This pre-print is published in:

Journal of Applied Microbiology, 2019, Volume 127, Issue 5, Pages 1349-1361

DOI: 10.1111/jam.14418

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

Bojan Papi , Majda Golob, Darja Kušar, Mateja Pate, Irena Zdovc*

University of Ljubljana, Veterinary Faculty, Institute of Microbiology and Parasitology, Ljubljana, Slovenia

**Corresponding author*: Dr. Irena Zdovc

University of Ljubljana, Veterinary Faculty

Institute of Microbiology and Parasitology

Gerbi eva 60, SI-1000 Ljubljana, Slovenia

Phone: +386 1 4779 166

Fax: +386 1 4779 352

E-mail: irena.zdovc@vf.uni-lj.si

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 (Stari *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).

75 *L. monocytogenes* is a genetically heterogeneous species with a clonal population
76 structure (Chenal-Francisque *et al*. 2011; Cantinelli *et al*. 2013). It is divided into four
77 phylogenetic lineages, which vary in terms of their ecological, evolutionary and phenotypic
78 characteristics; lineage I is associated with a clinical origin, whereas lineage II is food-
79 associated (Maury *et al*. 2016). Recently, hypovirulent and hypervirulent clones have been
80 described (Maury *et al*. 2016). *L. monocytogenes* is widely distributed in the farm environment;
81 however, its ecology and transmission dynamics are complex and remain poorly understood
82 (Nightingale *et al*. 2004; Dreyer *et al*. 2016; Castro *et al*. 2018). The dairy farm environment
83 and cattle harbor a large diversity of *L. monocytogenes* strains, including genotypes involved
84 in human listeriosis (Borucki *et al*. 2004; Rocha *et al*. 2013). Asymptomatic carriers, in
85 particular cattle, are an important reservoir of *L. monocytogenes*, enabling its multiplication in
86 host cells and transmission into the farm environment through fecal shedding (Nightingale *et*
87 *al*. 2004; Esteban *et al*. 2009). Feed quality and storage, animal health, hygiene practice and
88 farm management practice are associated with *L. monocytogenes* occurrence in the farm
89 environment and animal listeriosis incidence (Sanaa *et al*. 1993; Nightingale *et al*. 2005; Castro
90 *et al*. 2018). Moreover, the farm environment is a possible source of contamination of meat and
91 dairy food processing facilities (Muhterem-Uyar *et al*. 2015).
92 Reliable and discriminatory methods for *L. monocytogenes* typing are crucial for
93 outbreak identification, transmission pathway elucidation and epidemiological surveillance
94 (Salipante *et al*. 2015). Pulsed-field gel electrophoresis (PFGE) was the "gold standard" typing
95 method for *L. monocytogenes* but has recently been replaced by whole-genome sequencing
96 (WGS) due to its superiority to PFGE and other genotyping methods with regard to the
97 discriminatory power and accuracy of phylogenetic inferences (Graves and Swaminathan 2001;
98 Deng *et al*. 2015; Salipante *et al*. 2015; Moura *et al*. 2016). Because of its ability to accurately
99 delineate outbreak clusters, WGS has been widely used in the epidemiological investigations

100 of human listeriosis cases (Jackson *et al*. 2016; Moura *et al*. 2016). On the contrary, in
101 veterinary surveillance (e.g. animal listeriosis outbreaks), molecular typing of
102 *L. monocytogenes* is not routinely performed (Wagner *et al*. 2005; Dreyer *et al.* 2015).

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

109

110 **Materials and Methods**

111

112 **Case description**

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

117 The farm utilized its own bull for natural breeding purposes. No history of abortion or
118 other reproductive disorders had previously been recorded. The herd consisted of 18 heifers and
119 50 dairy cows for milk distribution. The animals primarily grazed pasture and were additionally
120 fed grass silage. The pasture had been fertilized with cattle slurry and manure, which
121 occasionally contained spoiled silage and other feed. The farm was surrounded by woods
122 inhabited by a large number of wildlife species, especially deer.

123 An extensive epidemiological investigation (reviewed in Table 1) was performed with
124 the aim of identifying *L. monocytogenes* infection source and elucidating possible on-farm

125 transmission pathways. Five days after neurolisteriosis confirmation, the first sampling of
126 lactating cows (47 milk samples pooled from all four quarters) was performed; in addition, their
127 silage was also sampled (one sample). As the presence of *L. monocytogenes* was demonstrated
128 in the raw milk from nine cows, they were identified as having subclinical mastitis, separated
129 from the herd and treated with antibiotics for one month.

130 Approximately three weeks after neurolisteriosis confirmation, the second sampling of
131 lactating and treated cows was performed (April 15, Table 1), in which the following samples
132 were collected: (*i*) individual and pooled (bulk tank) milk samples from lactating cows; (*ii*)
133 individual and pooled milk samples from treated cows; (*iii*) fecal samples from treated cows;
134 (*iv*) bull and deer fecal samples; and (*v*) water samples from the farm environment (barn pipe
135 and trough, pond). The farm had a private water supply, providing household and barn drinking
136 water. Because the barn water samples were positive for *L. monocytogenes* and the same water
137 supply was also used for the household, the farmer additionally collected drinking water
138 samples (50 ml) from the household kitchen, barn milk room and barnyard well (April 24, Table
139 1).

140 After the treatment (i.e. six weeks after neurolisteriosis confirmation), mastitic cow milk
141 samples were once again examined. Moreover, the bulk tank milk (receiving milk from the
142 remaining cows) and drinking water from the household were sampled at the same time (May
143 4, Table 1).

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

147

148 **Sample collection**

149 Approximately 10 ml of milk from each quarter was collected in sterile plastic containers and
150 analyzed separately (four samples per cow). For bulk tank or pooled milk, 25-ml samples were
151 collected. Silage was sampled at five different sites, pooled to a total sample of 3 kg and mixed
152 well. Approximately 25 g of fecal samples were collected into sterile bags; for cows with
153 listerial subclinical mastitis, fecal samples were rectally collected, whereas for deer and bull,
154 ground fecal samples were collected. Water samples, other than those collected by the farmer,
155 were directly collected in sterile containers from each sampling site, and after their
156 transportation to the laboratory in cooling boxes, approximately one liter of the samples was
157 vacuum-filtered through sterile 0.22-μl bottle-top filters ("rapid"-Filtermax system; TPP,
158 Switzerland). The analysis was initiated on the day of sample collection.
159

160 **Bacteriology**

161 All collected samples except silage were regarded as clinical or environmental, thus
162 *L. monocytogenes* isolation was performed according to the World Organization for Animal
163 Health Manual (OIE 2004), and identified according to standard method ISO 11290-1 (ISO
164 1996; ISO 2004). The brain tissue sample taken from the cow with meningoencephalitis was
165 subjected to direct detection on blood agar (Blood agar base No.2; Oxoid, UK) and two-step
166 enrichment method using selective liquid (Fraiser broth; Oxoid) and solid media ALOA
167 (Biolife, Italia) and PALCAM (Oxoid) agar plates. Fecal and feed samples (25 g) as well as
168 water filters and raw milk samples (10 ml) were also examined according to OIE and ISO
169 methods. For brain tissue, milk and silage, one *L. monocytogenes* isolate per sample was stored
170 and typed. For water and fecal samples, five isolates per sample were stored and typed with the
171 aim of investigating *L. monocytogenes* diversity in environmental samples where high genetic
172 diversity is expected.
173

174 **PFGE typing**

175 All isolates (*n*=36) were PFGE-typed according to the PulseNet standardized protocol (Graves
176 and Swaminathan 2001). Briefly, *L. monocytogenes* genomic DNA was digested with *Apa*I and
177 *Asc*I restriction endonucleases. The generated DNA fragments were separated using the CHEF-
178 DR II system (Bio-Rad, USA) in accordance with recommended electrophoretic protocol.
179 PFGE profiles were analyzed using BioNumerics v7.6.2 software (Applied Maths, Belgium).
180 A combined *Asc*I-*Apa*I profile analysis was performed as previously described (Félix *et al.*
181 2018). Due to high genetic stability of *L. monocytogenes* and limited time span of the study,
182 isolates differing in one or more bands were considered to belong to different pulsotypes
183 (Barrett *et al*. 2006).

184

185 **WGS**

186 In total, 16 *L. monocytogenes* isolates with different pulsotype and isolation origin were
187 selected for WGS typing. Illumina MiSeq or Ion Torrent PGM platforms were employed to
188 >30× coverage. A list of all sequenced strains and associated metadata is given in Table S1.

189 Twelve isolates underwent WGS with Ion Torrent Technology (Thermo Fisher
190 Scientific, USA), which was performed at the Veterinary Faculty, Slovenia. Genomic DNA was
191 extracted using the DNeasy Blood & Tissue Kit (Qiagen) and sheared enzymatically to ~400
192 bp fragments using the Ion Xpress Plus gDNA Fragment Library Kit (Thermo Fisher
193 Scientific). WGS libraries were prepared according to IonXpress Plus gDNA Fragment Library
194 Preparation Protocol (Thermo Fisher Scientific). Template amplification and enrichment was
195 performed on the Ion OneTouch 2 System using the Ion PGM Template OT2 400 Kit System
196 (Thermo Fisher Scientific). Sequencing was performed on the Ion PGM System using the Ion
197 PGM Hi-Q Sequencing Kit (Thermo Fisher Scientific) according to the manufacturer
198 instructions.

199 In total, 7/16 isolates underwent WGS with Illumina Technology (Illumina, USA),
200 which was performed at the Brain & Spine Institute (Institut du Cerveau et de la Moelle
201 épinière, ICM), France. Genomic DNA was extracted using the Wizard Genomic DNA
202 Purification Kit (Promega, USA). Paired-end sequencing (2×100 or 2×150 bp) was performed
203 using TruSeq automated library preparation (Illumina) and NextSeq 500 System (Illumina).

204

205 ***In silico* MLST typing**

206 *In silico* multilocus sequence typing (MLST) was performed based on WGS data. Firstly, reads
207 were quality-trimmed using Cutadapt v1.17 (option –q 20) and assembled into contigs using
208 SPAdes v3.11.1 (Bankevich *et al*. 2012). MLST types were assigned based on assembled reads
209 according to the Institut Pasteur *Listeria* MLST scheme (http://bigsdb.web.pasteur.fr/listeria/).
210 According to a 7-gene MLST scheme, *L. monocytogenes* isolates were classified into sequence
211 types (STs), sharing all seven alleles, and clonal complexes (CCs), sharing at least six alleles
212 (Ragon *et al*. 2008).

213

214 **Core genome MLST typing**

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

219

220 **Virulence potential assessment**

221 Isolates were screened for the presence of 151 confirmed and candidate *L. monocytogenes*
222 virulence genes. The virulence gene panel consisted of 111 virulence genes extracted from the
223 literature (Camejo *et al.* 2011; Faralla *et al.* 2016; Maury *et al.* 2016; Aguilar-Bultet *et al.* 2018)

224 and publicly available databases (Table S2). Additionally, 40 recently described candidate
225 virulence factors associated with clinically relevant phenotypes (Maury *et al*. 2016; Aguilar-
226 Bultet *et al*. 2018) were included in the panel. For characterization of internalin A (InlA,
227 encoded by *inlA* gene), gene sequences were aligned and screened for premature stop codon
228 (PMSC) mutations using CLUSTAL W v2.1 (Thompson *et al*. 1994). The gene
229 presence/absence pattern was determined using the BLASTN algorithm with the following
230 parameters: 70% sequence similarity threshold, 70% sequence coverage threshold and word
231 size of 11.

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

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

243

244 **Results**

245

246 **Bacteriology**

247 Seven days after the cow with severe neurological symptoms was euthanized, neurolisteriosis
248 was microbiologically (March 18, Table 1) and histopathologically (data not shown) confirmed.

249 The farm was temporarily banned from selling raw milk and an extensive epidemiological
250 investigation was performed. Five days after neurolisteriosis confirmation, raw milk samples
251 from each quarter were collected from 47 dairy cows and examined for the presence of listeria
252 (March 30, Table 1); 9/47 cows (19.1%) were positive for *L. monocytogenes*. The nine detected
253 subclinical mastitis cases, defined here as cows excreting *L. monocytogenes* in milk and
254 exhibiting an increased somatic cell count, were separated from the other cows and treated with
255 antibiotics. Milk samples from the treated cows were examined twice for the presence of
256 *L. monocytogenes* (during and after the treatment; April 15 and May 4, Table 1), as well as the
257 pooled milk sample and individual fecal samples taken during treatment. Since all results were
258 negative, the farm was allowed to sell raw milk. Additionally, one sample of the silage used to
259 feed the animals before the onset of neurolisteriosis was collected and tested positive for
260 *L. monocytogenes* (March 30, Table 1).

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

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

272 In summary, 10 clinical and 26 environmental *L. monocytogenes* isolates were obtained.
273 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 *Asc*I-*Apa*I 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 *Apa*I profile and one band in *Asc*I profile from the L698 isolate from water (water pipe in the barn), resulting in 94.5% pairwise *Asc*I-*Apa*I 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

299 WGS. The isolate from the neurolisteriosis case exhibited 50 allelic differences in comparison
300 with the isolate from the pipe water and was therefore considered as epidemiologically
301 unlinked. Their lack of epidemiological relatedness was further confirmed by adding isolate
302 L666 to the analysis, which was an epidemiologically unrelated clinical isolate originating from
303 a concurrent case of caprine neurolisteriosis from another farm, thus serving as an outgroup
304 (Figure 2).

305

306 **Virulence potential assessment**

307 *Listeria* pathogenicity island 1 (LIPI-1) was fully conserved in all isolates (Figure 3). With the
308 exception of *inlF*, internalin-coding (*inl*) genes were present in all isolates (Figure 3). L699
309 isolate (ST21 and CC21), originating from pond water, had a premature stop codon (PMSC)
310 mutation in *inlA*, whilst the remaining 15 isolates encoded a full-length InlA. LIPI-3 was present
311 in four lineage I isolates, belonging to CC1 and CC4. LIPI-4 was present in two CC4 isolates
312 (Figure 3). The remaining 80 previously described virulence genes were generally conserved
313 in all isolates. Amongst the less-conserved genes, an association between phylogenetic lineage
314 and virulence gene presence was observed: *vip*, *aut* and *tagB* were present in isolates of
315 phylogenetic lineage I, whereas *aut_IVb* and *gltB* were present in isolates of phylogenetic
316 lineage II (Figure S1). Forty recently described candidate virulence genes associated with
317 clinically relevant phenotypes (Maury *et al*. 2016; Aguilar-Bultet *et al*. 2018) were also
318 frequently encountered in the analyzed isolates (Figure S1). All isolates infected the BoMac
319 cell line; however, they differed significantly in their invasion and replication efficiency (File
320 S1). No clear correlation between isolation origin or phylogenetic lineage and invasion or
321 intracellular replication capacity was observed. One noticeable exception was the isolate
322 belonging to ST29 (CC29), which had a decreased invasion and intracellular replication
323 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

349 the L691 isolate from water (pipe), which also belonged to CC1, exhibited 50 allelic differences
350 in comparison to the L628 isolate from the neurolisteriosis case, and was thus considered
351 microbiologically unlinked. In the scope of the present study, only one isolate from silage was
352 obtained and analyzed; therefore, a more extensive sampling of feed and environment may have
353 revealed possible epidemiological links.

354 In total, 9/47 (19.1%) dairy cows excreted *L. monocytogenes* with raw milk due to
355 subclinical mastitis. This surprisingly high percentage of dairy cows with subclinical mastitis
356 within a herd contrasts with the findings of other studies, in which *L. monocytogenes* excretion
357 from the infected udder into milk was generally not considered an important source of milk
358 contamination due to its low incidence (Jensen *et al*. 1996; Castro *et al*. 2018). Rather, milk
359 contamination is primarily believed to result from environmental/fecal contamination of the
360 udder surface (Sanaa *et al*. 1993; Hassan *et al*. 2001; Castro *et al*. 2018), or biofilm formation
361 in the milking system (Latorre *et al*. 2009; Latorre *et al*. 2010). Nevertheless, cases of raw milk
362 contamination due to subclinical mastitis have been reported (Hunt *et al.* 2012; Hasegawa *et al*.
363 2013). Results of the present study suggest that listerial (subclinical) mastitis should not be
364 neglected as a source of raw milk contamination in dairy farm environment.

365 Isolates from the nine subclinical mastitis cases belonged to three different
366 *L. monocytogenes* genotypes according to PFGE and WGS typing (6/9 to CC11, 2/9 to CC4
367 and 1/9 to CC2), which suggests that udder infection came from a common source, most likely
368 via fecal contamination of the immediate cattle environment, such as the stable, grazing
369 surfaces or milking devices. Of the identified CCs, CC2 and CC4 have been previously reported
370 as hypervirulent (Dreyer *et al*. 2016; Maury *et al*. 2016). Moreover, CCs identified in mastitis
371 cases have already been associated with human listeriosis outbreaks, further confirming their
372 virulence potential in humans (Chen *et al*. 2016). Results of the present study indicate that
373 subclinical listerial mastitis can lead to the contamination of raw milk with hypervirulent

374 *L. monocytogenes* CCs, which is in accordance with the study conducted by Kim *et al*. (2018),
375 in which isolates belonging to hypervirulent CCs were reported in the bulk milk, milk filters
376 and milking equipment. The presence of strains with increased infectious potential is of
377 particular importance in terms of public health as they represent a direct threat to the consumer
378 and suckling animals. Moreover, it calls for the implementation of effective measures to prevent
379 raw milk contamination, such as milking hygiene improvement and listerial mastitis control
380 (Walland *et al*. 2015). In this study, the epidemiological investigation was performed after the
381 occurrence of neurolisteriosis; therefore, subclinical mastitis cases may be overlooked if
382 neurolisteriosis is absent.

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

398 filtration of larger quantities (100 ml or more) of water (Arvanitidou *et al*. 1997; Lyautey *et al*.
399 2007).

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

411 In the present study, gentamicin protection assay showed that all isolates had the
412 capacity to infect and multiply in the BoMac cell line. This is in accordance with virulence gene
413 presence/absence results. Namely, the six key virulence-associated genes encoded by the LIPI-
414 1 island, involved in the crucial steps of *L. monocytogenes* intracellular cycle (Vázquez-Boland
415 *et al*. 2001), were fully conserved in all isolates. The observed difference in invasion and
416 intracellular replication capacity could partially be explained by the observed difference in
417 virulence gene presence/absence. The 80 less-conserved previously described virulence genes
418 were mostly associated with phylogenetic lineage rather than the origin of isolation, which is
419 in correspondence with previous findings (Moura *et al*. 2016; Painset *et al*. 2019). A full-length
420 InlA and LIPI-3 island (encoding listeriolysin S) associated with increased infectious potential
421 in humans at the population level (Maury *et al*. 2016), were commonly present in the analyzed
422 isolates. The LIPI-4 island, a recently described pathogenicity island involved in cerebral and

423 placental tissue tropism in a humanized mouse model (Maury *et al.* 2016), was present in both
424 CC4 isolates from subclinical mastitis cases (2/16 isolates). Hence, a substantial number of
425 analyzed isolates harbored genes associated with increased infectious potential and/or invasive
426 listeriosis (cerebral/placental tropism). This is in congruence with the study conducted by Kim
427 *et al*. (2018), in which a significant number of strains from the dairy farm environment encoded
428 virulence traits associated with increased infectious potential in humans.

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

442 The present study also showed that isolates with identical or highly similar virulence
443 gene profiles can have different isolation origins (clinical or non-clinical). Moreover, isolates
444 associated with subclinical mastitis belonged to different phylogenetic lineages and CCs, and
445 had a different virulence gene pattern. This suggests that infectious potential and/or clinical
446 outcome of the disease cannot be fully explained solely by the presence of known virulence

447 genes and/or *in vitro* cell-based assay results. Thus, larger-scale population studies are needed
448 to identify significant association between genotypes and phenotypes.

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

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

Acknowledgements

Benjamin Félix from ANSES (Maisons-Alfort, France) is warmly acknowledged for fruitful discussions, his help with the bioinformatics tools employed in this study and for providing the Illumina sequencing data. Maja Kavali and Alenka Usenik are acknowledged for performing PFGE typing, Dr Polona Juntes for providing the results of histopathological examination and Nina Sluga for her help during gentamicin protection assay. The bovine macrophage (BoMac) cell line was kindly provided by Dr Matthias Schweizer from the Institute of Virology and Immunology, VetSuisse Faculty, University of Bern, Switzerland. The authors would also like to express their gratitude to Dr Andrej Pengov, the veterinary practitioner and the farm owner for their cooperation and assistance throughout the sampling.

Conflict of interest declaration

No conflict of interest declared.

References

Aguilar-Bultet, L., Nicholson, P., Rychener, L., Dreyer, M., Gözel, B., Origgi, F.C., Oevermann, A., Frey, J. and Falquet, L. (2018) Genetic separation of *Listeria monocytogenes* causing central nervous system infections in animals. *Front Cell Infect Microbiol* **8**, 20.

Arvanitidou, M., Papa, A., Constantinidis, T.C., Danielides, V. and Katsouyannopoulos, V. (1997) The occurrence of *Listeria* spp. and *Salmonella* spp. in surface waters. *Microbiol Res* **152**, 395-397.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V.,

497 Vyahhi, N., Tesler, G., Alekseyev, M.A. and Pevzner, P.A. (2012) SPAdes: a new
498 genome assembly algorithm and its applications to single-cell sequencing. *J Comput*
499 *Biol* **19**, 455-477.
500 Barrett, D.T.J., Gerner-Smidt, P. and Swaminathan, B. (2006) Interpretation of pulsed-field gel
501 electrophoresis patterns in foodborne disease investigations and surveillance.
502 *Foodborne Pathog Dis* **3**, 20-31.
503 Blom, J., Kreis, J., Spanig, S., Juhre, T., Bertelli, C., Ernst, C. and Goesmann, A. (2016)
504 EDGAR 2.0: an enhanced software platform for comparative gene content analyses.
505 *Nucleic Acids Res* **44**, W22-28.
506 Borucki, M.K. and Call, D.R. (2003) *Listeria monocytogenes* serotype identification by PCR.
507 *J Clin Microbiol* **41**, 5537-5540.
508 Borucki, M.K., Reynolds, J., Gay, C.C., McElwain, K.L., Kim, S.H., Knowles, D.P. and Hu, J.
509 (2004) Dairy farm reservoir of *Listeria monocytogenes* sporadic and epidemic strains.
510 *J Food Prot* **67**, 2496-2499.
511 Bundrant, B.N., Hutchins, T., den Bakker, H.C., Fortes, E. and Wiedmann, M. (2011)
512 Listeriosis outbreak in dairy cattle caused by an unusual *Listeria monocytogenes*
513 serotype 4b strain. *J Vet Diagn Invest* **23**, 155-158.
514 Cabanes, D., Sousa, S., Cebriá, A., Lecuit, M., García del Portillo, F., and Cossart, P. (2005)
515 Gp96 is a receptor for a novel *Listeria monocytogenes* virulence factor, Vip, a surface
516 protein. *EMBO J* **24**, 2827-2838.
517 Camejo, A., Carvalho, F., Reis, O., Leitao, E., Sousa, S. and Cabanes, D. (2011) The arsenal of
518 virulence factors deployed by *Listeria monocytogenes* to promote its cell infection
519 cycle. *Virulence* **2**, 379-394.

520 Cantinelli, T., Chenal-Francisque, V., Diancourt, L., Frezal, L., Leclercq, A., Wirth, T., Lecuit,
521 M. and Brisse, S. (2013) "Epidemic clones" of *Listeria monocytogenes* are widespread
522 and ancient clonal groups. *J Clin Microbiol* **51**, 3770-3779.
523 Castro, H., Jaakkonen, A., Hakkinen, M., Korkeala, H. and Lindström, M. (2018) Occurrence,
524 persistence, and contamination routes of *Listeria monocytogenes* genotypes on three
525 Finnish dairy cattle farms: a longitudinal study. *Appl Environ Microbiol* **84**, 4.
526 Chen, Y., Gonzalez-Escalona, N., Hammack, T.S., Allard, M.W., Strain, E.A. and Brown, E.W.
527 (2016) Core genome multilocus sequence typing for identification of globally
528 distributed clonal groups and differentiation of outbreak strains of *Listeria*
529 *monocytogenes*. *Appl Environ Microbiol*. **82**, 6258-6272.
530 Chenal-Francisque, V., Lopez, J., Cantinelli, T., Caro, V., Tran, C., Leclercq, A., Lecuit, M.
531 and Brisse, S. (2011) Worldwide distribution of major clones of *Listeria*
532 *monocytogenes. Emerg Infect Dis* **17**, 1110-1112.
533 Cole, M.B., Jones, M.V. and Holyoak, C. (1990) The effect of pH, salt concentration and
534 temperature on the survival and growth of *Listeria monocytogenes*. *J Appl Bacteriol*
535 **69**, 63-72.
536 Deng, X., Shariat, N., Driebe, E.M., Roe, C.C., Tolar, B., Trees, E., Keim, P., Zhang, W.,
537 Dudley, E.G., Fields, P.I., Engelthaler, D.M. and Ledeboer, N.A. (2015) Comparative
538 analysis of subtyping methods against a whole-genome-sequencing standard for
539 *Salmonella enterica* serotype Enteritidis. *J Clin Microbiol* **53**, 212-218.
540 Dreyer, M., Aguilar-Bultet, L., Rupp, S., Guldimann, C., Stephan, R., Schock, A., Otter, A.,
541 Schupbach, G., Brisse, S., Lecuit, M., Frey, J. and Oevermann, A. (2016) *Listeria*
542 *monocytogenes* sequence type 1 is predominant in ruminant rhombencephalitis. *Sci*
543 *Rep* **6**, 36419.

544 Dreyer, M., Thomann, A., Böttcher, S., Frey, J. and Oevermann, A. (2015) Outbreak
545 investigation identifies a single *Listeria monocytogenes* strain in sheep with different
546 clinical manifestations, soil and water. *Vet Microbiol* **179**, 69-75.
547 ECDC (European Centre for Disease Prevention and Control) (2018) Fifth external quality
548 assessment scheme for *Listeria monocytogenes* typing. Stockholm: ECDC.
549 Esteban, J.I., Oporto, B., Aduriz, G., Juste, R.A. and Hurtado, A. (2009) Faecal shedding and
550 strain diversity of *Listeria monocytogenes* in healthy ruminants and swine in Northern
551 Spain. *BMC Vet Res* **5**, 2.
552 Faralla, C., Rizzuto, G.A., Lowe, D.E., Kim, B., Cooke, C., Shiow, L.R. and Bakardjiev, A.I.
553 (2016) InlP, a new virulence factor with strong placental tropism. *Infect Immun* **84**,
554 3584-3596.
555 Félix, B., Feurer, C., Maillet, A., Guillier, L., Boscher, E., Kerouanton, A., Denis, M. and
556 Roussel, S. (2018) Population genetic structure of *Listeria monocytogenes* strains
557 isolated from the pig and pork production chain in France. *Front Microbiol* **9**, 684.
558 Ghosh, P., Halvorsen, E.M., Ammendolia, D.A., Mor-Vaknin, N., O'Riordan M.X.D., Brumell,
559 J.H., Markovitz, D.M., Higgins, D.E. (2018) Invasion of the brain by *Listeria*
560 *monocytogenes* is mediated by InlF and host cell vimentin. *mBio* **9**, e00160–18.
561 Graves, L.M. and Swaminathan, B. (2001) PulseNet standardized protocol for subtyping
562 *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. *Int J*
563 *Food Microbiol* **65**, 55-62.
564 Haley, B.J., Sonnier, J., Schukken, Y.H., Karns, J.S. and Van Kessel, J.A. (2015) Diversity of
565 *Listeria monocytogenes* within a U.S. dairy herd, 2004-2010. *Foodborne Pathog Dis*
566 **12**, 844-850.

567 Hasegawa, M., Iwabuchi, E., Yamamoto, S., Esaki, H., Kobayashi, K., Ito, M. and Hirai, K.
568 (2013) Prevalence and characteristics of *Listeria monocytogenes* in bovine colostrum
569 in Japan. *J Food Prot* **76**, 248-255.
570 Hassan, L., Mohammed, H.O. and McDonough, P.L. (2001) Farm-management and milking
571 practices associated with the presence of *Listeria monocytogenes* in New York state
572 dairy herds. *Prev Vet Med* **51**, 63-73.
573 Henri, C., Leekitcharoenphon, P., Carleton, H.A., Radomski, N., Kaas, R.S., Mariet, J.F.,
574 Felten, A., Aarestrup, F.M., Gerner Smidt, P., Roussel, S., Guillier, L., Mistou, M.Y.
575 and Hendriksen, R.S. (2017) An assessment of different genomic approaches for
576 inferring phylogeny of *Listeria monocytogenes*. *Front Microbiol* **8**, 2351.
577 Ho, A.J., Ivanek, R., Grohn, Y.T., Nightingale, K.K. and Wiedmann, M. (2007) *Listeria*
578 *monocytogenes* fecal shedding in dairy cattle shows high levels of day-to-day variation
579 and includes outbreaks and sporadic cases of shedding of specific *L. monocytogenes*
580 subtypes. *Prev Vet Med* **80**, 287-305.
581 Hof, H. (2017) *Listeria* infections of the eye. *Eur J Ophthalmol* **27**, 115-121.
582 Hunt, K., Drummond, N., Murphy, M., Butler, F., Buckley, J. and Jordan, K. (2012) A case of
583 bovine raw milk contamination with *Listeria monocytogenes*. *Ir Vet J* **65**, 13.
584 ISO (International Organization for Standardization). (1996). *Microbiology of food and animal*
585 *feeding stuffs -- Horizontal method for the detection and enumeration of Listeria*
586 *monocytogenes -- Part 1: Detection method, Amendment 1: Modification of the*
587 *isolation media and the haemolysis test, and inclusion of precision data (ISO standard*
588 *11290-1:1996/Amd 1:2004)*. Geneva, Switzerland: ISO.
589 ISO (International Organization for Standardization). (2004). *Modification of the isolation*
590 *media and the haemolysis test, and inclusion of precision data (ISO standard 11290-*
591 *1:1996/Amd 1:2004).* Geneva, Switzerland: ISO.

592 Jackson, B.R., Tarr, C., Strain, E., Jackson, K.A., Conrad, A., Carleton, H., Katz, L.S., Stroika,
593 S., Gould, L.H., Mody, R.K., Silk, B.J., Beal, J., Chen, Y., Timme, R., Doyle, M.,
594 Fields, A., Wise, M., Tillman, G., Defibaugh-Chavez, S., Kucerova, Z., Sabol, A.,
595 Roache, K., Trees, E., Simmons, M., Wasilenko, J., Kubota, K., Pouseele, H., Klimke,
596 W., Besser, J., Brown, E., Allard, M. and Gerner-Smidt, P. (2016) Implementation of
597 nationwide real-time whole-genome sequencing to enhance listeriosis outbreak
598 detection and investigation. *Clin Infect Dis* **63**, 380-386.

599 Jensen, N.E., Aarestrup, F.M., Jensen, J. and Wegener, H.C. (1996) *Listeria monocytogenes* in
600 bovine mastitis. Possible implication for human health. *Int J Food Microbiol* **32**, 209-
601 216.

602 Kim, S.W., Haendiges, J., Keller, E.N., Myers, R., Kim, A., Lombard, J.E., Karns, J.S., Van
603 Kessel, J.A.S. and Haley, B.J. (2018) Genetic diversity and virulence profiles of
604 Listeria monocytogenes recovered from bulk tank milk, milk filters, and milking
605 equipment from dairies in the United States (2002 to 2014). *PLoS One* **13**, e0197053.

606 Kuch, A., Goc, A., Belkiewicz, K., Filipello, V., Ronkiewicz, P., Goł biewska, A., Wróbel, I.,
607 Kiedrowska, M., Wa ko, I., Hryniewicz, W., Lomonaco, S. and Skoczy ska, A. (2018)
608 Molecular diversity and antimicrobial susceptibility of *Listeria monocytogenes*
609 isolates from invasive infections in Poland (1997–2013). *Sci Rep* **8**, 14562.

610 Latorre, A.A., Van Kessel, J.A., Karns, J.S., Zurakowski, M.J., Pradhan, A.K., Zadoks, R.N.,
611 Boor, K.J. and Schukken, Y.H. (2009) Molecular ecology of *Listeria monocytogenes*:
612 evidence for a reservoir in milking equipment on a dairy farm. *Appl Environ Microbiol*
613 **75**, 1315-1323.

614 Latorre, A.A., Van Kessel, J.S., Karns, J.S., Zurakowski, M.J., Pradhan, A.K., Boor, K.J.,
615 Jayarao, B.M., Houser, B.A., Daugherty, C.S. and Schukken, Y.H. (2010) Biofilm in

616 milking equipment on a dairy farm as a potential source of bulk tank milk
617 contamination with *Listeria monocytogenes*. *J Dairy Sci* **93**, 2792-2802.
618 Letunic, I. and Bork, P. (2016) Interactive tree of life (iTOL) v3: an online tool for the display
619 and annotation of phylogenetic and other trees. *Nucleic Acids Res* **44**, W242-245.
620 Low, J.C. and Donachie, W. (1997) A review of *Listeria monocytogenes* and listeriosis. *Vet J*
621 **153**, 9-29.
622 Lundén, J., Tolvanen, R. and Korkeala, H. (2004) Human listeriosis outbreaks linked to dairy
623 products in Europe. *J Dairy Sci* **87**, E6-E12.
624 Lyautey, E., Lapen, D.R., Wilkes, G., McCleary, K., Pagotto, F., Tyler, K., Hartmann, A.,
625 Piveteau, P., Rieu, A., Robertson, W.J., Medeiros, D.T., Edge, T.A., Gannon, V. and
626 Topp, E. (2007) Distribution and characteristics of *Listeria monocytogenes* isolates
627 from surface waters of the South Nation River watershed, Ontario, Canada. *Appl*
628 *Environ Microbiol* **73**, 5401-5410.
629 Maury, M.M., Tsai, Y.H., Charlier, C., Touchon, M., Chenal-Francisque, V., Leclercq, A.,
630 Criscuolo, A., Gaultier, C., Roussel, S., Brisabois, A., Disson, O., Rocha, E.P.C.,
631 Brisse, S. and Lecuit, M. (2016) Uncovering *Listeria monocytogenes* hypervirulence
632 by harnessing its biodiversity. *Nat Genet* **48**, 308-313.
633 Mohammed, H.O., Stipetic, K., McDonough, P.L., Gonzalez, R.N., Nydam, D.V. and Atwill,
634 E.R. (2009) Identification of potential on-farm sources of *Listeria monocytogenes* in
635 herds of dairy cattle. *Am J Vet Res* **70**, 383-388.
636 Moura, A., Criscuolo, A., Pouseele, H., Maury, M.M., Leclercq, A., Tarr, C., Björkman, J.T.,
637 Dallman, T., Reimer, A., Enouf, V., Larsonneur, E., Carleton, H., Bracq-Dieye, H.,
638 Katz, L.S., Jones, L., Touchon, M., Tourdjman, M., Walker, M., Stroika, S., Cantinelli,
639 T., Chenal-Francisque, V., Kucerova, Z., Rocha, E.P., Nadon, C., Grant, K., Nielsen,
640 E.M., Pot, B., Gerner-Smidt, P., Lecuit, M. and Brisse, S. (2016) Whole genome-based

641 population biology and epidemiological surveillance of *Listeria monocytogenes*. *Nat*
642 *Microbiol* **2**, 16185.
643 Moura, A., Tourdjman, M., Leclercq, A., Hamelin, E., Laurent, E., Fredriksen, N., Van
644 Cauteren, D., Bracq-Dieye, H., Thouvenot, P., Vales, G., Tessaud-Rita, N., Maury,
645 M.M., Alexandru, A., Criscuolo, A., Quevillon, E., Donguy, M.P., Enouf, V., de Valk,
646 H., Brisse, S. and Lecuit, M. (2017) Real-time whole-genome sequencing for
647 surveillance of *Listeria monocytogenes*, France. *Emerg Infect Dis* **23**, 1462-1470.
648 Muhterem-Uyar, M., Dalmasso, M., Bolocan, A.S., Hernandez, M., Kapetanakou, A.E.,
649 Kuchta, T., Manios, S.G., Melero, B., Minarovi ová, J., Nicolau, A.I., Rovira, J.,
650 Skandamis, P.N., Jordan, K., Rodríguez-Lázaro, D., Stessl, B. and Wagner, M. (2015)
651 Environmental sampling for *Listeria monocytogenes* control in food processing
652 facilities reveals three contamination scenarios. *Food Control* **51**, 94-107.
653 Nightingale, K.K., Fortes, E.D., Ho, A.J., Schukken, Y.H., Grohn, Y.T. and Wiedmann, M.
654 (2005) Evaluation of farm management practices as risk factors for clinical listeriosis
655 and fecal shedding of *Listeria monocytogenes* in ruminants. *J Am Vet Med Assoc* **227**,
656 1808-1814.
657 Nightingale, K.K., Schukken, Y.H., Nightingale, C.R., Fortes, E.D., Ho, A.J., Her, Z., Grohn,
658 Y.T., McDonough, P.L. and Wiedmann, M. (2004) Ecology and transmission of
659 *Listeria monocytogenes* infecting ruminants and in the farm environment. *Appl*
660 *Environ Microbiol* **70**, 4458-4467.
661 Oevermann, A., Zurbriggen, A. and Vandevelde, M. (2010) Rhombencephalitis caused by
662 *Listeria monocytogenes* in humans and ruminants: a zoonosis on the rise? *Interdiscip*
663 *Perspect Infect Dis* **2010**, 632513.

664 OIE (World Organisation for Animal Health) (2004) Manual of diagnostic tests and vaccines
665 for terrestrial animals, Section 3.9, Chapter 3.9.6. *Listeria monocytogenes* (updates
666 adopted in May 2014). Paris, France: OIE.
667 Painset, A., Björkman, J.T., Kiil, K., Guillier, L., Mariet, J.F., Félix, B., Amar, C., Rotariu, O.,
668 Sophie Roussel, S., Francisco Perez-Reche, F., Brisse, S., Moura, A., Lecuit, M.,
669 Forbes, K., Strachan, N., Grant, K., Møller-Nielsen, E., and Dallman T.J. (2019)
670 LiSEQ – whole-genome sequencing of a cross-sectional survey of *Listeria*
671 *monocytogenes* in ready-to-eat foods and human clinical cases in Europe. *Microb*
672 *Genom* **5**, 1–11.
673 Portmann, A.C., Fournier, C., Gimonet, J., Ngom-Bru, C., Barretto, C. and Baert, L. (2018) A
674 validation approach of an end-to-end whole genome sequencing workflow for source
675 tracking of *Listeria monocytogenes* and *Salmonella enterica*. *Front Microbiol* **9**, 446.
676 Rabozzi, G., Bonizzi, L., Crespi, E., Somaruga, C., Sokooti, M., Tabibi, R., Vellere, F.,
677 Brambilla, G. and Colosio, C. (2012) Emerging zoonoses: the "One Health Approach".
678 *Saf Health Work* **3**, 77-83.
679 Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., Le Monnier, A. and Brisse, S.
680 (2008) A new perspective on *Listeria monocytogenes* evolution. *PLoS Pathog* **4**,
681 e1000146.
682 Reimer, A., Weedmark, K., Petkau, A., Peterson, C.L., Walker, M., Knox, N., Kent, H., Mabon,
683 P., Berry, C., Tyler, S., Tschetter, L., Jerome, M., Allen, V., Hoang, L., Bekal, S.,
684 Clark, C., Nadon, C., Van Domselaar, G., Pagotto, F., Graham, M., Farber, J. and
685 Gilmour, M. (2019) Shared genome analyses of notable listeriosis outbreaks,
686 highlighting the critical importance of epidemiological evidence, input datasets and
687 interpretation criteria. *Microb Genom* **5**, 1-16.

688 Rocha, P.R., Lomonaco, S., Bottero, M.T., Dalmasso, A., Dondo, A., Grattarola, C., Zuccon,
689 F., Iulini, B., Knabel, S.J., Capucchio, M.T. and Casalone, C. (2013) Ruminant
690 rhombencephalitis-associated *Listeria monocytogenes* strains constitute a genetically
691 homogeneous group related to human outbreak strains. *Appl Environ Microbiol* **79**,
692 3059-3066.
693 Salipante, S.J., SenGupta, D.J., Cummings, L.A., Land, T.A., Hoogestraat, D.R. and Cookson,
694 B.T. (2015) Application of whole-genome sequencing for bacterial strain typing in
695 molecular epidemiology. *J Clin Microbiol* **53**, 1072-1079.
696 Sanaa, M., Poutrel, B., Menard, J.L. and Serieys, F. (1993) Risk factors associated with
697 contamination of raw milk by *Listeria monocytogenes* in dairy farms. *J Dairy Sci* **76**,
698 2891-2898.
699 Stamatakis, A. (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of
700 large phylogenies. *Bioinformatics* **30**, 1312-1313.
701 Stari , J., Križanec, F., Zadnik, T. (2008) *Listeria monocytogenes* keratoconjunctivitis and
702 uveitis in dairy cattle. *Bull Vet Inst Pulawy* **52**, 351-355.
703 Stasiewicz, M.J., Oliver, H.F., Wiedmann, M. and den Bakker, H.C. (2015) Whole-genome
704 sequencing allows for improved identification of persistent *Listeria monocytogenes* in
705 food-associated environments. *Appl Environ Microbiol*. **81**, 6024-6037.
706 Steckler, A.J., Cardenas-Alvarez, M.X., Townsend Ramsett, M.K., Dyer, N. and Bergholz,
707 T.M. (2018) Genetic characterization of *Listeria monocytogenes* from ruminant
708 listeriosis from different geographical regions in the U.S. *Vet Microbiol* **215**, 93-97.
709 Stein, H., Stessl, B., Brunthaler, R., Loncaric, I., Weissenbock, H., Ruczizka, U., Ladinig, A.
710 and Schwarz, L. (2018) Listeriosis in fattening pigs caused by poor quality silage - a
711 case report. *BMC Vet Res* **14**, 362.

712 Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the
713 sensitivity of progressive multiple sequence alignment through sequence weighting,
714 position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-
715 4680.
716 Tienungoon, S., Ratkowsky, D.A., McMeekin, T.A. and Ross, T. (2000) Growth limits of
717 *Listeria monocytogenes* as a function of temperature, pH, NaCl, and lactic acid. *Appl*
718 *Environ Microbiol* **66**, 4979-4987.
719 Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel,
720 W., González-Zorn, B., Wehland, J. and Kreft, J. (2001) *Listeria* pathogenesis and
721 molecular virulence determinants. *Clin Microbiol Rev* **14**, 584-640.
722 Wagner, M., Melzner, D., Bagò, Z., Winter, P., Egerbacher, M., Schilcher, F., Zangana, A. and
723 Schoder, D. (2005) Outbreak of clinical listeriosis in sheep: evaluation from possible
724 contamination routes from feed to raw produce and humans. *J Vet Med B Infect Dis*
725 *Vet Public Health* **52**, 278-283.
726 Walland, J., Lauper, J., Frey, J., Imhof, R., Stephan, R., Seuberlich, T. and Oevermann, A.
727 (2015) *Listeria monocytogenes* infection in ruminants: is there a link to the
728 environment, food and human health? A review. *Schweiz Arch Tierheilkd* **157**, 319-
729 328.
730 Wiedmann, M., Mobini, S., Cole, J.R., Watson, C.K., Jeffers, G.T. and Boor, K.J. (1999)
731 Molecular investigation of a listeriosis outbreak in goats caused by an unusual strain
732 of *Listeria monocytogenes*. *J Am Vet Med Assoc* **215**, 369-371.
733

734 **Supporting information**

735 **Figure S1** Virulence gene presence/absence pattern of 120 confirmed and candidate virulence
736 genes, mapped onto the core genome phylogenetic tree. A total of 16 *Listeria monocytogenes*

737 isolates that underwent WGS were screened for the presence of virulence genes. For easier
738 visualization, virulence genes were divided into 80 previously characterized virulence factors
739 (A) and 40 recently described candidate virulence factors associated with clinically relevant
740 phenotypes (B). The colors of the leaves on the tree represent isolation origin: red, clinical
741 origin; blue, non-clinical origin. Bar, number of amino acid substitutions per site.
742 **File S1** Gentamicin protection assay in the bovine macrophage (BoMac) cell line.
743 **Table S1** Availability of raw sequencing data.
744 **Table S2** List of 151 confirmed and candidate *Listeria monocytogenes* virulence genes used in
745 this study.

Table 1: Overview of samples collected in 2014 at the organic dairy farm after bovine neurolisteriosis case confirmation.

Date of sampling	**Source**	**Sample type/No.**	***L. monocytogenes* cultivation**	**Isolate ID, selected for typing*****
March 18	Euthanized cow	Brain tissue (*n*=1)	Positive	***L628***
March 30	Lactating cows I*	Milk (*n*=47)	Positive 9/47	***L630***, **L631**, L632, L633, **L634**, **L635**, **L636**, ***L637***, ***L638***
	Feed	Grass silage (*n*=1)	Positive	**L639**
April 15	Lactating cows II**	Milk (*n*=44)	Negative	-
		Bulk tank milk (*n*=1)	Negative	-
		Individual milk samples (*n*=9) and pooled milk (*n*=1) from the treated cows	Negative	-
	Cows	Feces of treated cows (*n*=9)	Negative	-
	Bull	Feces (*n*=1)	Negative	-
	Water	Water–pipe (barn) (*n*=1)	Positive***	***L667***, L689, L690, ***L691***, L692
		Water–trough (barn) (*n*=1)	Positive***	L668, L693, **L694**, L695, L696
		Water–pond (*n*=1)	Positive***	***L669***, L697, L698, **L699**, L700
	Deer	Pooled fecal sample (*n*=1)	Positive***	***L670***, L701, L702, L703, L704
April 24	Water	Water (household, milk room, well) (*n*=3)	Negative	-
May 4	Treated cows	Individual milk samples (*n*=9)	Negative	-
		Bulk tank milk (*n*=1)	Negative	-
	Water	Water–household (*n*=1)	Positive	L705, ***L706***, L707, L708, L709
	Total no.	132 samples	16 positive samples	36 *L. monocytogenes* isolates

* 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 *Asc*I-*Apa*I 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 *Asc*I-*Apa*I analysis, as previously described (Félix *et al*. 2018). Bar, average similarity of *Asc*I-*Apa*I 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.