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RESEARCH ARTICLE

Characteristics of *Listeria monocytogenes* isolated from tonsils of slaughtered fattening pigs in Switzerland

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Abstract Of 504 tonsil samples from slaughtered fattening pigs, 5.6 % were positive for *Listeria monocytogenes* by culture after enrichment. The 28 *L. monocytogenes* isolates were analyzed to gain insights into genetic relationships and virulence-associated traits. Of the 28 isolates, 57 % belonged to serotype 1/2a (genetic lineage II), 25 % to serotype 4b (genetic lineage I) and 18 % to serotype 1/2b (genetic lineage I). These serotypes are commonly associated with human listeriosis cases. Multilocus sequence typing assigned the 28 isolates to 16 clonal complexes (CCs) and three singletons, including one new sequence type (ST768). Several of these CCs were also found in strains from human infections. Sequence analysis of the whole internalin A gene (*inlA*) showed that all but one isolate (CC6/serotype 4b) encoded full-length proteins. Clinical strains from human patients commonly harbor full-length *inlA*. On the other hand, genes for benzalkonium chloride tolerance were not found and all but one isolate lacked genes for the stress survival islet (SSI-1). Thus, tonsils of slaughtered fattening pigs can be colonized with *L. monocytogenes* of public health impact. To counteract this threat during slaughter, prevention of contamination of carcasses and the environment is of major importance, in particular adherence to good slaughter hygiene practice. With regard to pig tonsils, special attention must be given to the handling and contamination of head meat and pig tongues.

Keywords *Listeria monocytogenes* · Pig tonsils · Serotypes · Multilocus sequence typing · Virulence factors

1 Introduction

Listeria monocytogenes is an important foodborne pathogen with a significant impact on public health and economy worldwide. *L. monocytogenes* has the potential to cause serious and potentially life-threatening conditions (including septicemia, meningitis, meningoencephalitis and abortion) in persons with diminished 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 1763 confirmed human cases of listeriosis (notification rate of 0.44 cases per 100,000 population) were reported in 2013 (EFSA/ECDC 2015). *L. monocytogenes* 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 slaughtered pigs, the animals might be asymptomatic carriers of important human pathogens such as *L. monocytogenes*. Several studies

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thereby reported that *L. monocytogenes* was isolated more frequently from pig tonsils than from fecal samples, but detection rates in the tonsils of slaughtered pigs varied considerably (Buncic 1991; Fredriksson-Ahomaa et al. 2009; Kanuganti et al. 2002). Pig tonsils may play a role in the contamination of pluck sets, carcasses and the slaughterhouse environment during slaughter (Fredriksson-Ahomaa et al. 2009). To evaluate the potential 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 determine the occurrence of *L. monocytogenes* in the tonsils from fattening pigs at slaughter and to further characterize isolated *L. monocytogenes*. Thereby, serotypes of isolated *L. monocytogenes* were compared with those associated with human infections, multilocus sequence typing (MLST) was performed to get information on genetic relationships (between isolates and potential human associations) and internalin A gene sequence profiling was used to detect mutations that might attenuate the virulence. Moreover, factors contributing to the persistence of *L. monocytogenes* in food-associated environments (benzalkonium chloride tolerance, stress survival islet) were examined.

2 Materials and methods

2.1 Abattoir and sampling

In an abattoir processing about 250 pig carcasses per hour, 504 tonsil samples were collected from 504 healthy fattening pigs. Sampled pigs originated from the north and central part of Switzerland and were about 6 months old. Sampling comprised two phases: in the first phase (March–May 2011), 250 carcasses from 126 batches were sampled; in the second phase (October 2014), 254 carcasses from 177 batches were sampled. Tonsil samples (*Tonsilla veli palatini*) were obtained at the end of slaughtering when the head had been cut off. Samples were excised using sterile forceps and scissors, placed into stomacher bags and transported cooled to the laboratory. Examinations in the laboratory were performed within 4 h after sampling.

2.2 Detection and identification of *L. monocytogenes*

Examination for *L. monocytogenes* was performed in accordance with ISO 11290-1:2004 using a two-step enrichment procedure (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 streaked onto sheep blood agar for appraisal of hemolysis.

2.3 Serotyping and lineage PCR

Listeria monocytogenes isolates were serotyped 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). The lineage-specific multiplex PCR assay was 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* and *actA3-plcB3*), 10 µl of GoTaq Green Master Mix (Promega, Madison, WI, USA) and 100 ng of extracted genomic DNA (DNeasy Blood and Tissue Kit; Qiagen, Hilden, Germany). The cycling conditions were: 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 a final step at 72 °C for 2 min.

2.4 Multilocus 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 cycling conditions were: for the *bglA, cat* and *ldh* loci: 94 °C for 4 min, followed by 35 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 http://www.pasteur.fr/mlst.

2.5 Internalin A gene (*inlA*) sequence profiling

To cover full-length *inlA*, four pairs of previously described primers were used for *inlA* profiling (Orsi et al. 2007). Amplifications were performed in 50-µl volumes using the Phusion Green Hot Start II High-Fidelity DNA Polymerase in accordance with the manufacturers' instructions (Thermo Fisher Scientific, Waltham, MA, USA). The cycling conditions were: 98 °C for 30 s, followed by 35 cycles of 98 °C for 5 s, 50 °C for 30 s, 72 °C for 1.5 min and a final step at 72 °C for 10 min. Amplicons were sequenced commercially by Microsynth and analyzed for mutations with CLC Main Workbench 6.9.1.

2.6 Benzalkonium chloride (BC) tolerance and stress survival islet (SSI-1)

BC tolerance was examined by testing for *bcrABC* genes using primers *p1* and *p2* (Elhanafi et al. 2010) and for the Tn*6188* transposon (Müller et al. 2013). Amplifications were performed using the GoTaq Green Master Mix (Promega). In addition, the presence of SSI-1 (*lmo0444-lmo0448*) was examined as described by Ryan et al. (2010). The Phusion Green Hot Start II High-Fidelity DNA Polymerase was used for the amplifications in accordance with the manufacturers' instructions (Thermo Fisher Scientific). The cycling conditions were (*lmo0444-lmo0448*): 98 °C for 30 s, followed by 35 cycles of 98 °C for 5 s, 59 °C for 15 s, 72 °C for 4.5 min and a final step at 72 °C for 10 min.

3 Results and discussion

3.1 Detection, serotypes and genetic lineages of *L. monocytogenes*

Listeria monocytogenes were detected by culture after enrichment in 28 (5.6 %) of the 504 tonsil samples obtained from 504 slaughtered fattening pigs. With regard to the two sampling phases (March–May 2011; October 2014), 3.2 % (8/250) of the animals and 6.3 % (8/126) of the batches tested positive in the first sampling phase and 7.9 % (20/254) of the animals and 11.3 % (20/177) of the batches in the second sampling phase. The results from the two sampling phases differed significantly at the animal level (Chi-square test, $P < 0.05$), but the cause for this difference could not be assigned. In other European studies examining pig tonsils, the prevalence of *L. monocytogenes* ranged from 14 to 45 % (Autio et al. 2004; Buncic 1991; Fredriksson-Ahomaa et al. 2009).

Of the 28 *L. monocytogenes* strains isolated from pig tonsils in the present study (Table 1), 16 (57 %) belonged to serotype 1/2a (genetic lineage II), seven (25 %) to serotype 4b (genetic lineage I) and five (18 %) to serotype 1/2b (genetic lineage I). Serotype 1/2a strains are frequently found in food products (Gianfranceschi et al. 2009; Martín et al. 2014; Parisi et al. 2010). The majority of human listeriosis cases are associated with serotype 1/2a, 1/2b and 4b strains and infections due to serotype 1/2a strains have increased in recent years (Althaus et al. 2014; Gianfranceschi et al. 2009; Lopez-Valladares et al. 2014). In a current Swiss study characterizing 93 *L. monocytogenes* strains isolated during 2011–2013 from human infections (Althaus et al. 2014), the majority of the strains belonged to serotype 1/2a (62.4 %), followed by serotypes 4b (30.1 %), 1/2b and 1/2c.

3.2 Multilocus sequence typing

MLST grouped the 28 *L. monocytogenes* isolates into 19 STs that were assigned to 16 clonal complexes (CC1, CC4, CC5, CC6, CC8, CC20, CC21, CC26, CC29, CC59, CC177, CC224, CC403, CC412, CC415, CC451) and three singletons (ST200, ST226, ST768). ST768 was thereby designated for the first time and the respective isolate belonged to serotype 1/2a. Table 1 shows the distribution of CCs among serotype 1/2a, 1/2b and 4b isolates of the two sampling phases. CC1 (serotype 4b isolates), CC6 (serotype 4b isolates), CC20 (serotype 1/2a isolates) and CC224 (serotype 1/2b isolates) were thereby identified among strains from both sampling phases. Overall, the results show the clonal heterogeneity of *L. monocytogenes* from pig tonsils. Great genetic diversity has also been described in human- and food-derived *L. monocytogenes* strains (Althaus et al. 2014; Haase et al. 2014; Parisi et al. 2010). Twelve of the CCs identified among the isolates from pig tonsils in the present study (CC1, CC4, CC5, CC6, CC8, CC21, CC26, CC29, CC59, CC224, CC403, CC412) were also found in *L. monocytogenes* strains from human patients in Switzerland (Althaus et al. 2014).

3.3 Internalin A

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 *inlA* mutations leading to premature stop codons (PMSCs) have been reported and these mutations might attenuate the virulence (Seveau et al. 2007; Van Stelten et al. 2010). Clinical *L. monocytogenes* strains from human patients commonly

Table 1 Characteristics of the 28 *L. monocytogenes* strains isolated from the tonsils of slaughtered fattening pigs in Switzerland

Sampling phase: year	No. of strains	Serotype	Genetic lineage	Clonal complex
2011	2	1/2a	II	CC20, CC451
	2	1/2b	I	CC59, CC224
	4	4b	I	CC1 (2x), CC4, CC6
2014	14	1/2a	II	CC8, CC20, CC21 (2x), CC26, CC29, CC177, ST200, ST226, CC403, CC412 (2x), CC415, ST768
	3	1/2b	I	CC5, CC224 (2x)
	3	4b	I	CC1, CC6 (2x)

harbor full-length *inlA* (Althaus et al. 2014; Van Stelten et al. 2010). In the present study, sequence analysis of the *inlA* gene showed that all but one isolate encoded full-length proteins. The respective CC6/serotype 4b isolate showed an *inlA* gene PMSC mutation caused by a three-codon deletion in amino acid position 738–740.

3.4 Benzalkonium chloride tolerance and stress survival islet

Increased BC tolerance and genes within the SSI-1 contribute to the persistence of *L. monocytogenes* in food-associated environments (Mullapudi et al. 2008; Ryan et al. 2010). Of the 28 *L. monocytogenes* isolates from pig tonsils, none harbored *bcrABC* genes or the Tn*6188* transposon. SSI-1 genes were only detected in one (CC224/serotype 1/2b) of the 28 isolates. Thus, these mechanisms promoting survival and growth of *L. monocytogenes* under food-associated stress conditions seem not to be widespread among isolates from tonsils of slaughtered fattening pigs.

4 Conclusions

Listeria monocytogenes were found in the tonsils of slaughtered fattening pigs in Switzerland (detection rate of 5.6 % at the animal level), but the prevalence was lower compared to the results from other European countries. Further characterization of the *L. monocytogenes* isolates from pig tonsils showed that the identified serotypes are commonly associated with human listeriosis cases, several of the clonal complexes were also found in strains from human patients and full-length internalin A genes predominated among them (as in clinical strains). Thus, tonsils of slaughtered fattening pigs pose a threat for the contamination of carcasses and the slaughterhouse environment with *L. monocytogenes* representing a potential health risk. To counteract 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 pig tonsils colonized with *L. monocytogenes* (and other important bacterial pathogens as e.g. *Yersinia enterocolitica*), special attention must be given to the handling and the potential contamination of the head meat, pig cheeks and pig tongues.

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