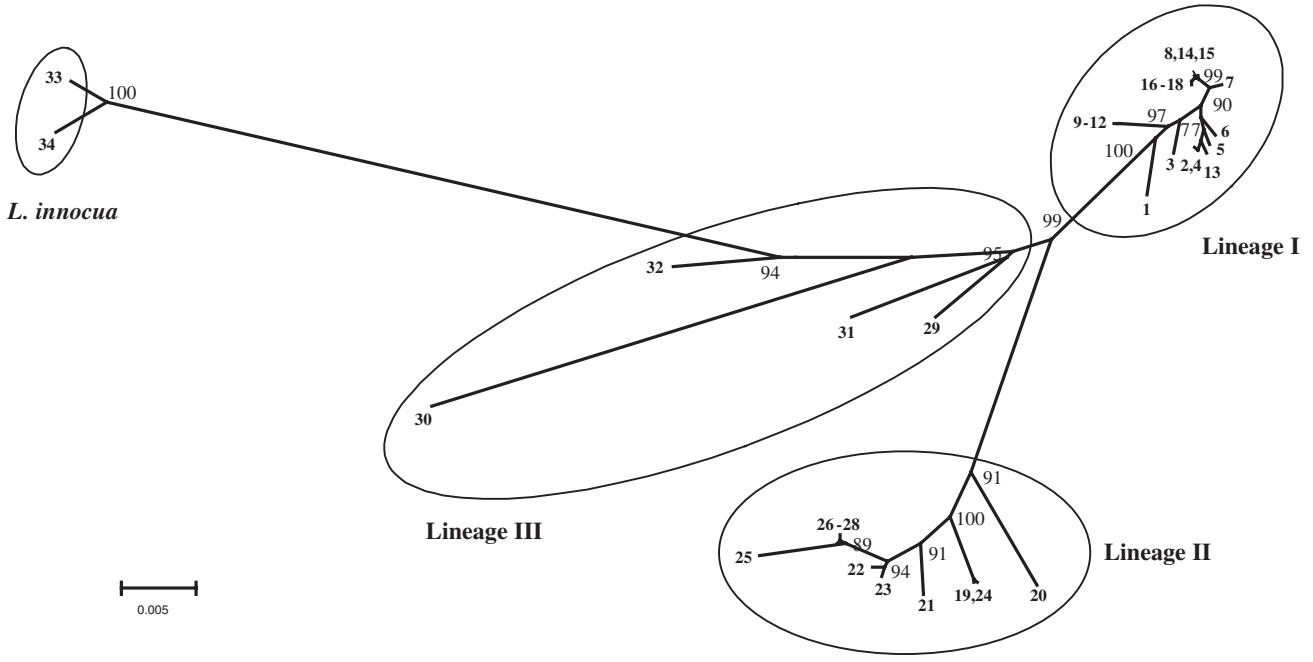

FIG. 1. Neighbor-joining cladogram of 23 and three . isolates from imported aquatic products, together with 12 food-related, three clinical, and six reference strains of . based on - - - - - - - - - concatenated gene cluster. Each number in bold represents a sequence type (ST). ST1 includes isolate NB9; ST2, isolate NB20; ST3, isolate NB26; ST4, isolate NB27; ST5, isolate M1; ST6, isolate S10; ST7, isolates NB1, NB14, NB15, NB16, S2, S4, S6, M5, V2, and ScottA; ST8, isolate NB4; ST9, isolate NB6; ST10, isolate NB7; ST11, isolate NB8; ST12, isolate NB10; ST13, isolate NB11; ST14, isolate NB13; ST15, isolates NB22 and NB23; ST16, isolate NB25; ST17, isolate NB29; ST18, isolates XJ90, 90SB1, and 125SL1; ST19, isolate NB12; ST20, isolate NB21; ST21, isolate NB30; ST22, isolate P3; ST23, isolate S11; ST24, isolate 10403S; ST25, isolate EGD; ST26, isolate NB28; ST27, isolate V1; ST28, isolate P19; ST29, isolate J1-168; ST30, isolate J1-158; ST31, isolate F2-208; ST32, isolate M7; ST33, isolates NB2 and NB3; and ST34, isolate NB24. The values above and below the branches (expressed as percentages) indicate the robustness of the corresponding branches, as determined by a bootstrap analysis evaluated from 1000 replications.

branched off from the lineage I main cluster in the cladogram (Fig. 1). This again highlights the possibility of - genome region being a potential clue for genome diversification and evolutionary history in . .

One 1/2b or 3b isolate (1/4, 25.0%) and seven 4b complex isolates (7/15, 46.7%) including ECI contained a deletion of 105 bp corresponding to one of the four PRRs of (Table 1). The PRRs in might be a potential marker to differentiate between ECI and ECII. In contrast to ECII isolates that contain an intact ActA protein, the ECI isolates all harbored a deletion of one PRR, which fell within the PRRs required for binding of the focal contact proteins VASP and Mena to stimulate actin-based motility. The deletion or absence of one or two PRRs might contribute to a pathogenicity lower than the wild-type strain (Chakraborty ., 1994). Nonetheless, despite having a partial deletion in its gene, ECI caused significant mortality in several major outbreaks throughout Canada, United States, Switzerland, and France (Herd and Kocks, 2001; Swaminathan and Gerner-Smidt, 2007). These findings suggest that, along with other invasion-associated proteins, three PRRs may be sufficient for the bacterium to spread intracellularly and intercellularly and cause listeriosis in humans and animals.

Of the 28 genes examined, . isolates (NB2, NB3, and NB24) only harbored 5 stress response genes, i.e., , , , , and .

V e ce e de

In the mouse virulence assay, all . isolates from imported aquatic products were almost as virulent as . lineages I and II reference strains, whereas lineage III reference strains exhibited relative virulence ranging from 0 to 100%. Further, three . isolates (NB2, NB3, and NB24) were nonpathogenic (Table 1).

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Disclosure Statement

No competing financial interests exist.

References

Chakraborty T, Ebel F, Wehland J, Dufrenne J, and Notermans S. Naturally occurring virulence-attenuated isolates of capable of inducing long term protection against infection by virulent strains of homologous and heterologous serotypes. FEMS Immunol Med Microbiol 1994; 10:1–9.

Chen J, Jiang L, Chen Q, Zhao H, Luo X, Chen X, and Fang W. *00* is involved in acid and heat stress responses and specific for . lineages I and II, and . . Foodborne Pathog Dis 2009a;6:365–376.

Chen J, Jiang L, Chen X, Luo X, Chen Y, Yu Y, Tian G, Liu D, and Fang W. serovar 4a is a possible evolutionary intermediate between . serovars 1/2a and 4b and . . J Microbiol Biotechnol 2009b;19:238–249.

Chen J, Luo X, Jiang L, Jin P, Wei W, Liu D, and Fang W. Molecular characteristics and virulence potential of isolates from Chinese food systems. Food Microbiol 2009c;26:103–111.

Chen J, Zhang X, Mei L, Jiang L, and Fang W. Prevalence of in Chinese food products from 13 provinces between 2000 and 2007 and virulence characterization of isolates. Foodborne Pathog Dis 2009d;6:7–14.

Chen Y and Knabel SJ. Multiplex PCR for simultaneous detection of bacteria of the genus , , and major serotypes and epidemic clones of . . Appl Environ Microbiol 2007;73:6299–6304.

Chen Y, Zhang W, and Knabel SJ. Multi-virulence-locus sequence typing identifies single nucleotide polymorphisms which differentiate epidemic clones and outbreak strains of . J Clin Microbiol 2007;45:835–846.

Chou CH and Wang C. Genetic relatedness between isolates from seafood and humans using PFGE and REP-PCR. Int J Food Microbiol 2006;110:135–148.

Doumith M, Buchrieser C, Glaser P, Jacquet C, and Martin P. Differentiation of the major serovars by multiplex PCR. J Clin Microbiol 2004a;42:3819–3822.

Doumith M, Cazalet C, Simoes N, Frangeul L, Jacquet C, Kunst F, Martin P, Cossart P, Glaser P, and Buchrieser C. New aspects regarding evolution and virulence of - revealed by comparative genomics and DNA arrays. Infect Immun 2004b;72:1072–1083.

Eifert JD, Curtis PA, Bazaco MC, Meinersmann RJ, Berrang ME, Kernodle S, Stam C, Jaykus LA, and Kathariou S. Molecular characterization of of the serotype 4b complex (4b, 4d, 4e) from two turkey processing plants. Foodborne Pathog Dis 2005;2:192–200.

Gerner-Smidt P, Ethelberg S, Schiellerup P, Christensen JJ, Engberg J, Fussing V, Jensen A, Jensen C, Petersen AM, and

genes and partially cross-hybridize with other species. Infect Immun 2001;69:3972–3979.
Hunter PR and Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol 1988;26:2465–2466.
Jiang L, Chen J, Xu J, Zhang X, Wang S, Zhao H, Vongxay K, and