

Characterization of *Listeria monocytogenes* Strains Isolated During 2011–2013 from Human Infections in Switzerland

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Abstract

Listeria monocytogenes, an emerging foodborne pathogen, can cause in the population at risk severe infections that are associated with high case fatality rates. A total of 93 *L. monocytogenes* strains isolated from different patients in Switzerland from July 2011 to September 2013 were further characterized. Septicemia was reported for 74.2% of the patients, meningitis for 10.8%, and abortion for 3.2%. The majority of the strains belonged to serotype 1/2a ($n = 58$) followed by serotype 4b ($n = 28$), 1/2b ($n = 5$), and 1/2c ($n = 2$). The strains represented 35 multilocus sequence typing sequence types, 8 of which were designated for the first time. Sequence analysis of the *inlA* gene in the 35 sequence types showed that most of the strains encoded full-length proteins. Screening for Listeriolysin S showed the presence of this virulence factor in 29 of the 33 genetic lineage I strains. By using *ApaI* and *AscI* for pulsed-field gel electrophoresis, most strains showed distinguishable patterns.

Introduction

LISTERIA MONOCYTOGENES IS AN IMPORTANT foodborne pathogen with a significant impact on public health and economy worldwide. Human infections are rare, but *L. monocytogenes* has the potential to cause serious and life-threatening disease. Clinical conditions primarily comprise septicemia, meningitis, meningoencephalitis, and abortion (Dogany, 2003). Because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to foodborne illnesses (Anonymous, 2009). In the European Union (EU), 1476 confirmed cases of listeriosis were reported in 2011 (Anonymous, 2013a). The overall EU notification rate was 0.32 cases per 100,000 population. In Switzerland, the annual incidence over the past 10 years ranged from 0.53 to 0.97 cases per 100,000 population. So far, three Swiss listeriosis outbreaks have occurred, including the latest one reported in 2011 caused by the consumption of contaminated cooked ham (Bula *et al.*, 1995; Bille *et al.*, 2006; Hächler *et al.*, 2013).

To gain insight into the relationships and virulence-associated characteristics of strains occurring in Switzerland, the aim of the present study was to characterize all clinical *L. monocytogenes* strains collected by the Swiss National Reference Laboratory from 2011 through 2013 by phenotyping and genotyping methods and to compare these results with data from other countries in Europe and the United States.

Materials and Methods

Strains

The 93 strains were isolated from July 2011 to September 2013 from human patients with a severe illness and a serious suspicion of infection with *L. monocytogenes*. Due to mandatory notification and the obligation of laboratories to forward human *L. monocytogenes* strains to the National Reference Laboratory, these strains are considered representative for the period screened.

Lineage polymerase chain reaction (PCR)

Lineage-specific PCR assays were performed using the primers and protocol described by Ward *et al.* (2008). Reactions were carried out in a 20- μ L volume that contained 1x GoTaq Green Master Mix (Promega, Madison, WI).

Serotyping

Serotyping was performed using the commercial set of *Listeria* O-factor and H-factor antisera from Denka Seiken (Pharma Consulting, Burgdorf, Switzerland).

Antimicrobial susceptibility testing

Strains were tested for antimicrobial susceptibility by the disc-diffusion method according to the protocols of the

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Clinical and Laboratory Standards Institute (Anonymous, 2013b). The antibiotics (Becton Dickinson, Sparks, MD) tested were the following: ciprofloxacin 5 µg (CIP5), rifampicin 5 µg (RA5), gentamicin 10 µg (GM10), ampicillin 10 µg (AM10), erythromycin 15 µg (E15), vancomycin 30 µg (VA30), clindamycin 2 µg (CC2), tetracycline 30 µg (TE30), and sulfamethoxazole 23.75 µg/trimethoprim 1.25 µg (SXT23.75/1.25).

PFGE subtyping and cluster analysis

Pulsed-field gel electrophoresis (PFGE) analysis of the strains was done in accordance with the CDC PulseNet protocol (<http://www.cdc.gov/pulsenet/pathogens/index.html>). DNA was digested separately with *ApaI* (100 U/plug; Roche Diagnostics, Rotkreuz, Switzerland) and with *AscI* (50 U/plug; Bioconcept, Allschwil, Switzerland). Electrophoresis was carried out at 14°C for 20 h at 6 V/cm under linear ramp with switch times from 4 to 40 s and 120°C included angle.

For pattern evaluation, BioNumerics v6.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used. Pairwise similarities between the *ApaI* and *AscI* PFGE patterns were calculated using the JACCARD similarity coefficient. Cluster analysis was performed on the unweighted pair-group method with averages. Tolerance and optimization parameters were set to 1.5% each. *Salmonella* serovar Braenderup strain H9812 (ATCC BAA 664) digested with *XbaI* (100 U/plug; Roche Diagnostics) was used as size standard. For each restriction enzyme, the different patterns were grouped into pulsotypes based on a cut-off value of 95% pattern similarity.

MLST

Multilocus sequence typing (MLST) was performed as described by Ragon *et al.* (2008) with the following modifications: Amplifications were performed in 50 µL volume using the HotStarTaq Master Mix (Qiagen) and 50 ng of genomic DNA. Following cycling, conditions were determined for amplification of the *bglA*, *cat*, and *ldh* loci: 94°C for 4 min, followed by 42 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 2 min; for the *abcZ*, *dapE*, *dat*, and *lhkA* loci: 94°C for 4 min followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 2 min. Amplifications were finalized with a 10-min 72°C step. Sequencing of the amplicons was performed employing the universal primers described by Ragon *et al.* (2008). Alleles and sequence types (STs) are publicly available at www.pasteur.fr/mlst.

Internalin A gene (*inlA*) sequence profiling

Internalin A profiling was performed on one representative strain of each ST identified in this study using PCR primers described previously (Orsi *et al.*, 2007). PCR amplifications were performed using the Phusion High Fidelity Taq Polymerase and conditions recommended by the supplier (Fermentas, St. Leon-Rot, Germany). Amplicons were sequenced commercially by Microsynth (Balgach, Switzerland) and analyzed for mutations with CLC Main Workbench 6.6.1.

Further strain characterization

The presence of Listeriolysin S was determined as described by Cotter *et al.* (2008). PCR reactions were per-

formed in 50-µL volume, using 1 x GoTaq Green Master Mix (Promega). PCR amplifications for the 50-kb *Listeria* genomic island (LGI1) (Gilmour *et al.*, 2010) were performed on serotype 1/2a strains using primers and PCR conditions described by Kovacevic *et al.* (2012). Reactions were carried out in 50-µL volume that contained 1 x GoTaq Green Master Mix (Promega). PCRs to determine the presence of the stress survival islet (a five-gene region consisting of *lmo0444-lmo0448*) were performed as described by Ryan *et al.* (2010). The Phusion High Fidelity Taq Polymerase was used for the PCR amplifications in accordance with the supplier's recommendations (Fermentas).

Results and Discussion

Origin of the strains

Most of the 93 human strains included in this study were isolated from patients with severe illness between July 2011 and September 2013. The origins of the strains for these patients are summarized in Table 1. Septicemia was reported for 74.2% of the patients, meningitis for 10.8%, and abortion for 3.2%. Furthermore, gastroenteritis was reported for 3.2% of the patients, colpitis for 1.1%, and inflammation of the knee joint for 1.1%. For 6.5% of the patients no medical history data were available. Five patients (5.4%) were aged < 10 years, 60 (64.5%) were aged > 60 years, and 24 (25.8%) patients were aged between 10 and 60 years. For four patients no data were available. Of the reported cases, 50.5% were female and 49.5% were male.

Lineages and serotypes

Lineage determination was achieved based on the MLST data and confirmed by lineage specific PCR. Strains belonged either to lineage II (60 strains) or to lineage I (33 strains).

The majority of the strains belonged to serotype 1/2a ($n=58$) followed by serotype 4b ($n=28$), 1/2b ($n=5$), and 1/2c ($n=2$) (Table 1). Serovar 1/2a was also reported as the most frequently isolated human serovar in other countries (Gianfranceschi *et al.*, 2009; Lyytikäinen *et al.*, 2006; Lopez-Valladares *et al.*, 2014). Strains of serotype 1/2a and 1/2c belonged to lineage II, whereas strains of serotype 1/2b and 4b belonged to lineage I. No specific correlation was found between lineage or serotype and clinical presentation.

Antimicrobial susceptibility testing

All 93 *L. monocytogenes* strains were susceptible to 8 antimicrobial agents (ciprofloxacin, rifampicin, gentamicin, ampicillin, erythromycin, vancomycin, tetracycline, sulfamethoxazole/trimethoprim), and all but 3 (different sequence types) strains were resistant to clindamycin.

PFGE subtyping and cluster analysis

The PFGE fingerprint patterns clustered to form 63 (*ApaI*) and 70 (*AscI*) distinguishable pulsotypes at a cut-off value of 95% pattern similarity, which highlights the heterogeneity among the 93 *L. monocytogenes* strains. Simpson's index of discrimination for *ApaI* and *AscI* was 98.7% and 98.8%, respectively. One cluster, formed by the six Swiss 2011 listeriosis outbreak strains (Hächler *et al.*, 2013), and 10 other clusters, formed by two to four strains

TABLE 1. NUMBER OF STRAINS SPLIT BY LINEAGES, SEROTYPES, MULTILOCUS SEQUENCE TYPING CLONAL COMPLEX, ISOLATION SOURCE AND OCCURRENCE OF DIFFERENT VIRULENCE FACTORS

	Total number of strains	Strain origin ^a				LLS ^b		SSI-1 ^c			
		B	L	P	O	+	-	+	-	DA	NA
Lineage I											
Serotype 1/2b											
Different CC ^d	5	5				2	3	4	1		
Serotype 4b											
CC1	14	8	2	2	2	14			14		
CC4	7	5	1		1	7			7		
CC6	4	4				4			4		
Different CC ^e	3	3				2	1		3		
Lineage II											
Serotype 1/2a											
CC8	15	10	2		3		15	12	1		2
CC21	6	3	1		2		6		2		4
Singleton 403	8	7	1				8	8			
Different CC ^f	29	22	3	1	3		29	13	5	3	8
Serotype 1/2c											
Different CC ^g	2	2					2	2			

^aB, blood; L, cerebrospinal fluid (liquor); P, placenta; O, other/no information.

^bLLS, Listeriolysin S; +, positive; -, negative.

^cSSI-1, stress survival islet; +, positive, -, negative; DA, SSI-1 polymerase chain reaction (PCR) amplicons obtained differ from those expected in either positive or negative strains; NA, the SSI-1 PCR did not give any amplicon.

^dOnly clonal complexes/singletons containing 4 strains or more are represented; all other CC (CC3, 5, 59, 224, 517) are summarized as "different CC."

^eOnly clonal complexes/singletons containing four strains or more are represented; all other CC (CC2, 54) are summarized as "different CC."

^fOnly clonal complexes/singletons containing 4 strains or more are represented; all other CC (CC7, 9, 14, 16, 26, 29, 36, 37, 101, 121, 155, 199, 204, 207, 394, 398, 412, 504, 671) are summarized as "different CC."

^gOnly clonal complexes/singletons containing four strains or more are represented; all other CC (CC9) are summarized as "different CC."

each, had identical patterns when analyzed with both enzymes. The few other strains, exhibiting identical patterns when analyzed with one enzyme (*ApaI*), usually yielded different patterns after the second (*AscI*) enzyme was applied. The high genetic diversity among the strains indicates that *L. monocytogenes* infections in Switzerland most often occur as single cases.

MLST typing

The 93 isolates were distinguished into 35 STs, 8 of which (ST671, 672, 673, 681, 682, 683, 684, 685) were designated for the first time. Simpson's index of discrimination was 93.9%. ST8 (14 strains, all serotype 1/2a) and ST1 (14 strains, all serotype 4b) were the most prevalent STs identified among these strains. No correlation was found between ST and clinical presentation. Figure 1 represents the diversity of Swiss strains superposed with the diversity described in previous studies (Ragon *et al.*, 2008; Chenal-Francisque *et al.*, 2011; Cantinelli *et al.*, 2013; Chenal-Francisque *et al.*, 2013). The STs found in this study are largely distributed across the clonal diversity of *L. monocytogenes*, including global collections. These results show that the population of Swiss *L. monocytogenes* strains is representative of the known diversity of this species, showing that the "everything is everywhere" paradigm of *L. monocytogenes* clones (Chenal-Francisque *et al.*, 2011; Cantinelli *et al.*, 2013) applies to Switzerland (Fig. 1) and demonstrates the presence of all major *L. monocytogenes* clones in Switzerland.

Internalin A

Internalin A is a cell-wall-anchored protein that is critical to the virulence of *L. monocytogenes* (Lecuit *et al.*, 2001; Seveau *et al.*, 2007). Several types of virulence-attenuating mutations causing premature stop codons (PMSCs) in the *inlA* gene have been documented (Jonquieres *et al.*, 1998; Nightingale *et al.*, 2005; Nightingale *et al.*, 2008; Ragon *et al.*, 2008; Cantinelli *et al.*, 2013). Of the 35 examined *L. monocytogenes* strains, representing each of the STs identified, there were only 3 (8.6%) with *inlA* gene PMSC mutations. The first strain (N13-1407) belonged to ST199/serotype 1/2a and had a deduced stop codon at amino acid position 9 caused by A deletion at nucleotide position 12 of the *inlA* gene. The second strain (N12-0367), belonging to ST121/serotype 1/2a, had a deduced stop codon at amino acid position 492 due to a C-to-T mutation at nucleotide position 1474. The third strain (N12-0486), belonging to ST9/serotype 1/2c, had a stop codon at amino acid position 577, precipitated by an A deletion at nucleotide position 1637. Similar PMSCs have been described previously among clinical *L. monocytogenes* strains isolated from other countries (Felicio *et al.*, 2007; Ragon *et al.*, 2008; Van Stelten *et al.*, 2010; Cantinelli *et al.*, 2013; Kovacevic *et al.*, 2013). All strains with PMSCs belonged to lineage II and were isolated from blood. The low frequency of PMSCs *inlA* as detected among the sequenced Swiss strains is consistent with previous reports among clinical strains from other countries and shows that isolates from clinical infections

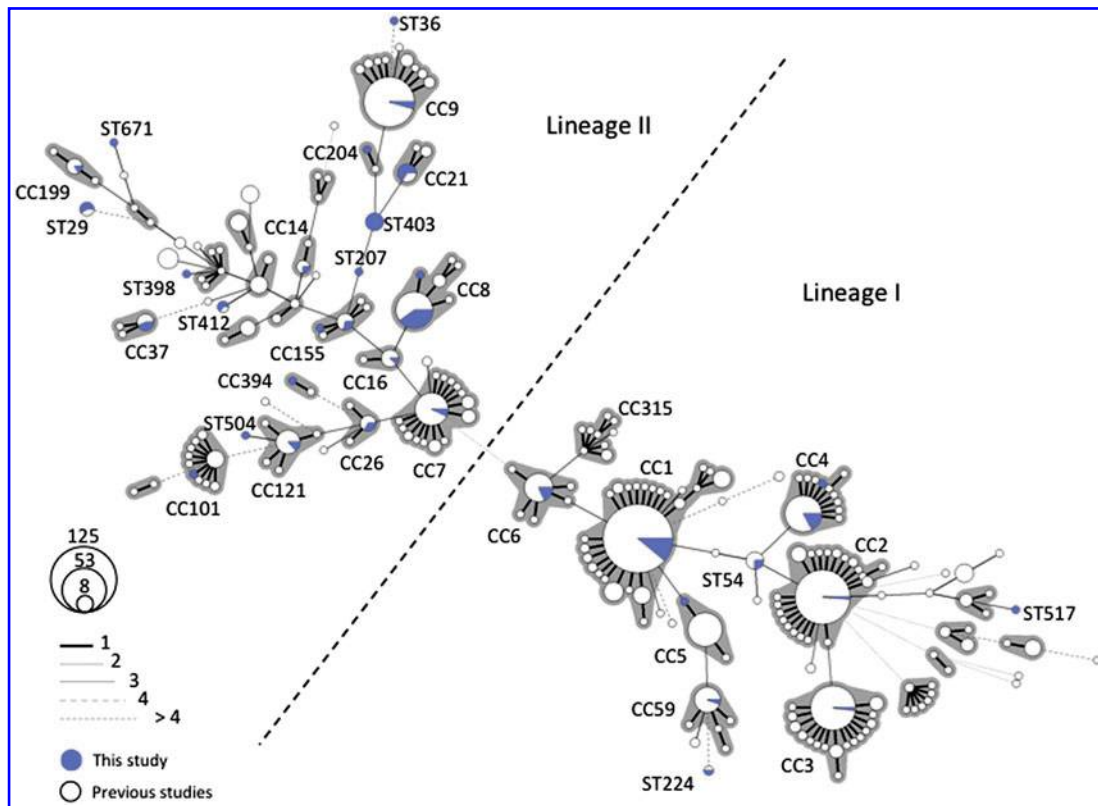


FIG. 1. Minimum spanning tree of multilocus sequence typing data for 956 *Listeria monocytogenes* strains. Each circle represents one sequence type (ST), the size of which is related to the number of strains with this ST. Blue sectors represent the 93 Swiss *L. monocytogenes* strains from this study, whereas white sectors represent previously published strains (Ragon *et al.*, 2008; Chenal-Francisque *et al.*, 2011; Cantinelli *et al.*, 2013; Chenal-Francisque *et al.*, 2013). Links between circles are represented according to the number of allelic mismatches between STs, as indicated. Gray zones surrounding groups of STs represent clonal complexes (CC).

most frequently are able to produce full-length InIA (Jacquet *et al.*, 2004; Van Stelten *et al.*, 2010).

Listeriolysin S (LLS)

Listeriolysin S (LLS) is an oxidative stress-inducible hemolysin that contributes to virulence and is present in a subset of lineage I strains (Cotter *et al.*, 2008). PCR screening for the arrangement and presence of *llsA*, *llsG*, *llsD*, *llsP* genes within the *Listeria* pathogenicity island 3 showed that LLS-positive strains accounted for 29 (87.8%) of the 33 genetic lineage I strains examined (Table 1). Similar to previous observations (Cotter *et al.*, 2008), this virulence factor was absent in lineage II strains. The positive strains were from serotypes 4b (93.1%) and 1/2b (6.9%), and included representatives from 7 STs. Most of the LLS-positive strains (86.2%) belonged to CC1, CC4, and CC6 (Table 1).

Listeria genomic island (LGI1)

The 50-kb LGI1 consists of genes encoding for putative type II and type IV secretion systems, pilus-like surface structures, a multidrug efflux pump homologue (EmrE), and an alternative sigma factor (Gilmour *et al.*, 2010). Although the exact role of this island has not yet been fully elucidated, it is suggested that it may play a role in bacterial persis-

tence and/or pathogenicity, and it was initially identified in *L. monocytogenes* serotype 1/2a strains of CC8 responsible for the 2008 Canadian listeriosis outbreak (Gilmour *et al.*, 2010). All of the 58 Swiss serotype 1/2a strains, including those of CC8, were negative for this genomic island using the LGI1 PCR (Table 1). This is worth mentioning as it suggests distinct subpopulations of CC8 strains.

Stress survival islet (SSI-1)

Genes within the stress survival islet (SSI-1) have been suggested to contribute to survival and growth of *L. monocytogenes* under stress associated with food environments (Ryan *et al.*, 2009). The distribution of this islet was examined among the Swiss listeriosis strains. Its presence was detected in 39 (42%) strains, of which most belonged to serotypes 1/2a ($n=33$) and 1/2b ($n=4$) (Table 1). The distribution of this islet among Swiss clinical strains seems similar to previous reports in which this islet was found more prevalent among nonserogroup 4 strains (Jacquet *et al.*, 2004; Kovacevic *et al.*, 2013). For 14 (15.1%) strains, all belonging to serotype 1/2a, there were no SSI-1 PCR amplicons detected, suggesting that the SSI-1 primer binding sites in these strains are incompatible (Table 1). Furthermore, in three serotype 1/2a strains from ST121 and ST504, the amplicon size obtained differed from that expected in the presence (9.7 kb)

or absence (1.1 kb) of the SSI-1 islet. These strains gave amplicons of about 2.2 kb in size. Amplicons of similar size were previously reported in some *L. monocytogenes* strains that belong to serotype 1/2a and ST121 that were found to harbor homologs of *L. innocua* genes instead of SSI-1 genes in this region (Hein *et al.*, 2011).

In conclusion, the present study delivers insights into the relationships and virulence-associated characteristics of clinical *L. monocytogenes* strains occurring in Switzerland. No links between serotypes, lineages, or MLST types on the one hand, and clinical origin of the strains on the other hand, were found. The MLST sequence types found in Switzerland are largely distributed across the global clonal diversity of *L. monocytogenes*. The results further highlight strain differences in the occurrence of several genetic elements, which are linked to bacterial persistence and/or pathogenicity.

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Disclosure Statement

No competing financial interests exist.

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