Source tracking on a dairy farm reveals a high occurrence of subclinical mastitis due to hypervirulent Listeria monocytogenes clonal complexes

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Running head: Tracking of L. monocytogenes on a dairy farm
Abstract

Aims: An extensive source investigation was conducted on a dairy farm with neurolisteriosis and subclinical mastitis cases to identify infection source and potential transmission routes of Listeria monocytogenes.

Methods and Results: A total of 36 L. monocytogenes isolates were obtained from animal clinical cases (neurolisteriosis and udder infection) and the farm environment (silage, feces, water). Isolates were typed using pulsed-field gel electrophoresis (PFGE) and whole-genome sequencing (WGS). Their virulence potential was assessed using the gentamicin protection assay and WGS-based identification of virulence genes. PFGE and WGS revealed a high genetic diversity of L. monocytogenes. An epidemiological link was confirmed for isolates from (i) several subclinical mastitis cases, (ii) silage and subclinical mastitis cases and (iii) different water sources. The neurolisteriosis isolate belonged to clonal complex (CC) 1, but infection source was not identified. A high occurrence (9/47 cows; 19.1%) of subclinical mastitis was observed with isolates belonging to CC2, CC4 and CC11.

Conclusions: The dairy farm environment was contaminated with diverse L. monocytogenes strains, including genotypes associated with human disease. Several isolates harbored genetic determinants associated with increased infectious potential in humans.

Significance and Impact of the Study: Results suggest that subclinical listerial mastitis should not be neglected as a potential source of milk contamination. The presence of hypervirulent CCs in subclinical mastitis cases calls for the implementation of improved mastitis detection.
Keywords
Listeria monocytogenes, food safety, WGS (whole-genome sequencing), PFGE (pulsed-field gel electrophoresis), virulence, dairy farm.

Introduction
Listeria monocytogenes is a ubiquitous Gram-positive bacterium that causes listeriosis in humans and in several animal species. It is characterized by its exceptional ability to grow under various adverse environmental conditions (Tienungoon et al. 2000). Although the incidence of listeriosis in humans is low, L. monocytogenes is recognized as an important foodborne pathogen due to the severity of the disease and a high (up to 30%) mortality rate (Hoelzer et al. 2012). In ruminants, which are the most susceptible farm animals, the disease usually manifests as neurolisteriosis or uterine infections (Low and Donachie 1997). The latter often lead to abortion, stillbirth or septicemia in neonates (Nightingale et al. 2004). Other, less frequent clinical forms of animal listeriosis are ocular infections, enteritis and mastitis (Low and Donachie 1997; Oevermann et al. 2010). A strong link between ingestion of contaminated feed, in particular low-quality silage, and listeriosis in ruminants has been established (Low and Donachie 1997; Nightingale et al. 2004), although other on-farm sources of infection, such as water, feeding troughs and bedding, are possible (Mohammed et al. 2009; Oevermann et al. 2010). In rare types of listeriosis, other (exogenous) infection routes are prominent, including ascendant infection of the udder in mastitis and direct eye inoculation in ocular listeriosis (Starič et al. 2008; Hof 2017). Listeriosis usually occurs sporadically, but cases of animal outbreaks have been reported (Wiedmann et al. 1999; Wagner et al. 2005; Bundrant et al. 2011; Dreyer et al. 2015).
L. monocytogenes is a genetically heterogeneous species with a clonal population structure (Chenal-Francisque et al. 2011; Cantinelli et al. 2013). It is divided into four phylogenetic lineages, which vary in terms of their ecological, evolutionary and phenotypic characteristics; lineage I is associated with a clinical origin, whereas lineage II is food-associated (Maury et al. 2016). Recently, hypovirulent and hypervirulent clones have been described (Maury et al. 2016). L. monocytogenes is widely distributed in the farm environment; however, its ecology and transmission dynamics are complex and remain poorly understood (Nightingale et al. 2004; Dreyer et al. 2016; Castro et al. 2018). The dairy farm environment and cattle harbor a large diversity of L. monocytogenes strains, including genotypes involved in human listeriosis (Borucki et al. 2004; Rocha et al. 2013). Asymptomatic carriers, in particular cattle, are an important reservoir of L. monocytogenes, enabling its multiplication in host cells and transmission into the farm environment through fecal shedding (Nightingale et al. 2004; Esteban et al. 2009). Feed quality and storage, animal health, hygiene practice and farm management practice are associated with L. monocytogenes occurrence in the farm environment and animal listeriosis incidence (Sanaa et al. 1993; Nightingale et al. 2005; Castro et al. 2018). Moreover, the farm environment is a possible source of contamination of meat and dairy food processing facilities (Muhterem-Uyar et al. 2015).

Reliable and discriminatory methods for L. monocytogenes typing are crucial for outbreak identification, transmission pathway elucidation and epidemiological surveillance (Salipante et al. 2015). Pulsed-field gel electrophoresis (PFGE) was the “gold standard” typing method for L. monocytogenes but has recently been replaced by whole-genome sequencing (WGS) due to its superiority to PFGE and other genotyping methods with regard to the discriminatory power and accuracy of phylogenetic inferences (Graves and Swaminathan 2001; Deng et al. 2015; Salipante et al. 2015; Moura et al. 2016). Because of its ability to accurately delineate outbreak clusters, WGS has been widely used in the epidemiological investigations
of human listeriosis cases (Jackson et al. 2016; Moura et al. 2016). On the contrary, in veterinary surveillance (e.g. animal listeriosis outbreaks), molecular typing of L. monocytogenes is not routinely performed (Wagner et al. 2005; Dreyer et al. 2015).

This study describes an extensive source investigation of L. monocytogenes on a small organic dairy farm with neurolisteriosis and subclinical mastitis. The objectives of the study were to investigate the genetic diversity and virulence potential of the isolates and to determine possible on-farm transmission patterns and listerial infection source using PFGE and WGS. To the best of our knowledge, this is the first case of an on-farm source investigation of animal listeriosis enhanced by WGS.

**Materials and Methods**

**Case description**

In March 2014, a 6-year old dairy cow on a small organic dairy farm displayed severe neurological symptoms. Due to their severity, the cow was euthanized and subjected to necropsy. Neurolisteriosis was histopathologically confirmed and L. monocytogenes was isolated from brain tissue.

The farm utilized its own bull for natural breeding purposes. No history of abortion or other reproductive disorders had previously been recorded. The herd consisted of 18 heifers and 50 dairy cows for milk distribution. The animals primarily grazed pasture and were additionally fed grass silage. The pasture had been fertilized with cattle slurry and manure, which occasionally contained spoiled silage and other feed. The farm was surrounded by woods inhabited by a large number of wildlife species, especially deer.

An extensive epidemiological investigation (reviewed in Table 1) was performed with the aim of identifying L. monocytogenes infection source and elucidating possible on-farm
transmission pathways. Five days after neurolisteriosis confirmation, the first sampling of lactating cows (47 milk samples pooled from all four quarters) was performed; in addition, their silage was also sampled (one sample). As the presence of L. monocytogenes was demonstrated in the raw milk from nine cows, they were identified as having subclinical mastitis, separated from the herd and treated with antibiotics for one month.

Approximately three weeks after neurolisteriosis confirmation, the second sampling of lactating and treated cows was performed (April 15, Table 1), in which the following samples were collected: (i) individual and pooled (bulk tank) milk samples from lactating cows; (ii) individual and pooled milk samples from treated cows; (iii) fecal samples from treated cows; (iv) bull and deer fecal samples; and (v) water samples from the farm environment (barn pipe and trough, pond). The farm had a private water supply, providing household and barn drinking water. Because the barn water samples were positive for L. monocytogenes and the same water supply was also used for the household, the farmer additionally collected drinking water samples (50 ml) from the household kitchen, barn milk room and barnyard well (April 24, Table 1).

After the treatment (i.e. six weeks after neurolisteriosis confirmation), mastitic cow milk samples were once again examined. Moreover, the bulk tank milk (receiving milk from the remaining cows) and drinking water from the household were sampled at the same time (May 4, Table 1).

In total, 36 L. monocytogenes isolates were obtained and subjected to molecular serotyping and PFGE typing (Table 1), of which 16 were subjected to WGS and nine to gentamicin protection assay (see File S1 in supplemental material).

Sample collection
Approximately 10 ml of milk from each quarter was collected in sterile plastic containers and analyzed separately (four samples per cow). For bulk tank or pooled milk, 25-ml samples were collected. Silage was sampled at five different sites, pooled to a total sample of 3 kg and mixed well. Approximately 25 g of fecal samples were collected into sterile bags; for cows with listerial subclinical mastitis, fecal samples were rectally collected, whereas for deer and bull, ground fecal samples were collected. Water samples, other than those collected by the farmer, were directly collected in sterile containers from each sampling site, and after their transportation to the laboratory in cooling boxes, approximately one liter of the samples was vacuum-filtered through sterile 0.22-µl bottle-top filters (“rapid”-Filtermax system; TPP, Switzerland). The analysis was initiated on the day of sample collection.

**Bacteriology**

All collected samples except silage were regarded as clinical or environmental, thus *L. monocytogenes* isolation was performed according to the World Organization for Animal Health Manual (OIE 2004), and identified according to standard method ISO 11290-1 (ISO 1996; ISO 2004). The brain tissue sample taken from the cow with meningoencephalitis was subjected to direct detection on blood agar (Blood agar base No.2; Oxoid, UK) and two-step enrichment method using selective liquid (Fraiser broth; Oxoid) and solid media ALOA (Biolife, Italia) and PALCAM (Oxoid) agar plates. Fecal and feed samples (25 g) as well as water filters and raw milk samples (10 ml) were also examined according to OIE and ISO methods. For brain tissue, milk and silage, one *L. monocytogenes* isolate per sample was stored and typed. For water and fecal samples, five isolates per sample were stored and typed with the aim of investigating *L. monocytogenes* diversity in environmental samples where high genetic diversity is expected.
**PFGE typing**

All isolates (n=36) were PFGE-typed according to the PulseNet standardized protocol (Graves and Swaminathan 2001). Briefly, *L. monocytogenes* genomic DNA was digested with ApaI and AscI restriction endonucleases. The generated DNA fragments were separated using the CHEF-DR II system (Bio-Rad, USA) in accordance with recommended electrophoretic protocol. PFGE profiles were analyzed using BioNumerics v7.6.2 software (Applied Maths, Belgium). A combined AscI-ApaI profile analysis was performed as previously described (Félix et al. 2018). Due to high genetic stability of *L. monocytogenes* and limited time span of the study, isolates differing in one or more bands were considered to belong to different pulsotypes (Barrett et al. 2006).

**WGS**

In total, 16 *L. monocytogenes* isolates with different pulsotype and isolation origin were selected for WGS typing. Illumina MiSeq or Ion Torrent PGM platforms were employed to >30× coverage. A list of all sequenced strains and associated metadata is given in Table S1.

Twelve isolates underwent WGS with Ion Torrent Technology (Thermo Fisher Scientific, USA), which was performed at the Veterinary Faculty, Slovenia. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) and sheared enzymatically to ~400 bp fragments using the Ion Xpress Plus gDNA Fragment Library Kit (Thermo Fisher Scientific). WGS libraries were prepared according to IonXpress Plus gDNA Fragment Library Preparation Protocol (Thermo Fisher Scientific). Template amplification and enrichment was performed on the Ion OneTouch 2 System using the Ion PGM Template OT2 400 Kit System (Thermo Fisher Scientific). Sequencing was performed on the Ion PGM System using the Ion PGM Hi-Q Sequencing Kit (Thermo Fisher Scientific) according to the manufacturer instructions.
In total, 7/16 isolates underwent WGS with Illumina Technology (Illumina, USA), which was performed at the Brain & Spine Institute (Institut du Cerveau et de la Moelle épinière, ICM), France. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, USA). Paired-end sequencing (2×100 or 2×150 bp) was performed using TruSeq automated library preparation (Illumina) and NextSeq 500 System (Illumina).

**In silico MLST typing**

*In silico* multilocus sequence typing (MLST) was performed based on WGS data. Firstly, reads were quality-trimmed using Cutadapt v1.17 (option –q 20) and assembled into contigs using SPAdes v3.11.1 (Bankevich et al. 2012). MLST types were assigned based on assembled reads according to the Institut Pasteur *Listeria* MLST scheme (http://bigsdb.web.pasteur.fr/listeria/).

According to a 7-gene MLST scheme, *L. monocytogenes* isolates were classified into sequence types (STs), sharing all seven alleles, and clonal complexes (CCs), sharing at least six alleles (Ragon et al. 2008).

**Core genome MLST typing**

For core genome MLST (cgMLST) typing, a cgMLST scheme consisting of 1748 alleles implemented in BioNumerics v7.6.2 software (Applied Maths, Belgium), was utilized. Isolates showing ≤7 allelic differences, i.e. belonging to the same cgMLST type, were considered to be potentially epidemiologically linked, as recommended by Moura et al. (2016).

**Virulence potential assessment**

Isolates were screened for the presence of 151 confirmed and candidate *L. monocytogenes* virulence genes. The virulence gene panel consisted of 111 virulence genes extracted from the literature (Camejo et al. 2011; Faralla et al. 2016; Maury et al. 2016; Aguilar-Bultet et al. 2018).
and publicly available databases (Table S2). Additionally, 40 recently described candidate virulence factors associated with clinically relevant phenotypes (Maury et al. 2016; Aguilar-Bultet et al. 2018) were included in the panel. For characterization of internalin A (InlA, encoded by inlA gene), gene sequences were aligned and screened for premature stop codon (PMSC) mutations using CLUSTAL W v2.1 (Thompson et al. 1994). The gene presence/absence pattern was determined using the BLASTN algorithm with the following parameters: 70% sequence similarity threshold, 70% sequence coverage threshold and word size of 11.

A core genome phylogeny of the analyzed isolates was inferred to map the virulence gene presence/absence pattern onto the phylogenetic tree. The assembled genomes of 16 strains that underwent WGS were uploaded to the EDGAR v2.3 pipeline (Blom et al. 2016). The pipeline was used to annotate draft genomes and perform core genome alignment of putative amino-acid residues. RAxML v8.1.22 (Stamatakis 2014) was used to infer core genome phylogeny under the PROTGAMMA substitution model and BLOSUM62 substitution matrix. The Interactive Tree Of Life (iTOL) tool v4.3 (Letunic and Bork 2016) was used to visualize and annotate the phylogenetic trees.

Furthermore, gentamicin protection assay in the bovine macrophage (BoMac) cell line was utilized to assess the invasion and intracellular replication efficiency of nine L. monocytogenes isolates belonging to different STs (File S1).

**Results**

**Bacteriology**

Seven days after the cow with severe neurological symptoms was euthanized, neurolisteriosis was microbiologically (March 18, Table 1) and histopathologically (data not shown) confirmed.
The farm was temporarily banned from selling raw milk and an extensive epidemiological investigation was performed. Five days after neurolisteriosis confirmation, raw milk samples from each quarter were collected from 47 dairy cows and examined for the presence of listeria (March 30, Table 1); 9/47 cows (19.1%) were positive for L. monocytogenes. The nine detected subclinical mastitis cases, defined here as cows excreting L. monocytogenes in milk and exhibiting an increased somatic cell count, were separated from the other cows and treated with antibiotics. Milk samples from the treated cows were examined twice for the presence of L. monocytogenes (during and after the treatment; April 15 and May 4, Table 1), as well as the pooled milk sample and individual fecal samples taken during treatment. Since all results were negative, the farm was allowed to sell raw milk. Additionally, one sample of the silage used to feed the animals before the onset of neurolisteriosis was collected and tested positive for L. monocytogenes (March 30, Table 1).

Approximately three weeks after neurolisteriosis confirmation, the following additional samples were collected: raw milk samples from 44 cows lactating at that time and bulk-tank milk sample, bull and deer fecal samples, and three water samples from the farm environment (April 15, Table 1). Of these, pooled deer feces and all water samples (3/3) were positive, resulting in 20 L. monocytogenes isolates as five isolates per sample were selected for typing (Table 1).

The first sampling of drinking water (household kitchen and barn milk room) and well water, performed by the farmer, tested negative for L. monocytogenes (April 24, Table 1). Subsequent sampling of the water from the household tested positive for L. monocytogenes, resulting in five L. monocytogenes isolates (May 4, Table 1). On the same day, the bulk-tank milk was also sampled and tested negative (May 4, Table 1).

In summary, 10 clinical and 26 environmental L. monocytogenes isolates were obtained. To establish possible epidemiological association, all isolates were subjected to PFGE. In
addition, 16 isolates covering all pulsotypes and both isolation origins underwent WGS and were assessed for virulence gene presence (Table S1). Nine selected isolates were also tested for invasion and replication efficiency using gentamicin protection assay (File S1).

**PFGE typing**

L. monocytogenes isolates exhibited nine distinct AScI-ApαI pulsotypes (Figure 1). Isolates with indistinguishable pulsotypes were combined into five clusters (clusters 1-5). The pulsotype of L628 isolate from the neurolisteriosis case differed in two bands in ApαI profile and one band in AScI profile from the L698 isolate from water (water pipe in the barn), resulting in 94.5% pairwise AScI-ApαI pulsotype similarity. Therefore, their epidemiological link could not be confirmed. The nine isolates from subclinical mastitis cases displayed four distinct pulsotypes. The following clusters of microbiologically associated isolates were identified: (i) Cluster 1, including isolates from different water sources (pond, pipe, trough and household); (ii) Clusters 2 and 4, respectively, including isolates from subclinical mastitis cases; and (iii) Cluster 4, including isolates from subclinical mastitis cases and silage. Isolates from the deer feces and water from the household (Clusters 3 and 5, respectively), each originated from a single sample and were thus considered epidemiological duplicates (Table 1 and Figure 1).

**WGS typing**

For easier comparison of PFGE and WGS typing results, isolates exhibiting ≤7 allelic differences were given the same cluster designation as determined by PFGE analysis. The three isolates sequenced with both WGS technologies exhibited 0–2 (average = 1) allelic differences (data not shown). Similar to PFGE classification, isolates were grouped into nine cgMLST types (clusters) according to the threshold of ≤7 allelic differences (Figure 2). In general, all the aforementioned epidemiological associations suggested by PFGE were also confirmed by
WGS. The isolate from the neurolisteriosis case exhibited 50 allelic differences in comparison with the isolate from the pipe water and was therefore considered as epidemiologically unlinked. Their lack of epidemiological relatedness was further confirmed by adding isolate L666 to the analysis, which was an epidemiologically unrelated clinical isolate originating from a concurrent case of caprine neurolisteriosis from another farm, thus serving as an outgroup (Figure 2).

**Virulence potential assessment**

Listeria pathogenicity island 1 (LIPI-1) was fully conserved in all isolates (Figure 3). With the exception of \textit{inlF}, internalin-coding (\textit{inl}) genes were present in all isolates (Figure 3). L699 isolate (ST21 and CC21), originating from pond water, had a premature stop codon (PMSC) mutation in \textit{inlA}, whilst the remaining 15 isolates encoded a full-length \textit{InlA}. LIPI-3 was present in four lineage I isolates, belonging to CC1 and CC4. LIPI-4 was present in two CC4 isolates (Figure 3). The remaining 80 previously described virulence genes were generally conserved in all isolates. Amongst the less-conserved genes, an association between phylogenetic lineage and virulence gene presence was observed: \textit{vip}, \textit{aut} and \textit{tagB} were present in isolates of phylogenetic lineage I, whereas \textit{aut}_{IVb} and \textit{gltB} were present in isolates of phylogenetic lineage II (Figure S1). Forty recently described candidate virulence genes associated with clinically relevant phenotypes (Maury et al. 2016; Aguilar-Bultet et al. 2018) were also frequently encountered in the analyzed isolates (Figure S1). All isolates infected the BoMac cell line; however, they differed significantly in their invasion and replication efficiency (File S1). No clear correlation between isolation origin or phylogenetic lineage and invasion or intracellular replication capacity was observed. One noticeable exception was the isolate belonging to ST29 (CC29), which had a decreased invasion and intracellular replication capacity (File S1).
Discussion

L. monocytogenes is a common raw milk contaminant and listeriosis outbreaks in humans are commonly associated with dairy products (Lundén et al. 2004). In Slovenia, no active surveillance of listeriosis in animals takes place; however, to limit animal and human disease, close cooperation between veterinarians, occupational health physicians and public health operators is established, recently referred to as the ‘one health approach’ (Rabozzi et al. 2012). The source investigation study presented herein aimed to identify L. monocytogenes transmission routes on a dairy farm with a case of bovine neurolisteriosis. During an extensive epidemiological investigation, L. monocytogenes isolates were obtained from clinical samples (brain and subclinical mastitis), deer feces, various water samples (pipe, trough, pond and household) and silage. Molecular typing showed high on-farm genetic diversity of L. monocytogenes and confirmed the epidemiological link between isolates from subclinical mastitis cases and silage as well as different water sources, but the source of infection for the neurolisteriosis case was not identified. Hypervirulent CCs (CC1, CC2 and CC4) were identified amongst clinical (neurolisteriosis and subclinical mastitis) and environmental (pipe) isolates. Gentamicin protection assay showed that isolates differed in terms of invasion and replication capacity. Accordingly, WGS was employed to improve our understanding of the genetic diversity, epidemiological associations and virulence profiles of L. monocytogenes isolates from the dairy farm with listeriosis.

In this study, the neurolisteriosis isolate was both microbiologically and histopathologically confirmed. It belonged to ST515 (CC1), a previously reported hypervirulent CC, the most common clone amongst the human clinical isolates in France (Maury et al., 2016), and significantly over-represented in bovine neurolisteriosis cases in Europe (Dreyer et al. 2016; Maury et al. 2016). The source of infection of the euthanized cow was not identified, as
the L691 isolate from water (pipe), which also belonged to CC1, exhibited 50 allelic differences in comparison to the L628 isolate from the neurolisteriosis case, and was thus considered microbiologically unlinked. In the scope of the present study, only one isolate from silage was obtained and analyzed; therefore, a more extensive sampling of feed and environment may have revealed possible epidemiological links.

In total, 9/47 (19.1%) dairy cows excreted L. monocytogenes with raw milk due to subclinical mastitis. This surprisingly high percentage of dairy cows with subclinical mastitis within a herd contrasts with the findings of other studies, in which L. monocytogenes excretion from the infected udder into milk was generally not considered an important source of milk contamination due to its low incidence (Jensen et al. 1996; Castro et al. 2018). Rather, milk contamination is primarily believed to result from environmental/fecal contamination of the udder surface (Sanaa et al. 1993; Hassan et al. 2001; Castro et al. 2018), or biofilm formation in the milking system (Latorre et al. 2009; Latorre et al. 2010). Nevertheless, cases of raw milk contamination due to subclinical mastitis have been reported (Hunt et al. 2012; Hasegawa et al. 2013). Results of the present study suggest that listerial (subclinical) mastitis should not be neglected as a source of raw milk contamination in dairy farm environment.

Isolates from the nine subclinical mastitis cases belonged to three different L. monocytogenes genotypes according to PFGE and WGS typing (6/9 to CC11, 2/9 to CC4 and 1/9 to CC2), which suggests that udder infection came from a common source, most likely via fecal contamination of the immediate cattle environment, such as the stable, grazing surfaces or milking devices. Of the identified CCs, CC2 and CC4 have been previously reported as hypervirulent (Dreyer et al. 2016; Maury et al. 2016). Moreover, CCs identified in mastitis cases have already been associated with human listeriosis outbreaks, further confirming their virulence potential in humans (Chen et al. 2016). Results of the present study indicate that subclinical listerial mastitis can lead to the contamination of raw milk with hypervirulent
L. monocytogenes CCs, which is in accordance with the study conducted by Kim et al. (2018), in which isolates belonging to hypervirulent CCs were reported in the bulk milk, milk filters and milking equipment. The presence of strains with increased infectious potential is of particular importance in terms of public health as they represent a direct threat to the consumer and suckling animals. Moreover, it calls for the implementation of effective measures to prevent raw milk contamination, such as milking hygiene improvement and listerial mastitis control (Walland et al. 2015). In this study, the epidemiological investigation was performed after the occurrence of neurolisteriosis; therefore, subclinical mastitis cases may be overlooked if neurolisteriosis is absent.

Isolates from different water sources (barn pipe and trough, pond and household) harbored a diverse population of L. monocytogenes, all sharing a common ST21 (CC21) strain, suggesting a common contamination source. Because water was coming from a common private supply to the household and barn, water supply was the most likely source of water contamination in the barn (pipe and trough) and household. Moreover, as the ST21 strain was also found in the farm environment (pond), the farm environment is also a likely source of water supply contamination. Two different genotypes were obtained from the pond, pipe and household; therefore, this study emphasizes the importance of typing multiple isolates from a single sample or source during source attribution. Water from the four different analyzed sources was positive for L. monocytogenes after filtration of one liter of water. Conversely, after the first sampling of 50 ml of drinking water collected by the farmer, all three water samples tested negative for L. monocytogenes. These results suggest that L. monocytogenes levels in the water samples were low; consequently, isolation procedure should include a filtration step to increase the rate of L. monocytogenes isolation. This is in accordance with previous publications, in which L. monocytogenes was successfully isolated from water samples after
filtration of larger quantities (100 ml or more) of water (Arvanitidou et al. 1997; Lyautey et al. 2007).

Improperly fermented silage is associated with increased L. monocytogenes fecal shedding and thus contributes to the maintenance and dispersal of listeria into the farm environment and fecal or environmental raw milk contamination (Sanaa et al. 1993; Nightingale et al. 2004; Kim et al. 2018). In this study, ST1279 (CC11) strain was isolated from the silage and milk of six mastitic cows. The transmission of ST1279 strain most likely occurred indirectly, i.e. through fecal contamination of the farm environment and/or silage, which subsequently contaminated the udder. L. monocytogenes fecal shedding in cattle was not confirmed, but this might be a result of the limited number of fecal samples collected on a single date. Although CC11 is not one of the most prevalent CCs in human (Maury et al. 2016; Kuch et al. 2018) and animal (Dreyer et al. 2016; Steckler et al. 2018) clinical isolates, it has been associated with ruminant neurolisteriosis cases (Dreyer et al. 2016).

In the present study, gentamicin protection assay showed that all isolates had the capacity to infect and multiply in the BoMac cell line. This is in accordance with virulence gene presence/absence results. Namely, the six key virulence-associated genes encoded by the LIPI-1 island, involved in the crucial steps of L. monocytogenes intracellular cycle (Vázquez-Boland et al. 2001), were fully conserved in all isolates. The observed difference in invasion and intracellular replication capacity could partially be explained by the observed difference in virulence gene presence/absence. The 80 less-conserved previously described virulence genes were mostly associated with phylogenetic lineage rather than the origin of isolation, which is in correspondence with previous findings (Moura et al. 2016; Painset et al. 2019). A full-length InlA and LIPI-3 island (encoding listeriolyisin S) associated with increased infectious potential in humans at the population level (Maury et al. 2016), were commonly present in the analyzed isolates. The LIPI-4 island, a recently described pathogenicity island involved in cerebral and
placental tissue tropism in a humanized mouse model (Maury et al. 2016), was present in both CC4 isolates from subclinical mastitis cases (2/16 isolates). Hence, a substantial number of analyzed isolates harbored genes associated with increased infectious potential and/or invasive listeriosis (cerebral/placental tropism). This is in congruence with the study conducted by Kim et al. (2018), in which a significant number of strains from the dairy farm environment encoded virulence traits associated with increased infectious potential in humans.

It has also recently been shown that internalin F (encoded by inlF gene) mediates L. monocytogenes invasion of the brain in mice (Ghosh et al. 2018). Four out of the seven isolates from subclinical mastitis cases subjected to WGS belonged to CC11 and lacked inlF, whereas the remaining clinical isolates, including the isolate from neurolisteriosis case, harbored inlF. Therefore, inlF presence may at least in part explain the difference in clinical outcomes (subclinical mastitis and neurolisteriosis) of the analyzed animal cases. Interestingly, vip gene was present in 8/8 clinical isolates, regardless of their phylogenetic lineage and clinical form of listeriosis, but absent in 4/8 non-clinical isolates. The vip gene codes for Vip surface protein, which is involved in L. monocytogenes invasion into the host cell and has been shown to be over-represented in isolates of clinical origin, suggesting its important role in pathogenesis (Cabanes et al. 2005; Painset et al. 2019). Additionally, several recently described candidate virulence genes over-represented in isolates of clinical origin (Maury et al. 2016; Aguilar-Bultet et al. 2018) were present in the analyzed isolates.

The present study also showed that isolates with identical or highly similar virulence gene profiles can have different isolation origins (clinical or non-clinical). Moreover, isolates associated with subclinical mastitis belonged to different phylogenetic lineages and CCs, and had a different virulence gene pattern. This suggests that infectious potential and/or clinical outcome of the disease cannot be fully explained solely by the presence of known virulence
genes and/or in vitro cell-based assay results. Thus, larger-scale population studies are needed to identify significant association between genotypes and phenotypes.

In this study, a high concordance of PFGE and cgMLST was observed. Due to the fact that isolates from each cluster were distant enough to be discriminated by PFGE, nine pulsotypes were identified, corresponding to nine cgMLST types with ≤7 allelic differences; thus, the same epidemiological links were established using both typing methods. Sequencing of three isolates with both WGS technologies confirmed previous findings that extraction kits and sequencing platforms do not markedly affect the results of epidemiological investigation (ECDC 2018; Reimer et al. 2019). High stability, repeatability, reproducibility, discriminatory power and epidemiological concordance of WGS for the typing of L. monocytogenes and other microbial species have been well established (Stasiewicz et al. 2015; Henri et al. 2017; Moura et al. 2017; Portmann et al. 2018).

In conclusion, the present source investigation study on a dairy farm identified large genetic diversity of L. monocytogenes, including the hypervirulent clones CC1, CC2 and CC4. A surprisingly high number of dairy cows with subclinical listerial mastitis were identified. Comprehensive virulence gene profiling enabled by WGS revealed that a substantial number of analyzed isolates, including mastitis isolates, belonged to the hypervirulent CCs and harbored genes associated with increased infectious potential and/or cerebral tropism (full-length InlA, LIPI-3 island and LIPI-4 island). The presence of hypervirulent CCs in milk from mastitic cows calls for efficient control measure implementation to enable early detection of listerial subclinical mastitis and thus prevent raw milk contamination, which represents a direct threat to the consumer. Although the source of infection for the cow with neurolisteriosis was not identified, an epidemiological link between the isolate from the farm environment (silage) and mastitis isolates belonging to CC11 was established. The present study improves our understanding of L. monocytogenes ecology on dairy farms.
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Conflict of interest declaration

No conflict of interest declared.

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milking equipment on a dairy farm as a potential source of bulk tank milk contamination with Listeria monocytogenes. J Dairy Sci 93, 2792-2802.


**Supporting information**

**Figure S1** Virulence gene presence/absence pattern of 120 confirmed and candidate virulence genes, mapped onto the core genome phylogenetic tree. A total of 16 *Listeria monocytogenes*
isolates that underwent WGS were screened for the presence of virulence genes. For easier visualization, virulence genes were divided into 80 previously characterized virulence factors (A) and 40 recently described candidate virulence factors associated with clinically relevant phenotypes (B). The colors of the leaves on the tree represent isolation origin: red, clinical origin; blue, non-clinical origin. Bar, number of amino acid substitutions per site.

**File S1** Gentamicin protection assay in the bovine macrophage (BoMac) cell line.

**Table S1** Availability of raw sequencing data.

**Table S2** List of 151 confirmed and candidate *Listeria monocytogenes* virulence genes used in this study.
Table 1: Overview of samples collected in 2014 at the organic dairy farm after bovine neurolisteriosis case confirmation.

<table>
<thead>
<tr>
<th>Date of sampling</th>
<th>Source</th>
<th>Sample type/No.</th>
<th>L. monocytogenes cultivation</th>
<th>Isolate ID, selected for typing***</th>
</tr>
</thead>
<tbody>
<tr>
<td>March 18</td>
<td>Euthanized cow</td>
<td>Brain tissue (n=1)</td>
<td>Positive</td>
<td>L628</td>
</tr>
<tr>
<td></td>
<td>Feed</td>
<td>Grass silage (n=1)</td>
<td>Positive</td>
<td>L639</td>
</tr>
<tr>
<td>April 15</td>
<td>Lactating cows II**</td>
<td>Milk (n=44)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bulk tank milk (n=1)</td>
<td>Negative</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Individual milk samples (n=9) and pooled milk (n=1) from the treated cows</td>
<td>Negative</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cows</td>
<td>Feces of treated cows (n=9)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bull</td>
<td>Feces (n=1)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>Water–pipe (barn) (n=1)</td>
<td>Positive***</td>
<td>L667, L689, L690, L691, L692</td>
</tr>
<tr>
<td></td>
<td>Water–trough (barn) (n=1)</td>
<td>Positive***</td>
<td>L668, L693, L694, L695, L696</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water–pond (n=1)</td>
<td>Positive***</td>
<td>L669, L679, L698, L699, L700</td>
<td></td>
</tr>
<tr>
<td>Deer</td>
<td>Pooled fecal sample (n=1)</td>
<td>Positive***</td>
<td>L670, L701, L702, L703, L704</td>
<td></td>
</tr>
<tr>
<td>April 24</td>
<td>Water</td>
<td>Water (household, milk room, well) (n=3)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>May 4</td>
<td>Treated cows</td>
<td>Individual milk samples (n=9)</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bulk tank milk (n=1)</td>
<td>Negative</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>Water–household (n=1)</td>
<td>Positive</td>
<td>L705, L706, L707, L708, L709</td>
<td></td>
</tr>
<tr>
<td>Total no.</td>
<td>132 samples</td>
<td>16 positive samples</td>
<td>36 L. monocytogenes isolates</td>
<td></td>
</tr>
</tbody>
</table>

* First sampling of lactating cows (five days after neurolisteriosis confirmation)
** Second sampling of lactating cows (approximately three weeks after neurolisteriosis confirmation)
*** Five colonies from each culture were subcultured, hence five isolates from one investigated sample were subjected to PFGE typing and molecular serotyping. Isolates subjected to WGS typing are depicted in bold. Isolates subjected to gentamicin protection assay are depicted in italics.
Figure legends

**Figure 1** PFGE dendrogram of *Listeria monocytogenes* isolates based on a combined Ascl-ApaI analysis. A total of 32 *L. monocytogenes* isolates obtained within the framework of this study were included in the comparison. The isolates with indistinguishable pulsotypes are designated as clusters. Isolates that had their MLST type assigned according to WGS data are highlighted in bold; other isolates had their MLST predicted according to the combined Ascl-ApaI analysis, as previously described (Félix et al. 2018). Bar, average similarity of Ascl-ApaI profiles.

**Figure 2** cgMLST dendrogram of *Listeria monocytogenes* isolates. A total of 16 *Listeria monocytogenes* isolates obtained within the framework of this study were included in the comparison. L666 isolate, which originated from a concurrent but epidemiologically unrelated caprine clinical case, was added to the analysis. The isolates exhibiting ≤7 allelic differences were considered epidemiologically associated and were given the same cluster designation as in PFGE analysis (Figure 1). Bar, number of allelic differences.

**Figure 3** Virulence gene presence/absence pattern, mapped onto the core genome phylogenetic tree. A total of 16 *Listeria monocytogenes* isolates that underwent WGS were included in the analysis and screened for the presence of 10 internalin-coding (*inl*) genes and 21 virulence genes encoded in the *Listeria* pathogenicity islands LIPI-1, LIPI-3 and LIPI-4. The *inlA* genes were screened for the presence of premature stop codon (PMSC) mutations; the L629 isolate with PMSC mutation is shown in light violet, whereas the remaining isolates had full-length InlA. The colors of the leaves on the tree represent isolation origin: red, clinical origin; blue, non-clinical origin. Bar, number of substitutions per site.