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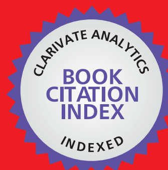

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Genetically Related Listeria Monocytogenes Strains Isolated from Lethal Human Cases and Wild Animals

Ruslan Adgamov[1], Elena Zaytseva[1,2],
Jean-Michel Thiberge[3], Sylvain Brisse[3] and Svetlana Ermolaeva[1*]
[1]Gamaleya Research Institute of Epidemiology and Microbiology, Moscow,
[2]Research Institute of Epidemiology and Microbiology, Vladivostok,
[3]Institut Pasteur, Genotyping of Pathogens and Public Health, Paris,
[1,2]Russian Federation,
[3]France

1. Introduction

The Gram-positive pathogenic bacterium *Listeria monocytogenes* is widely spread in nature [1; 12; 16; 21; 22; 55]. *L. monocytogenes* belongs to the group of pathogens that can be classified as infectious agents of sapronoses (or saprozoonoses) because their infectious reservoir is the environment [34; 37]. Characteristic features of this and similar pathogenic bacteria are abilities to grow effectively outside of the host and to infect a relatively wide range of hosts (polyhostality) [29; 32]. *L. monocytogenes* presence in natural ecosystems is supported by its isolation from a variety of animal species including mammals, birds, fish and mollusks [12; 16; 17; 22; 55]. Virulence factors, which are prerequisite to infection in humans, are involved in interactions with members of natural ecosystems including protozoa [38].

L. monocytogenes is a common contaminant of food products and causes food-borne infection in humans [10; 23; 45]. *L. monocytogenes* isolates divide into at least three distinct phylogenetic lineages. Serovar 4b strains together with strains of serovars 1/2b, 4e and 4d form phylogenetic lineage I, which has been responsible for the majority of outbreaks of food-borne listeriosis in humans [4; 23; 35; 40; 52]. Lineage II comprises strains of serovars 1/2a, 1/2c, 3a, 3c, which are associated with some recent large epidemics and many sporadic cases of foodborne listeriosis. Lineage III includes serovars 4a, 4c and a few 4b strains and is more rarely isolated from human clinical cases [52]. Epidemiologically important *L. monocytogenes* clones were identifed as being associated with listeriosis outbreaks in different countries and continents [9; 23; 35; 39; 47; 53]. The epidemic clones designated ECI, ECII and ECIV belong to lineage I, whereas ECIII belongs to lineage II [23].

Different molecular typing methods such as multilocus enzyme electrophoresis, Pulsed-Field Gel Electrophoresis (PFGE), ribotyping, random amplified polymorphic DNA (RAPD),

* Corresponding author

or amplified fragment length polymorphism have been used to demonstrate that food-associated and clinical *L. monocytogenes* isolates formed two distinctive although overlapping groups [2; 14; 15; 47]. In other words, although food is a major source of human infection, not all strains that contaminate food possess a similar virulence potential to cause invasive infection in humans. Analysis of domestic animal and farm environmental isolates revealed that some strains associated with human infection circulate in the agricultural complexes [20]. Although the role of the wild nature as an original source of listerial infection was suggested, the information about clone distribution among *L. monocytogenes* disseminated in natural ecosystems and their phylogenetic relationships with epidemiologically important clones is scarce [13; 19; 54].

Slowly evolving markers are necessary to analyze phylogenetic relationships and long-term epidemiology of pathogenic bacteria. Multilocus sequence typing (MLST) has been developed for the study of clonal relationships and has been successfully used for population genetics and global epidemiological analysis of different bacterial species including *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Campylobacter jejuni*, *Salmonella enterica*, *Clostridium difficile* [7; 8; 11; 28; 30; 39; 43; 50]. In *L. monocytogenes,* MLST currently distinguishes more than 430 STs, many of which are grouped into large clonal groups that were shown to be globally distributed (Ragon et al 2008, Chenal-Francisque et al 2011). MLST offers several advantages over other molecular typing methods. First, the DNA sequences are unambiguous and readily comparable between different laboratories, and can be stored in a shared central database to provide a broader resource for epidemiological and global population studies. Second, evolutionary genetics studies can be performed, since MLST describes variation believed to be neutral [7; 28; 30].

In this study, we applied a previously described MLST scheme [39; 43] to characterize clonal diversity of *L. monocytogenes* isolates from maternal-fetal cases of human listeriosis and from internal organs of wild animals captured in the forest territory of Russian Far East [55]. The study was aimed to reveal genetic relationships among invasive *L. monocytogenes* strains isolated from anthropogenic and wild environments and to compare isolates from Russia with those from other regions of the world, which are shared in a web database hosted at Institute Pasteur (www.pasteur.fr/mlst). Virulence of isolates belonging to the same MLST sequence type was checked by using the model of intravenous infection of laboratory mice. To evaluate diversity of virulence associated factors among isolates obtained from humans and wild animal hosts, we characterized internal sequences of the *inlA*, *inlB*, *inlC* and *inlE* genes encoding *L. monocytogenes* proteins of the internalin family, which comprises a number of surface and secreted proteins including established *L. monocytogenes* invasion factors InlA and InlB [3; 31].

2. Materials and methods

L. monocytogenes *isolates and culture maintenance.* In total, 40 *L. monocytogenes* isolates were included in the study (Table 1S). Some isolates of 1993-2005 were described previously [55], while others were isolated in a similar way as described [55]. The isolates were associated with infection in humans (15 isolates from maternal-fetal cases), wild small rodents (11 isolates obtained from the liver and spleen of northern red-backed vole (*Cletrionomus rutilus),* grey red-backed vole *(Cletrionomus rufocanus),* striped field mouse *(Apodemus*

No	strain	CC	ST	source	year	serovar	lineage	internalin gene alleles			
								inlA	*inlB*	*inlC*	*inlE*
1	VIMHA 009	**1**	1	stillborn	2005	4b	I	1	9	1	4
2	VIMHA 012	**1**	1	stillborn	2005	4b	I	1	9	1	4
3	VIMHA 015	**1**	1	stillborn	2005	4b	I	1	9	1	4
4	VIMHA 017	**1**	1	stillborn	2005	4b	I	1	9	1	4
5	VIMHA 010	**1**	1	stillborn	2005	4b	I	1	10	1	3
6	VIMHA 011	**1**	1	stillborn	2005	4b	I	1	9	1	3
7	VIMPA k23	**1**	64	stillborn	1998	n.d.	I	1	16	2	3
8	VIMHA 004	**2**	2	stillborn	2005	4b	I	1	1	1	1
9	VIMHA 005	**2**	2	stillborn	2005	4b	I	1	1	1	1
10	VIMHA 006	**2**	2	stillborn	2005	4b	I	1	1	1	1
11	VIMHA 007	**2**	2	stillborn	2005	4b	I	1	3	1	1
12	VIMVR 081	**2**	145	rodent	2005	4b	I	1	1	1	1
13	VIMVR 082	**2**	145	rodent	2005	4b	I	1	1	1	1
14	VIMVR 084	**2**	145	rodent	2005	4b	I	1	1	1	1
15	VIMVR 090	**2**	145	rodent	2005	4b	I	1	1	1	1
16	VIMVR 092	**2**	145	rodent	2005	4b	I	1	1	1	1
17	VIMVW 037	**2**	145	sludge	1998	4b	I	1	4	1	1
18	VIMVW 039	**2**	145	sludge	1998	4b	I	1	1	1	1
19	VIMVG 047	**19**	19	scallop	1993	1/2a	II	9	14	9	6
20	VIMPR750	**19**	314	rodent	1965	n.d.	II	9	14	6	6
21	VIMPA 064	**7**	7	stillborn	1997	n.d.	II	5	14	6	8
22	VIMPR 134	**7**	7	rodent	1952	n.d.	II	4	14	6	8

23	VIMPR 422	**7**	7	rodent	1952	n.d.	II	4	14	6	8
24	VIMCR 474	**155**	155	rodent	2006	1/2a	II	10	14	8	7
25	VIMUR 211	**155**	155	rodent	2006	1/2a	II	10	14	8	7
26	VIMHA 034	**313**	313	stillborn	2004	1/2a	II	9	14	9	6
27	VIMHA 036	**313**	313	stillborn	2004	1/2a	II	9	14	9	6
28	VIMHA 038	**313**	313	stillborn	2004	1/2a	II	9	14	9	6
29	VIMVR 062	**315**	315	rodent	2004	4b	I	2	1	4	1
30	VIMVG 061	**315**	315	scallop	1993	4b	I	2	1	4	1
31	VIMVG 062	**315**	315	scallop	1993	4b	I	2	1	4	1
32	VIMVG 064	**315**	315	scallop	1993	4b	I	2	1	4	1
33	VIMVG 065	**315**	315	scallop	1993	4b	I	2	5	4	1
34	VIMVG 067	**315**	315	scallop	1993	4b	I	2	1	4	1
35	VIMVG 077	**315**	315	scallop	1993	4b	I	2	1	4	1
36	VIMVG 100	**315**	315	fish	1993	4b	I	2	6	4	1
37	VIMVG 102	**315**	315	sea urchin	1993	4b	I	2	2	4	1
38	VIMVG 104	**315**	315	starfish	1993	4b	I	2	1	4	1
39	VIMVG 106	**315**	315	starfish	1993	4b	I	2	1	4	1
40	VIMVG 108	**315**	315	starfish	1993	4b	I	2	1	4	1

Table 1S. Strains used in the study

agrarius), korean field mouse *(Apodemus peninsulae*) and marine animals (12 isolates obtained from scallops, sea urchins and starfish). Two sludge isolates obtained on the territory where rodents were captured were also included [55]. Three historical rodent strains isolated in 1952 and 1965 were obtained from internal organs of wild rodents with the same methods as other rodent isolates were obtained in latter years [1]. The strains were kept frozen at -70°C. The cultures were plated on Brain-Heart Infusion (BHI, BD) agar and grown overnight immediately before an experiment.

PCR analysis and sequencing Overnight *L. monocytogenes* colonies were subjected to lysis by lysozyme/Proteinase K treatment as described [55]. The PCR was run with 1 µl of lysate in the "Tertsik" thermocycler (DNA Technology, Russia). PCR amplification and sequencing for MLST typing was performed as described [39] with following changes in the PCR running conditions: 20 s at 94°C, 20 s at 55 °C, 20 s at 72°C for the first 5 rounds followed by 30 cycles with timing reduced up to 5 s. For PCR amplification and sequencing of internal fragments of *inlA, inlB, inlC* and *inlE* gene, the primers were designed on the basis of the *L. monocytogenes* strain EGDe genome sequence (Glaser et al., 2001) as following: inlAF: 5′ – TAACGGGACAAATGCTCAGGC; inlAR: 5′ – TGTTAAACTCGCCAATGTGCC; inlBF: 5′ - TTTTCAGATGATGCTTTTGC; inlBR: 5′ – ATAGCGGGTTAAGTTGACTGC; inlCF: 5′ – TTTCCAGATCCCGGCCTAGC; inlCR: 5′ – ATAGCCTCAGTCTCCCCAACG; inlEF: 5' – TCGGAAAAGCGGATGTAACAG; inlER – 5' TGAAGCTGTTTAAATCCCACG. The PCR was performed as described above. The final elongation for 10 min was carried out for the samples used for sequencing. PCR products were purified with the Wizard® PCR Preps DNA purification kit (Promega). Sequencing was performed at the Center "Genome" (http://www.genome-center.narod.ru).

Sequence analysis Sequences were proofread and assembled in Chromas version 1.45 (Copyright© 1996-1998 Conor McCarthy, http://www.technelysium.com.au/chromas.html). DNA alignment was done with the ClustalW1.83.XP [46]. Descriptive analysis of the sequence polymorphism was performed using DnaSP version 4.10 [42]. Dendrograms were constructed with Mega version 3.1 [25]. Results of MLST typing are available from the *Listeria monocytogenes* Institut Pasteur MLST database at http://www.pasteur.fr/mlst. Sequence data of internalin gene fragments are available in the GenBank/EMBL/DDBJ databases under the accession numbers EU408789- EU408802, EU40880, EU408813, EU408816- EU408819, EU408822- EU408830, EU408830, EU408833- EU408837, EU408839- EU408844, EU408847, EU408848, EU408855- EU408864, EU408866, EU408871, EU408874- EU408878, EU408880, EU408887- EU408900, EU408902, EU408914- EU408928, EU408928, EU408931- EU408942, EU408945, EU408946, EU408953- EU408967, EU408969, EU408981- EU408984, EU408987- EU408993, EU408995, EU408998- EU409002, EU409004- EU409009, EU409012, EU409013, EU409020, EF056170- EF056174, EF056188- EF056191.

Statistics The mean values and standard errors were calculated with the use of Excel software, a part of Miscrosoft Office 2003 package. The t-test included in the same software was used for assessment of statistical significance.

3. Results

3.1 MLST typing of L. monocytogenes isolates

To define the genetic relationships of the 40 isolates included in this study, we applied the multilocus sequence typing (MLST) scheme based on sequences of the internal fragments of

seven housekeeping genes that was initially developed by Salcedo and co-workers [43] and further modified [39] (Table 1). Phylogenetic analysis of concatenated sequences revealed two major branches, which corresponded to lineages I and II, as deduced based on serotype and correspondence with data for reference strains (Ragon et al., 2008). There were no lineage III strains among studied isolates. The majority of substitutions were fixed differences between lineage I and II, i.e. nucleotide sites at which all sequences in lineage I were different from all sequences in lineage II [18]. Intralineage variability was low with the exception of the *ldh* gene.

Gene	Number of substitutions (nonsynonymous)			Between lineages		Number of alleles		
	total collection	lineage I	lineage II	fixed differences	shared mutations	total colle-ction	lineage I	lineage II
abcZ	21 (2)	3 (0)	3 (1)	15	0	5	2	3
bglA	14 (0)	4 (0)	7 (0)	3	0	6	2	4
cat	27 (4)	2 (1)	8 (2)	17	0	7	3	4
dapE	34 (7)	4 (3)	2 (0)	28	0	5	3	2
dat	57 (8)	2 (0)	2 (0)	56	0	6	3	3
ldh	30 (4)	3 (3)	20 (2)	8	1	8	4	4
lhkA	16 (2)	1 (0)	0	15	0	3	2	1

Table 1. Sequence polymorphism of the seven housekeeping gene fragments

ST	lineage	CC	Serovar	Source			
				Stillborns Number of isolates (year of isolation)	Rodents Number of isolates (year of isolation)	Sea animals Number of isolates (year of isolation)	Sludge Number of isolates (year of isolation)
1	I	1	4b	6 (2005)	-	-	-
64	I	1	n.d.	1 (1998)	-	-	-
2	I	2	4b	4 (2005)	-	-	-
145	I	2	4b	-	5 (2005)	-	2 (1998)
315	I	315	4b	-	1 (2004)	11 (1993)	-
19	II	19	n.d.	-	-	1 (1993)	-
314	II	19	1/2a	-	1 (1965)	-	-
7	II	7	n.d.	1 (1997)	2 (1952)	-	-
155	II	155	1/2a	-	2 (2006)	-	-
313	II	313	1/2a	3 (2004)	-	-	-

Table 2. Source distribution of sequence types (STs) and clonal complexes (CCs).

The isolates belonged to 10 sequence types (STs) (Table 2). Seven STs were previously described, and three novel STs (ST313, ST314, ST315) are described here for the first time. Three pairs of closely related STs were revealed, as single nucleotide polymorphisms in the *ldh* gene distinguished ST1 and ST64, ST2 and ST145, and ST19 and ST314, respectively. Based on the definition of a clonal complex (CC) as “a group of profiles differing by one

gene from at least one other profile of the group" [39], ST1 and ST64 belonged to the same CC, which was designated CC1 [39], ST2 and ST145 belonged to CC2, and ST314 and ST19 formed a novel clonal complex, which was designated CC19 (Table 2). Screening of ST313 and ST315 against the *L. monocytogenes* MLST database revealed that ST313 is closely related to ST20, and ST315 forms a clonal complex with ST95, ST96, ST102 and ST194. Single nucleotide substitutions in the *ldh* gene distinguished sequence types within each of these clonal complexes (data not shown).

3.2 Phylogenetic characterization of L. monocytogenes clinical isolates obtained in Russia

The *L. monocytogenes* strains isolated from the cases of human maternal-fetal infection belonged to five distinct clones (Table 2). Six and four 4b serovar isolates belonged to ST1 and ST2, respectively. The isolates were obtained in the city of Khabarovsk in the year 2005 [55]. Notably, strain F2365, the reference strain for epidemic clone I (ECI), belongs to ST1 [33; 39]. One more strain isolated from a maternal-fetal listeriosis case in 1998 belonged to ST64, a member of CC1 (Tables 1S and 2). Three serovar 1/2a clinical isolates belonged to ST313, which was so far uniquely described in Russia. The last clinical strain belonged to ST7, which was shown to be associated with multiple cases of human listeriosis in Europe as well as in Australia [39]. Thus, a majority of human isolates from Russia belonged to globally distributed clonal complexes.

3.3 Phylogenetic characterization of isolates obtained from small wild rodents and marine animals

Four distinct MLST profiles were revealed among eleven wild rodent isolates (Table 2). Only two profiles were found among twelve isolates obtained from marine animals. ST7 was revealed in strains isolated from wild small rodents (Table 2). The isolates were not related neither temporally nor territorially (Table 1 S). The profile ST145 belonging to CC2 was found in strains isolated from wild small rodents. There were no established epidemiological links between isolates from humans belonging to ST2 and these animals. ST145 strains were also isolated from sludge of the river that was territorially linked with the area of rodent capturing [51]. Still, isolates from rodents and from sludge were obtained in different years (Table 2).

ST315, which prevailed among marine isolates, has also been isolated from a rodent (Table 2). The rodent and marine animal isolates were obtained in different years, although from samples collected at closely located territories (Table 1 and [55]). In contrast, closely related rodent and marine animal isolates, which belonged to CC19, were separated by long distance and about 30 years (Table 1S).

3.4 Internalin gene diversity in L. monocytogenes isolates

To get a deeper insight into *L. monocytogenes* diversity and particularly into features that might distinguish the closely related strains isolated from different hosts, we performed partial sequencing of genes, which encode proteins of the internalin family [6]. The internal fragments of the internalin genes *inlA, inlB, inlC, inlE*, which encode functionally important LRR domains, were sequenced (Table 3). Sequences of whole LRR-coding fragments were

determined for *inlB, inlC* and *inlE* genes. The sequenced fragment covered 42 % of the LRR-domain coding region of the *inlA* gene, which carries the longest LRR domain.

Gene	Fragment length	Encoded amino acids	Number of substitutions (nonsynonymous)			Between lineages (nonsynonymous)		Number of alleles		
			total colle-ction	lineage I	lineage II	fixed diffe-rences	shared mutati-ons	total colle-ction	lineage I	lineage II
inlA	648	54-269	18 (6)	8 (2)	12 (5)	0	5 (2)	6	2	4
inlB	618	64-269	52 (18)	25 (10)	0	30 (9)	0	10	9	1
inlC	586	70-264	21 (7)	6 (3)	2 (0)	14 (4)	1 (0)	7	4	3
inlE	558	82-267	99 (45)	6 (3)	4 (2)	80 (30)	0	7	4	3

Table 3. Sequence polymorphism of the internalin gene fragments

The total amount of substitutions was comparable for the internalin genes and the MLST genes (Table 1 and Table 3). However, the amount of non-synonymous substitutions differed significantly: non-synonymous substitutions accounted for 30 – 50 % and 0 – 14 % of substitutions for internalins and housekeeping genes, respectively ($p<0,05$). The fixed nucleotide differences between two phylogenetic lineages prevailed among housekeeping genes and *inlB, inlC* and *inlE*. In contrast, there was no fixed difference for *inlA*. Shared mutations, carried by a few strains of both lineages, were found for *inlA* and *inlB* (Table 3). All lineage I human isolates carried the same *inlA* allele even though they belonged to different clonal complexes (Table 4 and Table 1S). Similarly, rodent isolates belonging to different clonal complexes were characterized by the same *inlB* allele. Analysis of the amino acid substitutions in the sequenced LRR-domains revealed that the majority of substitutions in InlC and InlE have a lineage-specific character (Table 4). In contrast, LRR-domains of InlA and InlB varied within lineages. There were no lineage-specific amino acid substitutions in InlA (Table 4).

Several alleles were revealed for at least one internalin gene within most STs with more than two isolates (Table 5). When the isolates were compared at all 11 markers (7 housekeeping and 4 internalin genes), the 40 isolates were distinguished into 17 genotypes (Fig. 1).

Human isolates belonging to ST1 differed in *inlB* and *inlE* gene sequences (Table 5 and Fig. 1). To get more evidence on relationships of these isolates with the epidemic clone I (ECI) strains, the *inlC* gene sequences were compared with the previously described *inlC* gene fragment, which distinguishes the ECI strains from other *L. monocytogenes* strains [5]. Six SNPs, which are characteristic for the ECI strains, were found to be conserved in all studied ST1 isolates . These results support the suggestion that ECI strains belong to CC1 and indicate that strains of ECI occur on the territory of Russia. The ST64 isolate, which belong to CC1, carried substitutions in the *inlB*, *inlC* and *inlE* genes that distinguished it from ST1

strains. The substitution in *inlC* was outside of the described *inlC* gene fragment, which is conserved among ECI strains (Chen *et al.,* 2007); hence, ST64 isolate could be considered as belonging to ECI as well.

ST	Strain[1]	InlA	InlB	InlC	InlE
		1111	111111222222	111122	1111111111111112222222222222222
		591458	6779136789045556	8124425	1244445666788990111222234455566
		448277	9231784617561272	8666743	5414567246828132046156968956912
		ALDSIS	LQSIALLLIESSMNKI	KKVFVRN	AGSMLHSTESATLYIIECIASSRNIIEDII
1	VIMHA009	......	....	....	....
1	VIMHA011	......	....	....	....
1	VIMHA012	......	....	....	....
1	VIMHA015	......	....	....	....
1	VIMHA017	......	....	....	....
1	VIMHA010	......	.P..............	....	....
64	VIMPHk23	......	.H..............	....	....
2	VIMHA004	......	..NV.IP.....T...	....	....
2	VIMHA005	......	..NV.IP.....T...	....	....
2	VIMHA006	......	..NV.IP.....T...	....	....
2	VIMHA007	......	.PNV.IP.....T...	....	....
2	VIMHA008	......	..NV.IP.....T...	....	....
145	VIMVR081	......	..NV.IP.....T...	....	....
145	VIMVR082	......	..NV.IP.....T...	....	....
145	VIMVR084	......	..NV.IP.....T...	....	....
145	VIMVR090	......	..NV.IP.....T...	....	....
145	VIMVR092	......	..NV.IP.....T...	....	....
145	VIMVW039	......	..NV.IP.....T...	....	....
145	VIMVW037	......	..NV.IP.....T.N.	....	....
315	VIMVR062	...T.N	..NV.IP.....T...	.NMC...	....
315	VIMVG098	...T.N	..NV.IP.....T...	.NM....	....
315	VIMVG108	...T.N	..NV.IP.....T...	.NM....	....
315	VIMVG106	...T.N	..NV.IP.....T...	.NM....	....
315	VIMVG104	...T.N	..NV.IP.....T...	.NM....	....
315	VIMVG077	...T.N	..NV.IP.....T...	.NM....	....
315	VIMVG067	...T.N	..NV.IP.....T...	.NM....	....
315	VIMVG064	...T.N	..NV.IP.....T...	.NM....	....
315	VIMVG061	...T.N	..NV.IP.....T...	.NM....	....
315	VIMVG062	...T.N	..NV.IP.....T...	.NM....	....
315	VIMVG102	...T.N	..NV.IP.....TT..	.NM....	....
315	VIMVG065	...T.N	..NV.IP.....T.Q.	.NM....	....
315	VIMVG100	...T.N	..NV.IP.....T.Q.	.NM....	....
313	VIMHA034	.VNTL.	A.NVT.PIVQAPS..T	Q...LKS	TRNTMDPSVTGFFSLTMSVTGNEKLVTNT.
313	VIMHA036	.VNTL.	A.NVT.PIVQAPS..T	Q...LKS	TRNTMDPSVTGFFSLTMSVTGNEKLVTNT.
313	VIMHA038	.VNTL.	A.NVT.PIVQAPS..T	Q...LKS	TRNTMDPSVTGFFSLTMSVTGNEKLVTNT.
7	VIMPA064	P..T.N	A.NVT.PIVQAPS..T	Q...LKS	KRNTMDPSVTGFFSLTMSVTGNEKLVTNT.
19	VIMVG047	.VNTL.	A.NVT.PIVQAPS..T	Q...LKS	TRNTMDPSVTGFFSLTMSVTGNEKLVTNT.
314	VIMPR750	.VNTL.	A.NVT.PIVQAPS..T	Q...LKS	TRNTMDPSVTGFFSLTMSVTGNEKLVTNT.
155	VIMUR211	.VNTL.	A.NVT.PIVQAPS..T	Q...LKS	TRNTMDPSVTGFFSLTMSVTGNEKLVTNTF
155	VIMCR474	.VNTL.	A.NVT.PIVQAPS..T	Q...LKS	TRNTMDPSVTGFFSLTMSVTGNEKLVTNT.
7	VIMPR134	...T.N	A.NVT.PIVQAPS..T	Q...LKS	KRNTMDPSVTGFFSLTMSVTGNEKLVTNT.
7	VIMPR422	...T.N	A.NVT.PIVQAPS..T	Q...LKS	KRNTMDPSVTGFFSLTMSVTGNEKLVTNT.

[1]strain sources are designated with a color as follows: human isolates are red, rodent isolates are black, marine animal isolates are blue and sludge isolates are green.

Table 4. Amino acid substitutions in internalins.

ST/CC	Number of alleles				Number of *inl* profiles
	inlA	*inlB*	*inlC*	*inlE*	
ST1/CC1 n=6	1	2	1	2	3
ST2/CC2 n=5	1	2	1	1	3
ST145/CC2 n=7	1	2	1	1	2
ST7 n=3	1	2	1	1	2
ST155 n=2	1	1	1	2	2
ST313 n=3	1	1	1	1	1
ST315 n=11	1	4	1	1	4

[1]only those STs are shown that are represented by 2 and more isolates

Table 5. Variability of internalin genes within STs[1]

One of the human ST2 isolates was distinguished by its *inlB* sequence from other ST2 isolates (Table 4 and Fig. 1). A nonsynonymous substitution was found, which brought about a Q72P substitution (Table 4). Three other ST2 isolates carried an identical *inlB* allele, and the same allele was found in six of seven closely related ST145 isolates (which belonged to CC2; Tables 5 and 1S). *inlA, inlC* and *inlE* were identical in ST2 and ST145 isolates. Therefore, both housekeeping and virulence gene markers were highly similar in human ST2 isolates and murine ST145 isolates, confirming that these human and wild mouse isolates are genetically closely related.

4. Discussion

Here we applied a previously described MLST scheme [39; 43] to determine the genetic diversity of isolates acquired from clinical human cases and internal organs of wild animals, and to compare the genotypes of Russian isolates with international genotypic data. Obtained results confirmed the worldwide distribution of large clonal complexes corresponding to so-called epidemic clones. Particularly, we demonstrate for the first time that strains closely related to epidemic clone ECI, which is responsible for a number of temporally and geographically distinct outbreaks of listeriosis in North America and Europe [23], were associated with fetal-maternal cases of listeriosis in Russia.

The wide distribution of certain clones among the human population might be supported by high rates of international food trade turnover. Alternatively, human activity might play a secondary role in spreading of *L. monocytogenes* clones, which might be widely distributed in natural ecosystems, where their distribution might be supported by parasitism in wild animals. To study *L. monocytogenes* distribution in natural ecosystems, periodical surveys were performed in Russia in different years [1; 55; 56]. Surveys included animals capturing and analysis of bacterial loads in the internal organs, therefore only invasive strains that are

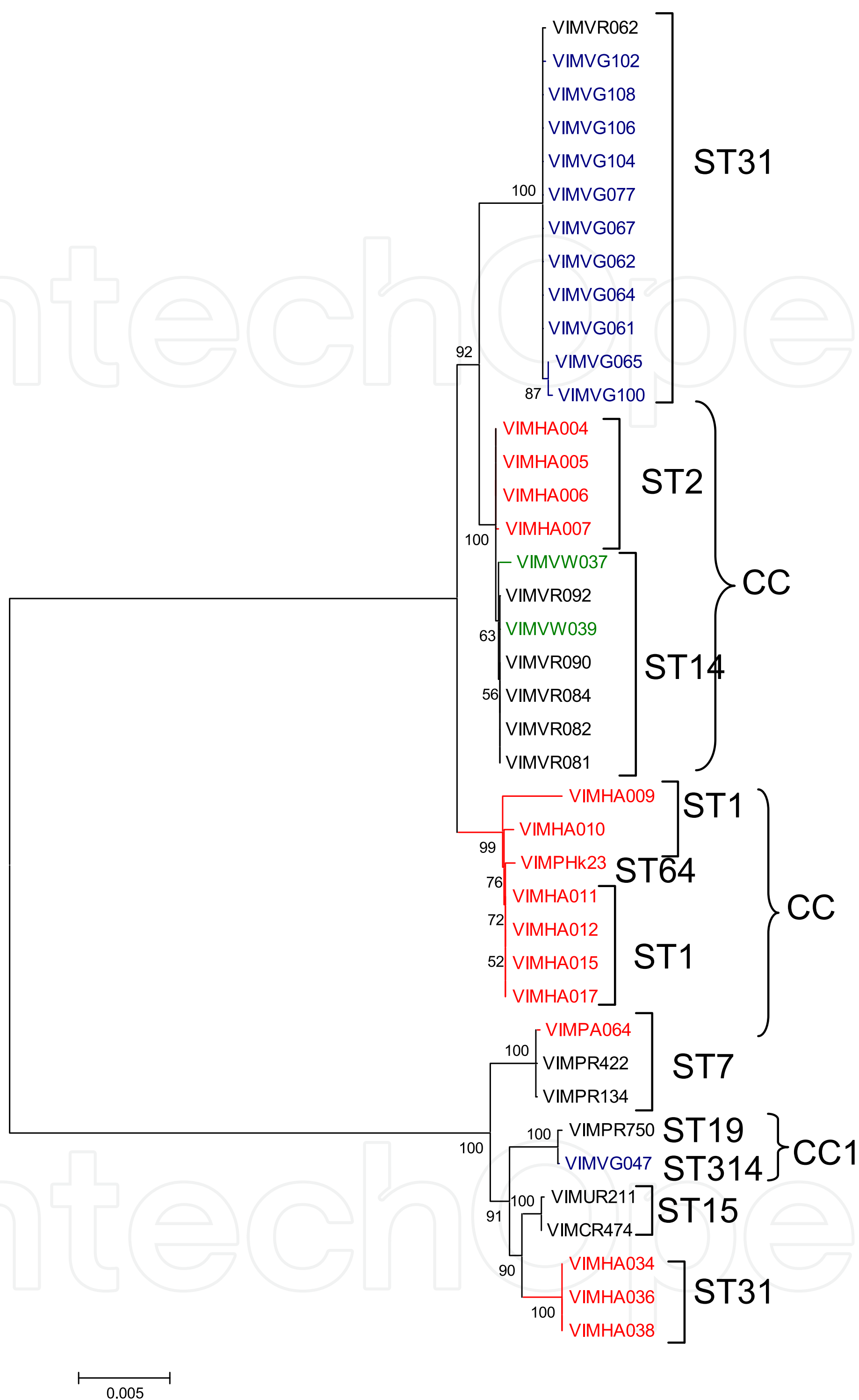

Fig. 1. The dendrogram was constructed based on the Neighbor-Joining method using concatenated sequences of the seven housekeeping and four internalin genes. The Kimura-2 parameters model was used for distance estimation. Square and curly brackets show MLST sequence types (ST) and clonal complexes (CC), respectively. The color of strain names designates the source of isolation: red for human isolates, green for rodent isolates, blue for isolates from marine animals, and black for sludge isolates. Bootstrap values obtained after 1,000 replicates are indicated at the nodes.

able to get to the internal organs were collected [52]. *L. monocytogenes* isolates studied in this work represent a few independent surveys among wild animals that were performed at different locations and in different years [1; 55]. All but one isolates, which were obtained in each survey, belonged to the same ST (see Table 2). The only exception was a survey among marine animals in 1993, when two STs were revealed: the majority of the isolates belonged to ST315, and a single isolate belonged to ST19 (Table 1S). The low diversity of animal isolates isolated each collection year might be due to the fact that the *L. monocytogenes* isolation from internal organs was successful only if outbreaks of listeriosis took place among wild animals, implying that successful surveys included epidemiologically related isolates. This suggestion is supported by the observation that secondary surveys sometimes failed to reveal *L. monocytogenes* at the same territory [55]. Still, the alternative hypothesis can not be excluded that STs found in rodents and marine animals might be prevalent in the wild environment.

Many epidemiologic studies demonstrated that lineage I is generally overrepresented among human clinical listeriosis cases, while lineage II strains are major contaminants of food products [2; 14; 15; 47]. These results led to the suggestion that lineage I strains have a higher infection potential due to intrinsic virulence features [23; 45]. Specific traits of epidemic clones include differences in both gene content and gene expression/regulation among strains [33; 44]. Similarly, particular genomic features might increase *L. monocytogenes* tropism for specific animal hosts.

Interestingly, that all lineage I *L. monocytogenes* isolates from human maternofetal listeriosis cases carried the identical *inlA* allele (Tables 1S and 4), and it is InlA that is required to cross the human maternofetal barrier and to cause fetal infection [27]. The lineage I isolates obtained from mice carried different *inlA* alleles but the same allele of the *inlB* gene. InlB was shown to be a major invasion factor for *L. monocytogenes* infection in mice [24; 26]. Mutations in InlB affect invasion efficiency and might influence *L. monocytogenes* virulence for mice [31; 49]. The fine tuning of internalin sequences might be one of the factors that influence *L. monocytogenes* tropism for specific animal hosts. The further study of naturally occurring internalin variants and their role in infection of different hosts is in progress at our laboratories.

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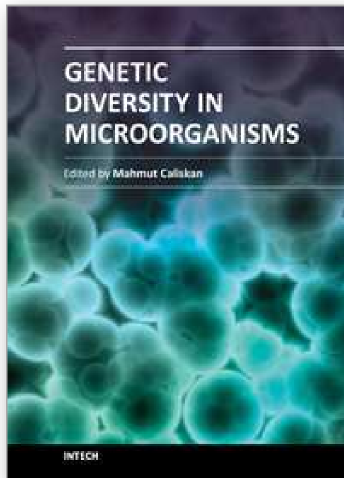

Genetic Diversity in Microorganisms
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Genetic Diversity in Microorganisms presents chapters revealing the magnitude of genetic diversity of microorganisms living in different environmental conditions. The complexity and diversity of microbial populations is by far the highest among all living organisms. The diversity of microbial communities and their ecologic roles are being explored in soil, water, on plants and in animals, and in extreme environments such as the arctic deep-sea vents or high saline lakes. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in microorganisms. The purpose of the book is to provide a glimpse into the dynamic process of genetic diversity of microorganisms by presenting the thoughts of scientists who are engaged in the generation of new ideas and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of microbial phylogeny, genetic diversity, and molecular biology.

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