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Ruminant Rhombencephalitis-Associated *Listeria monocytogenes* Strains Constitute a Genetically Homogeneous Group Related to Human Outbreak Strains

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Listeriosis is a disease that causes significant economic losses at the farm level because of high morbidity and mortality in ruminants. This study was performed to investigate the role of ruminants in the epidemiology of listeriosis in northern Italy and the possible association of animal-adapted strains of *Listeria monocytogenes* with strains associated with human disease. Twenty ruminant rhombencephalitis isolates previously confirmed as *L. monocytogenes* by bacteriology and PCR were characterized by serotyping, pulsed-field gel electrophoresis, multi-virulence-locus sequence typing (MVLST), and multiplex single nucleotide polymorphism (mSNP) typing for the detection of epidemic clones. Subtyping results were subsequently compared with those obtained from human, food, and environmental isolates of *L. monocytogenes*, including 311 isolates from the University of Turin, Grugliasco, Italy, and 165 isolates representing major human listeriosis outbreaks worldwide, in addition to other unrelated isolates. Both mSNP typing and MVLST showed that 60% of the isolates analyzed belonged to epidemic clone I (ECI), which has been epidemiologically linked to several human outbreaks of listeriosis. In particular, the 1981 Canada outbreak was linked to the use of sheep manure and the 1985 California outbreak was linked to the use of raw cow's milk. In our study, ECI isolates were collected from different ruminant species on geographically and temporally distinct occasions for the last 13 years. Our results support the hypothesis that ruminants represent possible natural reservoirs of *L. monocytogenes* strains capable of causing epidemics of listeriosis in humans.

Listeria monocytogenes is a Gram-positive, facultative, saprophytic, food-borne pathogen that causes listeriosis most commonly in immunocompromised mammals (1, 2). The main clinical manifestations of listeriosis in both humans and animals include encephalitis, septicemia, and abortion (1, 3). In ruminants, listeriosis is the most important central nervous system disease in various European neuropathological surveillance programs (4). The disease is most commonly acquired through the consumption of contaminated feed and food, particularly silage by ruminants and ready-to-eat (RTE) foods by humans (1, 5–7).

In the past few years, several phenotypic and genotypic typing systems have been able to subtype *L. monocytogenes* at various levels of taxonomic resolution, from genetic lineages (3) to serotypes (1) to clonal complexes (8) to epidemic clones (ECs) (5, 9) to strains (5). In particular, ECs of *L. monocytogenes* are defined as groups of genetically related strains that have been implicated in geographically and temporally unrelated outbreaks (5, 9). Although new typing methods have been developed (10–13), pulsed-field gel electrophoresis (PFGE) is still considered the gold standard method for the subtyping of *L. monocytogenes* (14) and, with serotyping, has been routinely used to characterize human and food isolates (15–20). Multi-virulence-locus sequence typing (MVLST) is a sequence typing method that has shown high discriminatory power, epidemiologic concordance, stability, typeability, and reproducibility. MVLST correctly identified all seven currently known ECs of *L. monocytogenes* (21–23).

PFGE and other molecular subtyping techniques have rarely been used to characterize animal isolates of *L. monocytogenes* (24–28). In fact, most isolates of *L. monocytogenes* from animal clinical cases have been characterized by serotyping and ribotyping (5, 20,

29). The latter has a lower discriminatory power than other fragment-based techniques, such as PFGE (14). Serotyping is able to identify 13 serotypes, but the majority of the strains associated with human listeriosis cases and outbreaks are represented by only three of them: serotypes 1/2a, 1/2b, and 4b (14). When a study used PFGE for the characterization of animal clinical isolates of *L. monocytogenes*, it presented preliminary data supporting a genetic correlation between individual strains responsible for veterinary clinical cases and strains recovered from cases of human listeriosis. It was therefore hypothesized that food animals could represent reservoirs of *L. monocytogenes* able to cause human infections (26). The role of ruminants as possible reservoirs of *L. monocytogenes* strains that are pathogenic to humans was also suggested by other studies (30–32). However, those studies did not systematically compare animal clinical isolates with those associated with human outbreaks.

Listeriosis is an important issue from a veterinary standpoint, considering the significant economic losses at the farm level due to the morbidity and high mortality it causes in animals (33). Veterinary listeriosis can also pose a potential risk to food safety and public health because of a link between the farm environment and

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TABLE 1 Description of the 20 *L. monocytogenes* isolates obtained from animal clinical cases

ID	Source	Yr isolated, location	Serotype	EC	Ascl pulsotype	ApaI pulsotype	Combined profile	MVLST VT
01	Bovine	2005, Lombardy	4b	ECI	S1	A3	P1	20
02	Bovine	2005, Lombardy	1/2b	— ^a	Unique ^b	A2	Unique	78
03	Bovine	2007, Piedmont	4e	—	S4	A4	P4	75
04	Caprine	2009, Piedmont	1/2a	—	S5	Unique	Unique	76
05	Ovine	2001, Aosta Valley	4b	ECI	S1	Unique	Unique	20
06	Bovine	2001, Piedmont	4b	ECI	S1	A3	P1	20
07	Bovine	2001, Piedmont	4b	ECI	S2	A2	P2	20
08	Bovine	2001, Lombardy	4b	ECI	S2	A2	P2	20
09	Bovine	2002, Lazio	4e	ECI	S3	A1	P3	20
10	Bovine	2002, Piedmont	4b	ECI	S2	A2	P2	20
11	Bovine	2002, Emilia Romagna	4b	—	Unique	A4	Unique	75
12	Bovine	2003, Veneto	4b	ECI	S3	A1	P3	20
13	Bovine	2009, Piedmont	4e	ECI	Unique	Unique	Unique	20
14	Ovine	2010, Piedmont	4b	—	S4	A4	P4	75
15	Bovine	2010, Piedmont	4b	ECI	S3	A1	P3	20
16	Ovine	2009, Piedmont	1/2b	—	Unique	Unique	Unique	08
17	Ovine	1999, Aosta Valley	4b	ECI	S1	Unique	Unique	20
18	Caprine	2000, Piedmont	4b	ECI	S3	A1	P3	20
19	Ovine	2002, Liguria	1/2a	—	S5	Unique	Unique	76
20	Caprine	2011, Piedmont	1/2a	—	Unique	Unique	Unique	77

^a —, not an epidemic clone.

^b Unique pulsotype not similar to any other observed in the database.

human infection (33). Studies aimed at characterizing isolates obtained from animals suffering from listeriosis are needed in order to better understand the pathophysiological, epidemiological, and genetic mechanisms involved in all clinical forms of the disease in different species. Additionally, further evaluation of the genetic diversity of *L. monocytogenes* isolates from ruminant rhombencephalitis and their degree of overlap with strains from human listeriosis cases is also desirable. To this end, *L. monocytogenes* isolates from ruminant rhombencephalitis cases collected over a 13-year period by the Center for Animal Encephalopathy (CEA) at the Istituto Zooprofilattico Sperimentale di Piemonte, Liguria, and Valle d'Aosta and within the Department of Veterinary Sciences of the University of Turin, Grugliasco, Italy, were characterized by serotyping, PFGE, multiplex single nucleotide polymorphism (mSNP) typing, and MVLST. Typed strains were compared with different sets of isolates collected from human outbreaks and sporadic cases and food and environmental sources previously characterized by PFGE (34) and MVLST (13, 21, 22). Molecular subtyping results were subsequently compared with microscopic brain lesions in order to determine possible associations between the severity and distribution of brain lesions and the location, animal species, and subtypes of *L. monocytogenes* strains isolated.

MATERIALS AND METHODS

Twenty cases of ruminant rhombencephalitis (12 cattle, five sheep, and three goats; Table 1) were diagnosed within the CEA and the Department of Veterinary Sciences, University of Turin, Grugliasco, Italy. The animals either died spontaneously or were sacrificed because of neurological signs and a poor prognosis. Brains were sampled and divided by an aseptic paramedian cut, and the larger portion was fixed in 10% buffered formaldehyde solution; the smaller portion was frozen at -20°C . Because of neurologic disease, all cases were also submitted for confirmatory tests for transmissible spongiform encephalopathies (TSE) (35).

Histopathologic and IHC evaluations. In each case, the obex; pons; mesencephalon; cerebellum; hippocampus; thalamus; basal nuclei; and occipital, parietal, and frontal cortex were trimmed. Subsequently, the tissues were routinely processed and embedded in paraffin and 4- μm

tissue sections were cut for histopathologic and immunohistochemical (IHC) evaluations. The sections used for histopathologic evaluation were stained with hematoxylin and eosin (H&E), histopathologic lesions were categorized by semiquantitative assessment of each anatomic brain region for the extent and size of microabscesses/perivascular cuffs by a previously established approach (36), and semiquantitative scores of 0 to 4 were assigned. IHC was performed by using the following protocol. For antigen retrieval, paraffin-included 4- μm brain sections were treated with citrate buffer (pH 6.0) at 100°C for 15 min. Subsequently, the sections were exposed to primary antibody (polyclonal anti-*Listeria* species antibody, code 43251; 1:250 dilution, Virostat, Portland, OR) and incubated overnight. The sections were then exposed to a secondary antibody and immunoperoxidase stained with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Microscopically positive antigen-antibody reactions were visualized after incubation with the chromogen 3,3'-diaminobenzidine (DAB) for 1 min at room temperature. Phosphate-buffered saline was used to wash the slides between the different preparation steps. H&E-stained and IHC slides were examined with a Leica DM LS2 light microscopic (Leica Microsystems, Wetzlar, Germany) at different magnifications. H&E-stained slides were evaluated at magnification levels of $\times 25$, $\times 100$, $\times 200$, and $\times 400$; a magnification of $\times 400$ was used to evaluate IHC immunopositivity.

Bacteriology, serotyping, and DNA isolation. The frozen brain samples were thawed, placed in liquid medium (Demi-Fraser broth and Fraser broth; Microbiol, Bagno a Ripoli, Italy), and incubated at 37°C for 48 h and at 4°C for 10 days. Subsequently, cultures were streaked onto *Listeria* Oxford agar (Microbiol) and incubated at 37°C for 48 h. Suspected *Listeria* species colonies were confirmed as *L. monocytogenes* with a miniaturized API System (bioMérieux, Marcy l'Etoile, France) and a colorimetric Vitek 2 identification System (bioMérieux, Bagno a Ripoli, Italy). All *L. monocytogenes* animal isolates were serotyped with antisera against the O and H antigens according to the manufacturer's instructions (Denka Seiken Co., Ltd., Tokyo, Japan). Serotypes were further confirmed with a multiplex PCR (37).

All isolates were stored at -80°C in cryogenic vials. Prior to DNA extraction, isolates were grown overnight at 37°C in tryptone soy broth (TSB; Acumedia, Lansing, MI). DNA was extracted by boiling with the following protocol. One milliliter of TSB culture (10^8 CFU/ml) was centrifuged at $12,000 \times g$ for 5 min; the pellet was subsequently resuspended

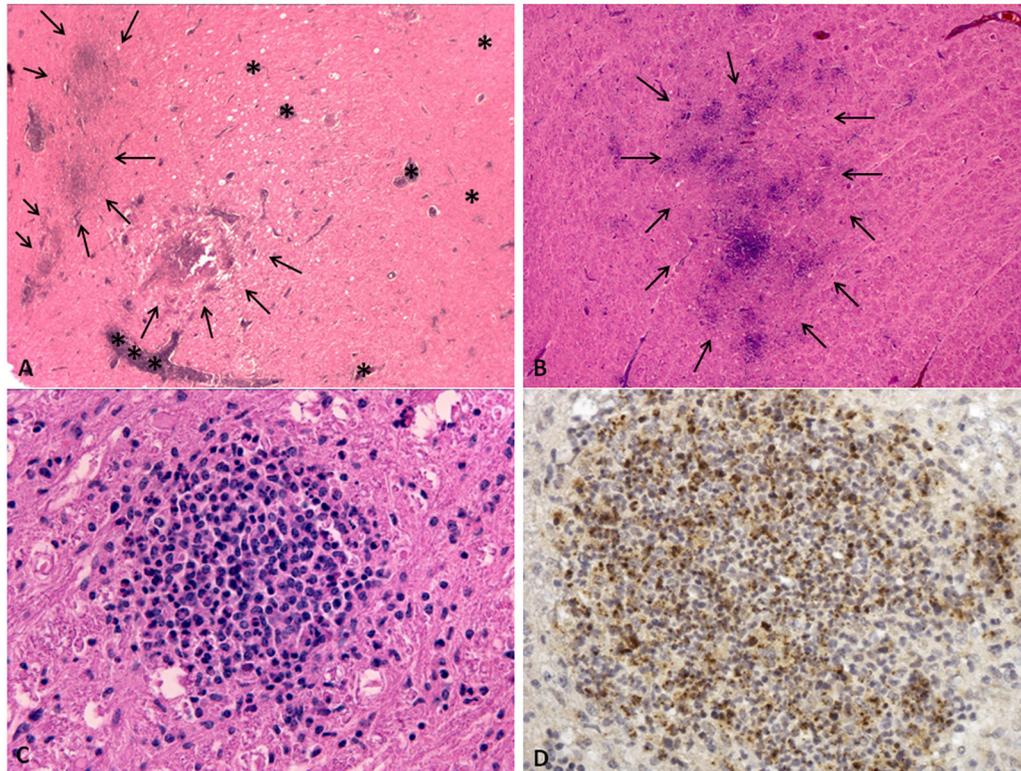


FIG 1 Histopathologic evaluations. (A) Bovine cerebellum. Multifocal-to-coalescing microabscesses (arrows) and mononuclear perivascular cuffing (asterisks) in the white matter. H&E staining. Magnification, $\times 25$. (B) Ovine brainstem. Malacic area (arrows) composed mainly of neutrophils. H&E staining. Magnification, $\times 25$. (C) Bovine brainstem. Microabscess composed mainly of macrophages and a few neutrophils. H&E staining. Magnification, $\times 400$. (D) Caprine brainstem. Immunopositivity (brown areas) for *Listeria* species in the cytoplasm of macrophages and neutrophils within a microabscess. DAB chromogen counterstained with hematoxylin. Magnification, $\times 400$.

in 1 ml of phosphate-buffered saline and boiled for 5 min. After centrifugation, the supernatant was stored at -20°C until use. DNA was quantified with a Bio-Photometer 6131 spectrophotometer (Eppendorf AG, Hamburg, Germany) and diluted to a concentration of 50 to 60 ng/ μl prior to PCR.

Duplex PCR, mSNP typing, PFGE, and MVLST. Isolates were confirmed as *L. monocytogenes* by a duplex PCR-minisequencing assay able to identify *L. monocytogenes* and five other *Listeria* species (38). Ruminant isolates were also analyzed with an mSNP typing screening assay able to identify five ECs (ECI, ECII, ECIII, ECIV, and ECV) of *L. monocytogenes* (39).

PFGE was performed by including genomic DNA in agarose plugs prior to digestion with the *AscI* and *Apal* restriction enzymes (New England BioLabs), followed by PFGE with a Chef DR III system (Bio-Rad, Hercules, CA) (40), and pulsotypes were analyzed as described elsewhere (34). Isolates were considered to belong to the same cluster when they showed a PFGE similarity level (SL) of at least 80%, while isolates with an SL of 100% were considered to be identical. PFGE profiles were subsequently compared with a set of 311 well-characterized *L. monocytogenes* strains isolated from environmental, food, and human clinical samples (34).

Intragenic regions of six virulence genes (*clpP*, *dal*, *inlB*, *inlC*, *lisR*, and *prfA*) were amplified, resolved, purified, and sequenced as previously described (21). Both forward and reverse strands were sequenced, and gene sequences were subsequently concatenated with Sequence Matrix 1.7.8 (41) and compared with sequences from 165 isolates representing major listeriosis outbreaks worldwide (13, 21, 22) with MEGA v5.0 (42). New allelic sequences (with at least one nucleotide difference) were assigned arbitrary virulence type (VT) numbers as previously described (23). The

neighbor-joining method in MEGA 5.0 was used to construct an unrooted neighbor-joining tree by using the number of nucleotide differences in the concatenated sequences of six loci with 1,000 bootstrap tests.

Statistical analysis. Brain lesion scores (microabscesses and perivascular cuffing lesion scores) were compared with geographic origins, ruminant species, and *L. monocytogenes* serotypes by using Kruskal-Wallis and Wilcoxon rank sum tests. Correlations between microabscesses and perivascular cuffing lesion scores were estimated with a Spearman rank correlation test. Test results were considered statistically significant when the *P* value was <0.05 . All statistical analyses were conducted with R statistical software (43).

Nucleotide sequence accession numbers. The gene sequences determined in this study were deposited in the GenBank database under accession numbers JX943890 to JX944009.

RESULTS

Histopathology and immunohistochemistry. No macroscopic lesions were observed in any of the cases analyzed in this study. However, microscopic brain lesions were observed and included mainly multifocal microabscesses, perivascular cuffing, and edema. Microabscesses (Fig. 1) were characterized by multifocal randomly distributed aggregates of neutrophils, small-to-coalescing foci of liquefactive necrosis, and edema. Microabscesses were observed in both gray matter and white matter but were more frequently observed in the white matter of rostral areas (cerebellum, mesencephalon, and thalamus) than in the brainstem. Multifocal gliosis, diffuse infiltration of neutrophils and microglial cells, and neuronal necrosis were observed in the areas adjacent to

microabscesses. The latter particularly involved the brainstem and cerebellum (data not shown). The rostral areas of the brain (thalamus, basal nuclei, and adjacent parietal cortex) were involved only when extensive microscopic lesions were present in the brainstem. Perivascular cuffings (Fig. 1A) were observed in all cases and were composed of at least one layer of inflammatory cells, most frequently lymphocytes and sporadically macrophages. In two cases, neutrophils predominated in the perivascular cuffings. The emergences of cranial nerves (mostly trigeminal and facial nerves) in the brainstem were affected by mostly microabscesses. All cases were positive by immunohistochemistry, thus confirming that *Listeria* species bacteria were present and associated with brain lesions. Immunopositivity was frequently observed in the cytoplasm of neutrophils in the microabscesses, in macrophages surrounding microabscesses (Fig. 1D), and in the neutrophils but less frequently in the perivascular cuffings and rarely in the cytoplasm of neurons. Immunopositivity was detected in the obex in all of the animals, followed by the brainstem (67% of the bovines and 100% of the small ruminants) and mesencephalon (50% of the bovines and 75% of the small ruminants). Confirmatory tests for TSEs were negative in all cases.

Bacteriology, serotyping, and EC screening. All isolates were confirmed as *L. monocytogenes*. Numbers of cases, animal species, serotypes, sources, and molecular subtyping results are summarized in Table 1. Serotype 4b was detected in 12 cases (60%; 8 bovine, 3 ovine, and 1 caprine), serotype 4e was detected in 3 cases (15%; all bovine), serotype 1/2a was detected in 3 cases (15%; 1 ovine and 2 caprine), and serotype 1/2b was detected in 2 cases (10%; 1 ovine and 1 bovine). The mSNP typing screening assay showed the ECI-specific profile in 12 isolates (60%).

PFGE profiles and MVLST VTs of the 20 animal isolates. Thirteen combined PFGE profiles and six MVLST VTs were identified in the 20 *L. monocytogenes* isolates analyzed here (Fig. 2 and Table 1). PFGE distinguished cluster I grouping 13 isolates (SL = 82.3%) and cluster II grouping 3 isolates (SL = 96.5%). Four isolates (no. 02, 16, 19, and 20) had an SL of 65.6% to all of the other isolates. Isolates were partially divided according to serotypes (Fig. 2).

A total concatenation length of 2,606 bp was observed for the six virulence genes analyzed by MVLST. New VTs were assigned for isolates 3, 11, and 14 (VT75); 4 and 19 (VT76); 20 (VT77); and 2 (VT78). Overall, MVLST identified three shared VTs (VT20, VT75, and VT76, composed of 12, 2, and 3 isolates, respectively) and three unique ones (VT8, VT77, and VT78). The MVLST neighbor-joining tree revealed three main clusters supported by bootstrap values of 92, 98, and 100%, respectively, clearly dividing isolates according to serotype. The 12 isolates presumptively identified as ECI by mSNP typing were confirmed as ECI (VT20) by MVLST. These 12 VT20 isolates showed four shared PFGE profiles and one unique profile (Table 1) and grouped at an 84.6% SL into cluster I. The VT75 isolates showed two shared profiles and one unique profile and grouped at a 96.5% SL into cluster II, whereas VT76 showed two unique profiles and grouped at a 68.9% SL.

Comparison with previously subtyped strains. None of the 20 *L. monocytogenes* ruminant clinical isolates had the same PFGE profile as any other sample in our PFGE database (data not shown). ECI isolates 9, 12, 15, and 18 had an SL of 90.1% with human clinical isolate U1, also previously identified as ECI (39).

The MVLST neighbor-joining tree revealed three main clusters

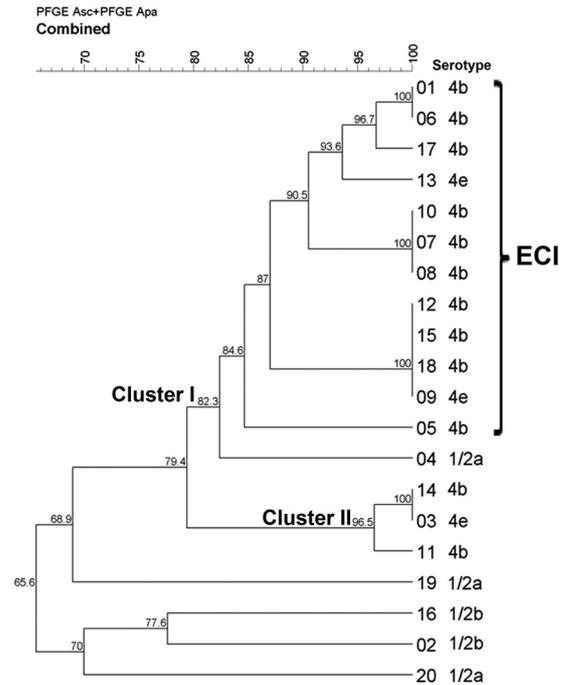


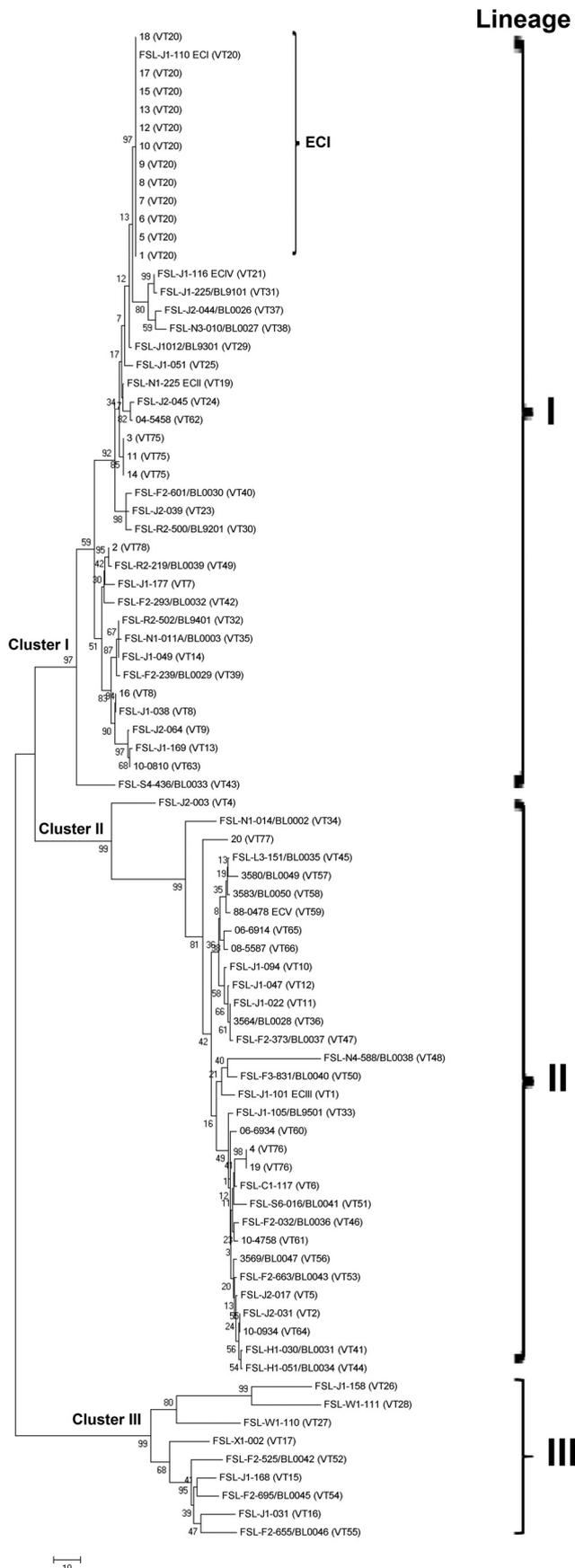
FIG 2 Dendrogram produced by the unweighted-pair group method using average linkages showing clustering by Dice coefficient of the combined PFGE (AscI and ApaI) profiles of the 20 *L. monocytogenes* isolates obtained from animal cases analyzed in this study. PFGE divided the *L. monocytogenes* animal isolates into nine unique and four shared profiles, P1 (two isolates), P2 (three isolates), P3 (four isolates), and P4 (two isolates).

that clearly separated serotypes, supported by bootstrap values of 97, 99, and 99%. Overall, clusters I, II, and III corresponded to lineages I, II, and III, respectively (Fig. 3). Only one isolate other than those belonging to VT20 showed a VT previously observed in human clinical cases, as isolate 16 (cluster I) had the same VT (VT8) as an *L. monocytogenes* strain of serotype 1/2b responsible for a human sporadic clinical case (13). VT78 differed by one SNP from VT49, observed in a food environmental isolate (21), and VT76 differed by three SNPs from a human clinical isolate assigned to VT6 (13). Isolate 20 was not closely related to any other isolate included in the comparison.

Statistical analysis. No significant associations between lesions, ruminant species, geographic origins, and *L. monocytogenes* serotypes were found (Kruskal-Wallis rank sum test; $P > 0.05$ in all cases). A significant positive correlation between the grade of microabscesses and perivascular cuffing was observed (Spearman rank correlation $\rho = 0.5702$; $P < 0.01$), particularly when considering only bovines (Spearman rank correlation $\rho = 0.8694$; $P < 0.001$). However, no significant correlation was detected when considering only small ruminants (Spearman rank correlation $\rho = 0.1443$; $P = 0.733$).

DISCUSSION

Based on the type and severity of histologic lesions and the statistical significance of more acute meningoencephalitis and rostral spread of lesions, small ruminants appear to be less capable than cattle of eliminating *L. monocytogenes* from their brains and thus are more susceptible to listeriosis once the pathogen reaches the brain, which is in line with previous observations (1, 4, 33). Our



findings support the hypothesis of pathogenesis of *L. monocytogenes* migration along the cranial nerves through the brains of mammals (4, 33, 44–46). However, the pathogenic mechanisms by which bacteria cause neuron injury by centripetally migrating into the brains of both humans and animals are still poorly understood (2, 46, 47) and thus require further investigation.

The prevalence of serotypes corresponding to lineage I reported in this study clearly supports this lineage as highly common in mammals (1, 3, 6, 9, 17, 19, 28, 30, 48). Lineage I has been frequently linked to human cases and associated with the encephalitic form of the disease in ruminants (6, 30, 49). Lineage II has been described as genetically more diverse and more frequently associated with both encephalitic and septicemic forms of listeriosis in ruminants (6, 49). Although reported to cause up to 10% of animal listeriosis cases (49), ruminant isolates belonging to lineage III were not identified in this study, as also previously observed (30). However, in both studies, no isolates obtained from other clinical forms of animal listeriosis (e.g., septicemia and/or abortion) were included.

PFGE is currently the gold standard method of characterizing *L. monocytogenes*, and it is still essential in epidemiologic investigations of listeriosis outbreaks and in the subtyping of food-borne pathogens (14, 50). The strains identified here as ECI showed four different PFGE profiles. Furthermore, isolates 3, 11, and 14 had the same MVLST VT (VT75), as did isolates 4 and 19 (VT76), but showed different PFGE profiles. It has been shown that epidemiologically linked *L. monocytogenes* strains isolated from the same outbreak can have different PFGE profiles (22, 51–54).

An MVLST approach was used in this study in addition to PFGE, as the former allows the clustering of *L. monocytogenes* strains with high epidemiologic concordance and discriminatory power (13, 21). Interestingly, ECI isolates were collected from different species at different places and at different times in northern Italy, suggesting that ECI isolates have been circulating in ruminants in the same territory for at least the last 13 years. Because of the absence of surveillance programs for ruminant listeric rhombencephalitis in Italy, farmers and veterinarians are not required to report the disease, thus potentially limiting the number of diagnosed cases.

A literature review was performed to assess the prevalence of ECI in samples from different sources. The ECI-associated ribotype was found in 10.5 to 12.8% of human sporadic cases (55, 56) and in 11.8% of animal cases (56). A recent study from Portugal found a prevalence of ECI markers in 13.58% of the analyzed *L. monocytogenes* isolates obtained from fermented meat sausages, processing plant environments, and other raw products (16). In Italy, ECI markers were found in 17.6% of the human clinical isolates obtained between 1994 and 2007, 18% of the food isolates, and 20% of the environmental isolates (48). ECI was also detected in one out of five human sporadic clinical strains originating in Piedmont from 2002 to 2006 (39). To further support our findings, 56 food and environmental strains of serotype 4b/4e selected

FIG 3 Unrooted neighbor-joining tree computed in MEGA 5.0 (52) based on the comparison of MVLST results for the *L. monocytogenes* ruminant isolates investigated in this study (20 clinical veterinary isolates) with 165 isolates previously characterized by MVLST (13, 21, 22). Statistical support was provided by bootstrapping with 1,000 replicates. The scale bar corresponds to the number of differences observed over the alignment of 2,606 bp.

from the set of 311 strains and isolated from a geographic area partially matching that of the veterinary clinical isolates analyzed here were screened by PCR for the presence of the ECI-specific marker (57). Seventeen isolates (30.3%) were identified as putative ECI strains (data not shown). When the prevalence of ECI strains in ruminant clinical cases (60%) was compared to the observed prevalence in various food types (30.3%), the results of the chi-square test showed a significant difference between the percentages: chi square = 4.3; $P = 0.03$. Therefore, we believe that the observed high prevalence of ECI is significant, especially considering the limited number of animals affected in such a long time period and in such a large geographic area.

Overall, our MVLST results suggest that *L. monocytogenes* strains isolated from ruminants with rhombencephalitis constitute a genetically homogeneous group related to human, food, and environmental isolates, as also previously highlighted by other authors (6, 49, 55). This is in contrast to what was observed in 183 *L. monocytogenes* isolates obtained mostly from ruminants and to a lesser extent from human clinical rhombencephalitis cases, food, and the environment analyzed with multilocus variable-number tandem-repeat analysis (MLVA) (30). Their findings revealed that *L. monocytogenes* isolates clustered according to serotype and whether they were obtained from clinical cases or from food/environmental sources. No matches between food isolates and ruminant isolates were observed; however, some human clinical isolates and ruminant rhombencephalitis isolates had identical MLVA types, indicating ruminants as possible reservoirs for human infection, as also previously suggested (26, 31, 58). The role of ruminants as possible reservoirs for human infection is also supported by our results and the comparison of 165 isolates (from both food and clinical cases) representing the major food-borne outbreaks of listeriosis reported worldwide in the last 40 years, in addition to other unrelated isolates from sporadic cases.

EC strains may possess a greater ability to persist in the environment, survive and replicate in foods, and be transmitted to animals and humans (9). For instance, dairy farms are more frequently contaminated with *L. monocytogenes* than are other environments (33). The amplification and spread of *L. monocytogenes* into the farm environment can be attributed to ruminants via shedding of the pathogen through feces and/or contamination of raw milk due to shedding or exogenous contamination from the environment (33). In particular, ECI has been epidemiologically associated with cross contamination (e.g., the use of sheep manure as fertilizer in the 1981 Canada outbreak), the use of raw materials (outbreaks in France in 1992 and California in 1985), or postpasteurization contamination (outbreak in Switzerland from 1983 to 1987) (9). Consequently, ECs that have been causing listeriosis in animals and humans (such as ECI) may live as saprophytes in the farm environment, and raw animal foods can then play a role in the contamination of RTE foods because of cross contamination and thus may result in sporadic cases and outbreaks.

This study compared the subtypes of *L. monocytogenes* isolates from clinically affected ruminants to those of a significant number of clinical and food isolates obtained from the major human outbreaks of listeriosis. Our findings suggest that ruminants represent an important natural reservoir of *L. monocytogenes* and a potential source of strains pathogenic to humans. Additional molecular subtyping data on a larger number of *L. monocytogenes* isolates from farm animals with and without neurological disease and from different geographical areas/continents are needed to con-

firm the listeriosis link between ruminants and humans. Such data may help in the development of intervention strategies for preventing listeriosis, thus diminishing economic losses for the food and livestock industries and enhancing food safety.

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