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Characteristics of *Listeria monocytogenes* strains isolated from fecal samples of healthy slaughtered livestock

Charakteristika von Listeria monocytogenes-Stämmen isoliert aus Kotproben von Schlachttieren

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Summary

Thirty-two *L. monocytogenes* strains isolated from fecal samples of slaughtered pigs, cattle, and sheep were characterized to gain insights into genetic relationships and virulence-associated traits. The majority (87.5 %) of the 32 strains belonged to serotype 1/2a (genetic lineage II), followed by serotype 4b (genetic lineage I). Multilocus sequence typing (MLST) grouped the 32 strains into 10 STs that were assigned to eight clonal complexes (CC1, CC4, CC6, CC8, CC29, CC37, CC54, CC90) and two singletons (ST36, ST200). ST 709 (CC90) was designated for the first time and comprised 20 serotype 1/2a strains from cattle and sheep. PCR screening for Listeriolysin S showed the presence of this virulence factor in the four lineage I/serotype 4b strains belonging to CC1, CC4, CC6, and CC54. Sequence analysis of the whole internalin A gene *(inlA)* of 10 strains representing the 10 STs showed that all but one strain (CC6/serotype 4b) encoded full-length proteins. Thus, slaughtered pigs, cattle, and sheep can be asymptomatic carriers of *L. monocytogenes* representing a potential health risk. To encounter this threat during slaughter, prevention of contamination of carcasses and the environment is of major importance, in particular adherence to good slaughter hygiene practices. With regard to slaughtered animals, process stages with an increased risk of direct or indirect fecal contamination deserve special attention.

Keywords: *Listeria monocytogenes,* livestock, serotypes, MLST, virulence factors

Zusammenfassung

Im Rahmen der vorliegenden Arbeit wurden aus Kotproben von geschlachteten Schweinen, Rindern und Schafen isolierte *L. monocytogenes*-Stämme weiterführend charakterisiert. Die Mehrheit (87.5 %) der 32 Stämme gehörte zum Serotyp 1/2a (genetische Linie II), gefolgt vom Serotyp 4b (genetische Linie I). Die 32 Stämme repräsentierten 10 MLST Sequenz-Typen, die sich acht klonalen Komplexen (CC1, CC4, CC6, CC8, CC29, CC37, CC54, CC90) und zwei Singletons (ST36, ST200) zuordnen liessen. ST 709 (CC90) wurde dabei zum ersten Mal beschrieben und umfasste 20 Serotyp 1/2a Stämme von Rindern und Schafen. In der PCR für Listeriolysin S erwiesen sich die vier Stämme der genetischen Linie I (Serotyp 4b; CC1, CC4, CC6, CC54) als positiv für diesen Virulenzfaktor. Die Internalin A *(inlA)* Sequenzanalyse bei 10 Stämmen, die die 10 MLST Sequenz-Typen repräsentierten, zeigte, dass bei den meisten Stämmen dieser Virulenzfaktor in der vollen Länge kodiert wurde. Schweine, Rinder und Schafe können daher asymptomatische Träger von *L. monocytogenes* sein, die für den Menschen ein Gesundheitsrisiko darstellen. Bei der Fleischgewinnung ist es daher von zentraler Bedeutung, die Kontamination von Schlachttierkörpern und der Schlachthof-Umgebung zu verhindern. Entscheidend ist hierfür die strikte Einhaltung der Schlachthygiene-Massnahmen. Besondere Aufmerksamkeit ist dabei Schlachtprozessstufen mit einem erhöhten (fäkalen) Kontaminationsrisiko für die Schlachttierkörper zu schenken.

Schlüsselwörter: *Listeria monocytogenes,* Schlachttiere, Serotypen, MLST, Virulenzfaktoren

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Introduction

Listeria monocytogenes is an important foodborne pathogen that has significant impacts on public health and economy worldwide. *L. monocytogenes* has the potential to cause serious and life-threatening conditions (including septicemia, meningitis, meningoencephalitis, and abortion) in persons with reduced immunity (Allerberger and Wagner, 2010). Because of its high case fatality rate in persons at risk, listeriosis ranks among the most frequent causes of death due to foodborne illness. In the European Union, a total of 1'763 confirmed human cases of listeriosis (notification rate of 0.4 cases per 100'000 population) have been reported in 2013 (EFSA/ECDC, 2015). In Switzerland, four big listeriosis outbreaks have occurred so far, including the latest one in 2013/2014 caused by contaminated ready-to-eat salad (Büla et al., 1995; Bille et al., 2006; Hächler et al., 2013; Stephan et al., 2015). *Listeria* spp. are widely distributed in the environment and certain strains may become established and persist in the processing environment (Blatter et al., 2010; Carpentier and Cerf, 2011; Larivière-Gauthier et al., 2014). Other reservoirs include domestic and wild animals, but their significance in view of foodborne diseases and potential transmission routes (during slaughter) remains to be elucidated.

With regard to meat production, food-producing animals were recognized in recent years as asymptomatic carriers of important human pathogens. The available literature on *L. monocytogenes* in healthy livestock is limited, but *L. monocytogenes* has been described in different species of food-producing animals (Esteban et al., 2009; Mohammed et al., 2010; Boscher et al., 2012; Sarno et al. 2012; Hasegawa et al., 2014). The carriage of such pathogens in livestock is correlated with the risk of carcass contamination and these pathogens might enter the food chain during slaughter (Hellström et al., 2010). To estimate the associated health risk for humans, data on the animals' probability of carrying *L. monocytogenes* must be complemented by analysis of genetic relationships and virulence-associated traits of strains. The aim of the present study was therefore to further characterize *L. monocytogenes* strains isolated from fecal samples of slaughtered livestock (pigs, cattle, sheep) by phenotyping and genotyping methods.

Materials and methods

L. monocytogenes strains

Thirty-two *L. monocytogenes* strains isolated from fecal samples of healthy slaughtered fattening pigs, cattle, and sheep (pigs: n = 6, cattle: n = 16; sheep: n = 10) were further characterized in this study. Briefly, 467 fecal samples from pigs, cattle and sheep were collected within a five-months period (August–December 2013) in a Swiss abattoir. Sampled animals originated from the north and central part of Switzerland. Fecal samples were collected after evisceration from the large intestine using swabs. For *L. monocytogenes* detection, the samples were enriched using a two-step procedure in accordance with ISO 11290-1:2004 (Half-Fraser broth, 24 h at 30 °C; Fraser broth 24 h at 37 °C; Oxoid, Pratteln, Switzerland). Subcultures were streaked onto Chromogenic Listeria Agar (Oxoid) supplemented with Listeria Selective and Differential Supplement and incubated for 48 h at 37 °C. Presumptive *L. monocytogenes* colonies (green-blue colonies surrounded by an opaque halo on the chromogenic agar) were confirmed, after incubation in Brain Heart Infusion Broth (Oxoid; 24 h at 37 °C), using the Assurance GDS® Tq assay for *L. monocytogenes* (BioControl Systems, Bellevue, WA, USA).

Serotyping and lineage PCR

Serotyping of strains was performed at the Swiss National Reference Center for Enteropathogenic Bacteria and Listeria (NENT; Institute for Food Safety and Hygiene, University of Zurich) using the commercial set of Listeria O-factor and H-factor antisera from Denka Seiken (Pharma Consulting, Burgdorf, Switzerland). Lineage-specific PCR assays were performed using the primers and protocol described by Ward et al. (2004), with minor modifications. Briefly, amplifications were performed in 20-µl volumes with 0.4 µM final primer concentrations (*actA1, plcB2, actA3-plcB3*), 10 µl of GoTaq Green Master Mix (Promega, Madison, USA), and 100 ng of extracted genomic DNA (DNeasy Blood and Tissue Kit; Qiagen, Hilden, Germany). The following cycling conditions were used: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and 72 °C for 2 min.

Multi-locus sequence typing (MLST)

MLST was performed as described by Ragon et al. (2008) with the following modifications: amplifications were performed in 50-µl volumes with 0.4 µM final primer concentrations, 25 µl of HotStarTaq Master Mix (Qiagen), and 50 ng of extracted genomic DNA (Qiagen). The following cycling conditions were used: for 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; and 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 concluded with a 10-min 72 °C step. Amplicons were purified using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, St Louis, MO, USA). Sequencing of the amplicons (Microsynth, Balgach, Switzerland) was performed using the universal primers described by Ragon et al. (2008). Alleles and sequence types (STs) are publicly available at www.pasteur.fr/mlst.

Listeriolysin S

The presence of Listeriolysin S was determined using three primer pairs as described by Cotter et al. (2008). Amplifications were performed in 50-µl volumes with 0.4 µM final primer concentrations *(1118, 1118deg, llsAint-llsAsoeD),* 25 µl of GoTaq Green Master Mix 2X (Promega) and 100 ng of extracted genomic DNA (Qiagen). The following cycling conditions were used: 94 °C for 3 min, followed by 35 cycles of 94 °C for 15 s, 56 °C for 30 s, 72 °C for 30 s, and 72 °C for 10 min.

Internalin A gene sequence profiling

Internalin A *(inlA)* profiling was performed on one strain of each ST using PCR primers described previously by Orsi et al. (2007). To cover full-length *inlA,* four pairs of primers were used. Amplifications were performed in 50-µl volumes using the Phusion Green Hot Start II High-Fidelity DNA Polymerase (Thermo Fischer Scientific, Waltham, MA, USA) and conditions recommended by the supplier (Fermentas, St. Leon-Rot, Germany). Amplicons were sequenced commercially by Microsynth and analyzed for mutations with CLC Main Workbench 6.9.1.

Results and Discussion

Of the 32 *L. monocytogenes* strains isolated from slaughtered pigs, cattle, and sheep, 28 (87.5 %) belonged to serotype 1/2a (genetic lineage II) and four (12.5 %) to serotype 4b (genetic lineage I). Serotype 1/2a strains thereby predominated in all three examined animal species (Tab. 1). Serotype 1/2a strains are frequently found in food products and food-processing environments (Gianfranceschi et al., 2009; Parisi et al., 2010; Kramarenko et al., 2013; Martín et al., 2014). Human clinical cases are frequently associated with strains of serotypes 1/2a, 1/2b, and 4b, and infections due to serotype 1/2a strains have increased in recent years (Gianfranceschi et al., 2009; Althaus et al., 2014; Lopez-Valladares et al., 2014). In a Swiss study examining 93 *L. monocytogenes* strains from human infections with mainly severe illness during 2011–2013 (Althaus et al., 2014), the majority of the strains belonged to serotype 1/2a (62.4 %; lineage II), followed by serotype 4b (30.1 %; lineage I), serotype 1/2b (lineage I), and serotype 1/2c (lineage II).

TABLE 1: *Characterization of 32 Listeria monocytogenes strains isolated from fecal samples of slaughtered food-producing animals in Switzerland.*

Origin	No. of strains	Serotype	Genetic lineage	Clonal complex/ singletons	Listeriolysin S
Pigs	4	1/2a	II	CC8, ST200 (3x)	–
	2	4b	I	CC4, CC6	+
Cattle	15	1/2a	II	ST36, CC37 (2x), CC90 (12x)	–
	1	4b	I	CC54	+
Sheep	9	1/2a	II	CC29, CC90 (8x)	–
	1	4b	I	CC1	+

MLST grouped the 32 *L. monocytogenes* strains into 10 STs that were assigned to eight clonal complexes (CC1, CC4, CC6, CC8, CC29, CC37, CC54, CC90) and two singletons (ST36, ST200). Serotype 1/2a (lineage II) strains were assigned to CC8, CC29, ST36, CC37, CC90, and ST200, while serotype 4b (lineage I) strains belonged to CC1, CC4, CC6, and CC54. Table 1 shows the distribution of CCs among the strains from the three examined animal species. CC90 (ST 709) comprised 20 serotype 1/2a strains isolated from both cattle and sheep. ST709 (CC90) was thereby designated for the first time in the present study. The remaining 12 strains showed a genetically heterogeneous picture (eight CCs, one singleton). In human-, food-, and environment-associated *L. monocytogenes* strains, great genetic diversity has been described (Parisi et al., 2010; Cantinelli et al., 2013; Althaus et al., 2014; Haase et al., 2014). Genotyping of 300 *L. monocytogenes* strains from five continents and diverse sources by Chenal-Francisque et al. (2011) showed the existence of few prevalent and globally distributed clones. CC1, CC2, and CC3 were thereby highly prevalent in lineage I strains (n = 199), while CC9 and CC7 predominated in lineage II strains (n = 98). The 10 STs found in our study were distributed across the global clonal diversity of *L. monocytogenes* (Ragon et al., 2008; Chenal-Francisque et al., 2011; Cantinelli et al., 2013). Comparisons of our livestock strains with Swiss clinical human strains showed that eight of the CCs/singletons identified among the *L. monocytogenes* strains from livestock (CC1, CC4, CC6, CC8, CC29, ST36, CC37, CC54) were also found in strains from human infections (Althaus et al., 2014).

Furthermore, to gain insights into virulence-associated traits, the presence of listeriolysin S and the internalin A gene sequence was determined. Listeriolysin is an oxidative stress inducible hemolysin and is present in a subset of lineage I strains (Cotter et al., 2008; Orsi et al., 2011). PCR screening showed the presence of listeriolysin S in the four lineage I/serotype 4b strains belonging to CC1, CC4, CC6, and CC54 (Tab. 1). As expected, this virulence factor was absent in the 28 lineage II/serotype 1/2a strains. Internalin A is a cell wall anchored protein, which facilitates the invasion of intestinal cells through interaction with E-cadherin receptors (Seveau et al., 2007). Multiple mutations leading to premature stop codons (PMSCs) in the *inlA* gene have been reported, and these mutations are associated with attenuated virulence (Seveau et al., 2007; Van Stelten et al., 2010). PMSCs are thereby observed more frequently in lineage II strains than in lineage I strains (Orsi et al., 2011). Clinical strains from human patients commonly harbor full-length *inlA* (Van Stelten et al., 2010; Althaus et al., 2014). Sequence analysis of the *inlA* gene from strains representing all 10 STs identified in the present study showed that all but one strain encoded full-length proteins. Only the CC6/serotype 4b strain originating from pig feces showed an *inlA* PMSC mutation caused by a three-codon deletion in amino acid position 738–740.

In conclusion, characterization of *L. monocytogenes* strains isolated from fecal samples of livestock (pigs, cattle, sheep) at slaughter showed that the strains belonged to serotypes commonly associated with human cases, factors critical to the virulence of *L. monocytogenes* were found, and several of the clonal complexes have also been described in isolates from human infections. Thus, with regard to meat production, there is a threat for the contamination of carcasses and the slaughterhouse environment with *L. monocytogenes* representing a potential health risk. To encounter this threat, prevention of contamination during slaughter is of major importance, in particular adherence to good slaughter hygiene practices and application of effective cleaning and disinfection procedures. With regard to slaughtered animals as carriers of *L. monocytogenes* (and other important bacterial pathogens), process stages with an increased risk of direct or indirect fecal contamination (e. g. dehiding when slaughtering cattle or sheep or evisceration) deserve special attention.

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Conflict of interest

The authors declare that no conflicts of interest exist, which could influence the content or opinions presented in the manuscript.

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